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Denitrification capacity and denitrifying bacteria in a restored bottomland hardwood forest, Mississippi River Alluvial Valley: hydrological impacts

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**DENITRIFICATION CAPACITY AND DENITRIFYING BACTERIA IN A RESTORED
BOTTOMLAND HARDWOOD FOREST, MISSISSIPPI RIVER ALLUVIAL VALLEY:
HYDROLOGICAL IMPACTS**

A Thesis

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

In
The Department of Environmental Studies

by
Yan Li
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ABSTRACT

Mississippi River Alluvial Valley (MAV) is one of the most important ecosystems in the United States, and bottomland hardwood forests (BLHs) are a major components of this ecosystem. The ecological and hydrological functions of BLHs can help maintain the diversity of species, groundwater balance, and nutrient removal etc. However, due to the increased human activities in the area, most of the BLHs were converted to agricultural land. Realizing the seriousness of the situation of the ecosystem, a series of programs have been carried out by the U.S. Fish and Wildlife Service (USFWS) and the U.S. Department of Agriculture (USDA) to restore the forested wetlands since several decades ago. However, the efficacy of these activities on denitrification is unclear. In this study, field and laboratory studies were carried out to determine the effects of hydrologic restoration (i.e. periodical flooding) on the denitrification capacity and denitrifying bacteria in a 30-year old restored BLH in the Red River Wildlife Management Area (RAWMA) of the MAV, and designed a better cultivation medium for denitrifying bacteria isolation from soils in this area. *In-situ* denitrification was estimated using the C₂H₂ block technique with a static chamber and potential denitrification rate was determined in the laboratory using a modification of the denitrification enzyme assay (DEA) method. Both *in-situ* and potential denitrification rates with amendment of nitrate (NO₃⁻) were significantly (P < 0.05) higher at flooded, lower elevation sites than those of higher elevation sites where the hydrology was not restored. Without amendment of NO₃⁻, no significant differences in potential and *in situ* denitrification rates were observed between low and high elevations. The population of denitrifying bacteria was measured by the most probable number (MPN) method, and denitrification gene *nirK* quantified by real-time quantitative PCR. The MPN and PCR results

were consistent with each other, without significant differences between the high and low elevation sites. The isolation rates of denitrifying bacteria from the designed medium No. 1 and No. 2 were higher than the classic cultivation medium. The numbers of species isolated by these two media still need to be determined, and compared to the classic one. Further improvement of the medium will be based on the evaluation of this first generation of media.

1. INTRODUCTION

The Mississippi River Alluvial Valley (MAV) is located downstream of the Mississippi River, with most of it historically covered by Bottomland Hardwood Forests (BLHs). The BLHs serve as wildlife habitat, timberland resources, groundwater balance regulator, and nutrient absorber. This ecosystem is essential for the regional environment. Most of the BLHs in the MAV disappeared after the emergence of agricultural activities in this area, especially during the last century. The conversion of BLHs to farmland brought several problems to the local and adjacent environment, including a decrease of species diversity and quantity of wildlife and trees, an increase of nutrient concentration in the surface and groundwater, and a loss of hydrological regulation.

With the restoration programs conducted by U.S. government agencies, millions of acres of farmland have been converted back to forests and researchers started to evaluate the success of the restoration. Species and function recoveries are the aspects that researchers have focused on.

Denitrification is a biogeochemical process associated with the nutrient reduction function of a forest ecosystem. It is mainly maintained by a group of microorganisms that have the ability to convert nitrate to gaseous nitrogen forms: N_2O or N_2 . With the emission of N_2O or N_2 , nitrate concentration in the soil is reduced. Denitrifying bacteria are the major members in this functional group.

Hydrological conditions may affect denitrification and denitrifying bacteria activities. Periodic flood may increase denitrification rate by affecting soil moisture, soil texture, available nitrate and organic carbon. High soil moisture and fine soil texture enhance denitrification. A

higher concentration of available nitrate and organic carbon also increase denitrification in those instances where nitrate or organic carbon is limited in the environment. Periodic flood may alter the community structure of denitrifying bacteria, but may not influence the population size. Under certain conditions, the community structure may be more important in determining denitrification rate and potential than the absolute population size alone. The primary purpose of this study is to determine the hydrological impacts on denitrification capacity and denitrifying bacteria in a restored BLH in the MAV.

In order to reveal the ecological functions and physiological characteristics of denitrifying bacteria in an environment, an appropriate medium is essential for isolation and cultivation of these organisms. So far, no a single medium has been approved that is good for all the denitrifying bacteria because of their wide phylogenetic distribution. Designing and optimizing a cultivation medium by using an evolutionary algorithm is another focus of this study.

2. LITERATURE REVIEW

2.1 Mississippi River Alluvial Valley (MAV)

The Mississippi River Alluvial Valley, one of the most extensive floodplains in the United States, is located in the historic floodplain of the Mississippi River that flows from Illinois through Missouri, Kentucky, Arkansas, Tennessee, Mississippi and Louisiana (Llewellyn et al., 1996) (Figure 2.1). It is a unique and important wetland ecosystem, consisting of bottomland hardwood forests (BLHs), oxbow lakes, and cypress swamps. Bottomland hardwood forests are floodplain forests, found along rivers and streams of the southeast and south central United States (Huffman and Forsythe, 1981; Mitsch and Gosselink, 1993). Historically, the largest concentrated BLHs in the U.S. were in the MAV (Sharitz and Mitsch, 1993).

Bottomland hardwood forests provide valuable hydrological and ecological functions, such as groundwater balance maintenance, flood control, water quality enhancement, and nutrient removal. They also provide timber products and serving as wildlife habitats (Walbridge, 1993). The variety of these ecosystems and their long growing season make them an important habitat for a wide diversity of wildlife species. Because of the fertile soil and healthy forests, however, these BLHs in the MAV became one of the first ecosystems used for logging and farming in the United States (Cowdrey, 1983).

Due to the extensive agriculture activities, the loss of these forests exceeded 120,000 ha per year during 1950s to 1970s (National Research Council, 1982) and by 1982, only 2.8 million ha of the original estimated 10 million ha BLH (at the time that Europeans arrived) remained (Hefner and Brown, 1985).

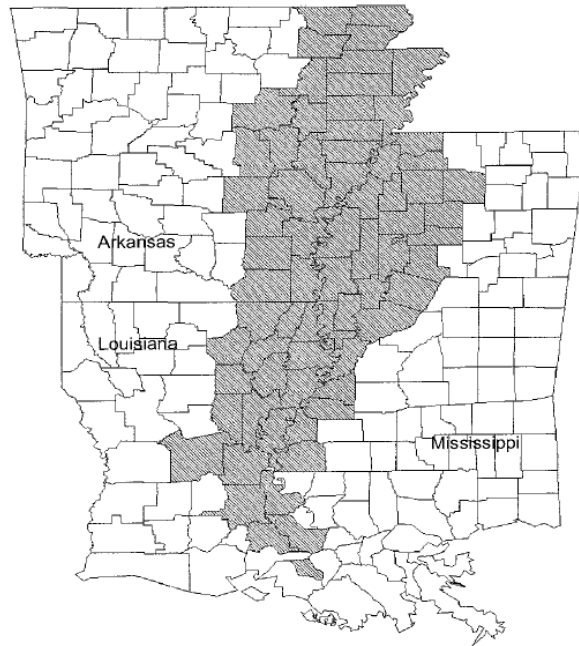


Figure 2.1 Counties and parishes in the lower Mississippi River Alluvial Valley in Arkansas, Mississippi and Louisiana (Schoenholtz et al., 2001).

The influences of BLH loss on the entire ecosystem have been recognized. A study by Rudis (1995) showed that regional forest fragmentation impacted community types, anthropogenic uses and resource values of bottomland hardwood. The author also found that wildlife habitat in the BLH was influenced. Sallabanks et al. (2000) suggested that BLH may be important to bird species preservation. Burdick et al. (1989) found that bird population and density were adversely affected by the reduction of BLH area. Corner et al. (2002) studied reptiles and amphibians in a BLH and suggested that certain logging activities may alter the herpetofaunal species composition. The abundance and quality of BLH also affect black bear and other wildlife species (Army Engineer Waterways Experiment Station, 1992). A study of a BLH riparian ecosystem indicated that a mature BLH is essential for water quality maintenance (Lowrance and Vellidis, 1995), and the ecosystem is an excellent nutrient sink and buffers the

nutrient discharge from the surrounding agricultural lands (Lowrance et al., 1984). BLH can also remove suspended sediment from water, which contributes to water quality improvement for the adjacent aquatic system. The distance from the river, flood duration, and tree basal area account for about 90% of the sedimentation rate (Kleiss, 1996). In the Cache River wetland, a BLH in Arkansas, the removal rate constant for inorganic suspended solid, total nitrogen and total phosphorous were 0.066 m/da, 0.048 da/super, and 0.0058 m/da (2.1 m/yr), respectively (Dortch, 1996). Ensign's study on a swamp showed that after clearcut, the swamp displayed significant higher suspended solid, total nitrogen, total phosphorous, total Kjeldahl nitrogen, and fecal coliform bacteria, but lower dissolved oxygen. Algal blooms also reoccurred after the clearcut (Ensign and Mallin, 2001). However, a restored BLH without the natural hydrologic regime reestablished cannot function as a natural BLH in the aspect of biogeochemical process, such as denitrification (Hunter and Faulkner, 2001). Loss of a BLH has caused hydrologic alteration in the area (Kolka, Singer et al., 2000). But with restoration of BHL near rivers or streams or on an erodible land, soil productivity could be protected (Williams, 1999).

During the last 200 years the MAV has lost about 80% of its BLHs, mainly to agriculture, making it one of the endangered ecosystems in the United States (Noss et al., 1995). This loss has radically changed the biogeochemical functionality of the altered ecosystems, in particular their ability to retain and process nitrogen (N). Since 1970's, programs have been undertaken by the U.S. government and state agencies to rebuild the hardwood forests (King and Keeland, 1999). In the 1970s and 1980s, the U.S. Fish and Wildlife Service (USFWS) aggressively reestablished BLHs on USFWS National Wildlife Refuges, private lands, and land transferred to the USFWS from the Farmers Home Administration (Haynes et al., 1995). The Conservation Reserve Program (CRP) and Wetland Reserve Program (WRP), carried out by the U.S.

Department of Agriculture (USDA) Natural Resources Conservation Service (NRCS), provided technical and financial assistance to landowners for wetland restoration and protection. The goal of CRP, the largest retirement program in the U.S. history (Szentandrasi et al., 1995), was to remove 40 – 45 million acres of highly erodible cropland from agricultural activity by 1990 and to rebuild it with trees (Ribaud, 1989). Landowners who participated in the program agreed to take the eligible land out of agriculture activity and put it into a reserve program for a 10-year period, and the landowners were compensated for the same period of time (Kennedy, 1990). By the mid 1990s, 36.5 million acres of cropland was enrolled in the program (Szentandrasi et al., 1995). The program of WRP restored over 8,000 acres of BLH in the lower Mississippi River area (NRCS, 2004).

After years of reforestation, a number of assessments and evaluations on the rebuilt wetland forests were conducted. Twedt et al. (2002) evaluated 2 to 10-year old rebuilt BLHs in Mississippi and Louisiana and found that the restoration benefited breeding birds. The author also found that the value of cottonwood reforestation for avian conservation was significantly greater than that of oak restoration in their first 10 years for avian conservation. Shear et al. (1996) compared 50-year old planted and naturally generated BLHs in Kentucky to mature forests and found that the restored forests adequately replaced the mature forests in terms of structure and function. Any remaining differences between natural and restored BLHs, the author speculated, would diminish over time.

The loss of BLHs has radically changed the biogeochemical functionality of the altered ecosystems, in particular their ability to retain and process N. However, little information is available regarding how a rebuilt BLH would restore its capacity of denitrification, which is one important function that removes N out of ecosystems (Henry et al. 2004).

2.2 Denitrification in Natural Ecosystems

2.2.1 Nitrogen cycle

Nitrogen is an essential element for humans, animals, plants, as well as microorganisms. It constitutes 16 % of proteins (Frink et al., 1999), which participate every process in cells as macromolecules. There is an abundance of nitrogen in the environment, of which nitrogen gas constitutes 78% (on the basis of volume) of the air. The nitrogen cycle is one of the key element cycles occurring on the earth. In this cycle, nitrogen gas is converted to ammonia mostly by a biological process, which is called nitrogen fixation. A portion of the ammonia converted from nitrogen gas is utilized by plants and microorganisms to provide the material for biosynthesis and growth support. An additional portion of the ammonia is converted to nitrate through the process of nitrification. Nitrate can be converted back to ammonia by a process known as ammonification or nitrate assimilation. Some of the nitrate is converted to nitrogen gas by a microbial process called denitrification (Zumft, 1997) (Figure 2.2), which is the major mechanism that removes nitrogen from an ecosystem.

Since the green revolution, much of the wetlands and forests have been converted to farmlands and nitrogen fertilizer has been widely used to increase the yield of crops and other plants to meet the needs of an increasing population (Tilman et al., 2001; Galloway et al., 2003). The excessive use of nitrogen fertilizer has caused serious environmental problems. The nitrate that cannot be utilized by plants enters into the surface water or ground water systems through surface flow or filtration, and becomes a source of pollution. High levels of nitrogen have been found in the Mississippi River. Model simulations and scientific

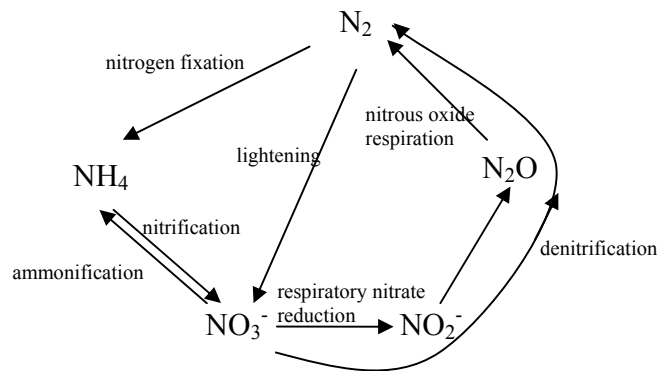


Figure 2.2 Global nitrogen cycle in natural ecosystems.

investigations have indicated that increasing nutrient loads from the Mississippi/Atchafalaya River systems are the main contributors to the recent decline in Gulf bottom water oxygen concentrations (Justic et al, 1993; Rabalais et al., 2002; Turner and Rabalais, 1994). The addition of anthropogenic nitrate has also caused hypoxia in western coast of India (Naqvi et al., 2000). In addition to causing ecological problems to the bodies of surface water, nitrate in groundwater can persist for decades and accumulate to a high level, which affects water quality and threatens the safety of drinking water (Hallberg, 1989; Nolan et al., 1997; Yang et al., 1998; Knobeloch et al., 2000).

2.2.2 Denitrification

Denitrification returns the fixed nitrogen into the atmosphere via gaseous nitrogen (i.e. NO , N_2O , or N_2). It plays a crucial role in the global cycling of nitrogen. The process of denitrification in natural ecosystems requires anaerobiosis and a supply of both NO_3^- and organic carbon. In an ecosystem, any soil property or environmental factor that can directly or indirectly influence one or more of these conditions may regulate *in situ* denitrification rates. Major possible regulation factors include soil moisture, soil texture, pH, anaerobiosis, available nitrate

and organic carbon. Peak denitrification rates were observed with an increase in soil moisture and the response time and duration were different depending on soil texture (Sexstone et al., 1985). Low soil moisture and mineral nitrogen slow down denitrification activity (Firestone and Davidson, 1989). The denitrification rate also decreases as the oxygen level increases and pH decreases (Cavigelli and Robertson, 2000).

Nitrous oxide (N_2O), which is produced during denitrification, is considered as a potential greenhouse gas in the troposphere. Although it is only responsible for about 6% of the global warming (Houghton et al., 1992), it is more potent than the other two common greenhouse gases, CO_2 and CH_4 , (due to its long lifetime about 150 years in the atmosphere (Pérez-Ramírez et al., 2003) and high global warming potential (GWP)). The GWP of N_2O is 5 and 280 times that of CH_4 and CO_2 over a 20-year time frame, 15 and 310 times in a 100-year frame, and 26 and 170 in a 500-year frame, respectively (IPCC, 1996). The concentration of N_2O in the atmosphere has increased from the pre-industry level of 285 ppb to present level of 310 ppb (Kavanaugh, 1987). The increase in the atmospheric concentration of N_2O contributes not only to global warming but also directly to destruction of stratospheric ozone layer (IPCC, 1997).

Recent estimates suggest that about 80% of the net increase in atmospheric N_2O is probably the result of agro-ecosystems (Mosier and Kroeze, 1999). It has been reported that the cultivation of soil and use of fertilizer contributed 57% of the total N_2O emission in European Union (Pérez-Ramírez et al., 2003). Drainage and cultivation of peatland increased N_2O emission from the soil, which used to be N sink (Kasimir-Klemetsson et al., 1997), while afforestation could decrease the emission of N_2O (Merino et al., 2004). A linear relationship between the amount of fertilizer applied and N_2O emission was found when mineral nitrogen

fertilizer was applied; the use of manure, both solid and liquid, also increased N₂O emission significantly (Gregorich et al., 2005).

2.3 Denitrifying bacteria

Denitrification is mainly sustained by denitrifying bacteria, although the ability of denitrification is also found in certain fungi (Zumft, 1997). Nearly 130 species of bacteria including archaeobacteria can reduce NO₃⁻ to N₂ (Zumft, 1992). Lateral gene transfer is a possible explanation to the wide existence of the denitrification ability (Bothe et al., 2000).

Most of the denitrifying bacteria can survive in the presence of oxygen. However, denitrification is only induced in anaerobic conditions in which microorganisms use nitrogen oxides (NO_x) as electron acceptors. It is an alternative pathway to aerobic respiration (Enwall et al., 2005).

Denitrifying bacteria reduce nitrate (NO₃⁻) to nitrous oxide (N₂O) or to nitrogen gas (N₂) under the catalysis of functional enzymes, through the following steps:



where Nar stands for nitrate reductase, Nir stands for nitrite reductase, Nor stands for nitric oxide reductase, and Nos stands for nitrous oxide reductase (Zumft, 1997; Rich and Myrold, 2004).

Denitrifying bacteria also have the ability to degrade toxic organic matter in the environment. Song et al. (2000) isolated 33 strains of halobenzoate-degrading denitrifying bacteria from halobenzoate enriched soil and sediment samples. Rudolphi et al. (1991) studied enzymatic steps of *Pseudomonas*-like and *Paracoccus*-like strains that can metabolize methylphenols and dimethylphenols in anaerobic condition along with reduction of nitrate. With the ability to degrade organic matter, denitrifying bacteria play a crucial function in reducing

organic carbon, thereby reducing nitrate in the wastewater and soils (Neef et al., 1996; Hallin and Pell, 1998; Pai et al., 1999; Song et al., 2000). Studying the group of denitrifying bacteria can contribute to the understanding of the nitrogen cycle in the environment. This can provide additional information that can be used in the decision making process related to natural ecosystem restoration and remediation of nitrate and organic pollutants in contaminated groundwater and soil.

Denitrifying bacteria are a group of heterotrophic bacteria and are phylogenetically diverse. They belong to over 50 genera and fall into all major physiological groups except for the *Enterobacteriaceae*, obligate anaerobes, and gram-positive bacteria except for *Bacillus* spp. (Zumft, 1992; Zumft, 1997). Traditional cultivation methods have been used to study denitrifying bacteria (Gamble et al., 1977; Ehrenreich et al., 2000; Brettar et al., 2001; Joseph et al., 2003). However, only a small portion of bacteria in the environment are culturable. Therefore, cultivation-based methods are not sufficient for studies such as microbial community characterization (Amann et al., 1995). To avoid the disadvantages of cultivation methods, 16S rDNA-based techniques have been widely used to characterize microbial community structure in environmental samples (Stephen et al., 1996; Felske and Akkermans, 1998). The 16S rDNA, however, is not suitable for studying the community of denitrifying bacteria due to their wide phylogenetic distribution (Zumft, 1992).

Studying functional genes can help solve this type of problem. Generally, sequences in functional genes are of generally greater variety than relatively conserved 16S rDNA, so it is more appropriate to use functional genes to discriminate between groups closely related but different in ecological functions (Palys et al., 1997). *NirK* and *nirS* are two functional genes encoding nitrite reductases. Nitrite reductase catalyzes the reduction of nitrite to NO, and it is

the first step to distinguish denitrifying bacteria from nitrate respiring bacteria that also have the ability to convert nitrate to nitrite, however, do not reduce nitrite to gaseous nitrogen (Priemé et al., 2002). This enzyme exists as two types of products from the encoding genes: one is from *nirK* gene, which contains copper; the other is from *nirS* gene, which contains cytochrome *cd₁* (Braker et al., 1998). These two types of genes do not exist within the same cell, but were discovered existing in different strains of the same species. *NirS* is more widely distributed, while *nirK* is only found in 30% of the denitrifying bacteria species. However, *nirK* gene has a wider distribution in different physiological groups (Coyne et al., 1989). Despite their different structures and distribution, the two types of genes are equivalent in terms of function and physiology (Glockner et al., 1993; Zumft, 1997). *NosZ* gene encodes nitrous oxide reductase. The reduction of nitrous oxide is the last step in the denitrification pathway. Some denitrifying bacteria cannot produce nitrous oxide reductase. Therefore, *nosZ* gene can be used to detect bacteria that have the ability to reduce nitrous oxide to nitrogen gas (Throback et al., 2004). Gene *nirK*, *nirS* and *nosZ* were recently used to detect denitrifying bacteria (Braker et al., 1998; Scala and Kerkhof, 2000; Rösch et al., 2002; Qiu et al., 2004; Rich and Myrold, 2004; Horn et al., 2006).

Polymerase Chain Reaction (PCR) is a basic and widely used technique for studying microbial community structure in environmental samples. The first trial of designing PCR primers for amplifying the *nirS* gene was done by Ward (1995), based on three sequences from two different species. Braker et al. (1998) developed a better PCR primer system for *nirK* and *nirS* genes using *Alcaligenes xylosoxidans* NCIMB 11015, *Hyphomicrobium zavarzinii* IFAM ZV-622^T (ATCC 27496), *Ochrobactrum anthropi* LMG 2136, *Rhizobium meliloti* Rm1021, *Rhizobium meliloti* 20115, *Rhodobacter sphaeroides* f. sp. *denitrificans*, *Alcaligenes* sp. strain

(DSM 30128), *Alcaligenes xylooxidans* subsp. *denitrificans* DSM 30026, and *Blastobacter denitrificans* IFAM 1005 (DSM 1113). With the combination of primers nirK1F and nirK3R, and nirK1F and nirK5R (Table 2.1), all the tested strains with a *nirK* gene were successfully amplified. With the combination of primers nirS1F and nirS6R, all the *nirS* genes in the test strains were amplified (Braker et al., 1998).

Table 2.1 Sequences of the primers used by Braker et al.

primer	sequence
nirK1F	GG(A/C)ATGGT(G/T)CC(C/G)TGGCA
nirK3R	GAACTTGCCGGT(A/C/G)G(C/T)CCAGAC
nirK5R	GCCTCGATCAG(A/G)TT(A/G)TGG
nirS1F	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T
nirS6R	CGTTGAACTT(A/G)CCGGT

The use of the cultivation-independent method has brought people new insights into community structure of the denitrifying bacteria in environment. However, estimating the abundance of denitrifying bacteria in environmental samples still relies on cultivation methods. Most Probable Number (MPN) has so far been the mostly used method. As discussed above, however, due to the limitation of cultivation-based methods, the MPN method can only provide an estimate of the cultivable portion of the entire population. For this reason, new approaches towards quantification of microbial populations were developed in the last decade, including hybridization- and PCR-based techniques. The former, such as Southern Hybridization, only compares the relative quantity of functional genes among different samples. The latter includes MPN-PCR, competitive PCR (cPCR), and real-time PCR (RT-PCR). The detection limit of the

MPN-PCR is lower than traditional cultivation methods; however, it is still partly dependent on cultivation, which introduces problems with the uncultivable microorganisms (Féray et al., 1999). For cPCR, target genes in samples and controls compete for amplification, and the initial quantity of the gene in samples is calculated by comparing the PCR products on an agarose electrophoresis gel (Johnsen et al., 1999; Changotra and Sehajpal, 2005). The real time-PCR (RT-PCR) is developed based on traditional PCR (Gruntzig et al., 2001; Philippot, 2005). It was first described by Holland et al. (Holland et al., 1991). The probe in the PCR reaction system is fluorescently labeled. The fluorescence emission, which logarithmically increases when PCR proceeds, is detected at each PCR cycle, and a threshold fluorescence intensity is defined within the logarithmic phase. Copy number of the targeted genes in the samples is calculated by comparison with standard curves constructed from amplifications of serial dilutions of standard DNA (Grüntzig et al., 2001). The real time-PCR (RT-PCR) has several advantages compared to other PCR-based quantification methods. Firstly, it focuses on the logarithmic phase rather than the end products. So it is more accurate when considering amplification efficiency and reagent limitation. Also, the detection range is rather wide to assure reliability. Last, there is no need for further treatment of the products to obtain result, which reduces the possibility of contamination (Heid et al., 1996; Grüntzig et al., 2001). In recent years real time-PCR (RT-PCR) has been applied to study the abundance of microorganisms in soil, water, and air (Hermansson and Lindgren, 2001; Guy et al., 2003; Makino and Cheun, 2003).

Application of molecular techniques to the study of denitrifying bacteria has been reported. Priemé et al. (2002) studied denitrifying bacteria communities in forest upland and wetland soils. They found that in both sites, there existed a large amount of denitrifying bacteria and most of the *nir* genes detected were not found in the cultivated denitrifying bacteria. Karin

Enwall et al (Enwall et al., 2005) detected *nosZ* genes by using denaturing gradient gel electrophoresis (DGGE) and *narG* genes by restriction fragment length polymorphism (RFLP), with subsequent cloning and sequencing, to study the composition of denitrifying bacterial communities in arable soil samples with different treatments. Braker et al. (2000) investigated genetic heterogeneity of denitrifying bacteria in sediment samples by amplifying *nirK* and *nirS*, and found high diversity of *nir* gene sequences in the samples by using RFLP.

There have been few quantitative studies of denitrifying bacterial population in environment samples by RT-PCR, however. A recent study by Henry et al. (2004) quantified *nirK* gene in different soil samples, in which *nirK* gene ranged from 9.7×10^4 to 3.9×10^6 copies per gram of soil. The detection limit was 10^2 copies with a linear relationship up to 7 orders of magnitude. Grüntzig et al. (2001) tested a PCR-based assay of *Pseudomonas stutzeri nirS* gene in vitro and applied it to marine sediment samples. The linear relationship was from 1 to 10^6 copy numbers of the gene (Grüntzig et al., 2001). Smith et al. (2007) quantitatively investigated the functional genes of denitrifying bacteria in estuary sediment by RT-PCR, and found that the copy numbers of the genes significantly decreased from estuary head to estuary mouth along a nitrate gradient.

2.4. Selective Medium for Denitrifying Bacteria

As early as one century ago, people started to realize that the number of colonies that could be obtained from a solid medium was far less than those existing in the environment, especially when soil samples were used to isolate bacteria (Rheims et al., 1999). In the past several decades, this problem was partially solved by the utilization of cultivation independent techniques. Many novel groups of bacteria that had never been detected by cultivation methods

were discovered (Felske and Akkermans, 1999; Axelrood et al., 2002; Furlong et al., 2002). However, some groups of these bacteria with relative importance in the environment, for example some numerically abundant groups, rarely got a chance to be cultivated on a solid medium (Felske and Akkermans, 1999; Axelrood et al., 2002; Furlong et al., 2002). This may be in part due to the lack of appropriate media. There are still many unidentified microorganisms in the environment, which need to be discovered. On the other hand, for most of the discovered “unculturable” bacteria, their physiological characteristics, study of which requires traditional cultivation methods, are in need of investigation (Joseph et al., 2003). Therefore, developing culture media that can grow higher numbers of colonies and cover a larger number of species is necessary. Tryptone soy agar (TSA) and tryptone soy broth (TSB) are standard and most commonly used growth media for denitrifying bacteria (Tiedje, 1988; Coyne et al., 1989; Smith and Tiedje, 1992). Widdel’s medium (Widdel and Bak, 1992) has also been widely used for denitrifying bacteria cultivation (Kniemeyer et al., 1999). Other media have been used under specific situations. For example, HYH-2 medium was used for *Halobacterium* species (Coyne et al., 1989). Yeast extract mannitol medium (YEM) was used for *Rhizobium* strains (Braker et al., 1998; Cheneby et al., 2000) and *Bradyrhizobium japonicum* (Coyne et al., 1989), and for isolation of denitrifying bacteria from acid forest soil (Rösch et al., 2002). Nutrient broth (NB) was used for denitrifying bacteria isolation from agriculture soil (Cheneby et al., 2000), activated sludge (Jørgensen and Pauli, 1995) and marine sediment (Braker et al., 2000), and for cultivation of *Pseudomonas*, *Alcaligenes*, *Ochrobactrum*, *Paracoccus*, and *Azospirillum* strains (Braker et al., 1998). Water from the environment supplemented with complex carbon and nitrate was also used for isolation of denitrifying bacteria (Ward and Priscu, 1997). Bromothymol blue (BTB) medium, screening medium (SM), denitrification medium (DM), artificial wastewater (AWW),

and Luria-Bertani (LB) medium were used by Takaya et al. (2003) for studying aerobic denitrifying bacteria. Oligotrophic medium (PYGV) was used for *Roseobacter denitrificans*, and peptone yeast extract glucose medium for *Blastobacter denitrificans* (Braker et al., 1998). Nitrate broth and nitrate agar were used to isolate denitrifying bacteria from soil, freshwater sediment and oxidized poultry manure to determine the population of dominant denitrifiers (Gamble et al., 1977). Acetate nutrient agar was used to isolate denitrifying bacteria from anaerobic-anoxic and anaerobic-aerobic sequencing batch reactors (Merzouki et al., 1999).

So far, no a single medium has been found suitable for all of the denitrifying bacteria, albeit various media have been used for different types of samples and different species of denitrifying bacteria. In order to maximize the number of colonies and species of denitrifying bacteria isolated from a specific sample, existing media have been modified to meet the need in different situations. D30 culture medium consisting of NB, supplemented with KNO_3 , sodium succinate and sodium acetate, was used for isolation of denitrifying bacteria from agriculture soil (Tiedje, 1982; Cheneby et al., 2000). An ethanol medium based on NB supplemented with ethanol, K_2HPO_4 , KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NH_4Cl , KNO_3 , and trace metal was used for isolation of immobilized denitrifying bacteria from soil and sediment for the development of nitrate sensor (Larsen et al., 1996). Mineral media supplemented with different carbon sources (methanol, sodium acetate, or ethanol) and NB were compared for the growth and nitrate consumption of *Paracoccus denitrificans*. Results showed that with NB, both growth and nitrate consumption rate were the highest, and the second highest was with addition of ethanol as carbon source. When ethanol was used, with thiamine and cobalamine, B group vitamins, and yeast extract in mineral medium, the doubling time was only a bit longer than with NB, but shorter than other combinations of components (Błaszczuk, 1993). A study by Gamble demonstrated that there

was no significant difference between the populations estimated by soil extract supplemented with yeast extract and nitrate broth (Gamble, 1976), TSB and a reported best soil extract-based medium (Martin, 1975).

Recently, an evolutionary algorithm (EA) was used for an evolutionary optimization of isolation conditions for denitrifying bacteria from activated sludge (Heylen et al., 2006). Eleven medium parameters were combined with different values and 15 growth media were generated by using The Simple Evolutionary Algorithm for Optimization (seao) software. Fitness of each medium in one generation was evaluated by the number of colonies and species of denitrifying bacteria isolated. And the values of fitness were used for producing the next generation of media. After cultivation on four generations of medium, Heylen et al. (2006) developed three media that had a higher fitness than the traditional TSA medium. This study provided a new direction for selective medium development, which can be utilized for the study of cultivation-dependent denitrifying bacteria.

3. MATERIALS AND METHODS

3.1 Study Area

The study sites were located in the 41,681 acres Red River Wildlife Management Area (RRWMA) (Figure 3.1) in lower Concordia Parish, Louisiana. This area is of low elevation with numerous rivers, streams, lakes, and ponds. It is easily flooded by the Red River and the Mississippi River as well as Cocodrie Bayou. Moreover, water in the area is difficult to drain. Before the Louisiana Department of Wildlife and Fisheries bought the land, timber density in most of the area was very low due to the extensive cutting activities. Since the area was designated as a wildlife management area in the 1960s, about 800 acres of former agricultural land were planted with about 265,000 hardwoods (Louisiana Department of Wildlife and Fisheries).

The RRWMA is dominated by BLHs of overstory and understory species (Why, 2003). It provides critical habitat for wildlife, including songbirds, waterfowls, and woodland water birds, interior and migrational birds, such as wild turkey, woodducks, woodpeckers, as well as white-railed deer, squirrels, and other small mammals. The first Louisiana black bear was moved to RRWMA in early March, 2001 to establish the population of the bear from their threatened status (Louisiana Department of Wildlife and Fisheries). But little study has been done in RRWMA on denitrification in the rebuilt wetland forest ecosystem.

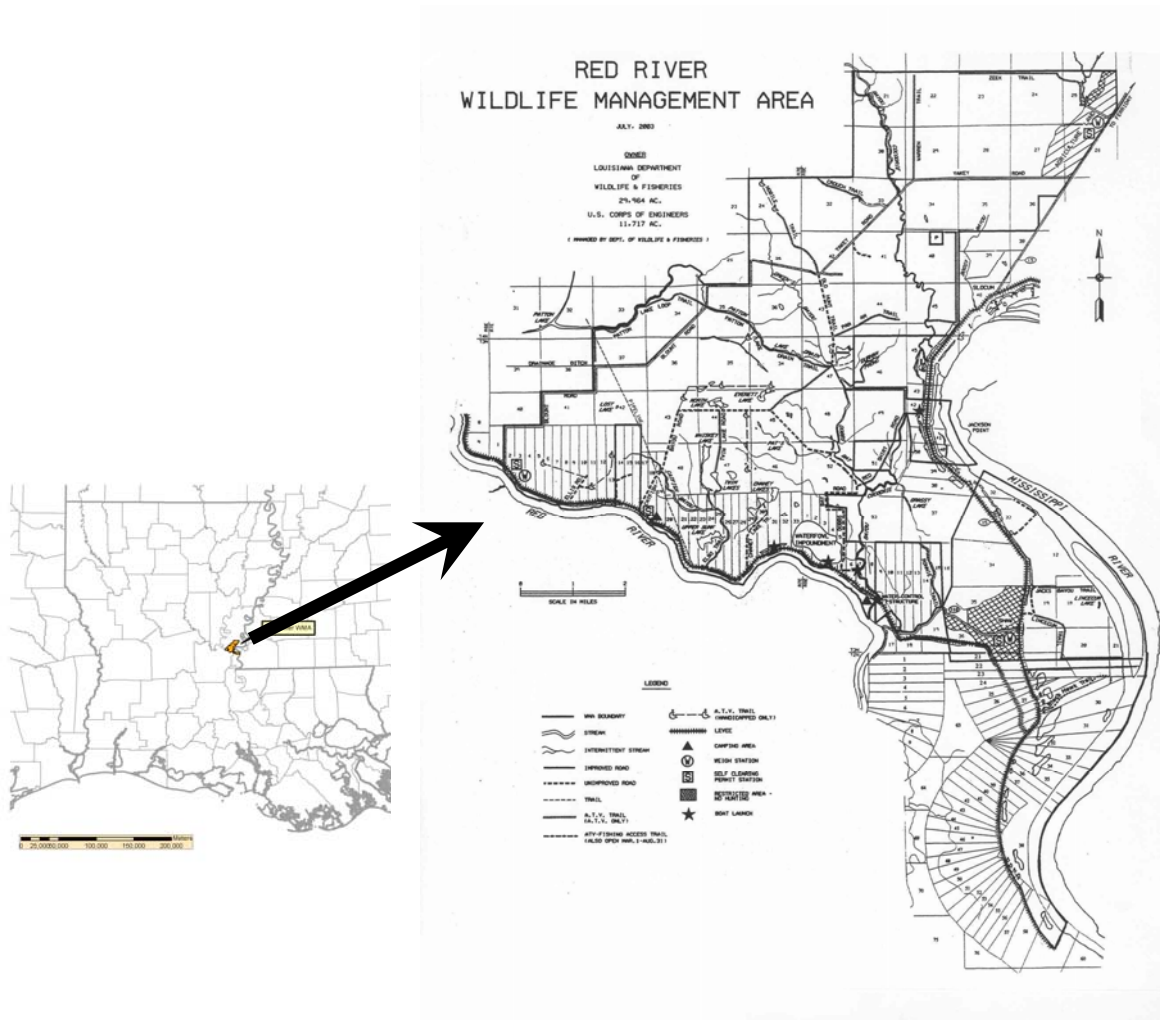


Figure 3.1 Location of Red River Wildlife Management Area (Louisiana Department of Wildlife and Fisheries)

3.2 Soil Sampling

The study area was a 30-year old restored forest surrounded by a levee to the southwest and a road to the north, and there were 12 sampling locations in the area (Figure 3.2). Six of the sampling locations of lower elevations were often flooded by the water coming out the levee in spring, and the other 6 were never flooded due to their higher elevations. Six duplicate soil cores from the surface of the soil from 0-5 cm were taken randomly within each sampling location by using a soil auger. Before the samples were taken, the leaves on top of the soil were gently

removed by hand. Six soil cores were combined into one polyethylene plastic bag at site. Air in the bag was driven out by rolling the bag. And the samples were put on ice before being sent back to the laboratory.

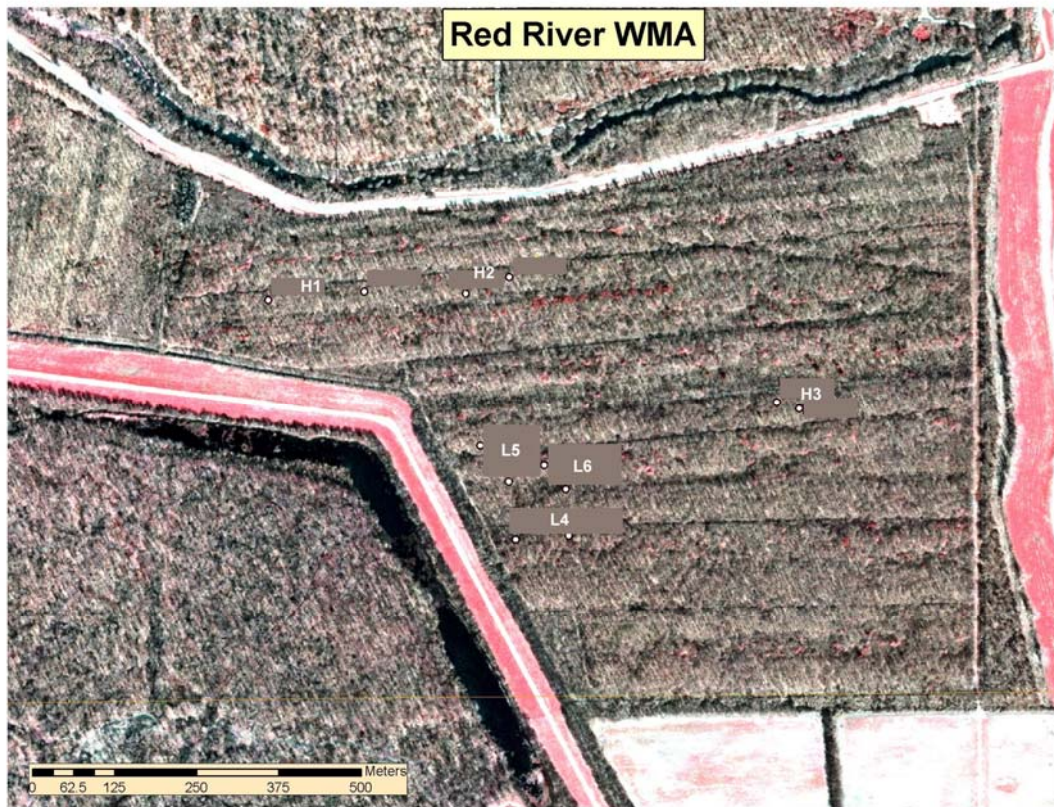


Figure 3.2 Sampling locations in Red River Wildlife Management Area. RR stands for Red River, H represents the sampling locations with higher elevations, and L represents those with lower elevations (courtesy of Dr. Faulkner).

Soil samples in each bag were mixed after they were taken back to the laboratory but before storage. The methods for mixing the soil samples were different for those from high and low elevation locations due to their different soil texture. For the samples from higher elevation sites, the zip bags were put onto a vertex for 1 minute. For the samples from lower elevation sites, the soils were mixed by hand. Each sample was divided into 2 parts and stored separately.

A small amount of mixed soil was stored at -20 °C for molecular analysis; the rest was stored at 4°C for soil and microbial incubation.

3.3 *In-situ* Denitrification Rate

The *in-situ* denitrification rate was studied by trapping N₂O emission from the soil by a static chamber. Two 30 cm × 30 cm × 35 cm chambers were setup at each sampling location. The lower edge of the chambers was inserted into the soil 5 cm deep from the surface. The leaves inside of the chambers were removed before the experiment. The soil inside of the chamber was first saturated with the flooding water near the sampling locations. Three liters of nitrate solution (3 mg/L NO₃⁻) was added to one of the chambers and to a second chamber, 3 L of distilled water was added as control for each sampling location. After the chamber was capped and sealed with ditch water to prevent the leakage of gas, 4.7 L of C₂H₂ was injected into the water layer in the chamber. Ten ml of headspace gas samples were taken at 0, 1, 2 hrs from each chamber by a syringe and stored in vacuumed vials. The N₂O concentrations in the gas samples were analyzed by a GC (Varian CP-3800) with an ECD detector.

3.4 *In-situ* N₂O Flux

On May 9, 2007, *in-situ* N₂O flux was measured by using static chamber without addition of C₂H₂. The chambers were the same as those used for *in-situ* denitrification rate study. Headspace gas inside of the chamber was flushed and 10 ml of the gas was collected by a syringe at time 0, 0.5 and 1 hour. The samples were stored in vacuumed vials and N₂O concentrations in the samples were analyzed by GC with an ECD detector.

3.5 Denitrification Potential

Denitrification potential of soil samples was measured by the denitrification enzyme activity (DEA) assay. DEA is a short-term assay measuring denitrifying enzyme activity existing in the soil under a certain environment at the time of sampling (Brooks et al., 1992; Luo et al., 1996). I modified the experiment by only amending the reaction system with NO_3^- , because I am interested in the denitrification potential with different hydrologic conditions with the existing carbon content (Ullah, 2005). Ten grams of homogenized soil sample from each sampling location was measured and added into each of two autoclaved 150 ml serum bottles. The bottles were sealed and left at room temperature overnight. The next morning, 15 ml of 10 mg/L NO_3^- solution and 5 ml of distilled water was added into one of the two bottles for each sample to deliver 15 $\mu\text{g NO}_3^-/\text{g dry soil}$. And 20 ml of distilled water was added into the second serum bottle with the same sample to be as control. The bottles were capped with rubber stoppers and kept airtight, and then purged with nitrogen gas for 20 minutes. Fifteen ml of C_2H_2 was injected into each bottle with a syringe. And the bottles were wrapped with aluminum foil and put onto a reciprocating shaker (GYROTORY Shaker Model G2) with a speed of 100 rpm/min at room temperature. Ten ml of gas samples from the headspace of the bottles were collected by syringe at 0, 2, 4, and 6 hours, and the samples were stored in vacuum vials. The concentration of N_2O in each sample was measured by GC (Varian CP-3800) with an ECD detector.

The total amount of N_2O produced in both headspace and dissolved in water was calculated with the following equation:

$$M = C_g(V_g + V_lD)$$

M: total amount of N_2O

C_g : concentration of N_2O in the headspace

V_g : volume of headspace in the bottle

V_l : volume of liquid phase

D: Bunsen absorption coefficient, which is 0.54 for N_2O at 25 °C (Hunter and Faulkner, 2001).

3.6 NO_3^- , NO_2^- , and NH_4^+ Concentrations in the Soil

The concentrations of NO_3^- , NO_2^- , and NH_4^+ in the soil samples were measured by the KCl extraction method. Five grams of soil samples were measured and added into centrifuge tubes, and 50 ml of 2 M KCl solution was added into each tube. The tubes were shaken for 1 hour, and centrifuged at 3,000 rpm for 5 minutes. Supernatant was collected by filtering through a Whatman[®] filter (47 mm diameter, 0.45 μm pore size) (Ullah, 2005). Samples were stored at -20 °C before analysis.

3.7 Quantification of Denitrifying Bacteria in the Soil

3.7.1 Culturing Method and Detection

The Most Probable Number (MPN) method was used in this experiment to estimate the population of denitrifying bacteria in the soil. MPN is a method to quantify the concentration of a target microbe based on a statistical estimation by testing a series of dilutions of the original sample.

Nine ml Tryptone Soya Broth (TSB) (Oxoid) with 10 mM KNO_3 was distributed into each Hungate tube, and a Durham tube was put upside down in the tube. No bubble was left inside the Durham tubes. After capping, each tube was purged with nitrogen gas for 3 minutes to

produce anaerobic condition. Then the tubes were autoclaved, and 0.5 ml of C₂H₂ (filtered with 0.25 µm pore size filter) was injected into each tube.

Soil suspension for each sample was made by adding the soil sample to autoclaved TSB with 10 mM KNO₃. A concentration series of soil suspension was made from 10⁻³ to 10⁻⁹. For each sample, there were 3 duplicate tubes at each concentration. The samples were incubated at 26 ± 1 °C for 1 week. Headspace gas samples were collected and analyzed by GC (Varian CP-3800) with an ECD detector to detect N₂O. The final population of denitrifying bacteria in each sample was obtained from a MPN table, and the Durham tubes with bubbles on the bottom were recorded.

Griess reagent was used for NO₂⁻ detection for the incubated samples in the MPN method. Griess reagent (Sigma) was mix 1:1 by volume with each incubated medium sample. After 15 minutes, mixtures with a pink color were recognized as positive for NO₂⁻ production, and those with no color change were recognized as negative.

3.7.2 Molecular Methods for Denitrifying Bacteria Quantification

3.7.2.1 DNA Extraction

DNA samples were prepared by using the UltraCleanTM Soil DNA Isolation Kit (MO BIO Laboratories, Inc.), following the protocol provided by the company.

One-half grams of each soil sample was added to the 2 ml Bead Solution tubes, and gently vortexed. Sixty µl of Solution S1 was added into each tube and the tubes were vortexed briefly. To each tube 200 µl of Solution IRS (Inhibitor Removal Solution) was added and vortexed 1 hr at 10x speed. The tubes were then centrifuged at 10,000 g for 30 s, and the supernatant in each tube was transferred to a microcentrifuge tube. To each tube 250 µl of

Solution S2 was added and vortexed for 5 s before being incubated in refrigerator at 4 °C for 5 min. After centrifugation for 1 min at 10,000 x g, 450 µl of supernatant was transferred to a new centrifuge tube. Then 900 µl of Solution S3 was added to each tube and vortexed for 5 s. The solution mixture from each tube was loaded onto a spin filter and centrifuged at 10,000 x g for 1 min, and the filtration was discarded, until all the solution was treated. Three hundred µl of Solution S4 was added into each tube and the tubes were centrifuged at 10,000 x g for 30 s. After the filtration was discarded, the tubes were centrifuged again for 1 min. Then the spin filter was transferred to a new tube and 50 µl of Solution S5 was added onto the filter. After centrifugation for 30 s, the spin filter was discarded, and the filtration in the tubes was the DNA sample. The tubes were stored at -20 °C for further analysis.

3.7.2.2 Real-Time PCR

The reaction mixture of real-time PCR consisted of 0.5 µl primer mixture (nirK1F and nirK5R); DNA template 1 µl, distilled water 11 µl, and 12.5 µl 2 x SYBR Green Supermix including 100 mM KCl, 40 mM Tris-HCl, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 50 units/ml iTaq DNA polymerase, 6mM MgCl₂, SYBR Green I, 20 nM fluorescein, and stabilizers, at pH 8.4 to make the total volume 25 µl (QIAGEN).

The real-time PCR conditions for *nirK* gene were modified from Henry et al. (2004) to obtain better amplification for the samples from our specific locations. Real-time PCR started at 50 °C for 2 min, and at 95°C for 15 min for enzyme activation. Six cycles of touchdown were then carried out, consisting of 15 s at 95°C for denaturation, 30 s at 63°C for annealing, and 30 s at 72°C for extension. From the second cycle, the temperature of annealing decreased 1°C per cycle until it reached 58°C. Fifty cycles were performed as it was in the previous six cycles except that the annealing temperature was 58°C in all the fifty cycles. The temperature was then

kept at 72 °C for 7 min. Finally, a 10 s step was repeated 80 times by decreasing the temperature by 0.5 °C each time to obtain a denaturation curve.

Experiments were performed with three duplicates for each sample. The copy numbers of the *nirK* gene in the soil sample were calculated by comparing the relationship between copy number and threshold cycle value to a standard curve from the standard DNA (cloning of DNA from environment samples from Philippot (Henry et al. 2005)).

3.8. Selective Medium Design

3.8.1 Basic Medium

The basic medium was based on Stanier et al. (1966). One liter of standard mineral base contains 20 ml of Hunter's vitamin-free mineral base, NH₄Cl 0.81 g, and yeast extract 5 g. Hunter's vitamin-free mineral base was prepared based on Cohen-Bazire et al. (Cohen-Bazire et al., 1957). The mineral base contains MgSO₄·7H₂O 29.59 g, CaCl₂·2H₂O 3.335 g, Na₂MoO₄·2H₂O 9.25 mg, FeSO₄·7H₂O 99 mg, Metal "44" solution 50 ml and distilled water with a total volume of 1 L. Metal "44" is prepared using Ethylenediaminetetraacetic acid (EDTA) 250 mg, ZnSO₄·7H₂O (250mg Zn) 1095 mg, FeSO₄·7H₂O (100 mg Fe) 500 mg, MnCl₂·4H₂O (180 mg Mn) 180 mg, CuSO₄·5H₂O (10 mg Cu) 39.2 mg, CoCl₂·6H₂O (5 mg Co) 20.2 mg, H₃BO₃ (2 mg B) 11.44 mg, 1 L distilled water and a few drops of sulfuric acid.

3.8.2 Parameters for the Medium

In order to design a selective medium for denitrifying bacteria, 7 parameters were used. They were pH with 4.5, 5, 5.5, 6, 6.5, and 7; carbon source with ethanol (2 carbon atoms), sodium succinate (4 carbon atoms), and glucose (6 carbon atoms); carbon: nitrogen ratio of 2.5, 5, 7.5,

10, 12.5, 15, 17.5, 20, 22.5; nitrogen (KNO₃) concentration with 3, 6, 9, 12, 15, and 18 mM; NaCl concentration with 0 and 5 g/L; Thiamine solution with 0, 1, and 2 ml/L (Kniemeyer et al. 1999); Cobalamin solution with 0, 1, and 2 ml/L (Kniemeyer et al. 1999). One ml of vitamin solution was added to each 1 L medium. The number of combinations of these parameters was 17496. It is impossible to test all these combinations. Therefore, a software package using an evolutionary algorithm was used to optimize the combination of the parameters.

3.8.3 Design by Software

The design was done by the Simple Evolutionary Algorithm of the Optimization (seao) Software, which can be downloaded free from the Internet (<http://www.cran.r-project.org>). The software has a graphic user interface which makes it easier to use for multiple users (Figure 3.3).

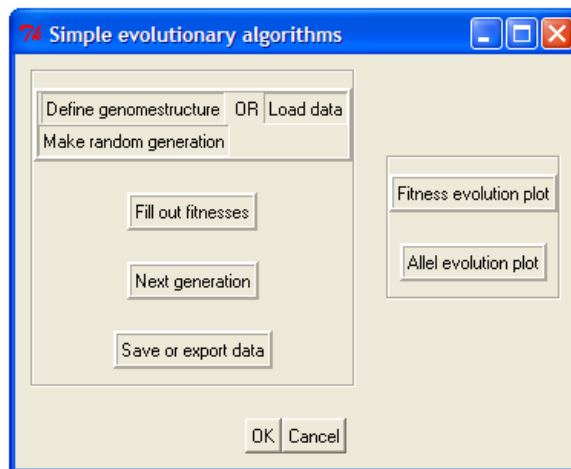


Figure 3.3 Interface of the graphic interface of the software of Simple Evolutionary Algorithm for Optimization (from R software)

The parameters for the next generation were setup based on Heylen et al.'s (2006) experiment: the number of individuals is 9, which was the largest number we can handle from the choices provided by the software, all the previous generations were used for producing a new

generation, the selection of parameters was based on fitness (rescaling = 0), the crossover rate is 90%, and the mutation was uniformly distributed, with a spread of 1.00 and a rate of 15. For the first generation of the media, the software randomly made the combination of parameter values to generate 9 different media.

Traditional medium for denitrifying bacteria TSA (Tryptone Soya Agar, Remel) and TSB (Tryptone Soya Broth, Remel) amended with 10 mM KNO₃ was used to compare the results from our design. Solid media were prepared with 1.5% agar.

3.8.4 Cultivation of Microorganisms

Soil suspensions were made with 5 g of combination soil from the sampling locations with lower elevation and 45 ml phosphate buffer. Soil suspensions were made with a soil concentration from 10⁻³ g/ml to 10⁻⁷ g/ml. A volume of soil suspension of 0.1 ml was dropped onto the solid media, and spread evenly on the whole surface of the media. The Petri-dishes were wrapped with petrifilm and incubated in anaerobic chamber with a temperature of 26 °C. After 1 week of incubation, for each medium, 50 colonies were picked up from each Petri-dish that had more than 50 separate single colonies growing on it. The single colonies were then transferred to a liquid medium with the same composition respectively. The liquid media were prepared with the same components for each medium without agar. The medium was distributed into Hungi tubes, and a Durham tube was put inside of each tube upside down. The tubes were then capped and headspace gas was exchanged by nitrogen gas for 3 min. Each single colony was transferred to 1 ml of the liquid medium and suspended. And the microbe suspension was transferred into the Hungi tube with the same medium. The tubes were then incubated at 26 °C

for 1 week. Each tube was checked for bubbling, and Griess reagent was used to detect the production of nitrite.

3.8.5 Identification of Denitrifying Bacteria

For the tube with both bubble and nitrite produced, the microorganism growing inside was identified as denitrifying bacteria. Tubes with no bubbles or nitrite were identified as not having denitrifying bacteria. For tubes with either bubbles or nitrite inside but not both, further identification was done by molecular methods.

The medium inside of the tube with either bubble or nitrite was centrifuged at 10,000 x g for 1 minute. The supernatant was discarded. Then DNA was extracted by using UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.), following the procedure provided by the company.

The centrifuged microorganism was resuspended in 300 µl of Bead Solution. After gentle vortexing, the mixture was transferred to a bead tube provided in the kit. Fifty µl of Solution S1 was added into each tube and the tubes were incubated at 65 °C for 10 min for the maximum yield. The tubes were then vortexed at 10x speed for 10 min. After the tubes were centrifuged at 10,000 x g for 30 s, the supernatant in each tube was transferred to a microcentrifuge tube. Three hundred µl of Solution S2 was added to each tube, and the tubes were vortexed for 5 s before incubation at 4 °C for 5 min. After centrifugation for 1 min at 10,000 x g, the supernatant was transferred to a new centrifuge tube, and 900 µl of Solution S3 was added and the tube was vortexed for 5 s. The solution from each tube was loaded onto a spin filter and centrifuged at 10,000 x g for 30 s, and the filtrate was discarded, until all the solution was treated. Three hundred µl of Solution S4 was added into each tube and the tubes were centrifuged at 10,000 x g

for 30 s. After the filtrate was discarded, the tubes were centrifuged again for 1 min. The spin filter was then transferred to a new tube and 50 µl of Solution S5 was added onto the filter. After centrifugation for 30 s, the spin filter was discarded, and the DNA was in the remaining filtrate. The tubes were stored at -20 °C for further analysis.

NirK genes were detected by PCR. The primer system was the mixture of nirK1F and nirK5R. The PCR reaction mixture contained 2.5 µl of the buffer with MgCl₂, 0.25 µl of dNTP (10 mM), 0.25 µl of primer mixture, 1 µl of DNA template, 0.15 µl of Taq Polymerase, and 20.85 µl of distilled water.

The PCR conditions started with 95 °C incubation for 4 min. Then 40 cycles were carried out with 40 s at 95 °C, 30 s at 58°C, and 40 s at 72 °C. The temperature was set at 72°C for 7 min, and then kept at 16 °C.

The PCR products were analyzed by electrophoresis on a 1 % agarose gel at 100 mV for 15 min. The products from cultured samples were compared to that from a standard strain with *nirK* gene.

3.9 Statistical Analysis

Analysis of covariance with split design was used for evaluating the impact of elevation, sampling time, or nitrate concentration on *in-situ* denitrification rate, denitrification potential and population size of denitrifying bacteria. Two-sample T test was used for comparing N₂O flux at sites of different elevations. Both of the analyses were done in SAS with adjustment by Tukey at a significance level of 5%.

4. RESULTS AND DISCUSSION

4.1 *In-situ* Denitrification Rate

In our *in-situ* denitrification rate study of May 17, 2006, both elevation and amendment of nitrate had significant influence ($P < 0.05$) on the rate (Table 4.1). Without an amendment of NO_3^- , denitrification rates at all the sampling locations were similar ($P > 0.05$). After adding NO_3^- , the sampling locations with lower elevations had significantly greater ($P < 0.05$) denitrification rates than those with higher elevations (Figure 4.1). The N_2O emission significantly increased ($P < 0.05$) at the lower elevation sampling locations after the addition of NO_3^- , and the average level of N_2O emission increased from 32.93 to 1062.67 $\mu\text{g N}/\text{m}^2/\text{hr}$. Results indicate that nitrate concentration is one compelling factor in these lower locations regulating the denitrification activities. In the sampling locations with higher elevations, there was no significant difference ($P > 0.05$) between those with the addition of NO_3^- and those without NO_3^- addition, but the average level was still numerically higher in the NO_3^- treatments (87.42 versus 7.02 $\mu\text{g N}/\text{m}^2/\text{hr}$) (Figure 4.1). Data suggest that, besides nitrate concentration, denitrification activities in these higher elevation locations might also be affected by other environmental factors such as soil moisture, anaerobic condition, and soil texture, etc.

Table 4.1 Statistical results of analysis of covariance for *in-situ* denitrification rate

Effect	Degree of freedom for numerator	Degree of freedom for denominator	F value	P
Elevation	1	3	123.85	0.0016
Nitrate	1	3	152.28	0.0011
Elevation*nitrate	1	3	111.36	0.0018

On May 9, 2007, *in-situ* N_2O flux was measured without C_2H_2 block and the addition of NO_3^- , there was no significant difference ($P > 0.05$) in terms of N_2O emission rate between

locations with low and high elevations (Figure 4.2). The average N₂O emission rates at locations with high and low elevations were -4.6484 and 8.4115 µg N/ m²/ hr respectively. It might be because the period of measurement of 1 hour was not long enough to detect the change of N₂O emission, which is usually at a low level in natural ecosystems. It might also be an indication that the environmental factors such as elevation, soil moisture, etc did not have significant impact on N₂O emission in this restored ecosystem.

In this ecosystem, nitrate was the major regulator of denitrification rate compared to other environmental factors where it was periodically flooded by river water. In places without flooding, nitrate was not the only regulator and might have minimal importance. The results demonstrated the importance of period flooding to the possible increase of denitrification rate. Also in Pfenning and McMahon’s study, the denitrification rate in river sediment was not limited by nitrate with nitrate concentration from 357 to 2142 µ mol/L (Pfenning and McMahon 1997).

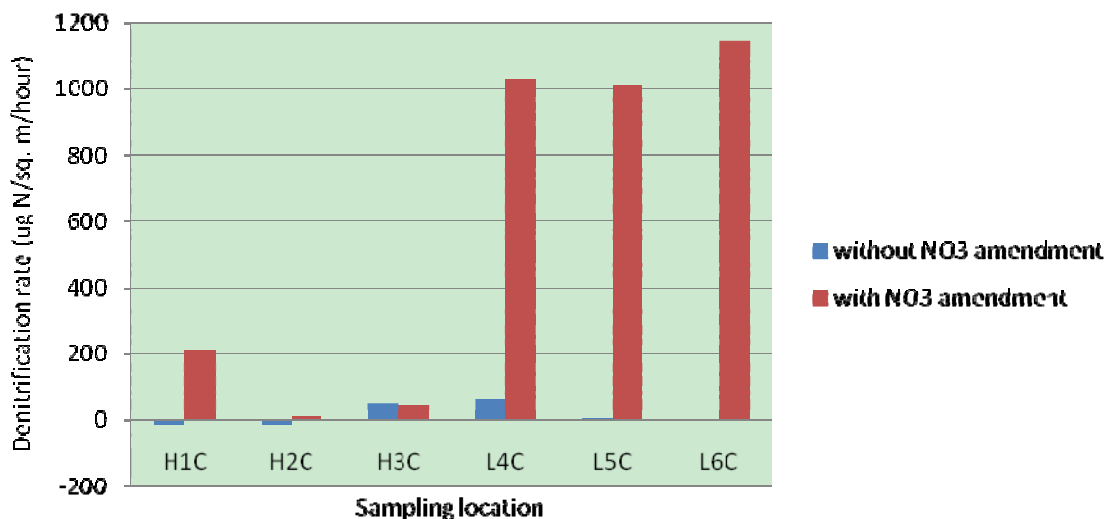


Figure 4.1 *In-situ* measurements of denitrification rate with and without the addition of NO₃⁻ at different sampling locations on May 17, 2006. (H stands for high elevation and L stands for low elevation)

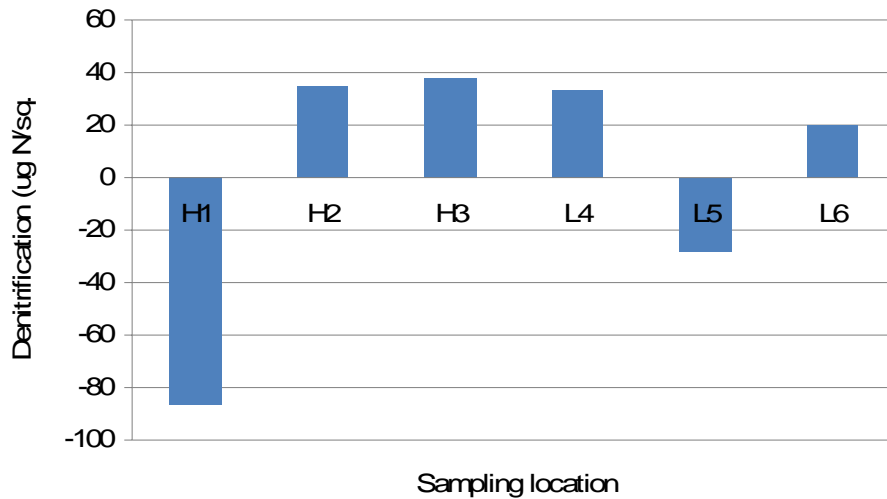


Figure 4.2 *In-situ* measurements of denitrification rate without the addition of NO_3^- at different sampling locations on May 9, 2007. (H stands for high elevation and L stands for low elevation)

4.2 Denitrification Potential

Addition of nitrate and sampling time influenced denitrification potential ($P < 0.05$). Without an amendment of NO_3^- , denitrification potentials were not significantly different ($P > 0.05$) between the low and high elevation locations for samples from both May 17, 2006 and May 9, 2007. With the amendment of NO_3^- , the low elevation locations demonstrated significantly greater ($P < 0.05$) denitrification potentials than the high locations on May 17, 2006; while on May 9, 2007, there was no significant difference ($P > 0.05$) between the two (Figure 4.3, Figure 4.4).

Denitrification potentials at sampling locations with higher elevations were not significantly different ($P > 0.05$) between with and without the NO_3^- amendment for samples from both May 17, 2006 and May 9, 2007. The average levels were 0.15 and 0.08 $\mu\text{g N} / \text{g dry}$

soil / hr for the sampling locations with and without the addition of NO_3^- on May 17, 2006. They were 0.27 and 0.07 $\mu\text{g N} / \text{g dry soil} / \text{hr}$ for the locations on May 9, 2007, respectively.

The results showed that nitrate concentration was not a single regulator for denitrification potential. The results of denitrification potential within different elevations at different sampling times were consistent with that of soil moisture between the high and low elevation locations at the two sampling times (Table 4.2). Soil moisture at low elevation locations was significantly greater ($P < 0.05$) than at high ones on May 17, 2006, while on May 9, 2007, there was no significant difference ($P > 0.05$) between the two. Soil moisture in high elevation locations was higher on May 9, 2007 than on May 17, 2006, though the difference was not significant ($P > 0.05$) (Table 4.2), while for low elevation locations, soil moisture was higher on May 17, 2006. This indicated that soil moisture affected denitrification potential with nitrate level $\sim 15\mu\text{g NO}_3^- / \text{g dry soil}$ in this ecosystem. A study on a seasonal dry tropical forest by Davidson et al. (1993) showed that soil moisture had a great impact on N_2O emission rate, while with high soil moisture, there was more N_2O emission. Brye et al. (2001) found that denitrification potential was limited by insufficient length of saturation for the soil in three ecosystems.

On May 17, 2006, the samples from low elevations amended with NO_3^- showed significantly greater ($P < 0.05$) denitrification potential than those without the amendment. For those samples from May 9, 2007, there was no significant difference ($P > 0.05$) between the two, which was due to the high denitrification potential without NO_3^- amendment at sampling locations of L4 and L6. And the two studies at different dates showed similar quantitative relationships among the sampling locations. For both with or without the addition of NO_3^- , H1 showed more denitrification potential than the other two locations at high elevations; L6 was with the highest denitrification potential in the low elevation locations; and L4 was the second.

This result was consistent with high level of nitrate and total nitrogen in these two locations (Table 4.3). The high level of denitrification potential at H1 both with and without addition of NO_3^- on May 9, 2007 was consistent with the high level of nitrate, total nitrogen and carbon at the location. This demonstrated the impact of nitrate concentration in soil to denitrification potential and available carbon may also influence denitrification potential. Pfenning and McMahon (1997) claimed that in riverbed sediment, organic carbon significantly increased N_2O emission, and the emission was also affected by the type of carbon source. In the study of Enwall et al (2005), the concentration of organic carbon was associated with the denitrification potential. Chabrierie et al. (2001) found that in estuaries, spatial soil heterogeneity has a major role in determining the spatial variation of denitrification potential.

Without NO_3^- , the N_2O emission, measured as the weight of N produced by unit gram of dry soil, has a linear relationship with time. With NO_3^- , the linear relationship starting time delayed, and the rate of production was larger than those samples without addition. This means that the denitrifying bacteria in the soil need some time to adapt to the increase of nitrate level. Once they adapt to the high concentration of nitrate, they tend to produce more N_2O per unit time.

Soil texture in locations with high elevations was mainly silt loam, and at low elevations mainly silty clay, although there was some difference between the two samplings (Table 4.3). In this study, the clay percentages at low elevation locations were significantly higher ($P < 0.05$) than those at high elevations both on May 17, 2006 and May 9, 2007. It indicated that the content of clay might affect the denitrification potential, however, it cannot regulate the potential independently. Groffman and Tiedje reported a strong negative relationship between the percentage of sand in the soil and denitrification rate (Groffman and Tiedje, 1989).

Soil temperature may affect denitrification rate (Bachand and Horne, 1999). The temperature of soil on May 9, 2007 ranged from 17.52 to 19.04 °C and from 20.19 to 21.33 °C on May 17, 2006. These values fell into the range of temperature that support the growth and activities of most denitrifying bacteria. Due to the limited sample size and the narrow temperature range, there was no relationship between denitrification rate and soil temperature observed in this study.

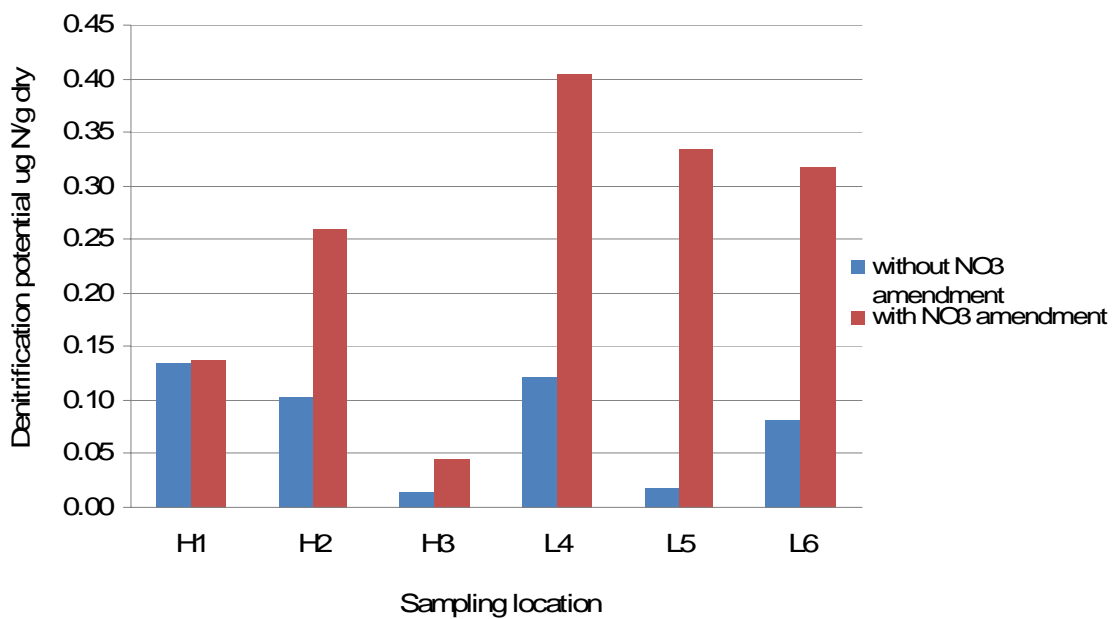


Figure 4.3 Denitrification potential at different sampling sites on May 17, 2006. (H stands for high elevation and L stands for low elevation)

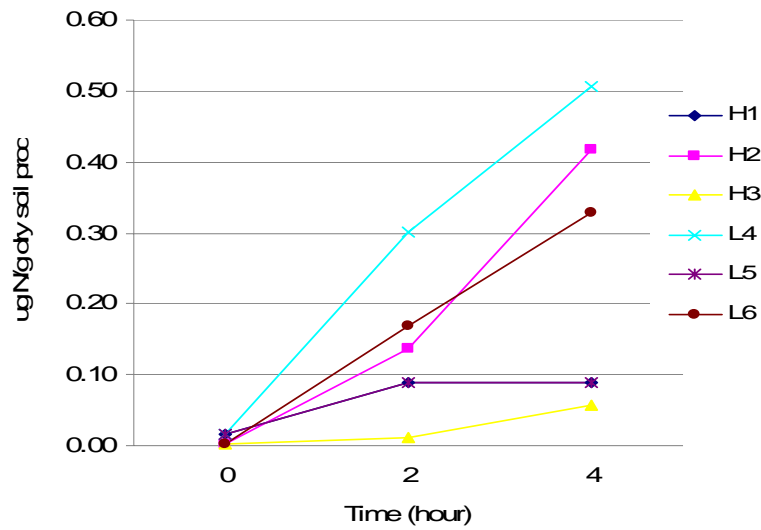


Figure 4.4 N produced in Denitrification Potential incubation from soil samples without NO₃⁻ amendment on May 17, 2006. (H stands for high elevation and L stands for low elevation)

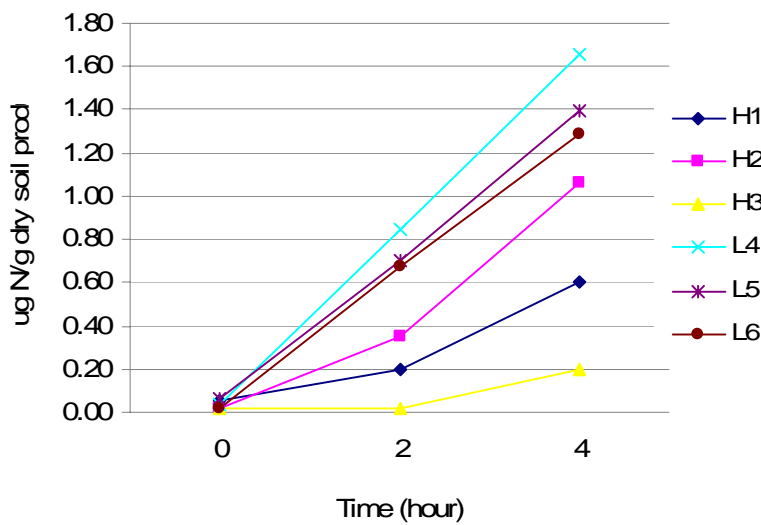


Figure 4.5 N produced in Denitrification Potential incubation from soil samples with NO₃⁻ amendment on May 17, 2006. (H stands for high elevation and L stands for low elevation)

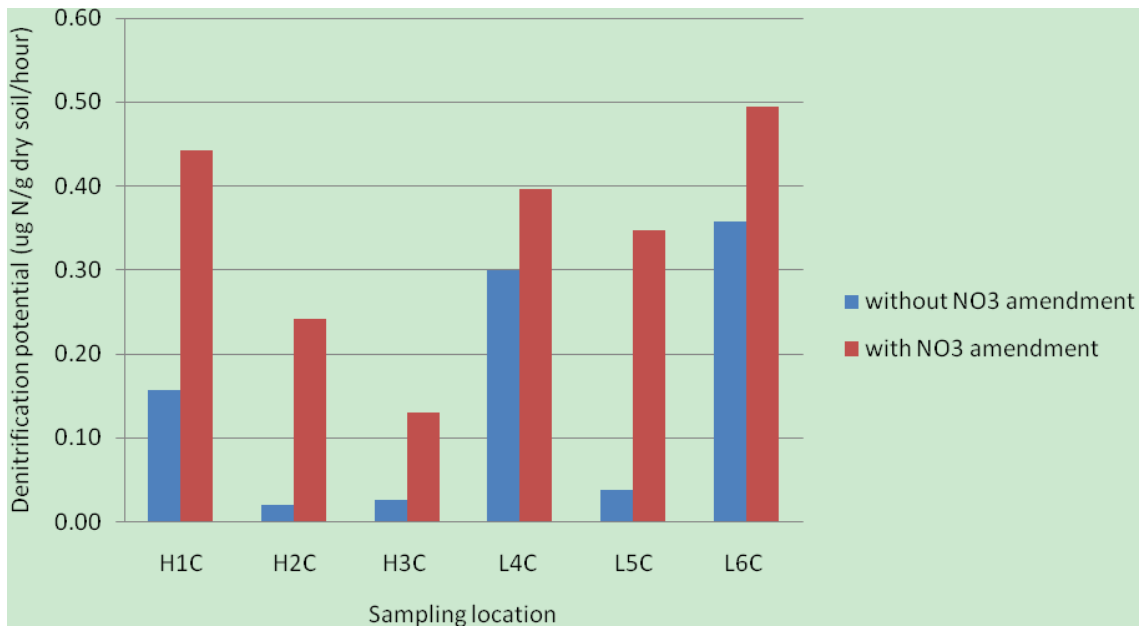


Figure 4.6 Denitrification potential at different sampling sites on May 9, 2007. (H stands for high elevation and L stands for low elevation)

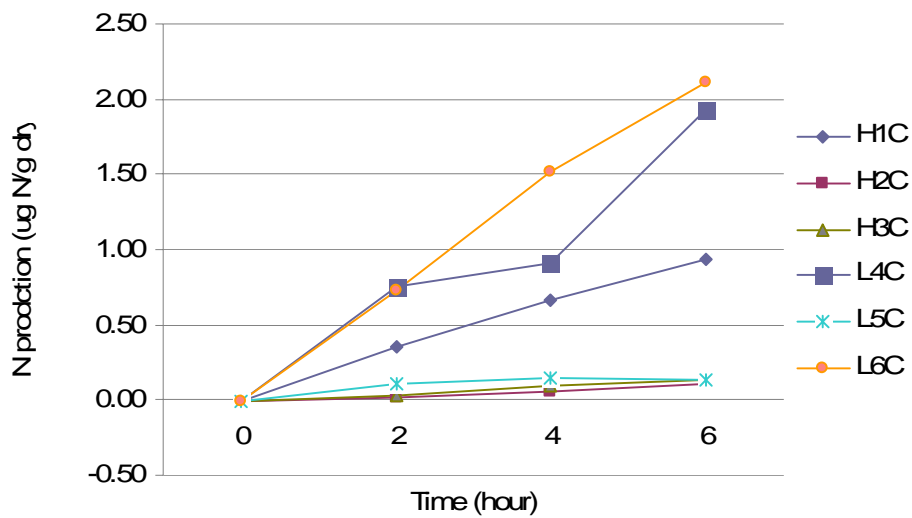


Figure 4.7 N production in Denitrification Potential incubation from soil samples without NO₃⁻ amendment on May 9, 2007. (H stands for high elevation and L stands for low elevation)

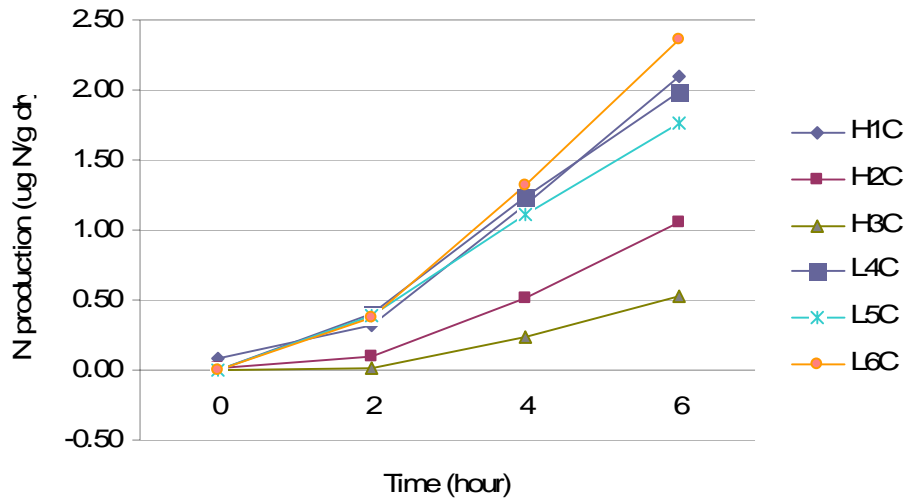


Figure 4.8 N production in Denitrification Potential incubation from soil samples without NO_3^- amendment on May 9, 2007. (H stands for high elevation and L stands for low elevation)

Table 4.2 Soil moisture at different sampling locations on different dates

Sampling date	H1C	H2C	H3C	L4C	L5C	L6C
May 17, 2006	23.96%	21.73%	22.86%	31.09%	30.81%	34.69%
October 3, 2006	11.57%	10.93%	9.71%	16.42%	15.64%	18.76%
May 9, 2007	31.53%	24.98%	23.05%	26.37%	25.58%	29.91%

Table 4.3 Types of soil in different sampling locations

		H1C	H2C	H3C	L4C	L5C	L6C
Clay (%)	May 17, 2006	11.51	11.10	11.48	41.94	38.47	58.96
	May 9, 2007	4.10	5.75	5.42	23.17	20.45	33.56
Sand (%)	May 17, 2006	38.48	23.05	23.03	8.09	9.69	5.30
	May 9, 2007	32.23	18.83	19.41	8.43	8.73	4.72
Silt (%)	May 17, 2006	50.00	65.86	65.49	49.97	51.84	35.74
	May 9, 2007	63.67	75.42	75.18	68.40	70.81	61.72
USDA Soil Classification	May 17, 2006	loam	silt loam	silt loam	silt loam	silt loam	silty clay loam
	May 9, 2007	silt loam	silt loam	silt loam	silt loam	silt loam	silty clay loam

Table 4.4 Soil temperature (°C) at different sampling locations on different dates

Sampling date	H1C	H2C	H3C	L4C	L5C	L6C
May 17, 2006	19.04	19.04	18.66	17.52	18.28	18.28
October 3, 2006	22.48	23.63	23.63	21.71	22.09	22.09
May 9, 2007	20.19	21.33	20.57	20.57	20.19	20.57

Table 4.5 NO₂⁻, NO₃⁻, NH₄⁺ concentration and total nitrogen and total carbon in soil samples at different sampling locations on May 9, 2007

	H1C	H2C	H3C	L4C	L5C	L6C
NO ₂ ⁻ (umol/L)	1.71	1.35	-0.01	1.58	0.94	1.65
NO ₃ ⁻ (umol/L)	1.881	0.127	0.065	3.298	0.457	3.607
NH ₄ ⁺ (umol/L)	71.62	38.18	32.84	7.54	27.36	35.13
Total nitrogen (%)	0.30	0.21	0.20	0.31	0.24	0.30
Total carbon (%)	4.56	3.02	2.89	4.24	3.07	3.66

4.3 Quantitative Study of Denitrifying Bacteria

4.3.1 Culturing Method

The population sizes of denitrification bacteria were similar ($P > 0.05$) at high and low elevations on October 3, 2006. The average population was 2.61×10^5 and 5.75×10^4 bacteria/g soil at high and low elevations, respectively (Figure 4.9).

On May 9, 2007, the populations of denitrification bacteria were determined in natural soil and in the soil after denitrification potential incubation with amendment of NO₃⁻. Two different detection methods were used for comparison (Table 4.5). The population sizes detected by bubbling were smaller than those from Griess positive identification. Part of the reason was that not all the denitrifying bacteria produce N₂ as a final product. Some of the bacteria finish their denitrification process with only N₂O production. And N₂O is soluble in water, with gas production, the pressure in the tube increased and the N₂O produced by microorganism might dissolve in water and not to be observed. The bubbles might also come from CH₄ or CO₂ which

can be produced during microorganism activities. By identifying Griess positive, population size of denitrifying bacteria that have the ability to convert nitrate to nitrite was estimated. For both natural soil and soil after denitrification potential incubation, the population sizes of denitrifying bacteria were not different ($P > 0.05$) between high and low elevation locations. This result was consistent with the one on May 17, 2007. This showed that the differences of hydrologic conditions did not affect the population size of denitrifying bacteria in this ecosystem. The estimated population sizes of denitrifying bacteria from natural soil and soil after denitrification potential incubation were also similar ($P > 0.05$), which indicated that the increase of nitrate concentration in a period of 6 hours will not change the proliferation activity of denitrifying bacteria.

The smaller population on October 3, 2006 was consistent with the lower denitrification potentials, comparing to May 9, 2007. The test was longer than 2 weeks because of the limit of available detection facility, which may cause an over growth of the denitrifying bacteria and convert nitrite to N_2O or N_2 , thereby lowering the positive results from the Griess reagent, and lowering the final estimate of the population. Gamble et al. (1977) studied soil samples using nitrate broth from 14 different locations all over the world, and got a population size of denitrifying bacteria between 10^4 and 10^6 bacteria/g soil. Chèneby et al. (2000) showed the denitrifying bacteria density of 3×10^6 and 4×10^6 bacteria/ g dry soil in luvisols, and 4×10^5 bacteria/ g dry soil in rendosol. These differences may reflect site-specific characteristics.

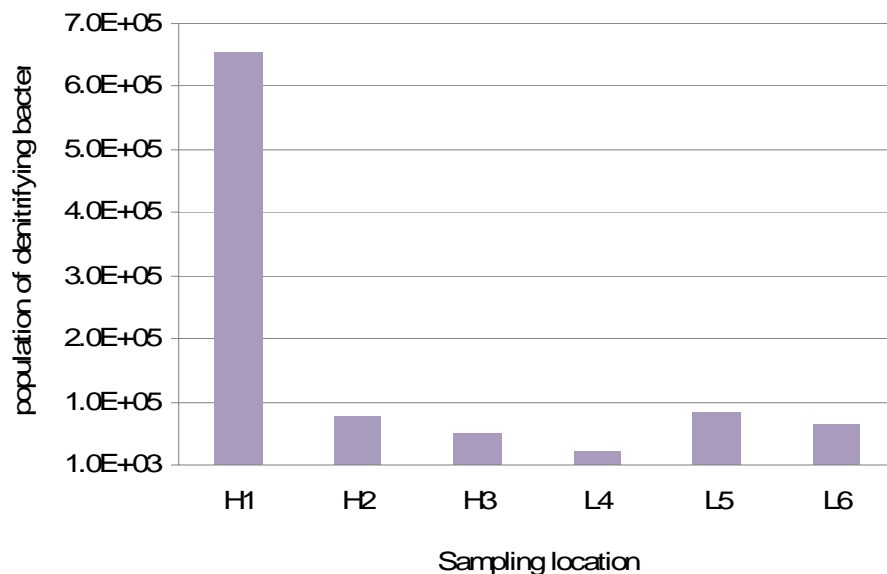


Figure 4.9 Estimated population size of denitrifying bacteria from soil samples on October 3, 2006. (H stands for high elevation and L stands for low elevation)

Table 4.6 Estimated population size of denitrifying bacteria from soil samples on May 9, 2007, after denitrification potential incubation without amendment of NO_3^- . (H stands for high elevation and L stands for low elevation)

Sampling location	Natural soil (/g soil)		Soil after DP incubation with NO_3^- (/g soil)	
	bubbling	Griess positive	bubbling	Griess positive
H1	4.3×10^6	1.5×10^7	7.4×10^4	4.3×10^7
H2	1.2×10^5	7.5×10^6	2.4×10^4	9.3×10^7
H3	4.3×10^4	7.5×10^6	2.4×10^5	1.5×10^6
average of H locations	1.49×10^6	1.00×10^7	1.13×10^5	4.58×10^7
L4	2.1×10^5	2.4×10^7	4.3×10^6	1.5×10^7
L5	9.3×10^4	4.4×10^6	1.5×10^5	1.2×10^8
L6	4.3×10^5	3.8×10^6	7.4×10^5	2.4×10^8
average of L locations	2.44×10^5	1.07×10^7	1.73×10^6	1.25×10^8

4.3.2 Molecular Approach by Real-time PCR

The amplification curves and melting curves indicated that the targeted *nirK* genes were well amplified and the amplification products were only from *nirK* gene (Figure 4.3.2.1 and

Figure 4.3.2.2). The curve from the standard DNA with *nirK* genes showed excellent linear relationship between ct value and logarithmic value of copy number within an order of magnitude of 10^2 - 10^5 (Figure 4.3.2.3).

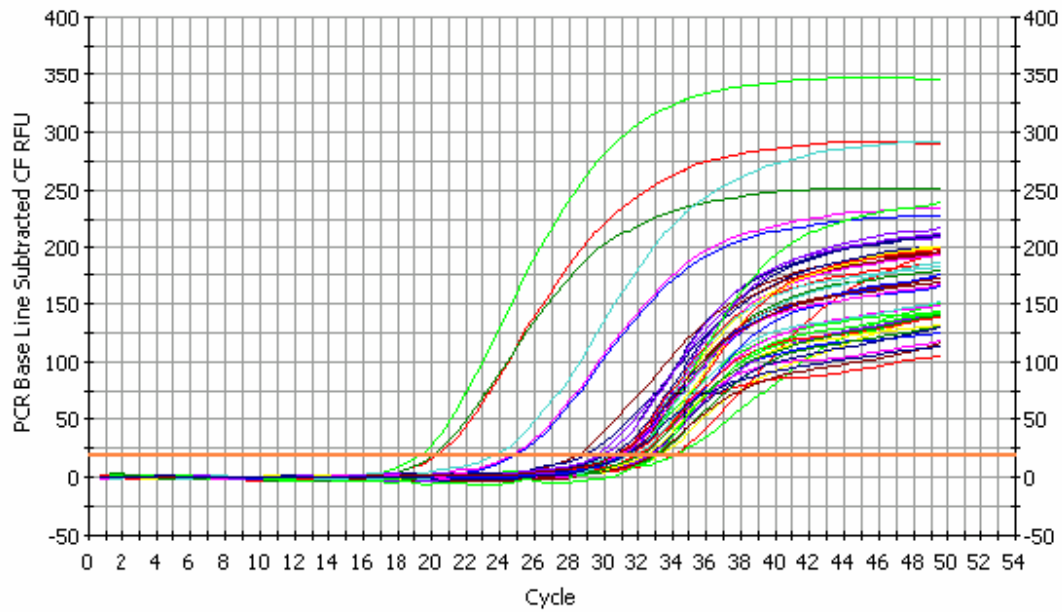


Figure 4.10 Amplification curves of of *nirK* genes from both standard DNA and soil samples.

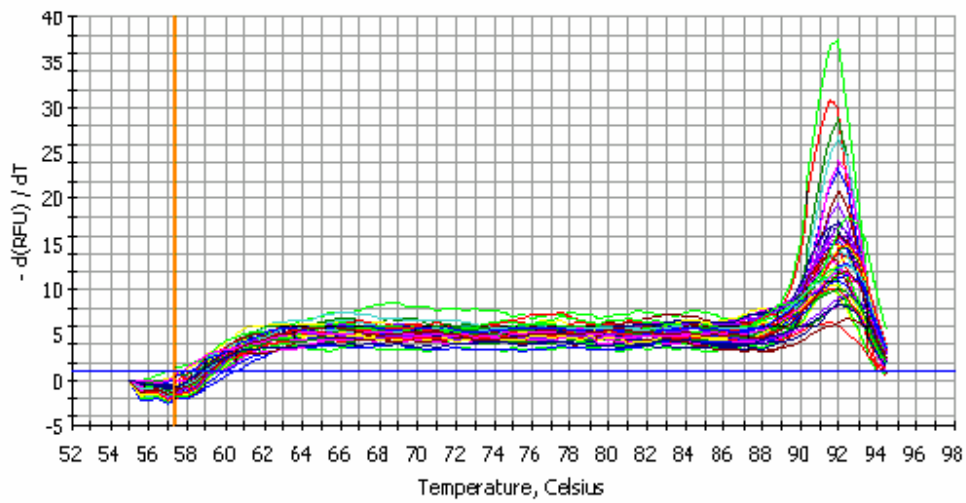


Figure 4.11 Melting curves of amplification of *nirK* gene from standard DNA with different concentrations and DNA from soil samples.

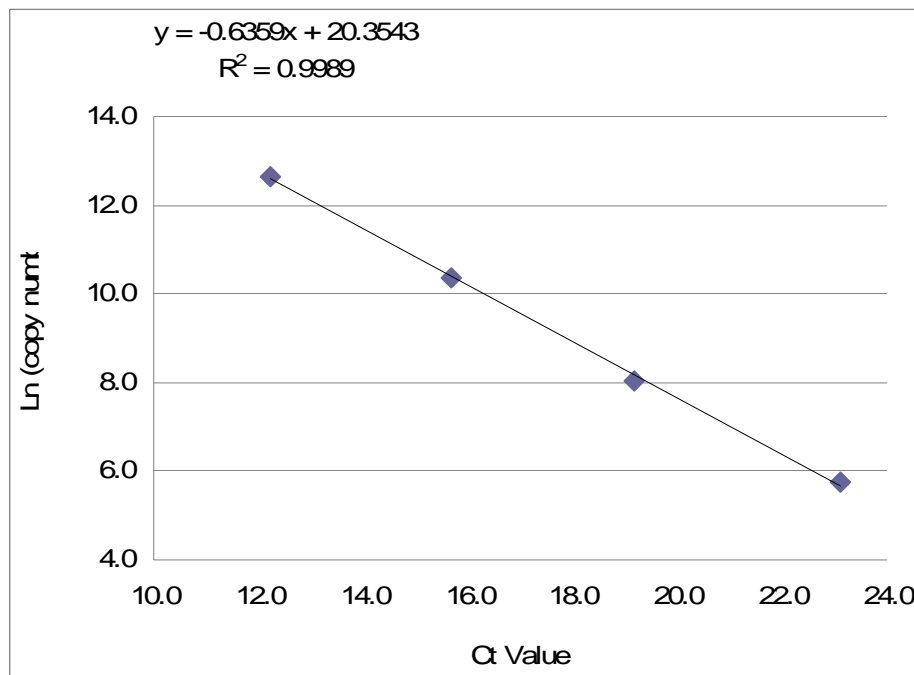


Figure 4.12 Relationship between ct value and logarithmic value of copy number of *nirK* gene by using the standard DNA.

In all the studies on natural soil at three different times, the population sizes from different sampling locations were not significantly different ($P > 0.05$). Furthermore, there was no significant difference ($P > 0.05$) between the thinned and unthinned locations in the study of May 17, 2006 and October 3, 2006. The average population sizes at high and low elevations on May 17, 2006, October 3, 2006, and May 9, 2007 were 5.17×10^6 and 5.00×10^6 , 6.83×10^5 and 4.68×10^5 , 8.29×10^6 and 2.20×10^7 cell/g dry soil, respectively (Figure 4.3.2.4, Figure 4.3.2.5, and Figure 4.3.2.6). This illustrated that the hydrological impact did not affect the population size of denitrifying bacteria.

On October 3, 2006, the population estimated by the culturing method was lower than that by molecular method. The cultivation methods only detect live and culturable cells while the molecular method is based on the amplification of genes including live and culturable cells, as well as dead but un-decomposed cells and live but not viable cells. The broader coverage of cells may cause the differences between the two methods. But on May 9, 2006, the estimated population sizes were a little larger by the cultivation method. The growth of fungi which have the ability to reduce nitrate by cultivation method may be the reason for the difference, since the molecular method only targeted *nirK* gene from bacteria. The assumption of one cell of denitrifying bacteria containing one *nirK* gene was the base of the comparison for the two methods. However, more study is needed to explain the difference.

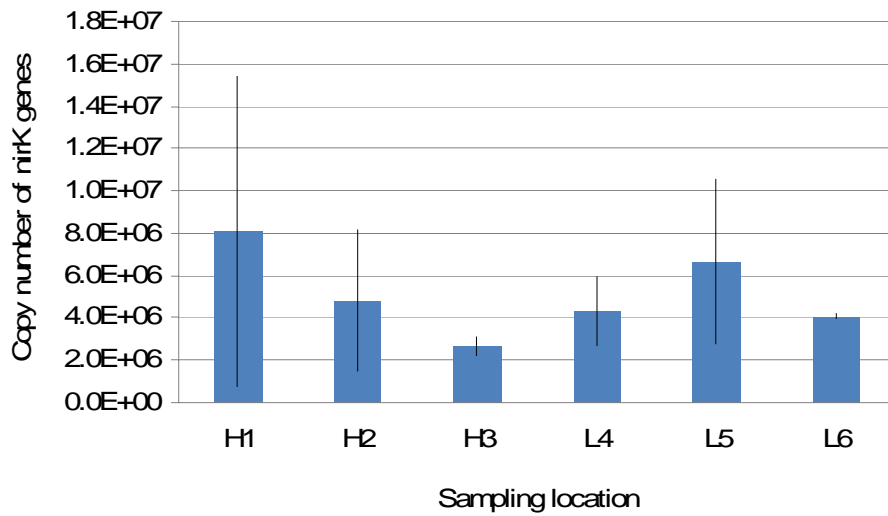


Figure 4.13 Copy numbers of *nirK* genes in soil samples from different sampling locations with standard error of the means on May 17, 2006. (H stands for high elevation and L stands for low elevation)

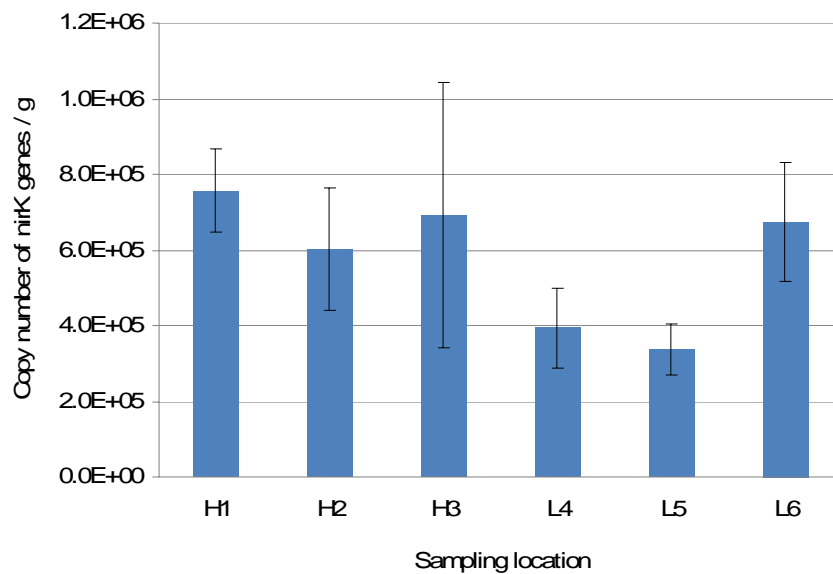


Figure 4.14 Copy numbers of *nirK* genes in soil samples from different sampling locations with standard error of the means on October 3, 2006. (H stands for high elevation and L stands for low elevation)

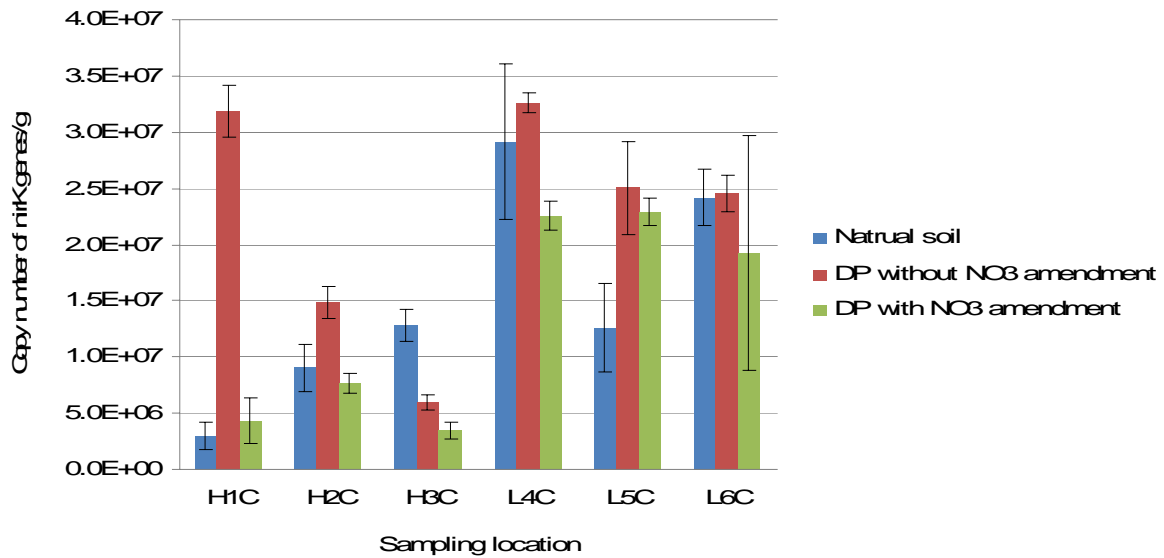


Figure 4.15 Copy numbers of *nirK* genes in natural soil samples, soil samples after denitrification potential incubation with and without addition of NO₃⁻ from different sampling locations with standard error of the means on May 9, 2007. (H stands for high elevation and L stands for low elevation)

On May 9, 2007, we also investigated the population sizes of denitrifying bacteria in soil after denitrification potential incubation (both with and without NO₃⁻ amendment) by molecular approach. The population sizes in these two different types of soil samples and natural soil samples were not significantly different ($P > 0.05$). The population sizes of denitrifying bacteria were close to each other ($P > 0.05$) between the low and high elevation locations in the soil after denitrification potential incubation without the amendment of NO₃⁻. With the amendment of NO₃⁻, the population sizes of denitrifying bacteria from low elevation were significantly higher ($P < 0.05$) than those from high elevation (Table 4.3.2.1). That indicated that the addition of NO₃⁻ changed the proliferation activity of denitrifying bacteria in the 6-hour incubation period, therefore changed the culturable community structure in situ.

Table 4.7 Average population size at different sampling locations with different treatment

	Natural soil (/g dry soil)	Soil after DP incubation without NO₃⁻ amendment(/g dry soil)	Soil after DP incubation with NO₃⁻ amendment(/g dry soil)
High elevation	8.29 x 10 ⁶	1.76 x 10 ⁷	5.19 x 10 ⁶
Low elevation	2.20 x 10 ⁷	2.74 x 10 ⁷	2.16 x 10 ⁷

The population sizes of denitrifying bacteria at high elevation were similar to each other on the sampling date of May 17, 2006, October 3, 2006, and May 9, 2007. But the population size on May 9, 2007 was significantly larger than that on May 17, 2006. It was significantly smaller on October 3, 2006 than on May 17, 2006. The larger population sizes were consistent with the higher denitrification potential levels on the same sampling day. Philippot (2005) also obtained the same results claiming that the density of denitrifying bacteria affected the denitrification potential. However, the similar population sizes cannot explain the different denitrification potential showed at high and low elevations. The community structure of denitrifying bacteria might play a key role determining N₂O emission, and affected denitrification potential in different locations. This was demonstrated by Cavigelli and Robertson (2000) and Holtan-Hartwig et al. (2000). Enwall (2005) stated that the denitrification potential was not coupled with the denitrifying bacteria community composition. The participation of fungal denitrifiers in the activity may also affect the denitrification potential and rate. What was the community structure in the sampling locations? How did they affect the denitrification potential? What was the role of the fungal denitrifiers in this study site? These questions still need to be answered by further investigation. Enwall (2005) reported that different nitrate sources and heavy metal concentrations may cause the difference in the denitrifying bacteria community structure in agriculture field. However, the results are not

comparable between different experiments. It may be due to site-specific characteristics and culturability and gene copy numbers per genome for each species.

The soil samples after denitrification potential incubation without amendment of NO_3^- showed a larger population than the natural soil and the soil from denitrification potential incubation with amendment of NO_3^- , except for the sample from H3C. With an amendment of NO_3^- , the population tended to be smaller than that in the natural soils. It might be because the environment in the incubation of denitrification potential study without addition of NO_3^- benefit proliferation of denitrifying bacteria, while with the addition of NO_3^- , denitrifying bacteria were more active in denitrification than proliferation.

4.4 Medium Design

The microorganisms isolated on different cultivation media showed different growth conditions. With a lower pH, more fungi grew on the medium, and the total number of microorganisms on one petri-dish was smaller than that with a higher pH at the same level of soil concentration. On medium 6 and 7 with pH 4.5, there were the least growth of microorganisms, and none of the isolated bacteria were denitrifying bacteria (Table 4.7). That indicated that a low pH of 4.5 is not suitable for denitrifying bacteria growth. Medium 1 and 2 had much higher isolation rate of denitrifying bacteria than the other media, and their combinations were similar to medium 8, which only had an isolation rate of 4%. This showed that a pH close to neutral (~7), was favorable to denitrifying bacteria growth. Medium 4 with a neutral pH did not have high isolation rate. It might be because the carbon source of ethanol cannot be utilized by most of the denitrifying bacteria or the high carbon / nitrogen ratio and high nitrate concentration affected the growth of the denitrifying bacteria.

Media with pH between 7 and 8 are often used to isolate or cultivate the denitrifying bacteria. A medium with pH = 8 was used by Braun et al. (1984). Seyfried et al. (1994) used pH = 7.5 for the isolation of denitrifying bacteria which can oxidize toluene and xylene in an aerobic condition. Gamble et al. (1977) adjusted the pH to 7.2 to isolate the denitrifying bacteria from soil and sediment. Because the pH value in a forest soil is between 4.5 and 7, we chose this range.

Glucose is widely used as a carbon source in the medium for denitrifying bacteria isolation and cultivation. Gamble et al. (1977) claimed that one third of the isolates cannot use glucose or other carbohydrates as a single carbon source. In our study, the number of colonies of denitrifying bacteria growing with single hydrocarbon was comparable to that with the complex medium. However, the culturability of denitrifying bacteria species in media with more than one carbon source in one medium needs to be further studied.

Table 4.8 Combinations of different parameters for the media with different values.

Medium	pH	C source	C/N (molar)	KNO ₃ (mM)	NaCl (g/L)	thiamine (ml/L)	cobalamin (ml/L)	Denitrifying bacteria isolated (%)
1	7	succinate	7.5	12	0	2	2	18
2	7	glucose	7.5	15	0	2	1	12
3	6	succinate	25	3	5	1	2	6
4	7	ethanol	22.5	18	0	1	1	6
5	6.5	glucose	5	3	5	0	0	2
6	4.5	glucose	2.5	6	5	0	2	0
7	4.5	succinate	12.5	12	5	1	2	0
8	5	succinate	7.5	18	0	2	2	4
9	6	glucose	10	9	5	0	2	2
TSA								6

The carbon nitrogen ratio in our sampling sites was between 14.23 and 16.86 (molar). The closest carbon nitrogen ratios in the designed media were in medium 7 and medium 9. With low pH, neither of them had a good isolation rate. The ratios in Medium 1 and medium 2, which

have the highest isolation percentage of denitrifying bacteria, were close to the *in-situ* carbon nitrogen ratio range.

Media containing 5 mg/L NaCl provided lower isolation rates compared to those without NaCl. A similar result was obtained from Heylen's study (2006), in which all the media with highest fitness did not contain NaCl. The sample size in our experiment was not large enough statistically support the exclusion of NaCl. It will be determined by further study.

The denitrifying bacteria isolated by Gamble et al. (1977) with nitrate agar from soils, freshwater lake sediments, and oxidized poultry manure were less than 10% of the total bacteria isolated on average. The isolation rate in our study showed similar results.

The concentration of thiamine in this study was 0 to 2 ml / L. Other studies used 1 to 5 ml / L (Evans et al., 1991; Blaszczyk, 1993; Chan and Wheatcroft, 1993; Kniemeyer et al., 1999). The results in our experiment suggested that the isolation rate was related to the concentration of thiamine, with $R^2 = 0.5241$, which means that the isolation rate is higher with more thiamine. However, this relationship was only true below the concentration of 2 ml / L in our study.

In this study, the concentration of cobalamin in the range of 0 to 2 ml / L and the isolation rate had no relationship ($R^2 = 0.0000$). Cobalamin is a common component of medium for denitrifying bacteria isolation and cultivation.

The media evaluated in this study was the first generation of the optimization of cultivation medium. The production of next generation media will be based on the results from this generation. This experiment is the basis for further optimization of the cultivation medium. The study by Heylen et al. (2006) for optimizing cultivation medium for denitrifying bacteria from active sludge showed that after 4 generations of cultivation with evolutive media, the

average fitness and the number of media with higher fitness of each generation increased. From the second generation, they found 3 best media with highest fitness at each generation.

Future study should address the effectiveness of the media in the recovery of denitrifying bacteria in terms of species numbers. In addition, the community structure of the isolated denitrifying bacteria on each medium needs to be investigated.

5. CONCLUSIONS

This study was the first time to evaluate hydrological impact on denitrification rate, denitrification potential and denitrifying bacteria population in a restored BLH. In this ecosystem, hydrological conditions affected the denitrification process of the soil. With periodical flood, the soil had higher denitrification potential, albeit with a low *in-situ* denitrification rate. The differences in hydrological conditions did not affect the population size of the denitrifying bacteria. *In-situ* denitrification rate and denitrification potential was affected but not mainly regulated by the population size of denitrifying bacteria. The community structure might be more important in regulating the denitrification rate and potential than the population size. This study demonstrated that restoring the hydrologic regime is important to a BLH's restoration. With restoration of hydrologic conditions, a BLH can perform its biogeochemical functions in terms of reducing nitrate from the ecosystem, which is meaningful for water quality maintenance.

The molecular approach to study denitrifying bacteria in the environment is less time-consuming than classic cultivation methods, and it is a promising method for microbial ecology study. However, a better cultivation media with higher isolation rate and species coverage is still needed. With the improvement of existing cultivation medium, more "unculturable" species can be isolated. The medium 1 and medium 2 from this study were better than the classic TSA medium when considering isolation percentage of denitrifying bacteria. This work is the base for further optimizing the cultivation medium for the isolation of denitrifying bacteria from environmental samples. An optimized medium will offer a higher possibility to discover the unknown species in the environment and provide a better approach to study them.

REFERENCES

- Allen, J. A. (1990). "Establishment of bottomland oak plantations on the yazoo national wildlife refuge complex." Southern Journal of Applied Forestry **14**(4): 206-210.
- Amann, R. I., W. Ludwig, and K.-H. Schleifer (1995). "Phylogenetic identification and in situ detection of individual microbial cells without cultivation." Microbiological Reviews **59**(1): 143-169.
- Army Engineer Waterways Experiment Station (1992). Wildlife habitat function of bottomland hardwood wetlands, Cache River, Arkansas. Vicksburg, MS.
- Axelrod, P. E., M. L. Chow, C. C. Radomski, J. M. McDermott, and J. Davies (2002). "Molecular characterization of bacterial diversity from British Columbia forest soils subjected to disturbance." Canadian Journal of Microbiology **48**(7): 655-674.
- Bachand, P. A. M. and A. J. Horne (1999). "Denitrification in constructed free-water surface wetlands: II. Effects of vegetation and temperature." Ecological Engineering **14**(1): 17-32.
- Blaszczyk, M. (1993). "Effect of medium composition on the denitrification of nitrate by *Paracoccus denitrificans*." Applied and Environmental Microbiology **59**(11): 3951-3953.
- Bothe, H., G. Jost, M. Schloter, and B. B. Ward (2000). "Molecular analysis of ammonia oxidation and denitrification in natural environments." FEMS Microbiology Ecology **24**: 673-900.
- Braker, G., A. Fesefeldt, and K-P. Witzel (1998). "Development of PCR Primer Systems for Amplification of Nitrite Reductase Genes (*nirK* and *nirS*) To Detect Denitrifying Bacteria in Environmental Samples." Applied and Environmental Microbiology **64**(10): 3769-3775.
- Braker, G., J. Zhou, L. Wu, A. H. Devol, and J. M. Tiedje (2000). "Nitrite Reductase Genes (*nirK* and *nirS*) as Functional Markers To Investigate Diversity of Denitrifying Bacteria in Pacific Northwest Marine Sediment Communities." Applied and environmental microbiology **66**(5): 2096-2104.
- Braun, K. and D. T. Gibson (1984). "Anaerobic degradation of 2-aminobenzoate (anthranilic acid) by denitrifying bacteria." Applied and Environmental Microbiology **48**(1): 102-107.
- Brettar, I., E. R. B. Moore, and M.G. Höfle (2001). "Phylogeny and abundance of novel denitrifying bacteria isolated from the water column of the central baltic sea " Microbial Ecology **42**(3): 295-305.

- Brooks, M. H., R. L. Smith, and D. L. Macalady (1992). "Inhibition of existing denitrification enzyme activity by chloramphenicol." Applied and environmental microbiology **58**(5): 1746-1753.
- Burdick D. M., D. Cushman, R. Hamilton, and J. G. Gosselink (1989). " Faunal changes and Bottomland Hardwood Forest loss in the Tensas watershed, Louisiana." Conservation Biology **3**(2): 282-292.
- Brye, K.R., J.M. Norman, L.G. Bundy and S.T. Gower (2001). " Nitrogen and Carbon Leaching in Agroecosystems and Their Role in Denitrification Potential." Journal of Environmental Quality **30**:58-70.
- Cavigelli, M. and G. P. Robertson (2000). "Dissimilatory nitrite reductase genes from autotrophic ammonia-oxidizing bacteria." Applied and Environmental Microbiology **67**: 2213-2221.
- Cavigelli, M. and G. P. Robertson (2000). "The functional significance of denitrifier community composition in a terrestrial ecosystem." Ecology **81**(5): 1402-1414.
- Chabrierie, O., I. Poudevigne, F. Bureau, M. Vincelas-Akpa, S. Nebbache, M. Aubert, A. Bourcier, and D. Alard (2001). "Biodiversity and Ecosystem Functions in Wetlands: A Case Study in the Estuary of the Seine River, France." Estuaries **24**(6B): 1088-1096.
- Chan, Y. K. and R. Wheatcroft (1993). "Detection of a nitrous oxide reductase structural gene in Rhizobium meliloti strains and its location on the nod megaplasmid of JJ1c10 and SU47." Journal of Bacteriology **175**(1): 19-26.
- Changotra, H. and P. K. Sehajpal (2005). "Quantitative detection of serum HBV DNA levels employing a new S gene based cPCR assay" Biomedical and Life Sciences **150**(3): 481-491.
- Cheneby, D., L. Philippot, A. Hartmann, C. Henault, and J.-C. Germon (2000). "16S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils." Microbiology Ecology **34**: 121-128.
- Cohen-Bazire, G., W. R. Sistrom, and R.Y. Stanier (1957). "Kinetic studies of pigment synthesis by non-sulphur purple bacteria." Journal of cellular competitive physiology **49**(1): 25-48.
- Coyne, M. S., A. Arunakumari, B. A. Averill, and J. M. Tiedje (1989). "Immunological identification and distribution of dissimilatory heme cd1 and nonheme copper nitrite reductases in denitrifying bacteria." Applied and Environmental Microbiology **55**(11): 2924-2931.
- Cowdrey, A.E.(1983). "This land, this South: an environmental history." Lexington, KY: University Press of Kentucky.

- Cromer, R.B., J.D. Lanham, and H.H. Hanlin (2002). "Herpetofaunal response to gap and skidder-rut wetland creation in a southern bottomland hardwood forest." Forest Science **48**(2): 407-413.
- Davidson, E. A., P. A. Matson, P. M. Vitousek, R. Riley, K. Dunkin, G. Garcia-Mendez, and J. M. Maass (1993). "Processes regulating soil emissions of NO and N₂O in a seasonally dry tropical forest." Ecology **74**(1): 130-139.
- Dortch, M. S. (1996). "Removal of solids, nitrogen, and phosphorus in the Cache River wetland." Wetlands **16**(3): 358-365.
- Ehrenreich, P., A. Behrends, J. Harder, and F. Widdel (2000). "Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria " Archives of Microbiology **173**(1): 58-64.
- Ensign, S. H. and M. A. Mallin (2001). "Stream water quality changes following timber harvest in a coastal plain swamp forest." Water Resources **35**(14): 3381-3390.
- Enwall, K., L. Philippot, and S. Hallin (2005). "Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization." Applied and environmental microbiology **71**(12): 8335-8343.
- Evans, P. J., D. T. Mang, and L. Y. Young (1991). "Degradation of toluene and m-xylene and transformation of o-xylene by denitrifying enrichment cultures." Applied and Environmental Microbiology **57**(2): 450-454.
- Felske, A. and A. D. L. Akkermans (1998). "Spatial homogeneity of abundant bacterial 16S rRNA molecules in grassland soils." Microbial Ecology **36**: 31-36.
- Felske, A. and A. D. L. Akkermans (1999). "Searching for the predominant soil bacteria: 16S rDNA cloning versus strain cultivation." FEMS Microbiology Ecology **30**: 137-145.
- Féray, C., B. Volat, V. Degrange, A. Clays-Josserand, and B. Montuelle (1999). "Assessment of three methods for detection and quantification of nitrite-oxidizing bacteria and nitrobacter in freshwater sediments (MPN-PCR, MPN-Griess, immunofluorescence) " Microbial Ecology **37**(3): 208-217.
- Firestone, M. K. and E. A. Davidson (1989). Microbiological basis of NO and N₂O production and consumption in soil. Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere. M. O. Andrea and D. S. Schimel: 7-21.
- Frink, C. R., P. E. Waggoner, and J. H. Ausubel (1999). "Nitrogen fertilizer: Retrospect and prospect." Proc. Natl. Acad. Sci. USA **96**: 1175-1180.
- Furlong, M. A., D. R. Singleton, D. C. Coleman, and W. B. Whitman (2002). "Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the

- burrows and casts of the earthworm *Lumbricus rubellus*." Applied and Environmental Microbiology **68**: 1265-1279.
- Galloway, J. N., J. D. Aber, J. W. Erisman, S. P. Seitzinger, R. W. Howarth, E. B. Cowling, and B. J. Cosby (2003). "The Nitrogen Cascade." Bioscience **53**(4): 341-356.
- Gamble, T. N. (1976). The Commonality of Numerically Dominant Denitrifier Strains Isolated from Various Habitats. Department of Microbiology and Public Health, Michigan State University. **M.S.**
- Gamble, T. N., M. R. Betlach, and J. M. Tiedje (1977). "Numerically Dominant Denitrifying Bacteria from World Soils." Applied and environmental microbiology **33**(4): 926-939.
- Glockner, A. B., A. Jüngst, and W. G. Zumft (1993). "Copper-containing nitrite reductase from *Pseudomonas aureofaciens* is functional in a mutationally cytochrome *cd1*-free background (NirS-) of *Pseudomonas stutzeri*." Archives of Microbiology **160**(1): 18-26.
- Gregorich, E. G., P. Rochette, A.J. VandenBygaart, and D.A. Angers (2005). "Greenhouse gas contributions of agricultural soils and potential mitigation practices in Eastern Canada." Soil & Tillage Research **83**(1): 53-72.
- Groffman, P. and J. Tiedje (1989). "Denitrification in north temperate forest soils: relationships between denitrification and environmental factors at the landscape scale " Soil Biology & Biochemistry **21**(6): 621-626.
- Grüntzig, V., S. C. Nold, J. Zhou, J. M. Tiedje (2001). "Pseudomonas stutzeri nitrate reductase gene abundance in environmental samples measured by real-time PCR." Applied and Environmental Microbiology **67**: 760-768.
- Guy, R. A., P. Payment, U. J. Krull, and P. A. Horgen (2003). "Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage " Applied and Environmental Microbiology **69**(9): 5178-5185.
- Hallberg, G. R. (1989). Nitrate in ground water in the United States. New York, Elsevier.
- Hallin, S. and M. Pell (1998). "Metabolic properties of denitrifying bacteria adapting to methanol and ethanol in activated sludge - I. Stationary cultures " Water Research **32**(1): 13-18.
- Haynes, R. J., R. J. Bridges, S. W. Gard, T. W. Wilkns, and H. R. Cook (1995). Bottomland hardwood reestablishment efforts of the U.S. Fish and Wildlife Service: southeast region. Proceeding of the national wetlands engineering workshop. Technical report-WRP-RE-8. J. C. Fischenich, C. M. Lloyd and M. R. Palmero. Vicksburg, Mississippi, U.S. Army Corps of Engineers, Waterways Experiment Station: 322-334.
- Hefner, J. M. and J. D. Brown (1985). "Wetland trends in the southeastern United States." Wetlands **4**: 1-11.

- Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams (1996). "Real time quantitative PCR." Genome Research **6**: 986-994.
- Henry, S., E. Baudoin, J. C. López-Gutiérrez, F. Martin-Laurent, A. Brauman, and L. Philippot (2004). "Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR." Journal of Microbiological Methods **59**(3): 327-335.
- Hermansson, A. and P. E. Lindgren (2001). "Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR." Applied and environmental microbiology **67**(2): 972-976.
- Heylen, K., B. Vanparys, L. Wittebolle, W. Verstraete, N. Boon, and P. D. Vos (2006). "Cultivation of denitrifying bacteria: optimization of isolation conditions and diversity study." Applied and environmental microbiology **72**(4): 2637–2643.
- Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand. (1991). "Detection of specific polymerase chain reaction product by utilizing the 5'- 3' exonuclease activity of *Thermus aquaticus* DNA polymerase." Proceedings of the National Academy of Sciences of the United States of America **86**(16): 7276–7280.
- Holtan-Hartwig, L., P. Dörsch, and L. R. Bakken (2000). "Comparison of denitrifying communities in organic soils: kinetics of NO₃⁻ and N₂O reduction." Soil Biology & Biochemistry **32**(6): 833-843.
- Horn, M. A., H. L. Drake, and A. Schramm (2006). "Nitrous oxide reductase genes (*nosZ*) of denitrifying microbial populations in soil and the earthworm gut are phylogenetically similar." Applied and Environmental Microbiology **72**(2): 1019-1026.
- Houghton, J. T., B. A. Callander, and S.K. Verney (1992). Climate change 1992, The supplementary report to the IPCC Scientific Assessment. IPCC. Cambridge.
- Huffman, R. T. and S. W. Forsythe (1981). Bottomland hardwood communities and their relation to anaerobic soil conditions. Wetlands of bottomland hardwood forests. J. R. Clark and J. Benforado. New York, Elsevier: 187-196.
- Hunter, R. G. and S. P. Faulkner (2001). "Denitrification potentials in restored and natural bottomland hardwood wetlands." Soil Science Society of America journal **65**: 1865-1872.
- IPCC (1996). Climate change 1995: The science of climate change. C. U. Press.
- Johnsen, K., Ø. Enger, C. S. Jacobsen, L. Thirup, and V. Torsvik (1999). "Quantitative selective PCR of 16S ribosomal DNA correlates well with selective agar plating in describing population dynamics of indigenous *Pseudomonas* spp. in soil hot spots " Applied and Environmental Microbiology **65**(4): 1786-1788.

- Jørgensen, K. S. and A. S. Pauli (1995). "Polyphosphate accumulation among denitrifying bacteria in activated sludge." Anaerobe **1**(3): 161-168.
- Joseph, S. J., P. Hugenholtz, P. Sangwan, C. A. Osborne, and P. H. Janssen (2003). "Laboratory cultivation of widespread and previously uncultured soil bacteria " Applied and Environmental Microbiology **69**(12): 7210-7215.
- Kasimir-Klemedtsson, Å., L. Klemedtsson, K. Berglund, P. Martikainen, J. Silvola, and O. Oenema (1997). "Greenhouse gas emissions from farmed organic soils: a review." Soil Use and Management **13**(4): 245-250.
- Kavanaugh, M. (1987). "Estimates of future CO, N₂O and NO_x emissions from energy combustion." Atmospheric Environment **21**(3): 463-468
- Kennedy, H. E. (1990). Hardwood reforestation in the south: Landowners can benefit from Conservation Reserve Program incentives. New Orleans, LA, Southern Forest Experiment Station.
- King, S. L. and B. D. Keeland (1999). "Evaluation of Reforestation in the Lower Mississippi River Alluvial Valley." Restoration Ecology **7**(4): 348-359.
- Kleiss, B. A. (1996). "Sediment retention in a bottomland hardwood wetland in eastern Arkansas." Wetlands **16**(3): 321-333.
- Kniemeyer, O., C. Probian, R. Rosselló-Mora, and J. Harder (1999). "Anaerobic mineralization of quaternary carbon atoms: isolation of denitrifying bacteria on dimethylmalonate." Applied and Environmental Microbiology **65**(8): 3319-3324.
- Knobeloch, L., B. Salna, A. Hogan, J. Postle, and H. Anderson (2000). "Blue babies and nitrate-contaminated well water " Environmental Health Perspectives **108**(7): 675-678.
- Kolka, R. K., J. H. Singerb, C. R. Coppockb, W. P. Caseyb, and C. C. Trettinb (2000). "Influence of restoration and succession on bottomland hardwood hydrology " Ecological Engineering **15**: 131-140.
- Larsen, L. H., N. P. Revsbech, et al. (1996). "A microsensor for nitrate based on immobilized denitrifying bacteria." Applied and environmental microbiology **62**(4): 1248-1251.
- Llewellyn, D. W., G. P. Shaffer, and S. J. Binnerup (1996). "A decision-support system for prioritizing restoration sites on the Mississippi River Alluvial Plain." Conservation Biology **10**: 1446-1455.
- Louisiana Department of Wildlife and Fisheries. from <http://www.wlf.louisiana.gov/experience/naturalheritage/currentprojects/>.

- Lowrance, R., R. Todd, J. Fail, O. Hendrickson, P. Leonard, and L. Asmussen (1984). "Riparian forests as nutrient filters in agricultural watersheds " Bioscience **34**(6): 374-377.
- Lowrance, R. and G. Vellidis (1995). "A conceptual model for assessing ecological risk to water quality function of bottomland hardwood forests " Environmental Management **19**(2): 239-258.
- Luo, J., R. E. White, P. Roger Ball, and R. W. Tillman (1996). "Measuring denitrification activity in soils under pasture: optimizing conditions for the short-term denitrification enzyme assay and effects of soil storage on denitrification activity." Soil Biology & Biochemistry **28**(3): 409-417.
- Makino, S.-i. and H.-i. Cheun (2003). "Application of the real-time PCR for the detection of airborne microbial pathogens in reference to the anthrax spores." Journal of Microbiological Methods **53**: 141-147.
- Martin, J. K. (1975). "Comparison of agar media for counts of viable soil bacteria." Soil Biology and Biochemistry **7**: 401-402.
- Merino, A. n., P. Pérez-Batallón, and F. Macías (2004). "Responses of soil organic matter and greenhouse gas fluxes to soil management and land use changes in a humid temperate region of southern Europe." Soil Biology & Biochemistry **36**(6): 917-925.
- Merzouki, M., J.-P. Delgenès, N. Bernet, R. Moletta, and M. Benlemlih (1999). "Polyphosphate-accumulating and denitrifying bacteria isolated from anaerobic-anoxic and anaerobic-aerobic sequencing batch reactors." Current Microbiology **38**: 9-17.
- Mitsch, W. J. and J. G. Gosselink (1993). Wetlands. New York, Van Nostrand Reinhold.
- Naqvi, S. W. A., D. A. Jayakumar, P. V. Narvekar, H. Naik, V. V. S. S. Sarma, W. D'Souza, S. Joseph , and M. D. George (2000). "Increased marine production of N₂O due to intensifying anoxia on the Indian continental shelf." Nature **408**(6810): 346-349.
- National Research Council (1982). Impacts of emerging agricultural trends on fish and wildlife habitat. Washington D.C., National Academy Press.
- Neef, A., A. Zaglauer, H. Meier, R. Amann, H. Lemmer, and K.-H. Schleifer (1996). "Population analysis in a denitrifying sand filter: Conventional and in situ identification of *Paracoccus* spp. in methanol-fed biofilms." Applied and Environmental Microbiology **62**: 12.
- Nolan, B. T., B. C. Ruddy, K. J. Hitt, and D. R. Helsel (1997). "Risk of nitrate in groundwaters of the United States-a national perspective." Environmental Science and Technology **31**(8): 2229-2236.

- Noss, R. F., E. T. LaRoe, and J.M. Scott (1995). Endangered ecosystems of the United States: a preliminary assessment of loss and degradation Biological Report Washington D.C., US Department of the Interior, National Biological Service.
- NRCS (2003). "WRP supporting data by state available in tabular format."
- NRCS (2004). "Wetland Reserve Program fact sheet."
- Pai, S.-L., N.-M. Chong, and C.-H. Chen (1999). "Potential applications of aerobic denitrifying bacteria as bioagents in wastewater treatment." Bioresource Technoloty **68**(2): 179-185.
- Palys, T., L. K. Nakamura, and F.M. Cohan (1997). "Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data." International Journal of Systematic Bacteriology **47**: 1145-1156.
- Pérez-Ramírez, J., F. Kapteijn, K. Schöffel, and J.A. Moulijn (2003). "Formation and control of N₂O in nitric acid production-Where do we stand today?" Applied Catalysis B: Environmental **44**(2): 117-151.
- Pfenning, K.S. and P.B. McMahon (1997). "Effect of nitrate, organic carbon, and temperature on potential denitrification rates in nitrate-rich riverbed sediments." Journal of Hydrology **187**(3): 283-295.
- Philippot, L. (2005). Use of functional genes to quantify denitrifiers in the environment. The 11th Nitrogen Cycle Meeting.
- Philippot, L., and S. Hallin. (2005). "Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community." Current Opinion in Microbiology **8**: 234-239.
- Priemé, A., G. Braker, and J. M. Tiedje (2002). "Diversity of nitrite reductase (nirK and nirS) gene fragments in forested upland and wetland soils." Applied and Environmental Microbiology **68**(4): 1893-1900.
- Qiu, X. Y., R. A. Hurt, L.Y.Wu, C.H. Chen, J.M. Tiedje, and J.Z. Zhou (2004). "Detection and quantification of copper-denitrifying bacteria by quantitative competitive PCR." Journal of Microbiological Methods **59**(2): 199-210.
- Rabalais, N. N., R. E. Turner, and D. Scavia (2002). "Beyond science into policy: Gulf of Mexico hypoxia and the Mississippi River." Bioscience **52**(2): 129-142.
- Rheims, H., A. Felske, S. Seufert, and E. Stackebrandt (1999). "Molecular monitoring of an uncultured group of the class Actinobacteria in two terrestrial environments." Journal of Microbiological Methods **36**: 65-75.

- Ribaudo, M. O. (1989). "Targeting the Conservation Reserve Program to maximize water quality benefits " Land Economics **65**(4): 320-332.
- Rich, J. J. and D. D. Myrold (2004). "Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA." Soil Biology & Biochemistry **36**: 1431-1441.
- Rösch, C., A. Mergel, and H. Bothe (2002). "Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil." Applied and Environmental Microbiology **68**(8): 3818-3829.
- Rudis, V. A. (1995). " Regional forest fragmentation effects on bottomland hardwood community types and resource values." Landscape Ecology **10**(5): 291-307.
- Rudolphi, A., A. Tschech, and G. Fuchs (1991). " Anaerobic degradation of cresols by denitrifying bacteria." Archives of Microbiology **155**(3): 238-248.
- Sallabanks, R., J. R. Walters, and J. A. Collazo (2000). " Breeding bird abundance in bottomland hardwood forests: habitat, edge, and patch size effects" The Condor **102**(4): 748-758.
- Scala, D. J. and L. J. Kerkhof (2000). "Horizontal heterogeneity of denitrifying bacterial communities in marine sediments by terminal restriction fragment length polymorphism analysis " Applied and Environmental Microbiology **66**(5): 1980-1986.
- Schoenholtz, S. H., J. P. James, R. M. Kaminski, B. D. Leopold, and A. W. Ezell (2001). "Afforestation of bottomland hardwoods in the Lower Mississippi Alluvial Valley: status and trends." Wetlands **21**(4): 602-613.
- Sexstone, A. J., T. B. Parkin, and J. M. Tiedje (1985). "Temporal response of soil denitrification rates to rainfall and irrigation " Soil Science Society of America Journal **49**(1): 99-103.
- Seyfried, B., G. Glod, R. Schocher, A. Tschech, and J. Zeyer (1994). "Initial reactions in the anaerobic oxidation of toluene and m-xylene by denitrifying bacteria." Applied and Environmental Microbiology **60**(11): 4047-4052.
- Sharitz, R. R. and W. J. Mitsch (1993). Southern floodplain forests. Biodiversity of the southeastern United States: lowland terrestrial communities. W. H. Martin, S. G. Boyce and A. C. Echternacht. New York, John Wiley and Sons: 311-372.
- Shear, T. H., T. J. Lent, and S. Fraver (1996). "Comparison of restored and mature bottomland hardwood forests of southwestern Kentucky." Restoration Ecology **4**(2): 111-123.
- Smith, C. J., D. B. Nedwell, L. F. Dong, and A. M. Osborn (2007). "Diversity and abundance of nitrate reductase genes (narG and napA), nitrite reductase genes (nirS and nrfA), and their transcripts in estuarine sediments." Applied and Environmental Microbiology **73**(11): 3612-3622.

- Smith, G. B. and J. M. Tiedje (1992). "Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria." Applied and Environmental Microbiology **58**(1): 376-384.
- Song, B., N. J. Palleroni, and M. M. Häggblom (2000). "Isolation and characterization of diverse halobenzoate-degrading denitrifying bacteria from soils and sediments " Applied and Environmental Microbiology **66**(8): 3446-3453.
- Stanier, R. Y., N. J. Palleroni, and M. Doudoroff (1966). "The aerobic pseudomonads: a taxonomic study." The journal of general microbiology **43**(2): 159-271.
- Stephen, J. R., A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley (1996). "Molecular diversity of soil and marine 16S rRNA gene sequences related to B-subgroup ammonia-oxidizing bacteria." Applied and Environmental Microbiology **62**: 4147-4154.
- Szentandrasi, S., S. Polasky, R. Berrens, and J. Leonard (1995). "Conserving biological diversity and the Conservation Reserve Program " Growth and Change **26**(3): 383-404.
- Takaya, N., M. A. B. Catalan-Sakairi, Y. Sakaguchi, I. Kato, Z. Zhou, and H. Shoun (2003). "Aerobic denitrifying bacteria that produce low levels of nitrous oxide." Applied and Environmental Microbiology **69**(6): 3152-3157.
- Throback, I. N., K. Enwall, A. Jarvis, and S. Hallin (2004). "Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE." FEMS Microbiology Ecology **49**(3): 401-407.
- Tiedje, J. M. (1982). Denitrification In: Methods of Soil Analysis. Madison, WI., Page, A.L., Ed.
- Tiedje, J. M. (1988). Ecology of denitrification and dissimilatory nitrate reduction to ammonium. Environmental microbiology of anaerobes. A. J. B. Zehnder. New York, NY, John Wiley & Sons: 179-244.
- Tilman, D., J. Fargione, B. Wolff, C. D'Antonio, A. Dobson, R. Howarth, D. Schindler, W. H. Schlesinger, D. Simberloff, and D. Swackhamer (2001). "Forecasting Agriculturally Driven Global Environmental Change." Science **292**(5515): 281 - 284.
- Twedt, D. J., R. R. Wilson, J. L. Henne-Kerr, and D. A. Grosshuesch (2002). "Avian response to bottomland hardwood reforestation: the first 10 years." Restoration Ecology **10**(4): 645-655.
- Ullah, S. and Louisiana State University (Baton Rouge La.). Dept. of Oceanography and Coastal Sciences. (2005). "Denitrification and greenhouse gas emissions from cultivated and wetland alluvial soils." from <http://etd.lsu.edu/docs/available/etd-01142005-095834/>

- Walbridge, M. R. (1993). "Functions and values of forested wetlands in the southern United States." Journal of Forestry **91**: 15-19.
- Ward, B.B. (1995). "Diversity of culturable denitrifying bacteria." Archives of Microbiology **163**(3): 167-175.
- Ward, B. B. and J. C. Priscu (1997). "Detection and characterization of denitrifying bacteria from a permanently ice-covered Antarctic Lake." Hydrobiologia **347**(1-3): 57-68.
- Why, K. R. V. (2003). Feasibility of restoring the Louisiana black bear (*Ursus Americanus Luteolus*) to portions of their historic range. The School of Renewable Natural Resources, Louisiana State University. **Master of Science**: 102.
- Widdel, F. and F. Bak (1992). Gram-negative mesophilic sulfate-reducing bacteria. Berlin, Germany, Springer.
- Williams, J. R. (1999). "Addressing global warming and biodiversity through forest restoration and coastal wetlands creation." The Science of the Total Environment **240**(1-3): 1-9.
- Yang, C.-Y., Shih-Chuan, M.-F. Cheng, S.-Chuan, and Y.-L. Hsieh (1998). "Calcium, magnesium, and nitrate in drinking water and gastric cancer mortality." Cancer Science **89**(2): 124-130.
- Zumft, W. G. (1992). The denitrifying procaryotes. The procaryotes. A. Balows, H. G. Tru"per, M. Dworkin, W. Harder and K.-H. Schleifer. New York, Springer-Verlag. **1**: 554-582.
- Zumft, W. G. (1997). "Cell biology and molecular basis of denitrification." Microbiology and molecular biology reivews **61**(4): 533-616.

VITA

Yan Li was born on January 11, 1982 in the city of Harbin in northeast China. Before going to college, she showed great interests in plants and animals, concerned a lot about the environment around her, and thought it would be great if she could solve some environmental problems with her knowledge. So she chose to study environmental sciences when she went to college at Nanjing University in China in September, 2000. After getting her bachelor's degree of science in June, 2004, she continued her study at Nanjing University for one year, and then came to the Department of Environmental Studies at Louisiana State University pursuing her master's degree in the same field under the supervision of Dr. Aixin Hou. Now she is a master's candidate and trying to explore the outside world utilizing her knowledge and skills.