ABSTRACT

WU, LIN. Denitrification and Molecular Detection in Riparian Buffer Soils. (Under the direction of Deanna Osmond and Owen Duckworth.)

Denitrification, the bacterially meditated conversion of nitrate (NO_3^-) into nitrogen gas, is an important biogeochemical process in riparian buffers that contributes significantly to the abatement of NO_3^- contamination. The overall goal of this study is to resolve uncertainties in the mechanism of denitrification in riparian buffer soils by quantitatively linking chemical reactivity and biological activity. Specific objectives include: (1) To assess the rate of denitrification as a function of dissolved carbon type and concentration in riparian buffer soils through continuous column experiments and (2) to quantify the gene copy number for the enzymes responsible for nitrite reduction by using real-time PCR (polymerase chain reaction). We treated soil columns with 3 carbon types at 3 levels of carbon concentrations: citric acid, alginic acid, and Suwannee River dissolved organic carbon (DOC) at 4, 8, 16 mg C L^{-1}, with a 0 mg C L^{-1} trial used as a control. To measure chemical reactivity, the NO_3^- loss rate and nitrous oxide (N_2O) production rate were measured in triplicate for each treatment. To estimate biological activity, the abundance of the denitrification genes *nirK* and *nirS,* as well as 16s rDNA copy number, in each soil column were quantified with real-time PCR. Citric acid promoted denitrification significantly, with NO_3^- loss rate increasing from 0.2 mg N hr^{-1} L^{-1} to 0.72 mg N hr^{-1} L^{-1} with increasing C concentration. Similarly, N_2O production rate was also enhanced from 4.3×10^{-4} mg N hr^{-1} L^{-1} to 5.4×10^{-3} mg N hr^{-1} g^{-1} as carbon concentration increased from 4 mg C L^{-1} to 16 mg C L^{-1}. Alginic acid and DOC did not contribute to denitrification, with stable low NO_3^- loss rate and N_2O production rate around

0.02 to 0.04 mg N hr^{-1} L^{-1} (not above limit of detection: 0.04 mg N hr^{-1} L^{-1}) and 5.0×10^{-4} mg N hr^{-1} L^{-1}, respectively. The *nirK*, *nirS,* and 16s rDNA abundances, in citric acid treatments ranged from 10^7 to 10^8 copies g^{-1} soil, and were significantly higher (5 to 10 fold) than those treated with alginic acid and DOC (ca. 10^6 to 10^7 copies g^{-1} soil, respectively). The *nirS* and 16s rDNA abundances exhibited correlation (R^2= 0.53 and 0.85) with denitrification rates in citric acid treatment while *nirK* did not. These results provide a quantitative linkage between biological activity and chemical reactivity, and further understanding about the influence of organic carbon on the denitrification rates in riparian buffers.

Denitrification and Molecular Detection in Riparian Buffer Soils

by
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BIOGRAPHY

Lin Wu was born in Qingdao, China where is a beautiful coastal city along Pacific Ocean. She experienced a wonderful childhood on the beach with her parents. She loved travelling when she was a young girl, and went to many places over China. After high school, she came to Beijing, the capital of China, attending China Agricultural University when she studied Ecology. Four years colorful college life widened her eyes and opened a new prospect in her mind. With the goal of reading ten thousand books and travelling ten thousand miles, she decided to pursue her graduate study abroad. In the beautiful autumn of 2008, she came to the United States and started her master study in Soil Science at NC State University. She appreciated this experience of two years, especially working with an outstanding group of people. As part of her career plan, she will pursue her Ph.D. in Environmental Science and Engineering at University of North Carolina at Chapel Hill.

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Chapter 1

Introduction

In North Carolina, riparian buffers are an important best management practice (BMP) in regulating the transport of nitrate in groundwater flow from uplands to streams and other water bodies. Denitrification, the bacterially meditated conversion of nitrate into gaseous forms of nitrogen, is a major biogeochemical process that contributes significantly to groundwater purification from nitrate contamination in riparian buffers. Denitrification also has received much attention because it may result in the emission of nitrous oxides (N_2O), which may contribute to global warming and destruction of the ozone layer. Many researchers have reported that denitrification potential in riparian buffer was affected by various factors at different scales, including organic carbon concentration, hydrology, pH, and vegetation type. With the aid of modern molecular biology techniques, extensive work has also detected the putatively responsible denitrifying bacteria and genes. However, the relationship between chemical denitrification rate and microbial activity is poorly understood. The connection between quantification of denitrification rate and microbial activity may help us better understand and control denitrification in riparian buffers.

This work presented in this thesis examined the effect of organic carbon type and concentration on denitrification rate by using continuous column experiments. We also utilized real-time PCR to measure the responsible gene copy number so as to elucidate the relationship between denitrification rate and denitrifying gene abundance. We linked the chemical denitrification rate to molecular biological detection to give a new perspective on biogeochemical function of riparian buffers.

1.1 Denitrification

Because nitrogen is the mineral nutrient most in demand to microorganisms and plants, the nitrogen cycle is the one of the best-studied and most complex elemental cycles (Maier, Pepper and Gerba 2009) (Figure 1.1). Nitrogen biogeochemistry is drawn by a number of catalyzed transformations, including nitrogen fixation, ammonium oxidation, assimilatory and dissimilatory nitrate reduction, ammonification, and ammonium assimilation. Dissimilatory nitrate reduction to ammonia (DNRA), where ammonium is the end product, and denitrification, where a mixture of gaseous products are formed, are two separate pathways for the dissimilatory nitrate loss from soils and waters. Nitrite, usually derived from nitrate reduction, can also be converted to N_2 via the anammox (AN) pathway, involving the simultaneous conversion of nitrite and ammonium to N_2(Dong et al. 2009).

Denitrification, the stepwise microbial reduction of the dissolved nitrogen (N) oxides, nitrate (NO_3^-) and nitrite (NO_2^-), to the gases nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2), is the primary type of dissimilatory NO_3^- reduction found in soil (Groffman et al. 2006). It serves to remove NO_3^- from the soil and aquatic ecosystems. In agricultural fields, denitrification can cause nitrogen loss from soils (Yoshida et al. 2009, Philippot, Hallin and Schloter 2007). In aquatic ecosystems, denitrification helps to relieve eutrophication and promote water purification. It is also important to the atmosphere because some of the gaseous intermediates formed, such as N_2O, can cause depletion of the ozone layer or serve as a greenhouse gas that may contribute to climate change (Maier et al. 2009).

1.1.1 Denitrification and NO_3^- Removal

Denitrification is a significant process for dissolved NO_3^- removal in ecosystems (Willems et al. 1997). NO_3^-, a soluble anion that is not commonly adsorbed onto exchangeable sites on clay and organic matter, can be removed by biological attenuation. Plant uptake, microbial immobilization, and denitrification are three main pathways for biological NO_3^- removal. Denitrification is performed by groups of bacteria that consume NO_3^- by respiration in the absence of O_2, and convert it into nitrogen gas (N_2O, N_2). Denitrification is a desirable NO_3^- removal mechanism because of its complete removal of N from the systems, whereas plant uptake and microbial immobilization will rerelease NO_3^- back to soil solution after mineralization (Groffman et al. 1991).

1.1.2 Denitrification and N_2O emission

In addition to its important role in groundwater purification, denitrification received considerable interest because it leads to N_2O emissions, an important gas that contributes to both global warming and the destruction of the protective ozone layer in the Earth's atmosphere.

Firstly, N_2O, as a trace gas, has a long residence time and is highly efficient in absorbing long-wave radiation. In fact, N_2O is about 200 times more effective than carbon dioxide (CO_2) as a greenhouse gas (Maier et al. 2009). Secondly, N_2O is a natural catalyst of stratospheric ozone degradation (Bange 2000). N_2O is photolytically converted into NO, which reacts with ozone (O_3) to produce NO_2 and O_2. Based on the series of reactions in the

atmosphere, NO is regenerated and a large number of ozone molecules can be destroyed for every molecule of N_2O released to atmosphere.

Soil has been identified as a major source of N_2O, whose production is associated with two processes - nitrification and denitrification. N_2O production from denitrification is favored in wet soils with restricted aeration (Maier et al. 2009). Magg and Vinther (1996) also investigated the effect of temperature and soil moisture on the N_2O emission from denitrification. It was shown that N_2O production in the sandy loam soil responded significantly to both increased soil moisture and increased temperature, whereas the coarse sandy soil only reacted to increased temperature.

1.1.3 Measurement of Denitrification

Denitrification is a difficult process to measure for a variety of reasons. The predominant reason is the high background concentration in the environment of the dominant end product (N_2) (Groffman et al. 2006). Commonly utilized methods to measure the denitrification rate include: (1) acetylene-based methods, (2) ^{15}N tracers, (3) direct N_2 quantification, (4) N_2: Ar ratio quantification, (5) mass balance approaches, (6) stoichiometric approaches, (7) methods based on stable isotopes, (8) in situ gradients with atmospheric environmental tracers, and (9) molecular approaches (Groffman et al. 2006). These methods have been used to detect denitrification in terrestrial, aquatic, and wetland ecosystems. The choice of methods for measuring denitrification rate is dictated by the environment studies and the scale of the measurement. The ^{15}N tracer method has been applied in studies of denitrification in soil and is considered as one of the best for soil studies.

However, this method still has its own shortcomings, including complicated procedures and expensive instrumentation. Gas flow core techniques allow for direct estimation of N_2 emission from soil due to denitrification but are still associated with complex technology to ensure air-tightness and uncontaminated sampling.

A useful laboratory approach is continuous flow soil column experiments, which have been successfully used by many researchers to explore the denitrification potential (Knies 2009, Pavel et al. 1996, Willems et al. 1997). These columns are microcosms designed to simulate the soil ecosystem in order to examine various influencing factors and measure the corresponding denitrification rate. Variables, including flow rate, temperature, soil depth, carbon source, and substrate supply, can be isolated and determined by controlling laboratory settings, making this approach attractive for examining denitrification mechanisms.

1.2 Riparian Buffers

Non-point source (NPS) pollution has caused increasingly serious water quality issues throughout the world. Agricultural activities, such as confined animal feeding operations, overgrazing of land, excessive and ill-timed application of wastes and fertilizers, and tillage of crop land, all contribute to non-point source pollution (Monaghan et al, 2008; Mayer et al, 2005) in the form of nutrients, pesticides, metals, and sediment.

Riparian buffers, vegetated regions adjacent to streams and wetlands, are thought to be effective at intercepting and reducing nitrogen loads entering water bodies (Mayer et al. 2007). Functionally, riparian buffers are areas of direct interaction between upland landscapes and lowing-lying bodies of water involving exchanges of energy and matter

(Gregory et al. 1991). In North Carolina, riparian buffers are widely implemented as a best management practice (BMP) to control non-point source pollution and reduce the NO_3^- load to the ground water system. Gilliam(1994) also considered riparian buffer to be the most important wetland for surface water quality protection. Riparian buffers serve as a sink of other nutrients, herbicides, pesticides and sediments that can be potential pollutants of surface and ground water. Several researchers have measured >90% reductions in sediment and NO_3^- concentrations in water flowing through the riparian areas (Gilliam 1994).

The use of riparian management zones is relatively well established as a BMP for water quality improvement in forestry practices (Lowrance et al. 1997), but has been much less widely applied as a BMP in agricultural areas, or in urban or suburban settings. Riparian ecosystems are especially important for small streams (first, second, and third order), which account for over three quarters of the total stream length in the United States (Mayer et al. 2007).

1.3 Denitrification in Riparian Buffers

Nitrogen removal effectiveness in riparian buffers has been evaluated as a function of factors such as vegetation type, climate, and hydrology. However, other factors may also affect overall effectiveness, such as buffer width, pH, and soil depth. A number of studies have examined the denitrification under different combinations of conditions. Riparian buffers may be very effective at nitrogen removal, but the underlying causes that control the mechanism are unclear.

1.3.1 Effects of Organic Carbon on Denitrification

Availability of carbon substrates usually acts as a limiting factor for microbial processes in soil and ground water, and may regulate overall microbial activity (Starr and Gillham 1993). Boyer and Groffman (1996) found that denitrification was higher in surface soils than in subsurface soil, indicating the potential influence of soil organic carbon (SOC). Comparison between the SOC in the surface and subsurface soils in the forest and agricultural soil profiles showed that concentrations of both water-extractable organic carbon (WEOC) and bioavailable dissolved organic carbon (BDOC) were significantly higher in surface layer than those in subsurface layer (Boyer and Groffman 1996). In subsurface layers, amount of total SOC, WEOC, and BDOC declined significantly with depth which indicated a low rate of microbial processes. This conclusion is also consistent with the research conducted in aquifers where denitrification potential is related to the water table depth. Denitrification tends to occur more rapidly in aquifers with a very shallow water table (Starr and Gillham 1993). Because organic carbon availability decreases with depth below the ground surface, denitrification may be less relevant to the insufficient labile organic carbon supply in aquifers with a deeper water table.

Although soluble carbon pools are the key driver of microbial activities, different soluble carbon types play different roles. In other words, differences in bioavailability lead to different influence on these microbial processes. Humic acid is more bioavailable than fulvic acid based on Boyer and Groffman's research (1996). However, it is widely accepted the high molecular weight organics are not as easily assimilated as low molecular weight

organics. One possible explanation indicates that the C:N ratio of organic matter is a factor effecting bioavailability of soil organic carbon (Boyer and Groffman 1996). However, the relationship between carbon type, bioavailability, and denitrification rate are not well understood, and this uncertainly motivated this research.

1.3.2 Effects of Hydrology on Denitrification

Differences between column experiments and on-site measurements indicate that the NO_3^- removal capacity could be affected by other important factors such as local hydrology and groundwater flow patterns. The influence of hydrology and associated pattern of electron transport was also examined by Hill (2000). It is generally believed that subsurface denitrification in deeper groundwater is not as significant as that in the shallower groundwater region under the riparian buffers. However, data from Hill's research suggested that, with the interaction of groundwater flow, and electron acceptor and donors, NO_3^- could be effectively removed at deeper depth below riparian buffers. Therefore, significant groundwater denitrification is confined mainly to narrow zones of high biogeochemical activity within the riparian aquifer. The research also strongly supports the hypothesis that the location of denitrification "hotspots" is linked to the supply of oxidizable organic carbon. It also agrees with research conducted in stream riparian zones where relationships between hydrology and NO_3^- removal were examined within the context of the spatially heterogeneous hydrologic setting (Hill 1996).Although effective removal of NO_3^- occurs worldwide in different areas, a similar hydrogeologic setting can be found at most sites. The effective hydrological setting usually includes several factors as follows (Hill 1996, Puckett

2004): long residence times along groundwater flow paths, dilution of NO_3-N rich waters by less concentrated older groundwater, the bypassing of riparian zones by tile drains or ditches, and the movement of groundwater along deep flow paths below shallower, organic rich reducing zones. Those riparian zones have permeable surface soils and an impermeable layer under them at a depth of 1 to 4 m. Such impermeable layers produce shallow subsurface flow of groundwater under the riparian area.

1.3.3 Effects of Other Factors on Denitrification

Many different chemical and environmental factors may affect denitrification rates. Meta-analysis was used to identify the trends between nitrogen removal effectiveness and buffer width, hydrological flow path, and vegetative cover (Mayer et al. 2007). Mean nitrogen removal effectiveness in buffers >50 m wide were significantly higher than in narrow buffers (0-25m), suggesting that buffer width is an important consideration for nitrogen management in watersheds (Mayer et al. 2007). However, the ideal buffer width required to maximize water quality benefits while minimizing unnecessary land utilization is difficult to determine (Smith, Osmond and Gilliam 2006). Another study conducted in Rhode Island (Groffman et al. 1991) to measure the denitrification in two grasses and two forested vegetated riparian buffers also suggested the ability of riparian buffers to support denitrification varies strongly with vegetation, soil type, and pH. Compared with grass riparian buffers, forested riparian buffers are generally more effective at reducing concentrations of NO_3^-(Osborne and Kovacic 1993). pH is sometimes considered a factor influencing denitrification, but Bradley (1992) observed no significant relationship between

groundwater pH and denitrification rate. Studies with Bibb soil columns under saturated–flow conditions showed that effluent NO_3^- concentrations could be described by a linear combination of temperature, flow rate and influent NO_3^- concentrations. NO_3^- removal was reduced with increasing flow rate and increased with increasing temperatures (Willems et al. 1997), which agrees to the observation that denitrification potential was highest in October and August. Denitrification in riparian buffers also exhibits spatial distribution. In a coastal plain riparian forest, denitrification potential was more than two orders of magnitude higher in the top 10 cm of soil than in the top 10 cm of the shallow aquifer, and also consistently highest in surface soil near the field and stream (Lowrance 1992).

Soil systems are highly complex, and denitrification is controlled by the net effectsof influencing factors. Long-term monitoring of a riparian buffer is a strategy to further unveil the mechanisms of effectiveness of riparian buffer in removing NO_3^- and purifying groundwater. Riparian buffers at the Center for Environmental Farming Systems (CEFS) located in Goldsboro, NC have been monitored for 12 years forNO_3^- dynamics. King (2005)studied the influence of vegetation types onNO_3^- concentrations. Effects of vegetation type, groundwater depth and buffer width on NO_3^- removal from groundwater were also investigated by Knies (2009) based on continuing monitoring. Because of low DOC in groundwater, it has been suggested that denitrification in these buffers may be carbon limited. To further investigate denitrification in riparian buffer soils, examination of the effects of carbon concentration and type on the biology and chemistry of denitrification are necessary to elucidate the effectiveness of riparian buffers in NO_3^- removal.

1.4 Biology of Denitrification

1.4.1 Responsible Genes for Denitrification

From a biological perspective, denitrification is the microbial process by which dissolved N oxides serve as terminal electron acceptors for respiratory electron transport resulting in the reduction of NO_3^- to gaseous products (Wallenstein et al. 2006). Most bacteria with this functional trait belong to a wide range of various subclasses of *Proteobacteria*. However, the ability to promote denitrification is widely distributed in the microorganisms, not only in large group of phylogenetically unrelated bacteria (Zumft 1997), but also can be found in mitochondria of certain fungi (Shoun et al. 1992) and some *Archaea*(Philippot 2002). Lateral gene transfer is the most likely explanation for this widespread ability to denitrify (Braker et al. 2001).

With developments of molecular biology towards analysis of functional genes for those organisms, *Archaea*, bacteria, and fungi, various denitrifying genes have been sequenced and exploited as biomarkers to discriminate between closely related but ecologically different populations (Throback et al. 2004). Although denitrification can be found within more than 50 genera (Zumft 1997), the functional genes for the denitrification pathways are common. Denitrification involves four enzymatically catalyzed reductive steps:NO_3^- reduction, nitrite reduction, nitric oxide reduction, and N_2O reduction (Philippot et al. 2002) (Figure 1.2).

The commonly investigated genes that code the responsible enzymes are *narG (*NO_3^- *reductase)*, *nirS* and *nirK (*NO_2^- *reductases), norB (*NO *reductase),* and *nosZ (*N_2O *reductase)*.

The reduction of nitrite to nitric oxide, the key step in this dissmililatory denitrification process, is the first step in the reaction that produces a gaseous product and is thus of most interest to this thesis. This reaction is central to denitrification and is catalyzed by two different types of nitrite reductases (*nir*). Probes and antibodies have been usedfor this enzyme to identify denitrifying isolates (Ward, Cockcroft and Kilpatrick 1993). These two evolutionarily unrelated forms of the nitrite reductase enzyme are different in terms of structure and the prosthetic metal: a copper and a cytochrome cd1-nitrite reductase, coded for by the *nirK* and *nirS* genes, respectively (Philippot 2002) (Figure 1.3). These two genes are the most common molecular markers for denitrifier community studies (Braker, Fesefeldt and Witzel 1998). These genes seem to be mutually exclusive in a given strain, but both of them have been found in different strains of the same species.

1.4.2 Application of quantitative PCR

With the aid of biotechnology, increasing numbers of microbial studies have relied on gene detection and other molecular level measurements. Molecular tools to investigate the diversity and community composition include terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), and quantitative polymerase chain reaction (qPCR). Quantitative PCR, also referred as real-time PCR, has emerged as a promising tool for studying soil microbes (Kabir et al. 2003).

In an optimized reaction, the target gene quantity will approximately double during each amplification cycle. In qPCR, the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule (such as a double-stranded DNA binding dye or a dye-labeled probe). With each amplification cycle, the fluorescence intensity increases proportionally to the increase in amplicon concentration, with the qPCR instrument system collecting data for each sample during each PCR cycle. The resulting plots of fluorescence intensity vs. cycle number for all the samples are then set with their background fluorescence at a common starting point. Then, a threshold level of fluorescence is set above the background but still within the linear phase of amplification for all the plots. The cycle number at which an amplification plot crosses this threshold fluorescence level is called the Ct or threshold cycle. This Ct value can be directly correlated to the starting target concentration of the sample. Therefore, based on real-time detection of the fluorescent molecules or fluorogenic probe during amplification cycles, qPCR can measure the relative abundance of any particular gene fragment given specific primers (Gruntzig et al. 2001, Fierer et al. 2005). This technique yields relatively rapid yet quantitative assessment of the abundances of specific genes.

This technology is not limited by the cultivability of bacteria, which also helps to quantify functional genes of uncultivated bacteria in the environment (Lopez-Gutierrez et al. 2004). Commonly used methods, such as most probable number (MPN), are biased by the unculturability of many microorganisms. The quantitative nature of the qPCR promises to be a valuable tool for soil microbial research (Whelan, Russell and Whelan 2003).

Henry (2004) showed that qPCR has a high sensitivity that can detect the significant changes in the denitrifying community density. Although it provides precise quantification of the target gene, no information on community composition is obtained because the estimated abundances of the different microbial groups from qPCR may not equal to the true percentages of these groups in the real soil samples due to extraction bias, heterogeneity, and other PCR inhibitions. However, qPCR when combined with other biotechnology procedures is still a very promising method to explore denitrifying communities.

1.4.3 Gene Detection and Denitrification

A system has been developed by the amplification of specific nitrite reductase gene (*nirK* and *nirS*) fragments with PCR. The PCR system for the *nirK* and *nirS* genes, using one generally amplifying primer combination for each *nir* gene, have been applied successfully to detect populations of denitrifying bacteria in aquatic systems. Detection of such populations from soils by using these PCR systems should be possible as well (Braker et al. 1998). The difference between aquatic and soil systems is due to the scale of process and impacts the sampling strategies. In aquatic sediments or terrestrial soils, localized zones of denitrification occur on the 10 cm scale (Groffman et al. 2006). Sediments and soil samples tend to have higher diversity of organisms, and the typical sample size is a few grams so care must be taken to sample the appropriate sediment layers (Groffman et al. 2006).

DNA probes and PCR primers for these genes have been used to study the distribution, abundance, diversity of the denitrifying microorganisms (Groffman et al. 2006). Specific primer sets have been developed for real-time amplification of *nirK* and *nirS*.

Reliable PCR primers are a prerequisite for denitrifier community surveys since they ultimately determine what is detected in the environmental sample. Braker et al.(1998) developed PCR primer systems for *nirK* and *nirS* by aligning available sequences to find conserved regions. His results suggested the suitability of the method for qualitative detection of denitrifying bacteria in environmental samples. Increasing number of partial *nir* sequences are deposited in the GenBank which indicates the importance of reassessment of more variable PCR primers. Throback and others re-evaluated PCR primers targeting *nirS* and *nirK* and combined new primers with existing primers (2004). Their work demonstrated that *nirS* denitrifiers were common in soil, and exhibited the promising application of Denaturing Gradient Gel Electrophoresis (DGGE) for screening and comparing denitrifying communities.

In the case of *nirK*, Henry (2004) has designed a set of degenerated primers to amplify *nirK*, *nirK* 876 and *nirK*1040, which showed high specificity. Commonly, 16s rDNA is used to estimate total bacteria (Lopez-Gutierrez et al. 2004). Lopez-Gutierrez et al (2004) found significantly different ratios of *narG* to 16S rDNA across environments, illustrating the different community composition of denitrifying bacteria. It may also demonstrate selective advantage of NO_3^- reducing genetic potential under various environmental conditions. Therefore, gene copy numbers from qPCR can provide a rough estimation of the actual number of organisms. When applying qPCR to analyze DNA isolated from complex environments, such as soil, inhibitors may interface with PCR amplification (Martin-Laurent et al. 2001). 16S rDNA here can be used as endogenous control because

both functional genes and 16s rDNA quantification are subjected to the same DNA extraction and amplification biases (Lopez-Gutierrez et al. 2004).

Several studies compared the distribution and diversity of these two genes. Bothe et al (2000) indicated that both genes are found in all environments but *nirS* appears to be dominant in most environments. This was in agreement with Braker (2000), who also reported *nirS* could be amplified from both Puget Sound and Washington continental margin while *nirK* could only be amplified from the continental margin. Subsequent T-RFLP analysis of *nirS* and bacterial rDNA also revealed a high level of functional and phylogenetic diversity (Braker et al. 2001). Reverse Transcription-PCR was incorporated to detect the expressed denitrification genes and indicated that *nirS* was the only one detected directly as a RT-PCR product. This study suggests that in estuarine sediments *nirS* is more active than *nirK*. Moreover, *nirS* sequences present a high diversity in two different sediments which underlines the differences of the compositions of responsible bacterial communities (Nogales et al. 2002). It was also reported different populations of organisms containing *nirS* developed under the selection of differing environmental conditions at distant geographic locations (Braker et al. 2001). However, *nirK* also presents high diversity in different soil environments. In rhizosphere samples of three economically important grain legumes (Sharma et al. 2005), *nirK* presented higher activity than *nirS*. The *nirK* gene and transcripts could be detected in all the rhizosphere samples while the *nirS* could not be detected. Prieme et al. (2002) showed surprisingly that there was very little overlap in *nirK* gene sequences between forested upland soils and wetland soils. They also found that the *nirK* gene pool in

marsh soils were more diverse than in upland soils. Also, *nirK* gene sequences in several wellcharacterized halophilic *Archaea* differ substantially from those identified inbacteria (Ichiki et al. 2001).

1.5 Summary

Despite this interest in denitrification rates and biological processes, few studies have sought to connect chemical measurement and biological detection. Our research will focus on column experiments using soils from the Center for Environmental Farming, an area with established riparian buffers that have been the subject of a longitudinal monitoring study for 12 years. It has been hypothesized that the efficacy of these buffers is limited by low concentrations of dissolved organic carbon (King 2005, Knies 2009), but the mechanisms of buffer function are currently unknown. In this research, we utilize column experiments to study the rates of NO_3^- loss and N_2O production as a function of dissolved organic carbon type and concentration. In addition, we link observations of chemical transformations to quantifiable molecular biology parameters, such as gene copy number, through the use of quantitative PCR.

Thus the overall goal of this study is to resolve the uncertainties in the mechanism of denitrification in riparian buffer soils by quantitatively linking chemical reactivity and biological activity. Specific objectives are: (1) *To assess the rate of denitrification as a function of dissolved carbon type and concentration in riparian buffer soils through continuous column experiments* and (2) *to quantify the gene copy number of the enzymes responsible for nitrite reduction with method of real-time PCR.*

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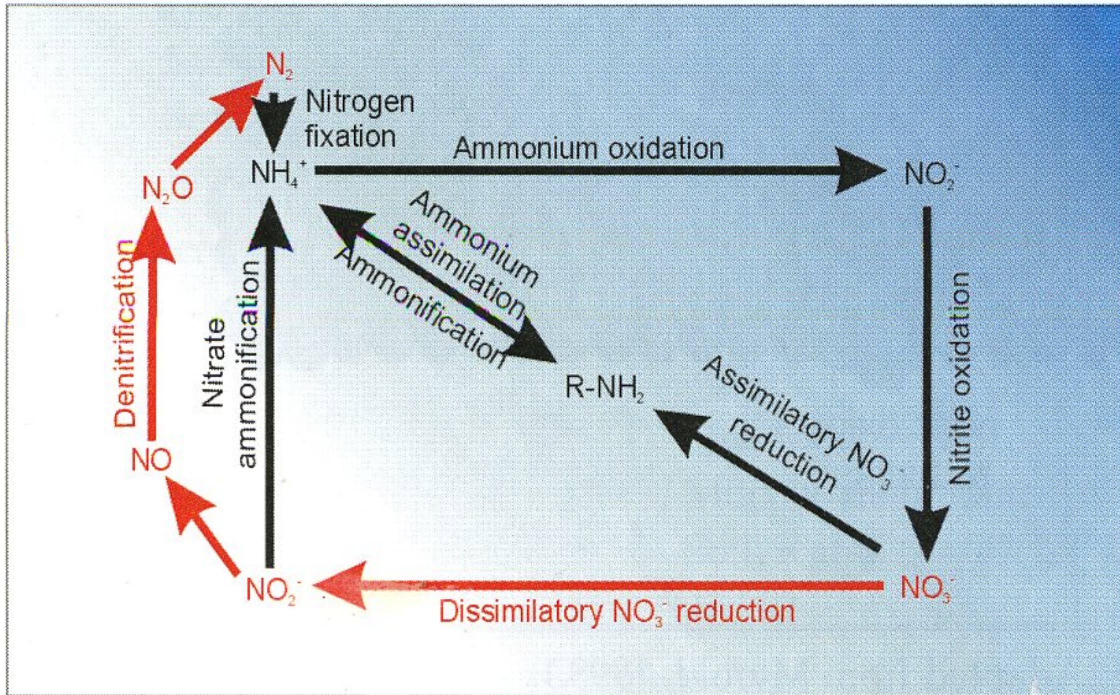

Figure 1.1 Schematic diagram of nitrogen cycle. Red arrows show stepwise denitrification.(Maier et al. 2009)

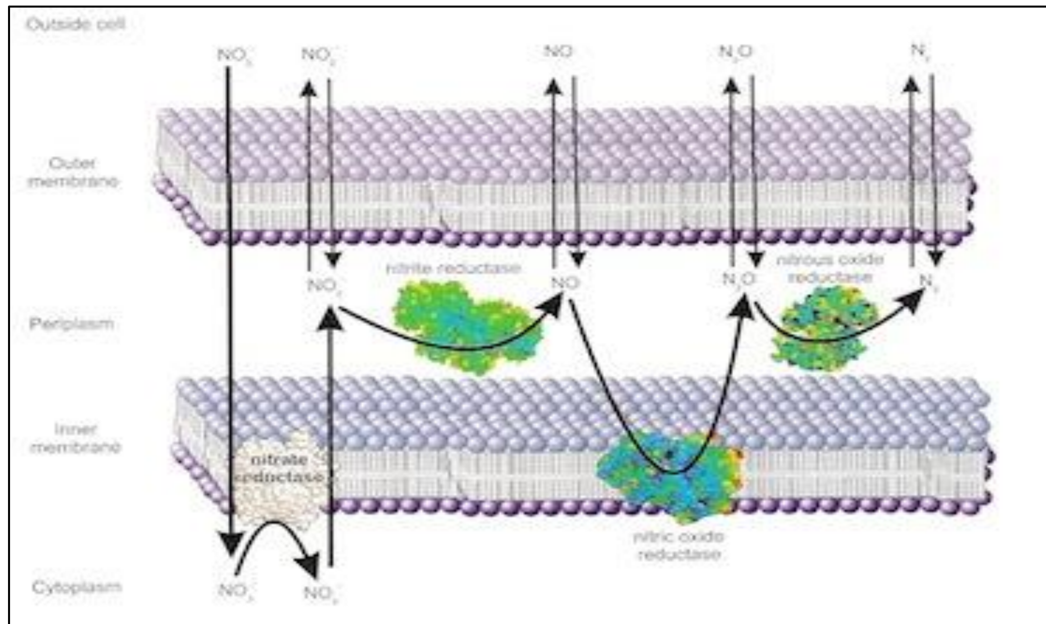

Figure 1.2 Denitrification pathways in cell membrane. (Maier et al. 2009)

(a)

(b)

Figure 1.3 (a) Cu-containing nitrite reductase from "*A. cycloclastes*" encoded by *nirK*, showing polypeptide fold of the CuNIR trimer. (Zumft 1997)
(b) Cytochrome cd_1 nitrite reductase from *Paracoccus denitrificans* GB17 encoded by *nirS*, showing the protein dimer with the heme C domain on top. (Zumft 1997)

Chapter 2

Chemical Measurement of Denitrification

2.1 Introduction

In North Carolina, riparian buffers are one of the most important best management practices (BMP) for water quality protection from non-point source pollution. NO_3^- is highly soluble and moves with precipitation or irrigation through the soil profile into groundwater (Osmond, Gilliam and Evans 2002). The role of riparian buffers in regulating the NO_3^- loading from uplands to the aquatic system has received much attention (Hill 1996). The capability of a riparian buffer to remove NO_3^- through denitrification is controlled by its physicochemical and biological characteristics. Understanding these characteristics will help to better guide beneficial use of riparian buffers in alleviating NO_3^- contamination.

Plant uptake and denitrification are primary fates of NO_3^- in riparian buffers (Hill et al. 2000). Denitrification occurs in the absence of oxygen when NO_3^- is used as substitute electron acceptor in soil microbial respiration. NO_3^- is converted to nitrous oxide (N_2O) and dinitrogen gas (N_2) through series of intermediate species. Most organisms cannot utilize molecular nitrogen, with the exception of nitrogen-fixing bacteria. Denitrification, therefore, is highly effective in NO_3^- removal from a readily available pool and produces long-term improvements in water quality. In contrast, NO_3^-, which is taken up and immobilized by plants, may eventually be mineralized during plant decomposition, and released into the soil.

Generally, it is accepted that availability of NO_3^- and organic carbon supply are two major limiting factors for denitrification in riparian buffers (Greenan et al. 2006). When sufficient NO_3^- is available to induce the denitrifying activity, an increase of soil organic

carbon drastically increases NO_3^- removal in organic C-limited soils. In anaerobic groundwater systems, significant relationships between potential denitrification and sediment total organic matter, and enhanced denitrification of sediments amended with glucose, indicate the importance of carbon limitation (Bradley, Fernandez and Chapelle 1992). Therefore, the bioavailability, and thus type of organic carbon, also influences the denitrification significantly (Myrold and Tiedje 1985). It has also been suggested that type of organic carbon also plays an important role in governing denitrification (Dodla et al. 2008). Experiments confirmed that simple organic carbon addition accelerates denitrification rates (Minami and Fukushi 1984), but denitrifying bacteria do not utilize all the organic carbon present in soil. The C/N ratio and chemical structure of organic carbon may play key roles in governing the denitrifying bacteria activities in soil (Dodla et al. 2008).

Nitrous oxide (N_2O) emission from riparian buffer soils is one of the most important anthropogenic sources of N_2O (Adouani et al. 2010). Although N_2O is present in the atmosphere in trace amounts, it contributes to climate change as the third most significant greenhouse gas (in terms of radioactive forcing) after CO_2 and CH_4(Olivier et al. 1998). It is released as an intermediate of the denitrification reaction that consumes organic substrates as electron donors. Many factors, such as substrate concentrations and C/N ratio, influence the N_2O production and emission. It has been reported that the use of different carbon sources may result in similar denitrification rates, but appreciably different N_2O emission (Adouani et al. 2010).

The NO_3^- dynamics of riparian buffers at the Center for Environmental Farming Systems (CEFS) located in Goldsboro, NC have been monitored for 12 years. Previous work has confirmed the efficiency of riparian buffers in regulating NO_3^- contamination (Knies 2009, King 2005). However, the soil ecosystems are highly complex, and a complicated web of interactions between environmental, chemical, and biological factors controls this processes in soil. Soil column methods can be incorporated to simplify and explore these complicated processes in soil (Obenhuber and Lowrance 1991). With precisely controlled laboratory experiments, column approaches have been has been successfully utilized by several studies to explore the influence of variables on denitrification (Obenhuber and Lowrance 1991, Pavel et al. 1996, Willems et al. 1997, Knies 2009).

This research seeks to elucidate the role of carbon source in biogeochemical cycling of nitrogen by determining the effects of carbon type and concentration on denitrification. Specifically, the present work quantifies the influence of carbon type and concentrations on denitrification rate by utilizing soil columns.

2.2 Material and Methods

2.2.1 Site Description and Soil Collection

Soil was collected from the R4W buffer at the Center for Environmental Farming Systems (CEFS) located in Middle Coastal Plain in Goldsboro (Wayne County), North Carolina (Figure 2.1). R4W buffer is located adjacent to the farm's drainage ditch network

along Neuse River. Based on *Soil Survey of Wayne County, North Carolina* (Barnhill 1974), the soil series found in R4W buffer are Lumbee sandy loam in the buffer, with Wagram loamy sand (Loamy, kaolinitic, thermic Arenic Kandiudults) in the adjacent field.

Soils were collected 2 m from the edge of the field-buffer interface. Soil was sampled from a depth of 3 m using an auger. Soil samples was kept on ice during transportation and stored at 4 °C until packed into soil columns. Soil samples were analyzed for texture and organic composition (Table 2.1). The soils were found to be course with a very low organic carbon content.

2.2.2 Soil Columns and Solution Preparation

Columns were built with PVC pipe of 13.2 cm length and 3.0 cm width. Collected soil was packed into columns to an approximate density of 1.60 g cm^{-3}. Columns were sealed by rubber stoppers and equipped with plastic tubing outlets which were filtered by PVC fabric (Figure 2.2).

Unless noted, all chemicals used are reagent grade. All solutions were made with type I deionized water with a resistivity of 18.3 MΩ·cm. The sample of river dissolved organic carbon (DOC) and alginic acid utilized in the study were purchased from International Humic Substances Society (IHSS) and Acros Orgics, respectively.

Solutions were made by different known concentrations of organic carbon and a single concentration of NO_3^- salt. KNO_3 was used as nitrogen source and set at a

concentration of 5.0 mg N L^{-1} for all solutions. Three types of organic carbon were chosen as carbon source: citric acid ($C_6H_8O_7$) [a low molecular weight (LMW) metabolite] (Figure 2.3A), alginic acid ($(C_6H_8O_6]_n$)[a large biopolymer composed of uronic sugar acids] (Figure 2.3B), and river dissolved organic carbon (Suwannee river 1R101N) (Table 2.3).

Concentrations of organic carbon were set as 4.0 mg C L^{-1}, 8.0 mg C L^{-1}, and 16.0 mg C L^{-1}. One reference treatment was set at 0.0 mg C L^{-1} and 5.0 mg N L^{-1} as a control (Table 2.2).

After boiling, anaerobic solutions were poured into carboys, which were wrapped with aluminum foil and continuously purged with humified argon gas to keep the solution anaerobic before delivery into columns. Solution pH in the citric acid treatments was adjusted to 6 by using NaOH solutions to avoid confounding the effects on denitrification from low pH, since low pH inhibits denitrification.

2.2.3 Sampling

Continuous flow column experiments were conducted to measure the denitrification rate from different concentrations of organic carbon. The inflow ends of the vertically positioned columns were connected to a light-shielded 25 L carboy where the anaerobic solutions were stored. A six channel peristaltic pump (Manostat Cassette) was used to transfer solution at constant flow rate of 0.3 mL min^{-1}. To minimize oxygen contamination, fluorinated ethylene propylene (FEP) tubing was wrapped in aluminum foil, except for the length of tubing used by the pump. The experimental set up is shown in Figure 2.4.

Triplicates of columns for each treatment were run for 6 days, which was sufficient time for the system to reach steady state. During operation, approximately 30 mL of effluent was collected in 50mL disposable plastic beakers every day. The mass of effluent was measured to determine specific flow rate. pH was measured using an Accumet Excel pH/conductivity meter (XL20). pH in alginic acid and DOC treatments changed less than 0.5 unit between inflow and effluent, while pH in citric acid changed 0.3 to 1 unit. Effluent samples were filtered through 0.22 μm HC Millipore syringe filters and were frozen at -20 °C until thawed for further analysis. Effluentfrom each respective column was also collected into sealed vacutainer tubes for determination of N_2O production.

For determination of NO_3^- loss, NO_3^- concentrations of the influent and effluent solutions were determined colormetrically with a sulfanilamide color reagent using an Automated Ion Analyzer (QuikChem Method 10107-01^{-1}-A, Lachat Instruments QuikChem brand 8000). Frozen samples were completed thawed and shaken well before measurement. The loss of NO_3^- was calculated by difference of NO_3^- concentration between inflow and effluent samples. In experiments with measureable NO_3^- loss (citric acid treatments), nitrite production was also measured colormetrically with same method as NO_3^-. In all cases, NO_2^- concentrations were less than1.00 mg N L^{-1}, and most were below the detection limit (0.05 mg N L^{-1}).

For analysis of N_2O production, 10 mL of gas sample was extracted from the headspace of vacutainer tube and injected into a gas chromatograph (Hewlett Packard 5890

GC-ECD, injector temperature 60 °C). The N_2O production was calculated by subtracting the ambient background concentration of N_2O from the measured sample concentration.

2.2.4 Data Analysis

The overall rate of denitrification can be expressed as rate of NO_3^- loss and rate of N_2O production. NO_3^- loss rate can be calculated using the following equation:

$$R\,(NO_3^-) = q \times \Delta C\,(NO_3^-) / m$$

with q as flow rate (L hr^{-1}), ΔC as the change of NO_3^- concentration (mg L^{-1}) between inflow and effluent, and m as the initial mass of soil packed into the column (g).

Similarly, N_2O production rate can be mathematically calculated as:

$$R\,(N_2O) = q \times \Delta C\,(N_2O) / m$$

where q is the flow rate (L hr^{-1}), ΔC (N_2O) is the concentration of N_2O dissolved in column effluent, and m is the initial mass of soil in the column (g).

2.3 Results and Discussion

2.3.1 NO_3^- Loss and N_2O Production

Concentrations of NO_3^- from triplicate column experiments at carbon concentration of 4, 8 and 16 mg C L^{-1} are shown in Figure 2.5 (a, b, c). For each carbon type, NO_3^- concentration in inflow and effluence are plotted against time. There is a slight decrease in 4

mg C L^{-1} columns fed with citric acid, showing the trend to reduce more NO_3^- in the last two days (Fig 2.5 a). Significant decreases of NO_3^- concentration were observed in citric acid treatments at the concentration of 8 mg C L^{-1} and 16 mg C L^{-1}, which decreased are up to 2.1 mg N L^{-1} and 3.8 mg N L^{-1}, respectively (Fig 2.5 a). However, alginic acid and DOC did not exhibit NO_3^- reduction, with no detectable decrease of NO_3^-concentration in DOC effluent and most alginic acid effluent (limit of detection = 0.1 mg N L^{-1}). No significant difference was found among difference carbon concentrations for alginic acid (Fig 2.5 b). With the exception of two days (day 3 and day 5) 16 mg C L^{-1} effluent NO_3^- concentration did not present detectable decrease (Fig 2.5 c).

Table 2.4 (a) shows the average NO_3^- loss rate given combinations of carbon type and carbon concentration. NO_3^- loss rates at concentration of 0 mg C L^{-1} are background from control experiments where no carbon source is added into the solution. Generally, in citric acid treatments, NO_3^- loss rate increase with higher C concentration. Mean NO_3^- loss rates are greater for 16 mg C L^{-1}columns (0.82 ± 0.21 mg N hr^{-1} L^{-1}) compared to either 8 mg C L^{-1} (0.38 ± 0.15 mg N hr^{-1} L^{-1}) or 4 mg C L^{-1} (0.12 ± 0.08 mg N hr^{-1} L^{-1}). However, low NO_3^- loss rates are observed in both alginic acid and DOC treatments at all carbon concentrations, which are stable at low level (0 mg N hr^{-1} L^{-1} to 0.04 mg N hr^{-1} L^{-1}) and not above limit of detection (LOD = 0.04 mg N $hr^{-1}L^{-1}$, calculated from detection limit of NO_3^- loss 0.1 mg N L^{-1}).Alginic acid and dissolved organic matter do not exhibit a promotion effect on NO_3^- reduction, where NO_3^- loss rates do not increase with higher carbon concentration. Our data

sets indicate that detectable NO_3^- loss can only be found in citric acid treatments; it is evident that citric acid promotes reduction of NO_3^- effectively compared with alginic acid and DOC.

N_2O production rates for different carbon sources are shown in Table 2.4 (b). All N_2O concentrations in effluent samples were above the limit of detection (50 ppbV). Similar to NO_3^- loss, mean N_2O production rate were greatest for the citric acid treatments at all concentrations, followed by the alginic acid and DOC treatments. In soil columns amended with citric acid, mean N_2O production rate was greatest from the 16 mg C L^{-1} treatment, which was ranged from 2.78×10^{-4} to 5.39×10^{-3} mg N hr^{-1} g^{-1} soil with an average at 3.79×10^{-3} mg N hr^{-1} g^{-1} soil. Citric acid treatments at the concentration of 4 mg C L^{-1} and 8 mg C L^{-1} had lower N_2O production rates, with average rates at 4.3×10^{-4} and 2.0×10^{-3} mg N hr^{-1} g^{-1} soil, respectively. The control treatment had the lowest N_2O production rate, which was stable at 3.8×10^{-4} mg N hr^{-1} g^{-1} soil. It was clear that N_2O production rates increased with increased citric acid concentration. In contrast, N_2O production rates in alginic acid and DOC treatments were low in all carbon concentrations, 5.0×10^{-4} mg N hr^{-1} L^{-1}, almost 10 fold lower than that given citric acid. No significant increase of N_2O production was observed with higher carbon concentration, suggesting alginic acid and DOC do not contribute to N_2O production, consistent with the lack of significant NO_3^- loss.

Citric acid enhanced both NO_3^- loss and N_2O production. Based on the denitrification pathway, part of nitrogen loss in NO_3^- reduction is emitted as gaseous form of N_2O. The percentages of N_2O-N production in total NO_3^--N loss are shown in Figure 2.6. Values are in

same range at all concentrations, suggesting a constant percentage of transformation of N_2 and N_2O.

2.3.2 Effects of Organic Carbon on Denitrification

Organic carbon availability is often the limiting factor of denitrification in riparian buffers (Greenan et al. 2006), and has been suggested to limit activity at the buffers at CEFS. Carbon type, as well as carbon concentration, has important influence on the organic carbon availability, and thus the denitrification rate. We selected to study three organic carbon types. Citric acid, which is LMW organic solute and an intermediate in metabolism, is most labile to microorganisms. Alginic acid is a larger linear copolymer with molar mass range of 10,000 – 600,000 g mol^{-1}. River DOC is the mid-size molecule with complex structure, but may be recalcitrant.

In aquifers, oxidation of organic carbon can result in reduced conditions beneath a frequently high water table, potentially followed by denitrification. This organic carbon also acts as the carbon source for the denitrifying microbes and regulates the denitrification rate in riparian buffers. Based on our results, citric acid, which is used as a model for LMW organic carbon, promoted denitrification significantly. Increased NO_3^- loss rate and N_2O production rate, can be observed to correlate with higher concentrations of citric acid supply (Figure 2.7). These results agree with in-situ observation (Smith, Osmond and Gilliam 2006) where organic carbon in groundwater flow was enriched by riparian vegetation and lead to enhanced NO_3^- loss. The release of low-molecular-weight organic acids, such as citric acid,

malic acid, and acetic acid, from root exudation and cell lysis, is well documented (Jones 1998, Jones et al. 2003). Generally higher concentrations are found in rhizosphere soil compared to those present in the bulk soil. Riparian vegetation, producing higher concentrations of LMW organic acids, may enhance denitrification by enriching liable carbon source to denitrifiers. The nature of organic carbon, may also be responsible for ecosystem-scale differences in denitrification (Ward et al. 2009). Stronger correlation between denitrification and soil organic matter that is readily decomposable has been demonstrated than for total organic carbon has been also observed (Stanford, Vanderpol and Dzienia 1975). These findings are consistent with our results (Figure 2.7). Denitrification enzyme activity (DEA), which is an index to evaluate the denitrification potential, also correlates with organic carbon, but the extent is largely influenced by the variety of organic carbon (Kamewada 2007).

Alginic acid, however, does not significantly promote denitrification (Figure 2.7). Many authors have suggested that soluble C sources are key drivers of microbial activity, although not all soluble C is equally bioavailable (Boyer and Groffman 1996, Cook and Allan 1992) The generally accepted dogma that high molecular weight organics are more refractory than low molecular weight organics (Tate 1987) could be a possible explanation for the low denitrification rate in our alginic acid column experiments. It agrees with research by Dendooven (1996), which showed the glucose enhanced the denitrification potential more effectively than cellulose, cattle manure, or grass litter.

It is somewhat surprising that river dissolved organic matter (DOC) does not stimulate denitrification either for NO_3^- loss or N_2O production. This is in contrast with work of Groffman et al (1992), Lowrance (1992), and Willems et al. (1997), who concluded that denitrification in riparian buffer soils is highest in surface horizons and depends on organic matter content, but in agreement with work of Starr (1993) where denitrification was limited even given abundant DOC. One possible reason is the DOC sample from IHSS has a high C/N ratio (47.7:1), which is not suitable for supporting denitrifier activity. Groffman et al. (1991) compared the denitrification potential in forest and grass vegetated filter strips, indicating the nature of soil organic matter, low C/N ratio (<10:1) in grass plots, was more suitable for supporting microbial activity in the grass plot though forest plots have higherlevels of total organic matter. However, the low solution pH (<5) with the DOC amendment may also have contributed to the low denitrification rate.

2.4 Conclusions

Denitrification rate varies with organic carbon concentrations and type. When sufficient NO_3^- is present in system, availability of liable organic carbon may limit denitrification under anaerobic condition. Citric acid, the most labile organic carbon in our study, exhibited greatest enhancement of denitrification rate. With higher concentrations, both NO_3^- loss rate and N_2O production rate increased significantly. Increased concentrations of alginic acid and dissolved organic matter, however, did not show significant positive effect on denitrification rate or N_2O production.

The results of this study indicate that the importance of vegetation in riparian buffers in promoting denitrification and water purification. A rich supply of liable organic carbon, such as citric acid, from root exudates and plant turnover, will significantly promote the microbial NO_3^- removal and thus provide better buffer function for pollution control.

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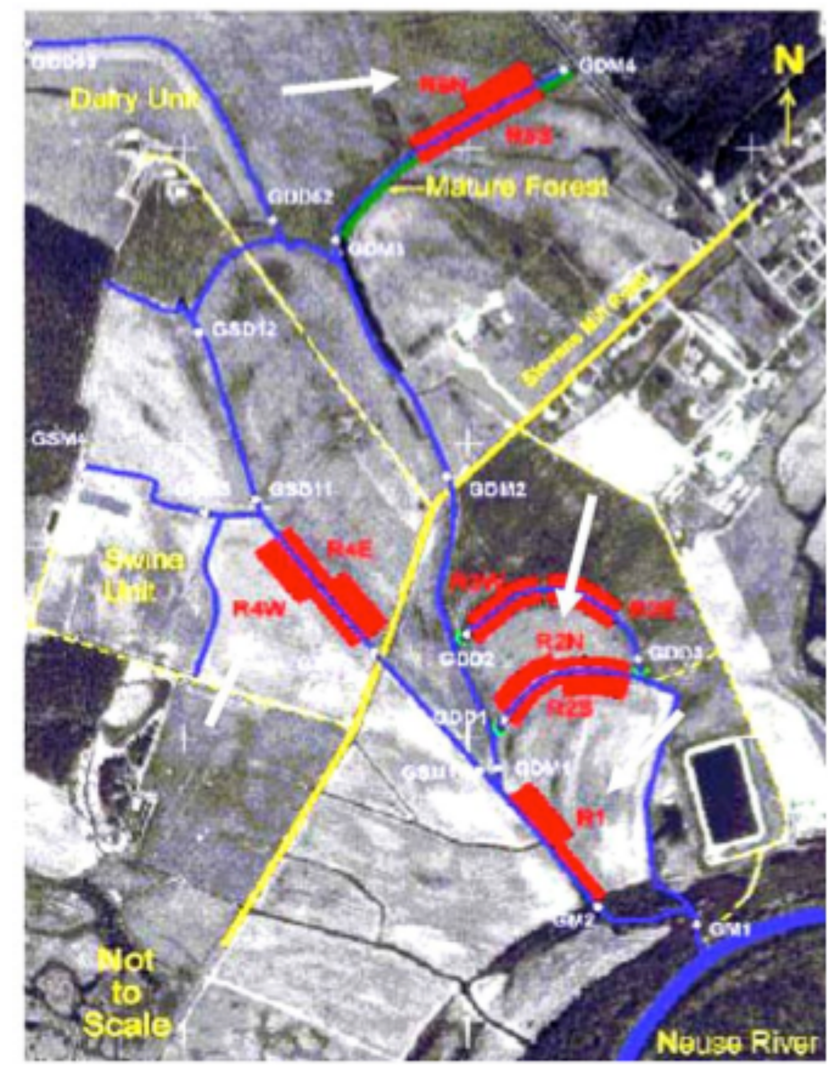


Figure 2.1 Location of buffer at CEFS. Arrows indicate buffers (R1, R2N, R4W, R5N) (Knies 2009). Not to scale.

Table 2.1 Soil Characteristics.

Soil Characteristics			
Texture	C %	N %	pH
Sandy	0.07	<0.02	5.19

Table 2.2 Experimental design of column experiments.

Carbon Concentration	Carbon type		
	Citric Acid	Alginic Acid	River DOC
0 mg C L^{-1}		Control	
4 mg C L^{-1}	CA 4	AA 4	DOC 4
8 mg C L^{-1}	CA 8	AA 8	DOC 8
16 mg C L^{-1}	CA 16	AA 16	DOC 16

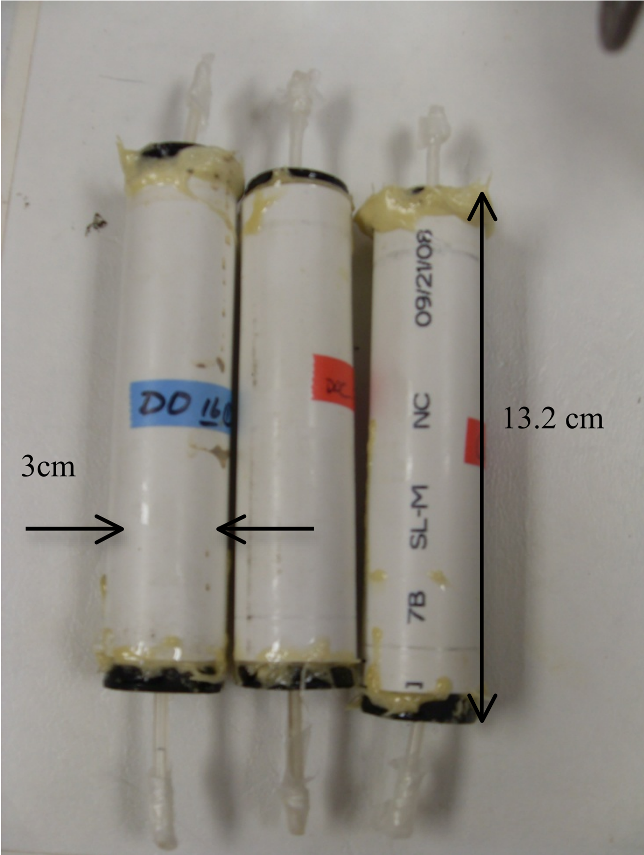
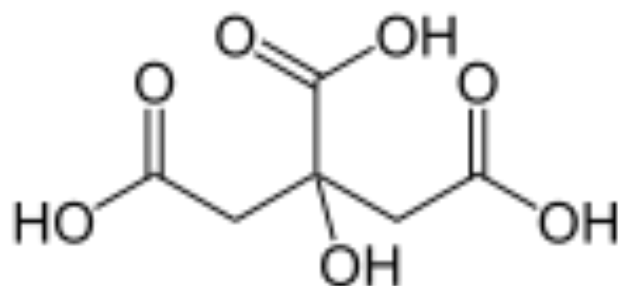
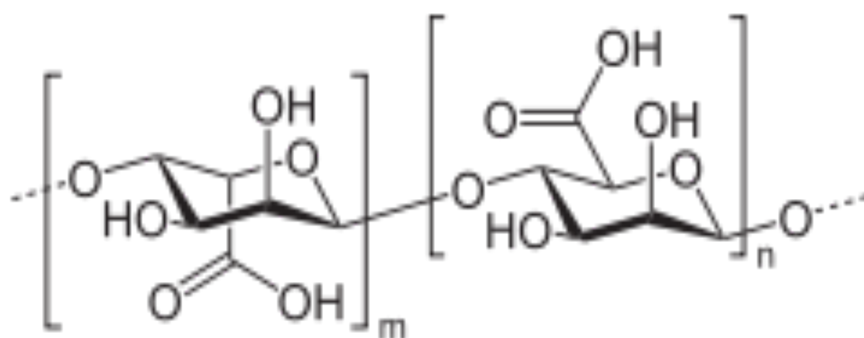


Figure 2.2 Soil columns.



(a)



(b)

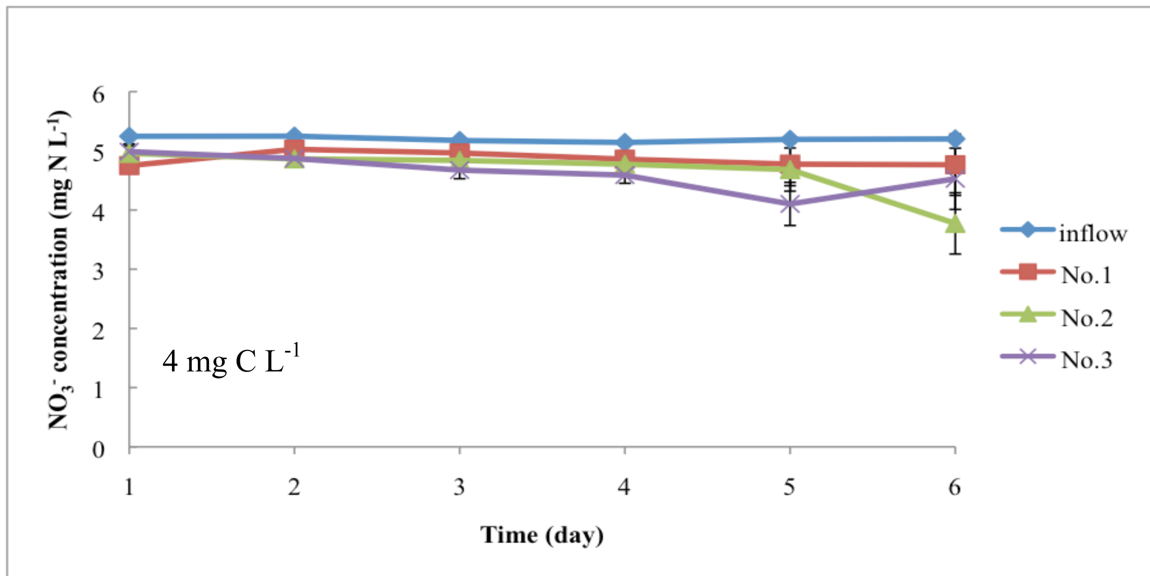
Figure 2.3 (a) Structure of citric acid. IUPAC name is 3-carboxy-3-hydroxy pentanedioic acid and (b) structure of alginic acid.

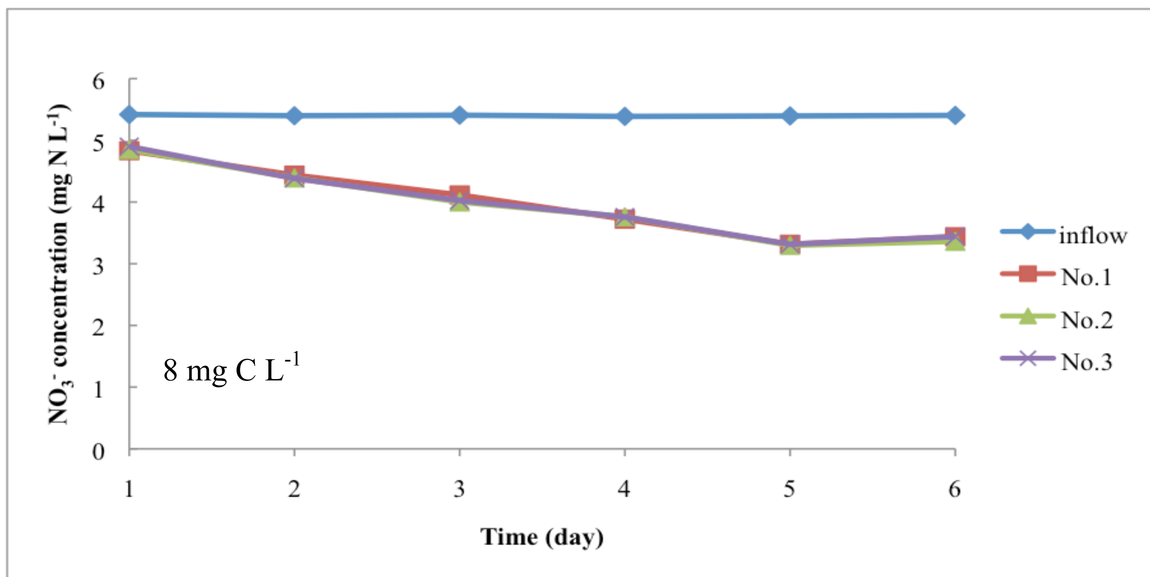
Table 2.3 Elemental composition of river DOC sample (1R101N) from IHSS.

Sample	C%	H%	O%	N%	H_2O%
Suwannee river 1R101N	52.47	4.19	42.69	1.10	8.15

Figure 2.4 Experimental setup.

Figure 2.5 (a) NO_3^- dynamics in inflow and effluence given citric acid at carbon concentration of 4, 8, 16 mg C L^{-1}.


6
5
4
3
2
1
0
NO3- concentration (mg N L-1)
4 mg C L-1
1
2
3
4
5
6
Time (day)
inflow
No.1
No.2
No.3


6
5
4
3
2
1
0
NO3- concentration (mg N L-1)
8 mg C L-1
1
2
3
4
5
6
Time (day)
inflow
No.1
No.2
No.3

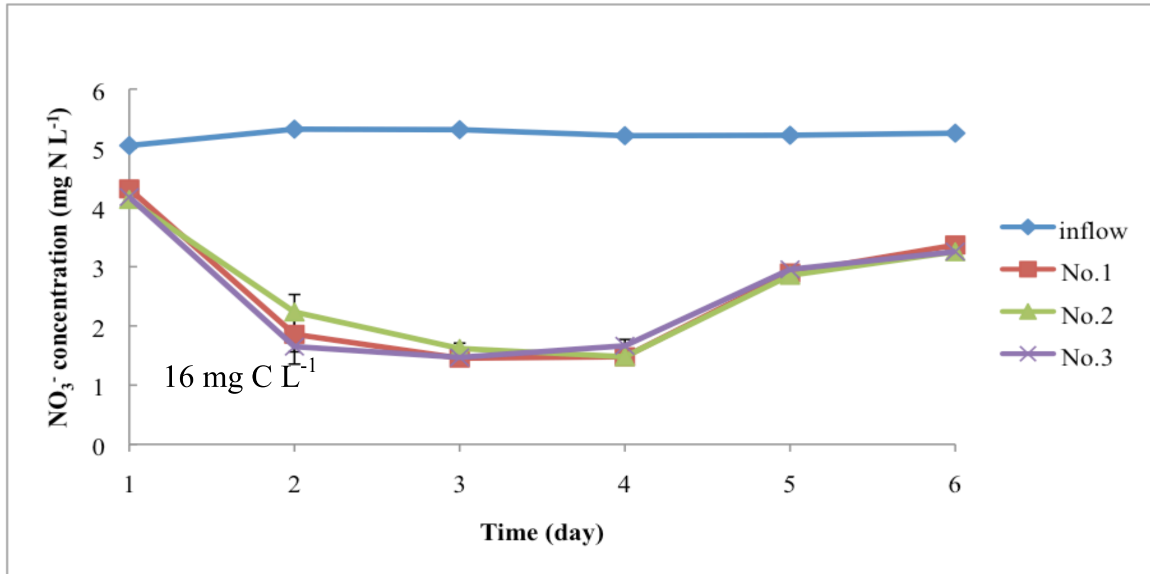
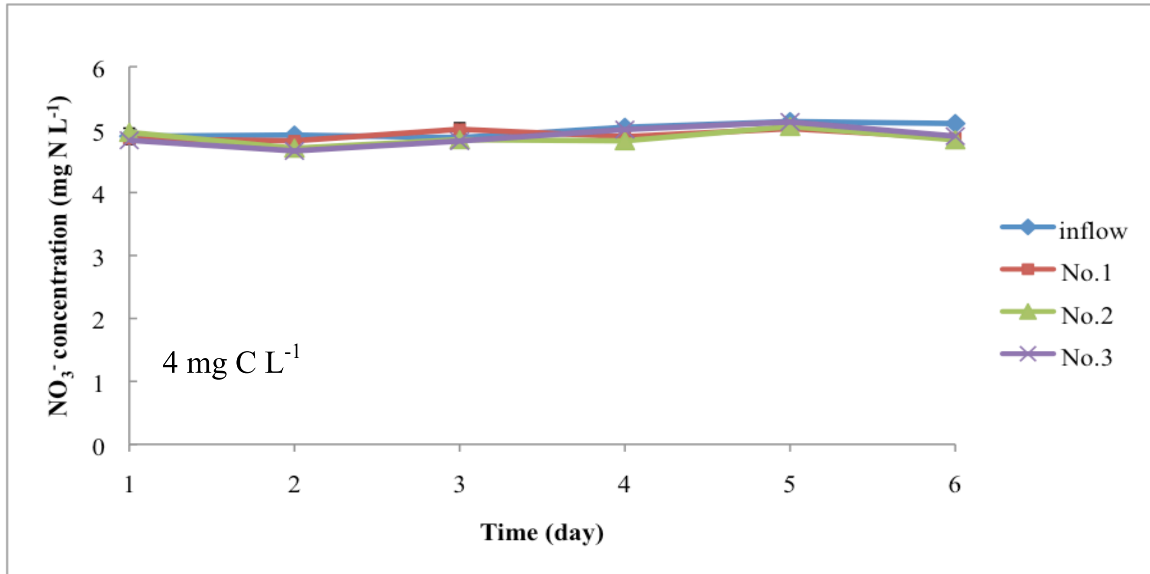
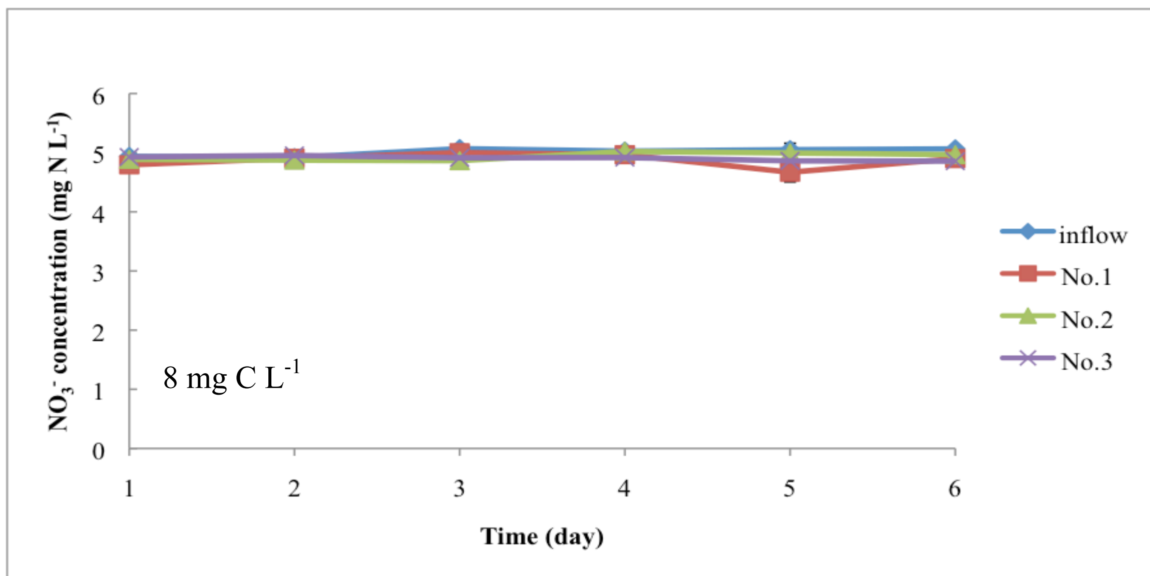

NO3- concentration (mg N L-1)
0
1
2
3
4
5
6
1
2
3
4
5
6
Time (day)
16 mg C L-1
inflow
No.1
No.2
No.3

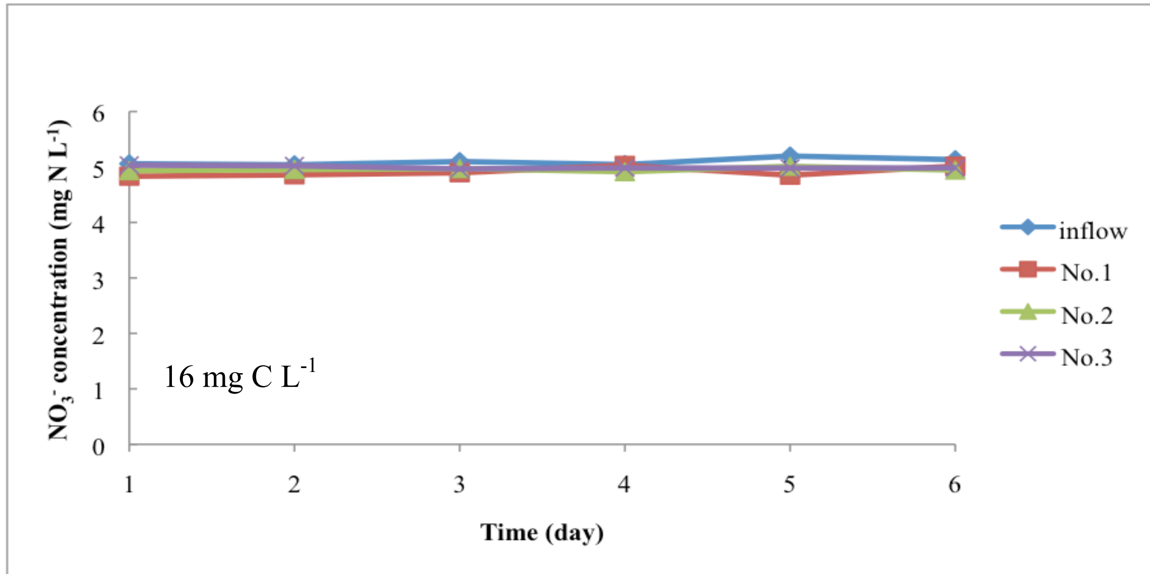
Figure 2.5 (b) NO_3^- dynamics in inflow and effluence given alginic acid at carbon concentration of 4, 8, 16 mg C L^{-1}.



6
5
4
3
2
1
0
1
2
3
4
5
6
Time (day)
NO3- concentration (mg N L-1)
4 mg C L-1
inflow
No.1
No.2
No.3

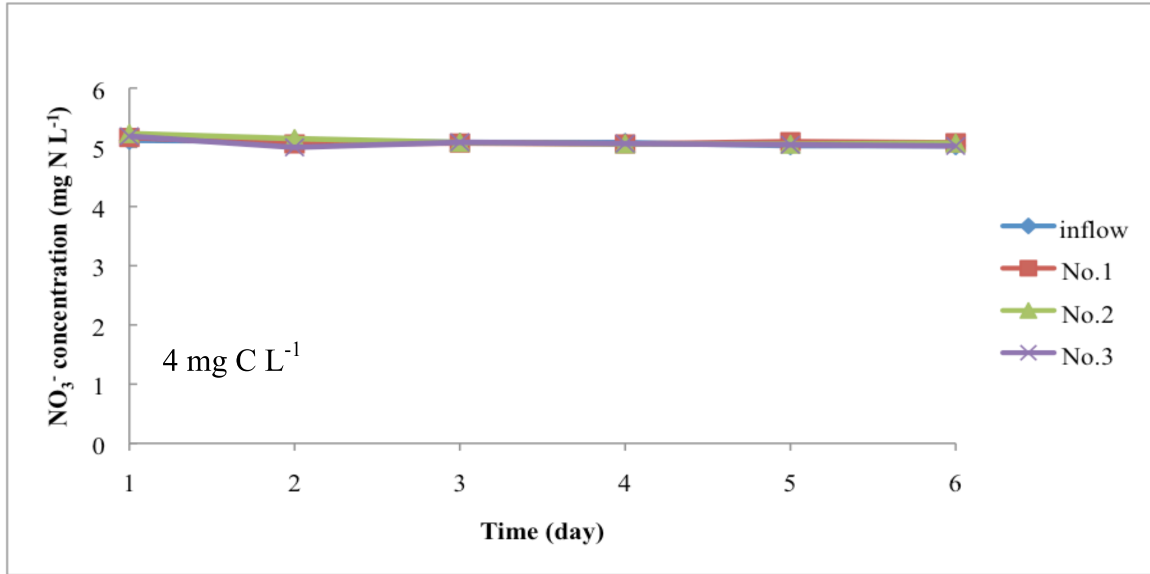


6
5
4
3
2
1
0
1
2
3
4
5
6
Time (day)
NO3- concentration (mg N L-1)
8 mg C L-1
inflow
No.1
No.2
No.3

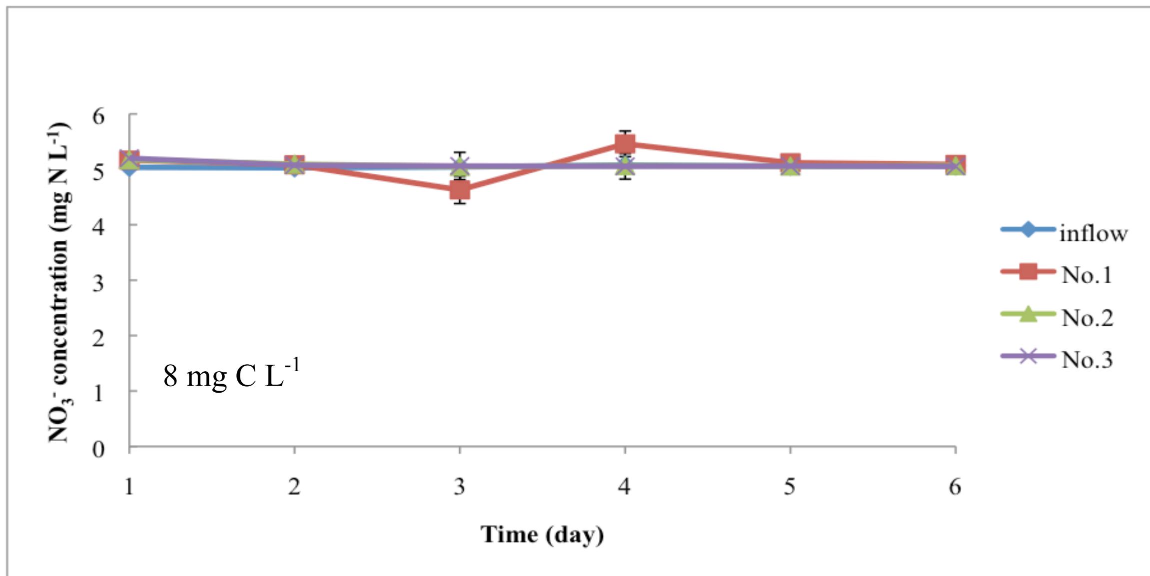


NO$_3^-$ concentration (mg N L^{-1})
6
5
4
3
2
1
0
16 mg C L^{-1}
1
2
3
4
5
6
Time (day)
inflow
No.1
No.2
No.3

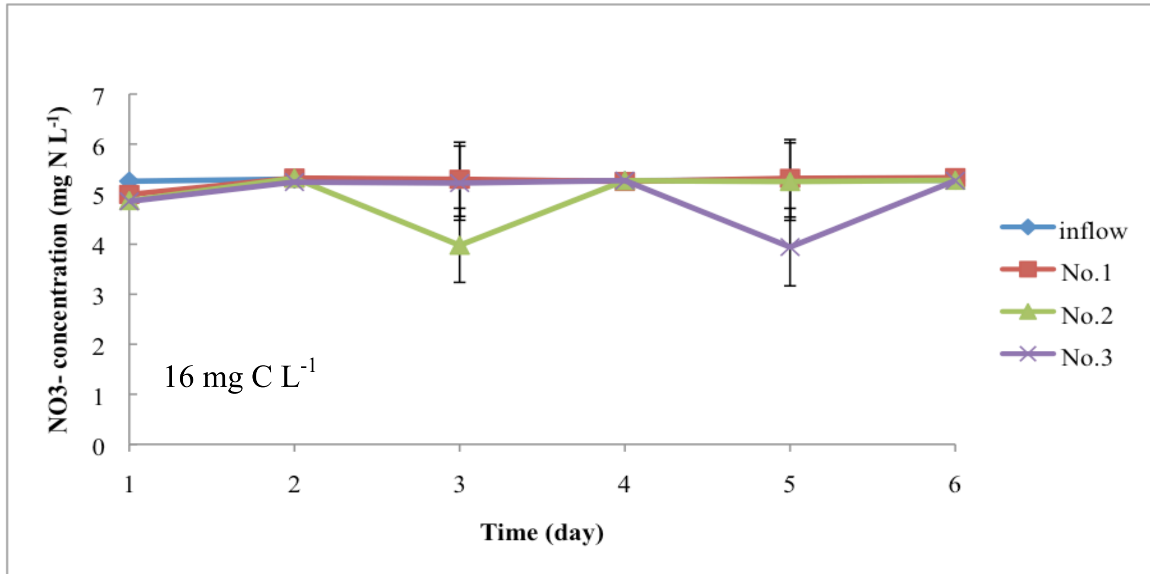
Figure 2.5 (c) NO_3^- dynamics in inflow and effluence given DOC at carbon concentration of 0, 4, 8, 16 mg C L^{-1}.



NO_3^- concentration (mg N L^{-1})
Time (day)
4 mg C L^{-1}
inflow
No.1
No.2
No.3



NO_3^- concentration (mg N L^{-1})
Time (day)
8 mg C L^{-1}
inflow
No.1
No.2
No.3


NO3- concentration (mg N L-1)
16 mg C L-1
Time (day)
inflow
No.1
No.2
No.3

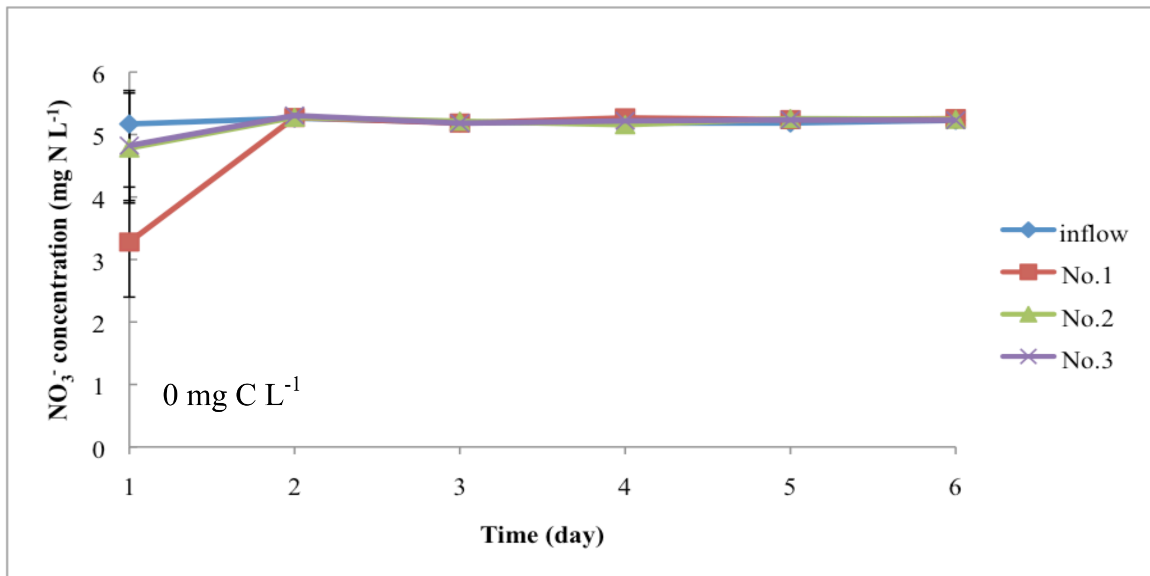

NO3- concentration (mg N L-1)
0 mg C L-1
Time (day)
inflow
No.1
No.2
No.3

Table 2.4 NO_3^- loss rate (a) and N_2O production rate (b) given combination of carbon type and concentration. For NO_3^- loss rate, limit of detection (LOD) = 0.04 mg N hr^{-1} L^{-1}, basedon reliable detection limit of NO_3^- loss (0.1 mg N L^{-1}). For N_2O production rate, limit of detection (LOD) = 10^{-5} mg N hr^{-1} L^{-1}, based on detection limit of N_2O production (50 ppbV).

Concentration (mg C L^{-1})	NO3-N Loss Rate (mg N hr^{-1} L^{-1})		
	Citric Acid	Alginic Acid	DOC
0	0.05(±0.06)	0.05(±0.06)	0.05(±0.06)
4	0.12(±0.08)	0.04(±0.03)	LOD
8	0.38(±0.15)	LOD	LOD
16	0.82(±0.21)	0.04(±0.02)	LOD

(a)

Concentration (mg C L^{-1})	N_2O Production Rate (mg N hr^{-1} L^{-1})		
	Citric Acid	Alginic Acid	DOC
0	$3.86\times10^{-4}(\pm1.28\times10^{-4})$	$3.86\times10^{-4}(\pm1.28\times10^{-4})$	$3.86\times10^{-4}(\pm1.28\times10^{-4})$
4	$4.33\times10^{-4}(\pm3.17\times10^{-4})$	$3.25\times10^{-4}(\pm8.54\times10^{-5})$	$3.84\times10^{-4}(\pm1.62\times10^{-4})$
8	$1.98\times10^{-3}(\pm1.01\times10^{-3})$	$4.15\times10^{-4}(\pm1.18\times10^{-4})$	$3.20\times10^{-4}(\pm1.01\times10^{-4})$
16	$3.79\times10^{-3}(\pm9.63\times10^{-4})$	$2.28\times10^{-4}(\pm8.75\times10^{-5})$	$4.54\times10^{-4}(\pm2.39\times10^{-4})$

(b)

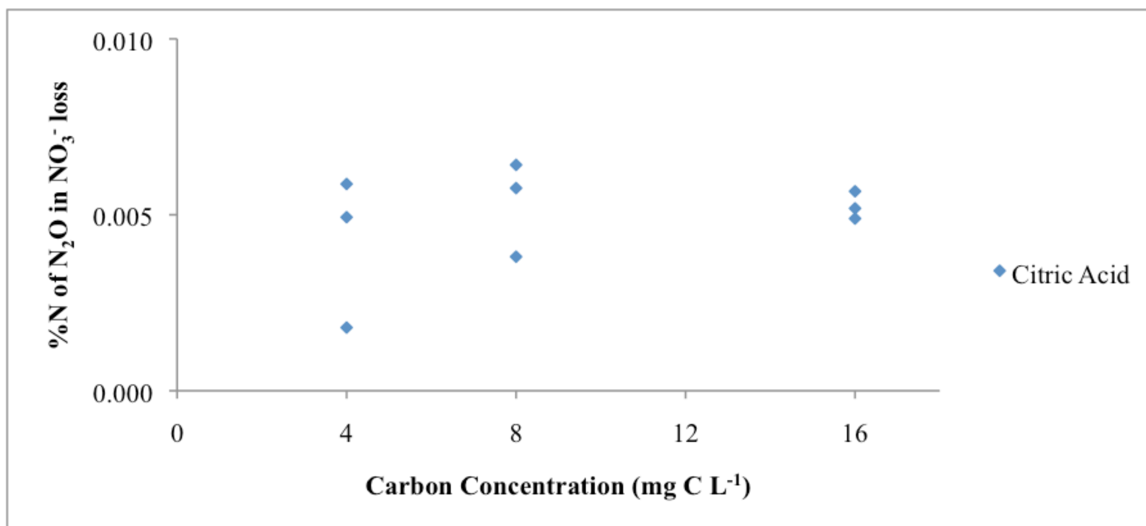
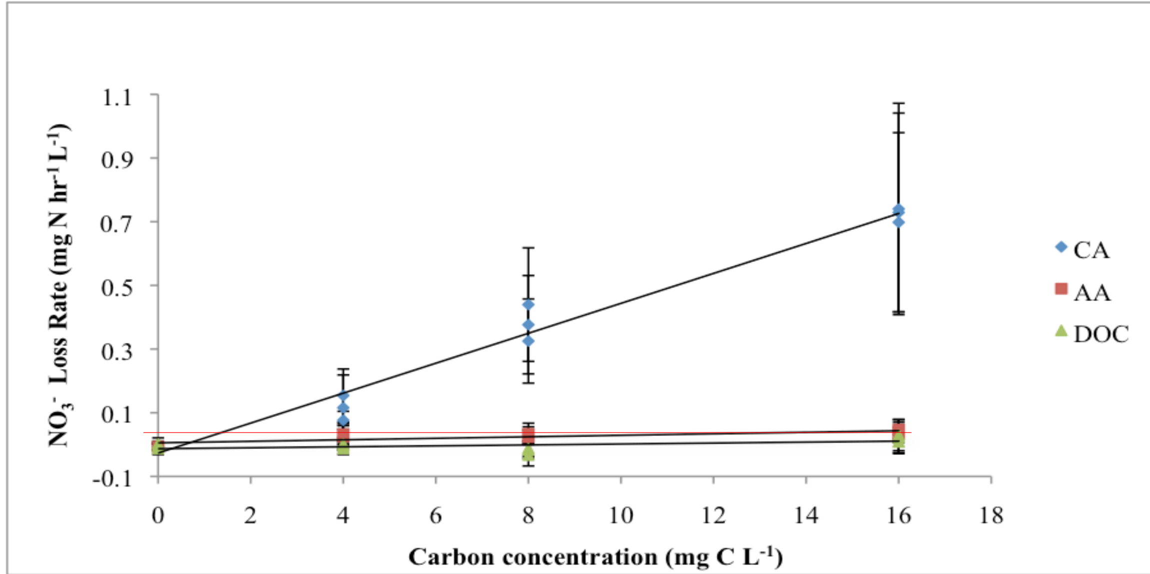
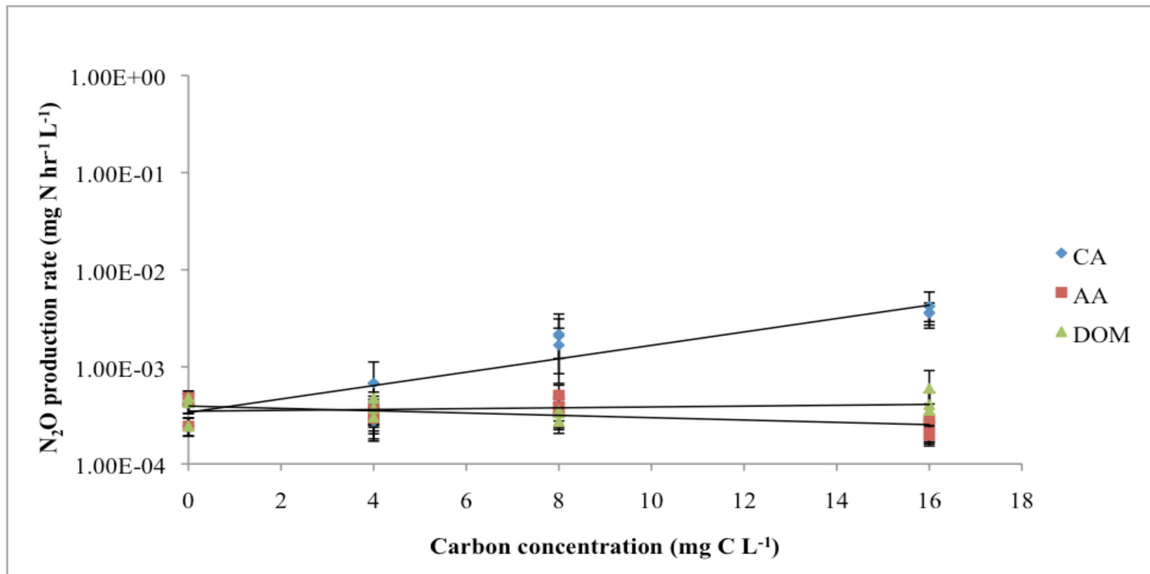


Figure 2.6 Percentage of N converted from NO_3^- to N_2O



(a)



(b)

Figure 2.7 NO_3^- loss rate (a) and N_2O production rate (b) as a function of carbon concentration. (Red line shows the limit of detection)

Chapter 3

Biological Detection of Denitrification

3.1 Introduction

Denitrification is a ubiquitous process in soils that involves a stepwise reduction of nitrogen oxides with evolution of the gases nitric oxide (NO), nitrous oxide (N_2O), and molecular nitrogen (N_2). It is of major importance to the cycling of nitrogen, and leads to a loss of fixed nitrogen from soil. Moreover, this process is of also global concern since its gases intermediates, NO and N_2O, could contribute to global warming (Crutzen 1970) and destruction of stratospheric ozone layer.

Denitrifiers can be found among a wide variety of taxonomic groups. Nearly 130 species of bacteria and archaea in more than 50 genera can perform denitrification (Zumft 1992). Most bacteria with this functional trait belong to a wide range of *Proteobacteria*. The ability to denitrify has also been found in some *Archaea*(Throback et al. 2004).

Functional genes that encode the responsible enzymes in the denitrification pathway, such as nitrite and nitric oxide reductases, can be exploited by targeting conserved regions of the genes. Although some studies have targeted the NO_3^- reductase genes (*napA* and *narG*) (Flanagan et al. 1999, Gregory et al. 2000,Mergel et al. 2001), most molecular investigations of bacterial denitrification in natural environments have utilized nitrite reductase genes (*nirK* and *nirS*) (Braker, Fesefeldt and Witzel 1998, Henry et al. 2004, Throback et al. 2004,Sharma et al. 2005, Yoshida et al. 2009). The reduction of NO_2^- to NO by nitrite reductase is the first step that reduces nitrogen oxides to gas, distinguishing it from parts of the denitrification process. Two structurally different nitrite reductases are found among

denitrifiers (Zumft 1997): a copper (Cu-Nir) enzyme encoded by the *nirK* gene; and a heme c and heme d1 (cd1-Nir) enzyme encoded by the *nirS* gene (Figure 1.3). Although they are mutually exclusively in the same cell, both types have been found in different strains of the same species. Significant functional differences between two nitrite reductases have not been reported (Zumft 1997, Prieme, Braker and Tiedje 2002).

The *nirK* and *nirS*genes have been exploited as biomarkers to analyze functional abundance for improving understanding of denitrification in difference environments. Quantitative PCR (qPCR),has been developed to quantify the denitrifying genes abundance instead of fastidious and time-consuming MPN-based approaches (Henry et al. 2004). This method allows for amplification and simultaneously quantification of targeted DNA sequences. Based on real-time detection of the fluorescent molecules or fluorogenic probe during amplification cycles, qPCR can measure the relative abundance of any particular gene fragment, given the appropriate specific primers. The advantage of the quantitative PCR method over other PCR-based quantification methods is that it focuses on the logarithmic phase of product accumulation rather than the endpoint product abundance. This technique thus is more accurate because it is less affected by amplification efficiency or depletion of a reagent.

Quantitative PCR methods have now become widely established for the quantification of denitrifying gene copy number from environmental samples. Denitrifiers across a glacier foreland were studied with quantitative PCR of denitrification genes

(Kandeler et al. 2006). Denitrifying genes abundance in forest soil and arable soil were also determined with quantitative PCR based method (Prieme et al. 2002, Throback et al. 2004). Relative abundance of different genes provided evidence of different denitrifier communities, with the significant effects on community stemming from the nature of organic substances.

The present work combines the molecular characterization with quantification of environmental factors and denitrification rates so as to evaluate abiotic controls responsible for N transformations. This research seeks to elucidate the relationships among denitrification rate in riparian buffer soils, the response of microbial communities to carbon supply, and the impact of changes of denitrifier abundance on the rate of denitrification.

3.2 Material and Methods

3.2.1 Sample Collection

After performing column experiments as described in Chapter 2, soils were collected in beakers, homogenized, and stored at 4 °C for genomic DNA extraction.

3.2.2 Total DNA Extraction

DNA was extracted in duplicated from each soil and purified from aliquots of 1.0 g (wet weight) of each homogenized soil sample. PowerSoil™ DNA Isolation Kit (MO BIO, USA) was used to perform DNA extraction, according to manufacturer's instruction. Samples were homogenized in 1 ml of extraction buffer in a beater tube during gentle vortexing. 60 µl of solution C1 containing sodium dodecyl sulfate (SDS) and other

disruption agents was added for complete cell lysis followed by vortexing at maximum speed for 10 minutes using a MO BIO Vortex Adapter (MO BIO, USA). Soil and cell debris were separated by centrifugation (10,000 × g for 30 s at room temperature). Afterwards, 250 µl of solution C2 was used to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins during incubation at 4 °C for 5 minutes. After 1 minute of centrifugation at 10,000 × g, up to 600 µl of supernatant was transferred to a clean tube and purified again with the aid of solution C3. Solution C4, which was a high concentration salt solution, was added to 750 µl of supernatant and only DNA could be bound tightly to silica membrane at high salt concentrations. Three loads into the Spin Filter, 675 µl of supernatant each, were required for complete DNA harvest where contaminants passed through the filter membrane, leaving only DNA bound to the membrane. Ethanol based wash solution was used to further clean the DNA with spin for 30 seconds at 10,000 × g, removing residual salt, humic acid and other contaminants. Another centrifugation at room temperature for 1 minute at 10,000 × g was required to remove residual ethanol wash solution. Avoiding splashing any ethanol wash solution, The Spin Filter was carefully placed in a clean tube. DNA was washed with 100 µl of sterile elution buffer where bound DNA was selectively released and collected in 10 mM TRIS solution after centrifugation for 30 seconds at 10,000 × g at room temperature.

After extraction, DNA was quantified by spectrophotometry at 260 nm using Nanodrop (Thermo scientific, USA). DNA was stored at -20 °C until used in qPCR.

3.2.3 Primers Design

Primers used for the quantification of the *nirS* (Kandeler et al. 2006), *nirK*(Henry et al. 2004) and 16s rDNA (Throback et al. 2004) were based on previous research (Table 3.1). The suitability of primers was checked against our standard gene by aligning sequences from GeneBank in BioEdit.

3.2.4 Quantitative PCR Assay

All chemicals were molecular grade, and DNAase and RNAase free. Three independent quantitative PCRs were performed for each gene and each soil replicate.

Amplification of qPCR products was carried out with a MasterCycler® ep realplex cycler (Eppendorf, USA). SYBR Green was used as the detection system. A reaction mixture of 25 µl contained: 2.5 µl of each primer for target gene, 12.5 µl of 2 × SYBR Green PCR master mix (including HotStar Taq *Plus* DNA polymerase, QuantiFast SYBR Green PCR buffer, dNTP mix with dUTP, SYBR Green I, ROX dye and 5 mM $MgCl_2$) (QuantiFast™ SYNR® Green PCR Kit, QIAGEN, USA), 2 µl of DNA template, and RNase-free water to complete the 25 µl volume.

The conditions for *nirS* qPCR were 300 s at 95 °C for enzyme activation as recommended by the manufacturer (QuantiFast™ SYBR® Green PCR Kit, QIAGEN, USA);

afterwards 35 cycles were added as followed: 45 s at 94°C for denaturation, 45 s at 57 °C for annealing, and 60 s at 72 °C for extension and data acquisition. The reaction was completed

after 300 s at 72 °C. One last step from 60 °C to 95 °C with an increase of 0.2 deg s^{-1} was added to obtain a specific denaturation curve (Fig. 3.1)

The conditions for *nirK* and 16s rDNA qPCR were similar as those for *nirS* except the annealing temperatures were 58 °C and 53 °C, respectively (Henry et al. 2004, Nogales et al. 2002).

The purity of amplified products was checked by the observation of a single melting peak (Fig 3.2) or the presence of a unique band of the expected size in a 2% (weight base) agarose gel stained with ethidium bromide.

3.2.5 Standard Curve Performance

Standard curves were obtained with serial dilutions of a known amount of genomic DNA containing a fragment of the 16s rDNA or *nirS* or *nirK* gene. 16s rDNA is the universal gene in bacteria and used to measure total bacteria abundance in out study. Standard genomic DNA used for denitrifying genes were selected from GeneBank and aligned with BioEdit (BioEdit, USA) (Table 3.2).

To draw the standard curve, Ct (threshold value) was plotted as a function of log of the copy number of the target DNA. Tenfold serial dilutions of standard genomic DNA ranging from 10^2 to 10^6 gene copies were used as template, by triplicate, to determine the standard curve. Three points of the standards were used as positive controls in each reaction. A control with no template was used as negative control.

3.2.6 Quantification and Correlation

Gene quantification was performed with the aid of standard curve. Absolute gene copy number was obtained from standard curve (Fig. 3.3).

A correlation analysis of the data set was used to examine the relationship between gene abundance and denitrification rate, as the samples for denitrification rate and gene abundance measurement were paired.

3.3 Results and Discussion

3.3.1 Production of standard curves

Standard genomes containing target genes were used to draw a standard curve with plotting Ct to the added number of gene copies. Linear responses were observed for 5 orders of magnitude, ranging from 10^2 to 10^6 gene copies. Figure 3.3 shows standard curves for *nirK* (a), *nirS* (b) and 16s rDNA (c).

3.3.2 Quantification of the 16s rDNA, *nirK* and *nirS* genes

The genomic DNA yields from the soil extractions of different treatments are listed in Table 3.3. Varied DNA yields from extractions suggest that extraction effectiveness may vary due to the DNA extraction bias. Gene copy numbers can be presented in two forms, copy number ng^{-1} DNA or copy number g^{-1} soil. For comparison with other studies, gene abundance will be expressed as copy number g^{-1} soil.

Real-time PCR assays are used to investigate variation in numbers of 16s rDNA, *nirK*, and *nirS,* genes in soil samples after different treatments. The 16s rDNA, *nirK,* and *nirS* abundances in the unit of copy number g^{-1} soil are plotted in groups of carbon concentrations (Fig 3.4 a, b, c). In all carbon concentrations, the highest 16s rDNA gene copy numbers are found in citric acid treatments, ranging from 3.8×10^{7} to 9.0×10^{7} copy g^{-1} soil. There is a lower 16s rDNA copy number in the other two carbon treatments, with a 5 fold significant difference between citric acid and alginic acid at 4 mg C L^{-1}. In 8 mg C L^{-1} and 16 mg C L^{-1} treatments, significant differences in 16s rDNA copy number are observed between all carbon types.

For *nirK*, the copper-contained denitrification gene, gene copy number for three carbon concentrations (4, 8, and 16 mg C L^{-1}) decreased from the citric acid to alginic acid and DOC. *NirK* copy numbers at each concentration are significantly higher at the citric acid, ranging from 1.05×10^{8} to 1.45×10^{8} copy g^{-1} soil, than the other two carbon types; there is no significant difference between *nirK* copy number in alginic acid and DOC treatments.

Gene copy numbers of *nirS* are lower in each treatment than those of *nirK*. For all carbon concentrations, gene copy numbers decreased from citric acid towards other two carbon sources, which is similar to *nirK*. *NirS* copy number of the 4 mg C L^{-1} treatment is higher in citric acid, but no significant difference between alginic acid and DOC is observed. Differences between gene copy numbers at three carbon sources are less pronounced for 8 and 16 mg C L^{-1} subgroups. For 16 mg C L^{-1} subgroup, although *nirS* gene copy number is

highest with citric acid, with decreasing gene abundances in alginic acid and DOC, there are no significant differences between nirS gene copy number at all three carbon sources.

Gene copy numbers are also plotted against carbon types (Fig 3.5 a, b, c). For 16s rDNA, although there is a clear trend of increasing gene copy numbers with increased carbon concentration in citric acid treatment, no significant difference is observed among different concentrations. In alginic acid and DOC treatments, 16s rDNA gene copy numbers are low, ranging from 5.79×10^6 to 1.32×10^7, and do not exhibit significant differences among concentrations.

Soil samples in citric acid treatment contain more *nirK* gene copies, ranging from 1.03×10^8 to 1.45×10^8 copy g^{-1} soil. However, there is no obvious trend in *nirK* gene copy numbers along with carbon concentrations as evidenced with 16 rDNA. Highest gene copy number is obtained from citric acid of 4 mg C L^{-1}, with the average of 1.45×10^8 copy g^{-1} soil, followed by 16 and 8 mg C L^{-1}. Soil samples with alginic acid and DOC contain less *nirK* gene copies for all carbon concentrations. No significant differences are observed for 4, 8 and 16 mg C L^{-1} for both carbon types.

Compared to *nirK*, *nirS* gene abundance is relatively lower in three carbon sources. In citric acid treatments, close *nirS* gene copy numbers are found in 8 mg C L^{-1} and 16 mg C L^{-1}, with the average of 4.38×10^7 copy number g^{-1} soil. Soil sample from 4 mg C L^{-1} treatment contains less *nirS* gene copies, 3.06×10^7 copy number g^{-1} soil. Similarly to *nirK*, *nirS* gene

abundance in alginic acid and DOC treatments are lower for all concentrations;there are no significant differences.

Comparison of *nirK*, *nirS* and 16s rDNA gene abundance in each treatment are shown in Fig 3.6. In soil columns supplied with citric acid, *nirK* has highest gene copy numbers in all four carbon concentrations, followed by *nirS* and 16s rDNA. Although there is significant differences between *nirK* and 16s rDNA gene copy numbers, no significant differences are observed between *nirS* and 16s rDNA abundance.

3.3.4 Correlation between gene abundance and denitrification rate

Correlations between denitrification rate and gene abundance are plotted in Fig 3.7. Denitrification rates correlate with 16s rDNA. In citric acid treatments, although a trend with increased *nirK* and *nirS* gene abundances is evident, where significantly higher denitrification rates are observed, there is no significant correlation between denitrification rates and *nirK* or *nirS* gene copy number.

3.3.5 Discussion

Our results from real-time PCR show density of *nirK* and *nirS* gene in soils after amendment with carbon sources are 10^6 to 10^8 gene copy number g^{-1} soil and 10^6 to 10^7 gene copy number g^{-1} soil, respectively. Previous studies have reported 10^5 to 10^6 copy g^{-1} soil of *nirK* in sandy soils (Henry et al. 2004), 10^7 copy g^{-1} soil of *nirK* and 10^6 copy g^{-1} of *nirS* in rice paddy field soil (Yoshida et al. 2009), and 10^7 copy g^{-1} soil of *nirK* in glacier soils (Kandeler et al. 2006). Our study presents higher abundance of *nirK* and *nirS* from

quantitative PCR, but lower than those observed by Qiu et al. (2004) using competitive PCR. Our *nirS* gene abundance is also higher than that in estuarine sediments, which ranges from 10^5 to 10^6 copy g^{-1} soil (Smith et al. 2007). These results may lead to the conclusion that additional supply of organic carbon may increase the denitrification gene abundance in soils. The *nirK* abundance is always larger than *nirS* in corresponsive treatments (2- to 5- fold; Table 3.4). Although multiple gene copies may be present in a single cell and the primer bias could not be excluded, our real-time PCR results suggest that the *nirK*-harboring denitrifiers might be more abundant than *nirS*-harboring denitrifiers in the riparian buffer soils, given the higher *nirK* abundance in control treatment. Philippot (2002) indicates only one copy of the *nirK* gene in bacteria but no further information about *nirS*. However, it disagrees with the previous observation that *nirS* appears to be numerically dominant in most environments (Bothe et al. 2000). It is also possible that *nirK* is more sensitive to carbon amendment than *nirS*, especially in citric acid treatments.

It is surprising that *nirK* exhibits higher abundance than 16s rDNA in all treatments, which, experiment biases excluded, represents the total number of bacteria. This runs counter to the previous studies which indicates only one copy of the *nirK* sequence in all the bacteria studied (Philippot 2002). However, This may be also result from methodological bias, because *nirK* primers might be more universal. He et al. (He et al. 2007) has found the abundance of total bacteria remained same regardless the carbon addition and Miller et al. (Miller et al. 2009) has detected the gene abundance in the range of 10^{11} copy g^{-1} soil. It is possible that the estimated abundances of total bacteria population from our very sandy

aquifer soil (10^7-10^8 copy g^{-1} soil) underestimates the true abundance of total bacteria population due to the primers. Moreover, it is also possible that our *nirK* primers quantify both bacteria and archaea, which may lead to the high gene copy number. Though it is previously thought that denitrification is a characteristic of bacteria, a few eukaryotes and archaea also have been confirmed with the denitrification genes (Philippot 2002, Kim et al. 2009). Functional *nirK* and *nirS* gene have been found in many archaea, such as *Haloarcula marismortui*(Cabello, Roldan and Moreno-Vivian 2004).

This study found a significant increase in *nirK* gene abundance in citric acid amended soil compared with that in untreated controls; neither alginic acid and DOC amendment induced a significant change in the abundance of *nirK*. Higher amounts of *nirK* copy number in citric acid correspond to increased denitrification rates, agreeing with our hypothesis. This observation suggests that denitrification rate is stimulated by simple carbon addition that increases the abundance of *nirK* gene. However, the lack of correlation between *nirK* abundance and denitrification rate is somewhat surprising. The *nirK* gene copy number is higher in citric acid treatments, which coincides with higher denitrification rate, but changes in copy number over carbon concentration are not significant. The reason for this finding is unclear, but it suggests that the *nirK*-harboring denitrifiers community is sensitive to citric acid supply and reaches the maximum (10^8 copy number g^{-1} soil) at relative low carbon concentration (4 mg C L^{-1}). Given increased carbon concentration, denitrifier population is no longer enlarged but kept at the limit range. Microbial communities may be altered by addition of labile organic carbon by selecting for populations that are most competitive in

terms of growth rates and their ability to utilize nutrients (Drenovsky et al. 2004). This *nirK*-harboring denitrifier community may be able to effectively compete for the labile citric acid-C under the induced denitrification conditions. *NirK* abundance and quantity of soil organic C have been studied in receding glacier foreland, showing strong correlation between each other (Kandeler et al. 2006), although little information is provided regarding the type of C. Groffman (Groffman et al. 2006) also has report that no correlation between denitrification rates and measurable features of denitrifying communities.

The most plausible explanation for the increased denitrification rate under higher concentration of citric acid supply is the higher metabolic activity, which could be measured by gene expression, rather than higher gene abundance. Although gene abundance is kept at the same range, gene expression level may be enhanced resulting in increased denitrification rates. Moreover, in terms of ecology, abundant supply of labile carbon stimulates their metabolic activity, increasing the percentage of active denitrifiers in the whole population, and enhancing the denitrification rats as a result.

In contrast to *nirK*, *nirS* gene abundance responded differently to organic carbon amendments. *NirS* gene abundance trends with the NO_3^- loss rate, although a linear correlation was not significant. Another study performed in estuarine sediments demonstrated the correlation between denitrification rate and *nirS* gene abundance (Dong et al. 2009). But the lack of correlation between *nirS* abundances and the denitrification rates in the Arabian sea may also suggest the role of environment factors (Ward et al. 2009).

In contrast to the increase *nirS* levels in citric acid amended soil, there is no significant increase in *nirS* levels in alginic acid and DOC treatments. The absence of increased *nirS* abundance in response to complex organic carbon source indicating the *nirS*-harboring denitrifiers can not effectively use complex organic carbon, compared to low-molecular-weight carbon addition. Low-molecular-weight carbon is readily utilized and stimulates the gene abundance, enhancing denitrification rate thereafter.

The 16s rDNA gene copy number was significantly correlated to denitrification rate, both NO_3^- loss rate (R^2=0.85) and N_2O production rate (R^2=0.72). The abundance of the 16s rDNA increases in response to citric acid addition, though the differences are not significant. Meanwhile, slightly changes of total bacteria community are observed given alginic acid and DOC treatments, indicating the stability of total bacteria community corresponding to complex C amendments. This result is in partial agreement with Miller et al. (2008) and He et al. (2007) who concluded that C additions is not sufficient to cause a measureable increased in the total bacteria community. Organic carbon addition can be in the position to affect total bacteria community, but also depends on the carbon type. Compared with alginic acid and DOC, citric acid, as an example of simply carbon addition, not only enhances denitrifying bacteria but also increase the total bacterial community. However, compared to previous study which quantified the 16s rDNA number in sandy low carbon soil (10^9 copy g^{-1} soil) (Lopez-Gutierrez et al. 2004), our 16s rDNA gene abundance is somewhat low (10^7-10^8 copy g^{-1} soil). A possible explanation may be the choice of primers. The primers in this study were different from the ones used by Lopez-Gutierrez (2004), and might be less universal.

Ratios of *nirK*/16s rDNA, *nirS*/16s rDNA, and *nirK*/*nirS* are showed in Table 3.4. *nirS* abundance is in the same range as 16s rDNA, but is only less than 50% as *nirK*. The fairly stable ratios of *nirS*/*nirK* indicate the stability of denitrifier community regardless of the carbon type and carbon concentration.

Although it should be recognized, as in any PCR-based study, that use of specific primers may not allow the quantification of entire target communities, our studies has nevertheless attempted to quantify the abundance of denitrification genes so as to demonstrate the effect of organic carbon addition on the denitrifiers population as well as the resulted denitrification rate.

3.4 Conclusion

This chapter presents the quantification of denitrification genes from column experiments. By measuring the gene abundance in riparian buffer soils after different treatments of organic carbon, it is possible to explain the influence of organic carbon on the denitrifier community and helpful to elucidate the correlation of denitrification rates and gene abundance. Gene abundances of *nirK*, *nirS*, and 16s rDNA are high (Fig 3.3) in the citric acid treatment, while those given alginic acid and DOC are lower. It is indicated that citric acid, as an example of low-molecular-weight carbon, contributes to the growth of denitrifier community and promote the denitrification indirectly. However, alginic acid and DOC, as complex carbon source, are not readily utilized by denitrifiers and do not enhance the gene abundances. Though lacking of correlation between *nirK* abundance and denitrification rates,

good correlation between denitrification rates from column experiments and *nirS* gene abundances illustrates the relationship between chemical and biological activity.

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Table 3.1 Primer sets used to detect denitrifying genes and 16s rDNA.

	Amplicon size (bp)	Primer		Q-PCR cycle anealing temperature (C)
		Name	Sequence (5'-3')	
nirS	425	nirSCd3aF	AACGYSAAGGARACSGG*	57
		nirSR3cd	GASTTCGGRTGSGTCTTSAYGAA	
nirK	165	nirK876	ATYGGCGGVAYGGCGA	60
		nirK1040	GCCTCGATCAGRTTRTGGTT	
16s rDNA	200	Eub338	ACTCCTACGGGAGGCAGCAG	53
		Eub518	ATTACCGCGGCTGCTGG	

* Y was a mixture of C and T. S was a mixture of C and G. R was a mixture of A and G.

Table 3.2 Bacterial strain genomes used for standard curve.

Strains	Source	gene types
Ralstonia eutropha	ATCC 17699-D	*nirS*/16s rDNA
Alcaligenes faecalis	ATCC 8750D	*nirK*

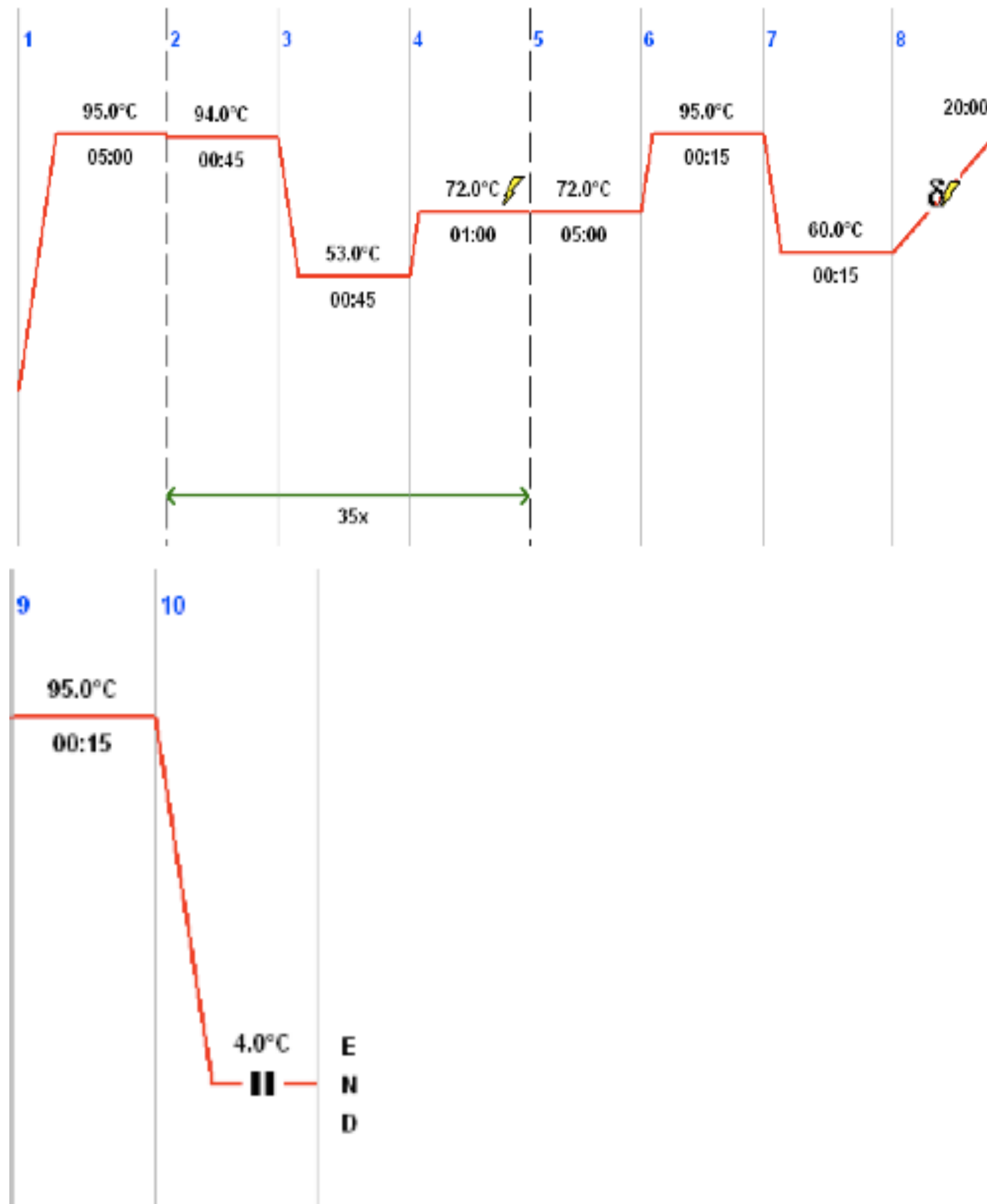
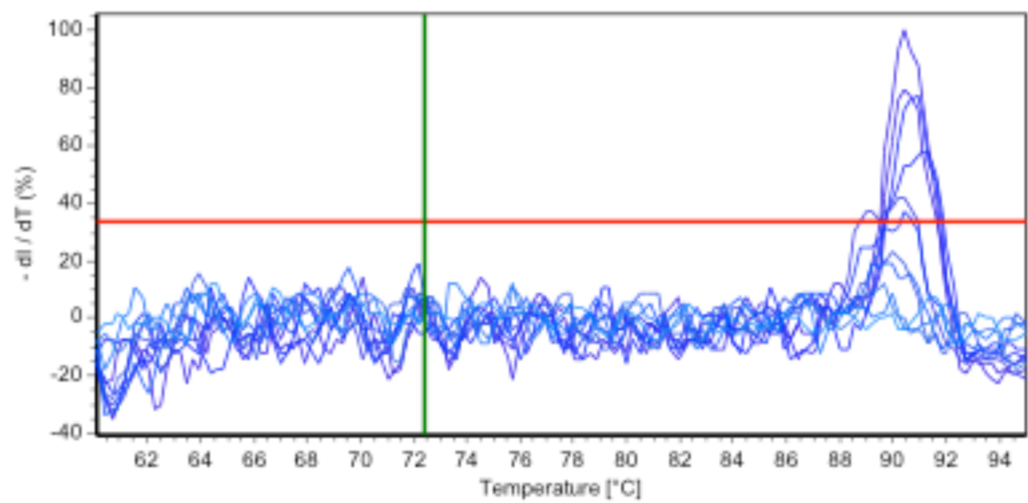
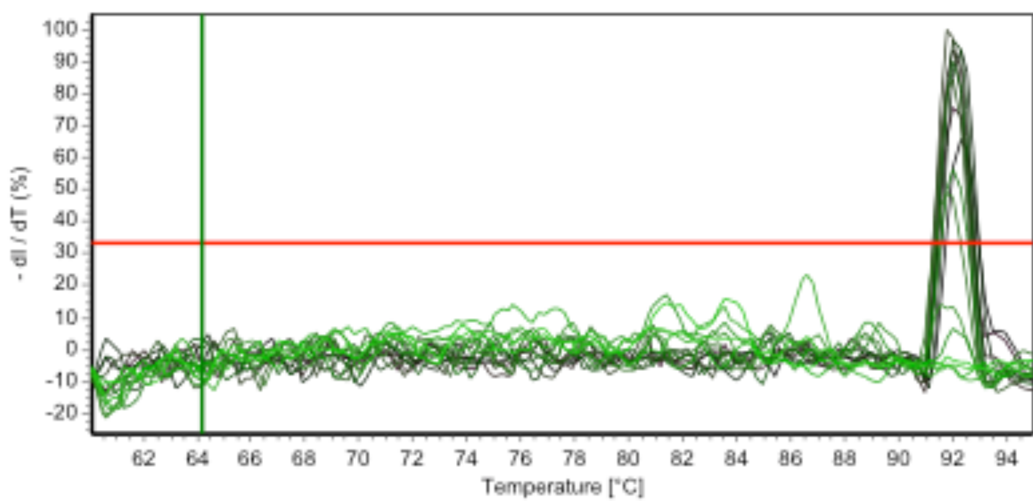


Figure 3.1 Quantitative PCR conditions for *nirS.*

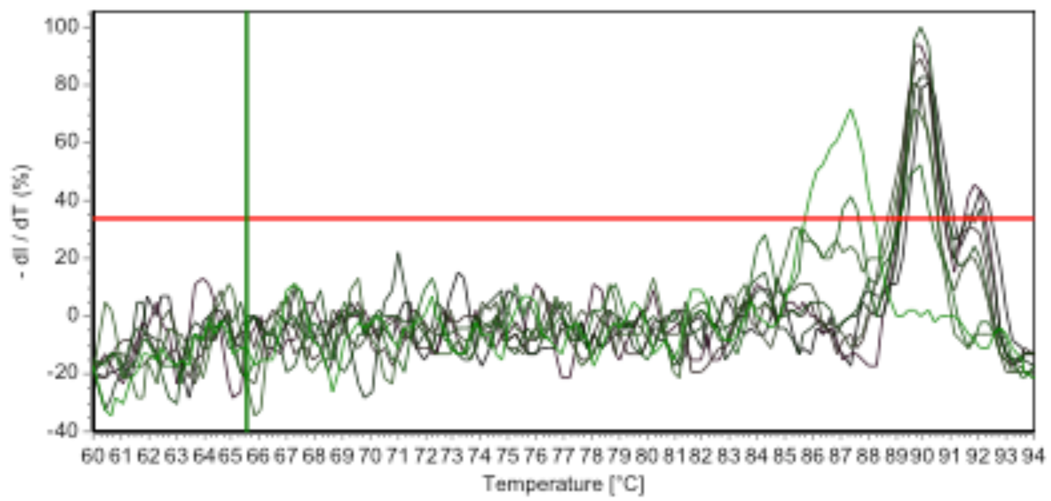
Figure 3.2 Profiles of melting curves: (a) *nirK*, (b) *nirS*, and (c) 16s rDNA.



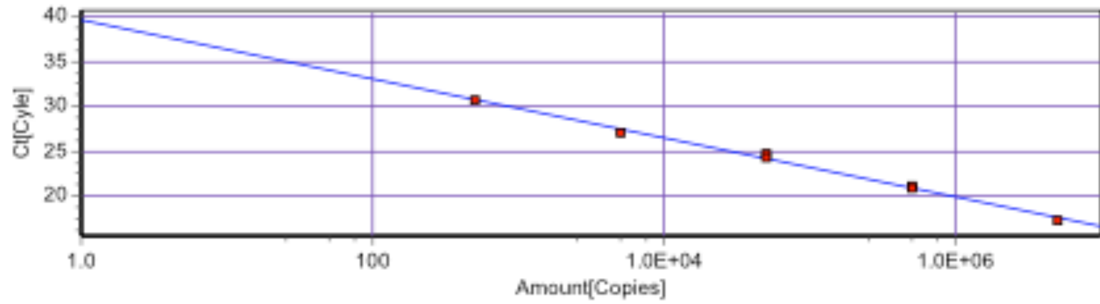
(a)



(b)

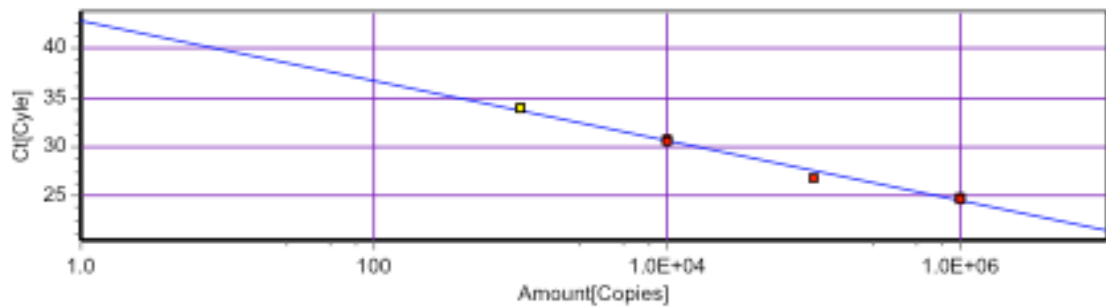


(c)



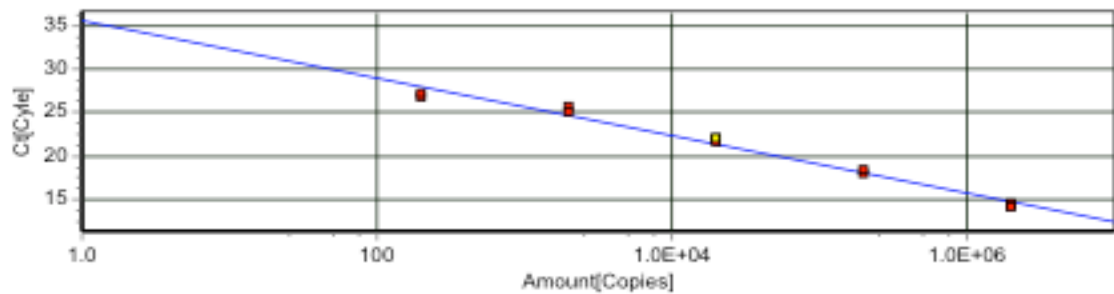
Slope	-3.294	R^2	0.995
Y-Intercept	39.63	Efficiency	1.01

(a)



Slope	-3.050	R^2	0.988
Y-Intercept	42.86	Efficiency	1.13

(b)



Slope	-3.311	R^2	0.984
Y-Intercept	35.56	Efficiency	1.00

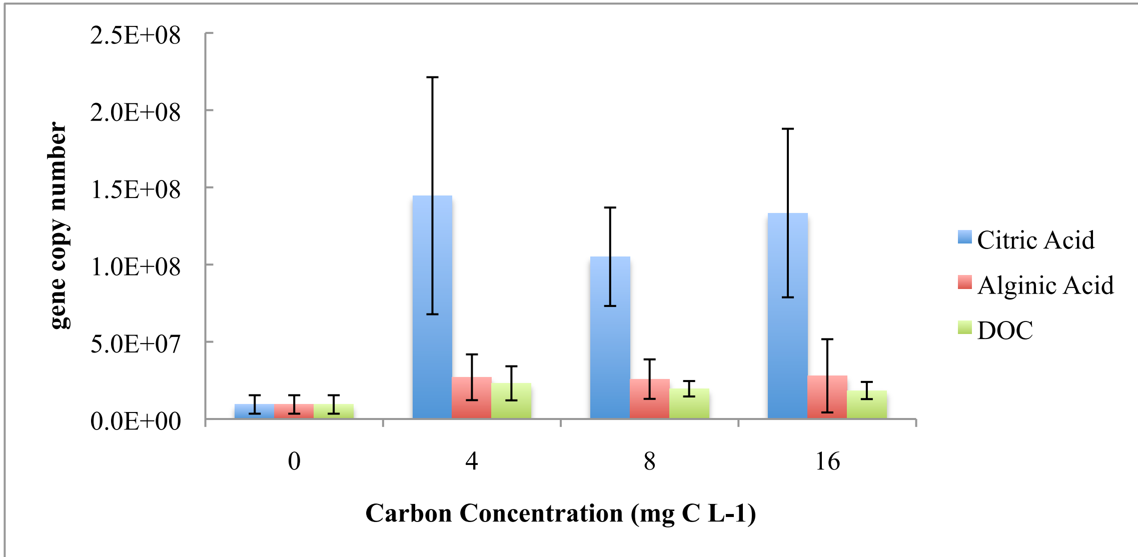
(c)

Figure 3.3 Standard curve for denitrification genes *nirK* (a), *nirS* (b) and 16s rDNA (c).

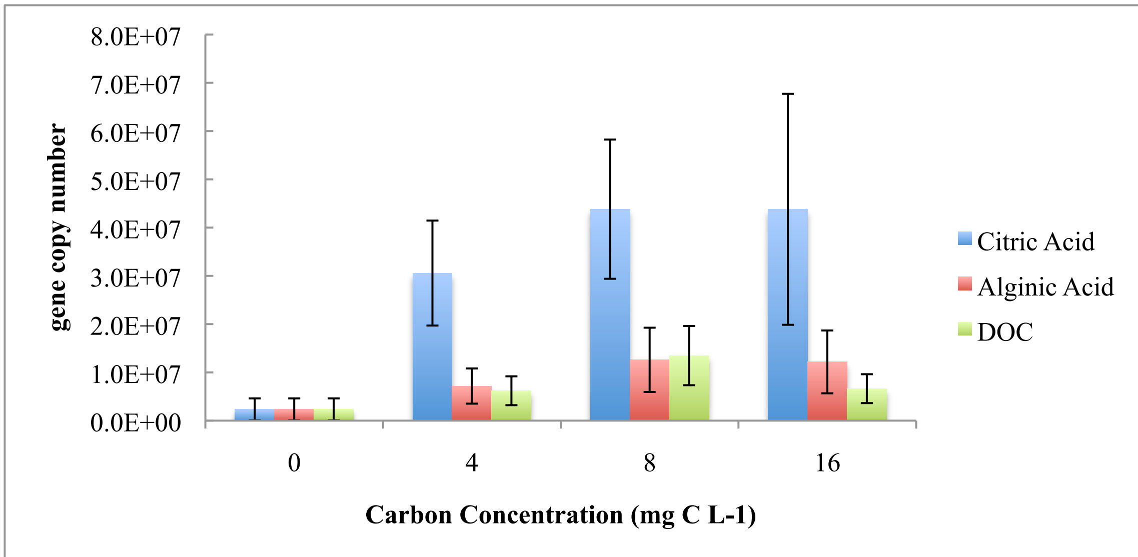
Table 3.3 DNA yields (ng DNA g^{-1} soil) from soil column samples.

Carbon Type	Column No.	Carbon Concentration			
		0 mg C^{-1}	4 mg C L^{-1}	8 mg C L^{-1}	16 mg C L^{-1}
		DNA yield			
	1	476.2	413.8	335.8	303.5
	2	315.8	448.3	401.9	416.7
Citric Acid	3	389.5	323.8	402	486.5
	1	476.2	198.5	174.6	569
	2	315.8	172.4	336.5	528.3
Alginic Acid	3	389.5	245.3	343.5	539.7
	1	476.2	640	437.9	644.6
	2	315.8	723.4	680.9	346.5
DOC	3	389.5	811.3	547.2	309.1

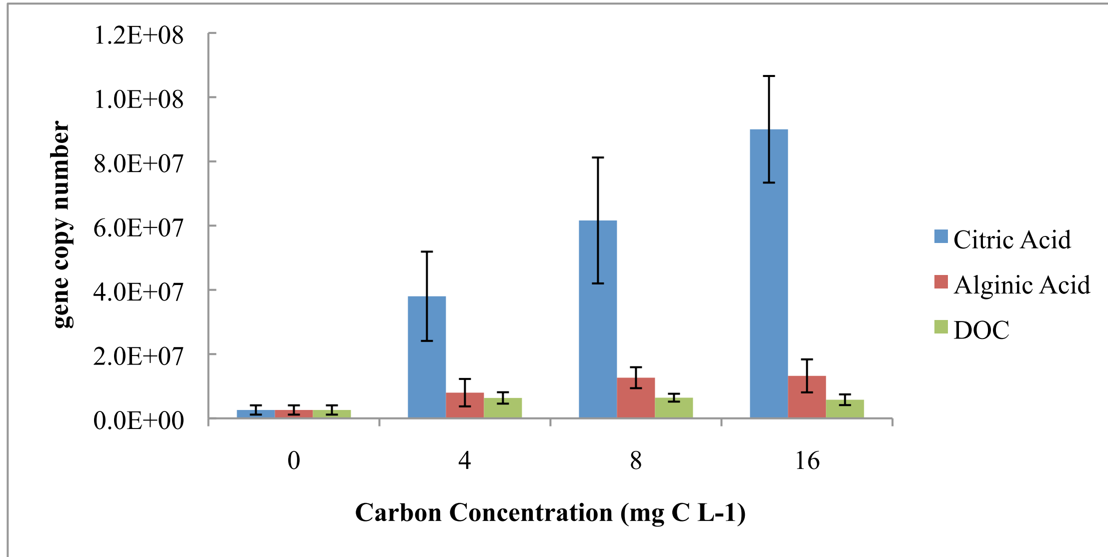
Figure 3.4 Gene copy abundance (a) *nirK*, (b) *nirS*, (c) 16s rDNA in groups of carbon concentrations.



(a)

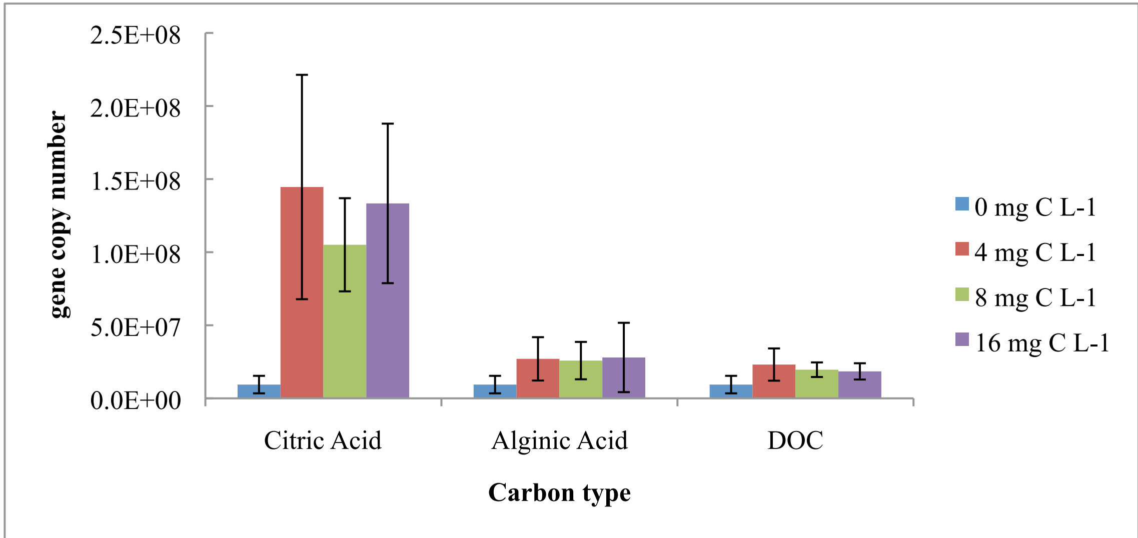


(b)

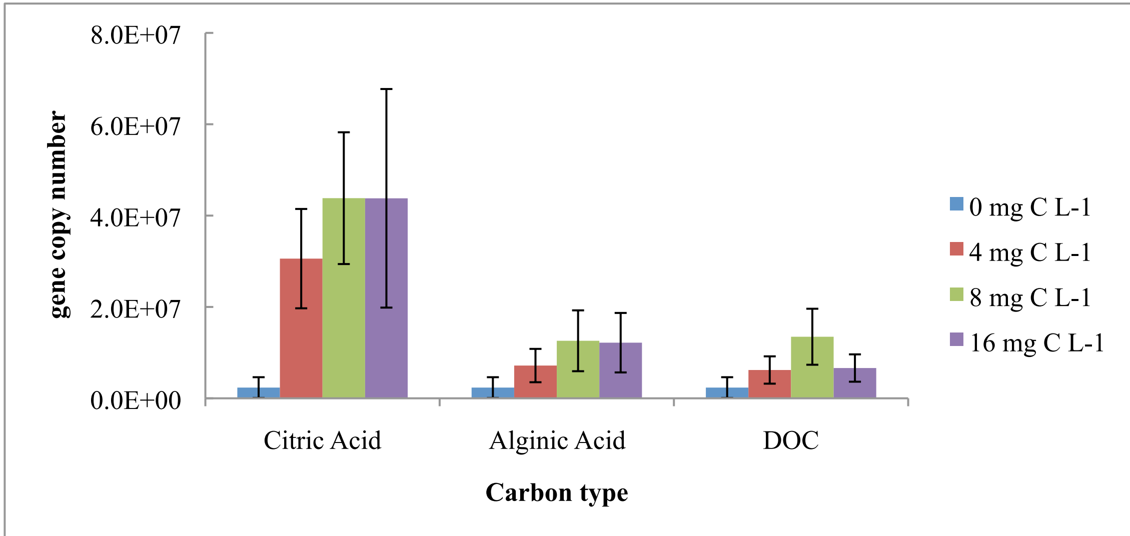


(c)

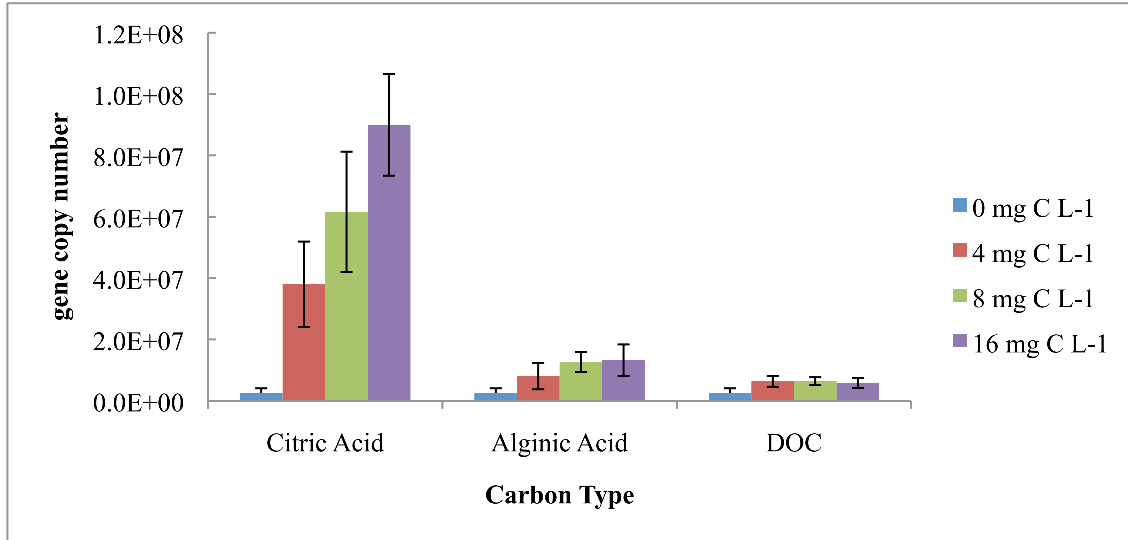
Figure 3.5 Gene copy abundance (a) *nirK*, (b) *nirS*, (c) 16s rDNA in groups of carbon types.



(a)

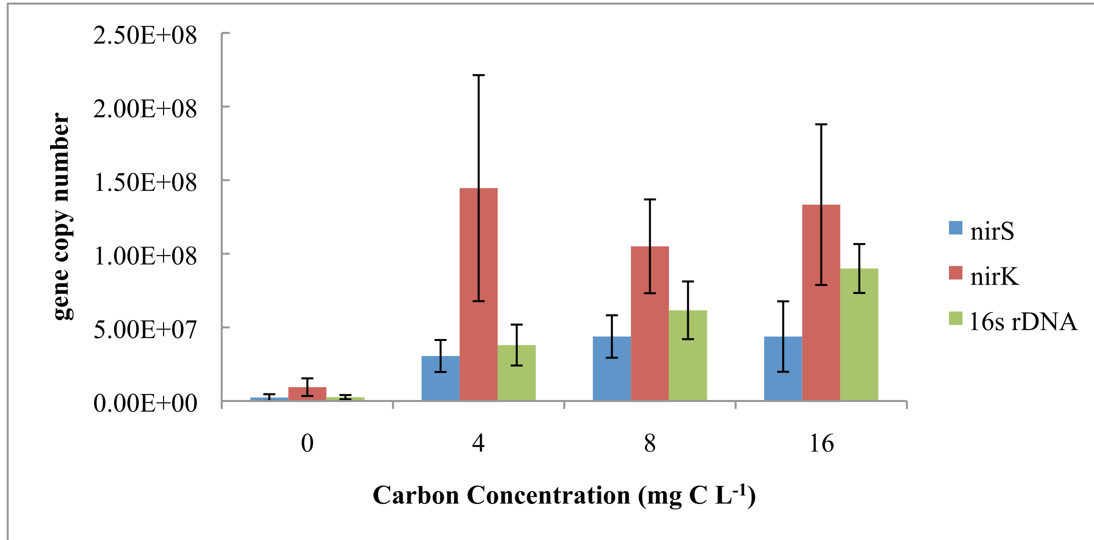


(b)

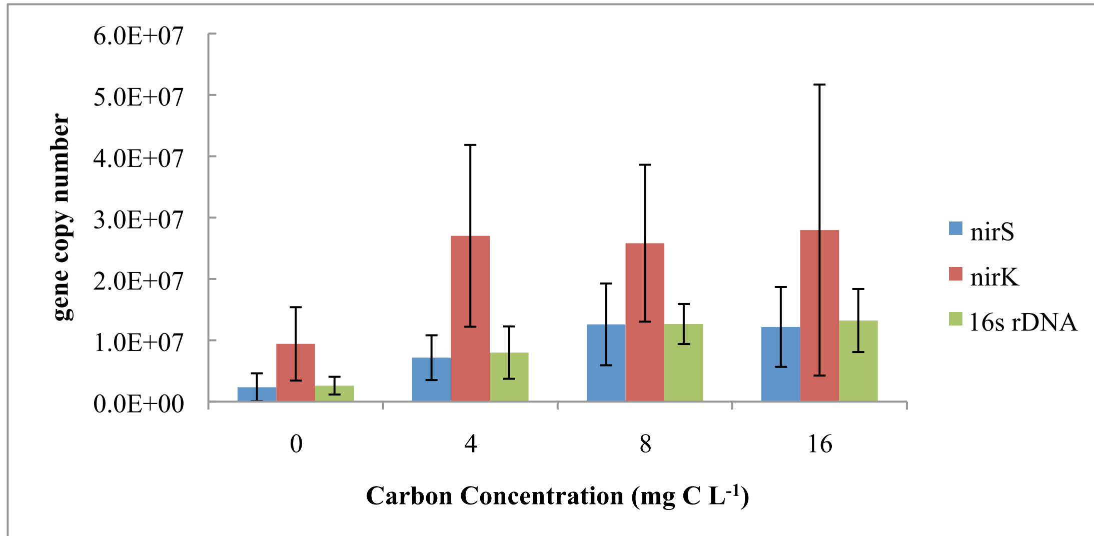


(c)

Figure 3.6 Gene abundance comparison of 16s rDNA, *nirK* and *nirS*.



(a)



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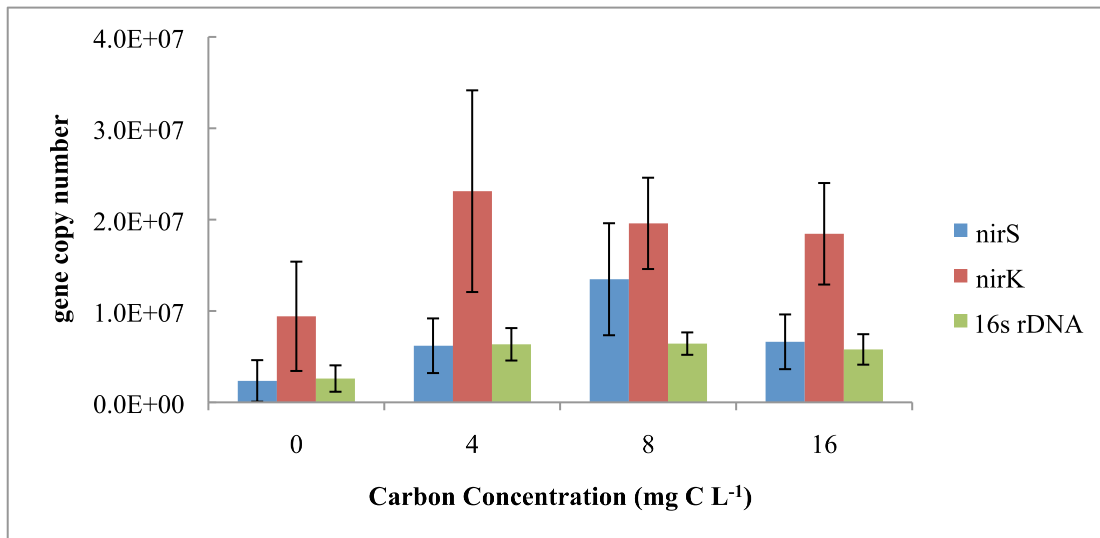
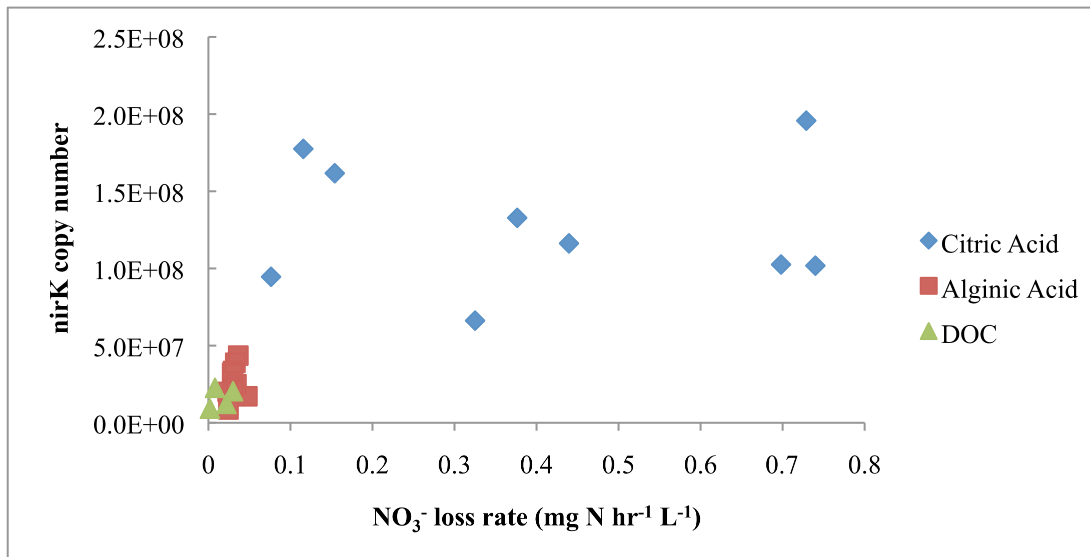
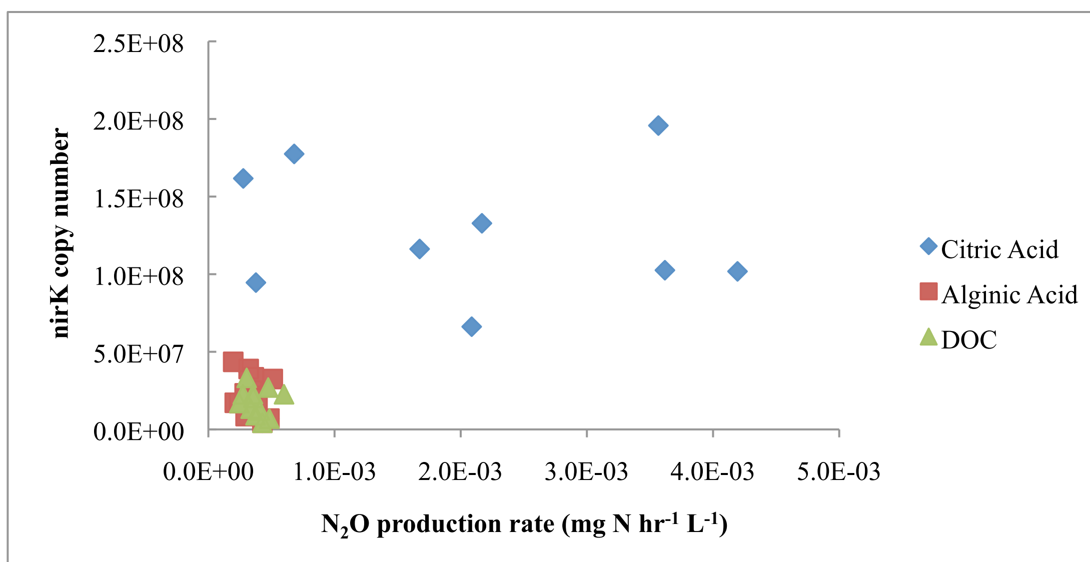


(c)

Figure 3.7 Correlation between denitrification rates and gene abundance: *nirK* (a) and (b); *nirS* (c) and (d); 16s rDNA (e) and (f).



(a)



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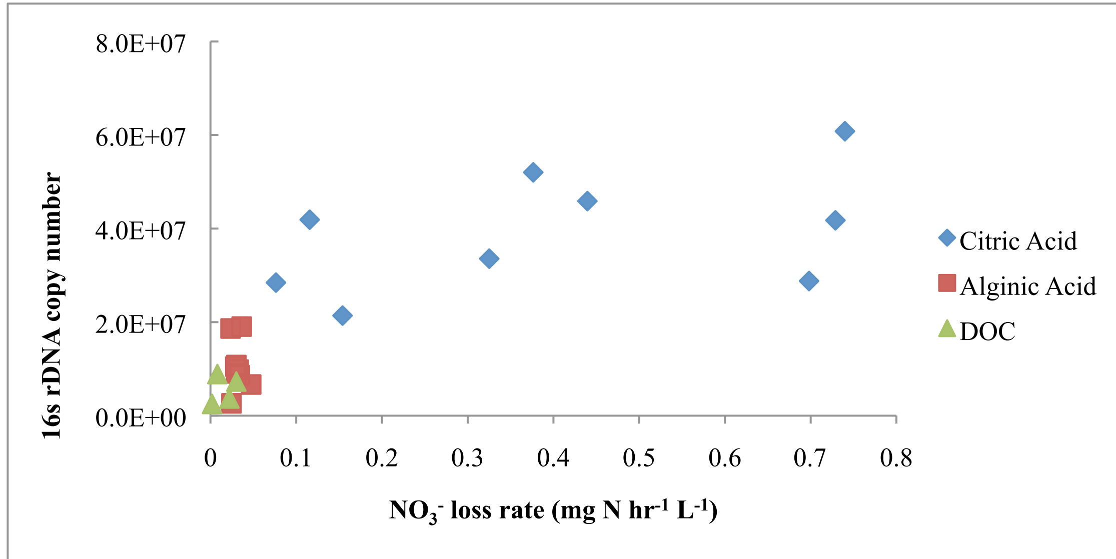
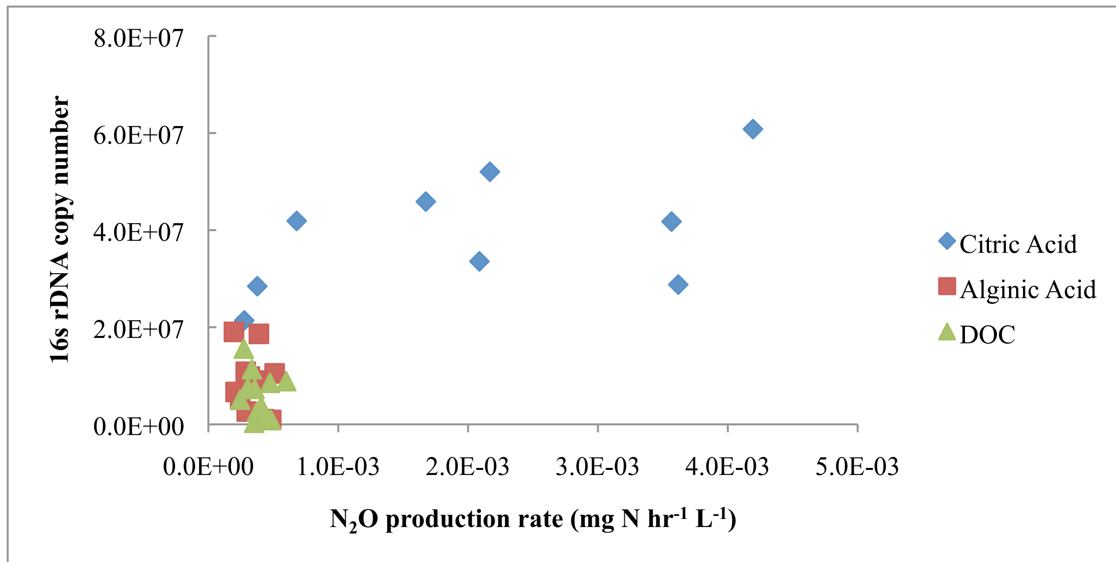
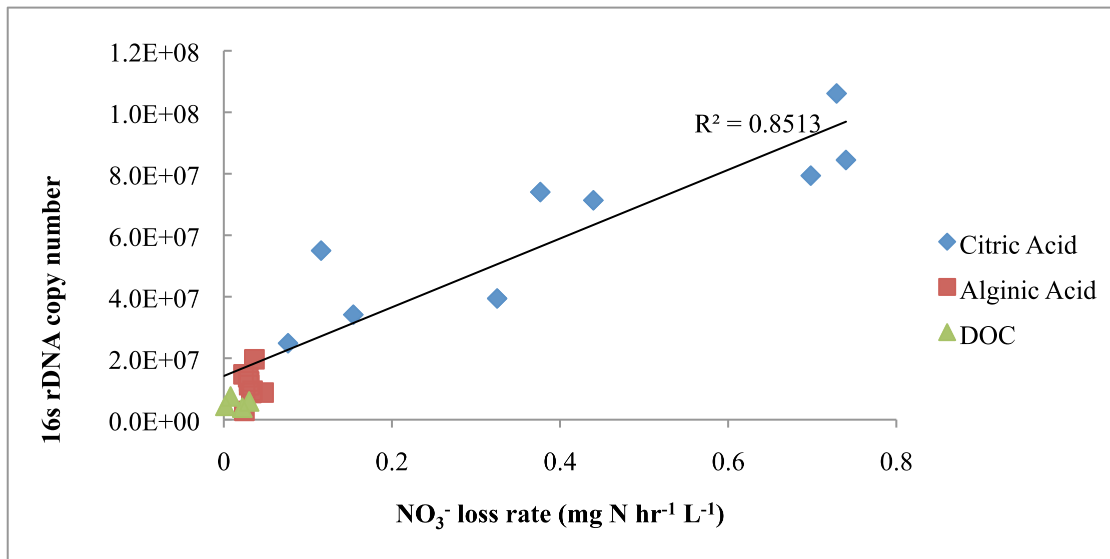


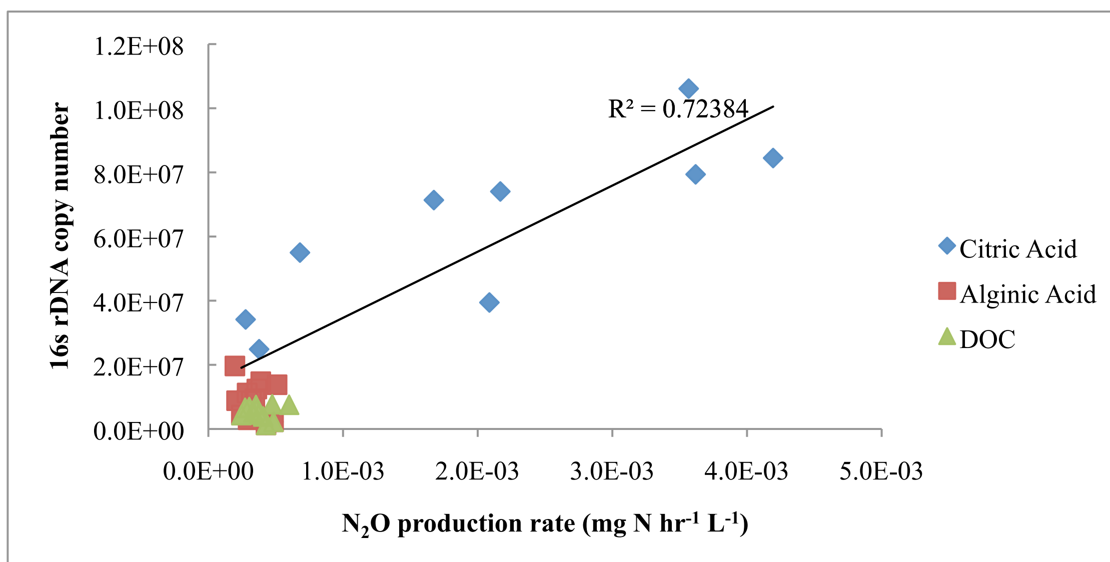
(c)



(d)



(e)



(f)

Table 3.4 Ratios of denitrification gene *nirK* and *nirS*.

Type	Concentration (mg C L^{-1})	*nirK*/16S	*nirS*/16S	*nirK*/*nirS*
Citric Acid	0	3.62	0.90	4.01
	4	3.80	0.80	4.73
	8	1.71	0.71	2.40
	16	1.48	0.49	3.05
Alginic Acid	0	3.62	0.90	4.01
	4	3.38	0.90	3.77
	8	2.04	1.00	2.05
	16	2.11	0.92	2.30
DOC	0	3.62	0.90	4.01
	4	3.64	0.98	3.73
	8	3.05	2.10	1.45
	16	3.19	1.14	2.78

Chapter 4

Summary and Conclusions

4.1 Summary of Major Findings

In this thesis, the chemical denitrification rates and biological denitrification genes abundances were investigated using continuous-flow column experiments and quantitative PCR, respectively. By linking responsible gene abundances with denitrification rates given different organic carbon source in riparian buffer soils, these approaches provided further understanding about the function of riparian buffer in remediation of NO_3^- contamination.

One major conclusions of this work is that both organic carbon type and carbon concentration have an influence on denitrification rate. Citric acid, as an example of a LMW carbon molecule, promotes both NO_3^- loss and N_2O production in riparian buffer soils, with the denitrification rate increasing with increasing carbon concentration. However, alginic acid and DOC, which are presumably less available to microorganisms, do not enhance denitrification rates, even at high carbon concentrations.

A second finding is that organic carbon supply may have effects on denitrification by influencing denitrification gene abundances in riparian buffer soils. For *nirK*, elevated gene abundance over a wide range of citric acid concentrations coupled with low gene copy numbers in alginic acid and DOC treatment indicate that enhanced *nirK* denitrifier population may be responsible for the high denitrification rates when labile organic carbon is abundant. A correlation between denitrification rates and *nirS* gene copy number also illustrates the potential importance of this mechanism of denitrification.

4.2 Broader Implication of Major Findings

4.2.1 Organic Carbon Supply and NO_3^- Remediation in Riparian Buffer

The work presented in this thesis has suggested a significant influence of organic carbon in promoting denitrification rates and increases denitrification gene abundance. Although DOC does not contribute to denitrification, which is surprising, the ability of LMW carbon to increase denitrification rate and denitrifier population may indicate a feasible method to regulate NO_3^- transportation in riparian buffers. It is possible that the efficacy of denitrification, which varies as a function of many factors, may also be estimated across different riparian buffers in the terms of organic carbon. Both organic carbon type and organic carbon concentration can be used as indicator to evaluate the NO_3^- removal effectiveness in riparian buffers.

It is also possible that LMW carbon supply can be enriched through vegetation management to enhance denitrification in riparian buffers. Plant root activity is the main source of citric acid and other low-molecular-weight carbon molecules(Jones 1998). The rhizosphere, as a result, often coincides with a hot spot of denitrification. By selectively planting vegetation which exudates high concentration of LMW carbon molecules from roots, we can increase denitrification rates and remove NO_3^-effectively.

4.2.2 Implication of Biological Detection in Riparian Buffers

Considering the linkage between chemical denitrification rate and responsible gene copy number, denitrification gene abundance may be used in riparian buffers to assess efficiency. Firstly, with the wide application of quantitative PCR technique and advanced instrumentation, fast qPCR assay can be performed in the field to give the estimate of denitrification gene copy number. Gene abundance, as an reflection of denitrification activity, can also be applied to evaluate riparian buffer function. Secondly, combined with DNA sequencing and phylogenetic analysis of denitrifiers, it is possible to further explore denitrifier community composition in riparian buffers. Microarrays, which aremultiplex technologies used in molecular biology, may also be applied in gene detection and quantification to determine relative abundance of nucleic acid sequences in the target. Because of the multiple probes in an array, microarray experiment can dramatically accelerate types of denitrification gene investigation.

4.3 Final Thoughts

4.3.1 Improvement for Present Work

Considering the problems confronted in this study, the improvements of present study may involve quantifying the denitrification gene in *Archaea* and the phylogenetic analysis of 16s rRNA. Due to the higher gene copy number of *nirK* and *nirS* than that of 16s rDNA, it is possible that *nirK* qPCR assays quantified part of gene sequences in *Archaea* besides bacteria. The ability to denitrify has been found in some *Archaea*(Throback et al. 2004).

Quantification of denitrification gene abundance in *Archaea* may be helpful to explain the high gene copy number and demonstrate the denitrifier community composition in riparian buffer soils.

4.3.2 Suggestions for Future Work

The results presented in this thesis improve our understanding of the influence of carbon type on denitrification and elucidate the relationship between chemical denitrification rate and biological gene abundance in riparian buffer soils. Gene expression, which is indicated by the mRNA copy number, is a perfect complement to gene abundance (Henderson et al. 2010). Quantitative reverse transcriptase (RT) PCR (qRT-PCR) can be incorporated to quantify the mRNA levels of genes involved in denitrification, which provides insight into active denitrifier communities. Our study presents *nirK* gene abundance in same range given different concentrations of citric acid addition. Higher gene expression level of *nirK*, though related to gene abundance, may be responsible for corresponding higher denitrification rate. Therefore, further detection of mRNA might be necessary to explain the biological mechanism of denitrification in riparian buffer thoroughly.

Furthermore, the target genes utilized in this thesis only catalyze nitrite reduction; more responsible genes may be needed to better demonstrate other nitrogen reduction steps such as anammox and dissimilatory NO_3^- reduction to ammonium (Dong et al. 2009). Interrelationships between these processes and the abundance of the responsible gene markers helps further clarify the mechanism of riparian buffer function.

4.4 References

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Henderson, S. L., C. E. Dandie, C. L. Patten, B. J. Zebarth, D. L. Burton, J. T. Trevors & C. Goyer (2010) Changes in Denitrifier Abundance, Denitrification Gene mRNA Levels, Nitrous Oxide Emissions, and Denitrification in Anoxic Soil Microcosms Amended with Glucose and Plant Residues. *Applied and Environmental Microbiology,* 76**,** 2155-2164.

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