# INORGANIC NITROGEN TRANSFORMATIONS AND MICROBIAL ASSEMBLAGE COMPOSITIONS IN SANTA FE RIVER TRIBUTARY SEDIMENTS

By

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To my husband, Jaemyeong and my parents dedicated in their support of my studies

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# INORGANIC NITROGEN TRANSFORMATIONS AND MICROBIAL ASSEMBLAGE COMPOSITIONS IN SANTA FE RIVER TRIBUTARY SEDIMENTS

By

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Inorganic nitrogen transformations in tributary systems have broad implications with respect to water quality, and carbon sequestration. Previous research has focused on nitrogen removal from small streams because of their higher efficiencies of nitrate removal compared to large rivers. I investigated relative importance of select inorganic nitrogen transformations, including nitrification, anammox, denitrification, and DNRA, and the microbial assemblages affecting nitrogen transformations in tributary sediments.

Results demonstrated that nitrification and denitrification mainly accounted for removal of ammonium and nitrate from tributary sediments, while Anammox and DNRA were insignificant to nitrogen cycling in tributary sediments. Potential nitrification, denitrification, and DNRA rates were influenced by pH, TC: TN ratios, and labile carbon contents in tributary sediments. Furthermore, the diversity of *nirS* (representing denitrifiers) was related to potential denitrification rates, and this relationship was regulated by organic carbon contents, emphasizing the importance of relationships between microbial structure, functions, and biogeochemical properties. Unlike denitrifiers, the diversity of *Archaeal amoA* (representing nitrifiers) was not related to

potential nitrification rates. However, the distribution of *Archaeal amoA* was correlated with pH of the systems, implying the importance of the relationship between microbial structure and biogeochemical properties.

# CHAPTER 1
# INORGANIC NITROGEN TRANSFORMATIONS AND ASSOCIATED MICROBIAL ASSEMBLAGE COMPOSITIONS IN TRIBUTARY SEDIMENTS

Inorganic nitrogen stored in ecosystems includes ammonium and nitrate, which are produced by biological nitrogen fixation, mineralization, and nitrification processes. Mineralization is the biological transformation of organic nitrogen to ammonium. In addition, inorganic nitrogen is added from external sources such as nitrogen fertilizer and agricultural activities. The ammonium produced by mineralization can be consumed by plants for growth (assimilation of nitrogen) or incorporated into microbial biomass (immobilization). Ammonium may also accumulate in anaerobic systems, such as may be present in riparian or wetland ecosystems. When oxygen is present, ammonium is oxidized to nitrate by aerobic *Bacterial* and *Archaeal* nitrifiers (Reddy and DeLaune, 2008). The nitrate produced by nitrifiers is reduced to nitrite, nitric oxide, nitrous oxide, and nitrogen gas by denitrification, such that nitrous oxide and nitrogen gas are released to the atmosphere. Thus, denitrification is considered to be a major process of nitrate removal in anaerobic environments.

It was recently observed that ammonium can be anaerobically oxidized to nitrogen gas in some marine and estuarine sediments (Thamdrup, 2002; Engström, 2005; Trimmer, 2003). ANaerobic AMMonium oXidation (anammox) accounted for up to 40% of nitrogen loss in the Black Sea (Kuypers, 2003), and between 19% and 35% in the Golfo Dulce coastal bay in Costa Rica (Dalsgaard, 2003). However, research on anammox in anoxic ecosystems other than marine ecosystems remains limited. Therefore, research on anammox in various ecosystems including stream, riparian, and freshwater ecosystems is needed to fully understand the role in removal of ammonium from anaerobic ecosystems.

Unlike the removal process of nitrate by denitrification, nitrate can be reduced to ammonium by Dissimilatory Nitrate Reduction to Ammonium (DNRA) under highly reduced conditions (Michael, 2003). This process can enhance the accumulation of ammonium in systems rather than promoting removal of nitrate (King, 1985). It has been reported that DNRA rates can be as high as denitrification rates in shallow estuarine and tidal systems (Kaspar, 1983; Rysgaard, 1996; Tobias, 2001). In addition, the pasture ecosystem impacted by livestock manure can be a hot spot for DNRA since the microbe performing DNRA was found in rumen and fecal materials (Maier et al., 2000).

Inorganic nitrogen transformation rates are regulated by biogeochemical properties such as pH and availability of electron acceptors and donors (Kaspar, 1983, Tobias et al., 2001; Tate, 2000). For example, lower pH caused by nitrogen fertilization, surrounding vegetation, or organic acids can reduce the availability of ammonia, which can affect the function of nitrifiers. Also, high organic matter content stimulates denitrification and DNRA rates via an increased supply of electron donors for denitrifiers and DNRA bacteria (Kaspar, 1983; Tobias et al., 2001; Yin et al., 2002). Nitrate concentrations can control denitrification and DNRA rates, and the concentrations of nitrite can control anammox rates via supply of electron acceptors. In addition, the change in TC:TN ratio due to nitrogen supplies from a live stock manure or nitrogen fertilization could shift decomposition rates, affecting the level of available organic carbon to denitrification and DNRA.

Previous research demonstrated that stream and riparian sediments are hotspots for inorganic nitrogen transformations in tributary ecosystems because these places

receive nitrogen and organic matter from uplands and stream water (Martin et al., 1999; Steinhart et al., 1998; Chatarpaul et al., 1980; Hill, 1983; Swank and Caskey, 1982; Holmes et al., 1996; Mulholland and Hill, 1997; Bowden et al., 1992; Pinay et al., 1993, 1995; Freeze and Cherry, 1979; Hill, 1996; Hill and Waddington, 1993; Likens et al., 1977). In addition, fluctuating water tables in riparian sediments creates anaerobic conditions, favoring denitrification (Cooper, 1990; Bowden et al., 1992; Lawrence, 1992; Schipper et al., 1993; Hanson et al., 1994; Pinay et al., 1995; Bowden et al., 1992; Pinay et al., 1993 and 1995). Thus, stream and riparian sediments can function as effective sinks for nitrogen in tributary ecosystems (Jones and Holmes, 1996). However, previous research has mainly focused on denitrification and nitrification rates in tributary sediments (Peterson et al., 2001; Lowrance et al., 1997; Hill, 1996; Likens et al., 1977). There is limited research on the potential roles of DNRA and anammox in removal of nitrate and ammonium from tributary sediments. Also, the biogeochemical factors affecting anammox and DNRA rates have not been well studied in tributary sediments.

Organic carbon availability is one of the most important factors regulating denitrification rates in tributary sediments. Carbon availability can vary depending on the vegetation types and quality of organic matter present in the systems (Martin et al., 1999). For example, the organic matter inputs to tributary systems surrounded by woody vegetation may consist of litter with higher lignin and lower cellulose contents than systems dominated by herbaceous vegetation. The low quality of litter and organic matter exhibits a slower rate of decomposition and is therefore a poor source of carbon for heterotrophic microbes (Chapin et al., 2002). Previous research demonstrated that grassy sites exhibited greater denitrification rates than woody sites (Schnabel et al.

1997; Groffman et al. 1991). Also, denitrification rates significantly correlated with respiration rates in planted sites, indicating that the decomposition of organic matter is a main factor regulating denitrification rates via a supply of available carbon to denitrifiers (Walton and Jiannino, 2005). However, most studies on denitrification have focused on the impact of vegetation and soil organic matter contents on denitrification rates (Knoepp, 1998; Gurlevik et al., 2004; Ebrecht and Schmidt, 2003; Garten and Van Miegroet, 1994), rather than on the quality of organic carbon. Therefore, research on the effects of organic matter decomposition rates associated with carbon quality is needed to better understand regulator for denitrification rates.

Nitrification and denitrification are main processes regulating nitrogen concentrations in tributary system, even though anammox and DNRA can be observed. Also, relative rates of denitrification were reported to be higher than those of anammox and DNRA in various ecosystems (Rich et al., 2008; Koop-Jakobsen and Gibli, 2009; Omnes et al., 1996; Revsbech et al., 2005). Therefore, many studies have focused on nitrification and denitrification processes related to nitrogen cyclings in tributary ecosystems. However, most studies have focused on how human activities, land use practices, and vegetation types influence nitrification and denitrification rates, rather than the microbial structures responsible for these processes. Also, little work has been done to determine how the microbial community structure might be related to nitrification and denitrification rates and how shifts in community structure may be linked to biogeochemical properties. For example, low pH can affect the microbial assemblage compositions of nitrifiers, through change in the diversity of nitrifier assemblage compositions. In case of denitrification, carbon and nitrogen concentrations affect

diversity of denitrifiers, influencing their rates. Therefore, research on relationships between biogeochemical factors, microbial assemblage compositions of nitrifiers and denitrifiers, and potential rates of these processes in tributary sediments is needed.

## Site Description

The Santa Fe River Watershed (SFRW) (3574 $km^2$) spreads across eight counties in North East Florida (Figure 1-1) and comprises the southeastern part of the Suwannee River Basin that drains into the Gulf of Mexico. Research has shown an increase in nitrate nitrogen concentration in the Suwannee River Basin covering southern Georgia and north-central Florida (Ham and Hatzell, 1996). Also, the increased nitrate nitrogen has been observed in spring, surface, and ground waters in the Suwannee River Basin area (Hornsby et al., 2001). Despite that the Santa Fe River Watershed comprises only 13% of the Suwannee River Basin area, the Santa Fe River Watershed is responsible for 22% of the total nitrogen input to the Suwannee River Basin (Suwannee River Water Management District, 2003). Therefore, research on nitrogen cycling in the Santa Fe River Watershed needs to be investigated in order to establish the management for nitrogen controls.

For land use, the pine plantation (23%) and agricultural land use (including crop and improved pasture 37%) occupied over the 60% of lands use, following the wetlands (18%), upland forest (11%), and urban (6%) (Sabesan, 2004). Comparing with the land use pattern in 1990, the forested area has shown to decrease, while the agricultural area increased (Sabesan, 2004). Therefore, nitrogen fertilizer from agricultural and timber production, and livestock manure from ranch activity are concerns in the Santa Fe River Watersheds. The soil type is classified as to Ultisols (37%), Spodosols (26%)

and Entisols (15%), and the soil texture is predominately composed of sandy, loamy, and organic soils (Lamsal et al., 2006).

The site for this research is tributary sediments at the Boston Farm Santa Fe Ranch Beef Unit Research Center (SFBRU) in the Santa Fe River Watershed, northern Alachua County. Land uses on this site include a low intensity cattle operation with approximately 300 heifers on 1,600 acres and a nursery operation using nitrogen fertilizer (Holly Factory Nursery) (Frisbee, 2007).

One of two tributaries in my research sites, Tributary 1 (T1) is located along CR 241roadway, flows into pond and finally into the Santa Fe River. T1 is surrounded by a improved pasture and affected by ranch activity. The sediment adjacent to the stream water (riparian sediments) contains relatively high organic matter washed from the upland soils containing livestock manure. Also, riparian sediments receive their water from groundwater and seepage water via a subsurface flow rather than stream water. However, the sediment in the stream water (stream sediments) receives water from stream, and contains sandy soil and relatively low carbon contents. The vegetation type is mixture of herbaceous and woody plants including *Carya* sp., *Pinus* sp., *Quercus* sp., *Magnolia grandiflora*, *Saururus cernuus*, *Juncus* sp., *Cephalanthus occidentalis*, *Hydrocotle umbellata*, and *Polygonum* sp.

The Up-stream region of Tributary 2 (T2-U) is affected by nitrogen fertilization from a nursery operation and surrounded with hardwood plants, including *Carya* sp., *Quercus* sp., and *Magnolia grandiflora,* and soft wood including *Pinus* sp. The Downstream region of Tributary 2 (T2-D) is influenced by a improved pasture and nitrogen fertilization from head water, and covered with grass including *Saururus cernuus*, *Juncus* sp. and

*Polygonum* sp., deciduous shrub plants including *Cephalanthus occidentalis*, and aquatic plants including *Hydrocotle umbellata* (Frisbee, 2007).

## Objectives

The overall objective of this research was to investigate the potential inorganic nitrogen transformation rates, biogeochemical factors regulating these rates, and associated microbial assemblage compositions in tributary sediments of the Santa Fe River. Specific objectives of this research were to:

- Investigate the relationship between potential inorganic nitrogen transformation rates and biogeochemical properties in tributary sediments.
- Investigate the relationship between extracellular enzyme activities associated with organic matter decomposition and potential denitrification rates in tributary sediments.
- Investigate the relationships between the biogeochemical factors affecting denitrification and nitrification and their microbial assemblage compositions in tributary sediments.

## Dissertation Format

This dissertation is composed of four main parts. The first part is the literature review (Chapter 2). The second part presents research on the biogeochemical properties and potential inorganic nitrogen transformation rates (Chapter 3), and research on the relationship between extracellular enzyme activities in tributary sediments and litter, and potential denitrification rates (Chapter 4). The third part of this dissertation describes research on relationships between biogeochemical properties and the microbial assemblage compositions associated with denitrification (Chapter 5) and nitrification (Chapter 6).

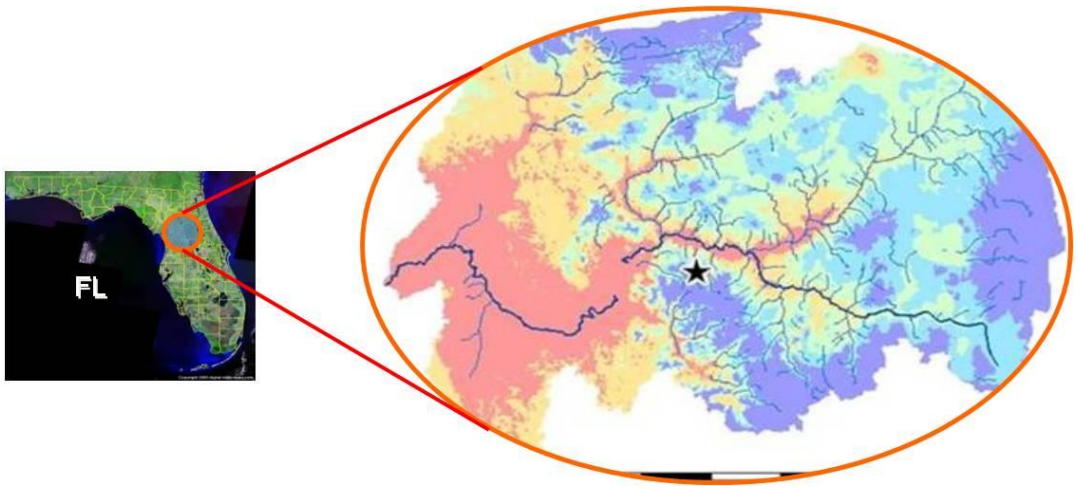



Figure 1-1. The Santa Fe River Watershed. The asterisk mark on the map is the research place.

# CHAPTER 2
# LITERATURE REVIEW

Increased nitrogen created by fertilization and improper disposal from ranch activity has been considerable concerns in agriculture ecosystems. The excessive input of nitrogen promotes eutrophication in aquatic ecosystems, which can cause a decrease in oxygen and sunlight available to other organisms. Also, nitrate is toxic to human health when consumed in drinking water. However, riparian ecosystems can reduce nitrate through denitrification, plant uptake, and microbial immobilization. In particular, since denitrification is the permanent removal mechanism of nitrate, this process is considered to be a desirable way of nitrate removal in watershed ecosystems. However, anaerobic ammonium oxidation (anammox) can also remove ammonium and nitrite from riparian ecosystems, and nitrification can increase denitrification rates via a supply of nitrate to denitrifiers. In addition, dissimilatory nitrate reduction to ammonium (DNRA) can accumulate ammonium in riparian ecosystems. Thus, to describe functions of riparian ecosystems for nitrogen removal, the comprehensive understanding of nitrification, anammox, denitrification, and DNRA is needed. This chapter reviewed previous research on importance of nitrification, anammox, denitrification, and DNRA in various ecosystems, biogeochemical factors affecting these rates, physiology of microbes associated with these processes, and analysis methods measuring each rate. Thus, this literature review will help to understand inorganic nitrogen transformations in riparian ecosystems.

## Nitrification

Nitrification is the biological oxidation of ammonium to nitrate using oxygen as an electron acceptor. Nitrification is an important step in the nitrogen cycle in soils because

nitrate produced by nitrification can be used as electron acceptors for denitrification. There are two types of nitrifiers: (1) autotrophic nitrifiers using ammonium as an energy source to fix carbon dioxide used in growth and maintenance and (2) heterotrophic nitrifiers using organic nitrogen as an energy source instead of ammonium (Chapin et al., 2002). This review mainly focuses on autotrophic nitrifiers because heterotrophic nitrifiers are not well studied in various ecosystems (Tate, 2000).

**Terrestrial Ecosystems**

Rapid rates of ammonification and nitrification are observed in tropical forest ecosystems because of relatively high concentrations of foliar and litter fall nitrogen (Vitousek and Matson, 1988). Temperate forest ecosystems can be expected to exhibit lower nitrification rates due to lower amounts of ammonium via a plant uptake relative to fertilized grasslands and freshwater ecosystems. It has been also suggested that increased nitrogen deposition due to fossil fuel burning and fertilizer utilization enhanced mineralization rate via a decrease in the TC:TN ratio of litter and forest soils, possibly increasing nitrification rates (Aber et al., 1998). The increased nitrification rate could lead to an increase of nitrate leaching from northern temperate forest ecosystems to adjacent watersheds (Aber et al., 1988). However, results from NITREX (Nitrogen Saturation experiment) demonstrated that the nitrogen retention efficiency was relatively high in NITREX sites, even after observing a linear relationship between forest floor nitrogen concentration and nitrification rate (Aber et al., 1995).

Disturbances in forest ecosystems, such as logging (Matson and Vitousek, 1984; Vitousek and Andariese, 1986), fires (Polygala et al., 1986; White, 1986; Weston and Attiwill, 1990), and addition of fertilizer (Adams and Attiwill, 1983) can affect mineralization rates, which in turn influence nitrification rates in forest soils. Additionally,

some research has observed heterotrophic nitrification and fungi using organic nitrogen as an electron donor instead of ammonium in acidic forest soils (Nishio et al., 1998; Robertson, 1982). These microbes were less sensitive to pH, implying that heterotrophic nitrifiers can play an important role in nitrification of acidic forest soils. However, their contribution to nitrification in forest ecosystems has not been well documented, because of the difficulty in distinguishing rates between autotrophic and heterotrophic nitrifiers.

**Ocean Ecosystems**

The majority of ammonium released by mineralization is consumed by phytoplankton (Harrison et al., 1992 and 1996), or is oxidized to nitrate at surface water (Yool et al., 2007). Also, the high flux of organic nitrogen supplied from sediments and upwelling deep ocean water is oxidized to ammonium in subsurface oxygen minimum zones (OMZ). The ammonium is oxidized to nitrate in OMZ (Ward, 2002), leading to denitrification (Codispoti et al., 1985; Ward and Zafiriou, 1988; Lipschultz, 1990; Naqvi and Noronha, 1991) and anammox (Lam et al., 2006; Kuypers et al., 2005 and 2006; Hamersley et al., 2007; Thamdrup et al., 2006). Thus, nitrification is indirectly responsible for the loss of nitrogen in ocean ecosystems. The recent discovery of *archaeal* nitrifiers has made it possible to explain a broader distribution of nitrifiers in ocean ecosystems, where nitrifiers have not been detected using molecular analysis. *Archaeal* nitrifiers were found to be much more abundant than bacterial nitrifiers in marine systems (Wüchter et al., 2006), implying that the versatile physiology and metabolism of *archaeal* nitrifiers could play a pivotal role in ocean nitrification.

**Coastal Wetlands**

Coastal wetlands are one of the most productive ecosystems due to tidal flooding frequency and import of nutrients from terrestrial and ocean ecosystems. Due to the limitation of oxygen by tidal flooding, most of the inorganic nitrogen is ammonium. Heterotrophic microbes, phytoplankton, and marsh vegetation consume this ammonium for their growths (Henriksen and Kemp, 1988). As a result, the nitrification process can be limited by ammonium and oxygen concentrations in coastal wetland ecosystems (Gűrel et al., 2005). Salinity can also influence nitrification in costal wetland ecosystems. Although a marine *Nitrosomonas* sp. was reported to survive in estuarine sediments at 15% salinity (Henriksen and Kemp, 1988), nitrifying bacteria exhibited lower rates at higher salinities (Rysgaard et al., 1999). Nitrification processes can occur in the surficial oxidized zone of sediments ranging from depths of 1 to 1.6 mm (Seitzinger, 1988; Kemp et al., 1990), as well as in plant root zones due to the release of oxygen via their roots, thereby stimulating coupled nitrification and denitrification processes (Rysgaard et al., 1996). Physical perturbation and bioturbation by macrofauna could create zones where oxygen can penetrate, supplying oxygen to nitrifiers (Rysgaard et al., 1996; Kemp et al., 1990).

**Streams**

Human activities can supply excess nitrogen to ecosystems, changing the balance between supply and demand for available nitrogen to plants, and leading to nitrogen saturation in terrestrial and aquatic ecosystems (Aber et al., 1998; Vitousek et al., 1994). Increased nitrogen deposition and agricultural activities can also increase nitrogen input to streams (David and Gentry, 2000), ultimately adding to the nutrient loads of large rivers (Seitzinger et al., 2002) and estuarine systems (Rabalais et al., 2002), and

causing water quality problems and eutrophication (Justic et al., 1993; Nixon, 1995). In addition, recent studies have shown that small streams can be important sites for nitrogen transformations and retention of nutrients. Despite their relatively small proportion of watershed surface area, small streams account for 85% of total stream length within a watershed and receive draining water and dissolved nutrients from adjacent terrestrial ecosystems (Fisher et al., 1998; Alexander et al., 2000). Therefore, nitrogen cycling in small streams is an important factor regulating nitrogen input to other systems. Nitrification in small streams has been a common subject of research because nitrate is a mobile chemical species (Starry and Valett, 2005). Previous research has suggested that nitrification rates account for 50% of the variability observed in stream nitrate concentrations, underscoring the importance of nitrification in river ecosystems (Peterson et al., 2001; Bohlen et al., 2001). Also, Peterson et al. (2001) have reported that 12 headwater streams, as part of the Lotic Intersite Nitrogen eXperiment (LINX), exhibited a high nitrification rate despite low ammonium concentration, indicating that small streams are potentially important sources of nitrate to other ecosystems. Nitrification in stream ecosystems can be limited by a variety of factors, including: (1) physical and chemical properties (e.g., dissolved oxygen concentration and pH) (Strauss et al., 2002); (2) agricultural activities adjacent to watersheds (Omernik, 1977); and (3) in-stream nitrogen adsorption or transformations (Triska et al., 1990; Jones and Holmes, 1996; Fisher et al., 1998).

**Wastewater Treatment Plants**

Each person contributes 8 to 12 pounds of nitrogen per year to wastewater treatment systems. Sixty percentage of the nitrogen in waste material is bound in the complex organic matter, and the remaining nitrogen form is ammonium (Tchobanolgous

and Burton, 1991). To remove inorganic nitrogen in wastewater plants, ammonium must be oxidized to nitrate before being reduced to nitrogen gas by denitrifiers. Nitrifiers tend to have long lives, reproduce much more slowly, and be limited by oxygen levels (Hammer and Mackichan, 1981). Wastewater plants are thus designed to have a longer average residence time to extend the contact time to biofilters and the aeration basin containing nitrifiers and dissolved oxygen (Weber, 1972). Because nitrifiers are sensitive to temperature, pH, and oxygen, calcium carbonate is applied to maintain the optimal pH (pH 7 to 7.5), oxygen is produced by rotating the biofilter, and warmer temperatures are maintained (Hammer and Mackichan, 1981).

**Factors Affecting Nitrification Rates**

**Ammonium**

The availability of ammonium could regulate nitrification (Triska et al., 1990), and thus the ammonification process, the biological conversion of organic nitrogen to ammonium, can influence nitrification rates (Jones et al., 1995). Previous research has found a positive relationship between ammonium concentrations and nitrification rates in streams (Kemp and Dodds, 2002; Mulholland et al., 2000; Triska et al., 1990), lakes (Hall, 1986), ground water (Strauss and Dodds, 1997) and soils (Davidson and Hackler, 1994) . However, plant roots and microbes also consume ammonium, and thus nitrifiers compete for ammonium in tundra ecosystems due to the lack of ammonium (Chapin et al., 2002).

**pH**

In a laboratory culture experiment, the optimal pH for nitrification rate ranges from pH 6.6 to 8.0, with negligible nitrification below pH 4.5 (Tate, 2000). The inhibitory effect of acidic pH to nitrification is due to the decreasing the ratio of ammonia to ammonium

at low pH; however, the exact mechanism of pH inhibition to nitrification is not yet completely understood (Bothe et al, 2007). Even though pH inhibition to nitrifiers in cultures has been observed, many acidic ecosystems have shown relatively high nitrification rates (Stark and Hart, 1997). Explanations for the increased nitrification rates include: (a) microsite variations in soil pH; (2) the presence of acidophilic autotrophic nitrifiers in acidic soils; (3) the presence of heterotrophic nitrifiers and fungi capable of the oxidation of organic nitrogen in acidic soils (Doxtander and Alexander, 1966; Eylar and Schmidt, 1959); and (4) adaption of nitrifiers to acidic conditions (Hankinson and Schmidt, 1988).

**Oxygen**

Nitrification rate is limited by oxygen, because nitrifiers use oxygen as an electron acceptor for oxidation of ammonium to nitrate. The minimum concentration of oxygen for nitrifier survival ranges from 1 to 6 μM (Henriksen and Kemp, 1988). Decreased nitrification in coastal ecosystems has been observed due to overgrowth of phytoplankton, which depletes oxygen availability for nitrifiers (Jenkins and Kemp, 1984; Hansen et al., 1981).

Some researchers have reported the presence of the nitrification process under lower oxygen concentrations. For example, *Nitrosomonas europaea* survived at oxic-anoxic interface conditions (Voytek and Ward, 1995); the growth rate of *Nitrosomonas marina* was the highest with 5% oxygen content (Goreau et al., 1980); and nitrification occurred with oxygen concentrations as low as 0.3 $\mu g \cdot ml^{-1}$ (Tate, 2000). In addition, nitrification can take place near the plant rhizosphere (Sand-Jensen et al., 1982; Wium-Andersen and Andersen, 1972), in oxygenated water of flooded soils, at the anoxic-oxic interface, or within soil microsites in sediments.

**Temperature**

The temperature for ammonium oxidation to nitrite in pure culture ranges from approximately 0 ºC to 65ºC, and nitrite oxidation to nitrate occurs at approximately 0 ºC to 40ºC (Tate, 2000). The optimal temperature for nitrification in laboratory settings ranges from 25 ºC to 30 ºC (Kadlec and Reddy, 2001). However, nitrification occurs from tropical to tundra soils at temperatures exceeding optimal ranges for ammonium oxidation. Temperature can affect other variables, including mineralization rates and oxygen concentrations, which in turn influence nitrification rate (Richardson 1985; Sheibley et al. 2003).

**Organic carbon**

Previous research has shown mineralization to have a positive relationship with nitrification because ammonium produced by mineralization can be used as an energy source for nitrifiers (Starry and Valett, 2005; Duff and Triska, 2000; Bianchi et al., 1999). Organic matter content could enhance nitrification rates via an increased mineralization rate. However, organic carbon content could limit nitrification rates because organic carbon could enhance heterotrophic microbes requiring ammonium for their growth (immobilization) in stream water (Bernhardt and Likens, 2002; Strauss et al., 2002), agricultural soils (Venterea and Rolston, 2000), forest soils (Montagnini et al, 1989; Ollinger et al., 2002), and wastewater treatment plants (Richardson, 1985). In addition, the response of nitrification to organic carbon could depend on the TC:TN ratio of organic matter, because the TC:TN ratio could determine the mineralization and immobilization rates. Thus, organic matter with a low TC:TN ratio could stimulate ammonification, increasing nitrification rate via the supply of ammonium. Organic matter with a high TC:TN ratio could enhance immobilization rates, resulting in competition

between heterotrophic microbes and nitrifiers for ammonium (Strauss and Lamberti, 2000; Verhagen and Laanbroek, 1991).

**Biochemistry of Nitrifiers**

Nitrification has two steps: (2-1) the oxidation of ammonium to nitrite mediated by an ammonium oxidizer; and (2-2) the oxidation of nitrite to nitrate mediated by a nitrite oxidizer (Schmidt, 1982).

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O;\ G^o = -272kJ\ mol^{-1} \quad (2\text{-}1)$$

The second reaction of nitrite to nitrate is;

$$2NO_2^- + O_2 \rightarrow 2NO_3^-;\quad G^o = -75kJ\ mol^{-1} \quad (2\text{-}2)$$

Recently, ammonia oxidizing archaea (AOA) were discovered in soils, oceans and estuarine sediments, with population sizes reported to be larger than those of ammonia oxidizing bacteria (AOB). Studies of the *Nitrosopumilus maritimus*, one of the *archaeal amoA* nitrifiers, genome revealed that *archaeal amoA* had electron transport different from other ammonia oxidizing bacteria. *N. maritimus* has also been shown to have a limited capacity for organic carbon assimilation, meaning that they can grow not only autotrophically but also mixtrophically, and thus they can influence nitrogen and carbon cycles in ocean ecosystems (Walker et al., 2010).

**Microbial Diversity of *Bacterial* and *Archaeal* Nitrifiers**

One environmental factor affecting the composition of nitrifiers in terrestrial ecosystems is pH, because ammonium which is unsuitable as a substrate for ammonia monooxygenase, is formed under acidic conditions (Kowalchuk and Stephen, 2001). However, previous research has shown that *Nitrosopira sp*. can tolerate acidic conditions (de Boer et al., 1995). Culture dependent techniques (MacDonald, 1986) and molecular surveys, such as 16S rRNA gene sequencing or Polymerase Chain Reaction-

Denaturing Gradient Gel Electrophoresis (PCR-DGGE), have demonstrated a dominance of *Nitrosopira* in acidic forest soils (Laverman et al., 2000) and acidic stream (Kowalchuk et al., 2000; Stienstra et al., 1994). However, the mechanisms for nitrification in acidic soils are not fully understood. Recent research has shown that *Crenarchaeota* was widely distributed and contributed significantly to nitrification rate in hot thermal springs (Hatzenpichler et al., 2008), marine ocean ecosystems (Francis et al., 2007), estuarine systems (Santoro et al., 2008), soils (Leininger et al., 2006), and wastewater treatment plants (Park et al., 2006).

Based on the sequences retrieved from previous studies, *archaeal* nitrifiers are divided into clusters representing soil and marine groups (Francis et al., 2005; Park et al., 2006). Soil groups are likely to be less sensitive to organic matter, while the marine group has been shown to be uninhibited by acidic pH (He et al., 2007). However, there is not yet enough information on which factors can influence the division between the marine and soil groups. *Archaeal* nitrifiers seem to be better adapted to environmental stress compared to bacterial nitrifiers, because they have low permeability membranes and several metabolic pathways capable of using dissolved organic carbon as a carbon source (Valentine, 2007).

**Measurement of Nitrification Rates**

The easiest way to measure the nitrification rate is to analyze concentrations of ammonium, nitrite, and nitrate in laboratory and environmental samples (Ferguson et al., 2007). Another method includes specific nitrification inhibitors (nitrapyrin and allylthiourea), which have been used to measure nitrification rates in coastal sediments (Macfarlane and Herbert, 1984; Hansen et al., 1981). However, it remains unclear if

these components inhibit *archaeal* ammonia monooxygenase, and if they also inhibit mineralization, which would result in underestimation of nitrification rates.

Nitrification rates are calculated by the difference in ammonium concentrations or nitrate accumulation rates in the presence and absence of the inhibitor. The $^{15}$N-nitrate isotope dilution technique is also widely used to measure nitrification rates. Following addition of $^{15}$N-ammonium and incubation, the concentration of $^{15}$N -nitrate oxidized from $^{15}$N-ammonium is analyzed (Koike and Hattori, 1978). However, added $^{15}$N-ammonium could possibly stimulate nitrification rates, thereby resulting in overestimation of nitrification rates relative to *in situ* rates. As the molecular approach, quantitative Polymerase Chain Reaction (qPCR) has been used to estimate quantification (as absolute number of copies) of both bacterial and *archaeal* amoA genes in marine waters, soils, and waste treatment systems (Harms et al., 2003; Geets et al., 2007; Kim et al., 2001 and 2004; Wüchter et al., 2006).

**Anaerobic Ammonium Oxidation (Anammox)**

In 2002, anaerobic ammonium oxidation (anammox) process was observed under anoxic conditions in marine and estuarine sediments in nature, playing an important role in nitrite and ammonium removals (Thamdrup and Dalsgaard, 2002; Engström et al., 2005; Trimmer et al., 2003). Most anammox research tended to focus on ocean and estuarine ecosystems, because the removal and accumulation of ammonium and nitrite were related to anoxic conditions in the deep water column. However, recently the identification of anammox has been extended to freshwater and arctic ecosystems (Schubert, 2006; Jetten et al., 2003; Rysgaard, 2004)

**Ocean Ecosystems**

Kuypers et al., (2003) discovered that anammox in the Black Sea removed inorganic nitrogen accounting for up to 40% of total nitrogen gas production in anoxic regions. Specially, at a 200m depth in the anoxic waters of Golfo Dulce, 19% to 35% of total nitrogen gas production was performed by the anammox process (Dalsgaard et al., 2003), and at Benguela OMZs, anammox produced 4.2 μg $N \cdot day^{-1}$ (Kuypers et al., 2005). The water chemistry of these oceanic ecosystems is very similar to that of oxygen depleted zones in the oceans, where 30% to 50% of the global nitrogen removal is expected to occur (Dalsgaard et al., 2003). Interestingly, anammox was detected in the Arctic Ocean, although the contribution was low relative to overall nitrogen gas production (less than 5%) (Rysgaard et al., 2004). Therefore, anammox may be a globally important removal process for oceanic nitrogen cycling.

**Estuarine Sediments**

The contribution of anammox to nitrogen production varies among ecosystems. In the estuarine ecosystems of the continental shelf sediments in the Skagerrak of the Baltic North Sea, anammox produces total 30 to 99 μM of $N_2$ per day, accounting for 24% to 67% of total nitrogen gas production (Thamdrup et al., 2002). In the Aarhus Bay, the anammox production of nitrogen gas reaches 83 μM of $N_2$ per day. However, the contribution of anammox to total nitrogen gas production was insignificant relative to denitrification in a eutrophic coastal bay, because high concentrations of organic matter enhanced activities of denitrifiers (Thamdrup et al., 2002). In sediments with high organic matter, anammox bacteria created 0.54 μg $N \cdot g\ soil^{-1} \cdot day^{-1}$ at Thames Estuary, UK and 2.8 μg $N \cdot g\ soil^{-1} \cdot day^{-1}$ at Chesapeake Bay, but the relative contribution of

anammox to total nitrogen gas production was very small (8%) (Engström et al., 2005; Rich et al., 2008).

**Wastewater Treatment Plants**

Most anammox research in freshwater ecosystems is performed in man-made systems, such as wastewater treatment plants (Sliekers et al., 2002; Strous et al., 1998; Fux et al., 2002; Third et al., 2005). Since bioreactors using anammox bacteria consume ammonium as an energy source and carbon dioxide as a carbon source (Egli et al., 2003), these systems can reduce the operational costs up to 90%, while still being environmentally friendly (Sliekers et al., 2003). Also, it has been reported that anammox bacteria were not affected by the chemical composition of waste water, surviving various types of waste water sludge (Pilcher et al., 2005). However, enrichment of anammox bacteria takes a relatively long time (11 to 30 days) (Strous et al., 1998; Van de Graff et al., 1996).

**Freshwater Systems**

In freshwater ecosystems, anammox has been identified in Lake Tanganyika, the second largest lake in the world. The lake produces 3.4 μg N per day (13%) (Schubert et al., 2006). Using 16S rRNA gene analysis, Penton (2006) found evidence for the widespread distribution of anammox, including at the Kellogg biological station (freshwater sediment), Wintergreen Lake (a small eutrophic lake), Sherriff's Marsh (rich organic wetland), and the Everglades WCA 2A (Water Conservation Area 2A subtropical wetlands). However, studies on the anammox process in freshwater, wetland ecosystems, or floodplains similar to anoxic oceanic conditions have not been well investigated.

## Factors Affecting Anammox Rates

### Nitrite and ammonium

Because anammox has been detected in natural ecosystems and proven to be important to nitrogen cycling, many researchers have begun to investigate factors controlling the anammox process. Dalsgaard (2002) observed that in anoxic incubation, anammox accounted for 65% of nitrogen gas formation. In addition, nitrite production from nitrate was faster than nitrite consumption, and therefore did not strain the rate of anammox. Engström (2005) suggested that competition between anammox and denitrification for nitrite is a significant determinant of absolute and relative anammox rates in coastal marine sediments. Trimmer (2005) also reported that anammox can be regulated by availability of nitrite, as well as the relative sizes or activities of anammox populations. The ammonium supplied by mineralization may be consumed by anammox bacteria as an electron donor (Kartal et al., 2007). However, there is little research on the effect of ammonium concentration on anammox rate. Therefore, research on the general regulators and controlling factors for anammox in broader ecosystems is needed.

### Organic carbon

Anammox bacteria are known to be autotrophic and use carbon dioxide as a carbon source. Thus, it was expected that the supply of organic carbon could not control anammox rates. (Strous et al., 1998). However, bioavailability of organic carbon enhances denitrification rates. Therefore, it can be assumed that higher amounts of organic carbon will stimulate denitrification. The enhanced denitrification rate may hinder the anammox process through competition for nitrite. However, previous studies have shown controversial results about the relationship between anammox rate and

organic carbon concentrations. In sediment from the Baltic North Sea transition, a negative correlation was observed between the relative importance of anammox and organic matter (Thamdrup and Dalsgaard, 2002), while in the Thames estuary, the anammox process was positively correlated with sediment organic content (Trimmer et al., 2003). Therefore, more research on relationship between anammox rate and organic carbon content is needed in various ecosystems.

**Oxygen**

The anammox process in a waste treatment plant was found to be inhibited by 1.1 μM oxygen, and occurred under anaerobic conditions (Strous et al., 1998). Although the anammox process exposed to oxygen, the removal of oxygen can revive the process (Jetten et al., 1998; Third et al., 2005). However, there are no reports on the effects of oxygen on anammox in natural systems. Presumably, anammox is also inhibited by oxygen in natural systems, because most naturally occurring anammox rate has been detected in anoxic conditions.

**Biochemistry of Anammox Bacteria**

Based on $^{15}$N-nitrogen experiments, the following anammox mechanism has been postulated: Anammox bacteria are chemolithoautotrophs and consume ammonium and nitrite in a ratio of 1 to 1. They reduce nitrite ($NO_2^-$) to hydroxylamine ($NH_2OH$) by a nitrite-reducing enzyme (NiR). Next, hydroxylamine ($NH_2OH$) and ammonium ($NH_4^+$) are condensed to hydrazine ($N_2H_4$) and water by a hydrazine hydrolase (HH). Finally, a hydroxylamine oxidoreductase (HAO) oxidizes hydrazine ($N_2H_4$) to dinitrogen ($N_2$) (Jetten et al., 1998; 2003). Overall process of anammox is following equation.

$$NO_2^- + NH_4^+ \rightarrow N_2 + 2H_2O \quad \Delta G0° = -358\ kJ \cdot mol\text{-}1 \qquad (2\text{-}3)$$

## Microbial Diversity of Anammox Bacteria

The first anammox bacteria, *Brocadia anammoxidans*, were detected using 16S rRNA gene sequence analysis and FISH with specific oligonucleotide probes in biofilm. *Brocadia anammoxidans* was found to be phylogenetically related to Planctomycetales (Strous et al., 1999; 2000). Using anammox specific 16S rRNA gene primers (mainly using Pla 46F primer) and anammox specific oligonucleotide probes, researches have reported the presence of at least three other anammox bacteria. These genera are *Brocade* (Kartal et al., 2004), *Kuenenia* (Schmidt et al., 2000), and *Scalindua* (Kuypers et al., 2003 and Schmidt et al., 2003). Recently, the *Candidatus Anammoxoglobus propionicus* species was found in a bioreactor (Kartal et al., 2007).

Penton et al., (2007) suggested a new primer that was 100% specific in the recovery of 700bp 16S rRNA gene sequence with 96% homology to the *Scalindua* group of anammox bacteria. This new primer detected anammox bacteria in 11 geographically and biogeochemically diverse freshwater and marine sediments (Penton et al., 2007).

## Measurement of Anammox Rates

### Isotope pairing method

The isotope pairing method involves additions of $^{15}N$-ammonium and $^{14}N$-nitrite to samples. After incubation, the ratio of $^{14}N^{15}N$ to$^{14}N^{14}N$ is analyzed using a gas chromatography-isotope ratio mass spectrometry and can be expressed as $\delta^{14}N^{15}N$ values following equation (Kuypers et al., 2003).

$$\delta^{14}N^{15}N = [(^{14}N^{15}N:^{14}N^{14}N)_{sample}]: [(^{14}N^{15}N:^{14}N^{14}N)_{standard}] -1$$

(Air can be used as the standard)

### Intermediate analysis

As mentioned above, hydrazine and hydroxylamine are intermediate products in the anammox process. Therefore, the intermediate production rate is related to the overall rate of anammox. Ammonium, hydrazine, and hydroxylamine are measured colorimetrically at time intervals after anoxic incubation of anammox bacteria with media (Jetten et al., 2005).

## Denitrification

Denitrifiers are heterotrophs and use nitrate and nitrite as electron acceptors and organic matter as an energy source. Most denitrifiers are facultative anaerobes and prefer to use oxygen when oxygen is available in the system (Chapin et al., 2002). The sequence of nitrate reduction is nitrate → nitrite → nitric oxide → nitrous oxide → nitrogen gas. The regulators for denitrification are organic matter availability, nitrate concentrations, anaerobic conditions, pH, and temperature in nature ecosystems (Tate, 1999).

### Forest Ecosystems

Forest ecosystems are usually limited by nitrogen, so that this system can have a substantial capacity to store excess nitrogen. The NITREX (Nitrogen saturation experiment) research in Europe reported that most nitrate amended to a forest ecosystem was retained in soils (Dise, 1995). Also, the rate of denitrification tends to be relatively low due to well-drained soil conditions (Nadelfohher, 2001). However, chronic nitrogen additions can saturate forest ecosystems with nitrogen, which can enhance the loss of retained nitrogen to the atmosphere by denitrification. At Hogwald Forest, Germany, significant amounts of nitrogen gas originated from nitrogen deposited on spruce and beech experimental plots (Butterbach et al., 2002). In forests having high

nitrate contents, the gaseous losses of nitrate were stimulated (Aber et al., 1995). Also, it was observed that tropical forests exhibited a higher rate of denitrification due to high moisture and nitrate contents. Therefore, increasing nitrogen deposition from the atmosphere and nitrogen fertilizer will stimulate denitrification rates in forest ecosystems, despite the overall nitrogen limitation.

**Agroecosystems**

Agroecosystems receive over 75% of their nitrogen from human activities. However, because this system has a low retention capacity for nitrogen, most nitrogen flows to other systems through denitrification or leaching (Galloway et al., 2004). Denitrification is a major process in nitrogen removal from agroecosystems; however, removal rates of nitrate by denitrification can vary substantially (Mosier et al., 2002). For example, the fraction of nitrogen removal via denitrification varied from 3% to 56% in flooded soils (Galbally et al., 1987; Freney et al., 1990). In an irrigated wheat field, 50% of the added nitrogen fertilizer was denitrified (Freney et al., 1992). Other research reported that only 1% to 4% of added nitrogen fertilizer was removed by denitrification (Mosier et al., 1986).

**Coastal Ecosystems**

Nitrogen input to coastal ecosystems has increased due to human activities. This increased nitrogen can influence the estuaries and coastal ecosystems, stimulating the growth of phytoplankton. Increased phytoplankton growth can suppress the growth of plants and benthic organisms due to reduced light availability and depleted oxygen. Thus, denitrification is an important mechanism for nutrient removal in coastal ecosystems (Canfield et al., 2005). Denitrification rates in coastal ecosystems are

regulated by organic matter loading, macrofauna, aquatic grasses, anoxic conditions, and nitrate (Cornwell et al., 1999).

**Freshwater and Riparian Ecosystems**

Frequent flooding, nitrogen inflow from terrestrial ecosystems, and the presence of vegetation in riparian ecosystems can affect denitrification rates. Green et al. (2004) estimated that globally, 50% of the nitrogen entering watersheds, including streams, is denitrified. Thus, denitrification is considered to be the most desirable ways of nitrate removal from watershed ecosystems.

In riparian ecosystems, seasonal variation in denitrification rates is important because the most concentrated nitrate inflow occurs during winter, coinciding with low microbial activity and plant uptake of nitrogen (Martin et al 1999). However, there is no typical relationship between denitrification rate and seasonality. In Chesapeake Bay, and the southwest of France, the denitrification rate tended to increase during winter (Weller et al 1994 and Pinay et al 1993). Other research found that denitrification rate was enhanced during spring and fall, and highest during summer in Kingston, Rhode Island (Groffman et al. 1996). This variability in denitrification rates is due to varying amounts of organic matter, the presence of vegetation, flooding times and oxygen levels (Reddy and Delaune, 2008). In addition, because denitrification rate is regulated by carbon availability, nitrification activity for nitrate supply, microbial activity, and optimal pH and temperature, it is expected that the lowest activity will be observed in deeper sediments (Martin et al., 1999). However, some research has found significant denitrification rates in deeper soils in riparian ecosystems (Sotomayor et al., 1996; Francis et al., 1989; Lind et al., 1989).

## Factors Affecting Denitrification Rates

### Nitrate

Forest ecosystems typically are not limited by oxygen, and thus microbes prefer to use oxygen rather than an alternative electron acceptor such as nitrate. In addition, plant and microbial growth typically suffers from nitrogen limitation in forest ecosystems, except for fertilized soils (Robertson and Tiedje, 1984; Davidson and Swank, 1987). However, in wetland ecosystems, nitrate is used more actively as an alternative electron acceptor due to absence of oxygen, and thus the nitrate availability can regulate denitrification rates in wetland soils (Reddy and DeLaune, 2008). Nitrate availability is also affected by the movement of nitrate into anaerobic sites from aerobic zones with nitrification, even though an external source of nitrate is present such as waste water, runoff, or precipitation.

### Organic carbon

The addition of carbon enhances denitrification rates because denitrifiers are heterotrophs, using organic carbon as an energy source (de Catanzaro and Beauchamp, 1985; Paul and Beauchamp, 1989). Research has shown that the denitrification rate is correlated with organic carbon concentrations (Reddy and DeLaune, 2008). However, carbon does not regulate denitrification rate in mineral soils with high organic matter, such as sludge-amended soil due to carbon saturation (Maier et al., 2000). Therefore, in carbon-limited conditions, the addition of a carbon source may enhance denitrification, while under high carbon conditions, the impact of nitrate addition on denitrification can be more apparent.

**Temperature**

Denitrification increases with temperature according to the $Q_{10}$ value. The optimal temperature ranges from 30ºC to 40ºC, although temperatures often fall outside this range (Tate, 2000).

**Plants**

The presence of plants can not only increase denitrification but also compete with denitrification. Plant uptake of nitrate can decrease the supply of electron acceptors for denitrifiers and inhibit the process. However, root exudates by plants can provide an energy source for denitrification, and oxygen released from plants can stimulate nitrification, enhancing the supply of nitrate to denitrifiers (Hopfensperger et al., 2009).

**Biochemistry of Denitrification**

Denitrification is the reduction of nitrate to nitrogen gas. Enzymes involved in the process include nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase. The common nitrate reduction process can be summarized as (Reddy and DeLaune, 2008):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O\ (gas) \rightarrow N_2\ (gas) \qquad (2\text{-}4)$$

Denitrifying bacteria are found in many divisions of the domain Bacteria. The majority of denitrifiers are heterotrophs and the overall process is following (Reddy and DeLaune, 2008):

$$5C_6H_{12}O_6\ (glucose) + 24NO_3^- + 24H^+ = 30CO_2 + 42H_2O + 12N_2$$

$$\Delta G0° = -14562.09\ kJ \cdot mole^{-1} \qquad (2\text{-}5)$$

**Microbial Community of Denitrifiers**

Denitrifiers are classified into three groups based on their energy source: organotrophs, which use organic matter, lithrotrophs, which use inorganic matter (e.g.

hydrogen or reduced sulfur compounds), and phototrophs, which use light as their energy sources. The most common denitrifiers are organotrophs (Tate, 2000). There are numerous bacterial genera that include strains capable of denitrification.

All molecular research on the denitrifying bacterial community uses functional gene coding for nitrate reductase such as *nirS* and *nirK*, and coding for nitrous oxide reductase such as *nosZ* (Bothe, 2000).

**Measurement of Denitrification Rates**

**Acetylene blocking method and measurement of nitrate consumption rates**

Direct measurement of nitrogen gas as the end product of denitrification is very difficult, because the atmosphere contains about 80% of nitrogen gas. Therefore indirect measurement methods are used. As the indirect method, acetylene blocking is the most extensively used technique of denitrification rate measurement because of its low cost, simplicity, and high sensitivity. The principle of this method is to measure the amount of nitrous oxide produced by denitrification in the headspace using gas chromatography after acetylene inhibits the reduction of nitrous oxide to nitrogen gas (Tiedje et al., 1988). However, acetylene inhibits nitrification and thus will underestimate denitrification rates when nitrate is low (Seitzinger et al., 1993). Another method is to measure the consumption rates of nitrate for a certain time interval. However, nitrate can be lost by immobilization, resulting in low sensitivity (Alef and Nannipieri, 1995).

**Measurement of the ratio of change relative to a conservative property**

This method is based on measuring the change in ratio of a conservative molecule (e.g. chloride or argon) to nitrate or nitrogen gas. When using chloride, the method assumes that nitrate and chloride have the same mobility. After the nitrate is consumed by denitrification, the ratio of nitrate to chloride will be decreased (Alef and Nannipieri,

1995). The measurement of the change in relative ratio of nitrogen to argon gas in the sample is premised on the fact that nitrogen gas production by denitrification can increase the ratio of nitrogen to argon gas. This method is not as sensitive compared to the methods mentioned above, but can give a representative measure of denitrification in large scale natural settings (Groffman et al., 2006).

**Isotope method**

Isotope method can improve the sensitivity and specificity of detection of denitrification. This method assumes a random association and homogenous distribution of $^{15}$N-nitrogen in the denitrification zone. After $^{15}$N-labeled nitrate is added to the sample and incubated, the isotopic composition of nitrogen gas is analyzed using a isotope ratio mass spectrometry following separation by gas chromatography (Groffman et al., 2006). The ratio of $^{15}$N-nitrogen gas ($^{14}N^{15}N$ to $^{14}N^{14}N$) is calculated as excess above their natural abundance (Thamdrup et al., 2000). However, enrichment of $^{15}$N-labeled nitrate can stimulate denitrification rates and it is difficult to mix $^{15}$N-labeled nitrate homogeneously into soils (Groffman et al., 2006).

## Dissimilatory Nitrate Reduction to Ammonium (DNRA)

Nitrite and nitrate are reduced to nitrogen gas in soils by anammox and denitrification, respectively. Thus, these processes play important roles in removal of nitrogen from soils. However, nitrate can be also reduced to ammonium by dissimilatory nitrate reduction to ammonium (DNRA) (Fewson et al., 1961). DNRA is performed by obligate anaerobes that live in highly reduced conditions such as lake sediments or permanently waterlogged wetlands (Reddy and DeLaune, 2008).

### Agricultural Soils

DNRA is desirable for agricultural soils because conserved nitrogen can be used by plants in a nitrogen limited environment (King et al., 1985). DNRA can also be a beneficial process in non-eutrophic environments as a supply of nutrient to organisms. Most research regarding DNRA in agroecosystems has focused on identifying favorable conditions for DNRA, in order to retain nitrogen in systems. For example, the addition of glucose and highly reduced conditions enhanced DNRA up to 5 times that of nitrate reduction in Chinese and Australian paddy soils (Yin et al., 2002). Also, in a cultivated field soil in France, the addition of carbon source with an increased TC:TN ratio was found to stimulate DNRA (Fazzolari et al., 1998). However, the contribution of DNRA to nitrate removal tends to be smaller than other processes (such as immobilization or denitrification) in agroecosystems.

### Freshwater and Riparian Ecosystems

Freshwater sediments and riparian wetlands have good conditions for DNRA due to the absence of oxygen and accumulation of organic matter (Matheson et al., 2002). For example, in unplanted riparian wetland soil, DNRA can be the principal mechanism of nitrate removal (49%), but with the presence of plants, denitrification was found to be the primary mechanism of nitrate removal (61% to 63%) due to increased nitrification via a supply of oxygen from root zone (Matheson et al., 2002). Typically, the overall proportion of nitrate removal due to DNRA is not larger (1% to 9%) than denitrification in freshwater and riparian ecosystems, except for in unplanted soils and sediments with high amounts of organic matter (Matheson et al., 2002).

### Estuarine and Coastal Ecosystems

Estuarine and coastal ecosystems have a mixture of freshwater and salt water. As a result, they may have relatively high amounts of organic matter flowing from the land. Denitrification in these ecosystems plays an important role in the removal of nitrate, preventing eutrophication of the ocean ecosystem. However, DNRA can adversely accelerate eutrophication through the accumulation of ammonium in coastal ecosystems. Estuarine and coastal ecosystems have higher amounts of organic matter and nutrients, and lower oxygen concentrations than terrestrial ecosystems, leading to favorable conditions for DNRA. Laguna Madre and Baffin and Concepcion Bays showed high rates of DNRA (0.656 to 32.94 mM $N \cdot m^{-2} \cdot day^{-1}$), resulting in eutrophication (An et al., 2002).

### Lakes

Organic matter accumulation through eutrophication in lakes leads to an increase in electron donor availability and a decrease in oxygen release, which can be favorable to DNRA. However, the presence of plants can increase the oxygen released into the rhizosphere, and this can stimulate denitrification via a supply of nitrate from nitrification rather than DNRA. Also, when nitrate is limited and the carbon is high, fermentative bacteria responsible for DNRA can be dominant due to the increased carbon contents. However, once nitrate was added, denitrifying bacteria became dominant due to the supply of an electron acceptor (Angelo et al., 1993).

## Factors Affecting DNRA Rates

### Nitrate

Under nitrate-limited conditions, the DNRA process is more efficient than denitrification, because DNRA consumes $8e^-$ per mole of nitrate reduced to ammonium

versus $5e^-$ for denitrification (Tiedje, 1988). In highly reduced and nitrate limited conditions, DNRA activity increased up to 20% (Ambus et al., 1992). However, in extremely nitrate-limited conditions such as a forest ecosystem, most nitrate was consumed by immobilization and less than 5% of nitrate was reduced via dissimilatory pathways such as DNRA (Bengtsson and Bergwall, 2000).

**Organic carbon**

The addition or increase of a carbon source such as glucose, DOC, or organic matter, increases DNRA rates (Fazzolari et al., 1998; Tobias et al., 2001; Kelso et al., 1999; Ambus et al., 1992). High DNRA rates can be expected in saturated and carbon-rich conditions, such as stagnant water, sewage sludge, high organic matter sediments, and rumens (Maier et al., 2000).

**Sulfate**

Reduced sulfur compounds (hydrogen sulfide, iron sulfide, and thiosulfate) can be used as electron donors for DNRA instead of organic matter. Thus, the addition of sulfide has been found to increase the oxidation of sulfide, while simultaneously reducing nitrate to ammonium (Brunet et al., 1996). In Laguna Madre and Baffin Bay, Texas, sulfide-induced DNRA contributed to the accumulation of ammonium (An et al., 2002).

**Plants**

Plants can provide oxygen to the soil profile via root respirations. However, DNRA microbes are fermentative and favor highly reduced conditions. Thus, the presence of plants can decrease DNRA rates. It has been reported that the presence of plants inhibits DNRA through the supply of oxygen in riparian and lake ecosystems (Matheson et al., 2002).

**Biochemistry of DNRA**

As mentioned above, the DNRA process consists of two steps: nitrate reduction to nitrite, and nitrite reduction to ammonium. Nitrate reduction to nitrite produces energy in most organisms (Betlach et al., 1982). However, the second step, reduction of nitrite to ammonium, does not create additional energy, but regenerates reducing equivalents through the reoxidation of $NADH_2$ to $NAD^+$. Theses reducing equivalents are then used to oxidize carbon substrates. Overall reaction is following (Tiedje, 1988):

$$C_6H_{12}O_6 + 3NO_3^- + 3H^+ = 6CO_2 + 3NH_3 + 3H_2O \quad (2\text{-}6)$$

The free energy is $\Delta G0' == -144$ kcal (Tiedje, 1988).

**Microbial Diversity of DNRA Bacteria**

Using a culture medium method, bacteria found to be capable of DNRA were *Clostridium* and *Bacillus* sp. (Caskey et al., 1979 and 1980). The ammonium producing isolates were *Clostridium* KDHS2, *Clostridium* KDHS3, and *Bacillus* KFHS6. Recent research has found that low nitrate concentrations resulted in a DNRA community composed of *Bacillus* strains in the rhizosphere of *Glyceria* maxima (Nijburg, 1997). It has also been reported that *Bacillus jeotgali*-related strains and two newly identified strains of GD0705 and GD0706 capable of DNRA are isolated in cultures from ammonium enriched soils (Fan et al., 2006). However, at present, research on microbial communities capable of DNRA has not been performed based on 16S rRNA or FISH (Fluorescent In Situ Hybridization). Therefore, the primer and probes used to detect DNRA bacteria need to be developed and used to investigate DNRA communities in natural ecosystems with DNRA rates.

### Measurement of DNRA Rates

When $^{15}N$-labeled nitrate is added to sediments, DNRA bacteria reduce the $^{15}N$ labeled-nitrate to $^{15}N$-ammonium. The $^{15}N$-ammonium contents are measured using a isotope ratio mass spectrophotometer equipped with an elemental analyzer.

## Summary

A review of the literature indicated that inorganic nitrogen transformation rates vary depending on ecosystem types (e.g. agriculture soils, forest soils, estuarine sediments, stream, or ocean water), plants, and biogeochemical properties (e.g. concentrations of nitrite, nitrate, ammonium, and organic carbon, and oxygen, pH, level of reduced conditions). Also, higher nitrification rates can lead to increased denitrification rates, while higher denitrification rates can inhibit anammox and DNRA rates due to competition for electron acceptors. However, most previous studies were performed in terrestrial soils, stream water, estuarine sediments, or ocean water. Research on inorganic nitrogen transformation rates in tributary sediments is limited, even though tributary sediments can function as sinks for nitrogen in watershed ecosystems. Also, all inorganic nitrogen transformation rates including nitrification, anammox, denitrification, and DNRA have not been thoroughly investigated. Furthermore, information on the relationships between microbial function and structure associated with these processes is limited. Thus, comprehensive research on inorganic nitrogen transformation rates and microbial assemblage structures associated with these processes is needed in tributary sediments.

# CHAPTER 3
# INORGANIC NITROGEN TRANSFORMATIONS IN TRIBUTARY SEDIMENTS

An excessive input of nitrogen to aquatic ecosystems can result in eutrophication, impairment of water quality, and human health problems such as a blue baby syndrome (Prasad and Power, 1995; Ward and Elliot, 1995; Sotomayor and Rice, 1996) and cancers (Prasad and Power, 1995; Starr and Gillham, 1993). It has been reported that riparian zones and tributaries in a watersheds can attenuate nitrogen through plant uptake, microbial immobilization, denitrification, and soil storage (Lowrance et al., 1997). Specifically, small streams have a higher efficiency of $NO_3^-$ removal than larger rivers because they have a smaller volume to surface ratio, increasing the possibility for benthic denitrification (Alexander et al., 2000).

Results from twelve headwater streams as parts of the Lotic Intersite Nitrogen eXperiment (LINX) demonstrated that $NH_4^+$ entering small streams was oxidized to $NO_3^-$ by nitrification, enhancing denitrification rates via a supply of $NO_3^-$ to denitrifiers (Peterson et al., 2001). Therefore, previous research by many scientists has focused on denitrification rates in small streams (Ceya et al., 1999; Mulholland et al., 2001 and 2004; Steinhart and Groffman, 1998). Several studies have documented that denitrification is a major contributor to nitrogen removal in tributary ecosystems (Steinhart and Groffman, 1998; Clausen et al., 2000; Lowrance et al., 1997; Korom, 1992). However, denitrification rates can be affected by other nitrogen transformations. For example, nitrification provides $NO_3^-$ to denitrifiers. Ammonification supplies $NH_4^+$ to nitrifiers, increasing nitrification rates. ANaerobic AMMonium OXidation (Anammox) can anaerobically oxidize $NH_4^+$ to $N_2$ (Dalsgaard et al., 2005), potentially decreasing $NH_4^+$ concentrations. Dissimilatory Nitrate Reduction to Ammonium (DNRA) potentially leads

to accumulation of $NH_4^+$ (An et al., 2002). Therefore, to understand the function of small streams with respect to N removal, further understanding is needed on relative rates of ammonification, nitrification, anammox, denitrification, and DNRA processes in tributary ecosystems.

Inorganic nitrogen transformation rates can be controlled by soil moisture content (Klemedtsson et al., 1988), hydraulic conductivity (Gilliam, 1994), temperature (William et al., 1997), different agricultural activities, (Martin et al., 1999), vegetation types (Haycock and Pinay, 1993), geomorphological properties (Opdyke et al., 2006), and seasonal variation (Rysgaard et al., 2005; Christensen et al., 1990). Many of these factors regulate the levels of electron donors and acceptors that can have profound influence on the relative rates of inorganic nitrogen transformation rates (Burford and Bremner, 1975; Reddy et al., 1982). For example, organic matter can increase denitrification and DNRA rates because these processes use organic matter as energy sources (Kaspar, 1983; Tobias et al., 2001; Yin et al., 2002). The concentrations of $NO_3^-$ and $NO_2^-$ can also control denitrification and anammox rates via supply of electron acceptors, respectively.

The critical zones for inorganic nitrogen transformations in tributaries are benthic sediments (Steinhart et al., 1998; Chatarpaul and Robinson, 1979; Hill, 1983; Swank and Caskey, 1982; Holmes et al., 1996, Mulholland and Hill, 1997) and riparian sediments (Bowden et al., 1992; Pinay et al., 1993 and 1995). Even though microbes in the water column of streams can participate in nitrogen cycling, the faster velocities of water flow and lower amounts of organic matter in stream waters can limit the removal of nitrate. However, stream sediments with high microbial activities can function as

effective sinks for nitrogen (Jones and Holmes, 1996). In addition, riparian sediments (the transitional area between upland and streams) can receive nitrogen and organic matter runoff from uplands (Freeze and Cherry, 1979; Hill, 1996; Hill and Waddington, 1993; Likens et al., 1977). Fluctuating water tables in riparian sediments creates alternative aerobic and anaerobic conditions that favor denitrification (Bowden et al., 1992; Pinay et al., 1993, 1995).

The Santa Fe River tributaries in the Boston Farm Santa Fe Ranch Beef Unit Research Center (SFBRU) receive nitrogen from nursery operations and carbon from dairy manure and surrounding vegetations. It was reported that a tributary with marsh and open water adjacent to the Santa Fe River had the buffer capacity for nitrogen removal through denitrification (Frisbee, 2007). Even though tributary systems have higher potential denitrification rates, their rates can be affected by other nitrogen transformations such as ammonification (Seitzinger, 1994; Gardner et al., 1987), nitrification (Jenkins and Kemp, 1984; Kemp and Dodds, 2002), anammox (Rysgaard et al., 2004; Rich et al., 2008), and DNRA (Rysgaard et al., 1996; Bonin et al., 1998; Michotey and Bonin, 1997). Thus, to understand nitrogen removal in tributaries of the Santa Fe River, I have developed the following objectives to determine relative potential rates of selected inorganic nitrogen transformations in tributary sediments.

The overall objective of this research was to investigate how biogeochemical properties influence potential inorganic nitrogen transformation rates in tributary sediments. The specific objectives were to:

- Determine potential rates of select inorganic nitrogen transformations in tributary sediments

- Investigate the relationships between potential rates of inorganic nitrogen transformations and biogeochemical properties in tributary sediments
- Compare potential rates of inorganic nitrogen transformations between stream and riparian sediments.

## Materials and Methods

### Site Description

The site for this research is tributary sediments (stream sediments and riparian sediments) at the Boston Farm Santa Fe Ranch Beef Unit Research Center (SFBRU) of the Santa Fe River Watershed, northern Alachua County, FL (Figure 3-1). Land uses on this site include a low intensity cattle operation with approximately 300 heifers on 1,600 acres and the nursery operation using nitrogen fertilizer (Holly Factory Nursery) (Frisbee, 2007).

One of two tributaries, Tributary 1 (T1) is located along the CR 241roadwaya and flows into a pond before entering into the Santa Fe River (Figure 3-1). The T1 system is surrounded by improved pastures and is impacted by ranch activity. The riparian sediment contains relatively high organic matter contents and receives water from groundwater and seepage water (Figure 3-1 (b)). The stream sediments receive water from stream and have relatively low organic matter contents (Figure 3-1 (b)). The vegetation type adjacent to the tributary sediments is a mixture of herbaceous and woody plants including *Carya* sp., *Pinus* sp., *Quercus* sp., *Magnolia grandiflora*, *Saururus cernuus*, *Juncus* sp., *Cephalanthus occidentalis*, *Hydrocotle umbellata*, and *Polygonum* sp.

Tributary 2 (T2) is affected by a nursery operation using nitrogen fertilizer ($NH_4NO_3$ and urea) at the headwater stream, drains cattle pasture on the SFBRU and flows into the Santa Fe River (Figure 3-1). The up-stream region of Tributary 2 (T2-U) is affected

by nitrogen fertilization and is surrounded with hardwood plants, including *Carya* sp., *Quercus* sp., and *Magnolia grandiflora* sp., and soft wood including of *Pinus* sp.

The downstream region of Tributary 2 (T2-D) is influenced by improved pastures and nitrogen fertilization from the head water (Figure 3-1). The land adjacent to T2-D is surrounded with grasses including *Saururus cernuus* sp., *Juncus* sp. and *Polygonum* sp., deciduous shrub plants including *Cephalanthus occidentalis* sp., and aquatic plants including *Hydrocotle umbellata* sp.(Frisbee, 2007).

**Sampling**

Samples were collected to a depth of 3 cm with a PVC core (diameter 7.5cm) at T1, T2-U and T2-D. Surface sediments (3 cm dept of sediments) were collected because surface sediments likely have much higher microbial activities compared to deeper sediments. Three samples from three locations (total of 9 samples) were collected in each tributary sediment in October 2007, January, April, and July 2008. Repeated sediment sampling (4 times) was considered as replicates for each sediment type. The sediments were transported to the laboratory on ice and stored at 4°C until analysis. Nine samples from each site were mixed to make a single composite sample. After mixing, triplicate sediments were distributed and prepared for the analysis. All roots and litter materials were removed from the sediment prior to analysis.

**Analyses of Biogeochemical Properties**

Sediment pH (1:1 ratio of sediments to water) was measured using a Fisher AR50 pH meter (Thomas, 1996). After sediment samples were extracted with 0.5 M $K_2SO_4$ (Bundy and Meisinger, 1994), extractable $NH_4^+$-N concentration was measured using a Seal AQ2+ automated Discrete Analyzer (EPA Method 350.1). Extractable $NO_3^-$-N concentrations was analyzed using an Alpkem Rapid Flow Analyzer 300 Series (EPA

Method 353.2). The $K_2SO_4$ extract was digested for total extractable organic nitrogen (Ext. Org N) via Kjeldahl block digestion. The concentration of Ext. Org N was analyzed using a Seal AQ2+ Automated Discrete Analyzer (EPA Method 351.1). Total extractable organic carbon concentration (Ext. Org C) was analyzed from the extract using a Shimadzu TOC-5050A Total Organic Carbon Analyzer equipped with a ASI-5000A auto sampler. Microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were extracted from sediments using a chloroform fumigation-extraction method (Brookes et al., 1985). Both the chloroform-fumigated and non-fumigated samples were extracted with 0.5 M $K_2SO_4$ for analyses of MBC and MBN. To measure MBC, each extracted sample was analyzed for extractable organic carbon (Ext. Org C) concentrations using a Shimadzu TOC-5050A Total Organic Carbon Analyzer equipped with an ASI-5000A auto sampler. To measure MBN, each extracted sample was digested for extractable organic nitrogen (Ext. Org N) via Kjeldahl digestion (Brookes et al., 1985). The concentrations of MBN were measured using a Seal AQ2+ Automated Discrete Analyzer (EPA Method 351.1). MBC and MBN concentrations were calculated by the difference between the fumigated and non-fumigated samples. The subsamples of sediments were dried at 70°C for 3 days and then ground using a ball grinder for total nitrogen (TN) and carbon (TC) analyses. TN and TC concentrations were measured on dry sediments using a Thermo Electron Corp. Flash EA 1112 Series NC Soil Analyzer (Nelson and Sommers, 1996; Bremmer, 1996).

**Potential Nitrogen Transformations Rates**

Potential rates were determined on triplicate sediments. For analysis of potential ammonification rates, 5 g of wet sediment and 5 ml of distilled de-ionized (DDI) water were added to 60 ml serum bottles. The sediments were purged with pure $N_2$ to create

anaerobic conditions and incubated for seven days at 24°C. After incubation, sediments were extracted using 0.5 M $K_2SO_4$ solution and extractable $NH_4^+$-N concentrations were measured using a Seal AQ2+ Automated Discrete Analyzer (Warning and Bremner, 1964) (EPA Method 350.1).

For analysis of potential nitrification rates, 10 g of wet sediment were added to an 160 ml open bottle. Sediments were amended with 1400 µg of $NH_4^+$-N (as $NH_4Cl$) followed by 30 ml DDI water. All samples were incubated in a Model 25 incubator shaker at 130 rpm (New Brunswick Scientific Co) for seven days at 24°C (Berg and Rosswall, 1995). After incubation, sediments were extracted using 0.5 M $K_2SO_4$ solution and extractable $NO_3^-$-N concentration was measured using an Alpkem Rapid Flow Analyzer 300 Series (EPA Method 353.2).

Potential anammox rates were determined using $^{15}N$-labeled $NH_4^+$-N (99 atom % $^{15}N$ as $^{15}NH_4Cl$) and $^{14}NO_2^-$-N (as $NaNO_2$) amended to sediments (Kuypers et al., 2003). Ten grams of wet sediments were added to a 160 ml serum bottle and purged with $N_2$ for 15 minutes to create anaerobic conditions. Sediments spiked with 1500 µg of $^{15}N$-labeled $^{15}NH_4^+$-N (99 at% $^{15}N$ as $^{15}NH_4Cl$ ) and 1400 µg of $^{14}NO_2^-$-N (as $NaNO_2$) using a syringe. All bottles (with sediments) were incubated in a Model 25 shaker incubator (New Brunswick Scientific Co.) at 130 rpm for seven days at 24°C. After incubation, the gas in the headspace was extracted into a 2 ml serum vacuum bottle. The ratios of $^{28}N_2$ to $^{29}N_2$ were measured using a isotope mass spec Costech Instrument Elemental Analyzer (Kuypers et al., 2003; Dalsgaard et al., 2005). The potential rates of anammox were calculated by the difference in the ratios of $^{28}N_2$ to $^{29}N_2$ between natural abundance and samples. The ratio of $^{15}N$-labeled $N_2$ ($^{28}N_2$ and $^{29}N_2$) was calculated as

excess above their natural abundance using the equation suggested by Hauck et al. (1958).

Potential denitrification rates were determined by an acetylene blocking method (Tiedje, 1999). Ten grams of wet sediments were added to a 160 ml serum bottle and purged with $N_2$ for 15 minutes to create anaerobic conditions. Each bottle was amended with 1400 µg of $NO_3^-$-N (as $KNO_3$) per 10 g of wet sediments and 20 ml of acetylene gas (12.5% of 160 ml serum bottle). $N_2O$ was measured at pre-determined time intervals up to 4.5 hours using a Shimadzu Gas Chromatograph 14-A (GC) (Tiedje, 1999). Potential denitrification enzyme activity (DEA) was also measured as above. However, 1.44 mg of $C_6H_{12}O_6$-C (glucose) and 280 µg of $NO_3^-$-N (as $KNO_3$) were added to 10 g of wet sediments for supply of sufficient carbon to denitrifiers (Tiedje, 1999).

For analysis of potential DNRA rates, 10 g of wet sediments were added to a 160 ml serum bottle and purged with $N_2$ for 15 minutes to create anaerobic conditions. Using a syringe, 1500 µg of $^{15}$N-labeled $NO_3^-$-N (99 at%$^{15}$N as $K^{15}NO_3$) per 10 g of wet sediments were added to each bottle and mixed with sediments. All bottles were placed in a Model 25 incubator shaker at 130 rpm (New Brunswick Scientific Co. INC) for seven days at 24°C. After incubation, sediments were extracted with 2 M KCl. The $^{15}NH_4^+$-N in the extracted solution was converted to $^{15}NH_3$ on an acid disk using a diffusion technique (Stark and Hart, 1996). The ratios of $^{15}NH_4^+$-N to $^{14}NH_4^+$-N on the acid disk were analyzed with a Thermo Finnigan MAT Delta Plus XL Mass Spectrophotometer equipped with a Costech Instrument Elemental Analyzer for flash combustion of solid material for N analysis (Rysgaard-Petersen and Rysgaard, 1993).

## Statistical Analysis

Statistical analysis was conducted using JMP version 8.0 (SAS 2007). One way analysis of variance test (ANOVA) was performed to investigate difference in biogeochemical properties and potential inorganic nitrogen transformation rates between sites. Least significant difference at the 5% confidence level was used for comparisons. All post comparisons of means were accomplished using a Tukey-Kramer HSD test, which adjusted for the overall error rates. Regression analysis was performed to determine if any relationships existed among these parameters using a Standard Least Squares model.

# Results

## Biogeochemical Properties

The lowest pH of stream sediments was observed in T2-U with an average pH of 5.0. The highest $NO_3^-$-N concentration was observed in stream sediments of T2-U and T2-D ($p<0.05$). However, there were no significant differences in $NH_4^+$-N, Ext. Org N, MBN, and TN concentrations among the sites (Tables 3-1 and 3-2). No differences in Ext. Org C, MBC, and TC concentrations among the sites were found in stream sediments (Tables 3-1 and 3-2). No significant differences between sites were observed for TC:TN and MBC:MBN ratios in stream sediments (Table 3-2).

In riparian sediments, the pH in T2-U (pH 4.6) was lower than that of T2-D (pH 6.2) ($p<0.05$). T2-U had higher $NO_3^-$-N, Ext. Org C, and MBC concentrations than those of T2-D ($p<0.05$). However, $NO_3^-$-N, Ext. Org C, and MBC concentrations of T2-U were not significantly different from those of T1. Ammonium and Ext. Org N concentrations were not different between sites (Tables 3-1). T2-D had the lowest concentrations of TN and TC in riparian sediments ($p<0.05$) (Table 3-2).

**Inorganic Nitrogen Transformations Rates**

To understand inorganic nitrogen transformation rates, potential ammonification, ammonium oxidation (nitrification and anammox) and nitrate reduction rates (denitrification and DNRA) were measured. The results of potential inorganic nitrogen transformation rates are summarized in Table 3-3.

When comparing potential inorganic nitrogen transformation rates between sites, potential nitrification rates in T2-U were lower than in T2-D of stream sediments ($p<0.05$) (Table 3-3). For riparian sediments, T2-U exhibited the lowest potential nitrification rates compared to T1 and T2-D ($p<0.05$). Potential denitrification rates were highest in T2-D compared to T1 and T2-U in riparian sediments ($p<0.05$). No differences in potential ammonification, anammox, and DNRA rates were observed between sites (Table 3-3).

When considering the relative importance of potential inorganic nitrogen transformation rates in stream sediments, 5%, 1% and 11% of added $NH_4^+$-N were aerobically oxidized to $NO_3^-$-N by nitrification in T1, T2-U and T2-D, respectively. Of the $NH_4^+$-N added, 0.07% to 0.08% was anaerobically oxidized to $N_2$ by anammox in T1, T2-U, and T2-D. Approximately 28% of added $NO_3^-$-N was reduced to $N_2$ by denitrification in stream sediments. Of the added $NO_3^-$-N, 0.54%, 0.23%, and 0.11% was reduced to $NH_4^+$-N by DNRA in stream sediments of T1, T2-U and T2-D, respectively (Table 3-4). Thus, most of added $NO_3^-$-N was reduced to $N_2$ rather than to $NH_4^+$-N in stream sediments.

For riparian sediments, 25% to 28% of added $NH_4^+$-N was aerobically oxidized to $NO_3^-$-N by nitrification in T1 and T2-D. However, only 7% of added $NH_4^+$-N was aerobically oxidized to $NO_3^-$-N by nitrification in T2-U. Approximately 0.09% of added $NH_4^+$-N was anaerobically oxidized to $N_2$ by anammox in riparian sediments. Thus, most

of added $NH_4^+$-N was aerobically oxidized to $NO_3^-$-N rather than to $N_2$ in riparian sediments. In case of nitrate reduction, 68% and 63% of added $NO_3^-$-N was reduced to $N_2$ by denitrification in T1 and T2-U, respectively; however, 95% of $NO_3^-$-N added to T2-D was reduced to $N_2$ by denitrification in riparian sediments. Between 0.54% to 1.11% of added $NO_3^-$-N was reduced to $NH_4^+$-N by DNRA in all sites (Table 3-4). Thus, most of added $NO_3^-$-N was reduced to $N_2$ rather than to $NH_4^+$-N in riparian sediments.

**Relationships between Inorganic Nitrogen Transformation Rates and Biogeochemical Properties**

Potential nitrification rates were positively correlated with pH in stream sediments ($R^2$=0.83, p=0.0001, Figure 3-2 (a)). Riparian sediments also exhibited a weak positive relationship with potential nitrification rates and pH ($R^2$=0.46, p= 0.015, Figure 3-2 (b)). Potential denitrification rates exhibited an exponentially negative correlation with TC:TN ratio of riparian sediments ($R^2$=0.48, p=0.013, Figure 3-3 (a)). Ext. Org C was weakly positively correlated with potential DNRA rates in riparian sediments ($R^2$=0.4, p= 0.0358, Figure 3-3 (b)). Potential ammonification rates were linearly correlated with $NH_4^+$-N concentrations in riparian sediments ($R^2$=0.58, p=0.0065, Figure 3-4).

**Comparisons of Inorganic Nitrogen Transformation Rates between Stream and Riparian Sediments**

In order to investigate how the location of tributary sediments affects potential inorganic nitrogen transformation rates, the rates of stream and riparian sediments were compared. The stream sediments are located in the stream water and the interface between sediments and stream water. The stream sediments contain an average of 0.4% of total carbon in T1, T2-U and T2-D. The riparian sediments are located at the transitional zones between stream water and upland soils, and thus receive the organic material from uplands. The total carbon was 4.1%, 4.8% and 2% in T1, T2-U and T2-D,

respectively. Therefore, the stream and riparian sediments can be categorized as low and high organic matter systems, respectively. Riparian sediments exhibited higher potential ammonification, nitrification, denitrification, anammox, and DNRA rates than those of stream sediments ($p<0.05$) (Tables 3-3).

## Discussion

### Inorganic Nitrogen Transformations

Ammonium added to stream and riparian sediments was primarily oxidized to $NO_3^-$ by nitrification. Also, denitrification was the dominant process involved in removal of added $NO_3^-$-N as compared to DNRA in stream and riparian sediments. Thus, nitrogen cycling in tributary sediments was mainly regulated by nitrification and denitrification rather than anammox and DNRA.

Potential nitrification rates in tributary sediments were lower in T2-U than in T2-D due to low pH. Other studies also reported the inhibition of pH to nitrification rates in various ecosystems (Srna and Baggaley, 1975, Ward, 1987; Engel et al., 1958; Wild et al., 1971; McHarness and McCarty 1973; Ste-Marie and Pare, 1999; Kyveryga et al., 2004). Nitrification rates in my study ranged from 0.2 to 8.8 mg N·kg dry sediment$^{-1}$·day$^{-1}$ and were higher than reported for stream sediments in Indiana, USA (ranging from 0.1 to 0.5 mg N·kg soil$^{-1}$·day$^{-1}$) (Strauss and Lamberti, 2002) (Table 3-5).

Added $NH_4^+$-N removal through anammox was insignificant compared to nitrification in tributary sediments. Potential anammox rates in my study ranged from 0.07 to 0.12 mg N·kg dry sediment$^{-1}$·day$^{-1}$, accounting for less than 0.1% removal from added $NH_4^+$-N. Rates in my study was lower than those reported for a tidal marsh (average of 0.36 mg N·kg soil$^{-1}$·day$^{-1}$), which were accounted for less than 1% of total nitrogen production (Table 3-5), and lower than those reported for estuarine sediments

of Chesapeake Bay (ranging from 2.9 mg N·kg soil$^{-1}$·day$^{-1}$), accounting for 0 to 22% of total nitrogen production (Rich et al., 2008; Koop-Jakobsen and Gibli, 2009) (Table 3-5). Nitrogen removal rates through anammox were significant in ocean water compared to my results; anammox accounted for 45% to 50% of total nitrogen productions in ocean ecosystems (Kuypers et al., 2003 and 2005). In addition, $\delta^{15}N$ values ($\delta^{15}N=[(R_{sample}/R_{air})-1]*1000$) of my samples range from 0.41 to 10‰ and this range could belong to minimum detection value of measurement. Thus, anammox rates of my samples can be overestimated because of low δ values and it needs to be caution to interpret the anammox rates in my study.

Potential denitrification rates were similar to those reported for riparian wetlands, constructed wetlands, and plant associated sediments (Clément et al., 2002; Hill et al., 2004; Sirivedhin and Gray, 2006; Schaller et al., 2004) (Table 3-6). Approximately, 26% to 95% of added $NO_3^-$-N was converted to $N_2$, suggesting the denitrification was the dominant process in tributary sediments.

Potential DNRA rates in my study were lower than those reported for estuarine sediments (ranging from 3.4 to 17mg N·kg soil$^{-1}$·day$^{-1}$) (Jørgensen, 1989). DNRA accounted for less than 1.2 % of from added $NO_3^-$-N in my research; however, other studies exhibited higher percentage of total nitrogen production by DNRA ranging from 4% to 75% in estuarine sediments, ocean water, and river (Omnes et al., 1996; Jørgensen, 1989; An and Gardner, 2002) (Table 3-6). Additionally, $NO_3^-$ removal through DNRA was less significant compared to denitrification in tributary sediments. Thus, denitrification plays a major role in reducing $NO_3^-$ compared to DNRA process in my research sites.

**Relationship between Nitrogen Transformations and Biogeochemical Properties**

Potential nitrification rates exhibited a positive relationship with increasing pH in stream sediments ($R^2$=0.83, p=0.0001, Figure 3-2 (a)). Generally, as pH decreases, the rate of nitrification also declines (Sharmmas, 1986). Since $NH_3$ (ammonia) rather than $NH_4^+$(ammonium) serves as an electron donor for the ammonia mono-oxygenase enzyme, the ratio of $NH_3$ to $NH_4^+$ can be a critical factor for determining the nitrification rate (Ward et al., 1987; Suzuki et al., 1974). The ratio of $NH_3$ to $NH_4^+$ is drastically reduced with decreasing pH (Ford et al., 1980). Thus, at a lower pH, the substrate may not be sufficient for the ammonia mono-oxygenase enzyme to function (Huesemann et al., 2002). Previous studies have confirmed the inhibition of nitrification at low pH values in marine ecosystems (Srna and Baggaley, 1973; Wickins , 1983; Ward et al., 1987; Engel et al., 1958; Wild et al., 1971; McHarness et al., 1972) and in forests and agricultural soils (Ste-Marie et al., 1999; Kyveryga et al., 2004). In thirty six streams in the Midwestern United States, a positive relationship between nitrification rate and pH was observed, suggesting that nitrification may be inhibited at low pH (Strauss et al.,2002). In addition**,** it was reported that nitrification began to slow in the pH range of 4 to 6 (Alexander,1965; Schmidt, 1982). The lowest limits for autotrophic nitrifiers have been reported to range from pH 4.0 and 4.7 in forest soils (Sahrawat, 1982; De Boer et al.,1989; Persson and Wiren, 1995). Thus, at pH from 4 to 6 in stream sediments, nitrification rates was strongly inhibited by pH, while after pH 6, nitrification rate could be increased. As a result, the potential nitrification rates could increase sharply after pH 6 in stream sediments. For riparian sediments, potential nitrification rates exhibited a weaker correlation with pH than stream sediments ($R^2$=0.46, p=0.015, Figure 3-2 (b)). Riparian sediments contain higher $NH_4^+$ concentrations than stream sediments (p<0.05,

Table 3-1). Thus, the nitrifiers in riparian sediments could have higher opportunities to use $NH_4^+$ than those in stream sediments. As a result, the effect of pH inhibition on nitrification rates was less in riparian sediments than stream sediments.

The factors regulating denitrification are organic carbon, $NO_3^-$, $O_2$, temperature, and vegetation types (Tate, 2000). Of these factors, organic carbon can be a critical factor for determining denitrification rate because organic carbon is the major sources of electron donors for denitrifiers. Previous researchers have exhibited a positive relationship between organic matter availability and denitrification rates in agricultural and forest soils (Burford and Bremner, 1975; Bijay-Singh et al., 1988; Boyer and Groffman, 1996), riparian buffer zones (Hill et. al., 2004; Rotkin-Ellman et al., 2004), and created and restored wetlands (Poe et al., 2003; Teiter and Mander, 2005; Sirivedhin et al., 2006). However, the quality of organic carbon is also important for determining denitrification rates in tributary ecosystems (Beauchamp et al., 1989; Hernadez and Mitsch, 2007; Bachand and Horne, 2000). In my study, extractable organic carbon contents were not higher in T2-D, even though it exhibited the highest denitrification rate. However, the TC:TN ratios of riparian sediments were negatively correlated with denitrification rates. This means that the quality of organic matter could influence the amounts of carbon available to denitrifiers, because TC:TN ratio is related to the degradation rate of organic matter. For example, research on the decomposition of crop residue has shown that plant material with a lower TC:TN ratio became an available carbon source to denitrifiers, increasing denitrification rates (Beauchamp et al., 1989). However, plant material with high lignin and low cellulose contents like higher TC:TN ratios, tended to supply less available organic carbon to denitrifiers because lignin is

more resistant to decomposition than cellulose. Studies in constructed wetlands indicated that plant species with high cellulose contents such as *Typha* or mixed vegetation of macrophytes and grasses, released more available carbon to denitrifiers than did bulrush plants such as *Schoenoplectus tabernaemontani* or *Scirpus* species with relatively high lignin and low cellulose contents, leading to higher rates of denitrification (Hernadez and Mitsch, 2007; Bachand and Horne, 2000). Schipper et al. (1994) also reported that the addition of watercress and fresh pine needles led to an increase in denitrification rate of up to five times higher than the addition of senescent pine needles in riparian soils, emphasizing the importance of organic carbon quality rather than carbon quantity. Bremner and Shaw (1958) suggested that TC:TN ratio of 3:1 for labile organic carbon sources optimized denitrification, whereas TC:TN ratio of 70:1 was required for more ligneous organic carbon sources.

A negative relationship between TC:TN ratio and denitrification rates can be also explained by increased immobilization. Previous research using $^{15}NH_4^+$-N and $^{15}NO_3^-$-N exhibited that microbes immobilized both $NO_3^-$ and $NH_4^+$, even though $NH_4^+$ uptake was greater (Vitousek and Matson, 1988; Fenn et al., 1998). Under high TC:TN ratio conditions, microbes prefer to immobilize inorganic nitrogen rather than mineralize organic nitrogen to $NH_4^+$ (Chapin et al, 2002). Thus, high TC:TN ratio in tributary sediments could lead to decrease in denitrification by removing $NO_3^-$ through immobilization. Previous research also reported that higher TC:TN ratio of substrates and additions of carbon sources enhanced immobilization of $NO_3^-$, resulting in decreased denitrification (Craswell, 1978; Rivera-Monroy and Twilley, 1996; Gők and Ottow, 1988).

DNRA regenerates reducing equivalents through the reoxidation of $NADH_2$ to $NAD^+$ simultaneously with oxidation of carbon substrates. Thus, it has been demonstrated that DNRA was favored by rich carbon conditions (Tiedje et al., 1988; Fazzolari et al., 1998; Sylvia et al., 2005). My research also indicated that Ext. Org C was weakly positively correlated with potential DNRA rates, implying that organic carbon content could be one of factors for determining DNRA rates in riparian sediments. However, other factors can also affect potential DNRA rates. For example, added $NO_3^-$ can be consumed by denitrification and immobilization by microbes. Thus, these other processes might affect DNRA rates, resulting in a weak correlation between potential DNRA rates and organic carbon concentrations in riparian sediments. Previous research on the DNRA process has examined coastal and estuarine sediments, demonstrating a 4% to 75% of $NO_3^-$ reduction by DNRA (Christensen et al., 2000; Jørgensen, 1989; An and Gardner, 2002). In tropical forest soils, the DNRA rate (0.6 mg $N \cdot kg\ soil^{-1} \cdot day^{-1}$) was three times greater than denitrification rate and resulted in a reduction of $NO_3^-$ availability to denitrifiers (Silver et al., 2001). In the hyporheic zone of streams (Storey and Williams, 2004), DNRA occurred and outcompeted denitrification for consuming $NO_3^-$, as well as in the paddy soils in China (Yin et. al., 2002), a riparian wetland in New Zealand (Matheson et al., 2002), and river sediments in the UK (Takeuchi, 2006).

### Comparison of Inorganic Nitrogen Transformation Rates between Stream and Riparian Sediments

All sites demonstrated that riparian sediments exhibited higher ammonification, nitrification, denitrification, anammox, and DNRA rates than those of stream sediments. Riparian sediments are characterized by high input of terrestrially derived water and

ground and seepage water, accumulation of dissolved $NO_3^-$ and $NH_4^+$, supply of carbon from upland soils, and long hydraulic residence times (Hedin et al., 1998; Peterjohn, 1984; Lowrance et al., 1985; Cooke and Cooper, 1988; Cooper, 1990; Hill et al., 1990; Mulholland et al.,1992). Thus, riparian sediments are the hotspot for nitrogen cycling in tributary ecosystems due to larger supply of electron donors and acceptors compared to stream sediments. In contrast, for the stream sediments, the rapid flow of stream water prevents organic matter from being accumulated and shortens the residence time of nutrients in the sediments (Jones and Holmes, 1996; Alan, 1995), resulting in lower rates of inorganic nitrogen transformation rates (Hill et al., 1996). Even though low denitrification, nitrification and DNRA rates in stream sediments compared to riparian sediments, previous research has suggested that hyporheic zone functioned as a inorganic nitrogen sink in stream ecosystems (Stanley and Jones, 2000). Mixing of downwelling surface water and upwelling groundwater in hyporheic zones creates the diffusions of inorganic nitrogen and organic carbon concentrations between stream sediments and stream water, which leads to increased denitrification rates. Hyporheic zones in N rich agricultural stream were reported to remove inorganic nitrogen from the stream water (Jones and Holmes, 1996; Hill et al., 1996). Thus, unlike stream sediments, hyporheic zone needs to be reconsidered a hot spot for inorganic nitrogen cyclings, linking to hydraulic conductivity between groundwater and surface water.

**Summary**

In the study tributaries, $NO_3^-$ and $NH_4^+$ concentrations in stream water (ranging 2 to 7 $mg \cdot l^{-1}$ for $NO_3^-$-N and 0.2 to 0.4 $mg \cdot l^{-1}$ for $NH_4^+$-N inT1, T2-U, and T2-D; Data not shown) exceeded the national background level (0.6 $mg \cdot l^{-1}$ for $NO_3^-$-N and 0.1 $mg \cdot l^{-1}$ for $NH_4^+$-N). However, tributary sediments can function as potential sinks for $NO_3^-$ through

denitrification. These results indicated that the relative rates of $NO_3^-$ removal through denitrification were higher than those of DNRA in these tributary sediments. Also, the $NH_4^+$ removal rates through nitrification were significant compared to anammox. Thus, removal rate of inorganic nitrogen in tributary sediments were mainly regulated by nitrification and denitrification rather than anammox and DNRA.

Potential nitrification rates in tributary sediments were lower with lower pH, likely due to the decreased ratio of $NH_3$ to $NH_4^+$ under acidic conditions. Potential denitrification rates were lower with higher TC:TN ratios in riparian sediments, implying that the quality of organic carbon can be one of the major factors controlling denitrification rates in tributary sediments. Also, sediments with higher TC:TN ratios could enhance immobilization of $NO_3^-$, resulting in decreased denitrification. In addition, potential DNRA rates were regulated by organic carbon contents in riparian sediments, implying that high organic carbon contents could enhance DNRA rates. Thus, when we add organic carbon to soils for removing $NO_3^-$ through denitrification, we have to consider the influence of organic carbon on immobilization, carbon quality, and DNRA rates in the systems.

Table 3-1. Summary of the pH, nitrate-N ($NO_3^-$-N), ammonium-N ($NH_4^+$-N), extractable organic nitrogen (Ext. Org N), microbial biomass nitrogen (MBN), extractable organic carbon (Ext. Org C), and microbial biomass carbon (MBC) of tributary sediments (n=4, $p<0.05$, Characters not labeled by same letter are significantly different at 95% confidence level).

| | | pH (pH) | $NO_3^-$N | $NH_4^+$-N | Ext. Org N | MBN | Ext. Org C | MBC |
|---|---|---|---|---|---|---|---|---|
| | | | | | (mg·kg dry sediment$^{-1}$) | | | |
| Stream sediments | T1 | 5.4 (±0.4) | 0.7 (±0.3)$^{b}$ | 5.9 (±0.7) | 8 (±2) | 19(±7) | 58 (±9) | 308 (±91) |
| | T2-U | 5.0 (±0.1) | 1.6 (±0.3)$^{a}$ | 5.2 (±0.7) | 6 (±3) | 13 (±7) | 63 (±28) | 329 (±88) |
| | T2-D | 5.8 (±0.3) | 1.6 (±0.2)$^{a}$ | 5.3 (±0.9) | 7 (±1) | 24 (±7) | 51 (±5) | 334 (±29) |
| Riparian sediments | T1 | 5.4 (±0.4)$^{ab}$ | 0.9 (±0.4)$^{ab}$ | 15 (±4) | 13 (±7) | 28 (±9)$^{b}$ | 95 (±24)$^{ab}$ | 812(±325)$^{ab}$ |
| | T2-U | 4.6 (±0.4)$^{b}$ | 1.4 (±0.5)$^{a}$ | 13 (±3) | 25 (±12) | 91 (±14)$^{a}$ | 133 (±14)$^{a}$ | 1184(±184)$^{a}$ |
| | T2-D | 6.2 (±0.1)$^{a}$ | 0.5 (±0.1)$^{b}$ | 19 (±5) | 7 (±2) | 38 (±20)$^{ab}$ | 84 (±24)$^{b}$ | 472 (±64)$^{b}$ |

Table 3-2. Summary of the total nitrogen (TN), total carbon (TC), ratio of carbon to nitrogen (TC:TN), and ratio of microbial biomass carbon to microbial biomass nitrogen (MBC:MBN) of tributary sediments (n=4 for each site, Characters not labeled by same letter are significantly different at 95% confidence level).

| | TN($g \cdot kg^{-1}$) | | TC($g \cdot kg^{-1}$) | | TC:TN | | MBC:MBN | |
|---|---|---|---|---|---|---|---|---|
| | Stream sediments | Riparian sediments | Stream sediments | Riparian sediments | Stream sediment | Riparian sediments | Stream sediments | Riparian sediments |
| T1 | 0.3 (±0.2) | 2.0 (±0.4)[a] | 3 (±1.2) | 41 (±7)[a] | 14 (±2) | 20 (±1) | 35 (±19) | 28 (±18) |
| T2-U | 0.4 (±0.2) | 2.6 (±0.5)[a] | 4 (±1.4) | 48 (±10)[a] | 12 (±2) | 18 (±1) | 23 (±7) | 14 (±2) |
| T2-D | 0.4 (±0.2) | 1.2 (±0.3)[b] | 5 (±1.5) | 20 (±3)[b] | 17 (±5) | 17 (±2) | 21 (±9) | 19 (±4) |

Table 3-3. Summary of the potential ammonification (PA), nitrification (PN), anammox (PAn), denitrification (PD), denitrification enzyme activity (DEA), and DNRA (DNRA) rates of the tributary sediments (n=4 for each site, * is the not detected value, Characters not labeled by same letter are significantly different at 95% confidence level).

| Sediments | Sites | PA | PN | PAn | PD | DEA | DNRA |
|---|---|---|---|---|---|---|---|
| | | | | (mg N·kg dry sediment$^{-1}$·day$^{-1}$) | | | |
| Stream sediments | T1 | 0.02 (±0.01) | 1.2 (±0.4)$^{ab}$ | 0.08 (±0.01) | 1.5 (±0.2) | 10 (±8) | 0.13 (±0.1) |
| | T2-U | * | 0.2 (±0.04)$^{b}$ | 0.07 (±0.02) | 1 (±0.3) | 3 (±2) | 0.06 (±0.04) |
| | T2-D | 0.1 (±0.1) | 2.6 (±0.7)$^{a}$ | 0.09 (±0) | 5.7 (±4) | 16 (±11) | 0.03 (±0.03) |
| Riparian sediments | T1 | 0.9 (±0.4) | 7.5 (±1)$^{a}$ | 0.11 (±0.03) | 15 (±2)$^{b}$ | 68 (±49) | 0.33 (±0.17) |
| | T2-U | 1.1 (±0.4) | 2.4 (±0.9)$^{b}$ | 0.12 (±0.01) | 14 (±1)$^{b}$ | 96 (±82) | 0.22 (±0.09) |
| | T2-D | 3.7 (±1.7) | 8.8 (±1.2)$^{a}$ | 0.1 (±0.03) | 39 (±12)$^{a}$ | 65 (±24) | 0.28 (±0.09) |

Table 3-4. Summary of the relative percentage of nitrogen retained in each inorganic nitrogen transformations from the $NH_4^+$-N or $NO_3^-$-N applied in tributary sediments of the T1, T2-U and T2-D systems (Additions of 1400μg of $NH_4^+$-N to 10g wet sediments for nitrification, 1500μg of $NH_4^+$-N to 10g wet sediments for anammox, and 1500μg of $NO_3^-$-N to 10g wet sediments for denitrification and DNRA).

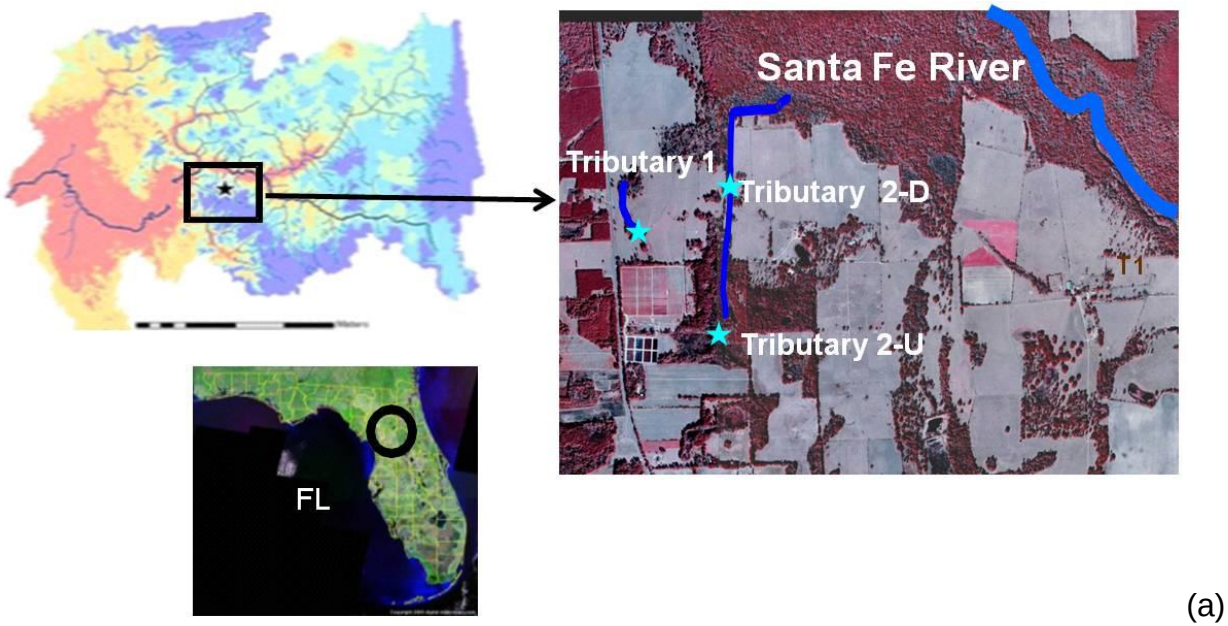
| Sediments | Sites | Nitrification | Anammox | Denitrification | DNRA |
|---|---|---|---|---|---|
| | | (% of added $NH_4^+$-N) | | (% of added $NO_3^-$-N) | |
| Stream sediments | T1 | 5 | 0.08 | 26 | 0.54 |
| | T2-U | 1 | 0.07 | 27 | 0.23 |
| | T2-D | 11 | 0.08 | 30 | 0.11 |
| Riparian sediments | T1 | 25 | 0.09 | 68 | 1.11 |
| | T2-U | 7 | 0.08 | 63 | 0.54 |
| | T2-D | 28 | 0.1 | 95 | 0.89 |

Table 3-5. Literature review for ammonium oxidation rates in various ecosystems.

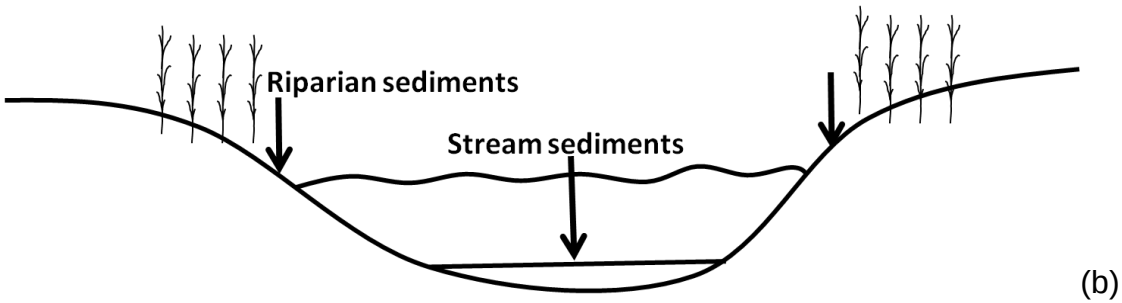
| | Type | Location | Rates ($mg\ N \cdot kg\ soil^{-1} \cdot day^{-1}$) | % of total N production | Reference |
|---|---|---|---|---|---|
| Nitrification | Lake sediments | Aarhus, Denmark | 0.001-0.73 ($g\ N \cdot m^{-2} \cdot day^{-1}$) | | Jensen et al., 1993 |
| | River | Colorado, USA | 0.01-0.29 ($mg\ N \cdot L^{-1} \cdot day^{-1}$) | | Sjodin et al., 1997 |
| | Lake sediments | New York, USA | 0.37 ($g\ N \cdot m^{-2} \cdot day^{-1}$) | | Pauer & Auer, 2000 |
| | Stream sediments | Ontario, Canada | 0-180 ($mg\ N \cdot m^{-2} \cdot day^{-1}$) | | Wayer, 1988 |
| | Stream sediments | Indiana, USA | 0.1-0.5 | | Strauss & Lamberti, 2002 |
| | Forest soils | Vermont, USA | 8 | | Kaur et al., 2010 |
| | Tributary sediments | Santa Fe River, FL | 0.2-8.8 | 1-28% | My study |
| Anammox | Marine sediments | Skagerrak, Norway Long Island Sound, USA | 0.18-1.2 ($mg\ N \cdot L^{-1} \cdot day^{-1}$) | 4-79% | Engström et al., 2005 |
| | Estuarine sediments | Chesapeake Bay, USA | 2.9 | 0-22% | Rich et al., 2008 |
| | Marine sediments | Greenland, Denmark | 0.01-1.3 ($mg\ N \cdot m^{-2} \cdot day^{-1}$) | 1-35% | Rysgaard et al., 2004 |
| | Lake | Tanganyika | 3.36 ($\mu g\ N \cdot m^{-2} \cdot day^{-1}$) | 13 % | Schubert et al., 2006 |
| | Tidal marsh | New England, USA | 0.36 | < 1 % | Koop-Jakobsen & Gibli, 2009 |
| | Tributary sediments | Santa Fe River, FL | 0.07-0.12 | < 0.1 % | My study |

Table 3-6. Literature review for nitrate reduction rates in various ecosystems.

| | Type | Location | Rates (mg N·kg soil$^{-1}$·day$^{-1}$) | % of total N production | Reference |
|---|---|---|---|---|---|
| Denitrification | Riparian sediments | River Morand, Swiss | 0.1-0.9 | | Cosandey et al., 2003 |
| | Constructed wetland | Illinois, USA | 1-22 | | Sirivedhin & Gray, 2004 |
| | Riparian sediments | Toronto, Canada | 2-30 | | Hill et al., 2002 |
| | Riparian wetlands | Brittany, France | 1-8.6 | | Clément et al., 2002 |
| | Forest wetland | Northeast, Spain | 1-3 | | Bernal et al., 2007 |
| | Plant associated sediments | Illinois, USA | 29 | | Schaller et al., 2004 |
| | Riparian wetland | Maryland, USA | 0.96-1.2 | | Vidon 2004 |
| | Tidal marsh | New England, USA | 3-10 | | Koop-Jakobsen & Gibli, 2009 |
| | Tributary sediments | Santa Fe River, FL | 1-39 | 26-95% | My result |
| DNRA | River | France | 9.8-35 (μg N·L$^{-1}$·day$^{-1}$) | 12-33% | Omnes et al., 1996 |
| | Riparian sediments | Denmark | 0.008-0.12 (μg N·m$^{-2}$·day$^{-1}$) | | Revsbech et al., 2005 |
| | Estuarine sediments | Denmark | 105 (mg N·m$^{-2}$·day$^{-1}$) | | Christensen et al., 2000 |
| | Estuarine sediments | Denmark | 3.4-17 | 4-21% | Jørgensen, 1989 |
| | Sea water | Texas, USA | 18-25 (mg N·m$^{-2}$·day$^{-1}$) | 15-75% | An & Gardner, 2002 |
| | Tropical forest soils | Puerto Rica, USA | 0.6 | 75% | Silver et al., 2001 |
| | Tributary sediments | Santa Fe River, FL | 0.03-0.33 | < 1.2% | My result |




(a)




(b)

Figure 3-1. Tributary 1 (T1) and Tributary 2 (T2-U and T2-D) in the Boston Farm Santa Fe Ranch Beef Unit Research Center (SFRBU) (a) and stream and riparian sediments (b) of the Santa Fe River Watershed, northern Alachua County, FL.

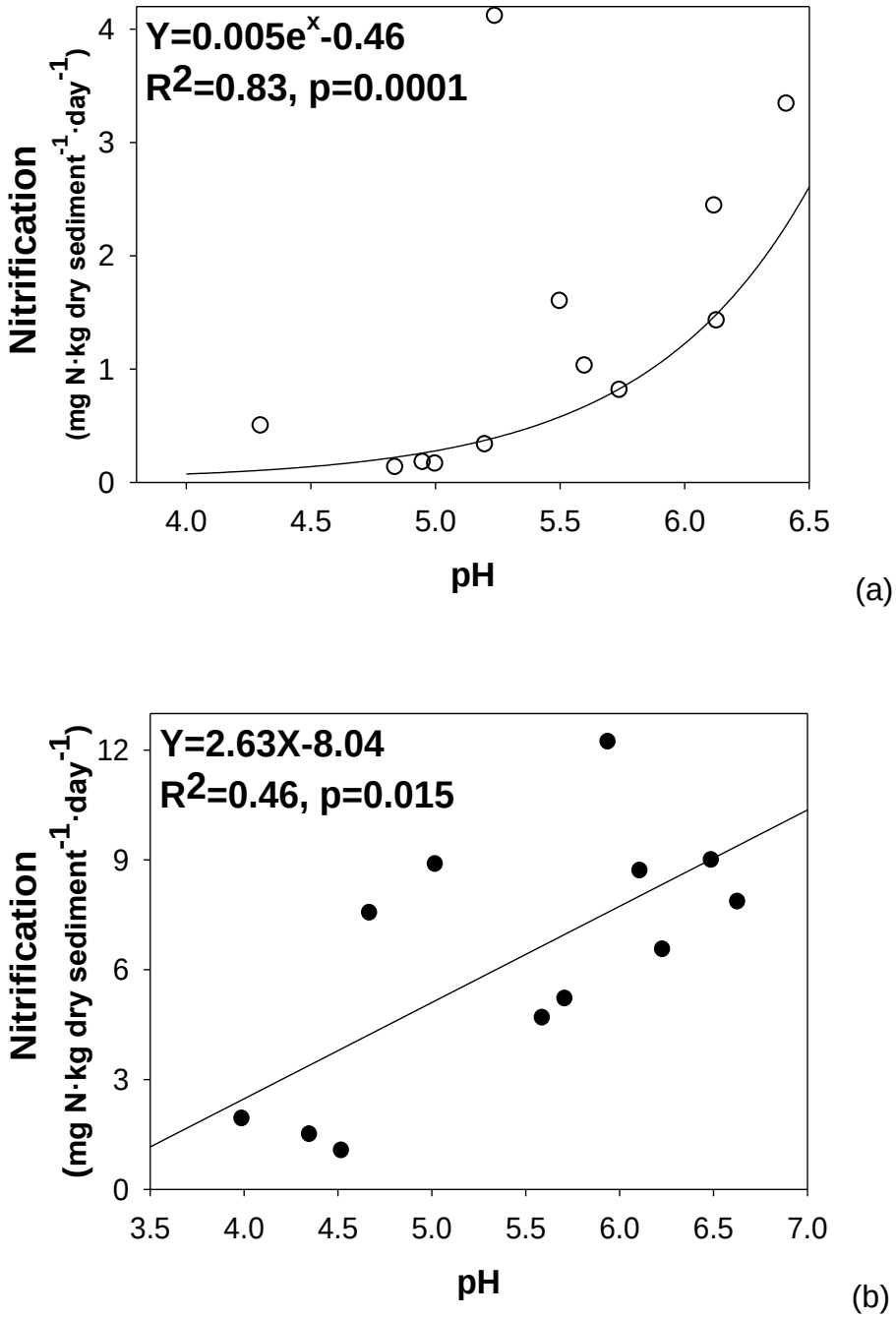
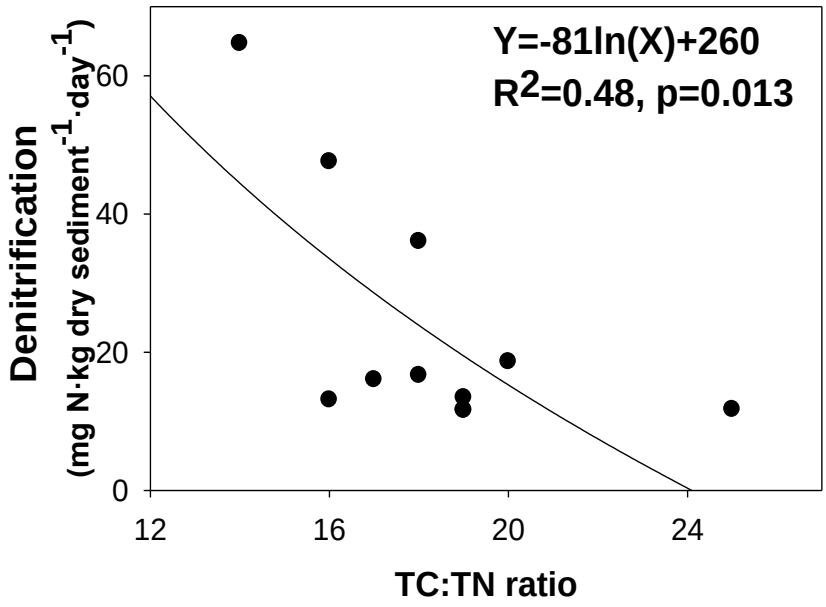
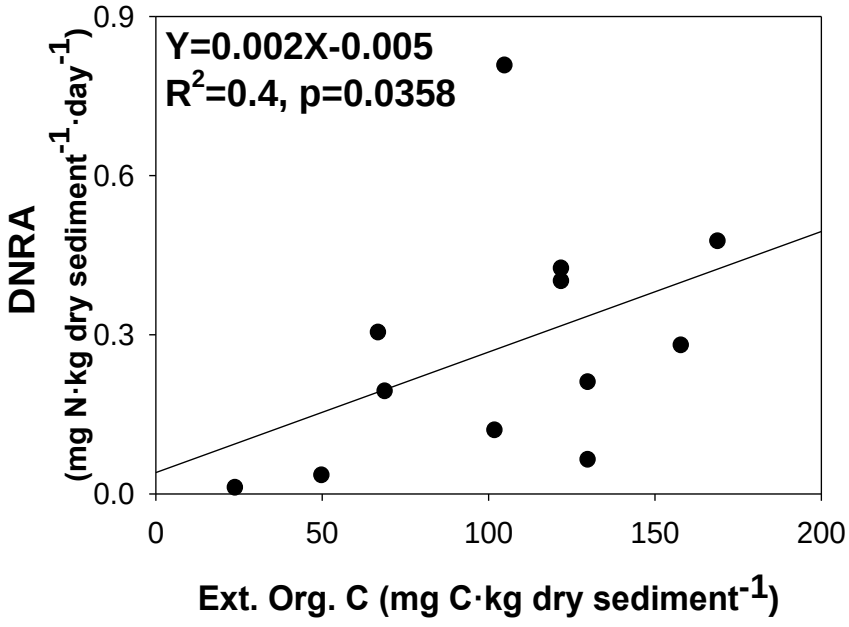



Figure 3-2. Relationships between pH and potential nitrification rates in stream sediments (a) and riparian sediments (b) (n=12).




(a)




(b)

Figure 3-3. Relationships between TC:TN ratio and potential denitrification rates (a) and extractable organic carbon (Ext. Org C) concentrations and potential DNRA rates (b) in riparian sediments (n=12).

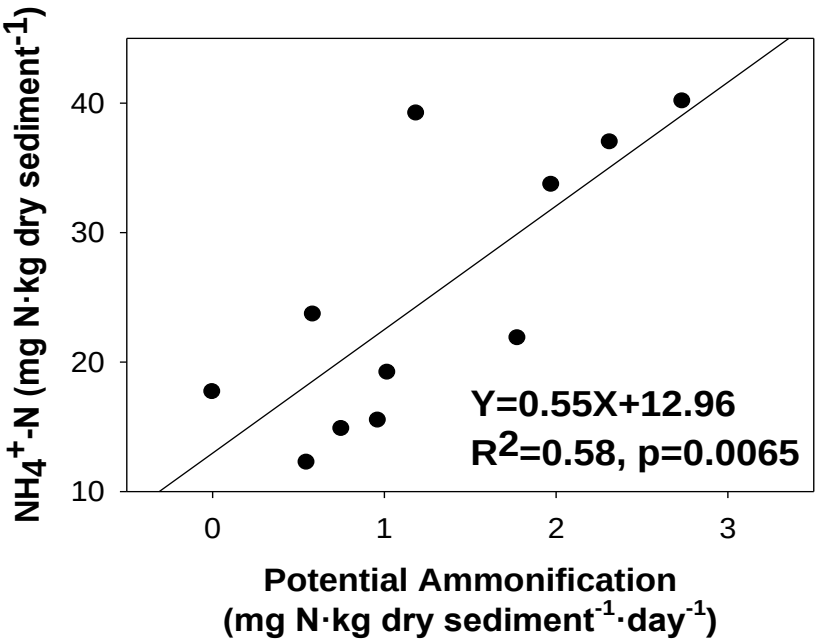



Figure 3-4. Relationships between ammonium concentrations and potential ammonification rates in riparian sediments (n=12).

# CHAPTER 4
# RELATIONSHIP BETWEEN EXTRACELLULAR ENZYME ACTIVITY AND DENITRIFICATION RATES IN TRIBUTARY SEDIMENTS

Organic carbon availability is one of the most important factors regulating the denitrification rate in sediments. For example, a low quality of organic matter, typically may be high in lignin and low in cellulose contents, exhibits a low rate of organic matter decomposition, supplying a poor source of carbon for denitrifiers. However, previous research mainly focused on the effect of organic carbon additions on denitrification rates (DeLaune et al., 1996; Davidson and Stahl, 2000; Kozub and Liehr, 1999; Gersberg et al., 1984; Ingersoll and Baker, 1998; Ragab et al., 1994).

Organic matter decomposition is associated with the activities of various extracellular enzyme produced by microbes (Weiss et al., 1991). Of various regulators affecting extracellular enzyme activities, including temperature, pH, moisture content, and availability of nutrients (King, 1986; Chamier and Dixon, 1982; Dillya and Munch, 1996; Sinsabaugh et al., 1992 and 1993), the quality of organic material is among the most important (Nannipieri et al., 2002). For example, phenol oxidase is associated with decomposition of lignin and is produced by microbes and fungi, and cellobiohydrolase and glycosidase are associated with degradation of cellulose (Nannipieri et al., 2002). Therefore, phenol oxidase and cellobiohydrolase enzyme activities can represent decomposition rates of lignin and cellulose components in soil organic matter and litter (Sinsabaugh, 1993 and 2002).

Soil organic matter is comprised of plant-derived complex polymers such as cellulose and lignin (Benner et al., 1984). Plant litter decomposition (expressed as mass loss from decaying litter) was found to be highly correlated with the activities of extracellular enzymes involved in lignocellulose degradation (Sinsabaugh et al., 1991

and 1993). In case of lignin degradation, a negative correlation between phenol oxidase and lignin contents was observed in wetland soils and riverine sediments (Freeman et al., 2004; Sinsabaugh and Linkins, 1989). Decreased phenol oxidase activity due to a high lignin content leads to the accumulation of phenolic compounds in soils (McLatchey and Reddy, 1998). This accumulation of phenolic compounds inhibit other extracellular enzyme activities such as β-glycosidase, chitinase, phosphatase, and sulfatase, resulting in the retardation of organic matter decomposition and the accumulation of recalcitrant organic matter in soils (Freeman et al., 2004; Wetzel, 1992; Appel, 1993). Therefore, phenol oxidase activities are linked with decomposition rates, and used for estimating carbon availability for heterotrophic microbes in soils (Carreiro et al., 2000). Excessive input of nitrogen increases urease, acid phosphatase, glycosidase, and N-acetyl-β-D-glucosaminase activities; however, high nitrogen input decreases phenol oxidase activities in organic soils (Saiya-Cork et al., 2002). It was observed that addition of nitrogen stimulated cellobiohydrolase activities in litters of dogwood and red maple. However, phenol oxidase activities substantially declined up on nitrogen addition to oak litter with high lignin content (Carreiro et al., 2000). Therefore, these variations of extracellular enzyme activities can determine the rates of decomposition of complex polymers. In turn, this will influence the amount of labile carbon sources available to heterotrophic microbes.

The tributaries of the Santa Fe River, Florida receive litter and organic matter from surrounding vegetation and the agricultural activities in the watershed. Thus, it can be expected that the different extracellular enzyme activities associated with litter and sediments would affect the amount of labile organic carbon. This will affect

denitrification rates in the Santa Fe River tributary sediments via a supply of available carbon to denitrifiers. Therefore, I have conducted a study to determine the relationship between extracellular enzyme activities and denitrification rates in tributary sediments.

Specific objectives were to:

- Investigate the relationship between extracellular enzyme activities and biogeochemical properties in tributary sediments.
- Investigate the relationship between extracellular enzyme activities and potential denitrification rates in tributary sediments.

## Materials and Methods

### Site Description

The site for this research is tributary sediments (stream sediments and riparian sediments) at Boston Farm Santa Fe Ranch Beef Unit Research Center (SFBRU) in the Santa Fe River Watershed, northern Alachua County, FL. The Tributary 1 (T1) is bordered with a pasture ecosystem that is vegetated with grasses and forest. The up-stream region of Tributary 2 (T2-U) is affected by N fertilization and is surrounded by hard wood species including *Carya* sp., *Quercus* sp., and *Magnolia grandiflora* sp. and soft wood including *Pinus* sp. The downstream region of Tributary 2 (T2-D) is affected by N fertilization from the headwater and bordered with a pasture that is covered with grass including *Saururus cernuus* sp., and *Juncus* sp., and deciduous shrub plants including *Cephalanthus occidentalis* sp. (Frisbee, 2007).

### Sampling

Samples were collected to a depth of 3 cm with a PVC core (diameter 7.5cm) at T1, T2-U and T2-D. Surface sediments (3 cm dept of sediments) were collected because surface sediments likely have much higher microbial activities compared to deeper sediments. Three samples from three locations (total of 9 samples) were

collected in each tributary sediment in October 2007, January, April, and July 2008. Repeated sediment sampling (4 times) was considered as replicates for each sediment type. The sediments were transported to the laboratory on ice and stored at 4°C until analysis. Nine samples from each site were mixed to make a single composite sample. After mixing, triplicate sediments were distributed and prepared for the analysis. All roots and litter materials were removed from the sediment prior to analysis. Litter from three locations at each site was also collected by hand. The litter was transported to the laboratory on ice and stored at 4°C until analysis. All soils were removed from the litter by hands. Litter was fragmented to an approximately 1cm quadrangle of samples with scissors for enzyme analysis.

**Analyses of Biogeochemical Properties**

Subsamples of sediments and litter were dried at 70°C for 3 days and ground using a ball grinder for total nitrogen (TN) and carbon (TC) analyses. Total N (TN) and C (TC) from the dry sediment and litter samples were measured using a Thermo Electron Corp. Flash EA 1112 Series NC Soil Analyzer.

Potential denitrification rates were analyzed by the acetylene blocking method (Tiedje, 1999). Ten grams of wet sediment were added to a 160 ml serum bottle and purged with $N_2$ for 15 minutes to create anaerobic conditions. Each bottle was amended with 1400 µg of $NO_3^-$-N (as the form of $KNO_3$) per 10 g of wet sediment and 20 ml of acetylene gas (12.5% of 160 ml serum bottle). $N_2O$ was measured at pre-determined time intervals of up to 4.5 hours using a Shimadzu Gas Chromatography 14-A (GC).

**Analyses of Extracellular Enzyme Activities**

For analysis of β-D-glucosidase activity, 500 µM 4-Methylumbelliferyl (MUF)-β-D-glucoside was used as the substrate model. One gram of wet sediment was diluted with

9 ml distilled de-ionized (DDI) water and mixed. For the litter, 1 g of chopped litter was mixed with 9 ml DDI water and mixed using a vortex mixer. Two hundred µl of suspension from the mixture were transferred into 96-well microplates, and 50 µl of substrate were added to each well. The 96-well microplate was incubated in the dark at room temperature for three hours. A Bio-Tek FL600 fluorometric plate reader measured the fluorescence of samples every 30 minutes for three hours using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Enzyme activities are expressed as mM substrate·kg dry sediment$^{-1}$·hr$^{-1}$ and mM substrate·kg litter$^{-1}$·hr$^{-1}$ (Hoppe, 1993).

For assay of cellobiohydrolase activities, 4-nitrophenyl-β-D-cellobioside was used as the model substrate. One gram of wet sediment was diluted with 9 ml DDI water and mixed with a vortex mixer. For litter, 1 g of chopped litter was mixed with 9 ml DDI water and mixed. One ml of homogenate was transferred to 2 ml amber centrifuge tubes, and 1 ml of substrate was added to samples. After the sample was incubated for one day at 24°C, the tubes were centrifuged for 1 minute at 10,000 rpm. Five hundred µl of suspendants were transferred to a new tube. Fifty µl of 1N NaOH and 2 ml of DDI water were added to samples to terminate the reaction. After mixing samples using a vortex mixer, the absorbance was measured at a wavelength of 410 nm using a Shimadzu UV160-Visible Recoding Spectrophotometer. Enzyme activities were expressed as mM substrate·kg dry sediment$^{-1}$·hr$^{-1}$ for sediment samples and mM substrate·kg litter$^{-1}$·hr$^{-1}$ for litter samples (Linkins et al., 1990).

For analysis of phenol oxidase activity, the model substrate L-dihydroxy phenylalanine (L-DOPA) was used. One gram of wet sediment or one gram of chopped

litter was added to 9 ml of 10 mM L-DOPA (Sigma) solution. Samples were incubated for 15 minute at 24°C. After incubation, samples were centrifuged for 10 minute at 6000 rpm and supernatants were filtered through #45 Whatman paper and transferred to new tubes. The absorbance was measured at 460 nm using a Shimadzu UV160-Visible Recoding Spectrophotometer. Enzyme activities were expressed as μM diqc (2-carboxy-2, 3-DIhydroindole-5,6-Quinone Compound from the enzymatic oxidation of L-DOPA) produced·kg dry sediment$^{-1}$·day$^{-1}$ and μM diqc produced·kg litter$^{-1}$·day$^{-1}$ (Pind et al., 1994).

**Statistical Analysis**

Statistical analysis was conducted using JMP version 8.0 (SAS 2007). One way analysis of variance (ANOVA) was determined to investigate differences in extracellular enzyme and denitrification activities between sites. Least significant difference at the 5% confidence level was used for comparisons. All post comparisons of means was accomplished using a Tukey-Kramer HSD test that protects the overall error rates. Regression analysis was performed to determine if any relationships existed among these parameters using a Standard Least Square model. Canonical analysis was used to investigate the interrelationship among sets of extracellular enzyme and denitrification activities across the sites.

## Results

**Extracellular Enzyme Activities**

Cellobiohydrolase (CBH) activities in litter were highest in T2-D as compared to T1 and T2-U. CBH activities in riparian sediments were higher in T2-D than in T1 (Figure 4-1(a), $p<0.05$). However, CBH activities in stream sediments were not different from each site (Figure 4-1(a)). β-D-glucosidase activities (Glu) were not significantly different

between sites (Figure 4-1(b)). Phenolic oxidase activities (PO) in stream and riparian sediments were highest in T1 compared to T2-U (Figure 4-1(c), $p<0.05$). However, no significant differences in PO activities were observed in litter between sites (Figure 4-1(c)). Extracellular enzyme activities (EEA) including the CBH, Glu, and PO activities were significantly higher in litter than stream and riparian sediments ($p<0.05$, Figure 4-1). Across all sites, EEA were found to be higher in riparian sediments than in stream sediments ($p<0.05$, Figure 4-1).

The EEA based on the amount of total carbon (TC) were calculated to test the carbon efficiency for EEA (Table 4-1). T2-D exhibited the highest CBH activities in riparian sediments and litter ($p<0.05$, Table 4-1). However, there were no differences in Glu and PO activities between T1, T2-U, and T2-D sites in stream and riparian sediments, and litter (Table 4-1).

**Correlation between Extracellular Enzyme and Potential Denitrification Rates**

Potential denitrification rates of tributary sediments were positively correlated with CBH activities of tributary sediments ($R^2=0.86$, $p=0.0083$, Figure 4-2 (a)). In addition, the TC:TN ratio of litter was weakly negatively correlated with CBH activity of litter ($R^2=0.4$ $p=0.032$, Figure 4-2 (b)).

I performed a canonical analysis with EEA of litter and riparian sediments, and potential denitrification rates of riparian sediments. Canonical correlation analysis is a multivariate statistical model that uses the interrelationships among sets of multiple dependent variables and multiple independent variables. Whereas regression analysis predicts a single dependent variable from a single independent variable, canonical correlation can predict multiple dependent variables from multiple independent variables (Hair, 1998). Therefore, this analysis can present how the relationship between EEA

and potential denitrification rate was linked with site variations. Results showed that T2-D was separated from T2-U and T1. This separation was determined by the interrelationships based on EEA and potential denitrification rates (Figure 4-3). In addition, T2-U showed a weak positive relationship between Glu and CBH activities in riparian sediments, while T2-D exhibited a strong relationship between potential denitrification rates in riparian sediments and CBH activities of litter.

## Discussion

### Extracellular Enzyme Activities

To describe the relationship between EEA and potential denitrification rates in tributary sediments, CBH, Glu and PO enzyme activities were measured in the stream and riparian sediments, and litter. My results demonstrated that T2-D exhibited higher CBH activities in litter than those of T1 and T2-U. Also, CBH activities in riparian sediments of T2-D were higher than those of T1.

Cellulose degradation is a major process in the supply of available carbon to heterotrophic microbes (Sinsabaugh, 1991). Cellobiohydrolase (CBH) is an enzyme that breaks down cellulose into cellobiose, and β-glycosidase (Glu) is an enzyme that hydrolyses cellobiose into glucose (Lawrence, 2000). CBH activity can be affected by substrate quality. For example, CBH is negatively correlated with the lignin content of litter (Keeler et al., 2009). Linkins et al. (1990) observed that heavily lignified litter such as oak exhibited a lower CBH activity than the less lignified litter such as dog wood. In addition, Hidaka (1984) showed that CBH activity of the fungus *Trichoderma viride* was significantly reduced by lignin extracted from soft and hard woods. In general, litter with a low TC:TN ratio tends to have less lignified materials, even though the TC:TN ratio of litter does not precisely represent the amount of lignin (Fogel and Cromack, 1977;

Herman, et al., 1977; Meentemeyer, 1978; Melillo et al., 1982). In my study, the litter of T2-D exhibited a lower TC:TN ratio (29) than those of T1 (31) and T2-U (39) and a weak negative relationship between CBH activities and TC:TN ratio in litter was observed. Therefore, a lower TC:TN ratio of litter could support increased CBH activities in litter of T2-D. In addition, CBH activities based on total carbon of litter were highest in T2-D. This implies that T2-D had a higher efficiency of enzyme activities for decomposition of litter. However, other factors including moisture contents, litter chemistry (i.e. ratio of lignin to nitrogen contents), and microbial community associated with litter decomposition, could also affect degradation rates of litter (Sinsabaugh et al., 1991, 1992 and 1993). Thus, these factors could result in a weak correlation between TC:TN ratio and CBH activities in litter.

**Relationships between Extracellular Enzyme and Potential Denitrification Rates**

EEA of soils and sediments is commonly used as an index of organic matter decomposition rates (Sinsabaugh et al., 2005; Asmar et al., 1994). The EEA is well correlated with the loss of litter and the amount of soil nutrients such C, N, and P (Sinsabaugh et al., 1991, 1992 and 1993). Therefore, systems exhibiting high CBH activities are favorable to microbes using organic carbon as an energy source. Even though T2-D contains less organic matter, the sediments and litter of this system showed high CBH and high carbon use efficiency for denitrification (Chapter 3). Therefore, it can be expected that the differing CBH activities might drive the rates of decomposition of litter and sediments which might influence the level of labile carbon available for denitrification.

To investigate the relationship between EEA and denitrification rates, I performed regression and canonical analyses using EEA of litter and sediments to predict potential

denitrification rate of sediments. The results indicated that potential denitrification rates of sediments were positively related with CBH activities of tributary sediments. Also, it was found that T2-D was associated with potential denitrification rates and CBH activities of litter. Thus, the litter of the T2-D system might be more easily decomposed due to the higher CBH activity of litter. This will result in an increased quality of carbon available to denitrifiers, enhancing their rates. Previous research has shown that vegetation type with high quality substrates, such as meadows (Rich et al., 2003), submerged plant (*E. canadensis* sp.) (Bastviken et al., 2004), grasslands (Lowrance et al., 1995), or emergent macrophytes (Hernadez and Mitsch, 2007) had higher soil denitrification rates than areas affected by plant species with a high TC:TN ratio, such as woody forests. Volokita (1996) and Nakajima-Kambe (2005) demonstrated that the addition of cellulose increased denitrification rates with isolated denitrifying microorganisms utilizing the cellulose as an energy source. In addition, Rich and colleagues (2003) reported that meadow soils with a lower TC:TN ratio harbored various groups of denitrifier assemblages and exhibited higher denitrification rates than forest soils with higher TC:TN ratios. They concluded that the carbon quality of soils influenced the biogeochemical properties of soil, such as the degradation rate and level of available organic carbon. In turn, these changes can affect the denitrifier community structure and their rates (Rich et al., 2003). Therefore, in my research sites, it can be expected that the difference in carbon quality of litter and organic matter in sediments could change the labile carbon availability in the system via differences in EEA, which can influence potential denitrification rates in tributary sediments.

## Summary

EEA can be used as an index of the decomposition rate because the EEA is well correlated with the loss of litter and organic matter, and the amount of soil nutrients such C, N, and P. Also, since decay rates of litter and organic matter are associated with the level of available carbon (Melillo et al., 1989; Howard and Howard, 1974; Swift et al., 1979), the analyses of EEA in the sediments and litter can explain the carbon availability to denitrifiers. Therefore, this research investigated the relationship between EEA and potential denitrification rates in tributary sediments. My results showed that tributary sediments with higher CBH activities in litter exhibited higher potential denitrification rates. A positive relationship between CBH activities and potential denitrification rates was observed in tributary sediments ($R^2$=0.86, p=0.0083). Also, CBH activities of litter were weakly negatively correlated with TC:TN ratios ($R^2$=0.4, p=0.032). Thus, the substrate quality representing the TC:TN ratio could affect CBH activities in litter. This will determine the availability of carbon source to denitrifiers, influencing their rates. Additionally, these results suggested that carbon quality affecting extracellular enzyme activities can be one of factors for determining potential denitrification rates in tributary sediments.

Table 4-1. Extracellular enzyme activities based on total carbon contents (TC) in tributary sediments (CBH is cellobiohydrolase; Glu is β-D-glucosidase; and PO is phenol oxidase activities). Characters not labeled by same letter are significantly different at 95% confidence level (n=4).

| | | CBH | Glu | PO |
|---|---|---|---|---|
| | | (M substrate·kg $TC^{-1}$·$hr^{-1}$) | | (μM diqc produced· kg $TC^{-1}$·$day^{-1}$) |
| Types | Sites | Ave | Ave | Ave |
| Stream sediments | T1 | 220 (±138) | 0.36 (±0.21) | 649 (±305) |
| | T2-U | 77 (±34) | 0.22 (±0.13) | 42 (±11) |
| | T2-D | 139 (±85) | 0.34 (±0.2) | 45 (±16) |
| Riparian sediments | T1 | 109 (±25)[b] | 1.65 (±0.25) | 52 (±25) |
| | T2-U | 134 (±26)[b] | 1.77 (±0.39) | 14 (±2) |
| | T2-D | 412 (±88)[a] | 2.11 (±0.66) | 79 (±27) |
| Litter | T1 | 12 (±4)[b] | 0.62 (±0.18) | 13 (±1) |
| | T2-U | 50 (±23)[b] | 1.71 (±0.82) | 18 (±4) |
| | T2-D | 146 (±41)[a] | 1.77 (±0.66) | 36 (±19) |

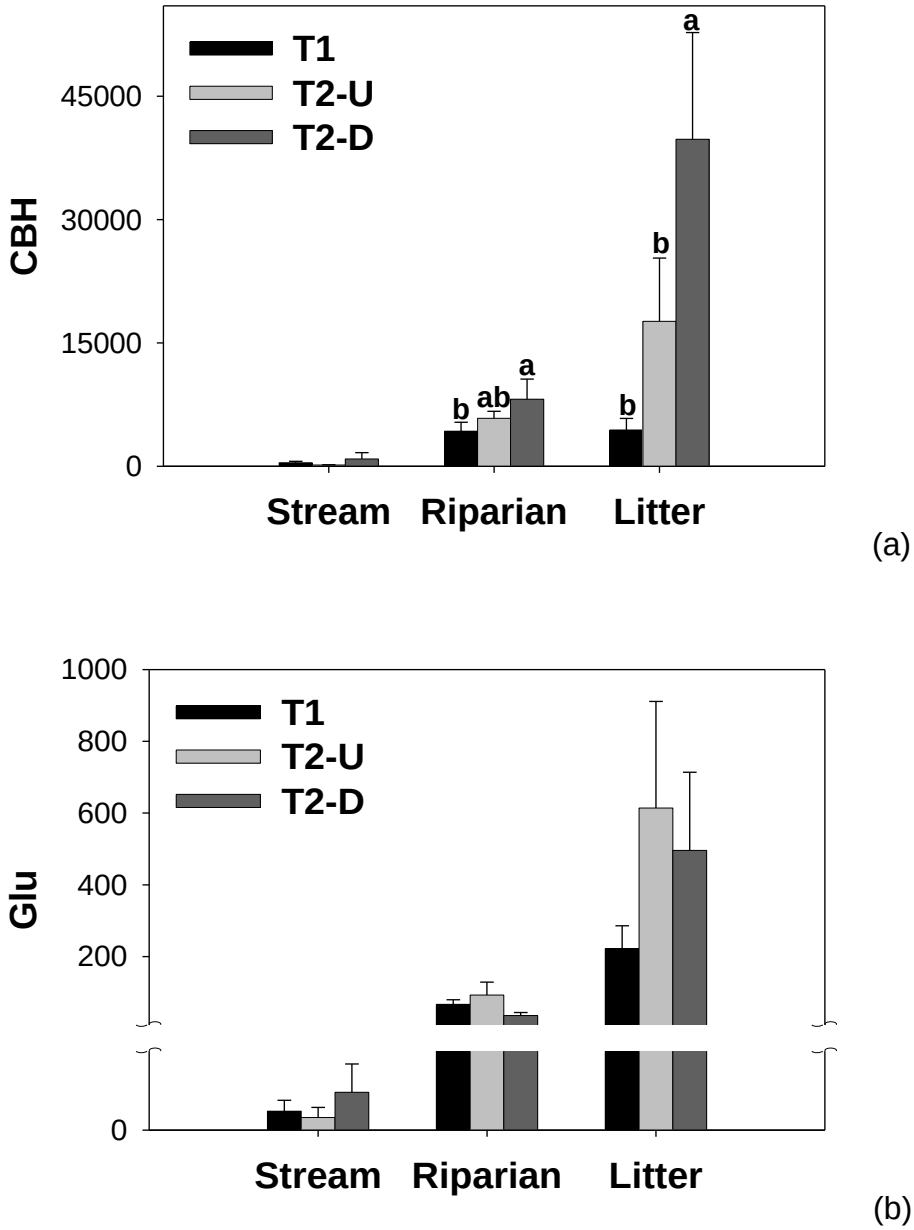
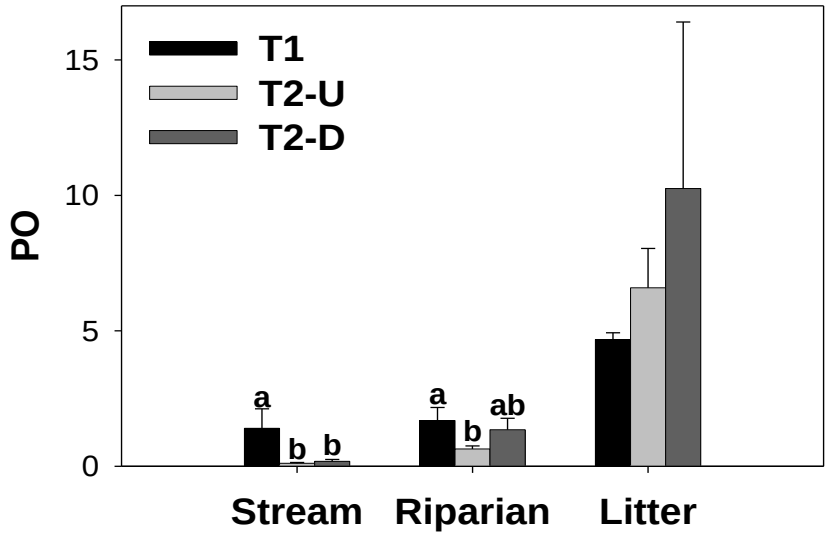
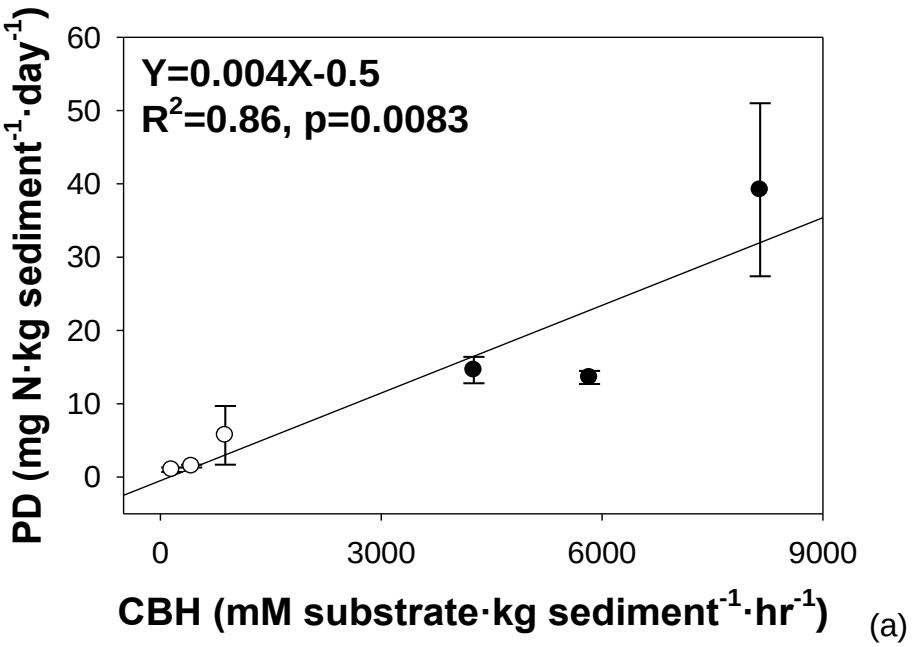



Figure 4-1. Cellobiohydrolase (CBH) (a), β-D-glucosidase (Glu) (b), phenol oxidase (PO) (c) activities in stream and riparian sediments, and litter (Characters not labeled by same letter are significantly different at 5% confidence level, n=4). The unit of CBH and Glu is mM substrate·kg dry sediment$^{-1}$·hr$^{-1}$ and mM substrate·kg litter$^{-1}$·hr$^{-1}$. The unit of PO is μM diqc produced·kg dry sediment$^{-1}$·day$^{-1}$ and μM diqc produced·kg litter$^{-1}$·day$^{-1}$.

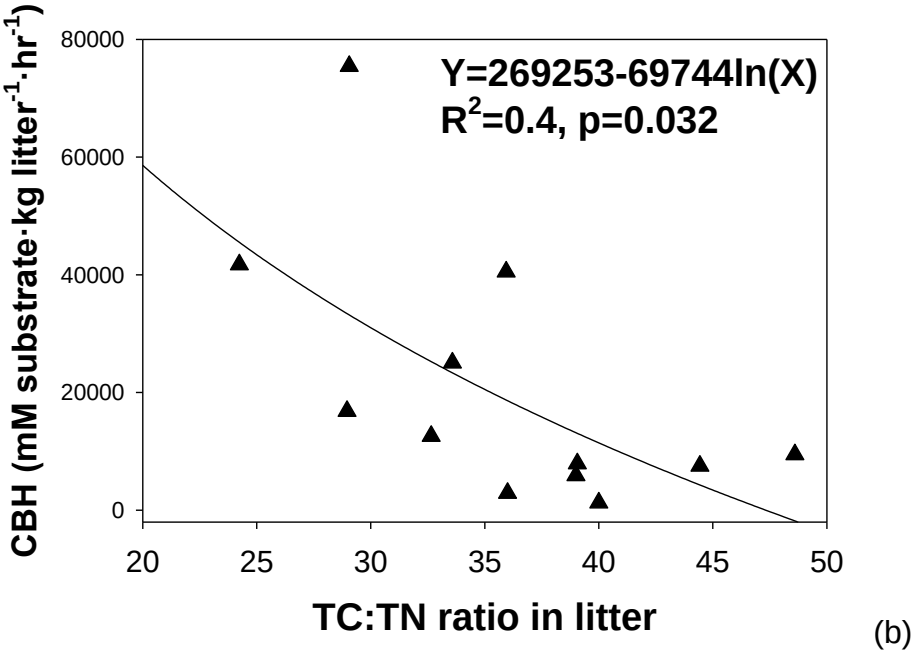



(c)

Figure 4-1. Continued.




(a)




(b)

Figure 4-2. Relationships between potential denitrification rates (PD) and cellobiohydrolase enzyme (CBH) activities of tributary sediments (Open circle=rates in stream sediments; Dark circle= rate in riparian sediments, Each data point is the average of 4 samples, n=24) (a), and TC:TN ratio and cellobiohydrolase enzyme (CBH) activity of litter (b) (n=12).

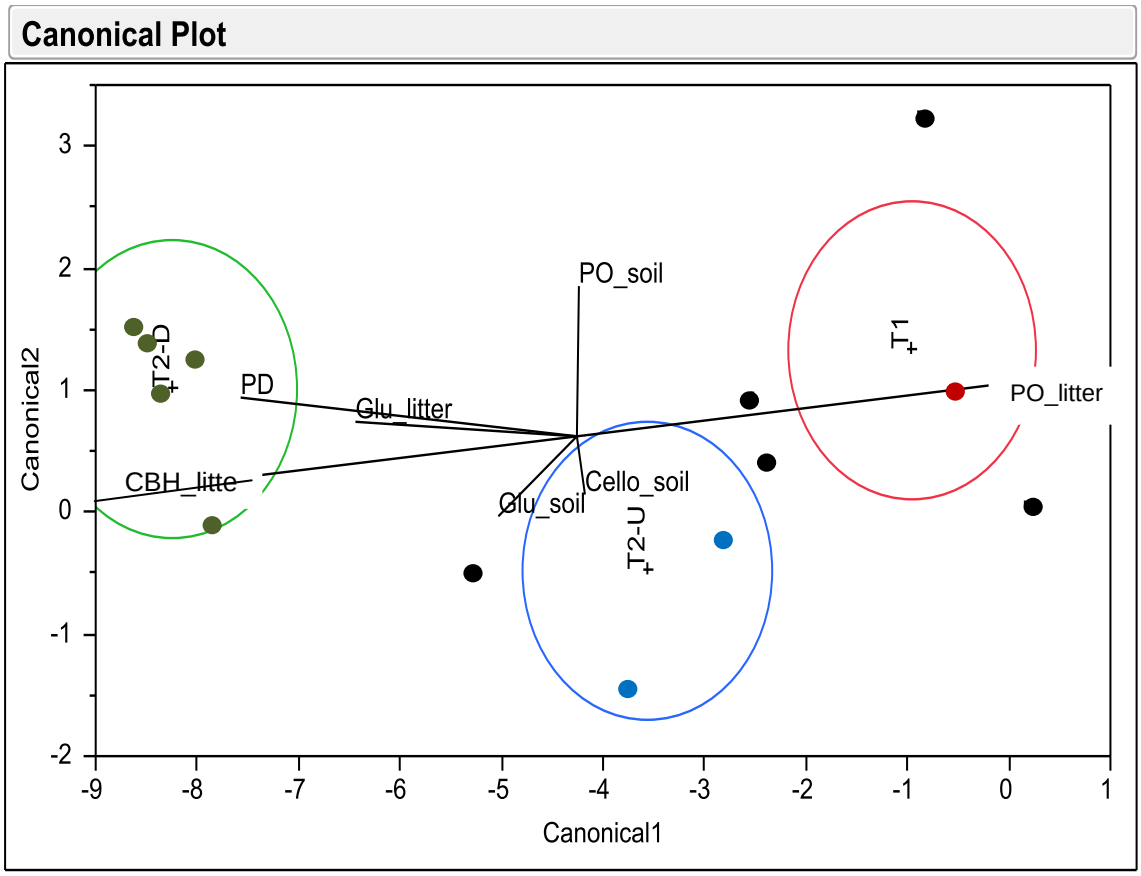



Figure 4-3. Canonical plot of extracellular enzyme activities and potential denitrification rates of riparian sediments and litter.

# CHAPTER 5
# RELATIONSHIPS BETWEEN BIOGEOCHEMICAL PROPERTIES, DENITRIFICATION, AND ASSOCIATED MICROBIAL ASSEMBLAGE COMPOSITIONS IN TRIBUTARY SEDIMENTS

Human activities such as fertilizer application and ranch activities can alter the physical and chemical properties of ecosystems in numerous ways, including changing pH (Kowalenko et al., 1978) and the availabilities of carbon and nitrogen, which in turn can regulate biogeochemical processes (Burkart, and James, 1999; Kohl et al., 1971; Lefebvre et al., 2007; Sanchez, 2001 and 2004; Christensen, 1992; Angers and N'Dayegamiye, 1991; Gerzabeck et al., 2001). These changes to biogeochemical properties can also change the structures and functions of microbial communities (Polymenakou et al., 2005; Torsvik and Øvreås, 2002; Balser and Firestone, 2005). For example, the amount of available carbon and nitrogen may affect the composition of the denitrifying assemblage in soils. Therefore, the organic matter and nitrogen contents affect not only the pathway of inorganic nitrogen transformations, but also the structures of microbial communities responsible for these processes (Henderson et al., 2010; Cao et al., 2008; Mills et al., 2008; Hunter et al., 2006; Santoro et al., 2006; Rich et al., 2003; Priemé et al., 2002). Most previous studies have focused on biogeochemical processes; however, much attention is currently being paid to the structures of microbial communities impacted by human activity. Little is known about the relationship between microbial function and structure responsible for nitrogen cycles. Research on how microbial community structure relates to inorganic nitrogen transformations is needed to better understand the impact of human activities on ecosystem function.

Most molecular ecological studies on denitrifier assemblages have focused on functional genes such as those that encode either nitrite reductase (*nirK* and *nirS*) or

nitrous oxide reductase (*nosZ*). Nitrite reductase converts nitrite to nitric oxide and may be encoded by two distinct metalloenzymes: *nirK* with a copper center; and *nirS* with a heme-based cytrochrome *cd* (Bothe et al., 2000). *nirK* and *nirS* are functionally equivalent; however, *nirS* is more widely distributed within *Archaea* and *Bacteria* than *nirK* (Bothe et al., 2000). *nirK* has been found to dominate in costal and marsh ecosystems, while *nirS* dominates in a broad range of environments (Braker et al., 2000). *nirS* clone libraries have been shown to exhibit higher diversities than those of *nirK* clone libraries from wetland soils (Priemé et al., 2002), ground waters (Yan et al., 2001; Santoro et al., 2006), and ocean sediments (Liu et al., 2003). Also, it was reported that *nirS* denitrifier communities responded differently than *nirK* denitrifier communities to environmental gradients, including ammonium and nitrate concentrations, and salinity in various ecosystems (Jones and Hallin, 2010). Wolsing and Priemé (2004) showed that sites treated with mineral fertilizers or dairy manure harbored different assemblages defined by *nirK* and *nirS* clone libraries. Thus, this study investigated how *nirS* and *nirK* genotypes are distributed according to various biogeochemical properties, and explored the relationships between denitrification rates and diversity of *nirS* and *nirK* clone libraries in tributary sediments of the Santa Fe River.

## Materials and Methods

### Site Description

The site for this research is tributary sediments (stream sediments and riparian sediments) at the Boston Farm Santa Fe Ranch Beef Unit Research Center (SFBRU) in the Santa Fe River Watershed, northern Alachua County, FL. Land uses on this site include a low intensity cattle operation with about 300 heifers on 1,600 acres and the nursery operation using nitrogen fertilizer (Holly Factory Nursery) (Frisbee, 2007).

Tributary 1 system (T1) is affected by a pasture ecosystem vegetated with grass and trees, while upstream region of Tributary 2 (T2-U) is affected by N fertilization, and both hard wood (*Carya* sp., *Quercus* sp., and *Magnolia grandiflora* sp) and soft wood including *Pinus* sp. The downstream region of Tributary 2 (T2-D) is affected by N fertilization from the headwater and a pasture ecosystem, and is covered with grass including *Saururus cernuus* sp., and *Juncus* sp., and deciduous shrub plants including *Cephalanthus occidentalis* sp. (Frisbee, 2007).

**Sampling**

Samples were collected to a depth of 3 cm with a PVC core (diameter 7.5cm) from three sites. Surface sediments (3 cm dept of sediments) were collected because surface sediments likely have much higher microbial activities compared to deeper sediments. Three samples from three places (total 9 samples) were collected from each tributary for January and July 2008. The samples were transported to the laboratory on ice. Nine samples from each site were mixed to make a single composite sample. After mixing, triplicate samples were taken and prepared for analysis. All roots and litter materials were removed from sediments prior to analysis. The samples were stored at -80°C until analysis.

**Nucleic Acid Extraction, PCR Amplification, Cloning, and Sequencing**

Nucleic acids were extracted from 0.25 g of sediments with the Power Soil DNA Isolation kit (MoBio, Carlsbad, CA, USA) following the manufacturer's instruction. DNA extracts were used as a template in Polymerase Chain Reaction (PCR) using primer sets designed by Yan et al. (2003), consisting of primers 583F (5'-TCA TGG TGC TGC CGC CKG ACG-3') and 909R (5'-GAA CTT GCC GGT KGC CCA GAC-3') which amplify an approximately 326 bp region of *nirK*, and 832 F (5'-TCA CAC CCC GAG CCG CGC

GT-3'), and 1606R (5'-AGK CGT TGA ACT TKC CGG TCG G-3'), which amplify an approximately 774 bp region of *nirS*. The amplification reaction mixture was composed of 25 µl GoTaq Green Master Mix (Promega, Madison, WI), 1 µl of each primer (100 $pmol \cdot \mu l^{-1}$), 13 µl of distilled de-ionized (DDI) water, and 10 µl of diluted DNA solution. An iCycler thermal cycler (BIORAD, Hercules, CA) was used for PCR amplification with the following conditions: initial enzyme activation and DNA denaturation of 15 min at 95°C, followed by 30 seconds at 94°C, 30 seconds at 60°C, 60 seconds extension at 72°C for 30 cycles, and a final extension of 72°C for 7min. The PCR products were analyzed by electrophoresis through 1.5% TAE agarose gels. For cloning, a pGEM®-T and pGEM®-T Easy Vector Systems (Promega WI) was used: the total volume of ligation reaction mixture was 10 µl, and ligation mixture contained 5 µl of 2X Rapid Ligation Buffer, 3 µl of fresh PCR-amplicons, and 2 µl DDI water. The reaction mixture was ligated into pCRII-TOPO cloning vector and transformed into chemically competent XL10-Gold® Ultracompetent Cells (Stratagene, CA) following the manufacture's protocol. Inserts within white colonies were confirmed by PCR amplification with the same primer set and PCR protocol described earlier, and their size was confirmed by electrophoresis on 1.5% TAE agarose gels. The insert-bearing clones were transferred to 96-well plates containing 200 µl of Luria Bertani broth and 8% (v/v) glycerol and kanamycin ($50 \mu g \cdot ml^{-1}$). Plates were incubated overnight at 37°C, covered with gas permeable membranes (Breath-easy, Diversified Biotech, USA), and total 326 clones were sent to the University of Florida Genome Sequencing Service Laboratory for sequencing.

**Construction of Phylogenetic Tree and Diversity Analysis**

The number of clones sequenced from each library is presented in Table 5-1. BLAST queries (Gen Bank, http://www.ncbi.nlm.nih.gov) were used to compare all DNA sequences of *nirS* with previous studies, as well as to check sequence relevance to the target genes. ClustalX2 was used to align the sequences with related sequences and one out-group species, and to check for appropriate alignment (Larkin et al., 2007). Phylogenetic trees were constructed using MEGA version 4 (Tamura et al., 2007) with a neighbor joining analysis using a Jukes-Cantor method for distance estimation. Bootstrap analysis (1000 re-sampling) was used to estimate reproducibility of phylogenic trees. The selected out-group for phylogenetic analysis was *nirN* from *Pseudomonas aeruginosa* (D84475) (Kawasaki et al., 1997).

For community analysis, DOTUR generated operational taxonomic units (OTUs) using a furthest neighbor algorithm. I used a cutoff of 10% difference in nucleic acid sequences because most of studies designated 5 or 10% as cut off and 10% is appropriate point for comparing diversity between species (Oakley et al, 2006, Priemé et al., 2002; Philippot, et al., 2009; Kandeler, et al., 2006). Based on rarefaction analysis using the numbers of clones per OTU, three indices (Shannon, Simpson, and Chao 1) were calculated for analyses of diversity (Shannon and Simpson) and richness (Chao 1) of *nirS* sequences (Schloss and Handelsman, 2005). Among diversity indices, Chao1 index considers the richness of species (number of the species present in the system) (Hughes et al., 2001), while the Shannon and Simpson indices take account of both richness and evenness (a measure of the relative abundance of the different species making up the richness of the system) of species present in the system (Schloss and Handelsman, 2005). Relative to the Shannon index, the Simpson index is more

sensitive to the abundance of the most common species in the system (Schloss and Handelsman, 2005). PHYLIP was used to calculate pair-wise distances between sequences (Felsenstein et al., 2004).

**Unifrac and Mantel Test**

In order to determine if denitrifier assemblages are significantly different from each other depending on environmental factors or location, Unifrac PCA (Principal component analysis) analysis was used (Lozupone and Knight, 2005). Phylogenetic tree was constructed by Mega version 4 using a Jukes-Cantor and maximum parsimony.

To investigate the relationships between microbial assemblages and environmental properties, Mantel test was used (Mantel et al., 1967; Mantel and Valand, 1970). Mantel test was performed in R -Vegan package (R Development Core Team, 2008; Oksanen et al., 2005) and is based on a nonparametric general regression model using distance matrices of phylogenetic trees and environmental factors (Dutilleul et al., 2000).

## Results

***nirS* Phylogenetic Tree**

Attempts to amplify *nirK* from sediment samples failed, likely due to lower abundance of these genes. However, *nirS* was amplified in all samples. *nirS* sequences were grouped into two distinct phylogenetic clusters for stream sediments (A and B) (Figure 5-1) and into three distinct phylogenetic clusters (A, B, and C) for riparian sediments (Figure 5-2). Genes from all clusters shared 70% to 98% similarity with sequences from previously obtained environmental clones in the Gen Bank.

Cluster A was composed of three sub-clusters (A-1, A-2, and A-3). Cluster A-1 consists of sequences cloned from all site samples. Clones in A-1 cluster with the *Beta*

and *Gammaproteobacteria*. However, Cluster A-2 is comprised only of clones from T2-D and clusters with *Alpha* and *Betaproteobateria*. Cluster A-3 consists of clones from T1 and T2-U and clusters with the *Gammaproteobacteria*. Cluster B consists of sequences from all site samples and clusters with to the *Alpha* and *Betaproteobateria* (Figure 5-1).

*nirS* sequences from riparian sediments grouped within three clusters (A, B and C). Sub-cluster A-1 was comprised of clones from all site samples, and is associated with the *Beta* and *Gammaproteobacteria*. However, Cluster A-2 is only composed sequences from T2-D. Sequences of Cluster A-2 belong to the *Alpha, Beta* and *Gammaproteobacteria*. Cluster B contains two sub-clusters (B-1 and B-2). Sub-cluster B-1 is comprised of clones from T2-U, while Sub-Cluster B-2 is composed of clones from T1 and T2-D, and cluster with the *Gammaproteobacteria*. Cluster C consists of sequences from T2-D (Figure 5-2).

**Diversity Indices for *nirS* Assemblage Composition**

In my study, OTUs were defined by DOTUR using a 90% DNA sequence similarity cutoff, assuming that sequence similarities greater than 90% represented the same species. The clone libraries from T1 and T2-U riparian sediments represented nearly all the *nirS* diversity when using 90% similarity of sequences as a cutoff point. However, rarefaction curves of stream sediments from all sites and T2-D riparian sediments did not reach a plateau at 10% cutoff. These rarefaction curves were steeply sloped at the 10% cut off points for sequences obtained from stream sediments and T2-D riparian sediments. When comparing rarefaction curves between stream and riparian sediments, libraries from stream sediments exhibited steeper nirS rarefaction curves than libraries from riparian sediments for all sites (Figure 5-3).

Results of diversity analyses indicated that Shannon and Simpson indices of stream sediments showed no difference in *nirS* libraries between sites. However, the Chao1 index was highest in T2-D, followed by T2-U, and T1 for stream sediments. For riparian sediments, the Shannon and Chao1 were highest in T2-D, followed by T1, and T2-U (Table 5-1).

To investigate the biogeochemical factors controlling microbial diversity and richness of *nirS*, Shannon, Simpson and Chao1 indices were correlated with biogeochemical properties. The Shannon index was negatively correlated with extractable organic carbon (Ext. Org C) contents of tributary sediments ($R^2$=0.8, p=0.017, Figure 5-4 (a)). The concentrations of microbial biomass carbon (MBC) of tributary sediments were also negatively correlated with the Shannon ($R^2$= 0.99, p<0.0001, Figure 5-4 (b)) and Simpson ($R^2$=0.79, p=0.018, Figure 5-4(c)) indices. In addition, a positive relationship between potential denitrification rates and richness index was observed in riparian sediments ($R^2$=0.99, p=0.0064, Figure 5-5); however this relationship was not detected in stream sediments.

**Relationship between Microbial Assemblages of the *nirS* and Biogeochemical Properties**

Results of the Unifrac PCA analysis indicated that the compositions for *nirS* in T2-D were significantly different from those of T1 and T2-U in stream sediments, explaining 21.34% of variation in assemblage composition (Figure 5-6). For riparian sediments, the microbial assemblage composition of the *nirS* in T2-D was separated from those of T1 and T2-U, explaining 26.82% of variation in assemblage compositions (Figure 5-6).

Results of the Mantel test demonstrated that extractable organic carbon (Ext. Org C) (Mantel r=0.7, p=0.047) and organic nitrogen (Ext. Org N) (Mantel r=0.89, p=0.009)

(Table 5-2) and microbial biomass carbon (MBC) (Mantel r=0.79, p=0.02) (Table 5-2) were the main factors affecting microbial assemblage compositions for *nirS* in tributary sediments. Thus the greater the difference in Ext. Org C, Ext. Org N and MBC concentrations between sites, the greater difference in microbial assemblage composition for *nirS*.

## Discussion

### *nirK* Denitrifiers

*nirK* was not amplified from sediments at my study sites, while *nirS* was amplified in all site samples. It is difficult to explain why *nirK* was not detected in my research sites because functions of *nirS* and *nirK* for nitrite reductase are equivalent and biogeochemical properties are significantly correlated with each other (Philippot et al., 2009). However, the absence of *nirK* in my site suggests that environmental conditions might select *nirS* rather than *nirK*. Previous research demonstrated that the levels of pH, nitrate, and soil moisture determined the *nirS* to *nirK* distribution ratio in grassland pasture (Philippot, et al., 2009). Also, organic carbon concentrations affected the copy numbers of *nirK*, while carbon, nitrate and pH did not affect the copy number of *nirS* in soils, indicating *nirS* may adapt to variations in environmental properties (Kandeler, et al., 2006). Thus, various biogeochemical properties in study sites might more exclude the *nirK*.

Furthermore, I used *nirK* primers designed for denitrifier in groundwater and Everglades soils (Yan et al. 2003; Smith, 2006). Thus, my primer set may not have detected *nirK* genes in tributary sediments, indicating that the *nirK* genes in my sites could be different from those of groundwater and Everglades soils.

### Relationships between Microbial Diversity and Richness of *nirS* and Biogeochemical Properties

Rarefaction curves of stream sediments from all sites and riparian sediments did not reach a plateau at the 10% cutoff. This implies that the number of clones sequenced from clone libraries did not represent the entire *nirS* diversity. Thus, more clones should be sequenced for these samples for covering the diversity of denitrifiers in my research sites. However, the number of clones sequenced in my research (from 29 to 69) was not lower than those of other research (30, 18, 50 to 75, 30 to 40) (Priemé et al., 2002; Braker et al., 2000; Santoro et al., 2006). In addition, previous research showed a steeper rarefaction curve at 5% to 10% cutoff (Braker et al., 2000; Santoro et al., 2006; Priemé et al., 2002). Thus, *nirS* assemblage composition might be very diverse so that numerous numbers of clones need to be sequenced for presenting diversity. However, expensive cost in analyzing sequences is also limitation for covering the diversity. Despite this limitation, rarefaction curves of riparian sediments were lower than those of stream sediments. It may not be surprising that the riparian sediments had a lower *nirS* diversity, because they contain higher carbon contents compared to stream sediments. Thus, high carbon content may likely lead to the dominant growth of a few species, resulting in less diverse group in riparian sediments compared to stream sediments.

Results from diversity analysis indicated that T2-D with highest denitrification rates also had the highest values of richness index scores in riparian sediments. Also, a negative relationship between the Shannon index and extractable organic carbon (Ext. Org C) contents was observed. This implies that higher carbon contents in tributary sediments may allow one species to outcompete others, which in turn decreases denitrifier richness and denitrification rates in the system. In contrast, more diverse

groups of denitrifiers such as that of T2-D seems to be related to higher denitrification rates, even though T2-D contained lower carbon contents than to T2-U. There are two non-mutually exclusive explanations linking diversity and productivity in ecology: the complementarity effect and sampling effect. Complementarity effect refers to the process by which species richness and diversity enhance productivity due to niche differentiation or positive interactions between species, resulting in a higher efficiency of resource utilization (Venail et al., 2008; Loreau et al., 2001). The sampling effect occurs when more diverse communities contain species with a higher average productivity than communities with lower diversity. In both situations, ecosystems with higher diversity have more opportunities to select prominent species, increasing productivity of the system, and increasing beneficial interaction for all species (Venail et al., 2008; Loreau et al., 2001). Thus, diverse group of denitrifiers in T2-D in riparian sediments could exhibit higher denitrification rates according to complementarity and sampling effects.

Sequences in T2-D riparian sediment cluster with *Pseudomonas stutzeri,* known to be a widely distributed denitrifier in natural environments. *P. stutzeri* is characterized as having high genetic diversity and occupies numerous ecological niches (Lalucat et al., 2006). *P. stutzeri* shows great metabolic versatility, capable of growing on a wide range of organic substrates utilized by few other *Pseudomonas* sp. (e.g., starch, maltose, and ethylene glycol). Therefore, the various metabolic capacities of these denitrifier species in T2-D riparian sediments could enhance microbial diversity and increase denitrification rates despite low carbon contents compared to T2-U riparian sediments.

Higher diversity indices in stream sediments compared to riparian sediments did not lead to increased denitrification rates. This can be explained in that relatively lower

carbon availability for denitrifiers could not support denitrification rates, despite of higher diversity.

### Relationships between Microbial Assemblages of the *nirS* and Biogeochemical Properties

From the PCA, *nirS* of T2-U was different from T2-D for riparian sediments. Mantel tests indicated that extractable organic carbon content was a significant factor determining *nirS* assemblage compositions. Thus, the larger difference in carbon contents between sites, the greater the difference in compositions of *nirS*. Thus, the difference of organic carbon contents between T2-U and T2-D could be linked to the difference in denitrifier assemblage compositions, which in turn will affect denitrification rates at each site. Microbial biomass carbon (MBC) was also found to be a main factor separating microbial assemblage compositions for *nirS* in my study. Higher MBC contents could imply a larger population of microbes. Thus, this may imply that the difference in population size also could affect distribution of microbial assemblage compositions for *nirS* in my sites.

Environmental factors known to affect denitrifier assemblage compositions are the level of salinity for *nirS* (Jones et al., 2010; Santoro et al., 2006), pH for *nosZ* (Enwall et al., 2005) and *nirS* (Priemé et al., 2002), and the amount of organic matter for *nirK* and *nosZ* (Kandeler et al., 2006). However, there has been no previous research on influence of carbon content on the microbial assemblage composition of *nirS*, even though carbon is one of the main regulators for denitrification rates. This study suggests the importance of organic carbon content in distributing the *nirS* in tributary sediments.

## Summary

The objective of this research was to investigate how denitrifiers coded by *nirS* functional genes were distributed according to various biogeochemical properties, and to observe relationships between biogeochemical factors affecting denitrification rates and denitrifier assemblage compositions in tributary sediments of the Santa Fe River. Results indicated that T2-D showed the highest diversity index of *nirS* clone libraries and denitrification rates in riparian sediments. In addition, the level of extractable organic carbon content was found to be one of the main regulators for diversity of *nirS*. T2-U had the highest organic carbon contents but the lowest denitrification rates, while T2-D had the lowest organic carbon contents but the highest denitrification rates. Generally, denitrification rates increase with available organic carbon contents in the system; however, my results do not follow this pattern. Therefore, diverse assemblage compositions of *nirS* could influence denitrification rates via the selection of denitrifiers capable of using carbon source more efficiently. In addition, my research showed that the difference in *nirS* assemblage compositions among sites was determined by the difference in organic carbon contents among sites. Therefore, the larger the difference in organic carbon levels between sites, the larger the difference of *nirS* assemblage composition. Additionally, the higher the content of organic carbon in the system, the lower the diversity of the *nirS* assemblage compositions, corresponding to a decrease in denitrification rate. These results imply that the microbial assemblage compositions of *nirS* can affect the microbial function, and these relationships can be regulated by the level of organic carbon in the systems.

Table 5-1. *nirS* diversity and richness in tributary sediments, estimated by the Shannon and Simpson indices, and the Chao 1 index.

| | Sites | No. of clones sequenced | No. of OTUs[¥] | Shannon index | Simpson index | Chao 1 index |
|---|---|---|---|---|---|---|
| Stream sediments | T1 | 60 | 42 | 3.6 (3.4, 3.8) | 0.99 | 79 (57,136) |
| | T2-U | 62 | 43 | 3.6 (3.4, 3.8) | 0.98 | 105 (67,201) |
| | T2-D | 66 | 45 | 3.6 (3.3, 3.8) | 0.98 | 135 (72,250) |
| | ALL | 188 | | | | |
| Riparian sediments | T1 | 40 | 24 | 3.0 (2.8, 3.3) | 0.97 | 33 (26,56) |
| | T2-U | 29 | 19 | 2.8 (2.6, 3.1) | 0.97 | 28 (21,55) |
| | T2-D | 69 | 43 | 3.5 (3.3, 3.8) | 0.98 | 121 (81,271) |
| | ALL | 138 | | | | |

[¥]Estimates of OTUs, the Shannon, Simpson and Chao1 indices are all based on difference of 10% or less in nucleic acid sequence alignments; values in parentheses are upper and lower bounds of 95% confidence intervals as calculated by DOTUR.

Table 5-2. Regression analysis of transformed data taken from distribution matrices; y-axis represent *nirS* assemblages and x-axis represents biogeochemical properties generated by the Mantel test.

| | pH | $NO_3^-$-N | Ext. Org N | MBN | Ext. Org C | MBC |
|---|---|---|---|---|---|---|
| Mantel r | 0.39 | -0.22 | 0.89 | 0.71 | 0.7 | 0.79 |
| p value | 0.16 | 0.89 | 0.009 | 0.18 | 0.047 | 0.02 |

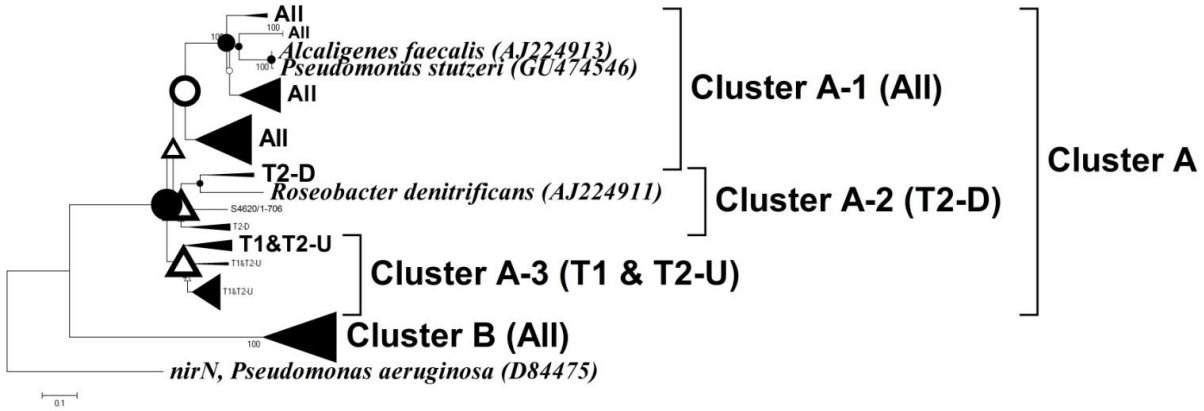



Figure 5-1. Neighbor-joining tree of *nirS* sequences obtained from stream sediments of tributaries (Dark circles indicate values over 90, empty circles indicate values from 70 to 90, and empty triangles indicate values from 50 to 70. Nodes are bootstrap scores based on percent occurrence of 1000 re-samplings).

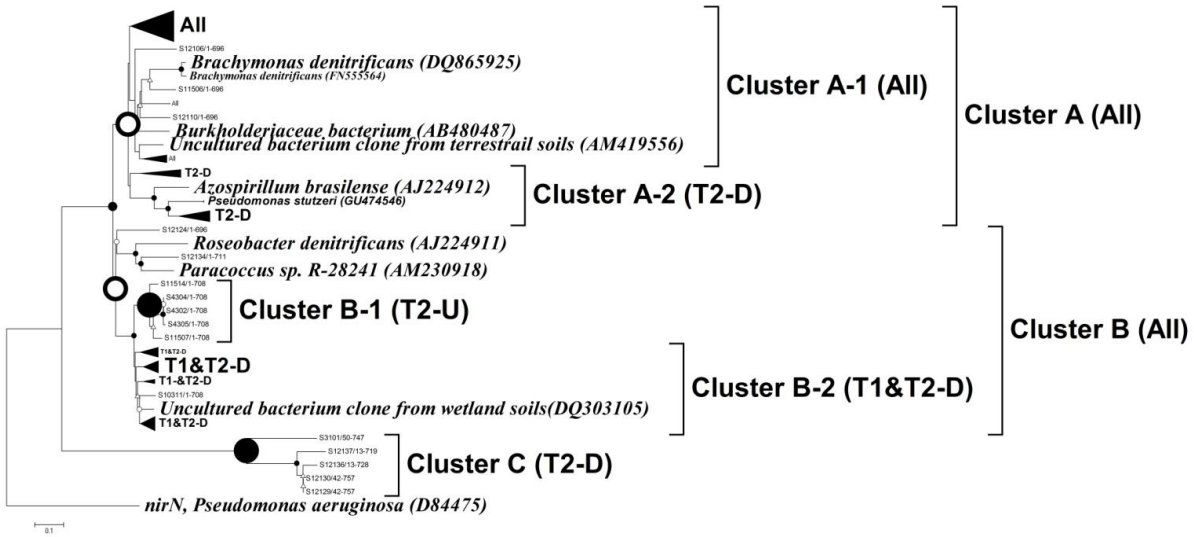
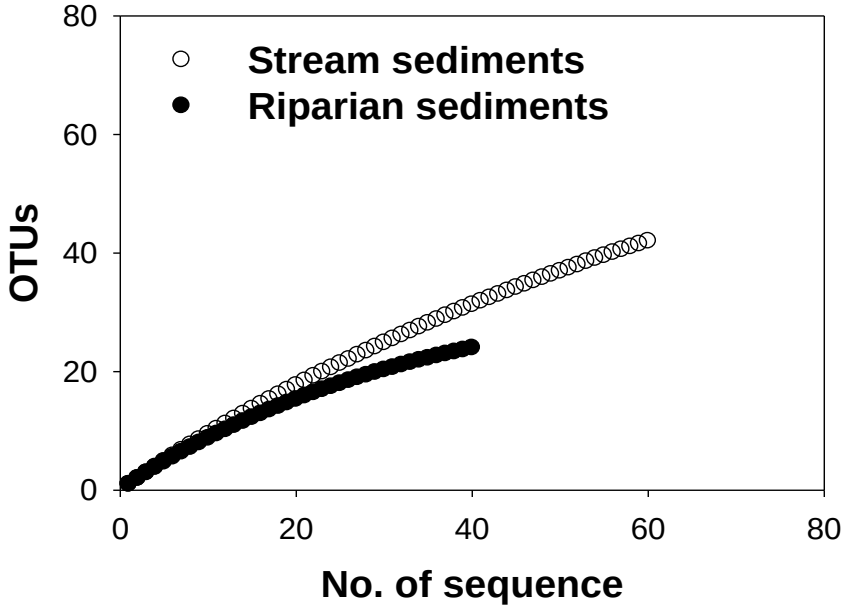
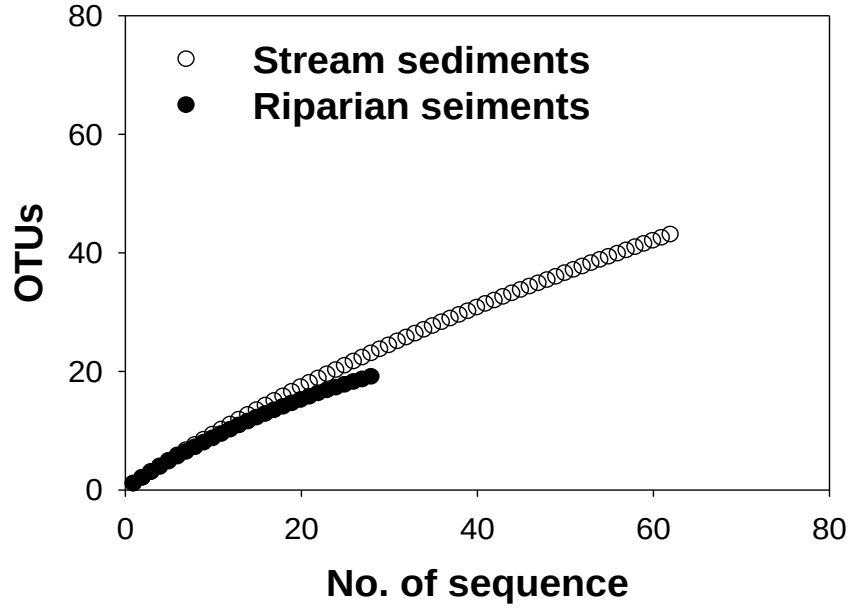



Figure 5-2. Neighbor-joining tree of *nirS* sequences obtained from riparian sediments of tributaries (Dark circles indicate values over 90, empty circles indicate values from 70 to 90, and empty triangles indicate values from 50 to 70. Nodes are bootstrap scores based on percent occurrence of 1000 re-samplings).

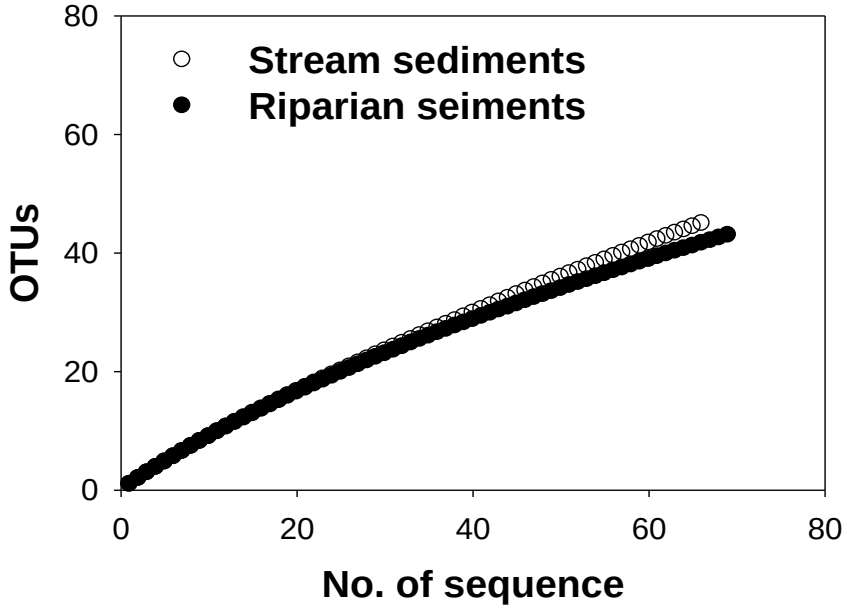



(a)



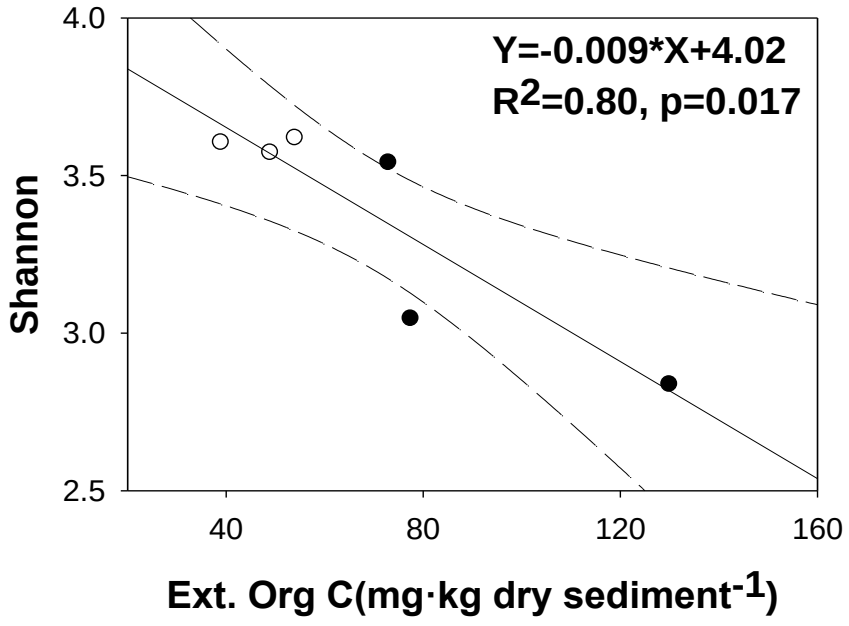

(b)

Figure 5-3. Rarefaction curves for *nirS* from DOTUR analysis using furthest neighbor assignment algorithm in tributary sediments in T1 (a), T2-U (b), and T2-D (c). Similarity cut off was 90%.

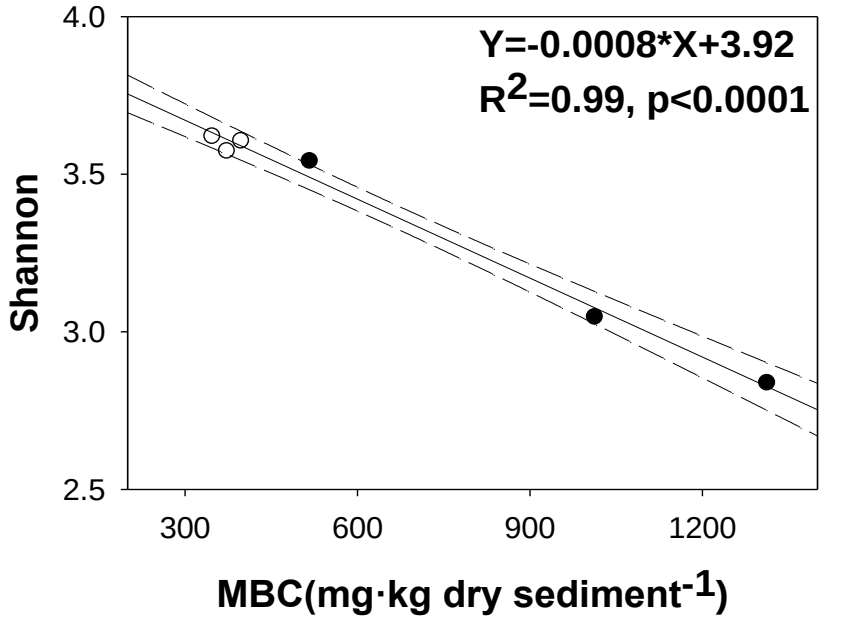



(c)

Figure 5-3. Continued.

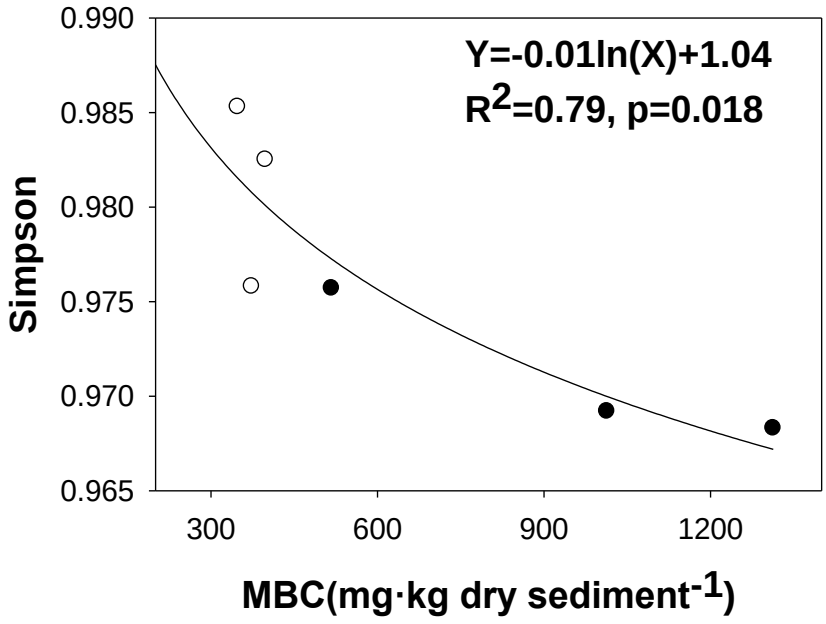



(a)




(b)

Figure 5-4. Relationships between concentrations of extractable organic carbon (Ext. Org C) and the Shannon index (a), and microbial biomass carbon (MBC) and the Shannon (b) and Simpson (c) indices for *nirS* in tributary sediments (Open circle= stream sediments, Dark circle=riparian sediments, Dashed lines are the 95% confidence intervals of the regression line).




(c)

Figure 5-4. Continued.

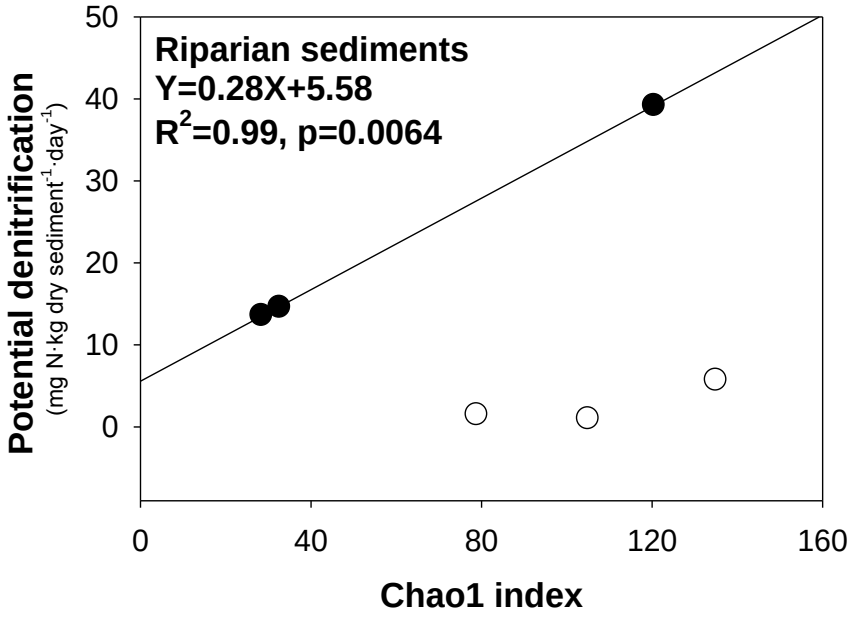



Figure 5-5. Relationship between potential denitrification rates and Chao 1 index in riparian sediments and stream sediments. (Open circle=stream sediments, Dark circle=riparian sediments)

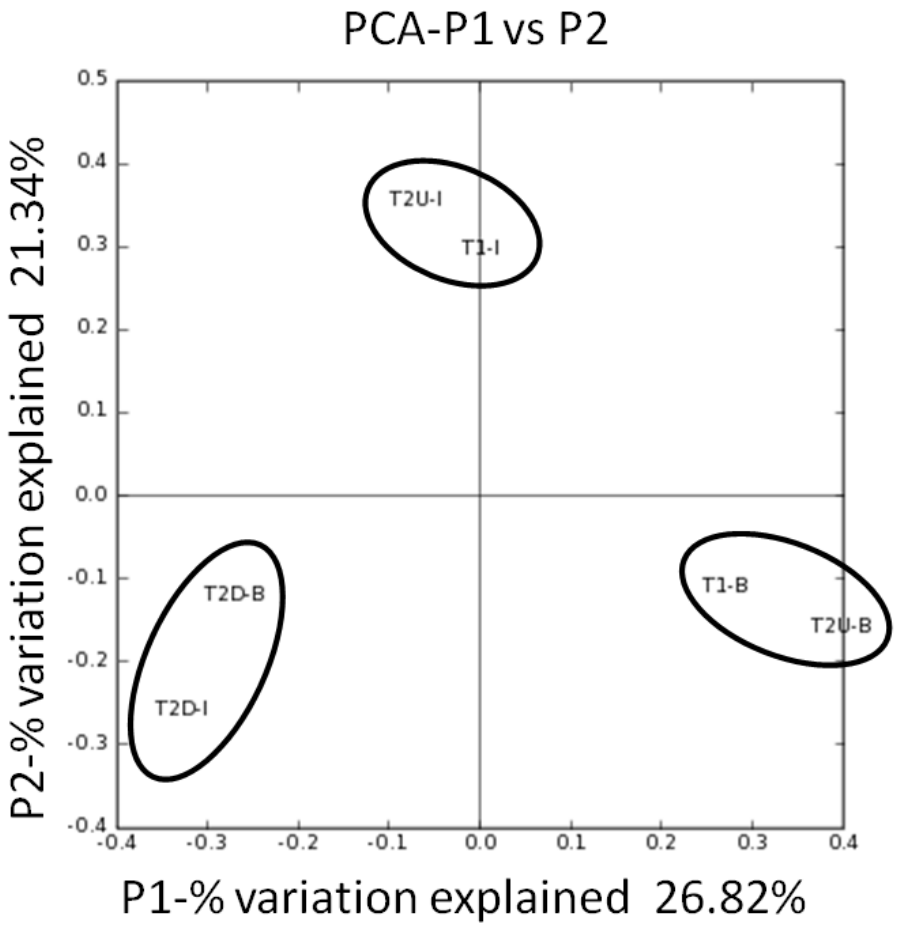



Figure 5-6. Principal component analysis for *nirS* assemblage composition using Unifrac in all tributaries sediments.

# CHAPTER 6
# RELATIONSHIPS AMONG BIOGEOCHEMICAL PROPERTIES, NITRIFICATION, AND ASSOCIATED MICROBIAL ASSEMBLAGE COMPOSITION OF TRIBUTARY SEDIMENTS

Nitrification is an important process in nitrogen transformations because nitrate produced from nitrification can be used as an electron acceptor by denitrifiers (Vanerborght and Billen, 1975; Vanerborght et al., 1977). Nitrification is a two step process: in the first step, ammonia oxidizing bacteria (AOB) oxidize ammonium to nitrite; and in the second step, nitrite oxidizing bacteria convert nitrite to nitrate (Tate, 2000). It was accepted that AOB were responsible for most nitrification in various ecosystems including acidic soils, terrestrial, and marine ecosystems (Pedersen et al., 1999; Boer and Kowalchuk, 2001; Kuai and Verstraete, 1998; Barraclough and Puri, 1995; Killham, 1986; Pennington and Ellis, 1993; Stroo et al., 1986). However, the presence of *Archaeal* ammonia oxidizers (AOA) was recently detected in oceans (Francis et al., 2005), estuarine sediments (Berman et al., 2006), marine sponges (Steger et al., 2008), terrestrial soils (Leininger et al., 2006), and hot springs (Zhang et al., 2008). AOA copy numbers exceed those of AOB in these systems (Leininger 2006; Wüchter 2006; Mincer 2007; Martens-Habbena 2009), suggesting that AOA account for most nitrification in a wide range of ecosystems. Thus, considerable research has focused on the role of *Archaea* in nitrogen cyclings in various ecosystems. One reason why AOA attract attention is their physiological characteristics. *Archaea* was considered to occupy niches from which *Bacteria* are excluded, such as low pH and high salinities (Weidler et al., 2007; Zhang et al., 2008; Pearson et al., 2004). The discovery of AOA in diverse ecosystems suggests that nitrification may be significant in a wide range of ecosystem types than previously recognized (Francis et al., 2007; Karl, 2007; Nicol and Schleper,

2006). However, little is known about the relationships among microbial diversity of AOA, nitrification rates, and environmental characteristics in tributary ecosystems, despite observations of their presence in various soils. Thus, this research investigated if AOB and AOA contribute to the nitrification rates in tributary sediments, and the relationship between nitrifier diversity, nitrification rates, and biogeochemical properties in tributary sediments. I hypothesized that variations in biogeochemical properties influence the microbial structure and assemblage compositions of nitrifiers.

## Materials and Methods

### Site Description

The site for this research is tributary sediments (stream sediments and riparian sediments) at the Boston Farm Santa Fe Ranch Beef Unit Research Center (SFBRU) in the Santa Fe River Watershed, northern Alachua County, FL. Land uses on this site include a low intensity cattle operation with about 300 heifers on 1,600 acres and a nursery operation using nitrogen fertilizer (Holly Factory Nursery) (Frisbee, 2007).

Tributary 1 (T1) is affected by a pasture ecosystem vegetated with grass and trees, while upstream region of Tributary 2 (T2-U) is affected by N fertilization and both hard wood (*Carya* sp., *Quercus* sp., and *Magnolia grandiflora* sp).and soft wood including *Pinus* sp. The downstream of Tributary 2 (T2-D) is affected by N fertilization from the headwater and a pasture ecosystem, and is covered with grass including *Saururus cernuus* sp., and *Juncus* sp., and deciduous shrub plants including *Cephalanthus occidentalis* sp. (Frisbee, 2007).

### Sampling

Samples (stream and riparian sediments) were collected to a depth of 3 cm with a PVC core (diameter 7.5cm) from three sites. Surface sediments (3 cm dept of

sediments) were collected because surface sediments likely have much higher microbial activities compared to deeper sediments. Three samples from three sites (total of 9 samples) were collected in each tributary sediment for December 2006, March, May, July, August, and October 2007, January, April, and July 2008. The sediments were transported to the laboratory on ice. Nine samples from each site were mixed to make a single composite sample. After mixing, triplicate samples were taken and prepared for analysis. All roots and litter materials were removed from sediments prior to analysis. The samples were stored at -80°C until analysis.

**Nucleic Acid Extraction, PCR Amplification, Cloning and Sequencing**

For all samples, nucleic acids were extracted from 0.25 g of sediments with the Power Soil DNA Isolation kit (MoBio, Carlsbad, CA, USA) following the manufacturer's instruction. An approximately 491bp fragment of AOB in T1 and T2-U for December 2006, March, May, July, and August 2007 samples was amplified using primer set *amoA*1F (5'-GGGGTTTCTACTGGTGGT-3') and *amoA*2R (5'-CCCCTCKGSAAAGCCTTCTTC-3'), developed by Rotthauwe et al. (1997). The Polymerase Chain Reaction (PCR) mixture was composed of 25 µl GoTaq Green Master Mix (Promega, Madison, WI), 1 µl of each primer (100 pmol·µl$^{-1}$), 13 µl of distilled de-ionized (DDI) water, and 10 µl of diluted DNA solution. An iCycler thermal cycler (BIORAD, Hercules, CA) was used for PCR amplification with the following conditions: initial enzyme activation and denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 30 seconds, 55°C for 45 seconds, and 72°C for 45 seconds, with a final extension step at 72°C for 7 min. PCR products were analyzed by electrophoresis through 1.5% TAE agarose gels.

Amplification of AOA was conducted using primer sets designed by Leininger (2006), consisting of primers A19F (5'-ATG GTC TGG CT(AT) AGA CG-3') and 643R (5'-TCC CAC TT(AT) GAC CA(AG) GCG GCC ATC CA-3'), which amplify an approximately 624bp region of AOA from samples collected from December 2006, March, May, July, August, and October 2007, January, April, and July 2008. The mixture for the PCR amplification was composed of 25 µl GoTaq Green Master Mix (Promega, Madison, WI), 1 µl of each primer (100 pmol·µl$^{-1}$), 13 µl of DDI water, and 10 µl of diluted DNA solution. An iCycler thermal cycler (BIORAD, Hercules, CA) was used for PCR amplification with following conditions: initial enzyme activation and DNA denaturation of 15 min at 95°C, followed by 30 seconds at 95°C, 45 seconds at 55°C, and 45 seconds extension at 72°C for 30 cycles, and a final extension of 72°C for 7min. PCR products were analyzed by electrophoresis through 1.5% TAE agarose gels.

For cloning, pGEM®-T and pGEM®-T Easy Vector Systems (Promega WI) were used. The total volume of ligation reaction mixtures was 10 µl and contained 5 µl of 2X Rapid Ligation Buffer, 3 µl of fresh PCR-amplicons, and 2 µl DDI water. The reaction mixture was ligated into the pCRII-TOPO cloning vector and transformed into chemically competent XL10-Gold® Ultracompetent Cells (Stratagene, CA) according to manufacturer's protocol. Inserts within white colonies were confirmed by PCR amplification with same primer set and agarose gel electrophoresis analysis. Insert-bearing clones were transferred to 96-well plates containing 200 µl of Luria Bertani broth and 8% (v/v) glycerol and kanamycin (50 µg·ml$^{-1}$). Plates were incubated overnight at 37°C, covered with gas permeable membranes (Breath-easy, Diversified

Biotech, USA) and sent to the University of Florida Genome Sequencing Service Laboratory for sequencing.

**Construction of Phylogenetic Tree and Diversity Analysis**

The number of clones sequenced from each library is presented in Table 5-2. All DNA sequences of AOA were compared to other sequences from previous research using BLAST (Gen Bank, http://www.ncbi.nlm.nih.gov), and to check for relevance to AOA genes. Sequences were aligned with related sequences and one out-group sequence using ClustalX2, and the alignment was manually edited (Larkin et al., 2007). The out-group for *amoA* selected was *amoA* from *Nitrosomonas* (AY958704) (Park and Noguera, 2007). Phylogenetic trees were conducted using MEGA version 4 with a maximum parsimony analysis using a Jukes-Cantor method for distance estimation (Tamura et al., 2007). Bootstrap analysis (100 re-sampling) was used to estimate reproducibility of phylogenetic trees. For analysis of microbial assemblage composition diversity, DOTUR generated operational taxonomic units (OTUs) using a furthest neighbor algorithm with a cutoff of 10% difference in nucleic acid sequences. The reason why I used a 10% cutoff in my research is that most of the studies designate 5% or 10% as cut off, and 10% is an appropriate point for comparing diversity between species (Francis et al., 2005; Leininger et al., 2006; Zhang et al., 2008). Richness and diversity indices including the Shannon, Simpson, and Chao1 indices were calculated using DOTUR (Schloss and Handelsman, 2005). PHYLIP was used to calculate pair-wise distance between sequences (Felsenstein et al., 2004).

**Unifrac PCA and Mantel Test**

To determine if clone libraries were significantly different from each other and related to environmental factors or location, Unifrac PCA (Principal Component Analysis)

analysis was conducted (Lozupone and Knight, 2005). The phylogenetic tree for Unifrac PCA analysis was constructed using Mega 4 and Jukes-Cantor and distance method with a maximum parsimony method for tree constructions. Also, to investigate the relationship between microbial assemblage compositions and environmental properties, the Mantel test was performed in R -Vegan (R Development Core Team 2008; Oksanen et al., 2010; Mantel, 1967; Mantel and Valand, 1970).

## Results

### AOA Phylogenetic Tree

AOA PCR bands were consistently more intense than those of AOB in T1 and T2-U samples for December 2006, March, May, July, and August 2007 (Table 6-1). Bands intensity is not truly a quantitative comparison but is frequently used as a semi-quantitative tool for comparisons. PCR bands from AOB were weak for October 2007, January, April, and July 2008 samples. However, PCR bands from AOA were intense for all samples, and AOA was amplified from all samples and sequenced. Phylogenetic trees were constructed for AOA genes with October 2007, January, April, and July 2008 samples. AOB was not studied further due to low abundance of AOB PCR bands in most samples.

AOA sequences were grouped into four distinct phylogenetic clusters for stream sediments. Cluster A was divided into two subgroups (A-1and A-2). Cluster A-1 was composed of clones sequenced from all sites and was similar to clones obtained from N fertilized soils (EF207208 and EF207209) (He et al., 2007), sandy soils (DQ534885, DQ534870, DQ148878 and DQ6534864) (Leininger et al., 2006), bioreactors (DQ278581) (Park et al., 2006), ocean water (DQ148587) (Francis et al., 2006), and hot spring (EU281321) (Zhang et al., 2008). Cluster A-2 was comprised of clones

sequenced from T2-D and contained sequences obtained from freshwater (EU309878 and EU309880) (Herrmann, 2009) and ocean ecosystems (AACY01575171) (Venter et al., 2004).This cluster also included *Nitrosopumilus maritimus*, cultured from the marine systems at Seattle (EU239959) (Kőnneke, 2005). Unlike Cluster A, Cluster B consisted of clones from T2-U and included clones sequenced from terrestrial soils impacted by N fertilization (EF207215) (He et al., 2007). Cluster C consisted of clones sequenced from T1 and T2-U, and included clones obtained from ocean sediments (DQ148789) (Francis et al., 2005). Cluster D included clones from all sites. In summary, sequences from stream sediments of T1 and T2-U clustered with sequences from soils, while sequences from stream sediments of T2-D clustered with sequences from both marine water and soils (Figure 6-1).

AOA sequences were grouped into three distinct phylogenetic clusters for riparian sediments. Cluster A included sequences from T1 and T2-U, and grouped with sequences from uncultured clones obtained from terrestrial soils receiving N fertilizer (EF207215) (He et al., 2007), and *Candidatus Nitrosocaldus yellowstonii*, cultured from a hot spring sediment (EU239961) (de la Torre et al., 2008). Cluster C consisted of sequences from T2-D and sequences obtained from a hot spring (EU553449) (Zhang et al., 2008), ocean (DQ148587) (Francis et al., 2005), freshwater systems (EU309880) (Herrmann et al., 2008), and sandy soils (DQ534854) (Leininger et al., 2006). Also, this cluster included *Nitrosopumilus maritimus* (EU239959) cultured from marine ecosystems (Kőnneke, 2005) and *Candidatus Nitrososphaera gargensis* cultured from a hot spring (EU281321) (Hatzenpichler, 2008). Cluster B included sequences from riparian sediments from all sites and were similar to uncultured clones sequenced from

ocean water, sediments (DQ148789) (Francis et al., 2005), and N fertilized soils (EF207221) (He et al., 2007). Therefore, sequences from T1 and T2-U were similar to sequences obtained from hot spring sediments or N fertilized soils, and sequences from T2-D tended to cluster with sequence from ocean and soil systems (Figure 6-2).

**Diversity Indices for AOA Assemblages**

Rarefaction curves reached a plateau at 10% cut off, implying that the sequences represented all the AOA diversity in clone libraries when using 90% similarity of sequences as a cutoff point. When comparing rarefaction curves between stream and riparian sediments, libraries from stream sediments exhibited steeper AOA rarefaction curves than libraries from riparian sediments for all sites (Figure 6-3).

T2-D *amoA* sequences exhibited the higher Shannon and Simpson indices in T2-D than those of T1 and T2-U in stream and riparian sediments; however, Chao1 richness index was highest in T1 stream sediments and T2-U riparian sediments (Table 6-2). I correlated the Shannon, Simpson and Chao1 indices with biogeochemical properties to investigate biogeochemical factors controlling microbial diversity and richness of AOA. pH was weakly positively correlated with the Simpson index in stream sediments ($R^2$=0.42, p=0.0229, Figure 6-4 (a)). Extractable organic carbon concentrations were weakly negatively correlated with the Shannon index in riparian sediments ($R^2$=0.41, p=0.0259, Figure 6-4 (b)).

**Relationship between the AOA Assemblage Compositions and Biogeochemical Properties**

Unifrac PCA analysis indicated that assemblage compositions for AOA in T2-D were separated from those of T1 and T2-U in tributary sediments, explaining 50.45% of variation (Figure 6-5). The Mantel test demonstrated that pH was a main factor

controlling the differences between microbial assemblage compositions in tributary sediments for all sites (Table 6-3, Mantel r=0.44, p=0.04). Thus, a greater difference in pH between sites is associated with greater difference in microbial assemblage composition for AOA.

## Discussion

### Bacterial *amoA*

Bacterial *amoA* (AOB) was not well amplified in my samples. This lack of amplification may be due to either low abundance or may indicate a methodological problem. Many previous studies reported that AOA were found in various ecosystems including ocean water, estuarine sediments, fertilized soils, hot springs, the guts of animals, and plant leaves (Moissl et al., 2008; Schleper, 2010). The copy numbers of AOA are higher than AOB in most environments (Schleper, 2010). In addition, *Nitrosomonas* growth, a AOB genus, is inhibited below pH 6.5 and bacterial nitrification rates are inhibited at pH 6.0 or lower (Tate, 1999; Bothe et al., 2007). Furthermore, I used AOB primers designed for sequences obtained from rice roots, activated sludge, a freshwater, and Everglades soils (Rotthauwe et al., 1997; Smith, 2006). Thus, my primer set may not have detected AOB genes in tributary sediments, indicating that the AOB genes in my samples could be different from those of rice roots, activated sludge, a freshwater, and Everglades soils.

### Relationships between Microbial Diversity and Richness of AOA and Biogeochemical Properties

Rarefaction curves of stream and riparian sediments from all sites were near plateau at 10% cutoff. Thus, the number of clones sequenced accounted for diversity of *amoA* in my libraries. Diversity indices showed a weakly positively correlation with pH in

stream sediments; however this relationship was not observed in riparian sediments. Soil pH is one of major factors influencing nutrient chemical forms, concentrations, availability of substrate, cell growth, and transformation rates (Kemmitt et al., 2006). A strong linear correlation between pH and microbial diversity has been observed in terrestrial (Fierer et al., 2006) and aquatic ecosystems (Lindström et al., 2005). In particular, nitrification rates are known to be low under acidic conditions (Boer and Kowalchuk, 2001; Tate, 2000). This phenomenon can be explained by reduction in $NH_3$ availability with decreasing pH through ionization to $NH_4^+$ (Frijlink et al., 1992). Thus, the availability of $NH_3$ under acidic conditions could limit nitrification rates and nitrifier diversity. However, most previous research focused on the effect of pH on AOA gene abundances rather than on diversity of AOA, and the results are also very controversial. Some research demonstrated a positive relationship between pH and abundances of AOA genes in lake sediments (pH 6.8-8.0), acidic soils (pH 3.7-5.8), and rivers, (Wu et al., 2010; He et al, 2007; Liu et al., 2010). Other studies showed either a negative relationship between AOA abundances and pH in acidic soils (pH4.5-7.0) (Nicol et al., 2008) and fresh sediments (pH 4.8-7.7) (Herrmann et al, 2009), or no correlation between them in estuarine sediments (pH 6.7-7.3) (Mosier and Francis, 2008) and fertilized soils (pH 8.3-8.7) (Shen et al., 2008). Thus, it is difficult to describe the relationship between AOA diversity and pH in my research. Despite this limitation, one possible explanation can be the level of ammonium concentrations. Stream sediments had a relatively higher pH, lower ammonium concentrations, lower nitrification and ammonification rates. Riparian sediments showed a relatively lower pH, higher ammonium concentrations, and higher nitrification and ammonification rates compared

to stream sediments (Chapter 3). Thus, under ammonium limited conditions such as stream sediments, the effect of pH on $NH_3$ availability could be significant for influencing AOA diversity, resulting in a weak positive relationship between pH and AOA diversity. However, under relatively high ammonium conditions such as riparian sediments, $NH_3$ could less limit the AOA diversity, because the continuous supply of ammonium to systems via ammonification or external supply from organic matter may maintain $NH_3$ availability to AOA, despite lower pH conditions. Also, AOA in riparian sediments might be more well adapt to acidic conditions for long term than that of stream sediments. Thus, a greater supply of ammonium and adaptation of AOA to acidic conditions could lead to the absence of a relationship between pH and AOA diversity in riparian sediments. Furthermore, oxygen concentrations and immobilization of ammonium could affect AOA assemblage compositions. Thus, these variations may result in the absence of a correlations between diversity index and pH in riparian sediments, and weak correlations between them in stream sediments.

Results indicated that high organic carbon contents led to a weakly decreased diversity of AOA in riparian sediments. Previous research demonstrated that the *Archaea Nitrosopumilus maritimus*, initially cultured from ocean water, grew chemoautotrophically via conversion of ammonia to nitrite using bicarbonate as a carbon source. However, the addition of organic carbon inhibited its growth rate (Kőnneke et al., 2005) and reduced the number of *amoA* gene copies (Hallin et al., 2009). Also, Wu et al., (2010) also demonstrated that AOA abundances were negatively correlated with accumulation of organic matter in lake sediments. This implies that inhibition by organic matter could prevent the AOA from becoming a more diverse group

in systems with higher amounts of organic matter. Therefore, higher carbon content in T1 and T2-U could restrict growth rates of AOA, resulting in a less diverse group. However, until now the mechanism linking the inhibition of organic matter to AOA remained unclear. Thus, correlations between Ext. Org C and diversity index might be regulated by other factors such as oxygen content, immobilization of ammonium, or ammonification rates capable of supplying ammonium to nitrifiers, resulting in a weak correlation between them.

I examined if diversity index of AOA correlates with nitrification rates in tributary sediments. However, there were no correlations between them, indicating that diversity of AOA was not related to potential nitrification rates in my samples. Therefore, unlike results from denitrification, nitrification rates could be more related to biogeochemical factors rather than microbial assemblage compositions.

### Relationships between Microbial Assemblages of AOA and Biogeochemical Properties

The AOA assemblage of T2-D was significantly different from those of T1 and T2-U. Also, the Mantel test showed that pH was a primary factor controlling microbial assemblage compositions for AOA in tributary sediments. Previous studies have described the effect of pH on the abundance of *amoA* gene and nitrification rates (He et al., 2007; Hallin et al., 2009); however, there is little study investigating the role of pH in the distribution of the AOA assemblage compositions. According to my results, the larger the difference of pH between sites, the larger the difference between AOA groups. Other factors reported to determine the distribution of AOA microbial assemblage in a subtropical macro-tidal estuary are salinity and the TC:TN ratio (Abell et al., 2010). In hot springs, geography had been found to be a main factor determining microbial

assemblages of AOA (Zhang et al., 2008). Therefore, more research is needed on distributions of AOA, comparing environmental factors across the globe, and mechanisms linking pH with *Archaeal amoA* gene distributions.

## Summary

Tributary sediments are transitional gradients between terrestrial and aquatic ecosystems, and thus may exert environmental controls over microbial community structure and function. Environmental characteristics can control microbial assemblage compositions in tributary ecosystems, which in turn could affect microbial functions in these systems. My research investigated the relationships between biogeochemical properties, nitrification rates, and *Archaeal amoA* (AOA) assemblage compositions in tributary sediments. Results indicated that a weak positive relationship exhibits between diversity index for AOA (Simpson index) and pH ($R^2$=0.42, p=0.0229) in stream sediments, possibly due to increased competition in the low pH. For riparian sediments, a weak negative correlations between diversity index for AOA (Shannon index) and organic carbon contents was observed ($R^2$=0.41, p=0.0259). However, until now, mechanism linking the organic matter contents and diversity of AOA remains unclear. Also, diversity index did not influence potential nitrification rates, implying that nitrification rates may be regulated by biogeochemical factors rather than microbial assemblage compositions in tributary sediments. The Mantel tests showed that pH was a significant factor affecting microbial assemblage compositions for AOA in tributary sediments (Mantel r=0.44, p=0.04). Thus, the larger the difference in pH between sites, the larger the difference of AOA assemblage compositions in tributary sediments.

Even though this research did not observe a significant relationship between diversity index (microbial structure) and nitrification rates (microbial function), the results

suggested that diversity index might be linked with pH and organic matter contents in tributary sediments. Also, my research suggested that the distribution of AOA was regulated by pH in tributary sediments.

Table 6-1. Relative abundances of PCR products of AOA (*Archaeal amoA*) and AOB (*Bacterial amoA*) in tributary sediments of T1 and T2-U for December 2006, March, May, July, and August 2007 samples. Comparisons were determined by relative intensities of EtBr (Ethidium bromide) stain.

| | Seasons | T1 Stream sediments | T1 Riparian sediments | T2-U Stream sediments | T2-U Riparian sediments |
|---|---|---|---|---|---|
| AOA | Dec 2006 | - | ++++ | ++++ | +++ |
| | Mar 2007 | + | + | + | + |
| | May 2007 | + | + | + | ++ |
| | Jul 2007 | + | + | +++ | ++ |
| | Aug 2007 | - | + | + | + |
| AOB | Dec 2006 | - | ++ | - | - |
| | Mar 2007 | - | + | - | - |
| | May 2007 | + | + | - | + |
| | Jul 2007 | - | + | - | - |
| | Aug 2007 | + | - | - | + |

Table 6-2. *Archaeal amoA* diversity and richness in tributary sediments, estimated by the Shannon, Simpson, and Chao 1 indices.

| | Sites | No. of clones sequenced | No. of OTUs[a] | Shannon index | Simpson index | Chao1 Index |
|---|---|---|---|---|---|---|
| Stream sediments | T1 | 174 | 24 | 2.4 (2.2-2.5) | 0.86 | 28 (25-46) |
| | T2-U | 138 | 26 | 2.5 (2.2-2.7) | 0.85 | 19 (27-42) |
| | T2-D | 132 | 23 | 2.7 (2.5-2.8) | 0.91 | 26 (33-40) |
| | All | 444 | | | | |
| Riparian sediments | T1 | 121 | 14 | 1.8 (1.6-2) | 0.75 | 19 (14-46) |
| | T2-U | 151 | 16 | 1.8 (1.6-2) | 0.75 | 21 (16-44) |
| | T2-D | 69 | 13 | 2.36 (2.2-.25) | 0.91 | 14 (13-21) |
| | All | 341 | | | | |

[a] Estimated average of OTUs, diversity and richness are all based on 10% or less differences in nucleic acid sequence alignments; upper and lower bounds of 95% confidence intervals as calculated by DOTUR.

Table 6-3. Regression analysis of transformed data taken from distribution matrices; y-axis represent *Archaeal amoA* assemblages and x-axis represents biogeochemical properties, generated by the Mantel test.

| | pH | $NH_4^+$-N | Ext. Org N | MBN | Ext. Org C | MBC |
|---|---|---|---|---|---|---|
| Mantel r | 0.44 | -0.3 | -0.12 | -0.06 | 0.03 | -0.002 |
| p value | 0.04 | 0.93 | 0.61 | 0.58 | 0.36 | 0.52 |

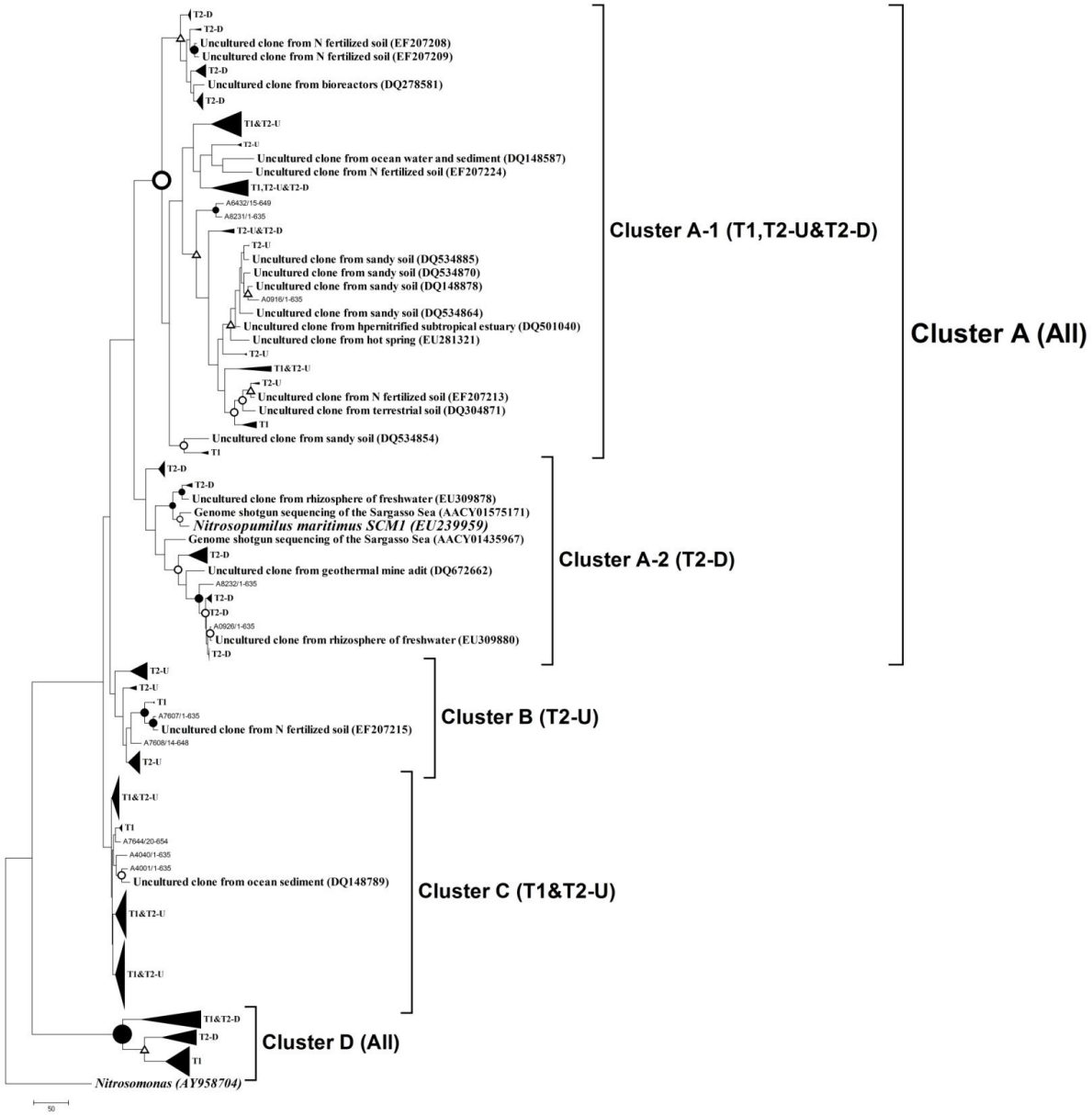



Figure 6-1. Maximum parsimony tree of *Archaeal amoA* sequences obtained from stream sediments. The dark circles (values over 90), empty circles (values from 70 to 90) and empty triangles (values from 50 to 70) on nodes are bootstrap scores based on percent occurrence of 100 re-samplings.

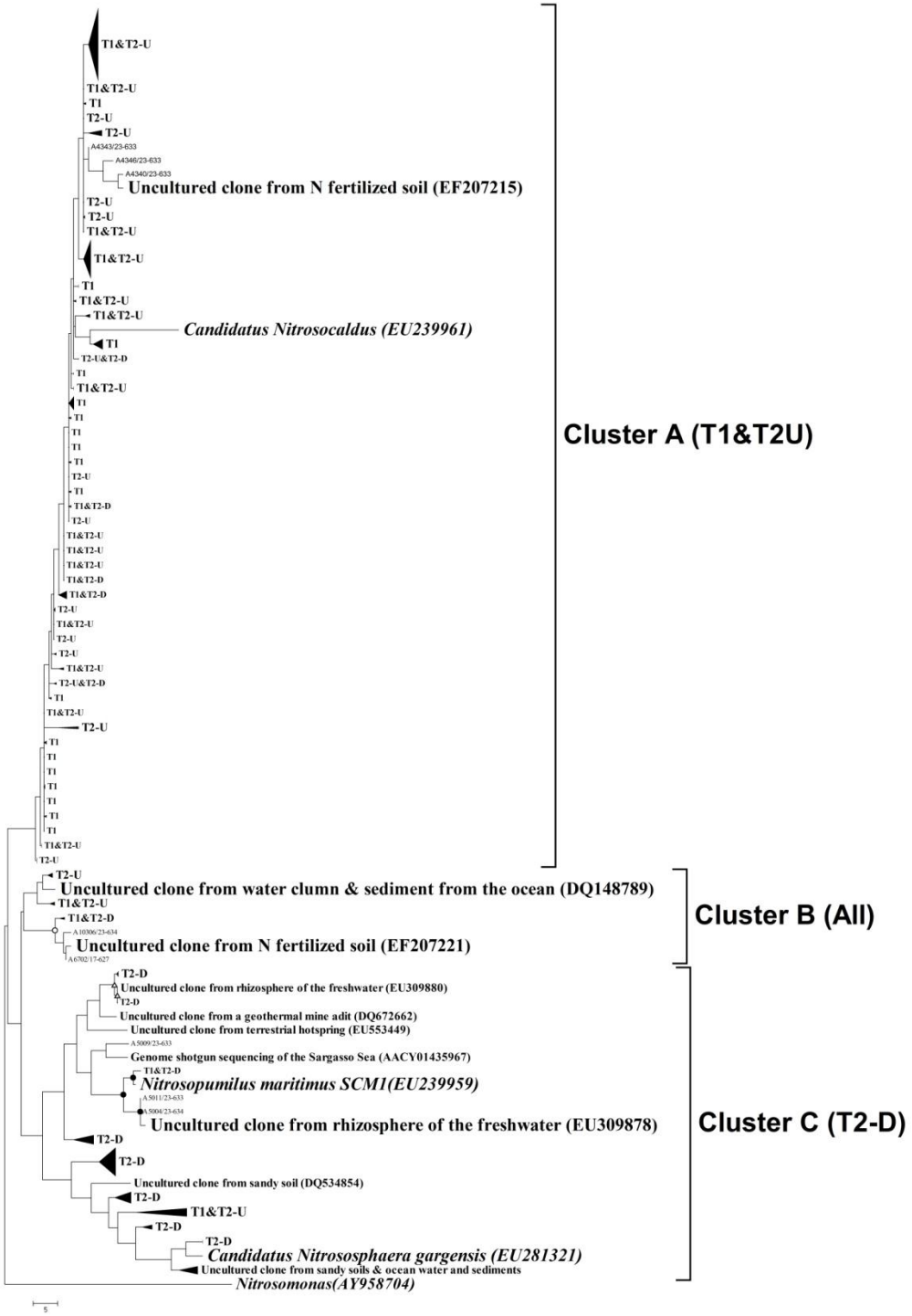
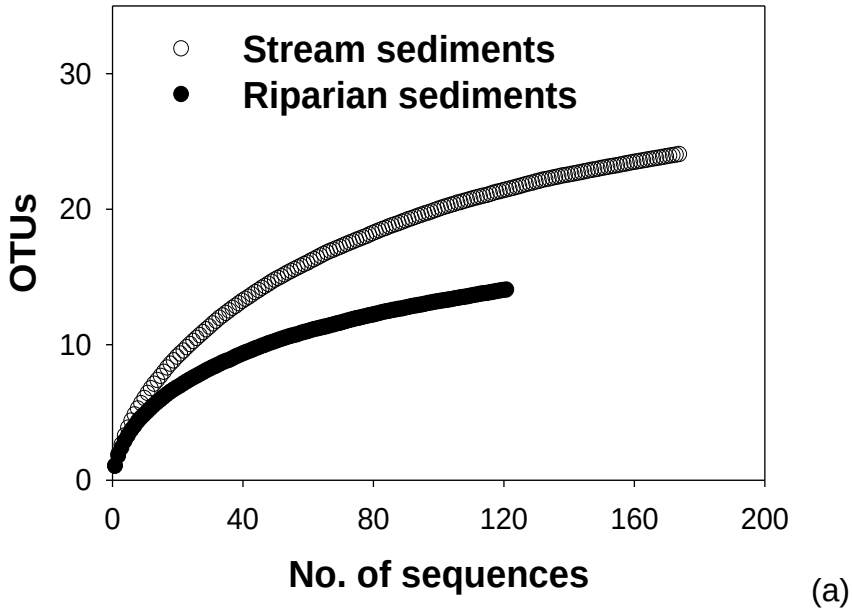
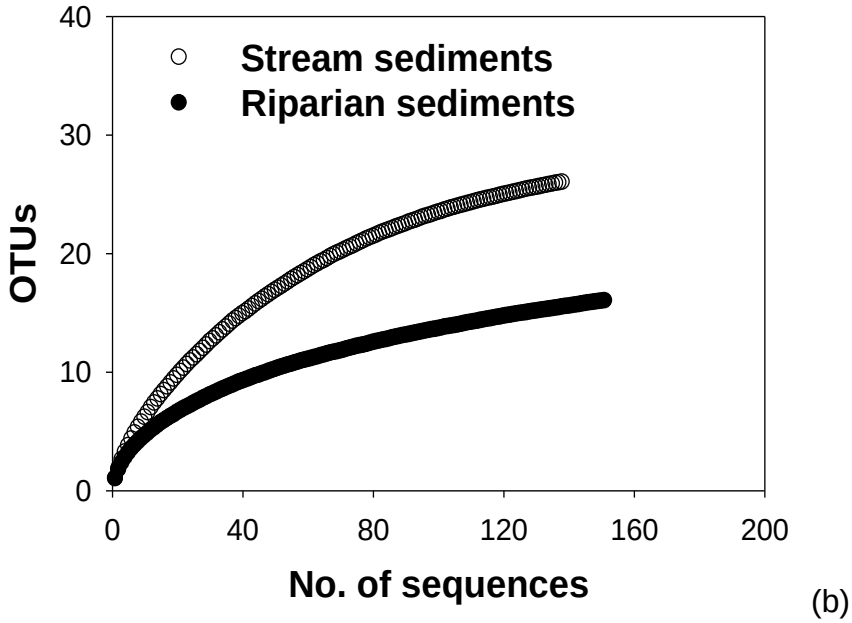



Figure 6-2. Maximum parsimony tree of *Archaeal amoA* sequences obtained from riparian sediments. The dark circles (values over 90), empty circles (values from 70 to 90) and empty triangles (values from 50 to 70) on nodes are bootstrap scores based on percent occurrence of 100 re-samplings.

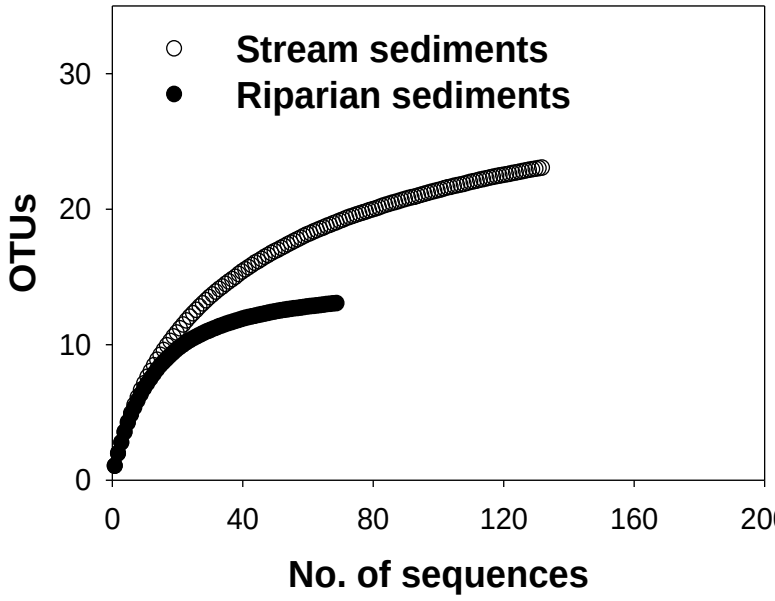



(a)




(b)

Figure 6-3. Rarefaction curves for *Archaeal amoA* from DOTUR analysis using a furthest neighbor assignment algorithm in T1 (a), T2-U (b) and T2-D (c) tributary sediments. The similarity cutoff was 90%.




(c)

Figure 6-3. Continued.

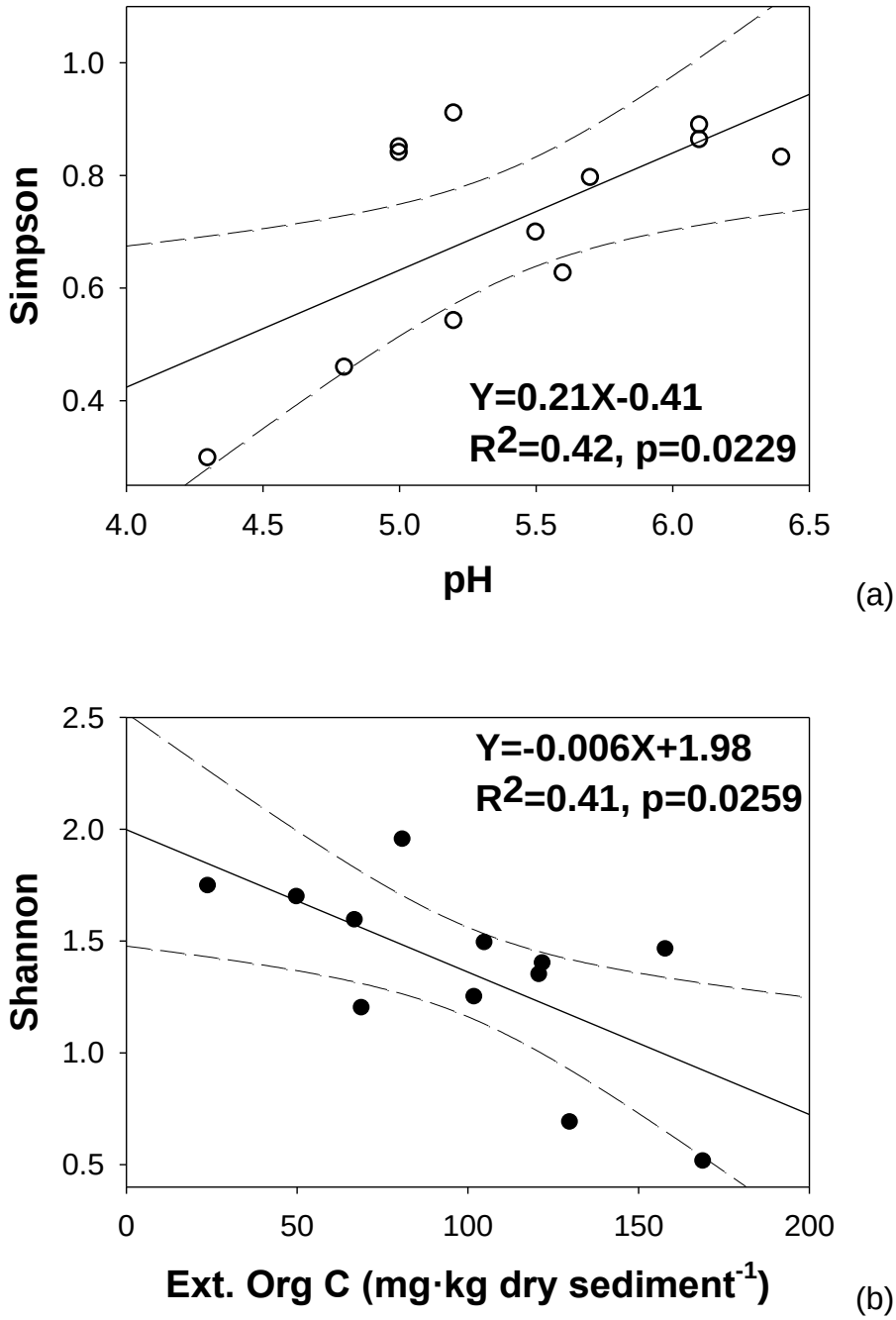



Figure 6-4. Relationships between pH and the Simpson index for *Archaeal amoA* in stream sediments (a), and extractable organic carbon concentrations (Ext. Org C) and the Shannon index for *Archaeal amoA* in riparian sediments (b) (Dashed line are the 95% confidence intervals of the regression line, n=12).

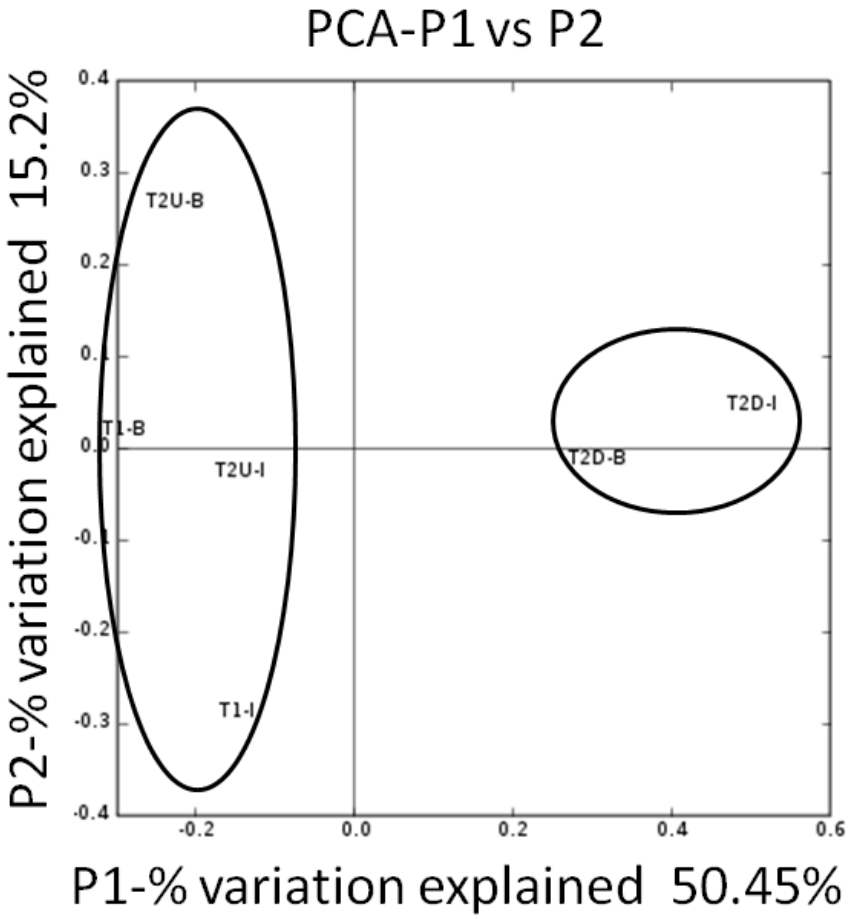



Figure 6-5. Principal component analysis for *Archaeal amoA* assemblage composition using the Unifrac in tributary sediments.

# CHAPTER 7
# CONCLUSIONS AND SYNTHESIS

Human activities have doubled the rate of reactive nitrogen input to the global nitrogen cycles, and the amount of nitrate input has been continuing to increase (Vitousek et al., 1997). This anthropogenic increase in nitrogen caused by fertilizer utilization is estimated to be 80Tg·yr$^{-1}$ (FAO, 1993; Schlesinger et al., 1992; Matthews, 1994). In pasture ecosystems, dairy manure is also considered to be a key source of excessive input of nitrogen (Adriano et al., 1971; Smith et al., 1980; Cooper et al., 1984; Burkart and James, 1999). Of the total nitrogen produced by human activities, including N fertilizer, agricultural activities, and N depositions, rivers are estimated to receive 20 to 25% of the N added to ecosystems (Galloway et al., 2004; Green et al., 2004). However, an estimated half of the nitrogen entering rivers is denitrified in buffer systems such as riparian, tributaries, and floodplains in watersheds (van Breemen et al., 2002; Galloway et al., 2004). In particular, small streams have a significantly higher efficiency of nitrate removal due to increased benthic denitrification rates (Alexander et al., 2000). However, denitrification rates in small streams can be affected by other inorganic nitrogen transformations such as nitrification, anammox, and DNRA. Nitrification can supply nitrate to denitrifiers, and DNRA and anammox can compete with denitrification for gaining nitrate and nitrite, respectively.

To better understand the function of tributary ecosystems for nitrogen removal, I investigated the potential inorganic nitrogen transformation rates including nitrification, anammox, denitrification and DNRA, and biogeochemical factors affecting these rates. I investigated how extracellular enzyme activities in litter and tributary sediments influenced potential denitrification rates, one of main processes for nitrate removal in

tributary ecosystems. Also, since the diversity of microbial community composition can affect microbial functions, I investigated if microbial assemblage compositions for nitrifier and denitrifier were related to their process rates, and explored the biogeochemical properties affecting microbial assemblage compositions.

**Inorganic Nitrogen Transformations and Biogeochemical Properties**

Relative rates of nitrate removal through denitrification were higher than those of DNRA in tributary sediments. Ammonium removal rates through nitrification are significant compared to anammox. Thus, removal of nitrate and ammonium in tributary sediments were mainly regulated by denitrification and nitrification rather than DNRA and anammox (Figure 7-1). In addition, riparian sediments exhibited higher potential rates of inorganic nitrogen transformations than those of stream sediments due to higher labile organic carbon contents. Thus, riparian sediments can be more important for inorganic nitrogen cycling in tributary ecosystems than stream sediments (Figure 7-1).

In order to explain the effect of biogeochemical properties on potential inorganic nitrogen transformation rates in tributary sediments, I conducted regression analyses with their rates and biogeochemical properties. Results showed a weak positive relationship between pH and potential nitrification rates in tributary sediments, implying that the decreased pH might suppress nitrification rates via a decreased ratio of $NH_3$ to $NH_4^+$ under acidic conditions. For denitrification, potential rates were negatively correlated with the TC:TN ratio in riparian sediments, implying that carbon quality may regulate denitrification rates. However, increased TC:TN ratio could enhance immobilization of nitrate, which in turn decrease nitrate availability to denitrifiers. In case of DNRA, potential rates increased with organic carbon contents in riparian sediments,

implying that high contents of organic carbon could enhance DNRA rates. Thus, when we add organic carbon to soils for removing nitrate through denitrification, we have to consider the influence of organic carbon on immobilization and carbon quality in the systems. Additionally, increased ammonification rates were associated with increased ammonium concentrations in riparian sediments.

### Extracellular Enzyme Activities and Denitrification Rates

To explain the effect of carbon quality on the supply of organic carbon to denitrifiers, cellulase, β-D-glucosidase, and phenolic oxidase enzyme activities were measured as an index of decomposition rate in tributary sediments and litter materials. Results indicated that the tributary system with lower cellulase activities in litter had lower denitrification rates than the tributary system with higher cellulase activities in litter. Cellulase activities in tributary sediments were positively correlated with potential denitrification rates in tributary sediments. Also, a weak negative relationship between cellulase activities and TC:TN ratio of litter was observed. Thus, the substrate quality representing the TC:TN ratio could affect cellulase activities in litter, affecting availability of organic carbon to denitrifiers and their rates.

### Microbial Assemblage Compositions for Denitrifiers and Nitrifiers, Biogeochemical Properties, and Rates

The riparian sediments with the highest organic carbon contents exhibited the lowest potential denitrification rates, while riparian sediments with the lowest organic carbon contents showed the highest potential denitrification rates. Generally, many studies have shown that denitrification rate increases with available organic carbon contents; however, results did not follow this pattern. Therefore, the diverse microbial assemblage composition of *nirS* (representing denitrifiers) could influence denitrification

rates via the selection of denitrifiers with genes capable of using the carbon source more efficiently. The diversity index for *nirS* was negatively correlated with organic carbon contents, and the systems with highest diversity index exhibited the highest denitrification rates. Thus, assemblage compositions for *nirS* may be linked with their microbial functions, and these relationships can be regulated by the level of organic carbon in the systems.

In case of nitrifiers, a weak positive relationship between diversity index for *archaeal amoA* (AOA) and pH was observed in stream sediments, likely due to increased competition in the low pH system. In addition, the distribution of AOA was regulated by pH in tributary sediments. However, the system with the more diverse AOA did not exhibit increased potential nitrification rates in tributary sediments. Thus, unlike results from denitrifiers, microbial assemblage composition for AOA was not directly related to the process.

## Synthesis

Tributary systems with relatively low pH and low quality of carbon such as forest streams or wetlands will exhibit low nitrification and denitrification rates. Acidic conditions may decrease the availability of $NH_3$, resulting in decreased nitrification rates. This reduction of nitrification rate could decrease denitrification rate via a lower supply of nitrate to denitrifiers. Accumulation of low quality carbon such as recalcitrant organic matter or lignin litter could retard the decomposition rates, resulting in lower supplies of labile organic carbon to denitrifiers. Thus, decreased nitrification and denitrification rates may lead to accumulations of ammonium and nitrate in the systems.

Tributary systems with relatively high pH, and relatively high quality of carbon such as grassland buffer strips will exhibit high nitrification and denitrification rates.

Nitrification will not be inhibited by pH at this site, as it is at the other sites due to their low pH, resulting in an increase of nitrification rates with relative high pH. In addition, high quality of carbon substrate, such as low lignin and high cellulase contents, will supply more labile carbon to denitrifiers via higher extracellular enzyme activities associated with decomposition rates of litter and organic matter. This will enhance denitrification rates, possibly resulting in the reduction of nitrate concentrations. Thus, to understand the buffer capacity of tributary ecosystems for nitrate removal, the relationships between overall inorganic nitrogen transformations and carbon quality should be considered.

## Future Studies

Future studies should focus on measuring *in situ* rates of nitrogen transformations to clarify the relationships between biogeochemical properties and inorganic nitrogen transformations at landscape scales. We need to investigate if these relationships in various tributary ecosystems are affected by human activities in order to establish appropriate management plans for nitrogen. Also, to maximize denitrification rates in tributary ecosystems, we should clarify the effect of carbon quality on availability of labile carbon using analyses of *in situ* litter decomposition rates and litter chemistry such as lignin, hemicellulose, and cellulose contents. Furthermore, to better understand how microbial structure affects microbial function, the research on enumeration of nitrifiers and denitrifiers is needed, as are studies on the diversity of anammox and DNRA microbes.

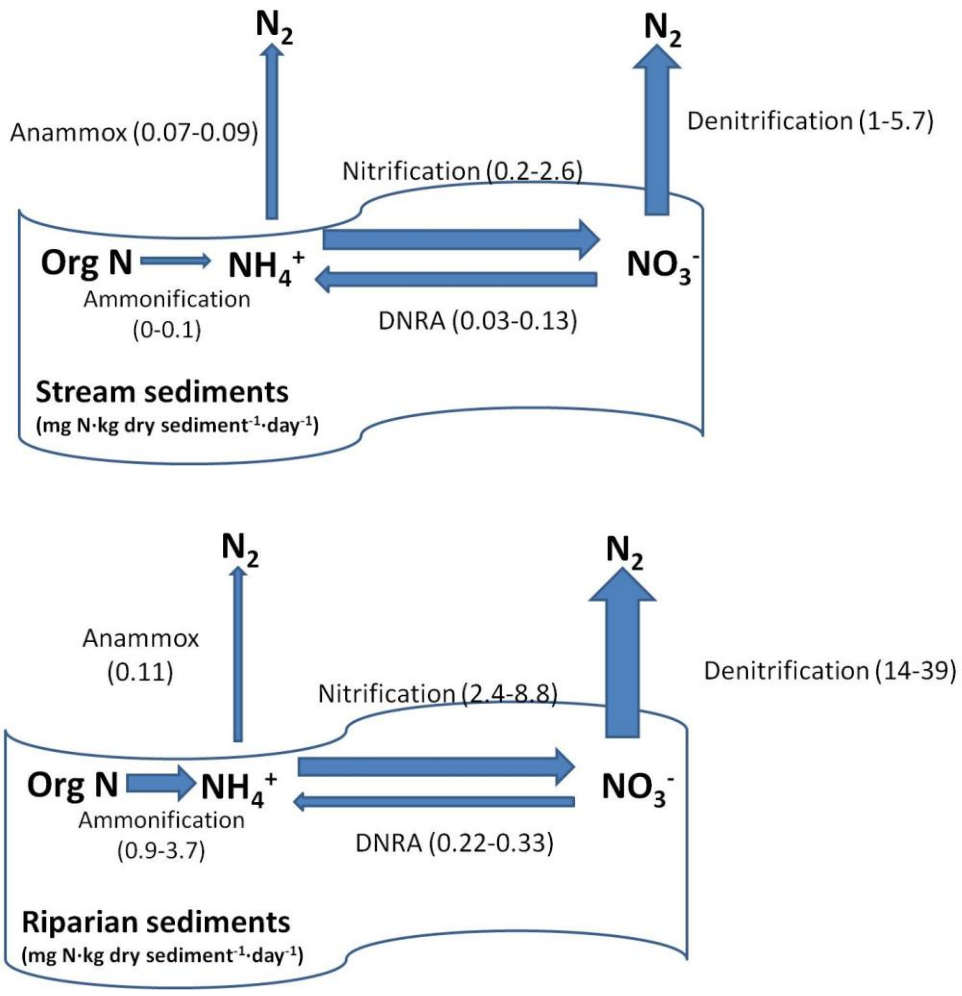



Figure 7-1. Potential rates of inorganic nitrogen transformations in (a) stream sediments and (b) riparian sediments.

# APPENDIX
# CACULATION OF ANAMMOX RATES

The potential rates of anammox are calculated by the difference in the ratios of $^{14}N^{15}N$ to ($^{14}N^{14}N$+$^{14}N^{15}N$+$^{15}N^{15}N$) between natural abundance and samples. Assuming the p is the atom fraction of $^{14}N$ and q is the atom fraction of $^{15}N$,

p=atom fraction of $^{14}N$, q=atom fraction of $^{15}N$

$p + q = 1$

$(p + q)^2 = p^2 + 2pq + p^2 = 1$ , so we can say

$p^2$=$^{14}N^{14}N$, 2pq=$^{14}N^{15}N$, $q^2$=$^{15}N^{15}N$

We can assume that $r = \frac{\text{ion current of mass 29}}{\text{ion current of mass 28}} = \frac{2pq}{p^2} = \frac{2q}{p}$ , $\frac{r}{2} = \frac{q}{p}$

$$\text{The atm \% of 15N} = 100 \times \frac{\text{No of 15N atmos}}{\text{No of (14N+15N) atoms}} = 100 \times \frac{q}{p+q} = \frac{q/p}{1+q/p} = 100 \times \frac{r/2}{1+r/2} = \frac{100r}{2+r}$$

From above equation, $r = \frac{2 \times atm\,\%\,15N}{(100 - \text{atm \% 15N})}$

With atm% of $^{15}N$, we can get the "r" value from above equation.

$$r' = \frac{\text{ion current of mass 30}}{\text{ion current of mass 28} + 29} = \frac{q^2}{p^2 + 2pq} = \frac{q/p}{p/q + 2} = \frac{r/2}{2/r + 2} = \frac{r^2}{4 + (1 + r)}$$

We know the "r" value from the above equation so that we can get the r'. The mole fraction of the various isotopic species in $N_2$ gas is (a+2b+c=1), where a=$^{14}N^{14}N$ to ($^{14}N^{14}N$+$^{14}N^{15}N$+$^{15}N^{15}N$), 2b=$^{14}N^{15}N$ to ($^{14}N^{14}N$+$^{14}N^{15}N$+$^{15}N^{15}N$), and c=$^{15}N^{15}N$ to ($^{14}N^{14}N$+$^{14}N^{15}N$+$^{15}N^{15}N$) can be expressed in terms of the measured rations, r and r', as follows (Hauk, 1958):

$r = \frac{2b}{a}$ $r' = \frac{c}{a+2b}$

$$\text{Fraction ratio of 14N14N to (14N14N + 14N15N + 15N15N)} = a = \frac{1}{(1+r)*(1+r')}$$

$$\text{Fraction ratio of } 14\text{N}15\text{N} \text{ to } (14\text{N}14\text{N} + 14\text{N}15\text{N} + 15\text{N}15\text{N}) = 2\text{b} = 1 - (\text{a} + \text{c})$$

$$\text{Fraction ratio of } 15\text{N}15\text{N} \text{ to } (14\text{N}14\text{N} + 14\text{N}15\text{N} + 15\text{N}15\text{N}) = \text{c} = \frac{\text{r}'}{1+\text{r}'}$$

With r and r', we can calculate a, 2b, and c. After total amount of $N_2$ in the sample is calculated, this amount is multiplied by 2b and divided by incubation days.

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# BIOGRAPHICAL SKETCH

Haryun Kim was born in Seoul, Korea. She received her bachelor's degree in environmental education in 2000 from the Korea National University of Education, Korea. She studied at the Department of Environmental Science and Engineering in Ewha Womans University in Seoul, Korea, which had an emphasis on ecology and gave her an opportunity to research nitrogen cycling. During her master's studies, Dr. Hojeong Kang was fascinated with the studies and research conducted in the lab. At first, she assisted the research on nitrogen cycling of constructed wetlands. The following semester, she received her own project about nitrogen cycling in forest ecosystems as a master's thesis. This research was funded by Korea Environment Ministry. Since she enjoyed working with Professor Dr. Hojeong Kang and loved conducting research with biogeochemistry and microbiology, she was looking to expand the scope of research to the entire system in which they operate. Biogeochemistry and microbial ecology attracted her because it enabled her to see the entire picture from the smallest details (e.g., genes) up to the biggest ones (the entire system). On 2005, she joined the Ph. D program at UF in the Soil and Water Science Department with Dr. Andrew Ogram and Dr. Reddy as her advisor. She wanted to learn more about biogeochemistry and how to apply this knowledge to the management and recovery of soil and river ecosystems.