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ISOLATION AND CHARACTERIZATION OF NITRATE REDUCING/DENITRIFYING BACTERIA FROM TALLGRASS PRAIRIE SOILS

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ISOLATION AND CHARACTERIZATION OF NITRATE REDUCING/DENITRIFYING BACTERIA FROM TALLGRASS PRAIRIE SOILS

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

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Abstract

This dissertation addresses the long term impact of crude oil/brine (e.g. salt water) spills on biogeochemical cycling of nitrogen in tallgrass prairie soils. The study sites were prairie soils that were accidently contaminated with crude oil and/or brine 5-10 years prior to this study. These sites had been partially bioremediated but still had low levels of oil and brine which could affect the abundance and species composition of denitrifying bacteria. The abundance of culturable nitrate reducing (NR) and denitrifying (DN) bacteria in the soil samples was estimated by 5-tube Most Probable Number (MPN) method using nitrate broth. The strength of the association of the abundance of NR and DN bacteria with various environmental factors including soil moisture, brine and/or crude oil, oxygen, and nitrate was estimated using multivariate statistics. The MPN tubes were the source of NR and DN isolates used to determine if molecular detection methods for NR and DN genes matched the ability of these strains to perform nitrate or nitrite reduction. My results showed that the NR and DN bacteria were as abundant in the long-term contaminated sites as in the uncontaminated sites. Soil moisture had a slight positive effect on the abundance of NR and DN bacteria in both contaminated and uncontaminated sites. In general, nitrate treatment did not produce an increase in numbers of NR and DN bacteria in contaminated sites. The degree of culturable bacterial diversity in the contaminated prairie sites was not lower than that in prairie uncontaminated sites. However, species composition of

nitrate-reducing bacteria varied among different sites where brine contaminated soils selected for salt tolerant bacteria like *Bacillus* while crude oil-contaminated sites selected for γ-Proteobacteria. Nitrate reducing and DN functional genes were detected in roughly half the strains that reduced nitrate or nitrite which suggests that the PCR-molecular detection methods underestimate the number of NR and DN bacteria. However, a high proportion of γ-Proteobacteria was correctly identified by PCR detection methods. These results indicate that both molecular and phenotypic methods are needed to correctly identify NR and DN bacteria. Examining a link to bioremediation through bioavailability of organic contaminants, I showed that naphthalene 1,2-dioxygenase (NDO) can enzymatically alter a variety of humic and fulvic acids and the extent of NDO-specific NADH oxidation paralleled the percent aromaticity of the humic and fulvic acids. Humic substances have not previously been known to be substrates for dioxygenases; even more significant was that dioxygenase enzymes can facilitate condensation between indole-like functional groups well-known to be present in humic and fulvic acids. The NDO enzyme retained activity for two weeks under ambient conditions suggesting prolonged extra cellular activity. These results illustrate how enzymes like NDO might alter the bioavailability of organic contaminants associated with soil when released into the environment upon microbial death.

Chapter 1: Introduction

Significance

Soil microorganisms are a fundamental part of biogeochemical cycles in general and biogeochemical cycling of nitrogen in particular. Denitrification is an important component of nitrogen cycling, in which some microorganisms (e.g. denitrifying bacteria) use nitrate or nitrite as alternative electron acceptors. In fact, several studies have focused on various aspects of nitrogen cycling. Philippot et al. (2009) linked the distribution of the fraction of bacteria with the genetic capacity to reduce N_2O to N_2 to areas with low potential N_2O emissions in a pasture. In addition, it was shown that a map of denitrification activity across a whole farm was reflected by maps displaying the community size and structure of a specific fraction of the denitrifyers at the site (Enwall et al., 2010). Since denitrification releases mineralized nitrogen in the soil ecosystem to the atmosphere, the balance between denitrification and N-fixation can determine the biologically available nitrogen for soils (Ollivier et al., 2011).

Denitrification could be affected by soil ecosystem contaminants such as crude oil and brine as they may alter the abundance and species composition of denitrifying bacteria in predictable ways. For example, γ-Proteobacteria are known to increase in crude-oil contaminated sites (Lee et al., 2002; Shim and Yang, 1999), and in fact, a wide diversity of γ-Proteobacteria including *Pseudomonas* and *Vibrio* species were shown to degrade hydrocarbons under nitrate reducing (NR)

conditions (Rockne et al., 2000). In addition, other studies (Vosswinkel et al., 1991; Song et al., 2000, Green et al., 2010) showed that strains for several genera of γ-Proteobacteria have the ability to denitrify. Therefore, I expect the loss of nitrogen via denitrification from crude oil contaminated sites may be greater compared to brine-contaminated or uncontaminated sites. I expect less nitrogen loss from brine contaminated soil if there are fewer NR and DN bacteria or decreased rates of activity. In fact, it was shown that nitrate and nitrite reduction rates were increasingly inhibited at increasing NaCl concentrations when comparing treatment of fishery wastewaters (Mariángel et al., 2008). Bacterial diversity in brine-contaminated sites is expected to be less because of selection for salt-tolerant genera such as *Bacillus* (Boch et al., 1997) and *Pseudomonas* (Egamberdiyeva, 2005).

Biogeochemical cycling of nitrogen

Nitrogen is an abundant element. Indeed, the volume of nitrogen gas (N_2) forms 78% of the atmospheric total volume. Although N_2 forms the highest percentage, nitrogen deficiency in soil is a common limiting factor for plant growth and productivity because vascular plants cannot combine N_2 directly into organic compounds (Nie et al., 2011). In fact, nitrate (NO_3^-) or ammonium ions (NH_4^+) are the main nitrogen forms absorbed by plants from the soil. Nitrogen circulates through the environment and living organisms including bacteria. Fallen leaves, animal feces, and dead plants or animals provide the soil with NH_4^+ by the soil

microorganisms that break down organic nitrogen in a process called ammonification. On the other hand, other bacteria obtain energy by oxidizing NH_4^+ to produce NO_3^- in a process called nitrification (Zumft, 1997). However, not all the nitrate produced is absorbed by plants. Some bacteria convert nitrate to nitrite and then to ammonia in a process called ammonifying nitrite reduction (Zumft, 1997). Other bacteria use nitrate and nitrite (NO_2^-) as alternative electron acceptors during respiration and convert them to the end products nitric oxide (NO), nitrous oxide (N_2O) and N_2 for energy production when oxygen is limiting, generally under anaerobic conditions. These end products are released to the atmosphere. This latter process is the traditionally defined pathway of denitrification (Braker et al., 2000) and is the focus of my research.

Physiology and taxonomic distribution occurrence of nitrate reducing/denitrifying bacteria

Nitrate reducing (NR) bacteria use nitrate as an alternative electron acceptor to obtain energy from dissimilatory reduction of nitrate into nitrite by nitrate reductase enzymes (Zumft, 1997). Nitrate reducing bacteria are facultative anaerobes that can use oxygen as their terminal electron acceptor (Chèneby et al., 2010). Nitrate reducing bacteria represent a diverse group with members among α, β, and γ-Proteobacteria, some members of Firmicutes, and even Archaea. Nitrate reducing bacteria are abundant in various environments such as human digestive tract (Bru et al., 2007), earthworm guts (Drake and Horn, 2007), and rhizosphere

(Brunel et al., 1992). Nitrate reduction is a facultative process, it depends primarily on the presence of nitrate, oxygen limitation, and electron donor availability (Tiedje, 1988).

In low O_2 environments, NO_3^- and NO_2^- are used as electron acceptors by some bacteria that perform a denitrification-like respiration (Bock et al., 1995; Braker et al., 2000). A series of enzymes direct the denitrification route in denitrifying bacteria: nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase. In anaerobic respiration, these enzymes consume NO_3^-, NO_2^-, NO, and N_2O, respectively, as terminal electron acceptors (Zumft, 1997).

In fact, after oxygen consumption (1), denitrification (2) is the highest-energy-yielding process as shown in equations 1 and 2 (Strohm et al., 2007).

$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$ ($\Delta G^{\circ\prime}$ = -2,870 kJ per mol glucose) (1)

$5C_6H_{12}O_6 + 24NO_3^- + 24H^+ \rightarrow 30CO_2 + 12N_2 + 42H_2O$ ($\Delta G^{\circ\prime}$ = -2,670 kJ per mol glucose) (2)

Denitrification is estimated to remove 40-50% of external inputs of dissolved inorganic nitrogen in marine coastal sediments (Seitzinger, 1990), causing an unbalance of nitrogen quantities in the ocean (Devol, 1991; Codispoti, 1995). The production and accumulation of NO and N_2O contributes to global warming and the destruction of the ozone layer (Dickinson and Cicerone, 1986;

Conrad, 1996). Since denitrification is common among phylogenetically unrelated microbial groups and closely related species vary in their ability to denitrify, it is very unsuitable to investigate communities of DN bacteria by using an approach based on 16S rRNA gene sequences (Zumft, 1992). So, to detect and to analyze denitrifying bacteria, gene sequences coding for the enzymes responsible for key steps in the denitrification pathway have been used (Braker et al., 1998; Scala and Kerkhof, 1998; Scala and Kerkhof, 1999).

Denitrification consists of four reaction steps in which nitrate is reduced to dinitrogen gas. The first step where nitrate is reduced to nitrite is common step to a taxonomically diverse group of bacteria (NR bacteria) and it is catalyzed by two different types of nitrate reductases, either membrane bound encoded by the *narGHJI* operon or periplasmic encoded by the *napABC* operon (Kandeler et al., 2006). Nitrite reductase is a significant enzyme in the denitrification pathway because it produces NO, the first gaseous product (Ye et al., 1994; Casciotti and Ward, 2001), thus nitrogen loss from soil. This enzyme is found as two different forms. The first one contains copper and is encoded by *nirK*, while the second contains cytochromes (hemes) *c* and d_1 and is encoded by *nirS* (Zumft, 1997; Braker et al., 2000**;** Casciotti and Ward, 2001**;** Gruntzig et al., 2001; Avrahami et al., 2002). These two forms are functionally similar (Casciotti and Ward, 2001; Avrahami et al., 2002**)** although structurally different (Adman et al., 1995; Casciotti and Ward, 2001; Avrahami et al., 2002). Many different bacteria contain *nirS*,

including *Paracoccus denitrificans* ATCC 19367, *Pseudomonas stutzeri* ATCC 14405, and *Roseobacter denitrificans* ATCC 33942^{T}. Some bacteria that possess *nirK* include *Hyphomicrobium zavarzinii* IFAM ZV-622 ^{T} ATCC 27496, *Alcaligenes* sp. strain DSM 30128, and *Alcaligenes xylosoxidans* subsp. *denitrificans* DSM 30026 (Braker et al., 1998). *nirS* appears to be more abundant in nature while *nirK* is found to be more widespread in different taxonomic groups (Coyne et al., 1989; Gruntzig et al., 2001). *nirK* was discovered in several ammonia oxidizing bacteria (AOB) such as *Nitrosomonas europaea* (Casciotti and Ward**,** 2001). In fact, physiological evidence suggests that *nirK* might be employed by AOB as a protection against NO_2^-, the toxic product of ammonia oxidation (Poth and Focht, 1985; Stein and Arp, 1998; Beaumont et al., 2004). The reduction of nitric oxide is catalyzed by nitric oxide reductase small and large subunits encoded by *norC* and *norB*, respectively (Braker and Tiedje, 2003). *norB* includes 2 classes: the first class encodes cytochrome *bc*-type complex (cNorB) while the second class encodes the quinol-oxidizing single-subunit class (qNorB) (Braker and Tiedje, 2003). The last step in the denitrification pathway is the reduction of nitrous oxide and is catalyzed by nitrous oxide reductase genes that are arranged in three transcriptional units consisting of the *nosZ* gene that encodes the catalytic subunit plus the *nosR* gene and the *nosDFYL* genes (Philippot, 2002).

Environmental factors influencing nitrate reduction/denitrification

Denitrification is an environmentally regulated process with respect to oxygen supply, the presence and nature of a nitrogen oxide, and possibly additional external factors such as metal ions (Philippot, 2002). Also, previous studies identified soil moisture and surface hydrology as important factors for supporting high denitrification activity (Groffman and Tiedje, 1989; Hunter et al., 2008). Moreover, the diversity of NR bacteria in a waste water treatment system was affected by salinity where the highest diversity of NR bacteria was observed at low salinity (Yoshie et al., 2004; Santoro et al., 2006). In this study, due to variation in the amount of crude oil, brine, soil moisture, and nitrate between contaminated and uncontaminated sites, I will focus on the effect of these environmental factors on the abundance and diversity of NR and DN bacteria since denitrifying enzymes are expressed at low oxygen or in the absence of oxygen, nitrate is needed for denitrification, and hydrocarbons may serve as an organic carbon source by heterotrophic bacteria possessing the requisite degradative pathways (Zumft, 1997).

Degradation of petroleum hydrocarbons by facultative anaerobic bacteria

Petroleum hydrocarbons (HC) consists mainly of saturated HC (e.g. alkanes), usaturated HC (e.g. alkenes and alkynes), cycloalkanes, mono-aromatic and polycyclic aromatic HC (Zhang et al., 2011). Low-molecular-weight molecules, such as straight, branched, cyclic alkanes and aromatic HC, have been shown to be readily degraded by many microorganisms, while long-chain alkanes

and polycyclic aromatic hydrocarbons are generally considered to be not as easily biodegraded due to their higher hydrophobicity (Zhang et al., 2011). Many different bacterial genera including *Pseudomonas***,** *Acinetobacter*, and *Rhodococcus* were shown to degrade alkanes (Atlas, 1981; Rojo, 2009). On the other hand, *Stenotrophomonas* and *Pseudomonas* species are among those shown to degrade toluene, benzene, ethylbenzene, and xylene (Lee et al., 2002; Shim and Yang, 1999). Also, a wide diversity of γ-Proteobacteria such as *Pseudomonas* and *Vibrio* species were shown to degrade naphthalene (Rockne et al., 2000). Therefore, the presence of crude oil might either positively or negatively affect the abundance of NR and DN bacteria and their ability to perform nitrogen transformations.

Different environmental factors (e.g., oxygen, temperature, pH, and nutrient levels) affect the degradation of petroleum hydrocarbons in soil. In addition, several physicochemical factors such as the number and types of microbial species present; the nature, amount, and bioavailability of contaminants (MacNaughton et al., 1999; Röling et al., 2002; and Smith et al., 2008) also play an important role in degradation of petroleum hydrocarbons.

The presence or absence of oxygen determines the pathway of biodegradation of hydrocarbons. In fact, Fuchs et al. (2011) summarizes 4 major pathways of aromatic hydrocarbon biodegradation. The first pathway comprises an attack by oxygenases that hydroxylate and finally cleave the ring with the help of

activated molecular oxygen. The second pathway is an anaerobic process that converts benzoyl-CoA to cyclic 1,5-dienoyl-CoA. The third pathway also occurs under anaerobic conditions in which fumarate can be added to toluene with the subsequent β-oxidation of the intermediate benzylsuccinate to benzoyl-CoA. And fourth is the anaerobic hydroxylation of ethylbenzene to 1-phenylethanol, and the ATP-dependent carboxylation of acetophenone that is involved in the conversion to benzoyl-CoA.

Humic substances: interaction with hydrocarbon degradation

Humic substances also affect hydrocarbon degradation. Humic substances comprise about 60 to 80% of the soil organic matter and consist of three chemical groups based on solubility in water adjusted to different acid - alkaline (pH levels) conditions: humic acids, fulvic acids, and humin (Brady and Weil, 2002). Dissolved organic matter (DOM) plays a critical role in determining the chemical and biological fate of organic contaminants in soils and sediments (Johnson and Amy, 1995). In fact, sequestration and irreversible binding of DOM has been thought to shield organic contaminants from degradation (Ragle et al., 1997; Engebretson and Wandruszka, 1999).

Some agricultural soil microorganisms were found to be nitrate-dependent humic acid (HA)-oxidizers. These microorganisms are phylogenetically diverse and included members of α-Proteobacteria, β-Proteobacteria, and γ-Proteobacteria **(**Van

Trump et al., 2011) which are some of the groups that I investigated in my study. Also, it was shown that microbial reduction of humic substances (HS) may play an essential role during the anaerobic oxidation of organic pollutants in anaerobic environments (Cervantes et al., 2008). In fact, some nitrate-reducing organisms also reduced hydroquinones within humic acids (HA) (Coates et al., 2002; Lovley et al., 1999). This reduction of HA by nitrate reducing bacteria may make hydrocarbons more accessible to this group of bacteria. I do not know whether my nitrate reducing strains reduce HA or not. However, Chapter 4 (previously published as AbuBakr et al., 2008) investigated if naphthalene 1,2-dioxygenase (NDO) is a humic-modifying enzyme that alters the bioavailability of organic contaminants associated with dissolved organic matter under aerobic conditions. Comparison of the accessibility of hydrocarbons associated with humic substances under nitrate-reducing versus oxygen-rich conditions would be an interesting future field of research.

Nitrogen as a limiting nutrient in the tallgrass prairie

Nitrogen limitation is an important regulator of plant growth (LeBauer and Treseder, 2008; Shaver et al., 2001; Aber et al., 1997). The effect of nitrogen on photosynthesis is simulated by most ecosystem models by using a relationship between leaf nitrogen content and photosynthetic capacity (Aber et al., 1997; Thornton et al., 2002). However, this relationship, in reality, may vary with different light, nitrogen availability, temperature, and CO_2 conditions (Reich et al.,

1995; Friend, 1991; Ripullone et al., 2003). Photosynthesis and respiration are main biological processes in plants in which nitrogen is a major constituent of proteins for these processes (Marschner, 1995). However, nitrogen is a limiting factor for plant growth (LeBauer and Treseder, 2008; Shaver et al., 2001; Aber et al., 1997). Therefore, denitrification may decrease soil nitrogen levels available for plants, thus affecting the growth and survival under specific environmental conditions (Friend, 1991; Verkroost and Wassen, 2005).

Atmospheric nitrogen is converted into ammonium that is available to organisms by biological nitrogen fixation. This process is an important natural input of available nitrogen in many terrestrial habitats (Zehr et al., 2003). Sublette et al. (2007b) showed that nitrogen is a critical nutrient in the tallgrass prairie soil where the addition of N-containing fertilizer was used to bioremediate a terrestrial crude oil spill, re-establish pre-spill N cycling and microbial diversity in order to accelerate the subsequent restoration. This microbial diversity is assumed to help in re-establishing the wide range of biogeochemical functions that are responsible for the recycling of soil nutrients and, as a result, supporting life in that area (Sublette et al., 2007b). A different study on the fate of the applied NH_4 through five growing seasons illustrated that the flow of nitrogen within native tallgrass prairie soils was controlled by the incorporation of nitrogen into soil organic matter (SOM). However, plants appeared to maintain productivity by firmly conserving immobilized nitrogen (Dell et al., 2005).

Burning of grasslands has long been recognized to maintain their plant diversity. Burning also affects the nitrogen pools. Although buring of grasslands causes loss of N from tallgrass prairie systems, burning can increase the total recovery of applied nitrogen due to greater N immobilization in the SOM where N is not available for plant uptake and needs to be mineralized first. The mechanism for increasing nitrogen immobilization was likely due to the greater microbial nitrogen demand in response to larger organic matter inputs with wider C:N ratios that is typical of burned prairie. This N immobilization increase microbial activity and aid rapid decomposition and turnover of organic matter, resulting in more available nutrients for plants over time (Dell et al., 2005). A different study showed that unburned prairie was wetter and had higher concentrations of NO_3^- in soil solution than annually burned sites. Also, although the rate of denitrification varied seasonally, denitrification was significantly higher ($P < 0.05$) in unburned sites than that in annually burned, annually burned and grazed, and cultivated sites. In fact, the denitrification enzyme activity (e.g. N flux rate) was highest in the unburned sites (e.g. 327 ± 69 µg. kg^{-1}. h^{-1}) and lowest in the cultivated sites (e.g. 30 ± 5 µg. kg^{-1}. h^{-1}) (Groffman et al., 1993).These results indicate that nitrate was released to the atmosphere and did not accumulate in soils in the annually burned sites. Also, these results indicate that the different bioremediation protocols used in my study, e.g. addition of water and/or fertilizer containing nitrate, may alter denitrification rates. As well, the high carbon levels present in the crude oil-contaminated sites are

expected to increase the denitrification rate if NR and DN bacteria in those sites can degrade hydrocarbons.

Site description overview and significance

Tallgrass prairies are characterized by tall grasses (e.g. typically 3 or more feet tall) including the native Big and Little Bluestem grasses, Gama Grass, Switch Grass, Indian Grass, and Prairie Cord Grass. In addition, shorter grasses, sedges, and several flowering plants are also common (Ladd and Oberle, 1995). In the United States, prairie grasslands were dominant ecosystems in the states in the central part of the country, including Oklahoma. Fires (e.g. burning), wind, drought, and bison grazing all together formed prairie lands over the years. However, just a few prairie lands remain today, as most grasslands were plowed for agriculture. Tallgrass prairie is especially rare, and great effort is made to restore the characteristic grasses (http://www.nature.org/ourinitiatives/regions/northamerica/unitedstates/oklahoma/placesweprotect/tallgrass-prairie-preserve.xml). Many studies of prairie restoration focus on the effects of burning and grazing and how those treatments affect biogeochemical cycling in prairie soils. On the other hand, not much is known about the effect of oil and/or brine contamination on prairie restoration.

To study the effect of oil/brine contaminantion, the Tallgrass Prairie Preserve (TPP), Osage County, OK was chosen as the study site. Tallgrass Prairie

Preserve, around 40,000 acres, is a Nature Conservancy preserve in Osage county, northeastern Oklahoma (Figure 1.1) (http://www.nature.org/idc/groups/webcontent/@web/@oklahoma/documents/document/prd_017760.pdf). A 29,000-acre cattle ranch was formerly located in this preserve (Jones and Cushman, 2004). The soils of the TPP originated from clays and shales of the Permian Red Beds. Those soils range from light sandy loams to heavier silt loams and clays (www.biosurvey.ou.edu). Based on the Soil Survey of Osage County (OK), one soil series found in the TPP is a complex mixture of Coweta series and Bates series where both series are formed from weathered sandstone. Other series are a boundary between Coweta-Bates complex and Steedman-Coweta complex, where Steedman soils are formed from weathered shale. All study sites, except J6 sites (Chapter 2, page 39), had an average soil texture of 30-38% sand, 38-45% silt, and 23-25% clay (Sublette et al., 2005). The J6 sites had soil texture of 30% sand, 51% silt, and 19% clay (Sublette et al., 2007a).

Osage County in northern Oklahoma has historically been the locale of oil production since the early 1900s, and spills of crude oil and brine have occurred from the beginning (Sublette et al., 2007b). The study sites include 5 contaminated sites and 4 adjacent uncontaminated sites chosen because they are close by, have similar soil type, and similar slope. Two of the four contaminated sites were contaminated by brine (salt water) spills and three were contaminated by crude oil.

The five contaminated sites were G5 and G7 (brine), J6-F, J6-NF, and LF (oil). The four adjacent uncontaminated sites were G5P, J6P, G7P, and LFP. LF site was contaminated in 1996, the G5 and J6 sites were contaminated in 1999, and G7 was contaminated in 2000. All contaminated sites decreased in crude oil and brine content during remediation, but were not free of crude oil and brine at the end of the treatment period. In 2005, another remediation treatment was applied to LF and G7 sites. A detailed description of each site and remediation treatment can be found in Chapter 2. Soils were sampled from the 9 sites in the fall 2005 and fall 2006 (e.g. 5- 10 years following contamination) which allowed us to study the long-term impacts of brine/crude oil contamination on nitrogen cycling bacteria. The purpose of the bioremediation/restoration was to improve grassland quality, in terms of percent plant cover and restore dominance by native tallgrass prairie plants (Sublette et al., 2007b).

Objectives

The overall objective of this study was to determine if there are long-term impacts of brine and crude oil contamination on nitrogen cycling in tallgrass prairie soils, specifically on NR and DN bacteria. The main objectives were: (1) to compare the abundance of culturable NR and DN bacteria in brine/oil-contaminated and uncontaminated soils through MPN estimates, (2) to determine the variation in species composition of culturable NR and DN bacteria isolated from contaminated and uncontaminated soils (3) to test if the detection of functional gene sequences

(e.g. nitrate reductase and DN genes) is a good indicator of NR and DN phenotype (4) to determine if naphthalene 1,2-dioxygenase (NDO) is a humic-modifying enzyme. NDO is the enzyme that catalyzes the first enzymatic step for the bacterial catabolism of a variety of mono and polycyclic aromatic compounds, and dissolved organic matter has been suggested to shield organic contaminants from degradation.

Summary of chapters' research foci

Chapter 1 is an introductory chapter that covers the significance, background, and the objectives of the study. Chapter 2 focuses on site description and sampling, the abundance of NR and DN in 5 contaminated vs. 4 tallgrass prairie uncontaminated soils, statistical analyses of the association of the presence of petroleum hydrocarbons, NaCl, nitrate, and soil moisture with the abundance of NR and DN bacteria. I hypothesized that NR and DN bacteria will be more abundant in oil contaminated and moist soils, due to more favorable environmental conditions (e.g. higher carbon, lower oxygen) in those sites. Chapter 3 determined the species composition of bacteria isolated from a nitrate broth enrichment containing soil from the study sites, and used PCR amplification to detect nitrate reductase genes (e.g. *napA* and *narG*) and denitrifying genes (e.g. *nirS*, *nirK*, *cnorB*, *qnorB*, and *nosZ*) from the isolates to see if detecting NR and DN genes is a good indicator of the NR and DN bacteria phenotype. I hypothesized that particular groups of bacteria (e.g. γ-Proteobacteria) will be relatively more abundant in crude

oil-contaminated sites and others (e.g. *Bacillus*, *Pseudomonas*) will be relatively more abundant in brine-contaminated sites. Chapter 4 describes testing naphthalene 1,2-dioxygenase (NDO) activity and the potential of dioxygenases released from lysed cells to be humic-modifying enzymes. I hypothesized that ‘‘cytoplasmic’’ enzymes, released by cell lysis, could participate in the modification of humic acids (HA) and fulvic acids (FA) and thus alter the bioavailability of organic contaminants in environments containing dissolved organic matter (DOM). Chapter 4 has been published as “Enzymatic transformation of humic substances by NDO” by Samer AbuBakr, Simone L. Macmil, Mark A. Nanny, and Kathleen E. Duncan, 2006 in *Soil Biology & Biochemistry*, 40: 2055–2062.

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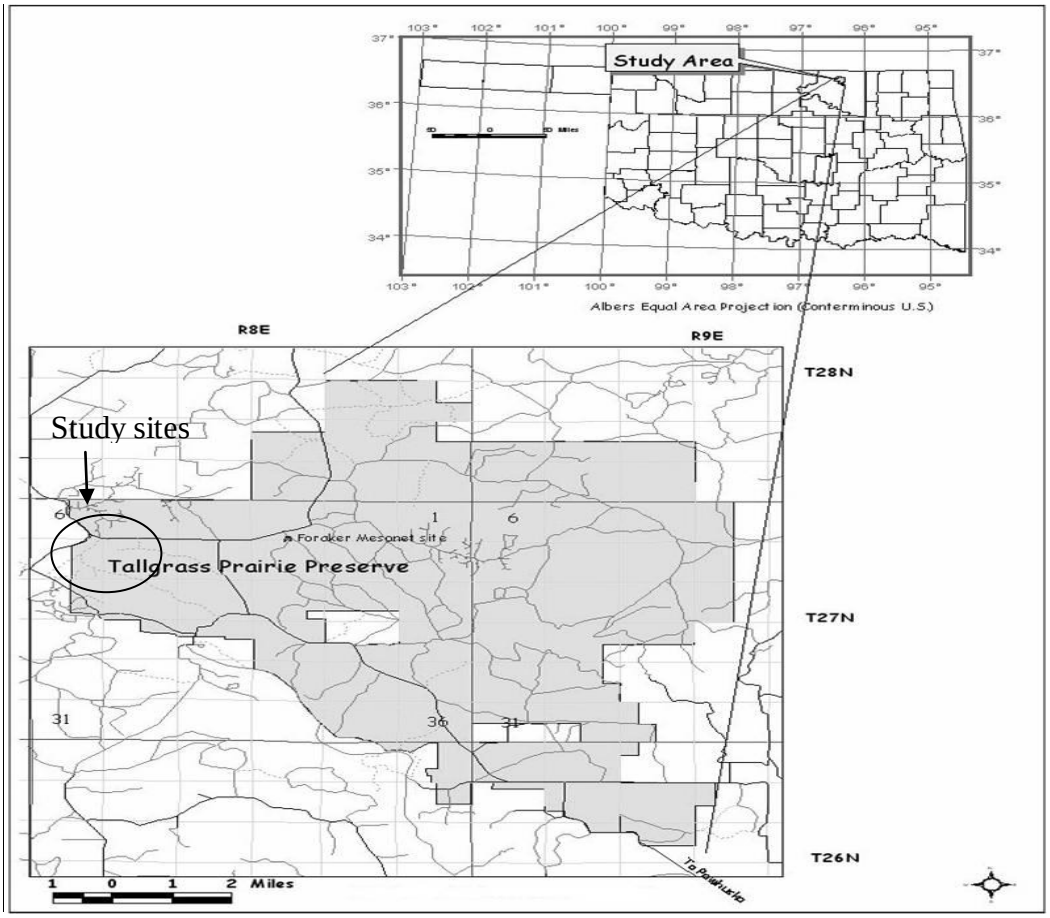
Figure 1.1. Tallgrass Prairie Preserve, Osage County, OK.

http://www.nature.org/idc/groups/webcontent/@web/@oklahoma/documents/document/prd_017760.pdf



Chapter 2: The effect of environmental factors on the abundance of culturable nitrate reducing/denitrifying bacteria from contaminated and uncontaminated tallgrass prairie soil

Abstract

Various environmental factors have been proposed, such as soil moisture levels, carbon, and nitrate sources to affect the abundance of nitrate reducing (NR) and denitrifying (DN) bacteria. In this Chapter, the strength of the association of the abundance of NR and DN bacteria with various environmental factors is estimated using multivariate statistics. Soil samples were collected from tallgrass prairie soils that had been contaminated with crude oil or brine (e.g. salt water) up to 10 years previously and from parallel uncontaminated sites. The abundance of culturable NR and DN bacteria in the soil samples was estimated by 5-tube MPN method using nitrate broth, while total petroleum hydrocarbons (TPH), sodium chloride, nitrate, and moisture were measured in the contaminated and the parallel uncontaminated sites. Viable heterotrophic bacteria and NR and DN bacteria from all sites were obtained from samples with a broad range of soil moisture (around 10-30% water/g soil) regardless of the source (e.g. site) of the isolates. My results showed that the abundance of NR and DN bacteria from the contaminated sites was not less than that from the uncontaminated sites and reflected soil moisture, with greater bacterial numbers from wetter soils. Also, current remediation treatments (e.g. nitrate, hay, watering) of contaminated sites sometimes, but not consistently,

were associated with greater abundance of NR and DN bacteria. Therefore, no long-term effect of contamination on the abundance of NR and DN bacteria was shown.

Introduction

Environmental factors affect the abundance and distribution of nitrate reducing and denitrifying bacteria in soil ecosystems

High levels of carbon, nitrate, and soil moisture (e.g. low oxygen concentration) are associated with higher denitrification rates when these environmental conditions occurred simultaneously (Groffman et al., 1993). In fact, it is not known whether these conditions favorable for denitrification persisted in formerly oil-contaminated sites and produced a greater abundance of NR and DN bacteria. However, it has been shown that denitrifying bacteria are not uniformly distributed in soils where the spatial heterogeneity of denitrification is influenced by spatial heterogeneity of carbon and N substrates in soils, and that denitrifying populations from select sites can be much greater than that indicated by MPN procedures of bulk soil (Martin et al., 1988; Murray et al., 1995, Harms et al., 2009)

A. Soil Moisture

The soil microbial community structure is thought to be one of the most sensitive indicators of biological properties of soil and reflects changes in the soil ecosystem (Kennedy and Smith, 1995; Yao et al., 2000). Different environmental factors, such as soil moisture, affect microbial activities and composition in soil by affecting the physiological status of bacteria (Harris, 1981). In fact, the osmotic

status of bacterial cells is affected by water availability that can regulate diffusion of gases, substrate accessibility, soil temperature, and pH. Moreover, moisture shortage may affect bacterial communities by stressing plants. Drought causes changes in nutrient distribution below ground and the release of organic compounds from plant roots into soils (Lynch and Whipps, 1990). Periods of moisture limitation may be a strong selective pressure on the structure and functioning of soil bacterial communities. Also, it has been shown that soil moisture is involved in controlling fluxes of important greenhouse gases such as nitrous oxide, even if these fluxes are not totally the result of microbial activity (Bollmann and Conrad, 1998). Sublette et al (2007a) found that soil moisture had a strong influence on viable biomass of soil microbes when phospholipid fatty acid (PLFA) concentrations from tallgrass prairie soil samples were measured. The PLFA concentration reflects the viable microbial biomass (White et al., 1997).

Soil moisture also impacts NR and DN bacteria by controlling oxygen diffusion to sites of microbial activity. When the moisture levels increases up to 60% of moisture holding capacity, the number of most microorganisms increase. However, when soil moisture increases to more than 60% of moisture holding capacity, oxygen diffusion and availability to microbes will decrease. As a result, facultative anaerobic microorganisms will be stimulated to use alternative electron acceptors such as nitrate. These conditions are expected to increase the number of

NR and DN bacteria with respect to that of strict aerobes, if these low oxygen conditions are maintained and nitrate is available (Paul and Clark, 1989).

B. Impact of crude oil and brine contamination

However, soil moisture is not the only environmental factor that could affect the abundance and activity of NR bacteria. The abundance of heterotrophic bacterial groups in any environment is related directly to the ability to utilize the available organic carbon sources as an energy source. In hydrocarbon-contaminated soils, hydrocarbons can be a source of carbon for those bacteria that possess pathways for hydrocarbon degradation. Nitrate reducing bacteria are typically facultative anaerobes, and thus they may possess the aerobic pathways of hydrocarbon degradation such as that found in *Pseudomonas putida* pG7, which contains the NAH7 naphthalene-degradation plasmid (Dunn and Gunsalus, 1973). In addition, however, they may have an advantage over aerobes in being able also to degrade hydrocarbons under NR conditions if they possess a pathway active under NR conditions. *Pseudomonas fluorescens* B-3468 may be such an organism, as it converted 2,4,6-trinitrotoluene under conditions of oxygen or nitrate respiration (Naumova et al., 1988). Various studies investigated the anaerobic degradation of polyaromatic hydrocarbons (PAHs) under NR and DN conditions. One laboratory study (al-Bashir et al., 1990) showed biodegradation of low molecular weight PAHs when denitrifying organisms were grown. Also, nitrate-dependent anaerobic degradation and mineralization of naphthalene by pure

cultures of *Pseudomonas stutzeri* and *Vibrio pelagius* has been demonstrated (Rockne et al., 2000). Therefore, I hypothesized that the abundance of hydrocarbon-degrading NR and DN bacteria will increase in the crude oil-contaminated sites. However, the range of organisms that degrade most low-molecular-weight components of mineral oil decreases with increasing salinity (Kleinsteuber et al., 2006). Therefore, soils contaminated with both brine and oil may be more difficult to be bioremediated, although it was shown that levels of TPH were reduced in the presence of brine contamination (Sublette et al., 2005).

C. Influence of nitrogen levels on NR and DN bacteria

One more environmental factor that affects NR and DN bacteria in soils is the availability of nitrate. It has been shown that the addition of plant residues, precious source of nitrogenous compounds, can lead to important long-term increase in potential nitrate reduction activity and size of a microbial community involved in nitrogen cycling. However, the effect of the type of plant residue itself was limited (Chèneby et al., 2010). Therefore, nitrogen-containing fertilizers are often used in bioremediation (Pope and Matthews, 1993). In fact the fertilizers used in this study were mixtures of ammonium nitrate (NH_4NO_3), diphosphorus pentoxide (P_2O_5), and potassium oxide (K_2O) (Sublette et al., 2007a).

Considering the effect of these environmental factors one by one, and in combination, since the amount of water available in soil (soil moisture) plays such

an important role in microbial activity and particularly for NR and DN bacteria by also affecting available oxygen levels, I hypothesize that bacteria numbers and the relative abundance of NR and DN bacteria increase with higher soil moisture. Also, since crude oil contamination alters bacterial communities in soils by providing alternative carbon sources and killing sensitive members of the microbial community (Sikkema et al., 1995) and many NR and DN are hydrocarbon degraders (Al-Bashir et al., 1990; Sharak Genthner et al., 1997; Rockne and Strand, 1998) I hypothesize that NR and DN bacteria will be more abundant in the crude oil-contaminated soils compared to that in the prairie uncontaminated soils, if the moisture levels are comparable. However, NR and DN bacteria will be less abundant in the sites that are contaminated with both oil and brine because salinity decreases the range of microorganisms that degrade most low-molecular-weight components of mineral oil (Kleinsteuber et al., 2006). Remediation treatments of nitrate fertilizer are expected to stimulate the activity and eventually increase the numbers of NR and DN bacteria. Therefore, I hypothesized the best conditions for NR and DN bacteria are soils contaminated with hydrocarbons, and remediated by application of nitrate containing fertilizer and watered. However, it is unknown whether the potentially stimulating effect on NR and DN bacteria of nitrate remediation of oil contaminated soils persists over a period of several years.

D. Sites Description

The sampling sites used in this work are located in the Tallgrass Prairie Preserve (TPP) in Osage County, Oklahoma. Nitrate reducing and denitrifying bacteria were isolated from a total of 5 contaminated sites designated as G5, G7, LF, J6-F, and J6-NF and 4 adjacent uncontaminated sites (G5P, J6P, G7P, and LFP). Leaks from oil pipelines had contaminated the sites with crude oil (J6-F, J6-NF, LF) or with a mixture of brine and oil (G5, G7). The sites had been partially remediated as described previously (Sublette et al., 2005; Sublette et al., 2007a; Duncan et al., 1998; Duncan et al., 1999). The history of each site is described in more detail in Materials and Methods (Section C, page 39). Briefly, the contaminated sites were contaminated during 1996-2000 but still contained low levels of TPH and Na^+ and Cl^- (Tables 2.1.a and 2.1.b). Sites G7 and LF underwent a further round of treatment during the course of this research as described in the Restoration phase section D of the Materials and Methods (page 40). The objective of this study was to determine the association of different environmental factors (e.g. TPH, NaCl, nitrate and soil moisture) with the abundance of NR and DN bacteria in long-term contaminated vs. uncontaminated prairie soils in order to determine which factors have an impact under field conditions.

Materials and Methods

A. Medium

Nitrate broth medium for detection of NR and DN bacteria consisted of 2.5 g of $NaNO_3$ added to 500 mL of nutrient broth (Difco Inc., Detroit, MI) before autoclaving. The antifungal agent, cycloheximide (Sigma Chemical Company, St. Louis, MO) (100 µg/mL final plate concentration) was added after autoclaving. Nitrate Reduction Broths containing beef extract, pancreatic digest of casein, or peptone, are commonly used to distinguish facultative heterotrophic bacteria based on whether they are able to reduce nitrate to nitrite or produce N_2 gas (Atlas, 1993).

B. Control strains

Control strains were used to confirm that the test conditions (e.g. detection of NR and DN bacteria in the presence of soil) correctly identified nitrate reducers and denitrifyers. These controls were: *Pseudomonas aeruginosa* ATCC 27853 (denitifyer), *P. putida* F1 ATCC 17485 (negative- neither a denitrifyer nor a nitrate reducer). *P. aeruginosa* S1-1 (a denitrifying bacterium isolated from tallgrass prairie), and *P. putida* pG7 (neither a denitrifyer nor a nitrate reducer). *P. aeruginosa* strains were grown at 37°C and *P. putida* strains were grown at room temperature (23-25°C).

C. Site History

The following is a brief summary of the timeline of contamination and remediation of each site. The reader may consult the referred sources for more details.

The G5 site was contaminated in the fall of 1999 by 3 separate breaks in the same line resulting in 3 lobes of contamination (e.g. G5N, G5M, and G5S). The amount spilled was unknown, but the water to oil ratio in this line was 10-15:1, and the brine had a total dissolved solids (TDS) of 105,000 mg/L. The initial TPH values were estimated as 1300-5200 mg/kg, Na^+ as 1100-1580 mg/kg, and Cl^- as 780-2450 mg/kg (Sublette et al., 2005). Remediation, as described in Sublette et al (2005), was performed from June 2000 to August 2003 and consisted of hay and fertilizer containing nitrate, and installation of a drainage pipe downhill from the contaminated area.

The J6 site was contaminated in January 1999 by a pipe break resulted in a spill of approximately 11 m^3 of dewatered crude oil. The initial Total Petroleum Hydrocarbon (TPH) concentration (EPA 418.1) was about 33,500 mg/kg (dry wt. basis) when remediation was initiated (following tilling) (Sublette et al., 2007a). Remediation, as described in Sublette et al (2007a), was performed from May 1999 to October 2001 and consisted of hay, fertilizer containing ammonium nitrate (NH_4NO_3), diphosphorus pentoxide (P_2O_5), and potassium oxide (K_2O), and tilling.

J6 was divided into two sections, fertilizer was applied in four increments throughout the 2 year-period of remediation on J6-F, while J6-NF was tilled but not fertilized.

The G7 site was affected primarily by brine. The original spill was in 2000, remediation treatments of hay and nitrate-containing fertilizer were performed, ending in 2004, and the sodium and chloride levels were reduced, but large expanses still had no plants in 2005. Sodium and chloride levels varied widely, but averaged 650 mg/kg of sodium and chloride (Sublette et al., 2004). On the other hand, LF, containing soil contaminated by a spill of crude oil, was remediated in 1996 and 1997 by mixing the contaminated soil with the uncontaminated soil plus nitrogen-containing fertilizer (Duncan 1998, 1999). During that time, TPH levels decreased to about a third of the original level and plants grew over most of the site, but they were primarily weedy, non-native, undesirable species.

D. Restoration Phase: G7 and LF

During the period of 2005-2006, treatments were performed on the G7 and LF sites in order to further decrease the contamination levels. The areas treated were approximately 45.72 m x 15.24 m (G7) and 27.43 m x 25.91 m (LF). Restoration treatments were started in the spring of 2005 by ripping the soil to a depth of 12 inches and tilling. Each site was divided into 4 blocks, and soil within each block was homogenized by mixing before being distributed into 36 enclosures

per block. The homogenized soil was placed inside a bag of bridal veil material, inside a 12 inch diameter PVC pipe enclosure with slots drilled into it for drainage. Each 12 inch (0.3 m) enclosure was centered in 2 m x 2 m area within the block (QAPP: Quality Assurance Project Plan, 2004). The soil inside each enclosure received one of the 4 following treatments: hay alone, fertilizer alone, hay plus fertilizer, and the fourth treatment being no fertilizer and no hay. The fertilizer was mixed into the top 6 inches of soil, and the hay was placed on the soil surfaces. Each of the treatments was originally subdivided as to whether earthworms were added or not. However, by the time of the first sampling, it was apparent that earthworms had escaped from their enclosures and had invaded non-treated enclosures, therefore earthworms were not considered as a treatment in this analysis. Each block contained 4 replicates of each treatment, and one of each treatment per block was destructively sampled on each sampling. An additional enclosure from LF, G7, or both, was collected on most sampling dates for quality control purposes giving a total of 33-34 samples. Soil for an MPN series was obtained from each destructed sample. After adding the amendments, the entire site was covered with hay. The enclosures were installed and the amendments added in May 2005 and watering began (Sublette et al., 2004).

E. Sample collection

All samples were obtained from the Tallgrass Prairie Preserve in Osage County, Oklahoma. Soil samples were collected from the following sites: G5

(brine/oil contaminated) (Sublette et al., 2005), J6-F (crude oil contaminated, treated with fertilizer) (Sublette et al., 2007a), and J6-NF (crude oil contaminated, not treated with fertilizers) (Sublette et al., 2007a) in March, 2005. In addition, soil samples were obtained from G7 (brine/oil contaminated) in July 2005, October 2005, and June 2006, and from LF (crude oil contaminated) in August 2005, October 2005, and June 2006. Samples from adjacent uncontaminated sites (G5P, J6P, G7P, and LFP) were collected at the same time as for contaminated sites. Briefly, for G5 and J6 sites, a trowel was used to remove 5 scoops of soil from 5 widely spaced points within a 25 m radius from approximately the top four inches of soil, beginning just below any loose litter layer, and placed in Whirl-pac® bags. Then, these soil samples from G5, J6-F, J6-NF and their parallel uncontaminated sites were homogenized in beakers using autoclaved spoons. The enclosures in LF and G7 were pulled out and the soil poured into stainless steel bowls, mixed and subsamples were taken out for a variety of assays. Approximately 300 g of homogenized soil from each of 33 or 34 enclosures was subsampled from LF and G7 sites for MPN assays (see Materials and Methods: section D, page 40 for more detailed information).

F. MPN series

Soils were initially mixed in Whirl-pac® bags using autoclaved spoons in the field and later in the lab were further homogenized in autoclaved beakers by mixing with autoclaved spoons. After homogenization, two grams of soil (wet

weight) from each sample was added to a sterile 50 ml centrifuge tube containing 18 mL of sterile isotonic saline (0.85% NaCl), vortexed for 1 minute and serially diluted (1:10) to 10^{-8} for all samples. Twenty microliters of each dilution was used to inoculate 180 µl of the nitrate broth (see A. Medium) in 5-fold replicate, making a five tube MPN series (Rodina, 1972). The inoculated microtiter plates were incubated at room temperature (23-25°C) in the dark for 14 days (Jones et al., 1991). All wells were examined for evidence of growth (turbidity) after 14 days, and those that were turbid were scored positive for aerobic/facultative heterotrophic bacteria. Subsequently, half of the volume of the wells was transferred to a fresh microtiter plate and tested for the presence of NR and DN bacteria using Griess reagents (Baron and Finegold, 1990; Smibert and Krieg, 1994). Briefly, 2 drops of reagent 1 (Sulfanilic acid) and 2 drops of reagent 2 (N, N-Dimethyl-1-naphthylamine) (bioMérieux Vitek, Inc., Hazelwood, MO) were added to every well. The presence of nitrite is detected by a red color after the addition of reagents 1 and 2, e.g, indicating nitrate reduction. If the medium remained colorless, e.g. no nitrite formed, it is due either to lack of reduction of nitrate, e.g. no nitrate reduction or the reduction of nitrite to other products, such as NO, N_2O, or N_2, hence denitrification. If the medium remained colorless, zinc metal dust (Mallinckrodt Chemical Works, St. Louis, MO) was used to detect if unreduced nitrate was present by reducing nitrate to nitrite and turning the medium pink or red. Therefore, pink or red after zinc dust indicates neither NR nor DN bacteria. If colorless after zinc dust, the well was assumed to contain DN bacteria. However,

since only the loss of nitrate and nitrite was demonstrated, bacteria in the colorless wells could be nitrite reducers rather than performing the entire denitrification pathway. Calculation of # of viable cells from MPN reading used the 5-tube MPN tables (Rodina, 1972), and were corrected for the % soil moisture to give estimated # cells per gram soil (dry weight). The original microtiter plates were the source of the isolates described in Chapter 3.

G. Detection of nitrate/nitrite reduction in the presence of soil

To test whether the presence of soil interferes with the ability to detect the reduction of nitrate/nitrite in my microtiter plate test format, the four *Pseudomonas* control strains of known phenotype previously described (see Materials and Method: Section B, page 38) were tested. Tests with *P. aeruginosa* strains were performed at 37°C, those with *P. putida* at room temperature (23-25°C). A small colony from each *Pseudomonas* strain was resuspended in 1.0 mL of sterile isotonic saline (0.85% NaCl) and vortexed gently. The inoculated saline was added to 1 g of autoclaved soil that came from G5P site. The inoculated saline and soil were added to 8 mL of sterile saline to produce a 1:10 dilution (e.g. 10^{-1}). An additional 1:10 dilution was performed and 20 µl diluted cell suspension added to wells of a microtiter plate containing 180 µl of nitrate broth. The inoculated microtiter plates were incubated at room temperature (23-25°C) in the dark for 14 days (Jones et al., 1991) and scored for growth and for the reduction of nitrate or nitrite as described previously.

H. Measuring soil moisture

Soil moisture for all samples was determined from the original soil mass by gravimetric measurements of two 10 g samples (wet weight) after oven drying (Keeney and Nelson, 1982). The % soil moisture was calculated as % soil moisture = (g water/g wet soil) x 100. Samples were taken from both the contaminated and the prairie sites. G7 and LF were regularly irrigated throughout the study. The other sites received only natural rainfall. Soil moisture for G5, J6-F, J6-NF, G5P, and J6P was measured for March 2005. Soil moisture for G7 and G7P was measured for July 2005, October 2005, and June 2006 at the time of sampling. Soil moisture for LF and LFP was measured for August 2005, October 2005, and June 2006 at the time of sampling.

I. Measuring total petroleum hydrocarbons, brine components, and NO_3

Brine components (Na^+, Cl^-) were extracted from oven-dried soil with deionized water, and concentrations were determined by ion chromatography (Harris, 1998). Continental Laboratories (Salina, KS, USA) performed TPH analysis of soil samples by using Environmental Protection Agency (EPA) method 418.1. Nitrate was measured in Oklahoma State University Laboratory (Stillwater, OK, USA). Metadata was collected from different studies that include TPH, Na^+, Cl^-, and NO_3 in contaminated-treated (G5, G7, LF, J6-F, and J6-NF) and prairie (G5P, G7P, LFP, and J6P) sites (Duncan, 1999; Sublette et al, 2005: Sublette et al, 2007a).

J. Statistical Analyses

Mean, standard deviation, coefficient of variation, and linear regression were calculated using Microsoft Excel version 2007. Also, one-way ANOVA, 2-way ANOVA, and Duncan's multiple range tests were used to analyze the data using Statistical Package for the Social Sciences (SPSS for Windows version 19.0, IBM, Chicago, Ill, USA). In addition, Student's *T* test and Welch's test were used to compare data using GraphPad Prism Ver. 3 (GraphPad Software, San Diego, CA, USA). The data were considered significantly different if the two-tailed *P*-value was <0.05. MPN values were converted to $\log_{10}$ values for statistical analysis in order to normalize the data for parametric statistical tests.

Results

Contaminant levels at the beginning of the study

Metadata was collected from different published studies and summarized in Table 2.1 to show the levels of TPH, Na^+, Cl^- and NO_3^- for sampling dates closest to those representing the beginning of the current study. Samples for the metadata were taken prior to site manipulation for G7 and LF. The metadata I collected show that there was a significant difference ($P < 0.05$) between TPH in G7 (primarily brine-contaminated) and that in LF (crude oil contaminated) when sampled in spring 2005 with G7 being lower (Table 2.1.a). (Welch's approximate t = 5.417, 43 d.f., mean difference = 3380.0, 95% CI = 2121.7 - 4638.3). On the other hand, there was no significant difference ($P > 0.05$) between TPH in J6-NF and that in J6-F during the period of April/June 2003. (Welch's approximate t = 1.471, 1 d.f., mean difference = -998.50, 95% CI = -9623.3 - 7626.3).

Nitrate-containing fertilizer was applied once in May 2005 to selected enclosures in the G7 and LF sites. Nitrate-containing fertilizer was last applied in June 2000 to J6-F samples taken in June 2003 and in April 2001 to G5 samples taken in April/June 2003. Nitrate levels varied in different sites (Table 2.1.b). When I compared nitrate levels in J6-NF, J6-F, and J6P, there was a significant difference ($P < 0.05$) in nitrate concentration among those 3 sites. Nitrate level in J6-F was higher than that in J6-NF or J6P ($P < 0.05$), with no significant difference

between J6-NF and J6P (Tukey-Kramer Multiple Comparisons Test. Mean difference (J6-NF vs J6-F), = -8.560, 95% CI = -17.034 to -0.08612; Mean difference (J6-NF vs J6P) = 3.480, 95% CI = -5.452 - 12.412; and Mean difference (J6-F vs J6P) = 12.040, 95% CI = 3.566 to 20.514). Finally, nitrate levels were significantly higher ($P < 0.05$) in G5 (e.g. G5N, G5M, and G5S) than that in G5P (Welch's approximate t = 10.248, 25 d.f., mean difference = -27.300, 95% CI = -32.786 - -21.814).

MPN assays

Table 2.2 summarizes MPNs for each site and each sample date, % NR and DN bacteria (e.g. "#NR and DN/ #heterotrophs" x 100), and soil moisture percentages. Although the MPN assays correctly indicated *Pseudomonas* control strains as denitrifyers in the absence of soil, when autoclaved soil was added to the dilution series, control DN strains were scored as nitrate reducers (NR), e.g. nitrate was consumed but not nitrite. Therefore, NR bacteria were not reliably distinguished from DN bacteria when scoring MPNs from the soil, and wells showing loss of nitrate and/or nitrite were reported as NR and DN bacteria.

The association of soil moisture levels with NR and DN bacteria: J6 and G5 sites

The J6-NF, J6-F, J6P, G5 (includes the N, M, and S lobes), and G5P sites were analyzed together as they represent contaminated sites that were not being

treated during the period of study and were sampled on the same dates. Figure 2.1 shows the % soil moisture [= (g water/g wet soil) x 100] in the J6 and G5 soils sampled in March 2005 (Figure 2.1, One-way ANOVA: $F(4, 9) = 89.988$, $p = 0.000$). Post Hoc tests (e.g. Duncan's multiple range test) group together the homogenous subsets showing that soil moisture in G5P was significantly lower than % soil moisture in the others, J6-NF next lowest, G5 and J6-F grouped together, and J6P with the highest % soil moisture (Figure 2.1, Appendix A).

The slope of the linear regression of $\log_{10}$ # heterotrophs, $\log_{10}$ # NR and DN bacteria, or %NR and DN bacteria/ heterotrophs on the % soil moisture was only slightly positive which indicates that $\log_{10}$ number of bacteria varies little over a fairly broad range of soil moisture (Figure 2.2). To illustrate, it was estimated there were 1.07×10^7 culturable heterotrophic bacteria/ g soil in G5P (13.6% soil moisture) and 1.78×10^7 culturable heterotrophic bacteria/ g soil in J6P (21% soil moisture). Figure 2.2 plots the %water/g wet soil vs $\log_{10}$ #heterotrophs (Figure 2.2.a), NR and DN bacteria (Figure 2.2.b), or %NR and DN bacteria (e.g. "NR and DN" /total heterotrophs) (Figure 2.2.c). When I plotted these relations without the J6F value, the R^2 increased just slightly (figures not shown, the parameter of the linear equations without J6F value are in Figure 2.2 caption) but the slope remained near 0.

Figure 2.3 plots $\log_{10}$ #heterotrophs (Figure 2.3.a), $\log_{10}$ #NR and DN bacteria (Figure 2.3.b), or %NR and DN bacteria/total heterotrophs (Figure 2.3.c) from different oil, brine, or prairie samples. There was no significant difference ($P > 0.05$) between numbers of heterotrophs, NR and DN bacteria, or the percentage of NR and DN bacteria to heterotrophs with the contaminant (e.g. G5 as brine, J6-NF and J6-F as oil, and G5P and J6P as prairie) (Figures 2.3.a, 2.3.b, and 2.3.c). (Figure 2.3.a, one-way ANOVA: $F(2,4) = 1.084$, $p = 0.420$). (Figure 2.3.b, one-way ANOVA: $F(2,4) = 2.109$, $p = 0.237$), and (Figure 2.3.c, one-way ANOVA: $F(2,4) = 2.571$, $p = 0.191$), i.e. at this point in time, the numbers of these culturable bacteria were similar in contaminated and uncontaminated sites.

Soil moisture association with NR and DN bacteria: G7 and LF sites

Figure 2.4 plots % soil moisture in G7 and LF that was sampled at different times during the experiment. Note that the uncontaminated samples were always drier, as expected, since the contaminated sites were watered throughout the study. My moisture results showed that % soil moisture in G7 that was sampled in July 2005, October 2005, and June 2006 is significantly ($P < 0.05$) greater than that in G7P that was sampled in October 2005, and June 2006 (Figure 2.4.a). Figure 2.4.a shows that G7P moistures in October 2005 and June 2006 were not significantly different from each other and grouped together. Also, G7 moisture in October 2005 and June 2006 were not significantly different from each other and grouped together. And finally, G7 moisture in July 2005 and October 2005 were members

of the same homogenous subsets (Duncan's multiple range test). (Figure 2.4.a, Appendix B.1, one-way ANOVA: F (4,103) = 31.341, $p = 0.000$).

Also, moisture results showed that % soil moisture in LFP was significantly lower than that in LF ($P < 0.05$) that was sampled in the same sampling date or in different sampling dates (Figure 2.4.b). LFP moistures in October 2005 and June 2006 were not significantly different from each other and grouped together (Figure 2.4.b). Also, LF moisture in October 2005 and June 2006 and LFP moisture in June 2006 were not significantly different and grouped together. Finally, LF moistures in August 2005, October 2005, and June 2006 were not significantly different from each other and grouped together as overlapping subsets using Duncan's multiple range test. (Figure 2.4.b, Appendix B.2, one-way ANOVA: F (4,103) = 3.420, $p = 0.011$). Figures 2.4.a and 2.4.b show that in spite of attempts to control water content, G7 generally was wetter than LF.

The range of soil moisture values varied among dates in G7 and LF sites (Figure 2.5), as shown by plotting MPN values and soil moisture for each sample individually. Soil moisture ranged from 23.2% to 29.6% from G7 site sampled in July 2005, 11.7% to 27.0% from LF site sampled in August 2005, 18.4% to 26.0% from G7 site sampled October 2005, 12.3% to 26.9% from LF site sampled in October 2005, 18.4% to 26.3% from G7 sampled in June 2006, and 11.6% to 26.0% from LF sampled in June 2006. On the other hand, soil moisture in G7P site

sampled in June 2006 ranged from 11.7% to 15.1%, which was similar to soil moisture (e.g. 11.4% to 15.5%) in the LFP site sampled on the same date (Figure 2.5). My moisture vs MPN results in G7 and LF sites showed that the slope of the line was very nearly zero indicating similar MPN net values over a fairly broad range of moisture levels (Figure 2.5, Appendix C). However, %NR and DN bacteria could vary from 0% to 100% in different samples with the same moisture level (Figures 2.5.c, 2.5.f, 2.5.i, 2.5.l, 2.5.o, 2.5.r).

MPN for G7 and LF

MPN values for all sample dates of G7 and G7P were analyzed together. As shown in Table 2.2 and Figures 2.6.a, 2.6.b, 2.6.c, there was great variation within any category (Appendices D.1, D.2, D.3). Also, MPN values for all sample dates of LF and LFP were analyzed together (Table 2.2 and Figures 2.6.d, 2.6.e, 2.6.f) and showed great variation within any category (Appendices D.4, D.5, D.6).

Figure 2.6 shows the abundance of heterotrophic bacteria, NR and DN bacteria, or %NR and DN with respect to G7 and G7P or LF and LFP sites that were sampled in different dates. Post Hoc tests (e.g. Duncan's multiple range test) group together the homogenous subsets. Figure 2.6.a shows the abundance of heterotrophic bacteria with respect to G7 and G7P soils sampled in different dates (Figure 2.6.a, Appendix D.1, One-way ANOVA: $F(4,103) = 11.292$, $p = 0.000$). Duncan's multiple range test showed that G7P sampled in June 2006 and G7

sampled in July 2005 were homogenous subsets and had the lowest abundance of heterotrophic bacteria, G7 sampled in July 2005, G7P sampled in October 2005, and G7 sampled in October 2005 were grouped together as subsets and were next to the lowest abundance of heterotrophic bacteria. G7P sampled in October 2005, G7 sampled in October 2005, and G7 sampled in June 2006 were homogenous subsets and had the highest abundance of heterotrophic bacteria.

Likewise, the $\log_{10}$ #NR and DN bacteria varied significantly among the sites, with G7 July 2005, G7P October 2005, and G7P June 2006 lower than that in the remaining samples. One-way ANOVA (NR and DN: G7, G7P): F (4,103) = 9.142, p = 0.000 (Figure 2.6.b, Appendix D.2). The % NR and DN bacteria varied greatly among samples taken from the same site, with the least variation and lowest value in G7P October 2005. One-way ANOVA (%NR and DN: G7, G7P): F (4,103) = 6.588, p = 0.000 (Figure 2.6.c, Appendix D.3). The $\log_{10}$ #heterotrophic bacteria value in LF October 2005 is significantly greater than that in the remaining samples. One-way ANOVA (Heterotrophs: LF, LFP): F (4,102) = 10.923, p = 0.000 (Figure 2.6.d, Appendix D.4). However, the $\log_{10}$ #NR and DN bacteria value in LFP October 2005 is significantly lower than the remaining samples. One-way ANOVA (NR and DN: LF, LFP): F (4,102) = 5.639, p = 0.000 (Figure 2.6.e, Appendix D.5). Finally, the % NR and DN bacteria varied greatly among samples taken from the same site, with the least variation and lowest value in LFP October

2005. One-way ANOVA (%NR and DN: LF, LFP): F (4,102) = 5.915, p = 0.000 (Figure 2.6.f, Appendix D.6).

Coefficient of variation (CV)

Table 2.3 shows the % coefficient of variation (CV) of NR and DN and heterotrophic bacteria in G7, G7P, LF, and LFP sites. Values of the CV for $\log_{10}$ # NR and DN bacteria and for $\log_{10}$ # heterotrophic bacteria ranged from 0-13%. The CV was higher for $\log_{10}$ #NR and DN bacteria than that for the log10 # heterotrophic bacteria for 2 out of 3 sample dates for G7, the same for June 2006, higher for 2 sample dates for LF and lower for October 2005. This indicates that there was proportionally more variation in abundance of NR and DN in LF and G7 than that for heterotrophic bacteria sampled at the same time.

Not as many samples were obtained from the uncontaminated sites, but in 2 out of 4 samples, the CV was higher for $\log_{10}$ #NR and DN than that for $\log_{10}$ # heterotrophic bacteria , and was lower for LFP sampled in October 2005.

Two-way ANOVA: effect of hay and/or fertilizer on bacterial numbers in G7 and LF

Two-way ANOVA calculations were made to determine if there was a significant effect of applying hay and nitrate-containing fertilizer on the abundance or proportion of NR and DN bacteria. My multivariate analyses (e.g. 2-Way

ANOVA) show the effect (p= 0.038) of hay and fertilizer together in enclosures on the relative abundance of NR and DN (%NR and DN) bacteria sampled from G7 in June 2006 (Table 2.4). Enclosures where no hay and no fertilizer were applied had the highest %NR and DN (69.5%), followed by plus hay plus fertilizer (56.7%), then plus hay no fertilizer (49.1%), and the lowest %NR and DN was the plus hay no fertilizer treatment (38.3%) (Table 2.4, Appendix E.3, Appendix F.3). Enclosures with fertilizer addition had higher %NR and DN bacteria sampled from LF site in October 2005 (p= 0.044) (Table 2.4, Appendix E.5, Appendix F.2) where %NR and DN was 13.9% with no fertilizer added and was 34.0% in enclosures when the fertilizer was added (Appendix F.2). The opposite was true for June 2006, the greatest relative abundance of NR and DN bacteria sampled from LF site (Table 2.4) was 66.3% from enclosures with no fertilizer added and was 45.1% in enclosures when fertilizer was added in May 2005 (p= 0.043) (Table 2.4, Appendix E.6, Appendix F.2). No significant effect of hay and/or fertilizer was shown on heterotrophic bacteria.

Also, near significant effects of hay in the G7 and LF sites on $\log_{10}$ NR and DN bacteria (p= 0.068 and p= 0.063, respectively) and the relative abundance of NR and DN bacteria (p= 0.057 and p= 0.098, respectively) sampled in October 2005 were shown (Table 2.4). Enclosures with Hay addition had lower $\log_{10}$ NR and DN bacteria than those with no hay addition sampled from G7 in October 2005 (e.g. 6.7 for no hay and 6.3 for the addition of hay) (Appendix E.2, Appendix F.1).

Also, enclosures with hay addition had lower %NR and DN compared to those with no hay addition (e.g. 31.0% for no hay and 14.7% for the addition of hay) sampled from G7 site in October 2005 (Table 2.4, Appendix E-.2, Appendix F.1). On the contrary, the enclosures with hay addition had higher $\log_{10}$ NR and DN bacteria compared to those that had no hay addition (e.g. 6.5 for no hay addition and 6.9 for the addition of hay) (Table 2.4, Appendix E.5, Appendix F.1) sampled from LF site in October 2005. Finally, the enclosures with hay addition had greater %NR and DN compared to those with no hay addition (e.g. 15.8% for no hay addition and 32.1% for the addition of hay) sampled from LF site in October 2005 (Table 2.4, Appendix E.5, Appendix F.1).

There was no significant effect on the hay and fertilizer treatments on the number or relative abundance of NR and DN bacteria for the remaining sample dates.

Discussion

This study investigated the effect of the long-term residual TPH and brine contamination on the abundance of NR and DN bacteria. My results suggested that the residual TPH and brine levels were not associated with differences in the abundance of NR and DN bacteria beyond that predicted by soil moisture levels in most cases. However, a previous study showed a decrease in the range of organisms that degrade most low-molecular-weight components of mineral oil with increasing salinity (Kleinsteuber et al., 2006). On the other hand, previous research on my J6 study sites at an earlier stage after contamination showed that nitrogen mineralization rates in a contaminated non-fertilized soil were significantly lower than that in a contaminated fertilized soil (Sublette et al., 2007b). These low rates of nitrogen mineralization (e.g. tendency toward net immobilization) reflect reduced availability of inorganic nitrogen to NR and DN bacteria that may lower their relative abundance. Although Sublette et al. (2007b) showed that contamination had an effect on nitrogen mineralization in my study sites at an earlier stage of contamination, MPNs were not performed at that time. On the other hand, in my study, my MPN results show that there were no fewer NR and DN bacteria in the contaminated sites compared to those in the uncontaminated sites 5-10 years post contamination. However, in my study nitrogen mineralization was not measured.

Although MPN values for heterotrophs and NR and DN bacteria were similar over a broad range of moisture levels, the relative abundance of NR and DN bacteria had a wide range (e.g. 0% to 100%) in different samples with the same moisture level which suggests that factors other than soil moisture controlled the % NR and DN bacteria.

However, previous studies (Martin et al., 1988; Murray et al., 1995, Harms et al., 2009) showed that denitrifying bacteria are not uniformly distributed in soils and that samples from bulked soils are not representative of the actual distribution. In fact, denitrifying populations from selected sites can be much greater than those from other sites. Note that, as explained in the Materials and Methods (Section D, page 40), our soil samples from G7 and LF sites were homogenized within each block and placed in the enclosures, and further mixing was performed before sampling from enclosures. This multi-step homogenization might be expected to lower the heterogeneity in the distribution of denitrifying bacteria. However, the heterogeneity in $\log_{10}$ abundance of NR and DN bacteria and heterotrophic bacteria were shown by estimates of the coefficient of variation to be greater for G7 and LF samples (% CV average for $\log_{10}$ of NR and DN = 8.5%, for $\log_{10}$ of heterotrophic bacteria = 6.3%, N = 6) than for the 4 samples taken from the uncontaminated sites (% CV average for $\log_{10}$ of NR and DN = 4.25%, for $\log_{10}$ of heterotrophic bacteria = 3.5%, N = 4). Even after soil homogenization, CV was still greater for NR and DN bacteria than that for heterotrophic bacteria, and greater for contaminated sites

than the CV for the uncontaminated sites. The different treatments (e.g. nitrate, hay) added to the enclosures may have contributed to spatial variation among enclosures. Since no subsamples were taken from individual enclosures, I do not know the degree of variation at a smaller scale within enclosures.

The G7 and LF sites were flooded with water, as a result, their soil moisture increased and the concentration of oxygen in those sites was expected to be low or even anaerobic conditions created. In fact, higher moisture was associated with greater heterotrophic and NR and DN abundance but the effect was slight. The amount of oxygen available in hydrocarbon contaminated soils determines the pathway by which those contaminates are degraded. For example, the biodegradation of PAHs has been studied under both aerobic and anaerobic conditions. The biotransformation of pollutants by microbial communities was observed under anaerobic conditions (Coates et al., 1996a, 1996b).

My results showed that although moisture levels varied among sites and among sampling dates, MPN values for NR and DN bacteria did not vary greatly. However, a different study showed that high soil moisture (29.76% ± 3.75), organic matter, and available nitrogen increased denitrifyer populations (Peralta et al., 2010). In addition, it was shown that phylogenetic composition of the archaeal and bacterial community and nitrogen cycling functional genes (e.g. *nirS* and *nosZ*) were tightly coupled with soil moisture and with seasonal alterations in labile

carbon and nitrogen pools where *nirS* was more abundant at higher moisture levels (Rasche et al., 2010).

Strong evidence for the effect of additional environmental parameters was shown by the higher nitrate level in J6-F that was accompanied with higher net NR and DN bacteria and %NR and DN bacteria in J6-F, assuming that MPN assays accurately enumerate culturable NR and DN. In fact, the amount of nitrate in soil (Tiedje, 1988) and soil moisture (Xia et al, 2004) are important regulators of denitrification. It was shown that a higher nitrate concentration in soil indicates a higher denitrification activity (Yu et al., 2012). In addition, soil moisture was identified as an important factor that increased denitrification activity (Orr et al., 2007). The study of denitrification in a tallgrass prairie in central Kansas (Groffman et al., 1993) emphasized that when multiple factors occur simultaneously, denitrification is promoted. These factors are high levels of water, nitrate, and available carbon source (Groffman et al., 1993). However, in in this study, nitrate was applied only one time, and the easily degraded hydrocarbon may have been already depleted. Therefore, the stimulation efforts may not have been successful since all important factors required for denitrification were not provided simultaneously. In addition, I am measuring the abundance of NR and DN bacteria. It may take a longer period of favorable conditions to increase the number of DN cells than to stimulate expression of denitrification.

A different study on the effect of environmental factors on denitrification of saline wetlands in semi-arid regions showed that a high exchangeable sodium percentage decreased the denitrification rate, apart from organic carbon, nitrate, and denitrifying bacteria (Huibin et al., 2012). Another study investigated the processes that inhibited the decomposition of organic material and affected the dynamics of mineral nitrogen (Dendooven et al., 2010). Dendooven et al. (2010) found that the high electric conductivity and pH of alkaline soil inhibited the decomposition of organic materials such as glucose. Also, the authors found that the reduction of nitrate and the formation of nitrite and ammonia in the glucose-amended alkaline soil was a result of aerobic assimilatory nitrate reduction (Dendooven et al., 2010), not dissimilatory nitrate reduction. However, my MPN results show that brine-contaminated sites did not have a lower abundance of NR and DN bacteria.

Chapter 2 provided an overview of the effect of several environmental factors such as hay and nitrate-containing fertilizer on the abundance of NR and DN bacteria in these soils. In fact, hay and fertilizer were shown to improve soil fertility while increasing the rate of salt leaching from the soil (Harris et al., 2005). However, the excessive use of fertilizer may increase salinity in arable soils (Quantin et al., 2008). I showed that in some cases, hay only, fertilizer only, or the interaction between hay and fertilizer were associated with lower abundance of NR and DN bacteria. This suggests that the carbon source provided (e.g. hay) may have increased the abundance of other groups of bacteria and/or fungi which, as a result

of competition, decreased the relative abundance of NR and DN bacteria. Since the amendments (e.g. hay and/or fertilizer) were associated with a greater abundance of NR and DN bacteria in some cases and lower in other cases, there was no conclusion about the effect of these amendments on the abundance of NR and DN bacteria. However, my results showed that sites that were contaminated 5-10 years previously by crude oil and/or brine did not show a decreased abundance of NR and DN bacteria.

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Table 2.1. Values for TPH, Na^+, Cl^-, and NO_3^- near MPN sampling dates.

Table 2.1.a. Total petroleum hydrocarbons (TPH).

Site	TPH[a]		
	Sampling date	# Samples	(mg/kg)(SD[b])
G7	Spring 2005	12	10941 (1068)
LF	Spring 2005	34	14321 (3163)
J6-NF	April/June 2003[*]	2	1957.5 (958.13)
J6-F	April/June 2003[*]	2	959 (59.4)
J6P	April/June 2003[*]	2	0 (0)
G5	April 2003[**]	3	315 (270)
G5P	April 2003	1	100 (N/A)

[a]Total Petroleum Hydrocarbons.

[b]Standard deviation.

[*]One sample was taken in April 2003, one in June 2003, and averaged.

[**]One sample was taken from G5N, one from G5M, one from G5S, and averaged.

N/A: Not applicable.

Data from Sublette et al 2005, 2007, and unpublished data.

Table 2.1.b. Brine components and NO_3^-.

Site	Sampling date	# Samples	Na^+ (mg/kg) (SD)	Cl^- (mg/kg) (SD)	NO_3^- (mg/kg) (SD)
G7	July 2005	32	288 (79.88)	99.8 (42.98)	1.7 (0.74)
LF	August 2005	32	50 (9.09)	BDL	12.4 (13.45)
J6-NF	June 2003	4	BDL	BDL	4.18 (3.22)
J6-F	June 2003	5	BDL	BDL	12.74 (6.73)
J6P	June 2003	4	BDL	BDL	0.70 (0.12)
G5	April/June 2003	3[a] or 26[b]	896 (402)[a]	1478 (859)[a]	28.12 (13.58)[b]
G5P	April/June 2003	1[a] or 9[b]	29[a] (N/A)	0[a] (N/A)	0.82 (0.17)[b]

a: Na^+, Cl^- samples (N= 3 for G5, N= 1 for G5P), samples were taken in April 2003.

For G5, one sample was taken from G5N, one from G5M, one from G5S, and averaged.

b: NO_3^- samples (N= 26 for G5, N= 9 for G5P), samples were taken in June 2003.

For G5, 9 samples were taken from G5N, 9 from G5M, 8 from G5S, and averaged.

For G5P, 9 samples were averaged.

BDL: below detected limits (e.g. the method detection limits were: 10 mg/kg for TPH, 1 mg/kg for brine components, and 0.01g/g for soil moisture)

N/A: not applicable.

Data from Sublette et al 2005, 2007, and unpublished data.

Table 2.2. Most Probable Numbers (MPNs) of NR and DN bacteria, heterotrophic bacteria, %NR and DN bacteria, and % soil moisture.

Site	Date	N	NR and DN* bacteria (SD)	Heterotrophs Mean (SD)	% NR and DN bacteria (SD)	% Soil Moisture (SD)
G5	Mar-05	3	5.10×10^6 (5.2×10^5)	1.50×10^7 (3.49×10^6)	35.08 (7.90)	18.02 (0.51)**
J6-NF	Mar-05	1	5.60×10^6 (N/A)	1.44×10^7 (N/A)	38.89 (N/A)	16.55 (0.07)**
J6-F	Mar-05	1	3.40×10^7 (N/A)	4.72×10^7 (N/A)	72.03 (N/A)	17.45 (0.35)**
G5P	Mar-05	1	2.80×10^6 (N/A)	1.07×10^7 (N/A)	26.17 (N/A)	13.6 (0.14)**
J6P	Mar-05	1	5.00×10^6 (N/A)	1.78×10^7 (N/A)	28.09 (N/A)	21 (0.14)**
G7	Jul-05	34	5.76×10^6 (6.04×10^6)	1.96×10^7 (2.68×10^7)	51.92 39.92	25.8 (2.78)
G7	Oct-05	34	7.29×10^6 (9.10×10^6)	3.66×10^7 (2.87×10^7)	22.40 (24.67)	23.3 (2.78)
G7	Jun-06	34	2.14×10^7 (2.39×10^7)	4.02×10^7 (2.42×10^7)	55.2 (27.40)	22.8 (2.17)
LF	Aug-05	34	4.92×10^6 (7.07×10^6)	7.06×10^7 (2.51×10^8)	31.52 (33.65)	20.7 (4.51)
LF	Oct-05	34	1.06×10^7 (1.26×10^7)	6.12×10^9 (3.23×10^{10})	24.44 (29.52)	18.9 (4.17)
LF	Jun-06	34	1.46×10^7 (1.70×10^7)	2.77×10^7 (2.71×10^7)	55.50 (30.29)	18.9 (5.31)
G7P	Oct-05	2	2.02×10^6 (7.78×10^4)	1.96×10^7 (0)	10.28 (0.40)	12.85 (0.21)
G7P	Jun-06	4	1.54×10^6 (9.79×10^5)	6.94×10^6 (2.61×10^6)	26.90 (19.89)	13.7 (1.49)
LFP	Oct-05	2	1.66×10^6 (1.06×10^6)	1.40×10^7 (1.44×10^7)	16.96 (9.84)	12.95 (0.07)
LFP	Jun-06	4	2.60×10^6 (2.09×10^6)	3.52×10^6 (1.56×10^6)	69.95 (35.11)	13.50 (1.74)

Date: Soil sampled collected for MPN estimation.

N: Number of samples.

N/A: Not applicable.

*Average per gram dry weight soil estimated from 5-tube MPN (Rodina, 1972).

**Moisture %: The average of 6 samples for G5, and an average of 2 for each J6-NF, J6-F, G5P, and J6P from soil dry weight for G7 and LF, N= the value listed.

% Soil moisture = (g water/g wet soil) x 100.

SD: Standard deviation.

NR and DN: Nitrate reducing/denitrifying bacteria.

Table 2.3. % coefficient of variation (CV) of NR and DN and heterotrophic bacteria in G7, G7P, LF, and LFP sites.

Site	Sample date	Mean log_{10} NR and DN	SD log_{10} NR and DN	% CV log_{10} NR and DN	Mean log_{10} Heterotrophs	SD log_{10} Heterotrophs	% CV log_{10} Heterotrophs
G7	Jul-05	6.43	0.82	13	7.06	0.43	6
LF	Aug-05	6.40	0.57	9	7.23	0.56	8
G7	Oct-05	6.51	0.66	10	7.47	0.29	4
G7P	Oct-05	6.30	0.02	0	7.29	0.00	0
LF	Oct-05	6.72	0.59	9	8.03	0.91	11
LFP	Oct-05	6.17	0.30	5	6.98	0.57	8
G7	Jun-06	7.2	0.31	4	7.5	0.32	4
G7P	Jun-06	6.1	0.40	7	6.8	0.18	3
LF	Jun-06	7.0	0.43	6	7.3	0.37	5
LFP	Jun-06	6.3	0.35	5	6.5	0.19	3

SD: standard deviation.

% CV: % coefficient of variation = (SD/Mean) x 100%.

Table 2.4. Two-way ANOVA: Summary of selected treatment effects.

Site	Sample date	Treatment	Dependent variable	F value	Sig
G7	Oct-05	Hay	log_{10} NR and DN	3.592	0.068
G7	Oct-05	Hay	%NR and DN	3.916	0.057
G7	Jun-06	Hay x Fertilizer interaction	%NR and DN	4.725	0.038
LF	Oct-05	Hay	log_{10} NR and DN	3.721	0.063
LF	Oct-05	Hay	%NR and DN	2.924	0.098
LF	Oct-05	Fertilizer	%NR and DN	4.436	0.044
LF	Jun-06	Fertilizer	%NR and DN	4.453	0.043

See Appendices E and F for complete ANOVA tables.

Figure 2.1. Percentage of soil moisture in J6 and G5 sites.

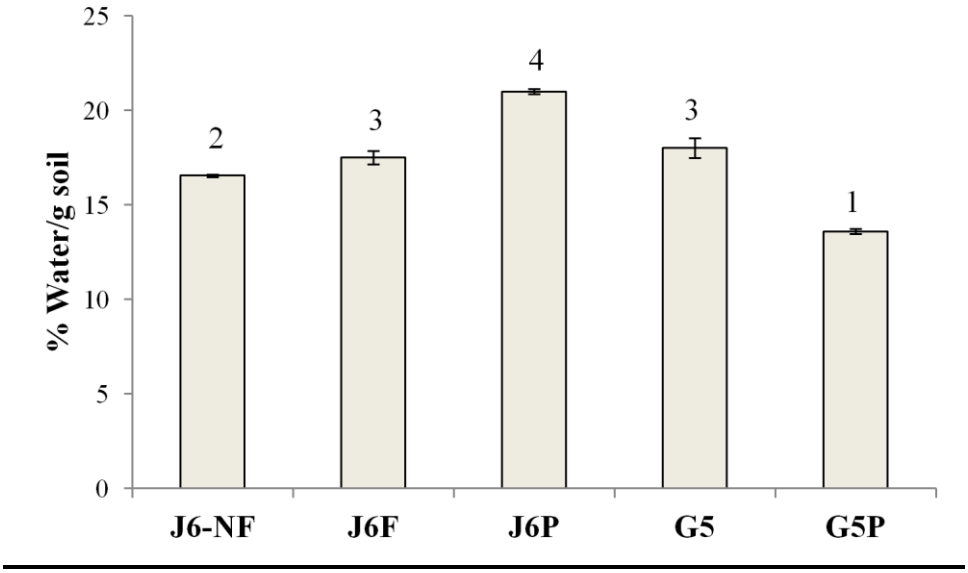


Figure 2.1. J6-NF crude-oil contaminated (not fertilized), J6-F crude-oil contaminated (fertilized), J6P (Prairie), G5 brine-contaminated (G5N, G5M, G5S), and G5P (Prairie). All values are an average of 2 samples, except for G5, which is an average of 6 samples 2 each from G5N, G5M, and G5S. Error bars indicate ±1 standard deviation. Soils were sampled in March 2005.

One-way ANOVA: F (4, 9) = 89.988, p = 0.000.

1, 2, 3, 4: Homogenous subsets (Duncan's multiple range test). See Appendix A for further details.

Figure 2.2.a. Abundance of heterotrophic bacteria (G5, J6) with respect to soil moisture.

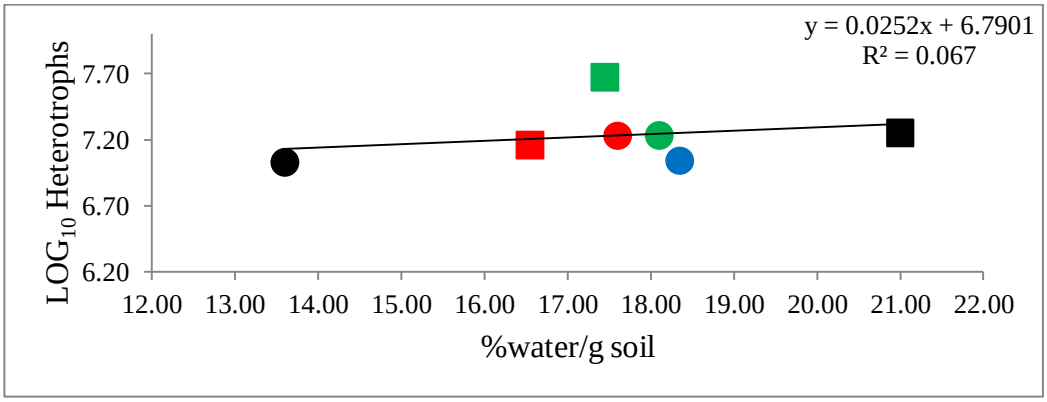


Figure 2.2.b. Abundance of NR and DN bacteria (G5, J6) with respect to soil moisture.

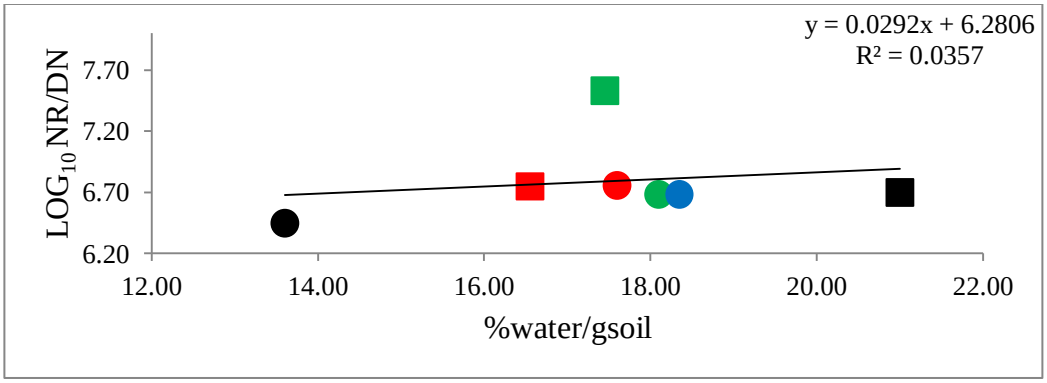


Figure 2.2.c. % NR and DN bacteria (G5, J6) with respect to soil moisture.

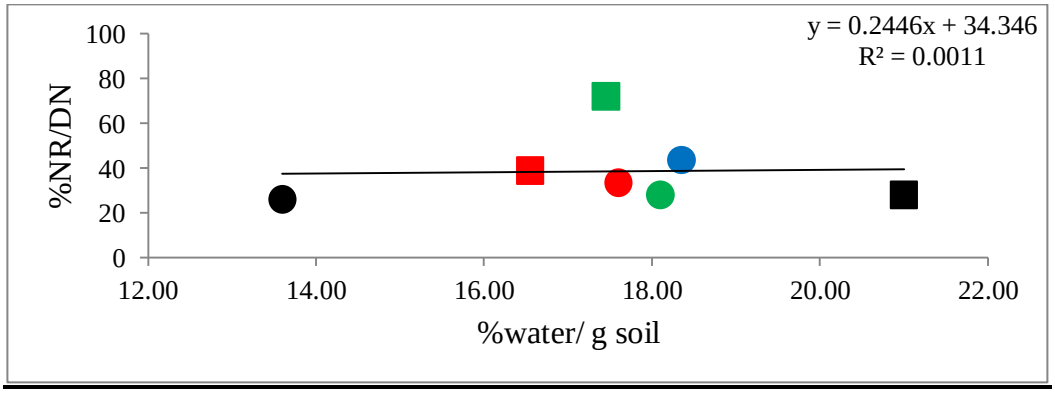


Figure 2.2. The relation between soil moisture and abundance of heterotrophs, NR and DN bacteria, and %NR and DN bacteria (March 2005). J6-NF, red square; J6-F, green square; J6P: black square; G5N: green circle; G5M: blue circle; G5S, red circle: and G5P: black circle. Linear regression for all samples is written on the figure.

Figure 2.2.a. Without J6-F: $y = 0.0264x + 6.6939$, $R^2 = 0.4148$.

Figure 2.2.b. Without J6-F: $y = 0.0313x + 6.1202$, $R^2 = 0.4488$.

Figure 2.2.c. Without J6-F: $y = 0.3391x + 27.117$, $R^2 = 0.0139$.

Soil moisture: % water content.

Figure 2.3.a. Abundance of heterotrophic bacteria with respect to contaminant (J6, G5).

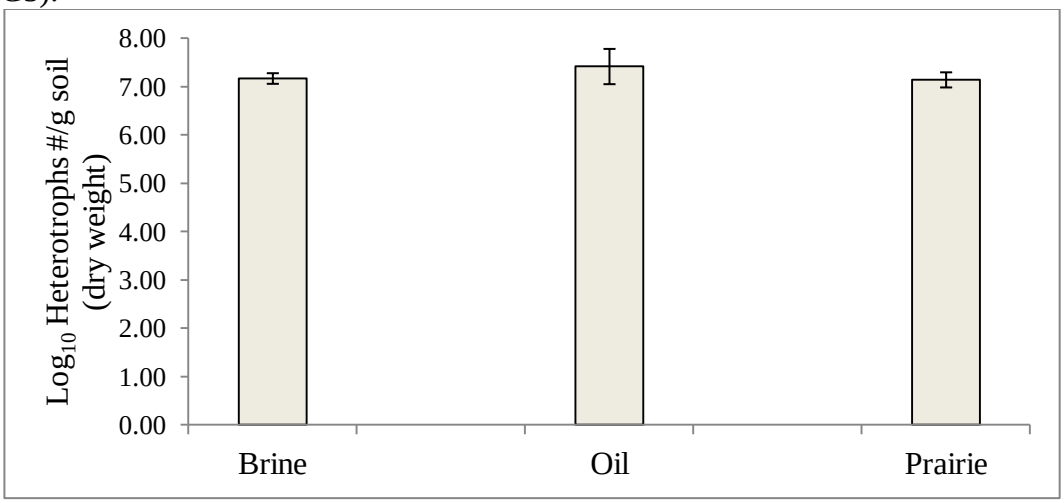


Fig 2.3.b. Abundance of NR and DN bacteria with respect to contaminant (J6, G5).

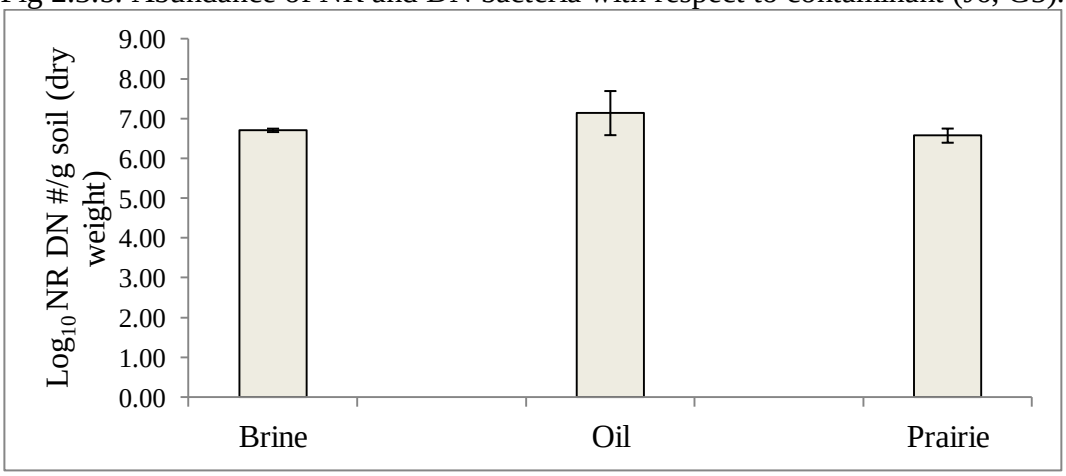


Fig 2.3.c. %NR and DN bacteria with respect to contaminant (J6, G5).

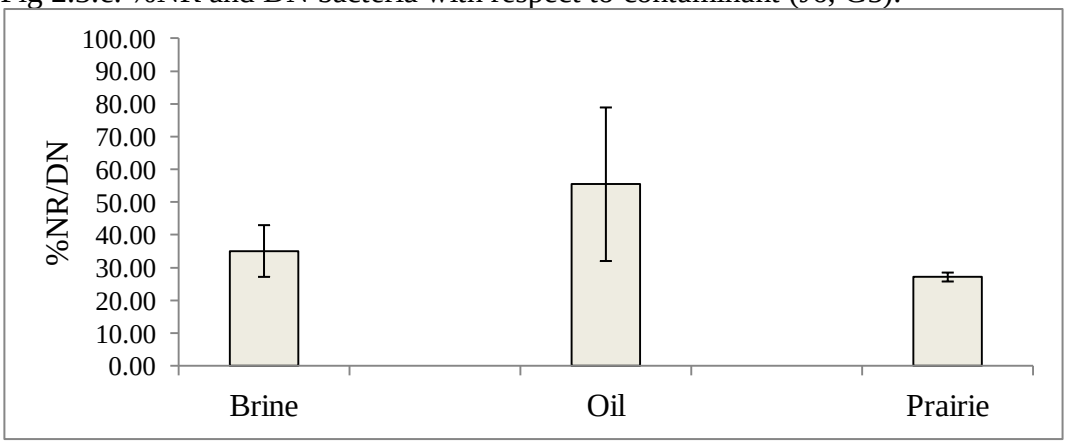


Figure 2.3. Aerobic heterotrophic, NR and DN bacteria, and % NR and DN bacteria to heterotrophs in brine-contaminated (G5N, G5M, and G5S), oil-contaminated (J6-NF and J6-F), and prairie (G5P and J6P) sampled in March 2005. Brine N= 3, Oil N=2, Prairie N= 2. Bars indicate average values. Error bars indicate ±1 standard deviation. Figure 2.3.a. One-way ANOVA: $F(2,4) = 1.084$, $p = 0.420$. Figure 2.3.b. One-way ANOVA: $F(2,4) = 2.109$, $p = 0.237$. Figure 2.3.c. One-way ANOVA: $F(2,4) = 2.571$, $p = 0.191$.

Figure 2.4.a. %Moisture in G7 and G7P.

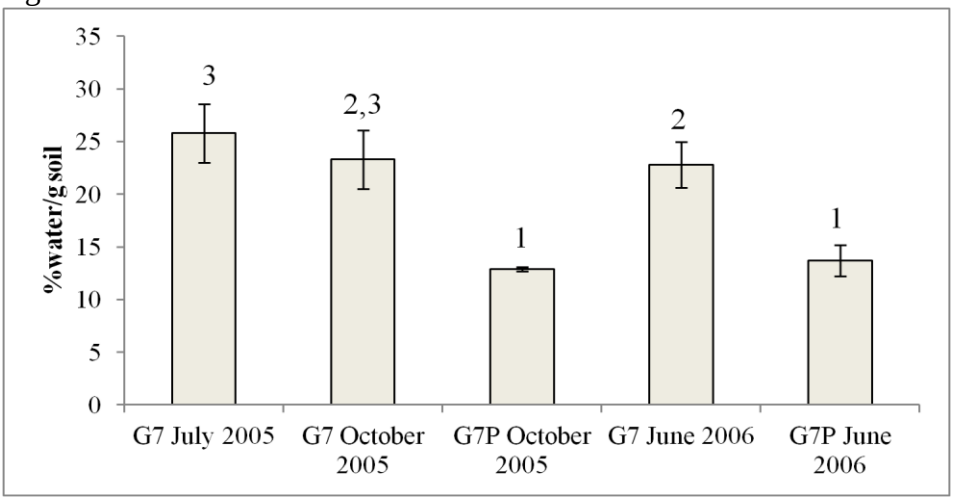


Figure 2.4.b. %Moisture in LF and LFP.

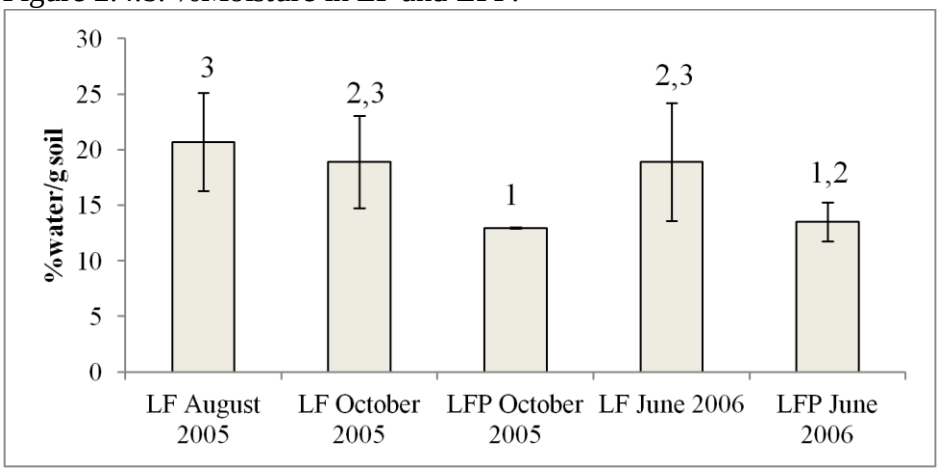
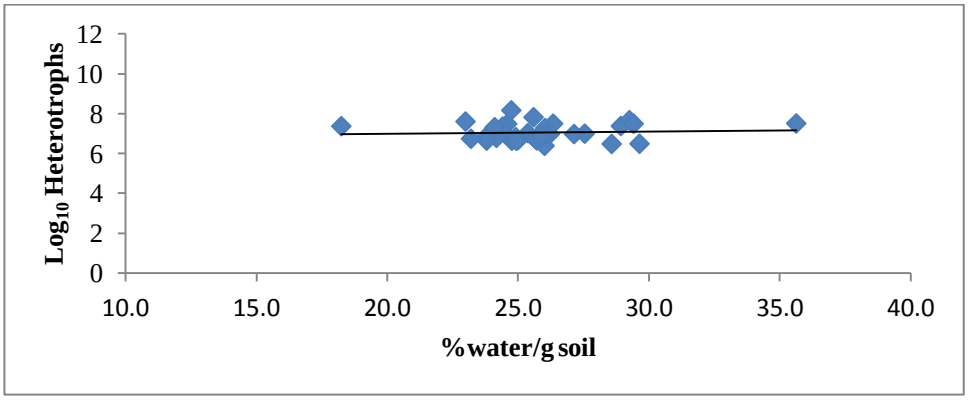


Figure 2.4. Moisture % in G7 and LF sites. G7 July 2005, G7 October 2005, G7 June 2006, LF August 2005, LF October 2005, and LF June 2006: N=34. G7P June 2006 and LFP June 2006:N=4. G7P October 2005 and LFP October 2005:N=2. Bars indicate average values. Error bars indicate ±1 standard deviation. Figure 2.4.a. 1, 2, 3: Homogenous subsets (Duncan's multiple range test) (Appendix B1). One-way ANOVA: F (4,103) = 31.341, p = 0.000. Figure 2.4.b. 1, 2, 3:

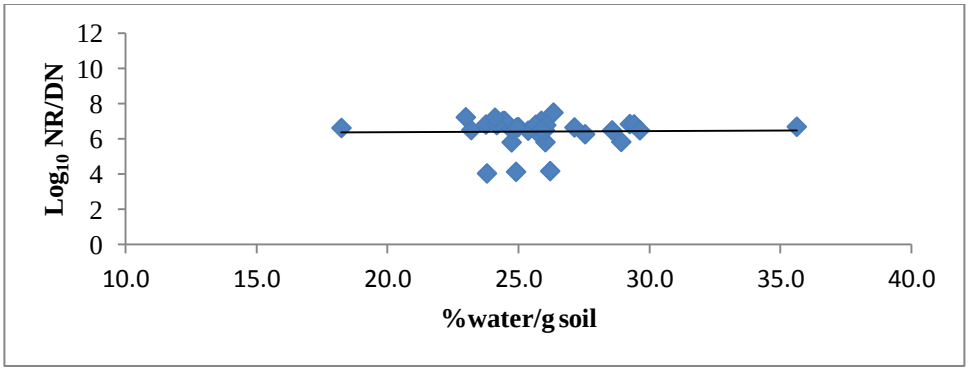
Homogenous subsets (Duncan's multiple range test) (Appendix B2). One-way ANOVA: F (4,103) = 3.420, p = 0.011.

Figure 2.5.

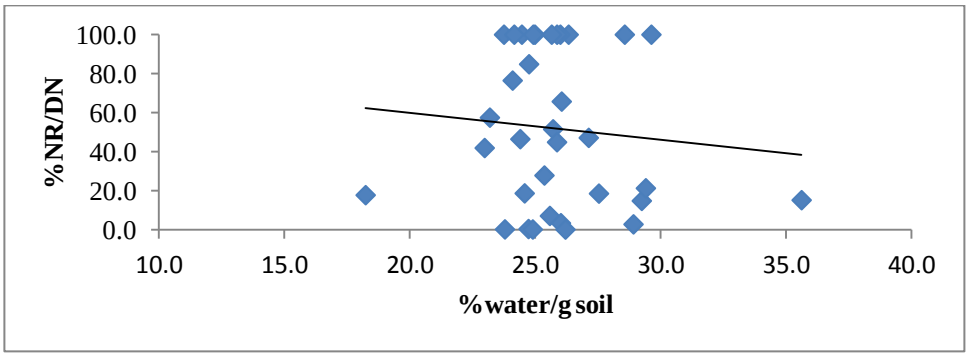
a. Abundance of heterotrophic bacteria (G7, July 2005) versus % soil moisture.



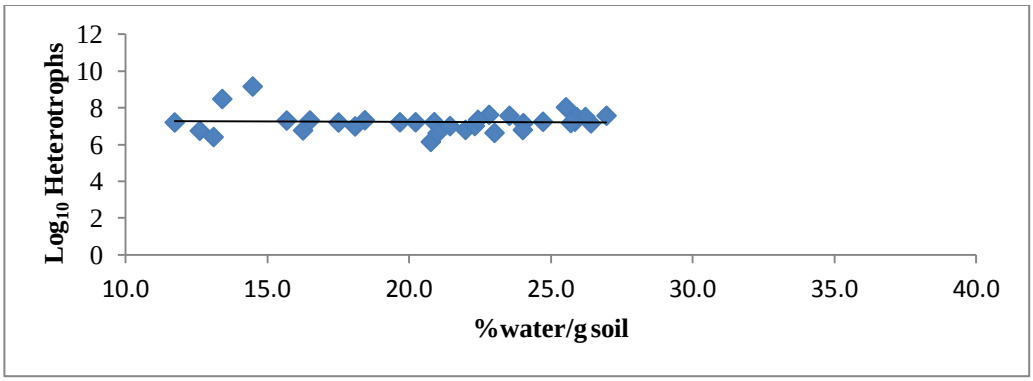
b. Abundance of NR and DN bacteria (G7, July 2005) versus % soil moisture.



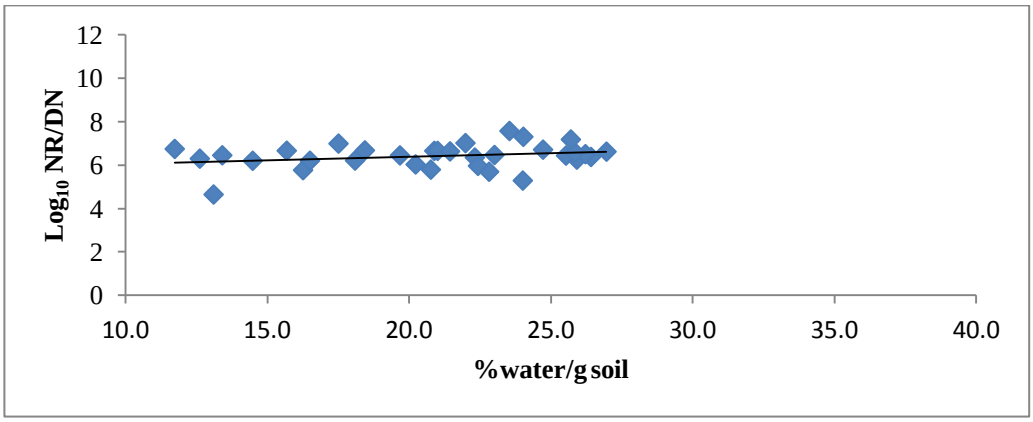
c. %NR and DN bacteria (G7, July 2005) versus % soil moisture.



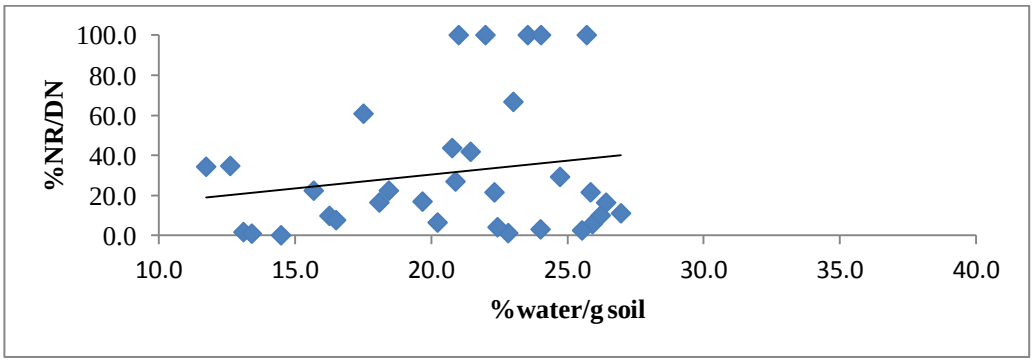
d. Abundance of heterotrophic bacteria (LF, August 2005) versus % soil moisture.



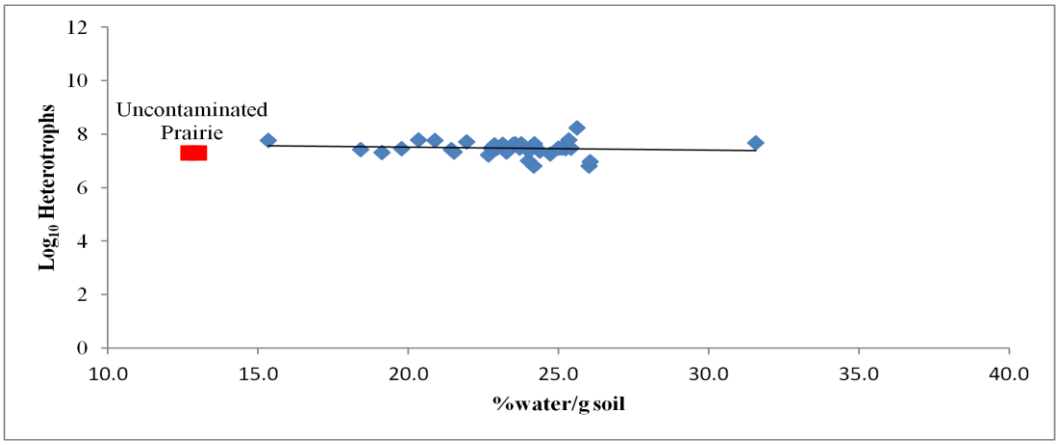
e. Abundance of NR and DN bacteria (LF, August 2005) versus % soil moisture.



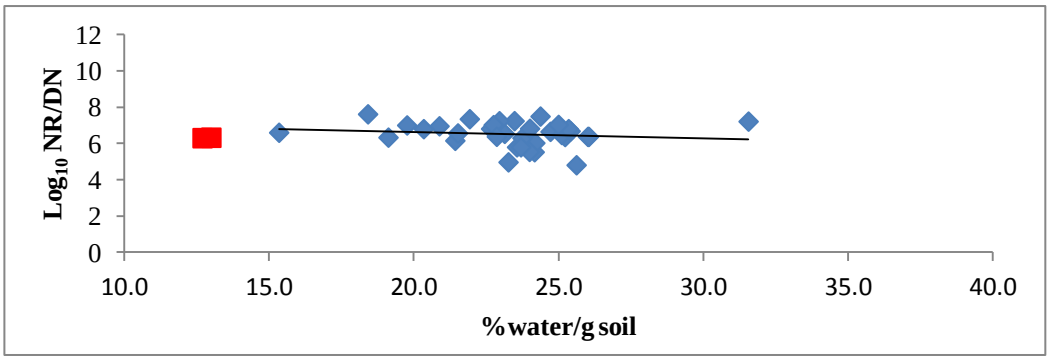
f. %NR and DN bacteria (LF, August 2005) versus % soil moisture.



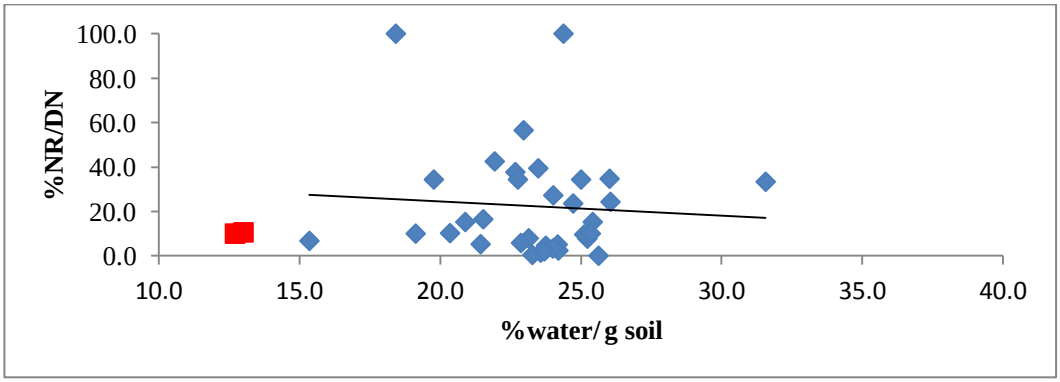
g. Abundance of heterotrophic bacteria (G7, October 2005) versus % soil moisture.



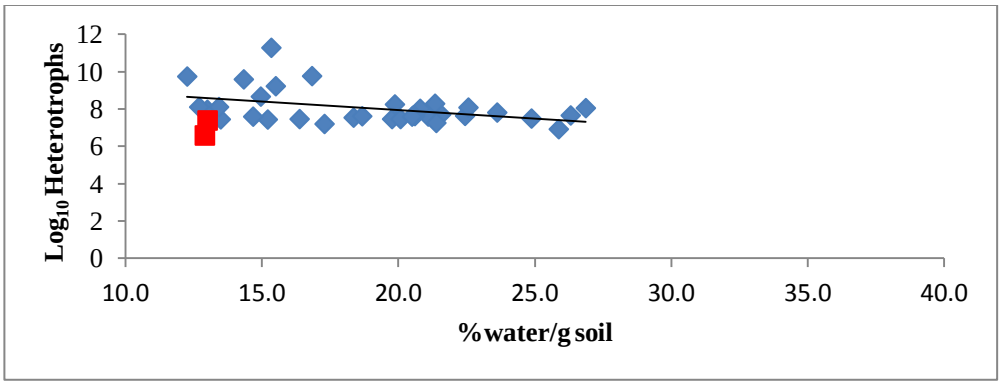
h. Abundance of NR and DN bacteria (G7, October 2005) versus % soil moisture.



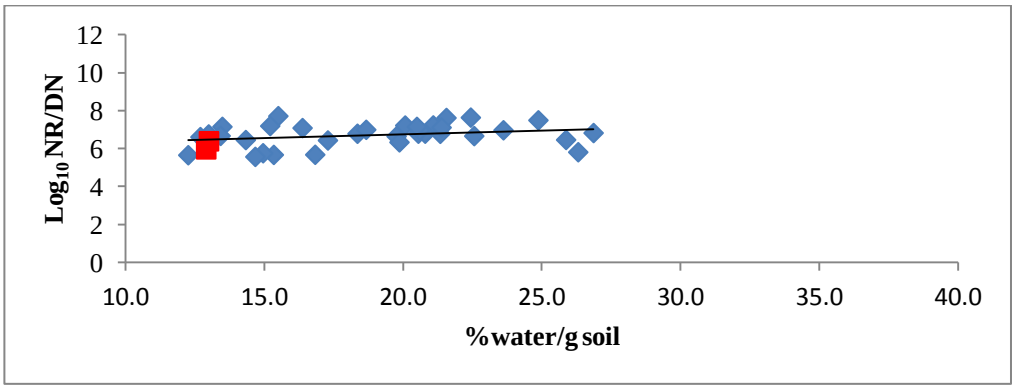
i. %NR and DN bacteria (G7, October 2005) versus % soil moisture.



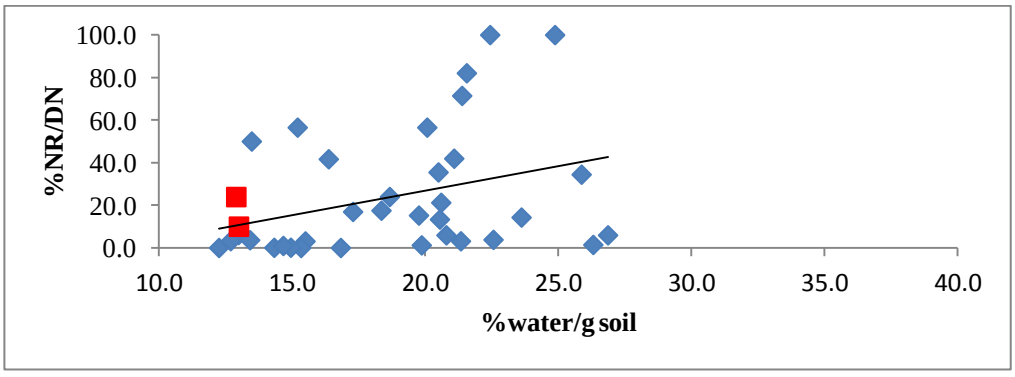
j. Abundance of heterotrophic bacteria (LF, October 2005) versus % soil moisture.



k. Abundance of NR and DN bacteria (LF, October 2005) versus % soil moisture.



l. %NR and DN bacteria (LF, October 2005) versus % soil moisture.



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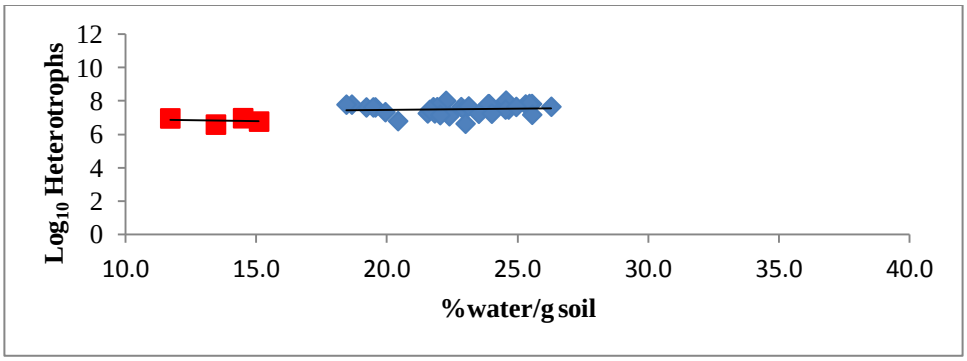
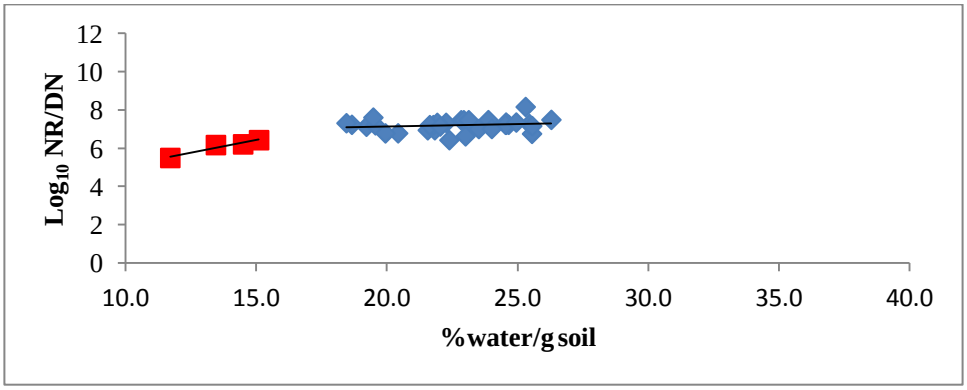
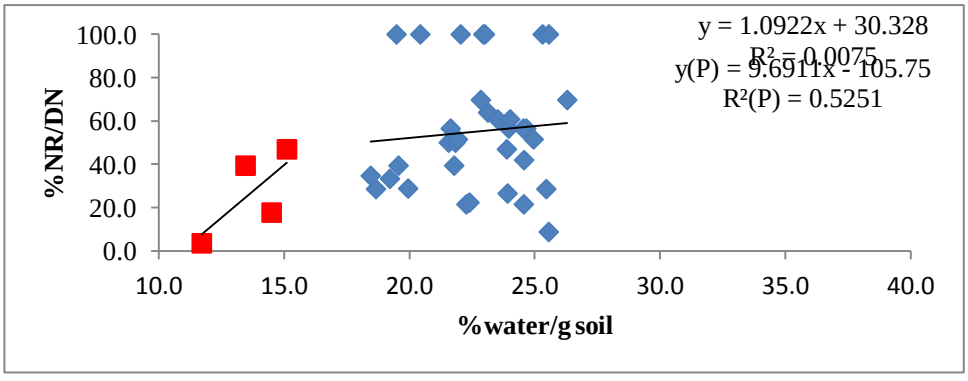
m. Abundance of heterotrophic bacteria (G7, June 2006) versus % soil moisture.



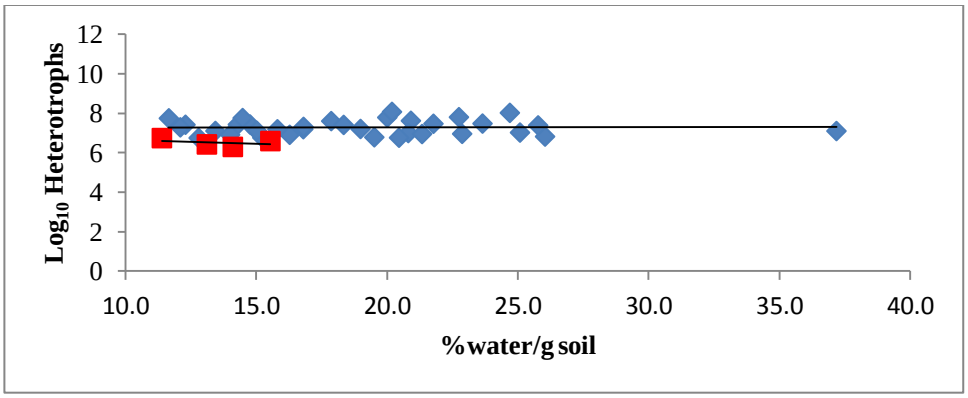
n. Abundance of NR and DN bacteria (G7, June 2006) versus % soil moisture.



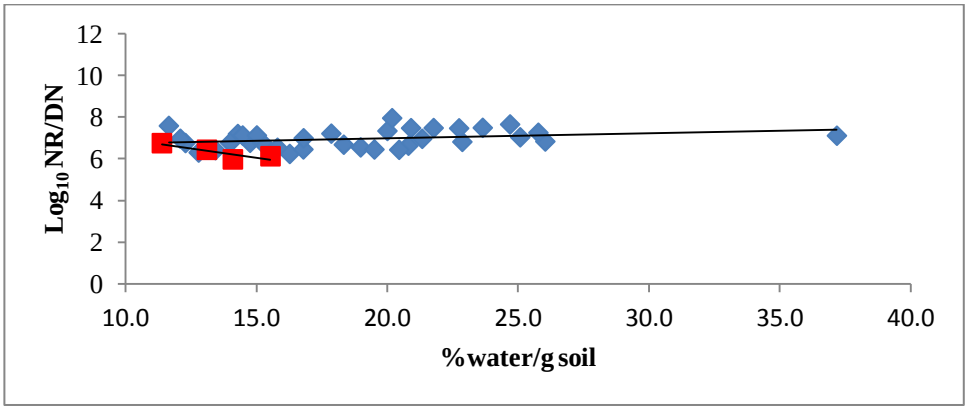
o. %NR and DN bacteria (G7, June 2006) versus % soil moisture.



p. Abundance of heterotrophic bacteria (LF, June 2006) versus % soil moisture.



q. Abundance of NR and DN bacteria (LF, June 2006) versus % soil moisture.



r. % NR and DN bacteria (LF, June 2006) versus % soil moisture.

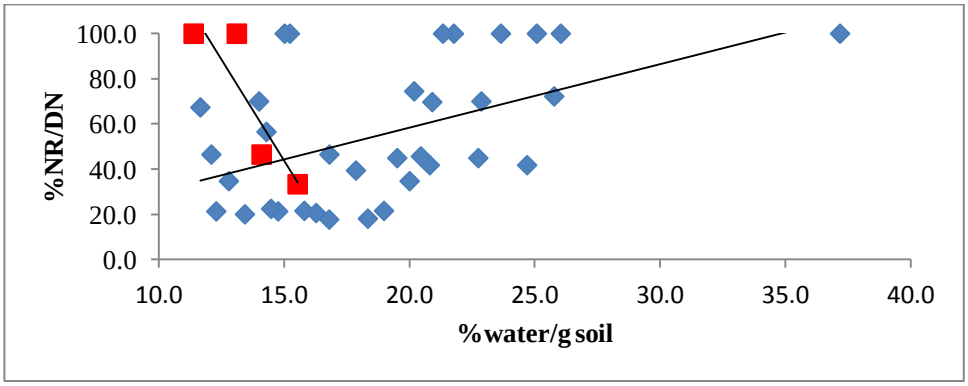


Figure 2.5. The relation between soil moisture and the $\log_{10}$ number of heterotrophic bacteria, $\log_{10}$ number of NR and DN bacteria, or %NR and DN bacteria in G7 or LF on different dates. Blue diamonds: soils from contaminated sites (N= 33 or 34). Red rectangles: soils from uncontaminated prairie sites (N= 2 or 4). Soils from contaminated and uncontaminated sites were sampled at the same date. See Appendix C for linear regression parameters.

a. Abundance of heterotrophic bacteria (G7, July 2005) versus % soil moisture.

b. Abundance of NR and DN bacteria (G7, July 2005) versus % soil moisture.

c. %NR and DN bacteria (G7, July 2005) versus % soil moisture.

d. Abundance of heterotrophic bacteria (LF, August 2005) versus % soil moisture.

e. Abundance of NR and DN bacteria (LF, August 2005) versus % soil moisture.

f. %NR and DN bacteria (LF, August 2005) versus % soil moisture.

g. Abundance of heterotrophic bacteria (G7, October 2005) versus % soil moisture.

h. Abundance of NR and DN bacteria (G7, October 2005) versus % soil moisture.

i. %NR and DN bacteria (G7, October 2005) versus % soil moisture.

j. Abundance of heterotrophic bacteria (LF, October 2005) versus % soil moisture.

k. Abundance of NR and DN bacteria (LF, October 2005) versus % soil moisture.

l. %NR and DN bacteria (LF, October 2005) versus % soil moisture.

m. Abundance of heterotrophic bacteria (G7, June 2006) versus % soil moisture.

n. Abundance of NR and DN bacteria (G7, June 2006) versus % soil moisture.

o. %NR and DN bacteria (G7, June 2006) versus % soil moisture.

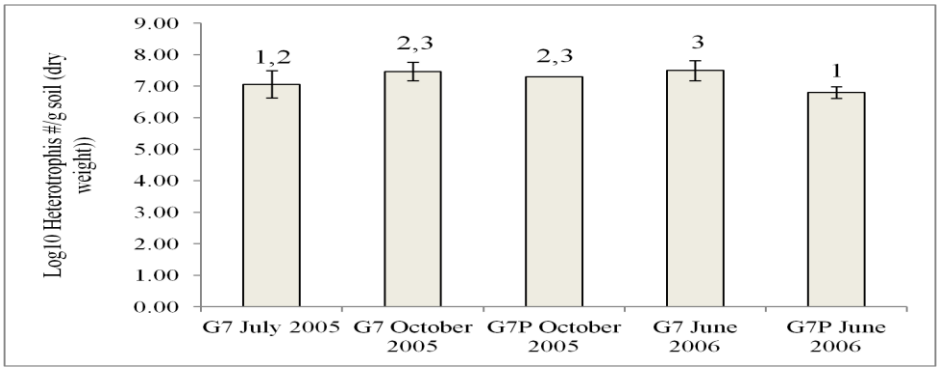
p. Abundance of heterotrophic bacteria (LF, June 2006) versus % soil moisture.

q. Abundance of NR and DN bacteria (LF, June 2006) versus % soil moisture.

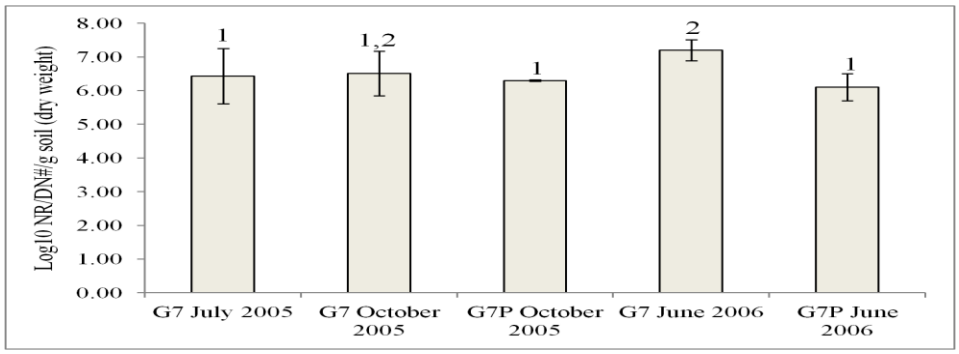
r. % NR and DN bacteria (LF, June 2006) versus % soil moisture.

Figure 2.6.

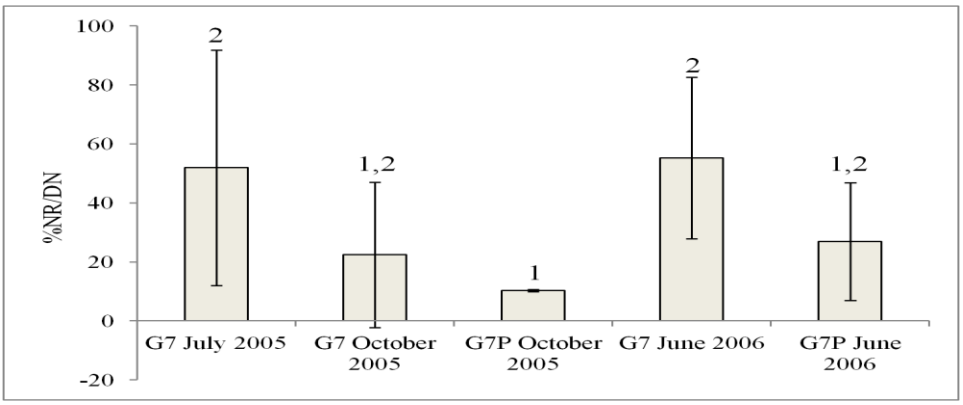
a. Abundance of heterotrophic bacteria (G7, G7P) with respect to site.



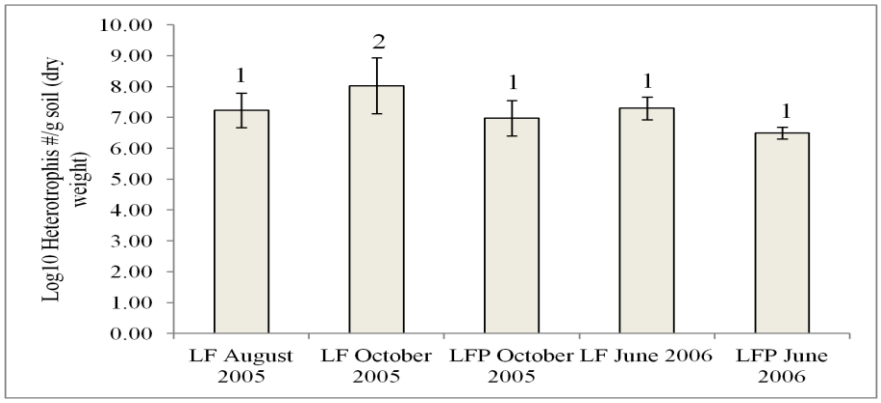
b. Abundance of NR and DN bacteria (G7, G7P) with respect to site.



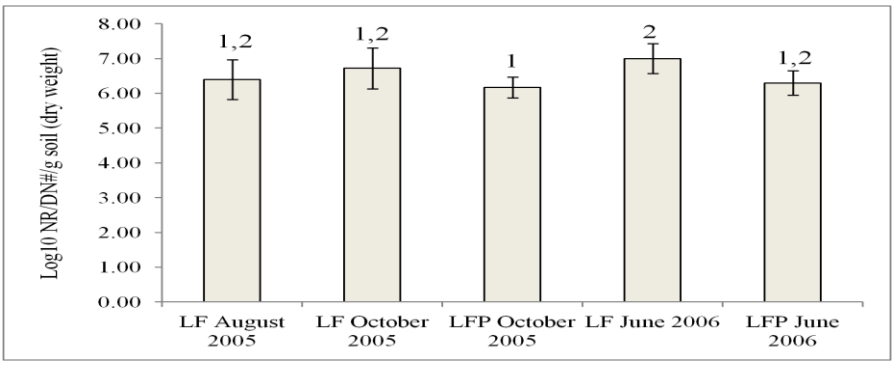
c. %NR and DN bacteria (G7, G7P) with respect to site.



d. Abundance of heterotrophic bacteria (LF, LFP) with respect to site.



e. Abundance of NR and DN bacteria (LF, LFP) with respect to site.



f. %NR and DN bacteria (LF, LFP) with respect to site.

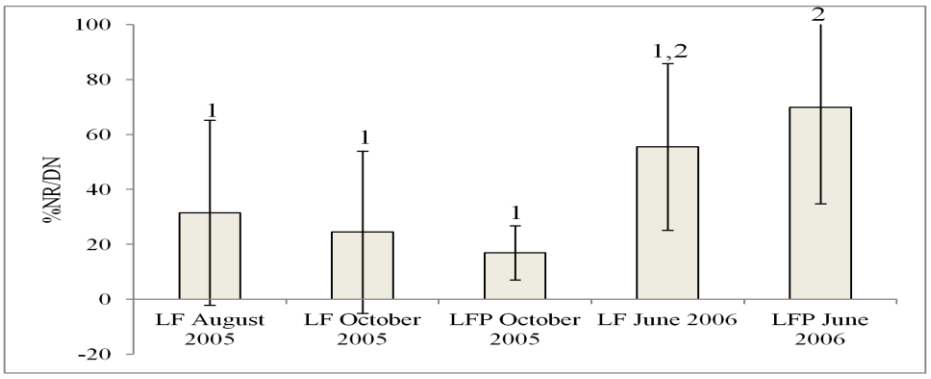


Figure 2.6. Log_{10} heterotrophic bacteria, log_{10} NR and DN bacteria, and %NR and DN in G7, G7P, LF, and LFP sampled in different dates.

a. Log_{10} heterotrophic bacteria in G7 and G7P. One-way ANOVA: F (4,103) = 11.292, p = 0.000 (Figure 2.6.a, Appendix D.1).

b. Log_{10} NR and DN bacteria in G7 and G7P. One-way ANOVA: F (4,103) = 9.142, p = 0.000 (Figure 2.6.b, Appendix D.2).

c. %NR and DN in G7 and G7P. One-way ANOVA: F (4,103) = 6.588, p = 0.000 (Figure 2.6.c, Appendix D.3).

d. Log_{10} heterotrophic bacteria in LF and LFP. One-way ANOVA: F (4,102) = 10.923, p = 0.000 (Figure 2.6.d, Appendix D.4).

e . Log_{10} NR and DN bacteria in LF and LFP. One-way ANOVA: F (4,102) = 5.639, p = 0.000 (Figure 2.6.e, Appendix D.5).

f. %NR and DN in LF and LFP. One-way ANOVA: F (4,102) = 5.915, p = 0.000 (Figure 2.6.f, Appendix D.6).

Chapter 3: Species composition of culturable nitrate reducing/denitrifying bacteria isolated from contaminated and uncontaminated tallgrass prairie soil

Abstract

Contamination of soil by spills of crude oil and oil field brine (e.g. salt water) is known to affect soil microbial communities. Hydrocarbon-degrading strains typically increase in numbers, while other strains may decrease. However, less well studied is the effect on the bacteria responsible for nitrogen cycling, an important biogeochemical cycle in tallgrass prairie soil. In this Chapter, I compare the species composition of nitrate reducing (NR) and denitrifying (DN) bacteria isolated from oil/brine tallgrass prairie soil contaminated 5-10 years previously versus those cultured from uncontaminated soil in order to see if contaminants select for particular groups of bacteria. Seventy-five strains were obtained by enrichment for NR bacteria from contaminated and uncontaminated tallgrass prairie soils. The strains were screened for the ability to reduce nitrate/nitrite by detection of nitrate/nitrite after growth in nitrate broth. Moreover, 16S rRNA gene sequences of these strains were used to determine their phylogenetic affiliation. The dominant genera were affiliated with γ-Proteobacteria, especially the genera *Stenotrophomonas* and *Pseudomonas*. All 75 strains were screened for the genes encoding the periplasmic nitrate reductase (*napA*), membrane-bound nitrate reductase (*narG*), copper nitrite reductase (*nirK*), and cytochrome cd_1-nitite

reductase (*nirS*) using primers specific for these genes. A subset of 32 strains that was confirmed to possess the NR or DN phenotype was chosen to be representative of the 16S rRNA phylogenetic diversity and was screened for other functional markers in the denitrification pathway, namely nitric oxide reductase gene (*norB*) and nitrous oxide reductase gene (*nosZ*). Out of the 75 strains, 58 reduced nitrate and/or nitrite and/or possessed at least one NR and DN gene. Around 40% of the strains shown to reduce nitrate/nitrite would not be identified as NR and DN bacteria by the battery of NR and DN primers used which indicates that PCR-based molecular detection underestimates culturable NR and DN bacteria. My results showed that a phylogenetically diverse group of NR and DN bacteria could be isolated from contaminated sites. However, proportionately more γ-Proteobacteria were detected in oil-contaminated sites, and more *Bacillus* in brine-contaminated sites than in the uncontaminated sites, suggesting a small residual effect of the contaminants on the NR and DN species distribution.

Introduction

Soil is a very complex environment with a high diversity of bacteria (Fierer and Jackson, 2006). After contamination of soil with crude oil and/or oil-field brine, certain groups of bacteria come to dominate the contaminated sites, particularly α-, β-, γ-, and δ Proteobacteria in the crude oil contaminated sites (Bragg et al., 1994; Harayama et al., 2004; and Head et al., 2006) and Firmicutes (Boch et al., 1997) and some *Pseudomonas* species (Egamberdiyeva, 2005) in the brine contaminated sites. The alteration in bacterial species composition and numbers might be expected to affect biogeochemical cycling in soils. In tallgrass prairie soils, nitrogen is a limiting nutrient for growth of plants and soil decomposition rates (Nie et al., 2011). Therefore, the effect of oil/brine contamination on nitrogen cycling bacteria is of particular concern in tallgrass prairie soil. In fact, NR and DN bacteria are known to be found in oil/brine contaminated soils (Song et al., 2000; Vargas et al., 2000; and An et al., 2004) including Tallgrass Prairie Preserve (TPP) soil contaminated by oil/brine (Duncan et al., 1999), but a detailed examination of their species composition and abundance in oil/brine contaminated TPP soil is unknown. In particular, the effects of long term residual oil and brine contamination on NR and DN species composition are not known.

Nitrate reducing bacteria represent a widespread group with members among α-, β-, and γ-Proteobacteria, some members of Firmicutes, and even

Archaea. These microorganisms use nitrate as an alternative electron acceptor that makes them able to obtain energy from dissimilatory reduction of nitrate into nitrite by nitrate reductase enzymes (Zumft, 1997). Then, the nitrite can be reduced into gaseous nitrogen compounds by denitrification or into NH_4 by dissimilatory nitrate reduction into ammonia (Philippot et al., 2002). Nitrate reduction to nitrite is catalyzed by two different types of nitrate reductases, either membrane bound encoded by the *narGHJI* operon or periplasmic encoded by the *napABC* operon (Philippot and Hojberg, 1999). In fact, NR bacteria can harbor either *narG*, *napA*, or both (Philippot,. 2002; Roussel-Delif et al., 2005).

Denitrification consists of four reaction steps in which nitrate is reduced to dinitrogen gas. Because nitrite reductase produces NO, the first gaseous product, nitrite reductase is a significant enzyme in the denitrification pathway (Ye et al., 1994; Casciotti and Ward, 2001) and signals the potential for loss of nitrogen from the soil ecosystem. Nitrite reductase is found as two different forms. The first form contains copper and is encoded by *nirK*, while the second contains cytochromes (hemes) *c* and d_1 and is encoded by *nirS* (Zumft, 1997; Braker et al., 2000**;** Casciotti and Ward, 2001**;** Gruntzig et al., 2001; Avrahami et al., 2002).

The reduction of nitric oxide is catalyzed by nitric oxide reductase, whose small and large subunits are encoded by *norC* and *norB*, respectively (Braker and Tiedje, 2003). Based on the European Molecular Biology Laboratory (EMBL)

sequence database, *norB* sequences grouped in two very distinct branches where the first class encodes the cytochrome *bc*-type complex (cNorB) while the second class encodes the quinol-oxidizing single-subunit class (qNorB) (Braker and Tiedje, 2003). The last step in the denitrification pathway is the reduction of nitrous oxide to N_2 and is catalyzed by nitrous oxide reductase encoded by the *nosZ* gene. Since denitrification is common among phylogenetically unrelated groups, it is very unsuitable to investigate communities of DN bacteria by using an approach based on 16S rRNA (Zumft, 1992; Braker et al., 2000). So, to detect and to analyze DN bacteria, genes coding for metabolic functions have been used for such key steps as nitrite and nitrous oxide reduction (Braker et al., 1998; Scala and Kerkhof, 1998; Hallin and Lindgren, 1999; Scala and Kerkhof, 1999; Braker et al., 2000).

Since nitrate reducers and denitrifyers can use nitrate as an alternative electron acceptor to oxygen to generate energy, and contain members from among many different groups of microorganisms, they are an excellent model system to investigate the response of an important component of the N-cycling soil community (e.g. nitrate reducers and denitrifyers) to the contaminants crude oil and brine. The effect of this contamination on microorganisms' abundance of culturable NR and DN bacteria was detailed in chapter 2. The objectives of this Chapter were (1) to determine if the species composition of culturable nitrate reducers and denitrifyers in contaminated and uncontaminated soils were as predicted by their tolerance to and/or utilization of the particular contaminants (2) evaluate the

detection of NR and DN genes in NR and DN strains using primers specific for a suite of NR and DN genes.

In this study, bacterial strains were isolated from crude oil- and oil/brine-contaminated and uncontaminated tallgrass prairie soils from TPP near Pawhuska, Oklahoma. The nature of the contaminant is hypothesized to alter the species composition of NR and DN bacteria in certain ways, e.g. I hypothesized that NR and DN bacteria in the crude oil-contaminated sites will contain a greater proportion of strains affiliated with groups, such as γ-Proteobacteria, known to increase in crude-oil contaminated sites (Lee et al., 2002; Shim and Yang, 1999). Certain strains of γ-Proteobacteria are noted for the ability to use crude oil components as sole carbon sources (Dandie et al., 2007b) although the ability to degrade petroleum compounds is widespread (Atlas, 1981). Since high levels of sodium chloride inhibit certain groups of bacteria (Soussi et al., 2001; Hung et al., 2005), I hypothesized that the NR and DN bacteria from brine contaminated sites will be biased towards those groups that are salt tolerant such as *Bacillus* (Boch et al., 1997) and *Pseudomonas* (Egamberdiyeva, 2005).

Although *nirS* and *nirK* have often been used to detect DN bacteria (Salles et al., 2012; Orlando et al., 2012), I was not able to amplify *nirS* or *nirK* from more than 4 strains out of the 75 I screened. This low percentage of *nirS*/*nirK* detection may be due to primers specificity limitations. To overcome primers specificity

limitations such as non-specific binding of primers which often increase when degenerate primers are used (Lexa and Valle, 2003; Qu et al., 2012) and as additional molecular indicators for DN bacteria, I tested primers for nitric oxide reductase gene (*norB*) and nitrous oxide reductase gene (*nosZ*) on a subset of 32 strains, including the 4 strains for which *nirS* or *nirK* amplicons were obtained. This subset of 32 strains was chosen as representative of the 16S rRNA phylogenetic diversity in the 75 strains. These additional molecular indicators may detect some NR and DN bacteria that were not detected by nitrate reductase and nitrite reductase primers. Sequencing of PCR-amplified products was used to characterize the 16S rRNA and functional NR and DN genes (*napA*, *narG*, *nirS*, *nirK*, *cnorB*, *qnorB*, and *nosZ*) from the isolates.

Materials and Methods

Medium and Microorganisms

Medium

Nitrate broth medium for detection of NR and DN bacteria consisted of 2.5 g of $NaNO_3$ added to 500 mL of nutrient broth (Difco Inc., Detroit, MI) before autoclaving. The antifungal agent, cycloheximide (100 µg/mL final concentration) (Sigma-Aldrich Corporation, St. Louis, MO, USA) was added after autoclaving. Nutrient agar plates (Difco Inc., Detroit, MI) were used for quadrant streak isolation of the individual strains. Nitrate Reduction Broths containing beef extract, pancreatic digest of casein, or peptone, are commonly used to distinguish bacteria based on whether they are able to reduce nitrate into nitrite or produce N_2 gas (Atlas, 1993).

Control strains for testing the ability to perform nitrate/nitrite reduction

Control strains were obtained from ATCC to confirm that the test conditions correctly identified nitrate reducers and denitrifyers. These controls were: *Stenotrophomonas maltophilia* ATCC 13637 (Nitrate reducer), *Pseudomonas aeruginosa* ATCC 27853 (Denitifyer), and *P. putida* pG7 (negative- neither a denitrifyer nor a nitrate reducer). *Pseudomonas aeruginosa* ATCC 27853 was grown at 37°C, *S. maltophilia* ATCC 13637 was grown at 30°C, and *P. putida* pG7

strain was grown at room temperature (23-25°C). Uninoculated wells were included as negative controls.

Origin of strains

The sites used in this study were located in the Tallgrass Prairie Preserve in Osage County, Oklahoma. The bacterial strains (N= #strains) described in this Chapter were obtained from soil samples collected from the following sites: G5 (brine/oil contaminated) (Sublette et al., 2005) (N=10), J6-F (crude oil contaminated, treated with fertilizer) (Sublette et al., 2007) (N=4), and J6-NF (crude oil contaminated, not treated with fertilizers) (Sublette et al., 2007) (N=3) in March, 2005. G7 (brine/oil contaminated) (N=10) in July, 2005, October, 2005, and June 2006. LF (crude oil contaminated) (N=5) in August, 2005, October, 2005, and June 2006. Samples from adjacent uncontaminated sites (G5P, N=10; J6P, N=6; G7P, N=12; and LFP, N=15) were collected at the same time as for contaminated sites. Briefly, a trowel was used to remove 5 shallow scoops of soil from 5 widely spaced points within a 25 m radius from approximately the top four inches of soil. These soil samples from G5, J6-F, J6-NF and their parallel uncontaminated sites were later homogenized. The enclosures in LF and G7 were pulled out and poured into stainless steel bowls, mixed, and subsamples were taken out for a variety of assays. The sampling scheme is described in more detail in Chapter 2 and the contamination and remediation history is described in detail in Chapters 1 and 2.

Soils were initially mixed in Whirl-pac® bags using autoclaved spoons in the field and later in the lab they were homogenized in autoclaved beakers by mixing with autoclaved spoons. The homogenized soils were then serially diluted in sterile isotonic (0.85%) saline and added to microtiter plates containing nitrate broth (Difco). After 2 weeks of incubation at room temperature (23-25°C) in containers that excluded light (Jones et al., 1991), all wells were examined for evidence of growth (turbidity) after 14 days, and those that were turbid were scored positive for aerobic/facultative heterotrophic bacteria. Subsequently, half of the volume of the wells was transferred to a fresh microtiter plate and tested for the presence of NR and DN bacteria using Griess reagents (Baron and Finegold, 1990; Smibert and Krieg, 1994) (see Chapter 2). The original microtiter plates were the source of the isolates described in this Chapter.

Obtaining pure cultures of the 75 strains

Based on positive scoring for growth and nitrate or nitrite reduction in the microtiter plates, nutrient agar plates were quadrant streaked with 25 µL from microtiter plate wells, chosen randomly from 10^{-2} to 10^{-5} dilutions (Appendix G.1) that scored positive for growth and nitrate reduction or denitrification. Positive results for nitrate and nitrite reduction were commonly obtained from the 10^{-2} to 10^{-7}. The nutrient agar plates were incubated at 30°C. A colony chosen from a plate was streaked at least 3 times in succession from a single colony to a fresh plate in

order to obtain pure cultures. Seventy five strains in total were obtained from microtiter plate wells for further testing.

Microtiter plate test of nitrate reduction and denitrification of pure cultures

After obtaining pure cultures of the 75 strains, each strain was retested using the microtiter plate format as described in Chapter 2 to determine the ability to reduce nitrate or nitrite in pure culture. Control strains (described under control strains section) of known phenotype were inoculated in triplicate in 96-well plate containing nitrate broth incubated for 2 weeks and tested for nitrate/nitrite reduction as described above.

DNA extraction from strains

Genomic DNA was isolated from cells using a bead beating method. Briefly, each of the 75 strains was streaked on nutrient agar plate incubated one to 3 days at 30°C until good colony growth was observed, then a single colony from each plate was used to inoculate 5 mL nutrient broth and incubated at 30°C until turbid growth was seen. A sufficient volume of culture (1 to 2 mL) was centrifuged at 6000xg for 10 minutes to provide a pellet size of approximately 10^9 bacteria. Pellets were resuspended in sterile isotonic saline (0.85%) to give a maximum suspension volume of 200 µL. A commercially available DNA extraction kit, FastDNA®SPIN Kit (QBIOgene, Solon, OH, USA), was used to extract DNA from the cell suspensions by following the manufacturer's directions. The protocol

combined mechanical (e.g., bead-beating) and chemical means to lyse the cells. Extracted DNA was stored at −20°C. Controls for contamination included processing one reagent control to which sterile water rather than cells was added.

Molecular detection of 16S rRNA gene sequences and nitrate reduction/denitrification functional genes

Determination of the 16S rRNA gene sequence and screening for nitrate reductase genes (e.g. *napA* and *narG*) and nitrite reductase genes (e.g. *nirK* and *nirS*) were performed for all 75 strains. The 16S rRNA gene sequence was amplified using primer pairs 27F/1492R (Wilson et al., 1990). Fragments from nitrate reductase genes were amplified using specific primer pairs as listed in Table 3.1. The *napA* F1/*napA* R1 primer pair was used to detect *napA,* and the primer pair *narG* F1/*narG* R1 was used to detect *narG* gene (Bedzyk et al., 1999). To amplify nitrite reductase genes, primer pairs *nirS* 1F/*nirS* 6R (Braker et al., 1998) and *nirK* F/*nirK* R (Qiu et al., 2004) were used to amplify fragments of *nirS* and *nirK*, respectively. Thirty-two strains that were shown to be NR and DN bacteria by reduction of nitrate and/or nitrite were chosen as a representative of the NR and DN 16S rRNA phylogenetic diversity to be screened for the presence of *qnorB, cnorB,* and *nosZ* functional denitrifying genes. Fragments from *cnorB* gene were amplified using primer pairs *cnorB* 2F/*cnorB* 6R, the *qnorB* gene using primer pairs *qnorB* 2F/*qnorB* 7R (Braker and Tiedje, 2003), and the *nosZ* gene using primer pairs *nosZ*-F-1181/ *nosZ*-R-1880 (Rich et al., 2003). Primers used for detecting denitrification

functional genes are listed in Table 3.1. PCR reaction mixtures consisted of 0.5 µL (10 mM stock) deoxynucleotide triphosphate mixture of all four nucleotides (dNTP), 1 µL (50 mM stock) $MgCl_2$, 2.5 µL(5.0 M stock) Betaine monohydrate (Henke et al., 1997) (Sigma-Aldrich Corporation, St. Louis, MO, USA), 5.0 ng/ µL- 50 pg/ µL DNA, 0.125 µL of Invitrogen Taq Polymerase (0.625 U). In addition, 24 µM of *napA*-F1 primer and 36 µM of *napA*-R1 primer; 33 µM of *narG*-F1 primer and 42 µM of *narG*-R1 primer; 5 pM/µL of *nirS* and *nirK* primers; 50 pM/µL of *cnorB* and *qnorB*, or 0.2 µM of *nosZ* primer were used in a buffer containing 50 mM Tris Cl, 50 mM KCL, and 0.01% Triton-X100 for a final volume of 25 µL. The master mix of the PCR reaction, e.g. all the above ingredients minus DNA, but with an additional 1 µL of PCR water was used as a negative control in all PCR reactions. All thermal cycling conditions included initial denaturation at 94°C for 4 minutes (exception: 5 minutes denaturation for *cnorB* and *qnorB* genes) and a final extension at 72°C for 10 minutes. Annealing temperatures for all reactions are listed in Table 3.1. Thermocyclers used were PE9600 thermocycler (Perkin Elmer Cetus, Gene Amp model PCR System 9600) Perkin Elmer, Foster City, CA, USA and RoboCycler Gradient 40 Thermal Cycler (Stratagene, Inc., La Jolla, CA, USA). Five strains were obtained from ATCC for use as positive controls for PCR detection of denitrification functional genes. *Escherichia coli* was used as the positive control for *napA* and *P. stutzeri* ATCC 17588 as positive control for *narG*, *P. aeruginosa* ATCC 27853 as the positive

control for *nirS* and *cnorB*, *Alcaligenes faecalis* ATCC 8750 as the positive control for *nirK* and *qnorB*, and *P. stutzeri* ATCC 17588 as the positive control for *nosZ*.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visually detect bacterial DNA PCR products. Mini 1% agarose gels in 0.5X TBE buffer (44.5 mM Tris Base, 44.5 mM Boric Acid, and 1 mM EDTA) were run at 94 volts for 20 minutes. SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) at 1µL (10000X in DMSO)/10 mL of agarose solution was added to the agarose mixture to aid visualization of the DNA. Lambda DNA Hind III (Promega, Madison, WI, USA) was used as a DNA molecular size marker. Agarose gels were visualized using a UV Intensity Transilluminator and documented with a Kodak DC 120 Zoom Digital Camera and Nucleo Tech Software (Nucleo Tech, San Mateo, CA).

DNA Sequencing and Analysis

PCR products were prepared for sequencing using ExoSAP-IT (USBiochemicals, Cleveland, Ohio) which contains Exonuclease I and Shrimp Alkaline Phosphatase (Bell, 2008). Briefly, 3 µL of ExoSAP-IT was directly added to 10 µL of the PCR product. The mixture was incubated in a RoboCycler Gradient 40 Thermal Cycler (Stratagene, Inc., La Jolla, CA, USA) at 37°C for 15 minutes to degrade remaining primers and nucleotides. Subsequently, the ExoSAP-IT enzymes were inactivated by heating at 80°C for 15 minutes.

The nucleotide sequences of ExoSAP-IT treated PCR products were determined at Oklahoma Medical Research Foundation (OMRF) at Oklahoma City, Oklahoma using 27F/1492R (Wilson et al., 1990) primers for 16S rRNA gene sequence PCR products and the primers listed in Table 3.1 for functional nitrate reductase genes and other genes of the denitrification pathway. Sequence chromatograms were analyzed using Sequencher (Windows version 4.2; Gene Codes Corp., Ann Arbor, MI) and Sequencher was used to produce a consensus sequence. The sequence of the 16S rRNA gene and nitrate reduction/denitrification functional genes were compared to the GenBank database using BLASTN searches (Basic Local Alignment Search Tool) (Altschul et al., 1990). The taxonomic assignation of 16S rRNA gene sequence was determined by the Naïve Bayesian rRNA Classifier of the Ribosomal Database Project II (RDP) (Wang et al., 2007). The 16S rRNA sequences were deposited in GenBank under accession numbers JQ917765-JQ917839.

16S rRNA gene sequences of the 75 strains were clustered as operational taxonomic units (OTUs) at an overlap identity cut-off of 98%, not 97%, to produce a finer discrimination among the strains, by MOTHUR software ver1.23 (Schloss et al., 2009). Unique sequences were aligned against the SILVA reference alignment database (Pruesse et al., 2007) using the NAST-aligner (DeSantis et al., 2006) and subsequently screened for chimeras using UChime (Edgar et al., 2011). A distance matrix was generated and used to cluster sequences into OTUs at 98% similarity. A

representative sequence from each OTU was assigned a taxonomic classification based on the naïve Bayesian classifier (Wang et al., 2007). The representative OTUs together with closely related sequences from GenBank database were aligned by Clustal-X software version 1.81 (Thompson et al., 1997). As implemented in CLUSTALX, the neighbor joining method of Saitou and Nei (1987) was used to construct the phylogenetic tree among the aligned sequences. Distances were calculated as % divergence between all pairs of sequences from the multiple alignment, alignment positions with gaps were excluded from the analysis and the Kimura two-parameter distance correction for multiple substitutions was applied (Kimura, 1980). The support for the tree branches was estimated from 1000 bootstrap replicates (Felsenstein, 1985). Only bootstrap values greater than 700 are shown on the phylogenetic trees. All richness and diversity measurements were calculated using the MOTHUR software package (Schloss et al., 2009) including Shannon and InvSimpson indeces:

Shannon index:

$$H_{shannon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} ln \frac{n_i}{N}$$

$$var(H_{shannon}) = \frac{\sum_{i=1}^{S_{obs}} \frac{n_i}{N} (ln \frac{n_i}{N})^2 - H^2{}_{shannon}}{N} + \frac{S_{obs} - 1}{2N^2}$$

$$LCI_{95\%} = H_{shannon} - 1.96 \sqrt{var\,(H_{shannon})}$$

$$UCI_{95\%} = H_{shannon} + 1.96 \sqrt{var\,(H_{shannon})}$$

InvSimpson index= $1/D_{simpson}$

$$D_{simpson} = \frac{\sum_{i=1}^{S_{obs}} n_i\,(n_i - 1)}{N\,(N-1)}$$

$$var(D_{simpson}) = \frac{\sum_{i=1}^{S_{obs}}(\frac{n_i}{N})^3 - (\sum_{i=1}^{S_{obs}}(\frac{n_i}{N})^2)^2}{0.25N}$$

$$LCI_{95\%} = D_{simpson} - 1.96\sqrt{var\,(D_{simpson})}$$

$$UCI_{95\%} = D_{simpson} + 1.96\sqrt{var\,(D_{simpson})}$$

where,

S_{obs} = the number of observed OTUs.

n_i = the number of individuals in the *i*th OTU.

N = the total number of individuals in the community.

Var = variance.

LCI = lower bound of confidence interval.

UCI = upper bound of confidence interval.

C: Good's measure of library coverage.

$$C = 1 - \frac{n_i}{N!}$$

where,

n_i = the number of OTUs that have been sampled once

N = the total number of individuals in the sample

Results

The purpose of this study was to compare the species composition of culturable nitrate reducers and denitrifyers in contaminated and uncontaminated tallgrass prairie soils. Specifically, whether there was a predictable alteration of the species composition of NR and DN bacteria due to crude-oil/brine contamination and whether the diversity of NR and DN bacteria was decreased in contaminated sites. In addition, a battery of primers were used to compare the detection of NR and DN genes to NR and DN phenotypes in strains isolated from contaminated and uncontaminated soils.

Microtiter plate assay results for the 75 strains and the distribution of NR and DN bacteria among the different sites

Seventy five strains were isolated from tallgrass prairie soils after enrichment in nitrate broth (Appendix G.2). Out of the 75 strains tested, 48 strains were identified as nitrate reducers, 6 as nitrite reducers, and 21 strains did not show any ability to reduce nitrate or nitrite (Table 3.2). Among the 54 that showed reduction of nitrate/nitrite, 17 were obtained from brine-contaminated soils, 9 from oil-contaminated soils, and 28 from uncontaminated soils. Therefore, the highest proportion of nitrate/nitrite reducers of the total isolated from a particular type of site was obtained from brine contaminated soil (85%), next highest from oil contaminated sites (75%), and the lowest proportion from the uncontaminated sites (65%) (Table 3.2).

The microtiter plate test conditions (Jones et al., 1991) correctly identified nitrate reducing control strains as nitrate reducers, but denitrifyers were misidentified as nitrite reducers. Therefore, for the purpose of this dissertation, nitrite reducers are designated as denitrifyers (DN), with the understanding that not all "DN" strains are capable of reducing nitrate to dinitrogen.

16S rRNA Phylogenetic affiliations of the strains

16S rRNA gene sequences were used to classify the 75 strains. The 75 strains consisted of 61% γ-Proteobacteria, 7% α-Proteobacteria, 7% β-Proteobacteria, 19% Bacilli, 5% Actinobacteria and 1% Flavobacteria (Figure 3.1.a). However, since strains were isolated from wells representing different dilutions (Appendix G.1), it is assumed that strains originating from the 10^{-4} or 10^{-5} wells represented species that were more abundant than those isolated solely from the 10^{-2} wells. Based on their origin from 10^{-4} or 10^{-5} wells, the most abundant bacteria were γ-Proteobacteria (59%), α-Proteobacteria (23%) Actinobacteria (12%), and Bacilli (1%). These abundance results corrected for the dilution show that γ-Proteobacteria were still most dominant group, but there were more α-Proteobacteria and fewer Bacilli than calculated from a direct count of the number of isolates (Appendix G.1).

γ-Proteobacteria were represented by 46 strains and were distributed as follows: 8 from brine contaminated, 10 from crude oil contaminated, and 28 from

uncontaminated prairie sites (Table 3.3, Figure 3.1.b). The two most abundant genera of γ-Proteobacteria were *Stenotrophomonas* (27 strains) and *Pseudomonas* (9 strains) (Table 3.3). Most strains (e.g. 19, 70%) of *Stenotrophomonas* were obtained from prairie while 4 strains of *Pseudomonas* were isolated from prairie, 4 from brine contaminated sites, and 1 from oil contaminated site. On the other hand, out of 5 α- Proteobacteria strains, 4 strains were obtained from brine contaminated and 1 strain was obtained from an uncontaminated prairie site, while out of 5 β-Proteobacteria strains, 1 strain was purified from a brine contaminated site and 4 strains were purified from uncontaminated prairie sites. There were 4 Actinobacteria strains, one was purified from the brine-contaminated site, one from the crude oil-contaminated site, and 2 from uncontaminated prairie sites. Also, one Flavobacteria strain was purified from crude oil contaminated site. Finally, 6 out of the 14 strains of Bacilli were purified from brine contaminated sites and the other 8 were purified from uncontaminated prairie sites (Table 3.3) (Figure 3.1.b), and none from crude oil-contaminated sites. The two most abundant genera of Bacilli were *Brevibacillus* (8 strains) and *Bacillus* (3 strains) (Table 3.3). Table 3.3 also shows that some genera (e.g. *Aeromonas, Serratia, Enterobacter, Ensifer*, *Bosea*, *Brevundimonas, Arthrobacter*, *Microbacterium*, *Paenibacillus*, and *Chryseobacterium*) were isolated from the contaminated sites, but not from the prairie sites. However, most of these genera (*Aeromonas, Serratia*, *Ensifer, Brevundimonas*, *Arthrobacter, Microbacterium*, *Paenibacillus*, and *Chryseobacterium*) were represented by single strains.

Most strains had 16S rRNA sequences highly similar (98%-100%) to those previously described isolates. The 75 strains were grouped into 29 OTUs based on 98% 16S rRNA similarity. Figures 3.2.a and 3.2.b show the phylogenetic affiliation of a representative sequence from each OTU, the total number of sequences in the OTU, and the NR and DN physiology of the strains in the OTU. Note that even grouping strains at the 98% level of similarity did not always create OTUs in which all strains had the same NR and DN physiology.

Figure 3.2.a shows the phylogenetic affiliation of the γ-Proteobacteria. The 9 *Pseudomonas* strains were grouped in 4 OTUs. The 3 *Pseudomonas* strains represented by strain I-1 that reduced nitrite after incubation in nitrate broth in the microtiter plate were members of the same OTU, most similar to the 16S rRNA sequence of *Pseudomonas frederiksbergensis* strain KOPRI 25689 (HQ824922), while the other 6 *Pseudomonas* strains were nitrate reducers with 5 of the 6 most similar to *P. putida* strains. Moreover, twenty out of the 27 *Stenotrophomonas* strains were clustered in one OTU. Out of these 20 *Stenotrophomonas* strains, 11 were nitrate reducers and 9 did not reduce nitrate or nitrite. Another *Stenotrophomonas* OTU had 5 strains (e.g. 4 nitrate reducers and one did not reduce nitrate or nitrite). On the other hand, the only *Stenotrophomonas* (e.g. I-57, JQ917821) that reduced nitrite after incubation in nitrate broth was clustered by itself with 99% similarity to *Stenotrophomonas* sp. FB206 (AY259519). Some strains appeared very similar in both 16S rRNA sequence and their ability to reduce

nitrate/nitrite. All 3 *Enterobacter* strains (nitrate reducers) were grouped together as one OTU. The five strains of *Acinetobacter* were grouped into two groups, a group of 3 (2 nitrate reducers and one did not reduce nitrate or nitrite) and a group of 2 (neither reduced nitrate or nitrite). Finally, I obtained single strains of genera *Aeromonas* (nitrate reducer) and *Serratia* (nitrate reducer).

Figure 3.2.b shows the remaining taxa. In the β-Proteobacteria, the 16S rRNA nucleotide sequence of *Achromobacter* strain I-45 was distinct at 98% level of similarity from the other 3 *Achromobacter* sequences. However, all 4 *Achromobacter* strains were nitrate reducers. All 8 *Brevibacillus* strains (7 nitrate reducers and one did not reduce nitrate or nitrite) were grouped together as one OTU. In addition, all 3 *Bacillus* strains (2 nitrate reducers and one did not reduce nitrate or nitrite) were grouped together with *Bacillus cereus* strains. However, the 2 *Lysinibacillus* strains (one nitrate reducer and one did not reduce nitrate or nitrite) were most similar to different species of *Lysinibacillus*. Some strains appeared very similar in both 16S rRNA sequence and their ability to reduce nitrate/nitrite. Both *Ensifer* strains (α-Proteobacteria) were grouped together and both reduced nitrite. Also, both *Bosea* strains (α-Proteobacteria) were grouped together and neither demonstrated nitrate reduction (Figure 3.2.b). Finally, I obtained single strains of the genera *Brevundimonas* (nitrate reducer), *Burkholderia* (did not reduce nitrate or nitrite), *Arthrobacter* (did not reduce nitrate or nitrite), *Rhodococcus* (did not

reduce nitrate or nitrite), *Microbacterium* (nitrate reducer), *Kocuria* (nitrate reducer), *Paenibacillus* (nitrate reducer), and *Chryseobacterium* (nitrate reducer).

Nitrate reducers grouped together or with the strains that were not able to reduce nitrate/nitrite in most of the OTUs. However, nitrite reducers grouped with just nitrite reducers in three OTUs (e.g. *Pseudomonas*, *Stenotrophomonas*, and *Ensifer*) (Figures 3.2.a and 3.2.b).

Distribution of the 75 strains by site of origin

The 75 strains were grouped into 29 OTUs based on 98% 16S rRNA similarity. The 29 OTUs and their source are shown in Table 3.4. In fact, OTU #1 (*Stenotrophomonas*) has the most strains (20 out of 75) in which 16 out of 20 were isolated from the uncontaminated prairie sites (Table 3.4). However, other OTUs have fewer numbers of sequences, with 16 OTUs having only one sequence. As shown in Appendix H strains belonging to OTU1 (*Stenotrophomonas*) were isolated from 5 of the 8 sites, OTU5 (*Stenotrophomonas*) from 4 sites, and OTU7 (*Brevibacillus*) from 3 sites. Of the OTUs containing 3 or more strains, some were found predominantly or exclusively in one type of site, e.g. OTU1 (*Stenotrophomonas*, 80% from Prairie), OTU2 (*Enterobacter*, 100% from oil), OTU3 (*Pseudomonas*, 100% from Prairie), and OTU26 (*Acinetobacter*, 100% from Prairie). Others were isolated from both contaminated and prairie sites; OTU5 (*Stenotrophomonas*: brine, prairie, and oil), OTU7 (*Brevibacillus*: brine and

prairie), OTU17 (*Bacillus*: brine and prairie), OTU19 (*Pseudomonas,* brine and prairie), OTU21 (*Sinorhizobium*/*Ensifer*: brine and prairie), OTU23 (*Achromobacter*: brine and prairie).

Out of the 29 OTUs, 6 (e.g. OTU4, OTU6, OTU8, OTU10, OTU12, and OTU 22) did not contain any NR or DN strains. Four of these 6 OTUs contained only strains isolated from uncontaminated sites, 2 (OTU12 and OTU 22) were isolated from brine-contaminated sites.

1. Molecular detection of nitrate reducing functional genes (e.g. *napA* and *narG*) in all 75 strains

Nitrate reductase is the enzyme that converts nitrate to nitrite. Primers designed to detect *napA* and *narG* nitrate reductase genes (Bedzyk et al., 1999) were used to screen for the presence of nitrate reducing genes in all 75 strains. Out of the 75 strains tested, *napA* only was detected in 8 strains while *narG* only was detected in 16 strains. Three strains had both *napA* and *narG* (Appendix I).

Nitrate reductase genes (e.g. *napA* and/or *narG*) were detected by PCR amplification and sequencing in 8 (40%) of the 20 strains from brine-contaminated sites. *narG* alone was detected in 2 (10%), *napA* was detected in 4 (20%), and both nitrate reductase genes were detected in 2 strains (10%). Of the 12 strains isolated from crude oil-contaminated soil, nitrate reductase genes were detected in 5 (42%)

and all five were *narG*. Finally, out of 43 strains that were obtained from the uncontaminated prairie, nitrate reductase genes were detected in 14 strains (33%). *narG* alone was detected in 9 (21%), *napA* alone was detected in 4 (9%), and both nitrate reductase genes were detected in 1 strain (2%). Accession numbers and % similarity of the closest match after BLASTN for each sequence are listed in Appendix J.

Phylogenetic affiliation of periplasmic nitrate reductase (*napA*) and membrane bound nitrate reductase (*narG*) genes

The *napA* nucleotide sequence was obtained from 11 strains out of the 75 screened (Appendix I). The *napA* phylogenetic tree (Figure 3.3) shows the 11 strains that possess *napA* and closely related previously described strains obtained from the GenBank database. All 11 strains were grouped in 3 clusters, α-Proteobacteria (3 strains: 2 *Bosea* and 1 *Ensifer*), β- Proteobacteria (1 strain: *Achromobacter*), and γ- Proteobacteria (7 strains: 6 *Pseudomonas* and 1 *Aeromonas*) (Figure 3.3). The match similarity between the *napA* sequences of these 11 strains and *napA* sequences of other strains from GenBank ranged from 89% to 93%.

A *narG* nucleotide sequence was obtained from 20 strains (Appendix I). The *narG* phylogram (Figure 3.4) shows the 20 strains that possess *narG* and closely related previously described strains. All strains were grouped in 3 clusters,

β- Proteobacteria (2 *Achromobacter* strains), γ- Proteobacteria (16 strains: 1 *Serratia*, 9 *Stenotrophomonas*, 3 *Pseudomonas*, and 3 *Enterobacter*), and Bacilli (2 *Bacillus* strains) (Figure 3.4). The match similarity between *narG* sequences of these 20 strains and *narG* sequences of other strains from GenBank ranged from 88% to 98%.

In most cases, the genus of the strain as determined by its 16S rRNA sequence matches the genus of strain from which the *narG* sequence was determined. However, the closest match (96%) for strain I-28 was *Bacillus thuringiensis* serovar *chinensis* CT-43 (CP001907) (Appendix J) when *narG* specific primers (Table 3.1) were used. Strain I-28 was isolated from the J6 crude oil-contaminated site. Its 16S rRNA gene sequence was 99% similar to that of the γ- proteobacterium *Stenotrophomonas maltophilia* strain 776 (EU430096). To resolve this discrepancy, the strain was restreaked on a nutrient agar plate to confirm pure culture. Subsequently DNA was extracted from a single colony as described in the DNA extraction section in the Materials and Methods section. The extracted DNA was used as a template to amplify 16S rRNA and *narG* genes using the primers previously described (Table 3.1). Restreaking from a single colony, DNA extraction, and PCR amplification were done twice. Sequence analysis for the obtained PCR products confirmed the previous results for strain I-28 (e. g. 16S rRNA = *Stenotrophomonas*, *narG* = *Bacillus*) and suggests that *narG* was acquired by horizontal gene transfer (Stolz and Basu, 2002). In addition, strain I-28 was

Gram stained after restreaking from a single colony. Gram staining after restreaking showed that this strain was a Gram negative rod, as expected for *Stenotrophomonas*.

The 9 *Stenotrophomonas* strains that possessed *narG* were grouped together in both 16S rRNA and *narG* phylogenetic trees. *Pseudomonas* strains I-1, I-2, and I-65 were grouped together in the 16S rRNA tree, however, the *narG* sequence of *Pseudomonas* strains I-1 and I-2 were more similar to that of *P. fluorescens* (U71398) in *narG* phylogenetic tree compared to *Pseudomonas* strain I-65 which was more similar to *P. aeruginosa* (Y15252). *Enterobacter* strains I-25, I-31, and I-32 were members of the same 98% similarity OTU for the 16S rRNA sequence. However, *Enterobacter* strains I-31 and I-32 were more closely related to each other in *narG* phylogenetic tree (e.g. 97%) compared to *Enterobacter* strain I-25 (89%). *Achromobacter* strains I-5 and I-49 were grouped together in the 16S rRNA tree but their *narG* sequences were just 89% similar. Finally, the 2 *Bosea* strains that possess *napA* were grouped together in both16S rRNA and *napA* trees. However, *Bosea napA* closest match (e.g. 89%) was *Starkeya novella* DSM 506 (CP002026), a member also of the α-Proteobacteria.

2. Molecular detection of denitrifying functional genes (e.g. *nirS, nirK, cnorB, qnorB,* and *nosZ*)

Primers designed to detect *nirS* nitrite reductase gene (Braker et al. 1998) and *nirK* nitrite reductase gene (Qiu et al. 2004) were used to screen all 75 strains for the presence of nitrite reductase gene(s). In addition, screening with *norB* and *nosZ* primers, as another indication of denitrification genes, was applied to a subset of the chosen 32 strains that showed the ability to reduce nitrate/nitrite in the microtiter plate assay and that represented the phylogenetic diversity of the 16S rRNA. The selected 32 strains are indicated in bold letters in Appendix G.2.

Table 3.5 summarizes the results for the eight strains in which one or more denitrification functional genes were detected by PCR amplification and confirmed by sequencing. Genes coding for nitrite reductase (*nirS* or *nirK*) were detected in 4 strains (Table 3.5). Nucleotide sequences from strains I-1 or I-2 were, respectively, 96% or 97% similar to *nirS* from *Pseudomonas migulae* (DQ518189). Also, a PCR product of the correct size was amplified from *Enterobacter* strain I-25 and *Achromobacter* strain I-49 when *nirK* specific primers were used. The nucleotide sequences obtained matched 78% to *nirK* from *Rhizobium* sp.R-24654 (AM230814) and 93% to *nirK* from *Achromobacter xylosoxidans* A8 (CP002287), respectively. However, the closest match (e.g. 99%) to the *nirK* PCR product from *Enterobacter* strain I-25 was *nirK* from uncultured bacterium clone CD-40 (GU270494).

In 11 strains, *nirS* primers were able to amplify amplicons of the expected size but when sequenced, those PCR products did not match sequences of nitrite reductase genes. The product of amplification using *nirS* primers for *Pseudomonas* strains I-67 and I-68 had no significant similarity to *nirS* sequence when either the nucleotide or amino acid translated sequence was used to interrogate the GenBank database. In fact, the PCR product in *Pseudomonas* strains I-67 and I-68 showed, respectively, 90% and 89% similarity to a putative exported protein in *P. fluorescens* Pf0-1 (CP00094). In addition, there was no significant similarity between *nirS* sequence from *Pseudomonas* strains I-1 or I-2 and the PCR product, obtained by *nirS* primers, from *Pseudomonas* strains I-67 or I-68. Also, DNA from 9 *Stenotrophomonas* strains (e.g. *Stenotrophomonas* strains I-18, 34, 35, 37, 38, 39, 40, 41, and 42) was amplified using *nirS* primers but the sequences obtained had no significant similarity to *nirS* sequence when either the nucleotide or amino acid translated sequence was used to interrogate the GenBank database. Instead, the PCR product in the 9 *Stenotrophomonas* strains was 94% similar to amino acid permease-associated region porin in *S. maltophilia* R551-3 (CP001111).

Gene coding for nitric oxide reductase (c*norB*) was detected in 3 *Pseudomonas* strains (e.g. strains I-1, I-2, and I-65) and *Ensifer* strain I-4 (Table 3.5). The sequences from strains I-1 or I-2 were, respectively, 90% or 96% similar to *cnorB* from *P. fluorescens* (AJ507356) (Appendix J). In addition, the *Pseudomonas cnorB* in strain I-65 was 87% similar to *cnorB* from *P. fluorescens*

(AF197467) (Table 3.5). *Ensifer* strain I-4 sequence showed 95% similarity (e.g. highest match) to *cnorB* from *Achromobacter cycloclastes* (AJ298324) and 93% similarity to *cnorB* from *Sinorhizobium fredii* NGR234 (CP001389). A previous study detected *cnorB* in *Ensifer adhaerens* PD29 using real-time PCR (Dandie et al., 2007b). However, its PCR product was not sequenced.

I was not able to detect *qnorB* sequence using primers specific for *qnorB* (Table 3.1) in any of the 32 strains that I screened for this gene.

Primers specific for a portion of the nitrous oxide reductase gene were successful in amplifying the *nosZ* fragment from 6 strains (Table 3.5). The *nosZ* sequence of *Pseudomonas* strains I-1 and I-2 was 96% similar to *nosZ* from *Pseudomonas* sp. PD 22 (DQ377794), but the highest match was 99% similar to the *nosZ* sequence from the uncultured bacterium clone Z30O20 (EF644937). *Pseudomonas* strain I-65 sequence was 96% similar to *nosZ* from *Pseudomonas brassicacearum* subsp. *brassicacearum* NFM421 (CP002585). In addition, the *nosZ* PCR product from *Achromobacter* strain I-5 was 91% similar to *nosZ* sequence from *Achromobacter xylosoxidans* A8 (CP002287) while strain I-6 was 85% similar to *nosZ* sequence from *P. stutzeri* DSM 4166 (CP002622). Finally, *Achromobacter* strain I-49 sequence was 94% similar to *nosZ* sequence from *A. xylosoxidans* A8 (CP002287) (Appendix J).

Detection of nitrate/nitrite reducing strains

Out of the 75 strains, 58 were found that had at least one NR gene (e.g *napA*$^+$ and/or *narG*$^+$ by sequence) and/or showed their ability to reduce nitrate or nitrite based on the microtiter plate results from pure cultures (Table 3.6). Fifty four strains were shown to be NR and DN bacteria based on reduction of nitrate and/or nitrite after incubation in nitrate broth in microtiter plates, while *napA* and/or *narG* were detected in 28 strains, of which 24 strains were *napA*$^+$ and/or *narG*$^+$ and also positive for NR and DN microtiter plate results. Only 4 strains (e.g. *Bosea* strains I-7 and I-8, *Stenotrophomonas* strain I-28, and *Bacillus* strain I-60) were *napA*$^+$ or *narG*$^+$ and negative for nitrate/nitrite reduction after incubation in nitrate broth in the microtiter plate assay.

Distribution of NR and DN strains

Multiple genera of NR and DN bacteria were isolated from contaminated and uncontaminated sites. In fact, 10 different genera were isolated from brine-contaminated sites, 6 from oil-, and 9 genera were isolated from prairie uncontaminated sites (Table 3.6). Even with some differences in the occurrence of various genera, contaminated sites were not less diverse than prairie. With 3 or more isolates, most genera occurred in more than one type of site, with the exception of *Enterobacter* (only in oil contaminated sites). However, isolates of Bacilli did not occur in the oil contaminated sites, but 6 strains were obtained from brine-contaminated sites (Table 3.6). Although library coverage was incomplete

with the highest coverage at 80% in G5P, and diversity was low; based on estimates of the classical Shannon diversity index and the inverse of the Simpson index, prairie sites were not more diverse, compared to the parallel contaminated sites where library coverage was comparable (Table 3.7, see G5 vs. G5P, G7 vs. G7P, and LF vs. LFP).

Discussion

This study represents an investigation of the diversity of NR and DN bacteria isolated from tallgrass prairie soils and their nitrate reduction/denitrification pathway genes. Nitrate reducing/denitrifying bacteria were readily isolated from oil and/or brine contaminated soils, after enrichment in nitrate broth, indicating that the contamination did not eliminate these important functional groups of bacteria. Although the use of nitrate broth containing high levels of proteinous components is biased towards isolation of certain genera, strains belonging to these genera have been traditionally used for studies of denitrification, and the PCR primers were developed using strains from these commonly cultured genera. Therefore, the primer sets were expected to detect these groups. However, no NR or DN functional genes were detected in many of these strains by PCR amplification with NR or DN functional gene primers.

When I combined the culture-based method (e.g. microtiter plate assay) and the molecular detection method (e.g. PCR amplification), the percentage of the detected NR and DN bacteria using the cultured and/or molecular detection was somewhat higher in the contaminated sites compared to that in the uncontaminated sites. The proportions of NR and DN bacteria were 95% (19 out of 20 strains) in the brine-contaminated sites, 83% (10 out of 12 strains) in the crude oil-contaminated sites, and 67% (29 out of 43 strains) in the uncontaminated prairie sites (Appendix G.2). The phylogenetic affiliation of the 75 strains supported my

hypothesis that some groups (e.g. Bacilli) that are known to be salt tolerant (Boch et al., 1997) were found in brine contaminated soils but not in crude oil contaminated soils. However, all of the Bacilli were isolated from 10^{-1} and 10^{-2} wells, suggesting that they were not the most abundant culturable NR and DN bacteria in the brine contaminated sites. In addition, no members of the α-, β-Proteobacteria, and Bacilli were obtained from crude oil contaminated sites. Instead, the majority of strains were members of the γ-Proteobacteria.

One of the reasons for the higher relative abundance in the proportions of γ-Proteobacteria NR and DN bacteria in the oil contaminated sites may be due to the ability of these groups of bacteria to utilize readily available carbon sources, in this case hydrocarbons, under nitrate reducing/denitrifying conditions. In fact, Dandie et al (2007b) have shown that a group of *cnorB*$^{+}$ *Pseudomonas mandelii* and closely related strains increased their relative percentage of the total population after the addition of glucose-C. This group of bacteria also was able to increase their proportion in the total population when they were incubated under denitrifying conditions after the addition of glucose-C. On the other hand, the same study showed that a different group of *cnorB*$^{+}$ bacteria (e.g. *Bosea*, *Bradyrhizobium*, and *Ensifer* spp, all α-Proteobacteria) was not able to increase its proportion in the total population in response to the addition of glucose-C. The relative percentage for this group of bacteria of the total population did not increase when they were incubated under denitrifying conditions after the addition of glucose-C (Dandie et al., 2007b).

Dandie et al (2007b) results suggest that different groups of denitrifying bacteria respond differently to the addition of carbon sources even under denitrifying conditions. Granted, the commonly utilized substrate, glucose, used in Dandie et al (2007b) is different from hydrocarbons in my study. However, the comparison was based on that both glucose and hydrocarbons are available carbon sources. In my NR and DN study, *Pseudomonas* strains (all NR or DN) were isolated from prairie sites (OTU3) as well as from oil and brine (OTU11 and OTU15), and from both oil and prairie (OTU19). The NR and DN isolates from prairie sites were dominated by γ-Proteobacteria, those that were most abundant were *Pseudomonas* (3 NR strains originated from 10^{-4} wells), not *Stenotrophomonas* (1 NR strain originated from 10^{-3} wells). *Stenotrophomonas* was often isolated from the nitrate broth enrichments, but from lower dilution wells (10^{-2}) or did not reduce nitrate or nitrite. My *Pseudomonas* strains were affiliated with *P. fluorescens*, *P. frederiksbergensis*, *P. putida*, and with *Pseudomonas* sp. WPCB087 (Figure 3.2.a), not with *P. mandelii* in Dandie et al (2007b). In my study, *Ensifer* and *Bosea* (both α-Proteobacteria) strains were isolated from brine-contaminated sites, not oil-contaminated sites. Thus, my results seem to fit that oil-contaminated sites were dominated by γ-Proteobacteria not α-Proteobacteria. However, it is not known whether hydrocarbons represent a readily usable carbon source for my NR and DN strains.

16S rRNA phylogeny and the reduction of nitrate/nitrite

The 16S rRNA sequence results showed that the 58 studied strains were well-represented among γ-Proteobacteria especially the genera of *Stenotrophomonas* (nitrate reducer) and *Pseudomonas* (nitrate/nitrite reducer). My results were consistent with previous studies regarding the presence of certain groups of NR and DN bacteria in oil/brine contaminated soils. In different culture-based studies on the composition of NR bacteria communities, *Bacillus, Moraxella,* and *Pseudomonas* were isolated from the sediment samples from the rhizosphere of the aerenchymatous plant *Glyceria maxima* **(**Nijburg et al., 1997). A larger diversity was observed when *Streptomyces, Bacillus,* and *Enterobacteriaceae* strains were isolated from abandoned and reclaimed mine soils (Shirey and Sextone, 1989). However, a recent study showed that DN bacteria were well-represented among members of the genus *Bacillus* under anaerobic conditions (Verbaendert et al., 2011).

Variation in nitrate/nitrite reduction capability within *Stenotrophomonas*

In the 58 strains that had at least one NR gene (e.g *napA*$^+$ and/or *narG*$^+$ by sequence) and/or showed the ability to reduce nitrate or nitrite based on the microtiter plate results, most of the strains among *Stenotrophomonas* (17 out of 18 strains) reduced nitrate. However, previous studies have shown that different species of *Stenotrophomonas* vary in their ability to reduce nitrate and/or nitrite, even among closely related species. For example, *S. acidaminiphila* (AF273080)

has the ability to reduce nitrate and nitrite (Assih et al., 2002), while *Stenotrophomonas maltophilia* strain c20 (AJ293469) (Minkwitz and Berg, 2001) and *S. rhizophila* strain e-p10 (AJ293463) (Minkwitz and Berg, 2001) are nitrate reducers. On the other hand, *S. humi* strain R-32729T (AM403587) and *S. terrae* type strain R-32768T (AM403589) reduce nitrate but they do not denitrify (Heylen et al., 2007). Whereas *S. chelatiphaga* strain LPM-5 (EU573216) (Kaparullina et al., 2009), *S. nitritireducens* strain L2 (AJ012229) (Finkmann et al., 2000), and *Stenotrophomonas* sp. YC-1 (DQ537219) (Yang et al., 2006b) do not reduce nitrate. Finally, *S. koreensis* (AB166885) does not reduce either nitrate or nitrite (Yang et al., 2006a).

Predictive power of detecting nitrate reducing genes

narG was detected in a wider phylogenetic range of NR and DN bacteria than was *napA*. Strains from which *napA* was amplified were all Proteobacteria (e.g. α-, β-, and γ- Proteobacteria) while strains with *narG* included Proteobacteria (e.g. β- and γ- Proteobacteria) and Firmicutes. Similar results were obtained from previous studies, e.g. *narG* was present in Proteobacteria, Firmicutes, Actinobacteria, and even Archaea while *napA* was present only in Proteobacteria (Philippot and Hojberg, 1999; Richardson et al., 2001). Both *napA* and *narG* were detected in 3 strains (γ- Proteobacteria *Pseudomonas* strains I-1 and I-2 and β-Proteobacteria *Achromobacter* strain I-49). Similar studies indicated that various NR Proteobacteria including *Pseudomonas* can harbor *napA* or *narG* or both

(Philippot, 2002; Zumft, 1997). One study indicated the presence of both *napA* and *narG* in the α- proteobacterium *Paracoccus denitrificans* (Bell et al., 1990).

The detection of *napA*/*narG* may be a good indicator of nitrate/nitrite reduction for only certain groups of the strains. While nitrate reductase gene(s) was/were detected in just 24 strains out of the 54 (44%) that reduced nitrate/nitrite, detection of *napA*/*narG* corresponded to the ability to reduce nitrate/nitrite in all 21 γ-Proteobacteria strains.

Detectable fragments of NR genes may not be sufficient to indicate the species composition of culturable NR bacteria. In fact, testing the phenotype of NR bacteria along with molecular detection of the functional NR genes may be a more comprehensive procedure to detect a broader range of these microorganisms. My results show that the microtiter plate assay is a better method to detect culturable NR and DN bacteria than the PCR method. In fact, 54 strains out of 75 were detected as NR and DN bacteria using the microtiter plate assay while just 28 strains out of 75 were detected as NR bacteria using the sequenced PCR product. Therefore, the *napA*/*narG* primers used would fail to detect roughly half of easily culturable NR and DN bacteria.

In most cases, the genus of the strain as determined by its 16S rRNA sequence is the same as the closest match for its *napA* sequence. *Bosea* (α-

Proteobacteria) stains I-7 and I-8 closest match (e.g. 89%) was *Starkeya novella* DSM 506 (CP002026) (α-Proteobacteria) (Appendix J) when *napA* specific primers (Table 3.1) were used. However, not all species have been surveyed for *napA*. *Bosea thiooxidans* (strain BI-42) gen. nov., sp. nov. was found to be able to denitrify and produce gas (e.g. N_2 or N_2O) in heterotrophic growth medium containing nitrate (Das et al., 1996). Nevertheless, no *napA* sequences of named *Bosea* species have been reported.

Underestimation of NR and DN bacteria by molecular detection

The lack of amplification of NR and DN gene fragments from strains known to be capable of nitrate reduction or denitrification has been previously reported (Dandie et al., 2007a; Klatte et al., 2011). In the microtiter plate assay, 4 strains did not reduce nitrate/nitrite although they possess at least one nitrate reductase gene (Appendix G.2). However, 30 strains reduced nitrate/nitrite when grown in nitrate broth in the microtiter plate assay although nitrate reductase genes were not detected in any of these 30 strains suggesting that the primers used in this study likely are not complementary to the nitrate reductase genes in these 30 strains. However, the presence of denitrification gene sequences is not adequate to conclude that denitrification activity exists. Even with having more than one gene in the denitrification pathway, microorganisms may still lack the ability to denitrify due to their inability to express the genes they possess because those genes must be transcribed and translated into the denitrification enzymes and these enzymes must

be active under the given environmental conditions. In addition, microbes may possess an incomplete denitrification pathway (e.g. the absence of one or more denitrification genes in the denitrification pathway) and as a result denitrification stops before the production of nitrogen gas (Fernandes et al., 2010). Also, the denitrification pathway genes may be borne on a plasmid. In fact, horizontal gene transfer (HGT) of denitrification genes was shown previously (Chan and McCormick, 2004) which indicates that bacteria can acquire or lose some denitrification genes.

Possible overestimation of nitrite reductase using *nirS* primers

Tallgrass Prairie sites with an abundance of *Stenotrophomonas* might overestimate *nirS* copies using *nirS* primers. PCR products of the correct size were amplified from 9 *Stenotrophomonas* strains. However, when those PCR products were sequenced, they were not nitrite reductase genes which indicates that a part of the amplicon has the same DNA sequence as the *nirS* primers. Although *Stenotrophomonas* was one of the most abundant culturable NR bacteria in this study, *nirS* gene was not detected in any *Stenotrophomonas* strain. These results suggest verification by sequencing of the PCR product if *nirS* primers are used to detect nitrite reductase in *Stenotrophomonas* strains.

cnorB best predictor of nitrite reducing phenotype

cnorB appeared to be the best representative of all tested denitrifying functional genes with respect to predicting the ability to reduce nitrite, followed by *nosZ* and finally *nirS*/*nirK*. *cnorB* was detected in all four strains (e.g. *Pseudomonas* strains I-1, I-2, and I-65 and *Ensifer* strain I-4) shown to reduce nitrite under my test conditions (e.g. microtiter plate assay). Previous studies showed that *cnorB* was amplified from *P. stutzeri* ZoBell ATCC 14405 (Z28384) (Viebrock and Zumft, 1988) and from *P. fluorescens* strain C7R12 (AF197467) (Philippot et al., 2001).

Nitrous oxide reductase (*nosZ*) gene was detected in 3 *Pseudomonas* strains that were all shown to reduce both nitrate and nitrite. In fact, *nosZ* was amplified previously from strains in the genus *Pseudomonas* such as *P. aeruginosa* DSM50071 (X65277) and *P. stutzeri* ATCC14405 (X53676) (Zumft et al., 1990). Moreover, *nosZ* gene was detected in 2 *Achromobacter* strains. Both strains were able to reduce nitrate, and *nosZ* has been cloned and sequenced previously from the denitrifying bacterium *Achromobacter cycloclastes* (Inatomi, 1998). Finally, *nosZ* gene was detected in *Aeromonas* strain I-6. To the best of my knowledge, a *nosZ* gene sequence has never been detected in *Aeromonas*. My 16S rRNA and *nosZ* gene sequence analysis were consistent in terms of phylogenetic affiliations. In fact, a different study showed that taxonomic and *nosZ* phylogenetic relationships were in a good agreement for most of the strains (Rich et al., 2003).

Effect of different electron acceptors on the detection of denitrifyers: a problem with *Bacillus*?

In my study, I used one electron acceptor, nitrate, to enrich for NR and DN bacteria. Since soil may contain different electron acceptors for denitrifyers, the use of several electron acceptors may increase the detection of denitrifyers. Using both nitrate and nitrite was crucial in detecting the denitrification potential for *Bacillus* reference strains (Verbaendert et al., 2011). For example, in Verbaendert et al (2011), 2 *Bacillus* strains were detected as denitrifyers when nitrite was used as an electron acceptor. These *Bacillus* strains were LMG 12363T whose 16S rRNA gene sequence was 100% similar to that of *Bacillus licheniformis* (X68416), and LMG 21831T whose 16S rRNA gene sequence was 98.7-100% similar to that of *Bacillus drentensis* (AJ542506). Using just one denitrifying electron acceptor (e.g nitrate) in this study may be a limiting factor that caused this low detection for denitrifyers in the Bacilli. Although 14 Bacilli were isolated after enrichment in nitrate broth, and 12 of those were among the 58 NR and DN strains, none of them was confirmed to be a nitrite reducer after testing in nitrate broth. An alternative pathway for some *Bacillus* strains is the reduction of nitrate via nitrite to ammonia. In fact it was shown that *B. subtilis* reduced nitrate via nitrite to ammonia during anaerobic nitrate respiration where benzyl viologen was the electron donor and nitrite was the electron acceptor (Hoffmann et al., 1998). The pathway uses the ResDE system coded by *resDE*.

Finally, my isolation of NR and DN bacteria shows that they were not eliminated by brine/oil contamination although certain groups were relatively more or less abundant in oil or brine sites than in the parallel prairie sites. However, whether this shift in species composition affects the loss of nitrogen from tallgrass prairie soil in the field is not known. Future research should investigate if an increased loss of nitrogen from soils via nitrate reduction and denitrification is a long-term impact of contamination by crude-oil and brine. Also, around 40% of the strains shown to reduce nitrate/nitrite would not be identified as NR or DN bacteria by the battery of the functional NR and DN primers used. This low detection indicates that PCR-based molecular detection underestimates the number of NR and DN bacteria and that a complementary method (e.g. physiology-based) is needed for a more sufficient detection of NR and DN bacteria.

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Table 3.1. Summary of primers used to detect denitrification functional genes.

Primer[a]	Primer sequence (5'-3')	Organism[b] (Accession #)	Nucleotide position[c]	Size of the PCR fragment (bp)	Annealing temperature (˚C)	Reference
napA-F1	CTGGACIATGGGYTTIAACCA	*Escherichi coli* O6	1107-1129	492	52˚C	1
napA-R1	CCTTCYTTYTCIACCCACAT	(Q8CVW4)	1579-1598			
narG-F1	ACICAYGGIGTIAACTGYAC	*Escherichi coli* W3110	145-164	523	52˚C	1
narG-R1	TCGSMRTACCAGTCRTARAA	(BAA36094)	649-668			
nirS 1F	CCTAYTGGCCGCCRCART	*Pseudomonas stutzeri* ZoBell	763-780	890	Touchdown	2
nirS 6R	CGTTGAACTTRCCGGT	(X56813)	1638-1653		56-51˚C	
nirK F	TCATGGTCCTGCCGCGYGACGG	*Alcaligenes faecalis*	1319-1337	329	Touchdown	3
nirK R	GAACTTGCCGGTNGCCCAGAC	(D13155)	1668-1648		63-53˚C	
cnorB 2F	GACAAGNNNTACTGGTGGT	*Pseudomonas denitrificans* Pd1222	553-571	389	Touchdown	4
cnorB 6R	GAANCCCCANACNCCNGC	(U28078)	942-925		57-52.5˚C	
qnorB 2F	GGNCAYCARGGNTAYGA	*Ralstonia eutropha* H16	1204-1220	637	Touchdown	4
qnorB 7R	GGNGGRTTDATCADGAANCC	(AF002661)	1841-1822		57-52.5˚C	
nosZ-F-1181	CGCTGTTCITCGACAGYCAG	*Pseudomonas stutzeri*	1463-1482	680	56˚C	5
nosZ-R-1880	ATGTGCAKIGCRTGGCAGAA	(M22628)	2162-2143			

[a]Forward and reverse primers are indicated by F and R as the last letter, respectively

[b] Strains used for PCR positive control as indicated in the references

[c]Positions in the nitrate reducing/denitrifying functional genes of the corresponding positive control microorganisms.

1. Alcántara-Hernández et al. (2009).

2. Braker et al. (1998).

3. Qiu et al. (2004).

4. Braker and Tiedje (2003).

5. Rich et al. (2003).

Table 3.2. Distribution of nitrate reducing (NR) and denitrifying (DN) strains isolated from different soil types.

Soil source	NR	DN	None	Total
Brine	14	3	3	20
Oil	9	0	3	12
Prairie	25	3	15	43
Total	48	6	21	75

* Nitrate/nitrite reduction determined using Griess reagents (Smibert and Krieg, 1994) after incubation in nitrate broth as described in Materials and Methods.

None: strain did not reduce nitrate or nitrite.

Brine: isolated from sites contaminated primarily by oil field brine, G5 and G7.

Oil: isolated from sites contaminated by crude oil, J6-F, J6-NF, and LF.

Prairie: isolated from uncontaminated sites, G5P, G7P, J6P, and LFP.

Table 3.3. Summary of the 75 strains by genus.

Taxonomic Group	Genus* (Total # of strains)	NR[a]	DN[b]	None	Brine	Oil	Prairie
γ-Proteobacteria	*Stenotrophomonas* (27)	16	1	10	3	5	19
	Pseudomonas (9)	6	3	0	4	1	4
	Aeromonas (1)	1	0	0	1	0	0
	Serratia (1)	1	0	0	0	1	0
	Enterobacter (3)	3	0	0	0	3	0
	Acinetobacter (5)	2	0	3	0	0	5
α-Proteobacteria	*Ensifer* (1)	0	1	0	1	0	0
	Bosea (2)	0	0	2	2	0	0
	Brevundimonas (1)	1	0	0	1	0	0
	Sinorhizobium (1)	0	1	0	0	0	1
β-Proteobacteria	*Achromobacter* (4)	4	0	0	1	0	3
	Burkholderia (1)	0	0	1	0	0	1
Actinobacteria	*Arthrobacter* (1)	0	0	1	1	0	0
	Rhodococcus (1)	0	0	1	0	0	1
	Microbacterium (1)	1	0	0	0	1	0
	Kocuria (1)	1	0	0	0	0	1
Bacilli	*Brevibacillus* (8)	7	0	1	3	0	5
	Bacillus (3)	2	0	1	2	0	1
	Paenibacillus (1)	1	0	0	1	0	0
	Lysinibacillus (2)	1	0	1	0	0	2
Flavobacteria	*Chryseobacterium* (1)	1	0	0	0	1	0
	Total: 75	48	6	21	20	12	43

*The Ribosomal Database Project (RDP) Classifier. All classifications were at the 98% threshold level or higher.

[a]Nitrate Reducers.

[b]Nitrite Reducers.

None: strain did not reduce nitrate or nitrite.

Brine: isolated from sites contaminated primarily by oil field brine, G5 and G7.

Oil: isolated from sites contaminated by crude oil, J6-F, J6-NF, and LF.

Prairie: isolated from uncontaminated sites, G5P, G7P, J6P, and LFP.

Table 3.4. Distribution of OTUs among sites.

OTU # (16S rRNA genus)*	Total # sequences	Brine	Oil	Prairie
1 (*Stenotrophomonas*)	20	1	3	16
2 (*Enterobacter*)	3	0	3	0
3 (*Pseudomonas*)	3	0	0	3
4 (*Burkholderia*)*	1	0	0	1
5 (*Stenotrophomonas*)	5	2	1	2
6 (*Rhodococcus*)*	1	0	0	1
7 (*Brevibacillus*)	8	3	0	5
8 (*Lysinibacillus*)*	1	0	0	1
9 (*Achromobacter*)	1	0	0	1
10 (*Acinetobacter*)*	2	0	0	2
11 (*Pseudomonas*)	2	1	1	0
12 (*Arthrobacter*)*	1	1	0	0
13 (*Chryseobacterium*)	1	0	1	0
14 (*Paenibacillus*)	1	1	0	0
15 (*Pseudomonas*)	1	1	0	0
16 (*Lysinibacillus*)	1	0	0	1
17 (*Bacillus*)	3	2	0	1
18 (*Stenotrophomonas*)	1	0	0	1
19 (*Pseudomonas*)	3	2	0	1
20 (*Stenotrophomonas*)	1	0	1	0
21 (*Sinorhizobium*/*Ensifer*)	2	1	0	1
22 (*Bosea*)*	2	2	0	0
23 (*Achromobacter*)	3	1	0	2
24 (*Serratia*)	1	0	1	0
25 (*Microbacterium*)	1	0	1	0
26 (*Acinetobacter*)	3	0	0	3
27 (*Kocuria*)	1	0	0	1
28 (*Aeromonas*)	1	1	0	0
29 (*Brevundimonas*)	1	1	0	0

*No strains in this OTU are NR or DN.

16S rRNA gene sequences of the 75 strains were clustered as operational taxonomic units (OTUs) at an overlap identity cut-off of 98% by MOTHUR software ver1.23.

Brine: isolated from sites contaminated primarily by oil field brine, G5 and G7.

Oil: isolated from sites contaminated by crude oil, J6-F, J6-NF, and LF.

Prairie: isolated from uncontaminated sites, G5P, G7P, J6P, and LFP.

Table 3.5. Strains containing denitrifying gene sequences.

Strain # (Genus)	Soil Type	*nirS*	*nirK*	*cnorB*	*nosZ*	NR and DN*
I-1 (*Pseudomonas*)	Brine	Yes	No	Yes	Yes	DN
I-2 (*Pseudomonas*)	Brine	Yes	No	Yes	Yes	DN
I-4 (*Ensifer*)	Brine	No	No	Yes	No	DN
I-5 (*Achromobacter*)	Brine	No	No	No	Yes	NR
I-6 (*Aeromonas*)	Brine	No	No	No	Yes	NR
I-25 (*Enterobacter*)	Oil	No	Yes	No	No	NR
I-49 (*Achromobacter*)	Prairie	No	Yes	No	Yes	NR
I-65 (*Pseudomonas*)	Prairie	No	No	Yes	Yes	DN

NR: Nitrate Reduction.

DN: Nitrite reduction/denitrification.

Genus: 16S rRNA sequence as classified by The RDP Classifier.

*Loss of nitrate (NR) or nitrite (DN) after incubation in nitrate broth.

nirS: Cytochrome cd1-nitrite reductase.

nirK: Copper nitrite reductase.

cnorB: Nitric oxide reductase gene.

nosZ: nitrous oxide reductase gene.

Table 3.6. Summary of 58 NR and DN strains by genus.

Taxonomic Group	Genus (RDP Classifier) (Total # of strains)	NR	DN	None	Brine	Oil	Prairie
γ-Proteobacteria	*Stenotrophomonas* (18)	16	1	1*	3	3	12
	Pseudomonas (9)	6	3	0	4	1	4
	Aeromonas (1)	1	0	0	1	0	0
	Serratia (1)	1	0	0	0	1	0
	Enterobacter (3)	3	0	0	0	3	0
	Acinetobacter (2)	2	0	0	0	0	2
α-Proteobacteria	*Ensifer* (1)	0	1	0	1	0	0
	Bosea (2)	0	0	2*	2	0	0
	Brevundimonas (1)	1	0	0	1	0	0
	Sinorhizobium (1)	0	1	0	0	0	1
β-Proteobacteria	*Achromobacter* (4)	4	0	0	1	0	3
Actinobacteria	*Microbacterium* (1)	1	0	0	0	1	0
	Kocuria (1)	1	0	0	0	0	1
Bacilli	*Brevibacillus* (7)	7	0	0	3	0	4
	Bacillus (3)	2	0	1*	2	0	1
	Paenibacillus (1)	1	0	0	1	0	0
	Lysinibacillus (1)	1	0	0	0	0	1
Flavobacteria	*Chryseobacterium* (1)	1	0	0	0	1	0
	Total: 58	48	6	21	20	12	43

NR: Nitrate Reducers.

DN: Nitrite Reducers.

*None: strain did not reduce nitrate or nitrite. Four strains (e.g. *Bosea* strains I-7 and I-8, *Stenotrophomonas* strain I-28, and *Bacillus* strain I-60) were *napA*$^+$ or *narG*$^+$ and negative for nitrate/nitrite reduction after incubation in nitrate broth in microtiter plate.

Brine: isolated from sites contaminated primarily by oil field brine, G5 and G7.

Oil: isolated from sites contaminated by crude oil, J6-F, J6-NF, and LF.

Prairie: isolated from uncontaminated sites, G5P, G7P, J6P, and LFP.

Table 3.7. Values for diversity indices for the 16S rRNA sequences from the 58 NR and DN strains.

Site (type)	# OTU (98%)*	# Sequences	% Library Coverage[a]	NpShannon[b]	InvSimpson[c]
G5 (brine)	6	10	70	2.15	9 (4.84-64.18)
G5P (prairie)	3	10	80	1	1.61 (1.03-3.61)
G7 (brine)	7	9	44	2.78	18 (7.85-61.65)
G7P (prairie)	5	7	42	2.32	7 (2.85-15.25)
J6 (oil)	4	5	40	2.28	10 (3.69-14.11)
J6P (prairie)	3	3	0	0	1 (1-1)
LF (oil)	4	5	40	2.28	10 (3.69-14.11)
LFP (prairie)	6	9	56	2.31	9 (4.21-65.52)

*98% 16S rRNA gene sequence similarity.

[a] C: Good's measure of library coverage.

$$C = 1 - \frac{n_i}{N!}$$

where,

n_i = the number of OTUs that have been sampled once

N = the total number of individuals in the sample

[b]NpShannon: non-parametric estimate of the classical Shannon diversity index (H^).

$$H_{shannon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} ln \frac{n_i}{N}$$

[c]InvSimpson index = inverse of the Simpson diversity index, $D_{simpson} = 1/D_{simpson}$

$$D_{simpson} = \frac{\sum_{i=1}^{S_{obs}} n_i\,(n_i - 1)}{N\,(N-1)}$$

See Materials and Methods.

OTU: sequences grouped at 98% similarity.

The MOTHUR software package (Schloss et al., 2009) was used to calculate observed richness (OTUs) and diversity (% library coverage, *H*^, Simpson).

G5: Brine; G5P: Prairie; G7: Brine; G7P: Prairie; J6: Oil; J6P: Prairie; LF: Oil; and LFP: Prairie.

Figure 3.1.a. Phylogenetic affiliation of 75 strains isolated from tallgrass prairie soils.

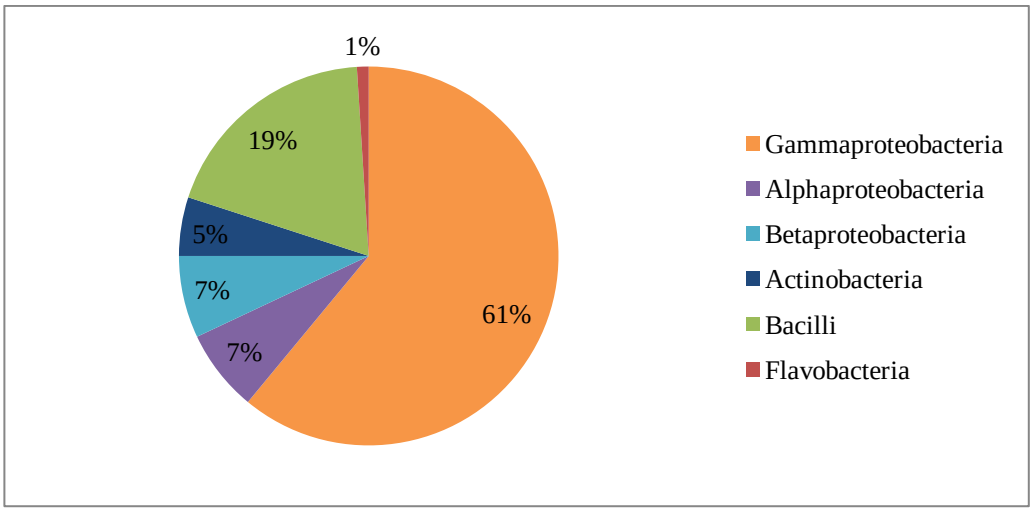

Figure 3.1.b. The relative abundance of the 75 strains from each sample type.

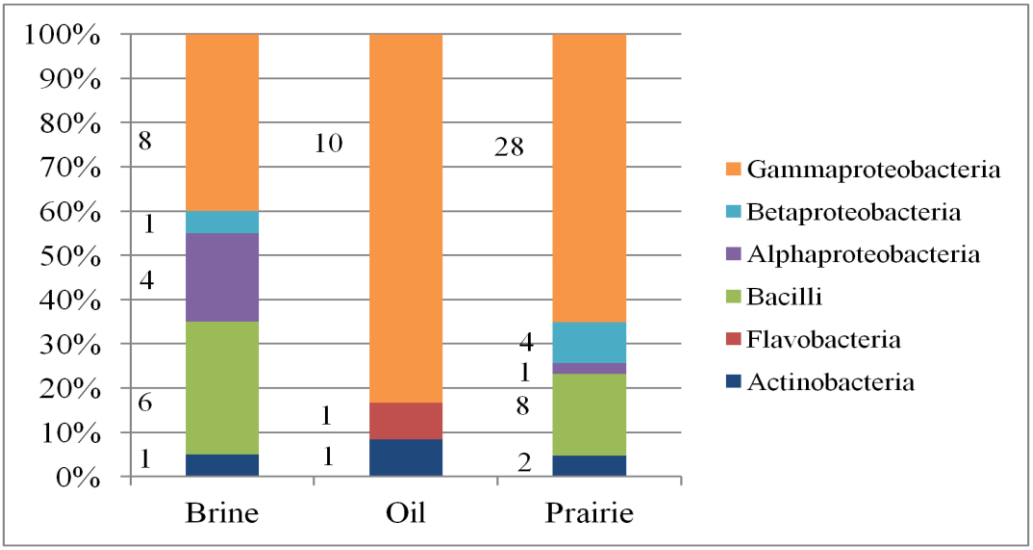

Figure 3.1. a. The relative abundance of the 75 strains in different bacterial taxonomic groups. b. Frequency and number of strains affiliated with different bacterial groups found in oil-, brine-, and prairie-soils. Seventy five strains were classified by the RDP Classifier tool. The number of strains corresponding to each group is indicated close to the graph bar (Figure 3.1.b).

Figure 3.2.a. γ-Proteobacteria phylogenetic tree based on 16S rRNA gene sequence.

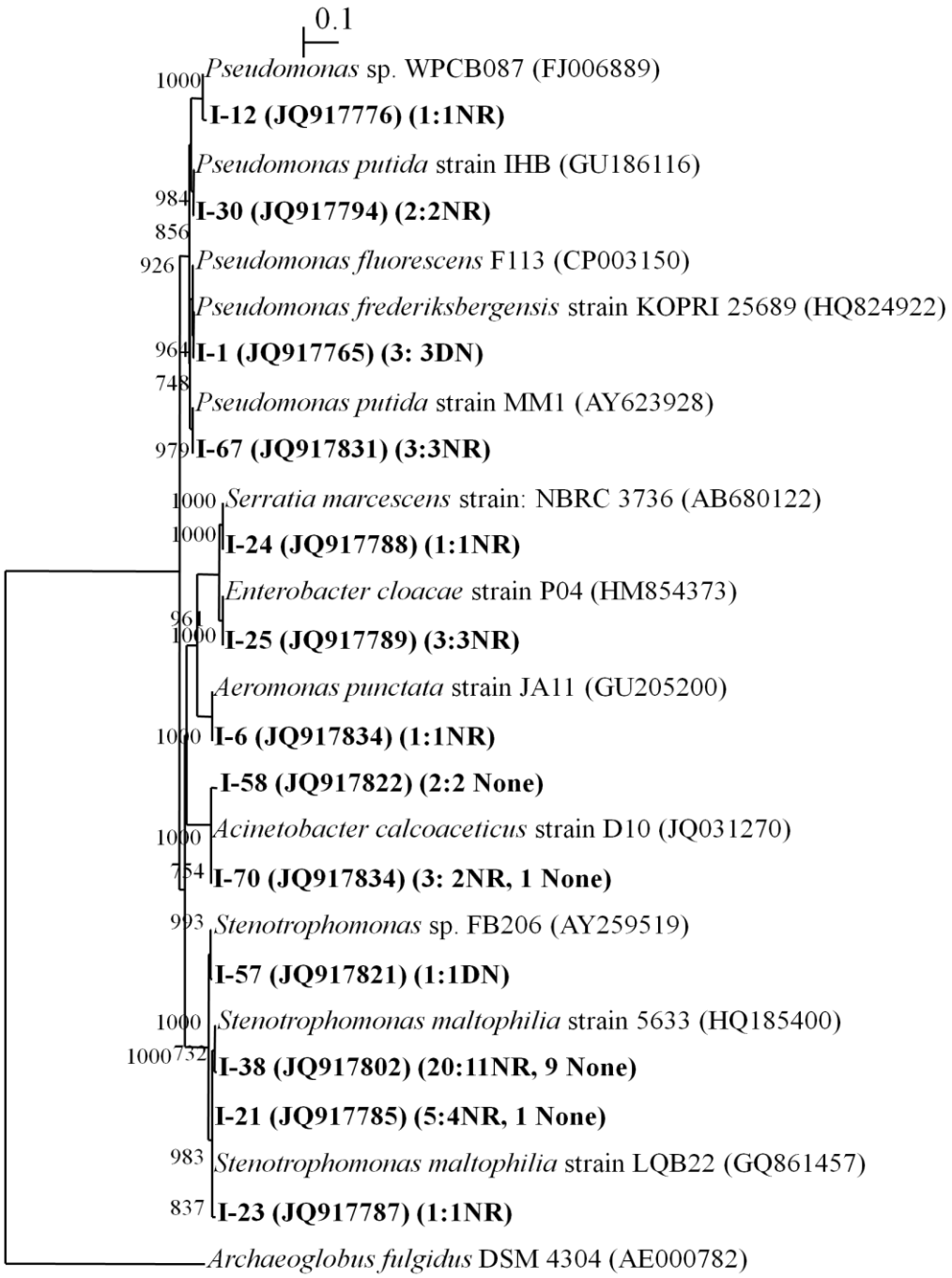

Figure 3.2.b. Phylogenetic tree based on 16S rRNA gene sequence of all groups except the γ-Proteobacteria.

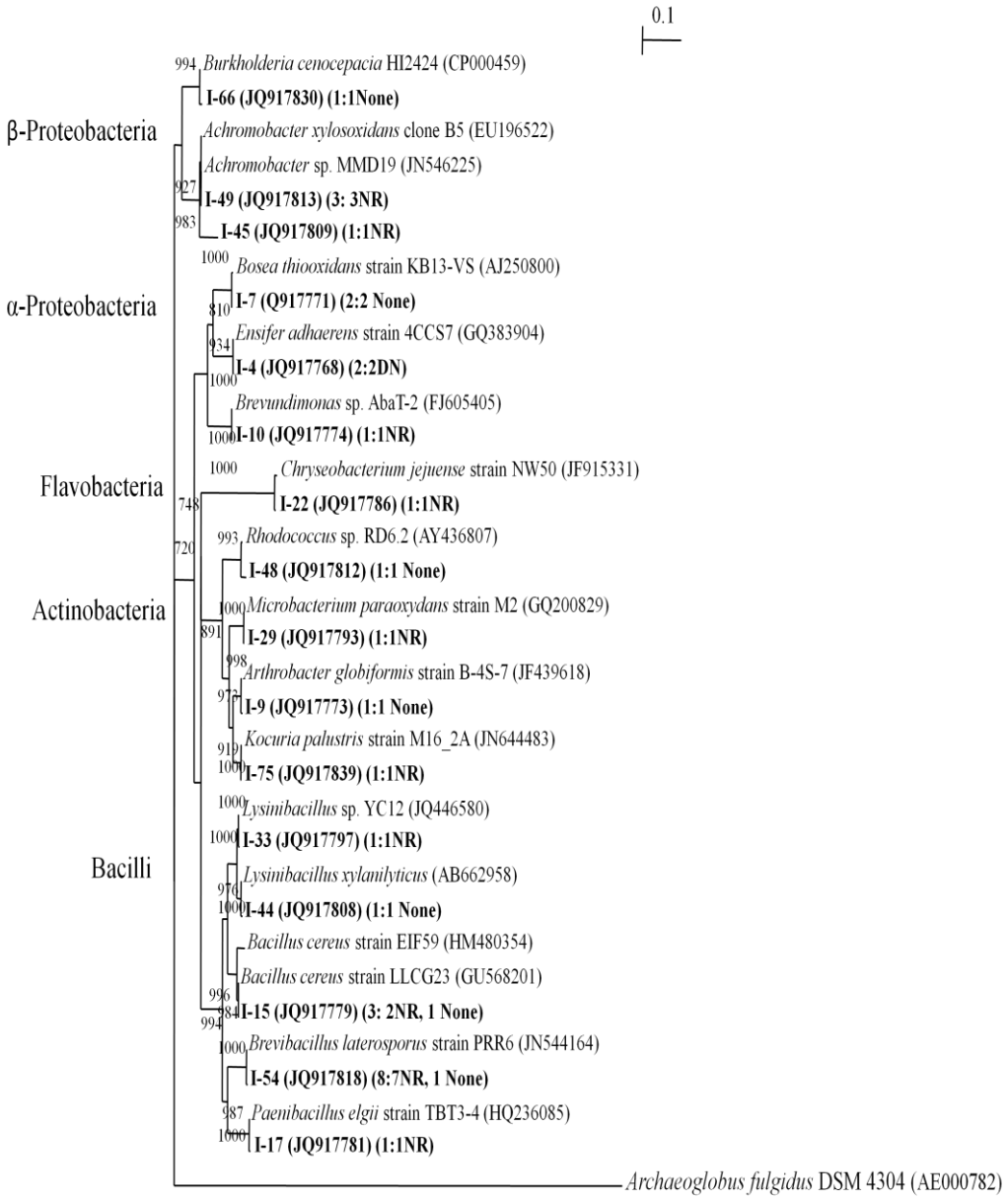

Figure 3.2. Phylogenetic tree of the 16S rRNA gene of 75 bacterial strains isolated from tallgrass prairie soils with respect to related sequences. There were 29 OTUs at the 98% level of similarity. One strain representing each OTU was used for phylogenetic analysis. The number of strains contained in each OTU is included between parenthesis next to the representative strain. The numbers of strains of NR (nitrate reducing) bacteria, DN (nitrite reducing) bacteria, and None (strains that did not reduce nitrate or nitrite) are also included next to the total number of strains included in the OTU. The tree is constructed from approximately 1400 bp 16S rRNA gene sequence using the neighbor-joining algorithm. One thousand bootstrap replications were performed; only values greater than 700 are shown. Figure 3.2.a (Bar: 0.1 nucleotide substitutions per nucleotide) shows the γ-Proteobacteria where the sequence of *Archaeoglobus fulgidus* DSM 4304 (AE000782) was included as the outgroup, while Figure 3.2.b (Bar: 0.1 nucleotide substitutions per nucleotide) shows the remaining groups where the sequence of *Archaeoglobus fulgidus* DSM 4304 (AE000782) was included as the outgroup.

Figure 3.3. Phylogenetic tree based on *napA* gene sequence.

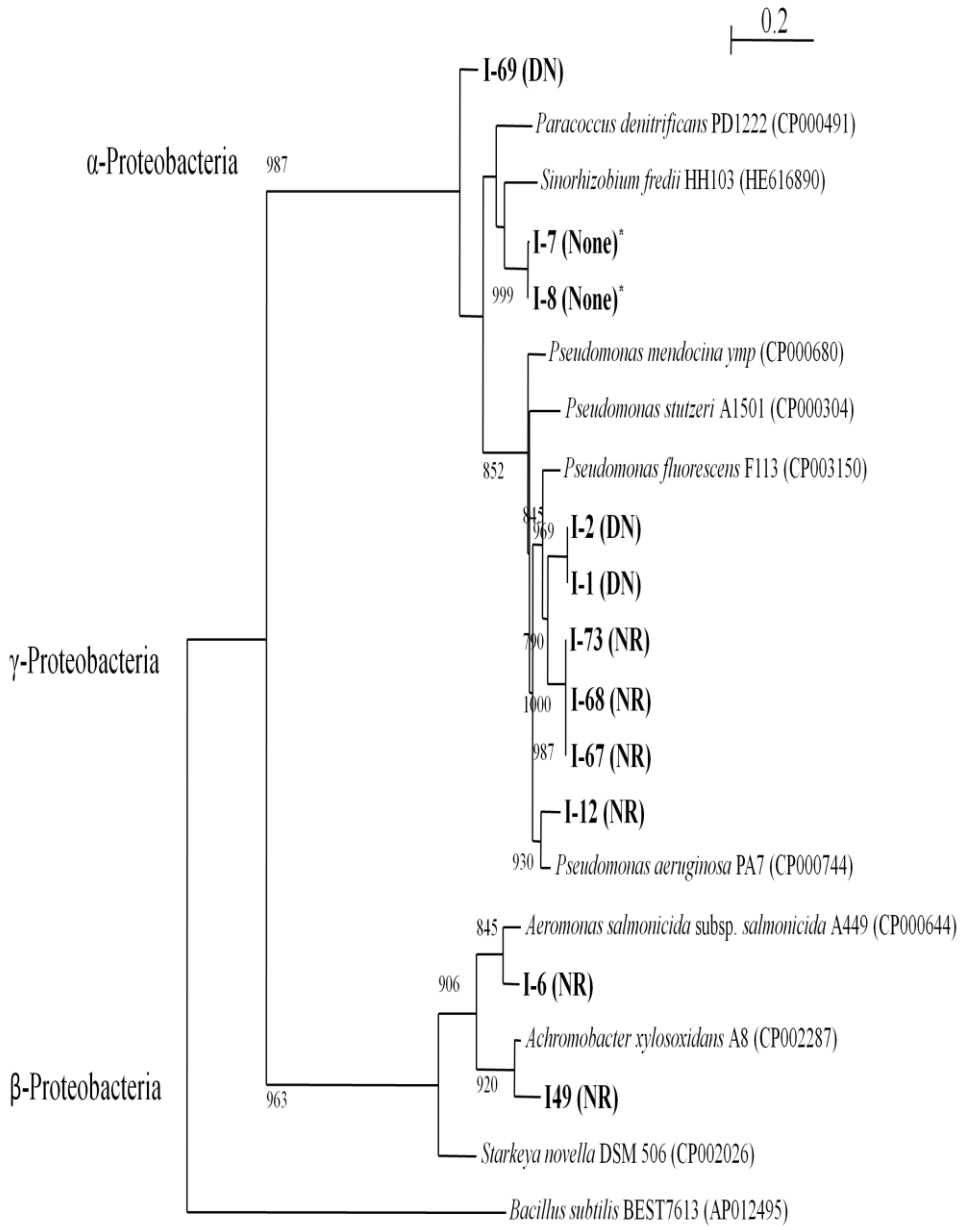

Figure 3.3. Phylogenetic tree of the *napA* gene of bacterial strains with respect to their related nucleotide sequences. The tree is constructed from approximately 492 bp *napA* gene sequence using the neighbor-joining algorithm. One thousand bootstrap replications were performed; only values greater than 700 are shown. NR: nitrate reducer, DN: nitrite reducer, and None: did not reduce nitrate nor nitrite based on the microtiter plate test. "I" is the strain number as shown in Appendix I. *100% similarity to 16S rRNA of *Bosea* based on 16S rRNA RDP classifier. Strain I-49 and *A. xylosoxidans* A8 (CP002287) contain both *napA* and *narG*. The sequence of *Bacillus subtilis* BEST7613 (AP012495) was included as the outgroup. Bar: 0.2 nucleotide substitutions per nucleotide.

Figure 3.4. Phylogenetic tree based on *narG* gene sequence.

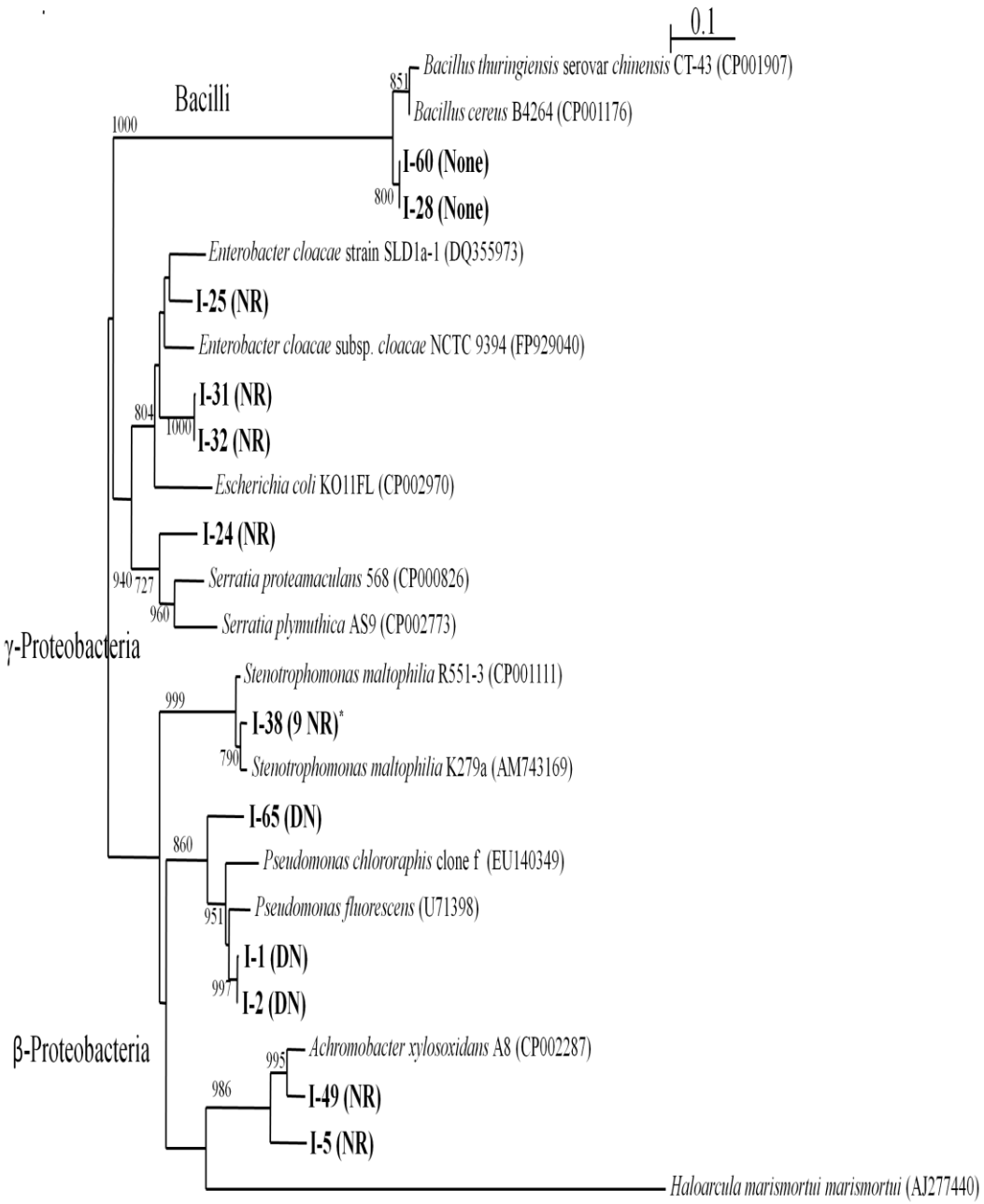

Figure 3.4. Phylogenetic relations of the *narG* gene bacterial strains with respect to their related nucleotide sequences. The tree is constructed from approximately 523 bp *narG* gene sequence using the neighbor-joining algorithm. One thousand bootstrap replications were performed; only values greater than 700 are shown. NR: nitrate reducer, DN: nitrite reducer, and None: did not reduce nitrate nor nitrite based on the microtiter plate test. "I" is the strain number as shown in Appendix I. Strain I-49 and *A. xylosoxidans* A8 (CP002287) contain both *narG* and *napA*. *I-38 is a representative strain for other 8 strains whose *narG* sequences are 98% similar to that of strain I-38. The sequence of *Haloarcula marismortui marismortui* (AJ277440) was included as the outgroup. Bar: 0.1 nucleotide substitutions per nucleotide.

Chapter 4: Enzymatic Transformation of Humic Substances by NDO

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Abstract

Enzymatic transformation of humic acids (HA), fulvic acids (FA) and indole was examined using naphthalene 1,2-dioxygenase (NDO). NDO was used as a model for dioxygenase enzymes found in various microbial species. Indole was used as a model substrate for NDO-catalyzed reactions resulting in condensation products. Although NDO is not classified as a soil enzyme, all HA and FA tested were susceptible to NDO-induced transformation. The extent of NDO-specific NADH oxidation in solutions containing HA and FA paralleled the percent aromaticity of the HA and FA. Furthermore, the UV-Vis absorptive properties of NDO-treated HA and FA were altered in a manner suggesting condensation reactions similar to the formation of indigo from indole. Condensation reactions were enhanced in NDO-treated mixtures containing indole and a FA. NDO retained activity for two weeks under ambient conditions, and retained some enzymatic activity for 9 days based on detection of specific metabolites by HPLC, suggesting prolonged extracellular activity. Humic substances have not previously been known to be substrates for dioxygenases; even more significant was that dioxygenase enzymes can facilitate condensation

reactions between indole-like functional groups well-known to be present in HA and FA. These results illustrate how dioxygenases can be potential humic-modifying enzymes when released into the environment upon microbial death and concurrent cell lysis which could alter the bioavailability of organic contaminants associated with dissolved organic matter through specific modulation of enzyme activity involving substrate competition.

1. Introduction

Humic substances comprise about 60 to 80% of the soil organic matter and consist of three chemical groups based on solubility: fulvic acid, humic acid, and humin (Brady and Weil, 2002). Humic substances are chemically very complex; they contain a variety of functional groups such as carbonyl, carboxyl, aromatic, acetal, heteroaliphatic, and aliphatic groups (International Humic Substance Society, 2005). Humic substances have been demonstrated to affect oxidative reactions of organic contaminants, e.g. the transformation of chlorinated phenols and anilines by phenol oxidases and metal oxides was altered by the addition of model humic compounds representative of specific functional groups present in soil organic matter (Park et al., 1999).

Dissolved organic matter (DOM) plays a vital role in determining the chemical and biological fate of organic contaminants in soils and sediments (Johnson and Amy, 1995). Dissolved organic matter has been traditionally thought to shield organic contaminants from degradation by sequestration and irreversible binding (Ragle et al., 1997; Engebretson and Wandruszka, 1999).

In this study, the enzymatic transformation of the model substrate indole in the presence of dissolved humic acids (HA) and fulvic acids (FA) was examined in order to assess how enzymatic degradation of organic contaminants is influenced by DOM composition and how DOM itself is modified by enzyme activity.

However, indole and related tryptophan and tryptophan derivatives are also normal components of DOM, having been previously detected in soil humic and fulvic acids (Sorge et al., 1993; Hatcher and Clifford, 1994; Wen et al., 2001; Knicker et al., 2002; Ikeya et al., 2004; Sierra et al., 2005), therefore we propose that enzymes that use indole as a substrate may also attack similar substrates in DOM.

Our study examines an enzyme, naphthalene 1, 2-dioxygenase (NDO) that, though considered to have a relaxed substrate specificity and capable of performing a variety of enzymatic reactions (Resnick et al., 1996), is more restricted with respect to substrates utilized and products formed than the non-specific and highly oxidative enzymes such as the laccases, tyrosinases, peroxidases and ligninases, all of which generate activated oxygen species that can engage in coupling reactions with DOM (Park et al., 1999; Claus, 2003). NDO holoenzyme consists of three subunits, e.g. an iron-sulfur flavoprotein reductase, ferredoxin, and iron-sulfur oxygenase, (Barriault and Sylvestre, 1999a; 1999b; Sylvestre et al., 1996). It is a member of the class III aryl-group-hydroxylating dioxygenases (Resnick et al., 1996) that catalyze the first enzymatic step for the bacterial catabolism of a variety of mono- and polycyclic aromatic compounds (Mason and Cammack, 1992; Menn et al., 1993; Kiyohara et al., 1994). NDO is capable of catalyzing monohydroxylation, desaturation, O-and N-dealkylation and sulfoxidation reactions, in addition to *cis*-dihydroxylations (Resnick et al., 1996). During oxygenation of a substrate by NDO to form a dihydrodiol product, there is a

stoichiometric oxidation of one molecule of NADH to NAD^+ and the consumption of one molecule of O_2. Although NDO from *Pseudomonas* species has been the focus of most studies (Resnick et al., 1996), DNA hybridizing to coding regions for NDO subunits have been found in a variety of naphthalene-degrading bacteria commonly found in contaminated soils, e.g. *Mycobacterium*, *Gordona, Sphingomonas*, and *Xanthomonas* (Hamann et al., 1999), suggesting there is a fairly broad distribution of NDO-like enzymes in soil bacteria.

In our study, indole was used as a model compound for the formation of condensation products by enzymatic activity of NDO. NDO oxidizes indole to the corresponding dihydrodiol, then two molecules of *cis*-indole-2,3-dihydrodiol undergo nonenzymatic dehydration to form indoxyl and condensation reactions to produce one molecule of indigo (Ensley et al., 1983; Eaton and Chapman, 1995). The formation of indigo is monitored by UV-Vis spectrophotometry at 600 nm (Eaton and Chapman, 1995).

The short-term kinetic activity of NDO, with naphthalene as the substrate, was measured and the production of the specific product naphthalenediol was identified using HPLC, in order to confirm the purification of enzymatically active NDO and test the activity of NDO after prolonged incubation. In addition to the HPLC, several different techniques, based on specific properties of the oxidation of

substrates by NDO were used to confirm the enzymatic activity of NDO on HA and FA. A decrease in absorbance at 340 nm, indicative of NADH oxidation, (Ensley et al., 1983; Eaton and Chapman, 1995) showed that NDO is enzymatically active on HA and FA. In addition, since molar absorptivity and relative absorbance values at specific wavelengths have been shown to be useful predictors of DOM aromaticity (Chin et al., 1994) and provide quantitative spectral characterization of HA and FA (Nurmi and Tratnyek, 2002), respectively, UV-Vis spectrophotometry was used to identify changes in the spectra of DOM after exposure to NDO.

This study is unique for two reasons. First, unlike the aforementioned enzymes, NDO is not normally an exoenzyme; in this study it is used as a model hydrocarbon-degradative enzyme to probe how cytoplasmic enzymes, released upon cell death and lysis, may act upon DOM as well as upon DOM-associated organic contaminants. Second, although the well-characterized reaction of NDO with indole is used as a model for the formation of condensation products, this study emphasizes enzyme reactions with humic and fulvic acids themselves rather than with model compounds representative of HA and FA functional groups. While the heterogeneity of HA and FA limits the ability to definitely characterize the molecular structure of the NDO-indole-HA/FA and NDO-HA/FA reaction products, standard UV-Vis characterization techniques clearly demonstrate the formation of condensation products.

2. Materials and Methods

2.1. Chemicals

NADH (β form), indole, indigo, naphthalene (CAS 91-20-3), 1,2-dihydro-1,2-naphthalenediol, and Aldrich Humic Acid (AHA) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). Suwannee River Humic Acid (SRHA), Elliott Soil Humic Acid (SHA), Suwannee River Fulvic Acid (SRFA), and Elliott Soil Fulvic Acid (SFA) were obtained from the International Humic Substances Society (IHSS).

2.2. Bacterial Strains and Culture Media

Bacterial strains used in this study were *E. coli* SG13009 (pREP4) and M15 (pREP4) (Barriault and Sylvestre, 1999a; 1999b) The *E. coli* strains (given by Dr. Michel Sylvestre, INRS-Sante, Universite du Quebec, Pointe-Claire, Quebec, Canada) were grown in Luria-Bertani (LB) broth. Media and media components were obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.3. Production and Purification of NDO Components

Recombinant *Escherichia coli* strains bearing cloned histidine-tagged genes were used to produce the three components of the NDO holoenzyme, e.g. an iron sulfur flavoprotein reductase, ferredoxin and iron-sulfur oxygenase, as previously reported (Barriault and Sylvestre, 1999a; 1999b; Sylvestre et al., 1996). After

over-night growth in LB broth containing kanamycin and ampillicin, cultures were diluted into fresh LB broth, and grown for two hours to mid-log phase. Cells were induced with 1mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 4 hours and lysed on ice by sonication. His-tagged proteins were purified from the crude extract using the BD Talon Protocol (BD TALON™ Metal Affinity Resins User Manual, BD Biosciences Clontech, Palo Alto, CA, USA) for purification of the oxygenase under native conditions and HIS-Select™Nickel Affinity Gel (Sigma-Aldrich) for the purification of the ferredoxin and reductase. The elution buffer contained 50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 250 mM imidazole, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10% (w/v) glycerol, 2% Tween 20.

2.4. NDO Enzyme Reaction Mixtures

Enzyme assays were performed in a 500 µl volume in (1:1) 50 mM 2-(N-morpholine)-ethanesulfonic acid (MES) buffer (pH 6.5): elution buffer (see composition above). The reaction mixture contained 100 nmol NADH, 100 nmol of substrate (indole or naphthalene, prepared as 100 µM stock in dimethyl formamide), and 1 nmol of each of the purified his-tagged NDO components (approximately 200 µg total). HA and FA when present, were at a final concentration of 4 mg/L. A mixture without NDO was used as a negative control. All reactions were performed in duplicate.

2.5. Measurement of Enzyme Activity

2.5.1. HPLC detection of the production of naphthalenediol

The ability of recombinant NDO to convert the substrate naphthalene to its corresponding 1,2-dihydro-1,2-naphthalenediol (e.g. "naphthalenediol") was measured by high performance liquid chromatography (HPLC, Beckman Coulter Inc., Fullerton, CA) with an Encosphere C18 5μ (250 mm x 46 mm) column (Alltech Associate, Inc. Deerfield, IL). The mobile phase was 20%:80% methanol-water at a flow rate of 1 mL/min (Allen et al., 1997). The UV absorbance detector was set at 254 nm to detect naphthalenediol (Sugiyama et al., 1999). The HPLC was calibrated with authentic standards obtained from Sigma-Aldrich.

2.5.2. Spectrophotometric measurements of indigo production and NADH oxidation

NDO catalyzes the conversion of indole to indigo, which can be readily measured owing to its absorbance at 600 nm (Ensley et al., 1983; Eaton and Chapman, 1995). Enzymatic activity of the purified reconstituted holoenzyme with indole as a substrate was assayed by monitoring the increase in absorbance at 600 nm using a Shimadzu 1601 Spectrophotometer. NDO activity was also monitored in certain experiments by measuring the decrease in absorbance of NADH at 340 nm or at 365 nm (Barriault and Sylvestre, 1999a; 1999b; Sylvestre et al., 1996). (Barriault and Sylvestre, 1999a; 1999b; Sylvestre et al., 1996). Reaction mixtures were incubated at 37^{o}C for 10 minutes before absorbance was measured. A mixture

without NDO was used as a negative control. All reaction mixtures were performed in duplicate.

2.5.3. Linear Detection Range.

Solutions of NADH, NDO, indole, and indigo were prepared over the range of concentrations used in this study and their absorbance at 340 nm, 365 nm, 465 nm, 600 nm, and 665 nm were measured (data not shown). Reaction mixtures were incubated at 37^{o}C for 10 minutes or as indicated in the individual experiments before measurements were made. Statistical analysis was performed using SPSS for Windows Version 11.5.

2.6. Experiments Testing the Long-term Activity of NDO

2.6.1. Incubation of NDO for 16 days with naphthalene as a substrate

The purified his-tagged NDO components (approximately 200 μg) were used individually or as the reconstituted holoenzyme (Barriault and Sylvestre, 1999a; 1999b; Sylvestre et al., 1996) in a set of reaction mixtures containing (1:1) 50 mM MES buffer (pH 6.5): elution buffer (pH 7.0), and 100 nmol NADH. NDO holoenzyme consists of 1 nmol reductase: 1 nmol, ferredoxin: 1 nmol oxygenase. Control reaction mixtures (in duplicate) contained holoenzyme NDO but no naphthalene, or 200 μM naphthalene and one of the three NDO components (e.g. reductase OR ferredoxin OR oxygenase). Reaction mixtures, except a naphthalene-minus control which included the holoenzyme, were injected initially with 200 μM

naphthalene and inoculated in duplicate into 10 mL serum vials with gray Teflon stoppers and crimp seals. Vials were incubated at room temperature (24˚C). Samples were removed for testing after 2 hours (e.g. "0 day"), 1, 4, 9, and 16 days of incubation. Stoppers and crimps were removed and sealed back immediately after each sampling. Samples removed for testing were diluted 1:1 with methanol before detection with the HPLC in order to fall within the linear range of detection. However, all the HPLC results refer to concentration in the reaction mixtures before dilution. Authentic standards for (1*R*,2*S*)-*cis*-1,2-dihydro-1,2-naphthalenediol (CAS 51268-88-3) were prepared on each sample date and run in parallel with the samples.

At time 0, the short-term kinetics of NDO activity with 200 μM of naphthalene as a substrate and approximately 200 μg of the holoenzyme was monitored by measuring the decrease in absorbance of NADH at 340 nm for 5 minutes (Barriault and Sylvestre, 1999a; 1999b; Sylvestre et al., 1996). A reaction mixture containing all components, as previously described, but without NDO, was used as a negative control. All reaction mixtures were performed on duplicate samples.

2.6.2. NDO activity over time with indole as a substrate

NDO holoenzyme purified as described above was incubated in (1:1) 50 mM MES buffer (pH 6.5): elution buffer (pH 7.0) at ambient temperature (23-

25^{o}C). NDO activity was measured immediately ("fresh NDO", e.g. time=0) and after 7 ("one week NDO") and 14 days ("two weeks NDO") of incubation after adding 100 nmol NADH and 100 nmol indole to aliquots of NDO in the incubation buffer. Reactions with NDO contain 1 nmol of each of the purified NDO components. Spectrophotometric measurements were made at 340 nm and 600 nm in order to detect the oxidation of NADH and production of indigo, respectively. All measurements were performed in duplicate samples, with the exception of the 7 day measurements, which were performed on triplicate samples. The rate of NADH oxidation was estimated from the slope of the decrease in absorbance at 340nm per minute over 4 minutes (fresh NDO) or 35 minutes (one week NDO and two week NDO). Indigo production was estimated after incubation with NADH and indole for 15 (no NDO), 30 (fresh NDO), or 35 minutes (one week NDO, two weeks NDO).

3. Results

3.1. Confirmation of NDO specific activity with naphthalene as a substrate

Recombinant *Escherichia coli* strains bearing cloned histidine-tagged genes were used to produce the three components of the NDO holoenzyme (Barriault and Sylvestre, 1999a; 1999b; Sylvestre et al., 1996). Short-term NADH kinetic measurements with naphthalene as a substrate showed a loss of absorbance at 340nm of -0.036 OD/min for a reaction mixture containing the reconstituted NDO holoenzyme, naphthalene, and all reaction components. The slope for the negative control (the reaction mixture without NDO) was -1.2×10^{-3} OD/min. Assuming a 1:1 relationship between number of moles of NADH oxidized per OD unit (Phillips, 1994) and moles of naphthalenediol produced (Dunn and Gunsalus, 1973; Ensley et al., 1982), 5.76×10^{-3} μM naphthalenediol were produced per min initially in the reaction mixtures containing the holoenzyme and naphthalene.

Using HPLC, a well-resolved peak was present for the naphthalenediol standards at 7.4 min within a linear range of detection of 2-40 μM ($r^2 = 0.9986$). The mean concentration of naphthalenediol in the reaction mixtures with the holoenzyme and naphthalene (Figure 4.1) after 2 hours of incubation was 0.09 μM, as measured by HPLC.

3.2. Incubation of NDO with naphthalene for 16 days

Incubation over a period of 16 days at room temperature (Figure 4.1) showed increasing amounts of naphthalenediol that were produced over time but only in the reaction mixtures containing the NDO holoenzyme and naphthalene. In these reaction mixtures, a hyperbolic relationship was detected between time vs. naphthalenediol concentration. The decrease in the rate of naphthalenediol accumulation indicates there may have been a substantial loss of enzyme activity after the 1st four days. The maximum concentration of naphthalenediol (70.74 and 65.86 μM for the replicate reaction mixtures) was measured on day 9. Based on the 1:1 stoichiometric relationship of naphthalene consumed to naphthalenediol produced (Dunn and Gunsalus, 1973; Ensley et al., 1982) we estimate that by day 9 approximately 34% of the naphthalene originally introduced (e.g. 200 μM) had been converted to naphthalenediol.

3.3. NDO retains the ability to oxidize NADH and transform indole for two weeks Reactivity of a purified preparation of NDO was measured at various intervals after incubation at room temperature (23-25^{o}C) in order to model some aspects of NDO activity after cell lysis. NDO holoenzyme was incubated in 1:1 MES: elution buffer for 7 days and 14 days, then 100 nmol NADH and 100 nmol indole were added and spectrophotometric measurements were made of the rate of oxidation of NADH and the production of indigo (Fig. 6.2). Incubation for one or two weeks, lessened, but did not eliminate the ability of NDO to oxidize NADH or

produce indigo from its substrate indole (Fig. 6.2). Further experiments are required to extend these findings to more environmentally realistic conditions, but these preliminary results support our hypothesis that "cytoplasmic" enzymes, released by cell lysis, could participate in the modification of HA and FA and thus alter the bioavailability of organic contaminants in environments containing DOM.

3.4. NDO Reactions with Indole

Reactivity of a purified preparation of NDO with indole was monitored spectrophotometrically by measuring changes in the absorbance of the reactants and products at specific wavelengths. NADH concentrations were monitored at 340 nm and 365 nm; indigo, a model condensation product or chromophore, was monitored at 465 nm, 600 nm, and 665 nm (Ensley et al., 1983). We found a high correlation (Pearson correlation coefficient R=0.977) between NADH readings at 340 nm and 365 nm. Therefore, NADH oxidation was measured at 365 nm rather than at 340 nm in reactions containing humic substances, as humic substances absorb strongly at 340 nm, but not at 365 nm. Calibration curves were made for all substances at the five wavelengths (data not shown) in order to accurately estimate changes in absorbance when various combinations of substances were used in reaction mixtures.

NDO oxidizes indole to the corresponding dihydrodiol, which undergoes nonenzymatic dehydration and condensation to form indigo (Ensley et al., 1983;

Eaton and Chapman, 1995). This reaction occurs with a subsequent decrease in NADH concentration and a corresponding increase in indigo concentration as illustrated in Figure 4.3 ("Indole"). A decrease in the absorbance readings at 340 nm and 365 nm were expected due to a decrease in NADH. Absorbance readings at 465 nm, 600 nm, and 665 nm increased as indigo was formed; absorbance readings at all three wavelengths were highly correlated with each other (Pearson correlation coefficient R: 0.980 to 0.996, $p<0.01$).

The NDO and indole reaction mixture had significantly different absorbance values than control reactions without indole (Figure 4.3, black bars) for all wavelengths measured (ANOVA, $p<0.05$). Comparing the difference in absorbance at 600 nm between the NDO plus indole reaction mixture versus the control mixture, approximately 10.7 nmol of indigo were produced by NDO. Based on the average of the difference in absorbance at 340 nm and 365 nm, it was calculated that approximately 65 nmol of NADH were oxidized assuming indigo does not absorb at these two wavelengths. However, our experiments showed (see Materials: Linear Detection Range, data not shown) that indigo does absorb at 365 nm, therefore subtracting the 365 nm absorbance expected to be produced by 10.7 nmol indigo gives a corrected absorbance for NADH of 0.216 at 365 nm and 0.259 at 340 nm, or an average of 73 nmol NADH oxidized. A stoichiometric relationship between NADH oxidization and conversion to indigo predicts a yield of 36.5 nmol

indigo, approximately three times that observed, indicating that our reaction conditions were not optimal for indigo production.

3.5. NDO Reactions with Humic and Fulvic Acids

Figure 4.3 (SRHA, AHA, SHA, SRFA, SFA) illustrates for reaction mixtures containing NDO and 4 mg/L of HA or FA, significant changes in absorbance readings were observed that were similar to absorbance changes observed in the indole experiments. Reactions containing NDO had higher absorbance readings for 465 nm, 600 nm, and 665 nm, and lower readings at 365 nm compared to control reactions without NDO. The extent of NADH oxidation in the presence of HA and FA was estimated by comparing the absorbance at 365 nm of reactions containing NDO to control reactions that contained HA and FA but did not contain NDO. Assuming no additional interference in the absorbance readings at 365 nm from the oxidized humic and fulvic acids, the greatest amount of NADH was oxidized in SHA (71.5 nmol) and the least in SRFA (58.3 nmol). Figure 4.4 shows that the quantity of NADH oxidized was roughly proportional to the percentage aromaticity (Thorn et al., 1989) of each humic substance (linear regression $y = 0.3644X + 49.664$, R squared = 0.8187) suggesting that aromatic functional groups in the humic substances may have been oxidized by NDO, although an effect on other functional groups cannot be ruled out.

The similar changes in absorbance readings at 365 nm, 465 nm, 600 nm, and 665 nm produced by reaction mixtures of NDO and either HA or FA compared to NDO and indole reaction mixtures suggest that purified, reconstituted NDO enzymatically modifies humic and fulvic acids. Increased absorbance at three longer wavelengths (e.g. 465 nm, 600 nm, and 665 nm) is consistent with the formation of HA and FA condensation products. Note that there is not a consistent relationship between quantities of NADH oxidized and greater absorbance at the three higher wavelengths, indicating that the degree of condensation does not increase linearly with the quantity of NADH oxidized (Figure 4.5). The increased absorbance values are also not those characteristic of the formation of indigo from indole. Although calculations suggest comparable quantities of NADH were oxidized when HA or FA were substrates as compared to indole as a substrate, there was less of an increase in absorbance at 465 nm, 600 nm, and 665 nm (e.g. the ratio of differences in absorbance, SRHA/indole; 0.7 at 465 nm, 0.55 at 600 nm, 0.38 at 665 nm).

3.6. NDO Reactions with SRFA and Indole

Figure 4.6 presents results from reaction mixtures containing NDO, SRFA, and indole. This experiment examined whether the characteristic changes in absorbance produced by active NDO plus a known substrate were enhanced or inhibited by the presence of a humic substance. Comparing control reactions containing SRFA but no NDO (white bars) to those containing SRFA and NDO but

no indole (black bars), show a lower absorbance at 365 nm indicative of NADH oxidation. Condensation of SRFA molecules is suggested by the absorbance increase at 465 nm, 600 nm, and 665 nm. The addition of indole to the reaction mix (grey bars) increased the absorbance at wavelengths previously found to correlate with indigo production and that of other chromophores: 365 nm ($p=0.020$), 465 nm ($p=0.020$), 600 nm ($p=0.058$), and 665 nm ($p=0.053$). Indigo production was estimated from the difference in absorbance between NDO-containing reactions with (grey bars) and without indole (black bars) at 600 nm as 23 nmol, at 665 nm as 26 nmol, and at 465 nm as 21 nmol. Based on these absorbance measurements at 465, 600, and 665 nm, approximately twice the quantity of indigo was produced when SRFA was present with indole in the reaction mix, than in the previous experiments in the absence of SRFA (i.e. NDO and indole experiments shown in Fig. 6.3, 10.7 nmol indigo). These results indicate that either SRFA enhances the efficiency of indigo formation or formation occurs of indole-SRFA condensation products containing chromophores with larger molar absorptivities than that of indigo or the enzymatically-modified SRFA. The evidence supports the latter: if indigo formation and enzymatic modification of SRFA were independent of each other, i.e. if no condensation products between indole and SRFA are produced, then the absorbance at wavelengths 465 nm, 600 nm, and 665 nm should be equal to, or less than, the sum of the absorbance values at these wavelengths for the NDO and indole experiments and the NDO and SRFA experiments. Instead, there was proportionally more absorbance at 600 and 665 nm

and less at 465 nm when NDO transformed a mixture of indole and SRFA than the sum of the separate reactions would predict.

4. Discussion

Our HPLC results (fig. 6.1) confirmed that our preparation of NDO has the expected enzymatic activity, ie. conversion of its substrate naphthalene to naphthalenediol. Also as predicted, naphthalenediol was detected only in the reaction mixtures containing the NDO holoenzyme and naphthalene. Our preparation of NDO retained activity for prolonged periods of incubation. Based on HPLC measurements, it is estimated by day 9 approximately a third of the naphthalene introduced had been converted to naphthalenediol. We also observed (Figure 4.2) that the purified reconstituted NDO holoenzyme is capable of NADH oxidation and indigo production from indole for up to two weeks when kept at room temperature.

Our results (fig. 6.3) demonstrate that NDO interacts with HA and FA resulting in a decrease in absorbance at wavelengths associated with NADH oxidation (used to assay NDO activity, Ensley et al., 1982; Simon et al., 1993), and increased absorbance at wavelengths characteristic of the condensation of HA and FA molecules. Furthermore, our results (fig. 6.6) show that an aromatic substrate (indole), a model for hydrocarbon contaminants degraded by NDO, is not only accessible to NDO in the presence of SRFA, but the formation of condensation products between indole and SRFA is enhanced, providing one explanation for observations of decreased bioavailability of hydrocarbon contaminants in soil or sediments over time (Ragle et al., 1997). NADH oxidation calculations for humic

and fulvic acid reaction mixtures (fig. 6.4) show that although the extent of NADH oxidation mostly parallels the percent aromaticity of the HA and FA as determined by ^{13}C nuclear magnetic resonance spectroscopy (International Humic Substance Society, 2005; Thorn et al., 1989; Perminova et al., 1999), as expected from previous research on NDO substrates (Resnick et al., 1996), increases in absorbance readings at 465 nm, 600 nm, and 665 nm demonstrate that HA and FA are not just being oxidized into dihydrodiols which would result in *lower* absorbance readings at these wavelengths. Instead, these absorbance *increases* indicate the presence of chromophores presumably formed through the condensation of HA and FA molecules in a reaction mechanism similar to that of NDO-initiated production of indigo from indole (Ensley et al., 1983; Eaton and Chapman, 1995; O'Connor et al., 1997), which shows similar increases in absorbance at these wavelengths. In indigo formation, indole is oxidized by NDO to the dihydrodiol product that then undergoes further non-enzymatic reactions to produce the condensation product indigo. Thus our observation of increased absorbance at multiple wavelengths when NDO reacts with HA and FA (fig. 6.5) may represent not only the initial products of the enzymatic reaction but also subsequent condensation reactions forming conjugated compounds.

4.1. Humic Acids Contain Indole- and Tryptophan-Type Functional Groups

Humic acids that contain a higher proportion of suitable aromatic substrates for oxidation by NDO may not necessarily undergo coupling reactions as readily,

as noted in by comparing Figure 4.4 to Figure 4.5 for SHA, which had the greatest amount of oxidation of NADH but the smallest increase in absorption at higher wavelengths. This indicates that although aromatic functional groups susceptible to NDO oxidation are present, for condensation of HA and FA molecules to occur, indole or tryptophan-type functional groups must also be present. Besides indole, NDO reacts with tryptophan derivatives to form products with a high molar absorptivity (Ensley et al., 1983; Kim et al., 2003). Indoles have been detected in humic materials isolated from soils and weathered coals using ^{15}N NMR (Wen et al., 2001; Knicker et al., 2002) and pyrolysis and thermochemolysis GC/MS techniques (Hatcher and Clifford, 1994; Ikeya et al., 2004). Likewise, tryptophan and tryptophan derivatives have been detected in soil humic acid (Sorge et al., 1993), fulvic acids from terrestrial, estuary, and marine sources (Sierra et al., 2005), DOM extracted from marine sediment pore waters (Kline and Chen, 2004), and in hydrophilic and hydrophobic fractions of wastewater effluent (Chen et al., 2003). This information coupled with the fact that NDO reacts with tryptophan and indole derivatives to form products with a high molar absorptivity, supports our hypothesis that the increased absorbance at 465 nm, 600 nm, and 665 nm, upon reacting NDO with HA and FA, either in the presence or absence of indole (fig. 6.6), results from the condensation of indole- or tryptophan-derivative functional groups inherently present in the HA and FA.

4.2. Environmental Implications of Enzymes Capable of Modifying DOM

Although fungi and certain strains of eubacteria are able to degrade humic material due to their secretion of non-specific exoenzymes such as the laccases, peroxidases, and ligninases, to our knowledge, the modification of specific moieties of humic acids and fulvic acids by dioxygenases has not been previously demonstrated. The fungal exoenzymes all generate activated oxygen species that engage in coupling reactions with humic constituents. In contrast, an electron pair is transferred to NDO via the iron-sulfur flavoprotein and Rieske ferredoxin, leading to subsequent oxygen activation at the mononuclear iron and catalysis (Resnick et al., 1996). Note that besides the classic *cis*-dihydroxylations, NDO has been shown to catalyze monohydroxylation, desaturation, O-and N-dealkylation and sulfoxidation reactions (Resnick et al., 1996). Furthermore, other oxygenase enzymes exist, such as toluene dioxygenase (Eaton and Chapman, 1995; Kim et al., 2003), styrene monooxygenase (O'Connor et al., 1997) and *p*-cumate dioxygenase (Eaton and Chapman, 1995) that are similar to NDO in their ability to catalyze indole and indole derivatives.

Although NDO is not an exoenzyme, lysed cells could be a source of NDO or similar oxygenase enzymes under natural conditions. We have observed that the purified reconstituted NDO holoenzyme is capable of enzymatic transformation of the model substrates naphthalene and indole when kept two weeks at room temperature. This illustrates that NDO released from lysed cells into an aqueous

environment might maintain some enzymatic activity over a significant period (e.g. days). However, these were laboratory conditions. Extracellular enzymes activities in soil are known to be affected by adsorption to clay and a pH-dependent modification that are mainly irreversible (Leprince and Quiquampix, 1996). Indeed, Renella et al. (2007) found that some enzymes have higher persistence values in soils under forest or set management, some have higher persistence values in the neutral and alkaline soils, and in others persistence values showed no obvious relationship with soil pH or management. Also, soil enzyme activity varies with soil type and is influenced by the texture and the content of organic matter of the soils (Tarafdar and Junggk, 1987). For example, there are fundamental differences in the relationships between soil chemistry and enzyme activities in light fraction soil and heavy fraction soil (Grandy et al., 2007). In some soils, minerals can stabilize or enhance enzyme activity, while humic acids inhibit it (Allison, 2006); however, Rillig et al. (2007) noted the existence of persistent, active, humic-enzyme associations. Enzymes released by lysed cells would be expected to show a similar range of response to specific soil conditions as do exoenzymes.

The modification of humic materials by dioxygenases, besides affecting the binding and bioavailability of contaminant aromatic hydrocarbons, could also play a role in the humification of organic matter into refractory HA and FA. Likewise, it is possible, based upon the ability of NDO to oxidize mono- and polycyclic aromatic compounds, for NDO to "prime" humic materials for subsequent

mineralization by microbial communities, and hence enhance the rate of carbon cycling of humic materials in aerobic sites. Again, specific soil conditions could play a role in determining the balance between enzymatic degradation and formation of soil organic matter, as noted for low organic matter accumulations in desert soils (Stursova and Sinsabaugh, 2008). Enzymes released by lysed cells may play a proportionately greater role in humic matter modification when conditions exist, such as alternating wet and dry conditions, which lead to periodic microbial die-offs.

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Figure Legends

Figure 4.1. Naphthalenediol formation over time in duplicate (indicated by diamonds and hollow squares) reaction mixtures containing the holoenzyme NDO and 200 μM naphthalene as measured by HPLC with a UV detector. Reaction mixtures were incubated at 37^{o}C and sampled after 2 hours ("day 0"), 1, 4, 9, and 16 days incubation. Control reaction mixtures showed no detectable production of naphthalenediol.

Figure 4.2. Absorbance values of reaction mixtures containing indole and NADH after incubation with NDO aged without NADH or indole for various periods at room temperature. Values with fresh NDO, NDO aged two weeks, and no NDO were the average of two reactions, values with NDO aged one week are the average of three reactions, with +/- one standard deviation (SD). A. Decrease in absorbance values of NADH at 340 nm over time (ΔA340 nm min^{-1}). B. Absorbance values of indigo at 600 nm.

Figure 4.3. Absorbance values of reaction mixtures containing indole, humic or fulvic acids after incubation with NDO. Values are averages of two reactions, with the range of absorbances indicated by bars. Black bars: control, contain substrate as indicated in the graph (e.g.100 nmol indole, or 4 mg/mL HA or FA) but no NDO. White bars: contain substrate and 1 nmol of each of the purified NDO components.

Figure 4.4. Differences among humic substances in % aromaticity versus oxidation of NADH when incubated with NDO. The % aromaticity (as determined by ^{13}C nuclear magnetic resonance spectroscopy, Thorn et al., 1989) is plotted as the X

value for each humic substance versus the quantity of NADH (in nmol, see Results) oxidized for those same reactions. An R squared of 0.8187 is shown by the linear regression equation $y = 0.3644X + 49.664$.

Figure 4.5. Differences between humic substances and indole in oxidation of NADH versus formation of condensation products. The quantity of NADH (in nmol, see Results) oxidized between a control reaction without NDO and the corresponding reaction containing NDO is plotted as the X value for each humic substance or indole versus the difference in absorbance values at 465 nm (diamonds), 600 nm (squares), and 665 nm (triangles) for those same reactions. The name (humic substance or indole) appears above its absorbance values.

Figure 4.6. Absorbance values of reaction mixtures containing SRFA and indole after incubation with NDO. Where indicated, reaction mixtures may contain 1 nmol of each of the purified NDO components ("NDO") or 100 mol indole ("indole"). Values are averages of two reactions, with the SD indicated by bars. White bars: control, contains SRFA but neither indole nor NDO. Black bars: contains SRFA and NDO. Grey bars: contains SRFA, NDO, and indole. Absorbance at 365 nm was not corrected for the contribution by indigo.

Figure 4.1.

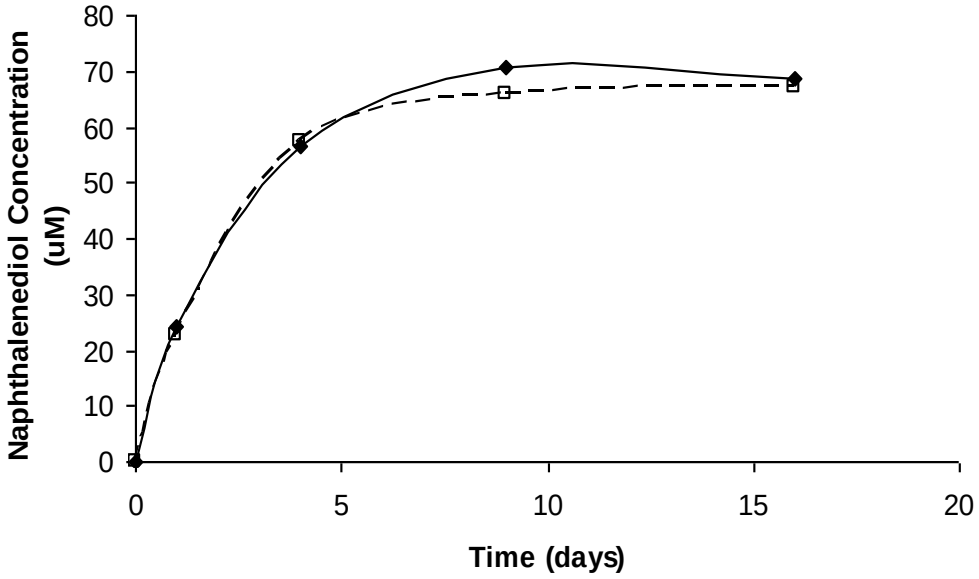

Naphthalenediol Concentration (uM)
80
70
60
50
40
30
20
10
0
0
5
10
15
20
Time (days)

Figure 4.2.

A

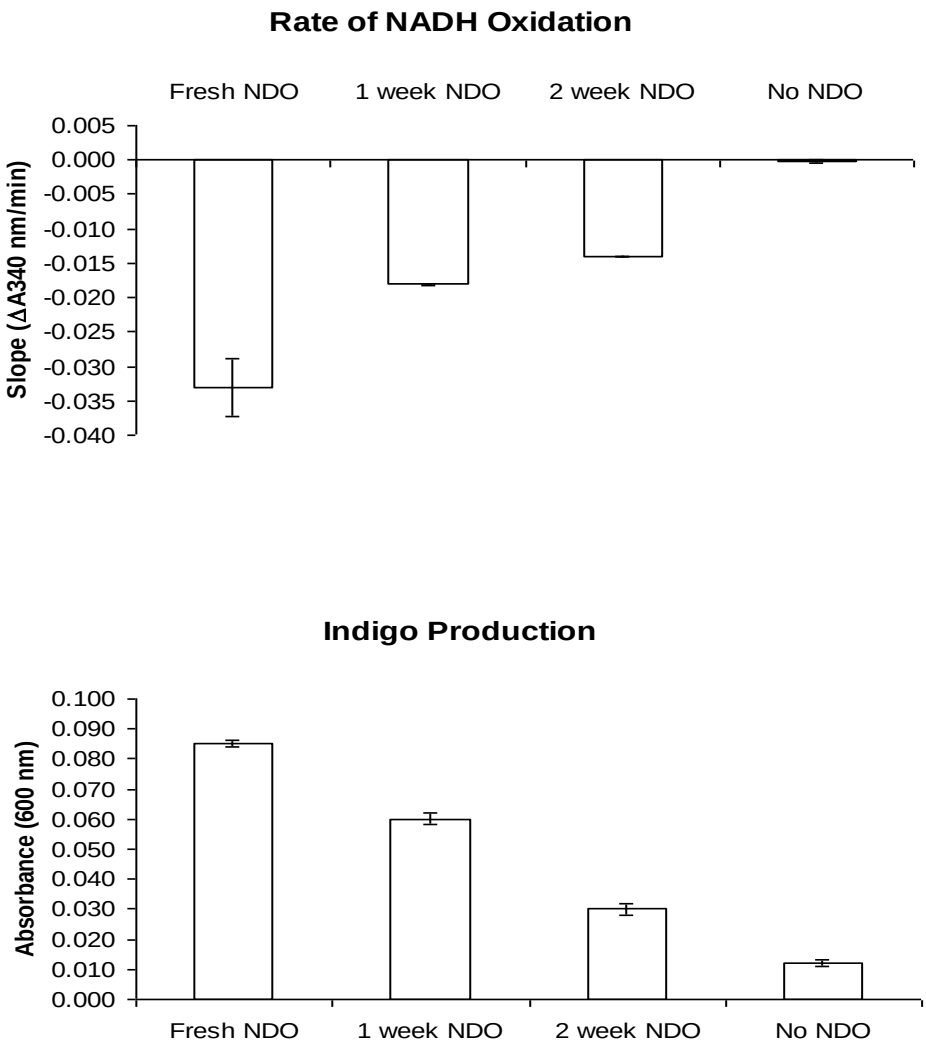
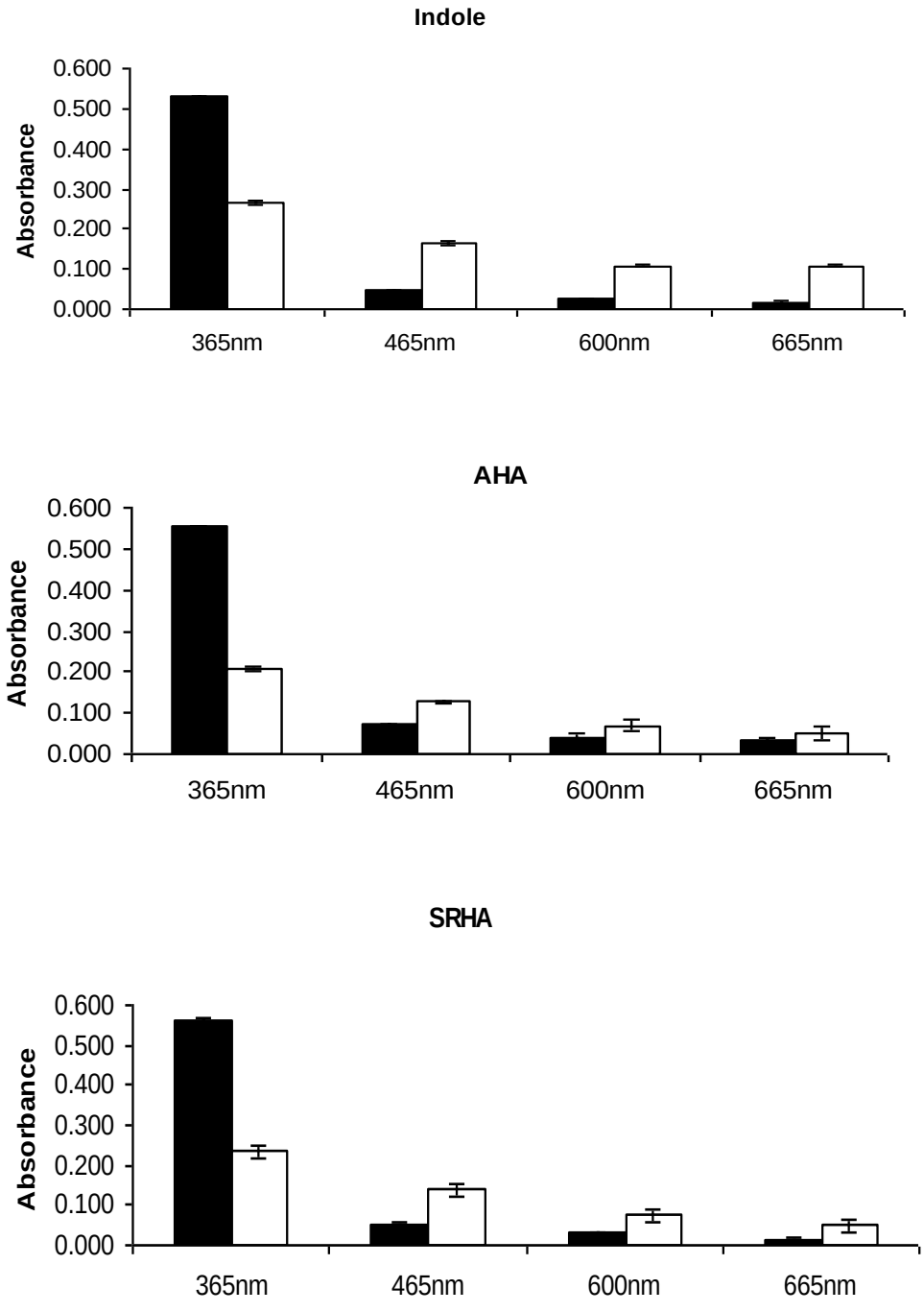
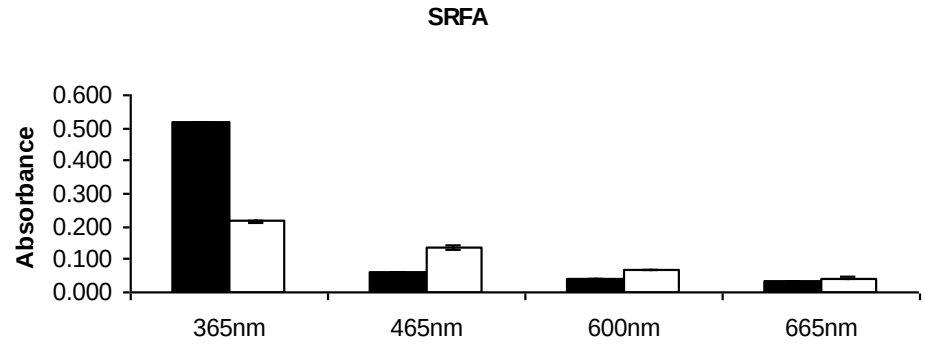
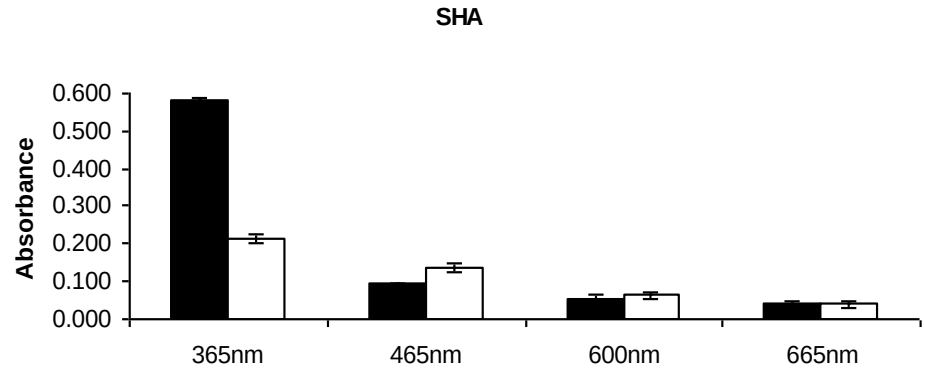

Rate of NADH Oxidation
Fresh NDO
1 week NDO
2 week NDO
No NDO
Slope (ΔA340 nm/min)
0.005
0.000
-0.005
-0.010
-0.015
-0.020
-0.025
-0.030
-0.035
-0.040
Indigo Production
Absorbance (600 nm)
0.100
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
Fresh NDO
1 week NDO
2 week NDO
No NDO

Figure 4.3.


Indole
Absorbance
0.600
0.500
0.400
0.300
0.200
0.100
0.000
365nm
465nm
600nm
665nm
AHA
Absorbance
0.600
0.500
0.400
0.300
0.200
0.100
0.000
365nm
465nm
600nm
665nm
SRHA
Absorbance
0.600
0.500
0.400
0.300
0.200
0.100
0.000
365nm
465nm
600nm
665nm


SRFA
Absorbance
0.600
0.500
0.400
0.300
0.200
0.100
0.000
365nm
465nm
600nm
665nm


SHA
Absorbance
0.600
0.500
0.400
0.300
0.200
0.100
0.000
365nm
465nm
600nm
665nm

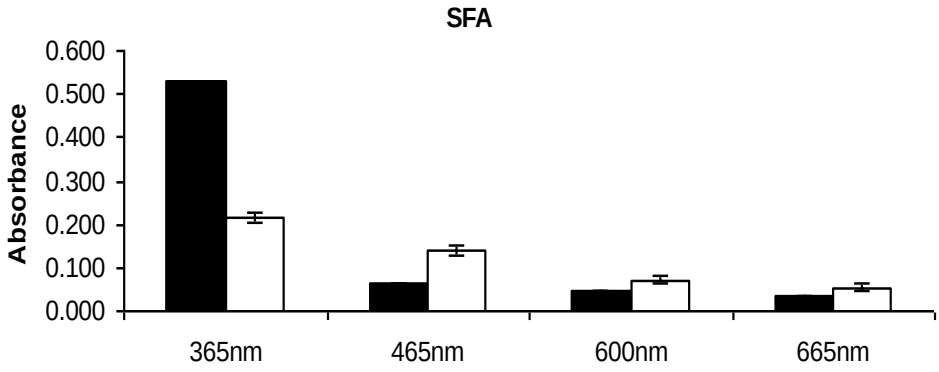

SFA
Absorbance
0.600
0.500
0.400
0.300
0.200
0.100
0.000
365nm
465nm
600nm
665nm

Figure 4.4.

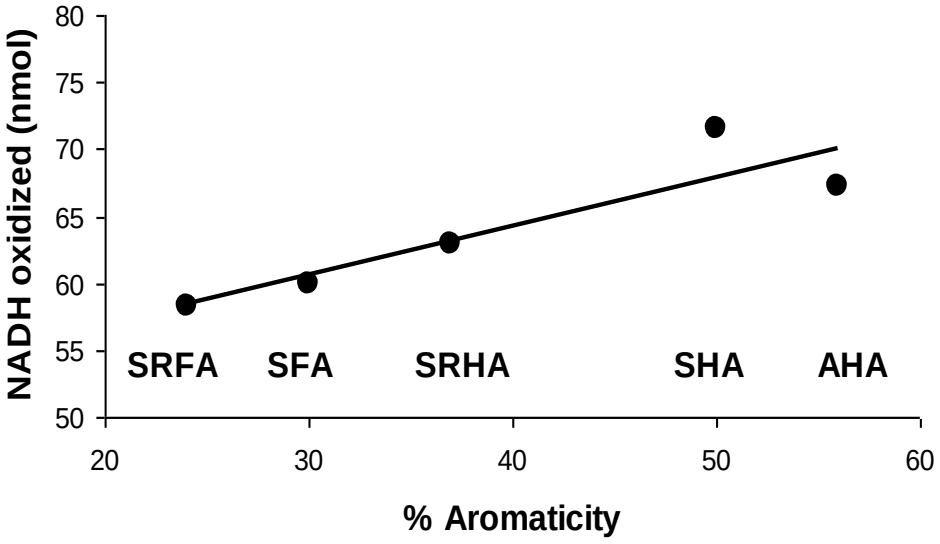

NADH oxidized (nmol)
80
75
70
65
60
55
50
SRFA
SFA
SRHA
SHA
AHA
20
30
40
50
60
% Aromaticity

Figure 4.5.

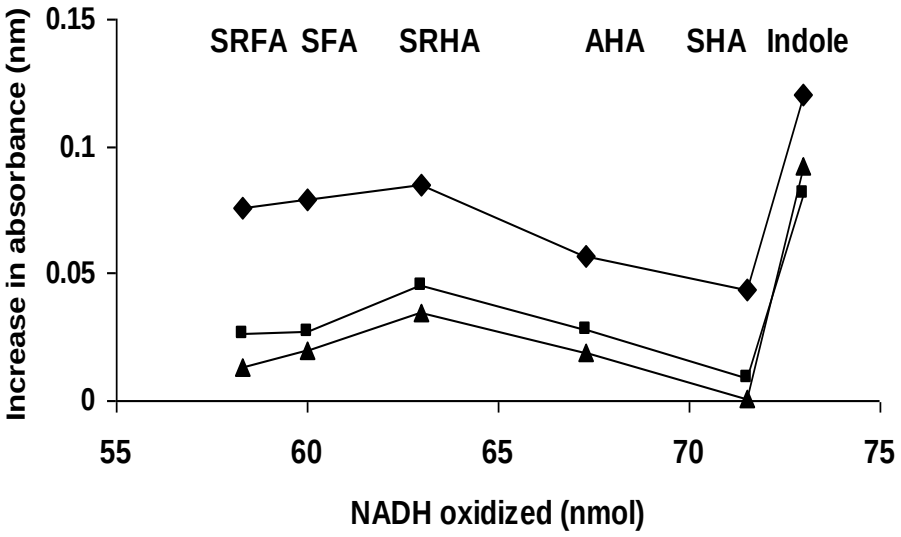
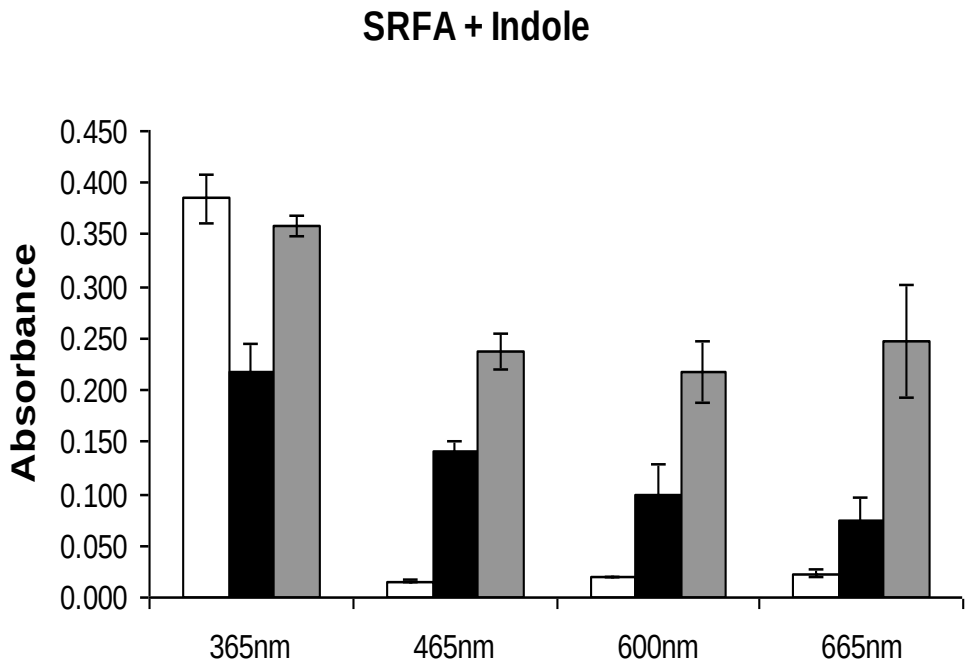

SRFA SFA SRHA AHA SHA Indole
Increase in absorbance (nm)
0.15
0.1
0.05
0
55
60
65
70
75
NADH oxidized (nmol)

Figure 4.6.


SRFA + Indole
Absorbance
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
365nm
465nm
600nm
665nm

Conclusions

This dissertation addressed the long term impact (e.g. 5-10 years) of crude oil/brine spills on biogeochemical cycling of nitrogen in tallgrass prairie soils that had been partially bioremediated. The effect of the long-term residual TPH and brine contamination on the abundance and diversity of nitrate-reducing (NR) and nitrite-reducing/denitrifying (DN) bacteria were assessed using molecular- and culture-based methods of detection.

The effect of contamination on the abundance of culturable NR and DN bacteria

Culturable NR and DN bacteria were as abundant in oil/brine contaminated sites as in prairie sites, at about 10^6-10^7 cells/g (dry weight) soil, showing that this important group of bacteria was not eliminated by contaminants. Although my results show an abundance of NR and DN bacteria in the tallgrass prairie contaminated sites, this dissertation did not address field measurement of rates of denitrification. Therefore, a "normal" abundance of NR and DN bacteria in the contaminated sites does not necessarily mean rates of denitrification in contaminated sites reflect those in uncontaminated sites. In addition, previous studies have shown a non-uniform distribution of denitrifying bacteria in soil. My sampling method did not compare the distribution of denitrification "hot spots" in contaminated and uncontaminated soils.

The effect of other environmental factors on the abundance of culturable NR and DN bacteria

It has been shown by other studies that high soil moisture, bioavailable carbon, and sufficient levels of nitrate are necessary for sustained denitrification. My research determined the effect of these factors singly and in combination on the abundance of NR and DN bacteria. There was a slight positive effect of soil moisture on the abundance of both heterotrophic and NR and DN bacteria in both contaminated and contaminated sites. However, the proportion of NR and DN bacteria varied widely for different samples containing the same soil moisture level due to generally higher %CV for the $\log_{10}$ number of NR and DN bacteria than that of heterotrophic bacteria. There were a few examples of elevated NR and DN bacteria abundance associated with nitrate treatment of oil-contaminated sites, but in general, the treatment did not produce a sustained increase in numbers of NR and DN bacteria.

Species composition, but not species diversity, was altered by oil/brine contamination

The nitrate broth assay showed that 54 of 75 strains isolated from contaminated or uncontaminated prairie soils reduced nitrate/nitrite. Four other strains possessed *napA* or *narG* (codes for nitrate reductase) but did not reduce nitrate or nitrite. My results for the 58 strains showed that the degree of NR and DN bacterial diversity in the contaminated areas was not lower than that from the

uncontaminated areas, but different genera were dominant. The dominant genera of the 58 strains were affiliated with γ-Proteobacteria, especially the genera *Stenotrophomonas* and *Pseudomonas*. Members of γ-Proteobacteria were dominant in oil contaminated sites while salt tolerant bacteria (e.g. *Bacillus*) were isolated from brine-contaminated, but not from oil-contaminated areas.

The power of molecular detection of NR and DN bacteria

The utility of molecular detection for NR (e.g. *napA* and *narG*) and DN (e.g. *nirS*, *nirK*, *cnorB*, *qnorB*, and *nosZ*) functional genes seem to vary among the gene used for screening with *cnorB* being the most strongly associated with nitrite reduction phenotype. My results suggest that if functional gene sequences (e.g. nitrate reductase and DN genes) are detected, then the strains are likely to have the NR or DN phenotype. However, in many strains, NR and DN genes were not detected, yet the strains still reduced nitrate/nitrite, indicating the molecular detection methods used underestimate the actual number of culturable NR and DN bacteria. The match between molecular detection and NR or DN phenotype was highest for γ-Proteobacteria. However, *nirS* gene presence was overestimated in *Stenotrophomonas* (γ-Proteobacteria) strains based on PCR amplification where PCR products of the correct size were amplified from 9 *Stenotrophomonas* strains. But when those PCR products were sequenced, they were not nitrite reductase genes which indicates that a part of the amplicon has the same DNA sequence as the *nirS* primers. Gene sequences for nitrate reductase were detected in a few

strains that did not demonstrate nitrate reduction under these assay conditions, indicating that the PCR-molecular detection and the phenotypic detection are complementary ways in detecting NR and DN bacteria.

Role for enzymatic modification of humic acids (HA)/fulvic acids (FA)

Although NDO is not classified as a soil enzyme, I showed that all HA and FA tested were susceptible to NDO-induced transformation. Also, the extent of NDO-sepcific NADH oxidation in solutions containing HA and FA paralleled the percent aromaticity of the HA and FA. The UV-Vis absorptive properties of NDO-treated HA and FA suggested condensation reactions occurred similar to those resulting in the formation of indigo from indole. Moreover, NDO retained activity for two weeks under ambient conditions suggested prolonged extracellular activity. These results demonstrate that NDO can chemically alter humic and fulvic acids, and that dioxygenases could be potential humic-modifying enzymes when released into the environment upon microbial death. This laboratory study suggests enzymes like NDO might alter the bioavailability of organic contaminants associated with soil. Fluctuating soil moisture conditions then might stimulate bioavailability of organic contaminants by lysing cells, releasing humic modifying enzymes.

Summary

My overall results suggest that the important physiological group of NR and DN bacteria are as abundant in formerly contaminated tallgrass prairie soils as in

uncontaminated soils. I recommend that both molecular and phenotypic detection of NR and DN bacteria be used. The PCR-based molecular detection of NR and DN bacteria was found to both under- and over-estimate different types of culturable NR and DN bacteria. And finally, as shown by the transformation of HA and FA by NDO, broad-substrate dioxygenases can be potential humic-modifying enzymes when released into the environment upon microbial death, thus altering the bioavailability of organic contaminants associated with soil.

Appendices

Appendix A: Duncan's Multiple Range Test for Figure 2.1, soil moisture for G5, G5P, J6-F, J6-NF, J6P, March 2005.

		Subset			
Site	N	1	2	3	4
G5P	2	13.6000			
J6NF	2		16.5500		
J6F	2			17.4500	
G5	6			18.0167	
J6P	2				21.0000
Sig.		1.000	1.000	0.165	1.000

Means for groups in homogeneous subsets are displayed based on observed means. Alpha = 0.05.
The error term is Mean Square (Error) = 0.162.
Uses Harmonic Mean Sample Size = 2.308.

Appendix B.1: Duncan's Multiple Range Test for Figure 2.4.a, soil moisture for G7, G7P.

Site	N	Subset 1	Subset 2	Subset 3
G7P October 2005	2	12.8500		
G7P June 2006	4	13.7000		
G7 June 2006	34		22.7588	
G7 October 2005	34		23.2824	23.2824
G7 July 2005	34			25.8471
Sig.		0.567	0.724	0.086

Means for groups in homogeneous subsets are displayed based on observed means. Alpha = 0.05.

The error term is Mean Square (Error) = 6.517.

Uses Harmonic Mean Sample Size = 5.965.

Appendix B.2: Duncan's Multiple Range Test for Figure 2.4.b, soil moisture for LF, LFP.

Site	N	Subset 1	Subset 2	Subset 3
LFP October 2005	2	12.9500		
LFP June 2006	4	13.5250	13.5250	
LF October 2005	34		18.9029	18.9029
LF June 2006	34		18.9353	18.9353
LF August 2005	34			20.6853
Sig.		0.829	0.056	0.531

Means for groups in homogeneous subsets are displayed based on observed means. Alpha = 0.05.

The error term is Mean Square (Error) = 20.992.

Uses Harmonic Mean Sample Size = 5.965.

Appendix C. Linear regression parameters for Figure 2.5.

Figure 2.5	Site	Sampling date	Dependent variable	Number	Y-intercept	Slope	R^2
a	G7	Jul-05	Heterotrophs	34	6.7967	0.0102	0.0044
b	G7	Jul-05	NR and DN	34	6.2344	0.0074	0.0006
c	G7	Jul-05	%NR and DN	34	87.355	-1.3711	0.0091
d	LF	Aug-05	Heterotrophs	33	7.327	0.0047	0.0014
e	LF	Aug-05	NR and DN	33	5.7196	0.0327	0.0678
f	LF	Aug-05	%NR and DN	33	2.749	1.3891	0.0346
g	G7	Oct-05	Heterotrophs	34	7.7373	-0.0114	0.0122
g	G7P	Oct-05	Heterotrophs	2	N/A*	N/A	N/A
h	G7	Oct-05	NR and DN	34	7.2899	-0.0335	0.0197
h	G7P	Oct-05	NR and DN	2	N/A	N/A	N/A
i	G7	Oct-05	%NR and DN	34	37.498	-0.6484	0.0053
i	G7P	Oct-05	%NR and DN	2	N/A	N/A	N/A
j	LF	Oct-05	Heterotrophs	34	9.7612	-0.0914	0.1766
j	LFP	Oct-05	Heterotrophs	2	N/A	N/A	N/A
k	LF	Oct-05	NR and DN	34	5.9641	0.0399	0.0783
k	LFP	Oct-05	NR and DN	2	N/A	N/A	N/A
l	LF	Oct-05	%NR and DN	34	– 19.09	2.3038	0.1057
l	LFP	Oct-05	%NR and DN	2	N/A	N/A	N/A
m	G7	Jun-06	Heterotrophs	34	7.1239	0.0171	0.0137
m	G7P	Jun-06	Heterotrophs	4	7.1668	-0.0257	0.0443
n	G7	Jun-06	NR and DN	34	6.5879	0.0272	0.0351
n	G7P	Jun-06	NR and DN	4	2.5096	0.2609	0.2609
o	G7	Jun-06	%NR and DN	34	30.328	1.0922	0.0075
o	G7P	Jun-06	%NR and DN	4	-105.75	9.6911	0.5251
p	LF	Jun-06	Heterotrophs	34	7.2679	0.0007	0.0001
p	LFP	Jun-06	Heterotrophs	4	7.1353	-0.046	0.169
q	LF	Jun-06	NR and DN	34	6.4948	0.0242	0.0891
q	LFP	Jun-06	NR and DN	4	8.6306	-0.1715	0.7489
r	LF	Jun-06	%NR and DN	34	2.2844	2.8083	0.2427
r	LFP	Jun-06	%NR and DN	4	313.08	-17.972	0.7965

*N/A: Not applicable.

Appendix D.1. Duncan's Multiple Range Test for Figure 2.6.a. MPN of Heterotrophs for G7, G7P.

Site	N	Subset 1	Subset 2	Subset 3
G7P Hetro June 2006	4	6.8150		
G7 Hetro July 2005	34	7.0612	7.0612	
G7P Hetro October 2005	2		7.2923	7.2923
G7 Hetro October 2005	34		7.4724	7.4724
G7 Hetro June 2006	34			7.5129
Sig.		0.219	0.053	0.301

Means for groups in homogeneous subsets are displayed based on observed means. Alpha = 0.05.
The error term is Mean Square (Error) = 0.118.
Uses Harmonic Mean Sample Size = 5.965.

Appendix D.2. Duncan's Multiple Range Test for Figure 2.6.b. MPN of NR and DN for G7, G7P.

Site	N	Subset 1	Subset 2
G7P NR and DN June 2006	4	6.0812	
G7P NR and DN October 2005	2	6.3041	
G7 NR and DN July 2005	34	6.4263	
G7 NR and DN October 2005	34	6.5096	6.5096
G7 NR and DN June 2006	34		7.2061
Sig.		0.288	0.057

Means for groups in homogeneous subsets are displayed based on observed means. Alpha = 0.05.
The error term is Mean Square (Error) = 0.391.
Uses Harmonic Mean Sample Size = 5.965.

Appendix D.3. Duncan's Multiple Range Test for Figure 2.6.c. %NR and DN for G7, G7P.

Site	N	Subset for alpha = 0.05	
		1	2
G7P %NR and DN October 2005	2	10.3000	
G7 %NR and DN October 2005	34	22.3882	22.3882
G7P %NR and DN June 2006	4	26.8750	26.8750
G7 %NR and DN July 2005	34		51.9088
G7 %NR and DN June 2006	34		55.1794
Sig.		0.388	0.098

Means for groups in homogeneous subsets are displayed based on observed means.

Uses Harmonic Mean Sample Size = 5.965.

Appendix D.4. Duncan's Multiple Range Test for Figure 2.6.d. MPN of Heterotrophs for LF, LFP.

Site	N	Subset	
		1	2
LFP Hetero June 2006	4	6.5134	
LFP Hetero October 2005	2	6.9814	
LF Hetero August 2005	33	7.2291	
LF Hetero June 2006	34	7.2811	
LF Hetero October 2005	34		8.0332
Sig.		0.061	1.000

Means for groups in homogeneous subsets are displayed based on observed means. Alpha = 0.05.

The error term is Mean Square (Error) = 0.413.

Uses Harmonic Mean Sample Size = 5.959.

Appendix D.5. Duncan's Multiple Range Test for Figure 2.6.e. MPN of NR and DN for LF, LFP.

Site	N	Subset 1	Subset 2
LFP NR and DN October 2005	2	6.1707	
LFP NR and DN June 2006	4	6.3109	6.3109
LF NR and DN August 2005	33	6.3963	6.3963
LF NR and DN October 2005	34	6.7175	6.7175
LF NR and DN June 2006	34		6.9533
Sig.		0.106	0.057

Means for groups in homogeneous subsets are displayed based on observed means. Alpha = 0.05.

The error term is Mean Square (Error) = 0.279.

Uses Harmonic Mean Sample Size = 5.959.

Appendix D. 6. Duncan's Multiple Range Test for Figure 2.6.f. %NR and DN for LF, LFP.

Site	N	Subset for alpha = 0.05 1	Subset for alpha = 0.05 2
LFP %NR and DN October 2005	2	16.9500	
LF %NR and DN October 2005	34	24.4324	
LF %NR and DN August 2005	33	31.5121	
LF %NR and DN June 2006	34	55.4559	55.4559
LFP %NR and DN June 2006	4		69.9500
Sig.		0.053	0.424

Means for groups in homogeneous subsets are displayed based on observed means.

Uses Harmonic Mean Sample Size = 5.959.

Appendix E.1. Two-way ANOVA for Figure 2.4. G7, July 2005.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	log_{10}denit	1.080[a]	3	0.360	0.516	0.675
	log_{10}hetero	0.226[b]	3	0.075	0.384	0.765
	perdenit	5751.055[c]	3	1917.018	1.227	0.317
Intercept	log_{10}denit	1401.782	1	1401.782	2007.932	0.000
	log_{10}hetero	1689.935	1	1689.935	8638.090	0.000
	perdenit	91740.276	1	91740.276	58.727	0.000
hay	log_{10}denit	0.332	1	0.332	0.476	0.496
	log_{10}hetero	0.012	1	0.012	0.062	0.805
	perdenit	155.208	1	155.208	0.099	0.755
fertiliz	log_{10}denit	0.416	1	0.416	0.596	0.446
	log_{10}hetero	0.155	1	0.155	0.794	0.380
	perdenit	3500.682	1	3500.682	2.241	0.145
hay * fertiliz	log_{10}denit	0.289	1	0.289	0.414	0.525
	log_{10}hetero	0.047	1	0.047	0.242	0.626
	perdenit	1781.965	1	1781.965	1.141	0.294
Error	log_{10}denit	20.944	30	0.698		
	log_{10}hetero	5.869	30	0.196		
	perdenit	46864.812	30	1562.160		
Total	log_{10}denit	1426.128	34			
	log_{10}hetero	1701.363	34			
	perdenit	144229.750	34			
Corrected Total	log_{10}denit	22.024	33			
	log_{10}hetero	6.095	33			
	perdenit	52615.867	33			

a. R Squared = .049 (Adjusted R Squared = -0.046).

b. R Squared = .037 (Adjusted R Squared = -0.059).

c. R Squared = .109 (Adjusted R Squared = 0.020).

log_{10}denit: log_{10} denitrifyers.

log_{10}hetero: log_{10} heterotrophic bacteria.

perdenit: %NR and DN.

Appendix E.2. Two-way ANOVA for Figure 2.4. G7, October 2005.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	log_{10}denit	2.348[a]	3	0.783	1.930	0.146
	log_{10}hetero	0.024[b]	3	0.008	0.088	0.966
	perdenit	2865.859[c]	3	955.286	1.664	0.196
Intercept	log_{10}denit	1441.154	1	1441.154	3552.577	0.000
	log_{10}hetero	1892.018	1	1892.018	21141.556	0.000
	perdenit	17717.631	1	17717.631	30.865	0.000
hay	log_{10}denit	1.457	1	1.457	3.592	**0.068**[*]
	log_{10}hetero	0.000	1	0.000	0.006	0.941
	perdenit	2247.867	1	2247.867	3.916	**0.057**[*]
fertiliz	log_{10}denit	0.636	1	0.636	1.569	0.220
	log_{10}hetero	0.014	1	0.014	0.157	0.695
	perdenit	533.493	1	533.493	.929	0.343
hay * fertiliz	log_{10}denit	0.209	1	0.209	0.515	0.479
	log_{10}hetero	0.008	1	0.008	0.086	0.772
	perdenit	61.117	1	61.117	0.106	0.746
Error	log_{10}denit	12.170	30	0.406		
	log_{10}hetero	2.685	30	0.089		
	perdenit	17220.936	30	574.031		
Total	log_{10}denit	1455.272	34			
	log_{10}hetero	1901.181	34			
	perdenit	37128.720	34			
Corrected Total	log_{10}denit	14.518	33			
	log_{10}hetero	2.708	33			
	perdenit	20086.795	33			

a. R Squared = .162 (Adjusted R Squared = 0.078).

b. R Squared = .009 (Adjusted R Squared = -0.090).

c. R Squared = .143 (Adjusted R Squared = 0.057).

log_{10}denit: log_{10} denitrifyers.

log_{10}hetero: log_{10} heterotrophic bacteria.

perdenit: %NR and DN.

[*]significant treatment effects (p<0.05) and near significant effects (p<0.1).

Appendix E.3. Two-way ANOVA for Figure 2.4. G7, June 2006.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	log_{10}denit	0.142[a]	3	0.047	0.496	0.688
	log_{10}hetero	0.095[b]	3	0.032	0.384	0.765
	perdenit	3913.493[c]	3	1304.498	2.012	0.134
Intercept	log_{10}denit	1703.378	1	1703.378	17810.685	0.000
	log_{10}hetero	1856.706	1	1856.706	22481.584	0.000
	perdenit	93079.617	1	93079.617	143.576	0.000
hay	log_{10}denit	0.013	1	0.013	0.132	0.719
	log_{10}hetero	0.002	1	0.002	0.020	0.888
	perdenit	8.180	1	8.180	0.013	0.911
fertiliz	log_{10}denit	0.002	1	0.002	0.016	0.901
	log_{10}hetero	0.059	1	0.059	0.714	0.405
	perdenit	1129.048	1	1129.048	1.742	0.197
hay * fertiliz	log_{10}denit	0.126	1	0.126	1.323	0.260
	log_{10}hetero	0.042	1	0.042	0.503	0.484
	perdenit	3063.133	1	3063.133	4.725	**0.038**[*]
Error	log_{10}denit	2.774	29	0.096		
	log_{10}hetero	2.395	29	0.083		
	perdenit	18800.582	29	648.296		
Total	log_{10}denit	1724.818	33			
	log_{10}hetero	1878.371	33			
	perdenit	118305.930	33			
Corrected Total	log_{10}denit	2.916	32			
	log_{10}hetero	2.490	32			
	perdenit	22714.075	32			

a. R Squared = .049 (Adjusted R Squared = -0.050).

b. R Squared = .038 (Adjusted R Squared = -0.061).

c. R Squared = .172 (Adjusted R Squared = 0.087).

log_{10}denit: log_{10} denitrifyers.

log_{10}hetero: log_{10} heterotrophic bacteria.

perdenit: %NR and DN.

[*]significant treatment effects (p<0.05) and near significant effects (p<0.1).

Appendix E.4. Two-way ANOVA for Figure 2.4. LF, August 2005.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	log_{10}denit	0.842[a]	3	0.281	0.866	0.470
	log_{10}hetero	0.210[b]	3	0.070	0.206	0.891
	perdenit	621.373[c]	3	207.124	0.110	0.954
Intercept	log_{10}denit	1329.133	1	1329.133	4099.355	0.000
	log_{10}hetero	1705.927	1	1705.927	5033.009	0.000
	perdenit	38389.118	1	38389.118	20.393	0.000
hay	log_{10}denit	0.795	1	0.795	2.453	0.128
	log_{10}hetero	0.027	1	0.027	0.080	0.779
	perdenit	386.916	1	386.916	0.206	0.654
fertiliz	log_{10}denit	0.002	1	0.002	0.006	0.941
	log_{10}hetero	0.138	1	0.138	0.406	0.529
	perdenit	23.308	1	23.308	0.012	0.912
hay * fertiliz	log_{10}denit	0.062	1	0.062	0.192	0.665
	log_{10}hetero	0.052	1	0.052	0.154	0.698
	perdenit	207.611	1	207.611	0.110	0.742
Error	log_{10}denit	9.403	29	0.324		
	log_{10}hetero	9.829	29	0.339		
	perdenit	54590.269	29	1882.423		
Total	log_{10}denit	1360.352	33			
	log_{10}hetero	1734.638	33			
	perdenit	94842.580	33			
Corrected Total	log_{10}denit	10.245	32			
	log_{10}hetero	10.039	32			
	perdenit	55211.642	32			

a. R Squared = .082 (Adjusted R Squared = -0.013).

b. R Squared = .021 (Adjusted R Squared = -0.080).

c. R Squared = .011 (Adjusted R Squared = -0.091).

log_{10}denit: log_{10} denitrifyers.

log_{10}hetero: log_{10} heterotrophic bacteria.

perdenit: %NR and DN.

Appendix E.5. Two-way ANOVA for Figure 2.4. LF, October 2005.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	log_{10}denit	2.109[a]	3	0.703	2.217	0.107
	log_{10}hetero	0.878[b]	3	0.293	0.335	0.800
	perdenit	5716.875[c]	3	1905.625	2.481	0.080
Intercept	log_{10}denit	1523.945	1	1523.945	4805.693	0.000
	log_{10}hetero	2187.802	1	2187.802	2502.049	0.000
	perdenit	19440.638	1	19440.638	25.312	0.000
hay	log_{10}denit	1.180	1	1.180	3.721	**0.063**[*]
	log_{10}hetero	0.054	1	0.054	0.062	0.805
	perdenit	2245.567	1	2245.567	2.924	**0.098**[*]
fertiliz	log_{10}denit	0.764	1	0.764	2.410	0.131
	log_{10}hetero	0.799	1	0.799	0.914	0.347
	perdenit	3406.613	1	3406.613	4.436	**0.044**[*]
hay * fertiliz	log_{10}denit	0.208	1	0.208	0.657	0.424
	log_{10}hetero	0.011	1	0.011	0.013	0.910
	perdenit	21.124	1	21.124	0.028	0.869
Error	log_{10}denit	9.513	30	0.317		
	log_{10}hetero	26.232	30	0.874		
	perdenit	23040.999	30	768.033		
Total	log_{10}denit	1545.869	34			
	log_{10}hetero	2221.226	34			
	perdenit	49053.830	34			
Corrected Total	log_{10}denit	11.622	33			
	log_{10}hetero	27.111	33			
	perdenit	28757.874	33			

a. R Squared = .181 (Adjusted R Squared = 0.100).

b. R Squared = .032 (Adjusted R Squared = -0.064).

c. R Squared = .199 (Adjusted R Squared = 0.119).

log_{10}denit: log_{10} denitrifyers.

log_{10}hetero: log_{10} heterotrophic bacteria.

perdenit: %NR and DN.

[*]significant treatment effects (p<0.05) and near significant effects (p<0.1).

Appendix E.6. Two-way ANOVA for Figure 2.4. LF, June 2006.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	log_{10}denit	0.294[a]	3	0.098	0.503	0.683
	log_{10}hetero	0.402[b]	3	0.134	0.968	0.421
	perdenit	4463.824[c]	3	1487.941	1.729	0.182
Intercept	log_{10}denit	1640.150	1	1640.150	8424.299	0.000
	log_{10}hetero	1796.773	1	1796.773	12976.045	0.000
	perdenit	105140.632	1	105140.632	122.182	0.000
hay	log_{10}denit	0.177	1	0.177	0.907	0.349
	log_{10}hetero	0.011	1	0.011	0.083	0.776
	perdenit	610.401	1	610.401	0.709	0.406
fertiliz	log_{10}denit	0.001	1	0.001	0.007	0.933
	log_{10}hetero	0.297	1	0.297	2.142	0.154
	perdenit	3831.503	1	3831.503	4.453	**0.043**[*]
hay * fertiliz	log_{10}denit	0.114	1	0.114	0.584	0.451
	log_{10}hetero	0.114	1	0.114	0.825	0.371
	perdenit	69.138	1	69.138	0.080	0.779
Error	log_{10}denit	5.841	30	0.195		
	log_{10}hetero	4.154	30	0.138		
	perdenit	25815.760	30	860.525		
Total	log_{10}denit	1649.971	34			
	log_{10}hetero	1807.032	34			
	perdenit	134841.650	34			
Corrected Total	log_{10}denit	6.134	33			
	log_{10}hetero	4.556	33			
	perdenit	30279.584	33			

a. R Squared = .048 (Adjusted R Squared = -0.047).

b. R Squared = .088 (Adjusted R Squared = -0.003).

c. R Squared = .147 (Adjusted R Squared = 0.062).

log_{10}denit: log_{10} denitrifyers.

log_{10}hetero: log_{10} heterotrophic bacteria.

perdenit: %NR and DN.

[*]significant treatment effects (p<0.05) and near significant effects (p<0.1).

Appendix F.1. Mean log_{10} Heterotrophs, log_{10} NR and DN, and %NR and DN for plus hay and no hay enclosures.

Site	Date	N	Hay	Log_{10}Het	Log_{10} NR and DN	%NR and DN
G7	Jul-05	16	No	7.081 (0.111)	6.531 (0.209)	54.175 (9.881)
		18	Yes	7.043 (0.104)	6.333 (0.197)	49.894 (9.316)
G7	Oct-05	16	No	7.477 (0.075)	6.729 (0.159)	31.013 (5.990)
		18	Yes	7.469 (0.071)	6.314 (0.150)	14.722 (5.647)
G7	Jun-06	16	No	7.534 (0.074)	7.243 (0.080)	53.896 (6.589)
		18	Yes	7.548 (0.068)	7.204 (0.073)	52.894 (6.001)
LF	Aug-05	15	No	7.257 (0.151)	6.224 (0.147)	30.848 (11.227)
		18	Yes	7.200 (137)	6.537 (0.134)	37.733 (10.226)
LF	Oct-05	15	No	8.076 (0.234)	6.520 (0.141)	15.813 (6.928)
		18	Yes	7.996 (0.220)	6.893 (0.133)	32.094 (6.532)
LF	Jun-06	15	No	7.301 (0.093)	7.030 (0.110)	59.950 (7.334)
		18	Yes	7.264 (0.088)	6.885 (0.104)	51.461 (6.914)

N: number of enclosures sampled.

Appendix F.2. Mean log_{10} Heterotrophs, log_{10} NR and DN, and %NR and DN for plus fertilizer and no fertilizer enclosures.

Site	Date	N	Fertilizer	Log_{10} Het	Log_{10} NR and DN	%NR and DN
G7	Jul-05	17	No	6.995 (0.107)	6.543 (0.203)	62.199 (9.603)
		17	Yes	7.130 (0.107)	6.321 (0.203)	41.870 (9.603)
G7	Oct-05	17	No	7.452 (0.073)	6.385 (0.155)	18.899 (5.821)
		17	Yes	7.493 (0.073)	6.659 (0.155)	26.835 (5.821)
G7	Jun-06	17	No	7.499 (0.070)	7.230 (0.075)	59.276 (6.186)
		17	Yes	7.584 (0.072)	7.216 (0.078)	47.514 (6.416)
LF	Aug-05	17	No	7.294 (0.141)	6.388 (0.138)	33.446 (10.541)
		16	Yes	7.164 (0.147)	6.373 (0.143)	35.136 (10.932)
LF	Oct-05	17	No	8.189 (0.227)	6.556 (0.137)	13.926 (6.733)
		16	Yes	7.882 (0.227)	6.857 (0.137)	33.981 (6.733)
LF	Jun-06	17	No	7.189 (0.090)	6.964 (0.107)	66.340 (7.127)
		16	Yes	7.376 (0.090)	6.951 (0.107)	45.072 (7.127)

Appendix F.3. Mean log_{10} NR and DN, log_{10} Heterotrophs, and %NR and DN in G7 subdivided by hay and/or fertilizer addition.

Dependent variable	Hay	Fertilizer	Mean (SE)	Date
Log_{10} Denit	No	No	6.550 (0.295)	Jul-05
		Yes	6.513 (0.295)	
	Yes	No	6.536 (0.279)	
		Yes	6.130 (0.279)	
Log_{10} Het	No	No	7.051 (0.156)	Jul-05
		Yes	7.112 (0.156)	
	Yes	No	6.938 (0.147)	
		Yes	7.148 (0.147)	
%NR and DN	No	No	57.088 (13.974)	Jul-05
		Yes	51.263 (13.974)	
	Yes	No	67.311 (13.175)	
		Yes	32.478 (13.175)	
Log_{10} Denit	No	No	6.671 (0.225)	Oct-05
		Yes	6.788 (0.225)	
	Yes	No	6.099 (0.212)	
		Yes	6.530 (0.212)	
Log_{10} Het	No	No	7.471 (0.106)	Oct-05
		Yes	7.482 (0.106)	
	Yes	No	7.433 (0.100)	
		Yes	7.504 (0.100)	
%NR and DN	No	No	28.388 (8.471)	Oct-05
		Yes	33.638 (8.471)	
	Yes	No	9.411 (7.986)	
		Yes	20.033 (7.986)	
Log_{10} Denit	No	No	7.312 (0.109)	Jun-06
		Yes	7.174 (0.117)	
	Yes	No	7.148 (0.103)	
		Yes	7.259 (0.103)	
Log_{10} Het	No	No	7.456 (0.102)	Jun-06
		Yes	7.612 (0.109)	
	Yes	No	7.542 (0.096)	
		Yes	7.555 (0.96)	
%NR and DN	No	No	69.463 (9.002)	Jun-06
		Yes	38.329 (9.624)	

	Yes	No	49.089 (8.487)	
		Yes	56.700 (8.487)	

Appendix F.4. Mean log_{10} NR and DN, log_{10} Heterotrophs, and %NR and DN in LF subdivided by hay and/or fertilizer addition.

Dependent variable	Hay	Fertilizer	Mean (SE)	Date
Log_{10} Denit	No	No	6.276 (0.201)	Aug-05
		Yes	6.173 (0.215)	
	Yes	No	6.500 (0.190)	
		Yes	6.573 (0.190)	
Log_{10} Het	No	No	7.362 (0.206)	Aug-05
		Yes	7.153 (0.220)	
	Yes	No	7.225 (0.194)	
		Yes	7.175 (0.194)	
%NR and DN	No	No	32.525 (15.340)	Aug-05
		Yes	29.171 (16.399)	
	Yes	No	34.367 (14.462)	
		Yes	41.100 (14.462)	
Log_{10} Denit	No	No	6.291 (0.199)	Oct-05
		Yes	6.749 (0.199)	
	Yes	No	6.821 (0.188)	
		Yes	6.965 (0.188)	
Log_{10} Het	No	No	8.211 (0.331)	Oct-05
		Yes	7.940 (0.331)	
	Yes	No	8.167 (0.312)	
		Yes	7.824 (0.312)	
%NR and DN	No	No	6.575 (9.798)	Oct-05
		Yes	25.050 (9.798)	
	Yes	No	21.278 (9.238)	
		Yes	42.911 (9.238)	
Log_{10} Denit	No	No	6.978 (0.156)	Jun-06
		Yes	7.081 (0.156)	
	Yes	No	6.950 (0.147)	
		Yes	6.821 (0.147)	
Log_{10} Het	No	No	7.149 (0.132)	Jun-06
		Yes	7.452 (0.132)	
	Yes	No	7.228 (0.124)	
		Yes	7.299 (0.124)	
%NR and DN	No	No	72.013 (10.371)	Jun-06
		Yes	47.888 (10.371)	
	Yes	No	60.667 (9.778)	

		Yes	42.256 (9.778)	

Appendix G.1. Dilution origin for 75 strains.

Strain #	Site	Genus (class/subclass)[a]	Dilution[b]	Phenotype[c]
1	G7	*Pseudomona* (γ)	10^{-3}	DN
2	G7	*Pseudomonas* (γ)	10^{-3}	DN
3	G7	*Pseudomonas*(γ)	10^{-3}	NR
4	**G7**	***Ensifer*(α)**	**10^{-4}**	**DN**
5	G7	*Achromobacter* (β)	10^{-3}	NR
6	G7	*Aeromonas* (γ)	10^{-3}	NR
7	**G7**	***Bosea* (α)**	**10^{-4}**	**None**
8	**G7**	***Bosea* (α)**	**10^{-4}**	**None**
9	**G7**	***Arthrobacter* (Actino)**	**10^{-5}**	**None**
10	**G7**	***Phenylobacterium* (α)**	**10^{-5}**	**NR**
11	G5	*Stenotrophomonas* (γ)	10^{-2}	NR
12	G5	*Pseudomonas* (γ)	10^{-3}	NR
13	**G5**	***Stenotrophomonas* (γ)**	**10^{-4}**	**NR**
14	G5	*Brevibacillus* (Bacilli)	10^{-2}	NR
15	G5	*Bacillus* (Bacilli)	10^{-3}	NR
16	G5	*Brevibacillus* (Bacilli)	10^{-2}	NR
17	G5	*Paenibacillus* (Bacilli)	10^{-2}	NR
18	G5	*Stenotrophomonas* (γ)	10^{-2}	NR
19	G5	*Brevibacillus* (Bacilli)	10^{-2}	NR
20	G5	*Bacillus* (Bacilli)	10^{-2}	NR
21	LF	*Stenotrophomonas* (γ)	10^{-3}	NR
22	LF	*Chryseobacterium* (Flavo)	10^{-3}	NR
23	LF	*Stenotrophomonas* (γ)	10^{-3}	NR
24	**LF**	***Serratia* (γ)**	**10^{-4}**	**NR**
25	**LF**	***Enterobacter* (γ)**	**10^{-5}**	**NR**
26	J6-NF	*Stenotrophomonas* (γ)	10^{-2}	None
27	J6-NF	*Stenotrophomonas* (γ)	10^{-3}	None
28	**J6-NF**	***Stenotrophomonas* (γ)**	**10^{-4}**	**None**
29	J6-F	*Microbacterium* (Actino)	10^{-2}	NR
30	J6-F	*Pseudomonas* (γ)	10^{-2}	NR
31	J6-F	*Enterobacter* (γ)	10^{-3}	NR
32	J6-F	*Enterobacter* (γ)	10^{-3}	NR
33	G5P	*Lysinibacillus*(Bacilli)	10^{-2}	NR
34	G5P	*Stenotrophomonas* (γ)	10^{-2}	NR

35	G5P	*Stenotrophomonas* (γ)	10^{-2}	NR
36	G5P	*Stenotrophomonas* (γ)	10^{-2}	NR
37	G5P	*Stenotrophomonas* (γ)	10^{-2}	NR
38	G5P	*Stenotrophomonas* (γ)	10^{-2}	NR
39	G5P	*Stenotrophomonas* (γ)	10^{-2}	NR
40	G5P	*Stenotrophomonas* (γ)	10^{-2}	NR
41	G5P	*Stenotrophomonas* (γ)	10^{-3}	NR
42	**G5P**	***Stenotrophomonas* (γ)**	**10^{-4}**	**NR**
43	J6P	*Achromobacter* (β)	10^{-2}	NR
44	J6P	*Lysinibacillus*(Bacilli)	10^{-2}	None
45	J6P	*Achromobacter* (β)	10^{-2}	NR
46	J6P	*Brevibacillus* (Bacilli)	10^{-3}	NR
47	J6P	*Brevibacillus* (Bacilli)	10^{-3}	None
48	**J6P**	***Rhodococcus* (Actino)**	**10^{-4}**	**None**
49	G7P	*Achromobacter* (β)	10^{-2}	NR
50	G7P	*Stenotrophomonas* (γ)	10^{-2}	None
51	G7P	*Stenotrophomonas* (γ)	10^{-2}	None
52	G7P	*Stenotrophomonas* (γ)	10^{-2}	None
53	**G7P**	***Brevibacillus* (Bacilli)**	**10^{-4}**	**NR**
54	G7P	*Brevibacillus* (Bacilli)	10^{-3}	NR
55	**G7P**	***Acinetobacter* (γ)**	**10^{-4}**	**None**
56	**G7P**	***Acinetobacter*(γ)**	**10^{-4}**	**NR**
57	G7P	*Stenotrophomonas* (γ)	10^{-2}	DN
58	G7P	*Acinetobacter* (γ)	10^{-2}	None
59	G7P	*Brevibacillus* (Bacilli)	10^{-3}	NR
60	G7P	*Bacillus* (Bacilli)	10^{-3}	None
61	LFP	*Stenotrophomonas* (γ)	10^{-2}	NR
62	LFP	*Stenotrophomonas* (γ)	10^{-2}	None
63	LFP	*Stenotrophomonas* (γ)	10^{-2}	None
64	LFP	*Stenotrophomonas* (γ)	10^{-2}	None
65	LFP	*Pseudomonas* (γ)	10^{-3}	DN
66	LFP	*Burkholderia* (β)	10^{-3}	None
67	**LFP**	***Pseudomonas* (γ)**	**10^{-4}**	**NR**
68	**LFP**	***Pseudomonas* (γ)**	**10^{-4}**	**NR**
69	LFP	*Ensifer* (α)	10^{-2}	DN
70	LFP	*Acinetobacter* (γ)	10^{-2}	NR
71	LFP	*Stenotrophomonas* (γ)	10^{-3}	None

72	LFP	*Acinetobacter* (γ)	10^{-3}	None
73	**LFP**	***Pseudomonas* (γ)**	**10^{-4}**	**NR**
74	LFP	*Stenotrophomonas* (γ)	10^{-2}	NR
75	LFP	*Kocuria* (Actino)	10^{-3}	NR

[a] Genus (class/subclass) as determined by The Ribosomal Database Project (RDS) Classifier program.

[b] Dilution corresponded to microtiter plate-wells from which strains were originated.

[c] Phenotype: NR: nitrate reduction, DN: nitrite reduction/denitrification, None: no reduction of nitrate or nitrite.

[*]The highly abundant strains (e.g. originated from wells with 10^{-4} or 10^{-5} dilution) are indicated in bold letters.

Appendix G.2.Summary of isolates, molecular detection of NR and DN genes, and NR and DN physiology.

Isolate #	Isolate name (16S rRNA Accession #)	Site type	Genus [% similarity]* (Class/subclas)	Closest GenBank match accession # (% similarity)	*nap A*	*nar G*	*nir S*	*nir K*	*cnor B*	*nos Z*	NR and DN
1	**G7-101B3B (JQ917765)**	**Brine**	***Pseudomonas* [100%] (γ)**	**DQ647192 (99%)**	+	+	+		+	+	**DN**
2	**G7-105B12A (JQ917766)**	**Brine**	***Pseudomonas* [100%] (γ)**	**HQ824922 (100%)**	+	+	+		+	+	**DN**
3	G7-129B8B (JQ917767)	Brine	*Pseudomonas* [100%] (γ)	JF703647 (99%)							NR
4	**G7-135C4 (JQ917768)**	**Brine**	***Ensifer* [99%] (α)**	**HM219616 (100%)**					+		**DN**
5	**G7-221B10A (JQ917769)**	**Brine**	***Achromobacter* [99%] (β)**	**JN629044 (99%)**		+				+	**NR**
6	**G7-221B10B (JQ917770)**	**Brine**	***Aeromonas* [100%] (γ)**	**GU205200 (99%)**	+					+	**NR**
7	G7-305C11A1 (JQ917771)	Brine	*Bosea* [100%] (α)	AJ250800 (99%)	+						None
8	G7-305C11B (JQ917772)	Brine	*Bosea* [100%] (α)	AJ250800 (99%)	+						None
9	G7-325D2 (JQ917773)	Brine	*Arthrobacter* [100%] (Actino)	JF439618 (99%)							None
10	**G7-401D6 (JQ917774)**	**Brine**	***Phenylobacterium* [100%] (α)**	**FJ605405 (99%)**							**NR**
11	**G5NA3A (JQ917775)**	**Brine**	***Stenotrophomonas* [100%] (γ)**	**FJ404810 (99%)**							**NR**
12	G5NB3B (JQ917776)	Brine	*Pseudomonas* [95%] (γ)	FJ006889 (99%)	+						NR
13	**G5NC6A2 (JQ917777)**	**Brine**	***Stenotrophomonas* [100%] (γ)**	**FJ404810 (99%)**							**NR**
14	G5MA8	Brine	*Brevibacillus*	AB112720							NR

	(JQ917778)		[100%] (Bacilli)	(99%)							
15	G5MB8A (JQ917779)	Brine	*Bacillus* [100%] (Bacilli)	GU568201 (99%)							NR
16	G5MA9A2 (JQ917780)	Brine	*Brevibacillus* [100%] (Bacilli)	DQ371289 (99%)							NR
17	G5MA10B (JQ917781)	Brine	*Paenibacillus* [100%] (Bacilli)	HQ236085 (99%)							NR
18	**G5SA2A1 (JQ917782)**	**Brine**	***Stenotrophomonas* [100%] (γ)**	**JN987861 (99%)**		+					**NR**
19	G5SA3A (JQ917783)	Brine	*Brevibacillus* [100%] (Bacilli)	FR686596 (99%)							NR
20	G5SA5A (JQ917784)	Brine	*Bacillus* [100%] (Bacilli)	JF496369 (99%)							NR
21	**LF-521B12 (JQ917785)**	**Oil**	***Stenotrophomonas* [100%] (γ)**	**JN867123 (100%)**							**NR**
22	LF-617B2A1A (JQ917786)	Oil	*Chryseobacterium* [100%] (Flavo)	JN208181 (99%)							NR
23	**LF-617B2A1B (JQ917787)**	**Oil**	***Stenotrophomonas* [100%] (γ)**	**HQ670707 (99%)**							**NR**
24	**LF-821C8A1 (JQ917788)**	**Oil**	***Serratia* [100%] (γ)**	**AB680122 (99%)**		+					**NR**
25	**LF-825D4 (JQ917789)**	**Oil**	***Enterobacter* [99%] (γ)**	**HM854373 (99%)**		+		+			**NR**
26	J6N-NFA4A (JQ917790)	Oil	*Stenotrophomonas* [100%] (γ)	EU430096 (99%)							None
27	J6N-NFB4B1 (JQ917791)	Oil	*Stenotrophomonas* [100%] (γ)	GQ280904 (99%)							None
28	J6N-NFC6A2 (JQ917792)	Oil	*Stenotrophomonas* [100%] (γ)	EU430096 (99%)		+					None
29	**J6N-FA10A**	**Oil**	***Microbacterium***	**HM569613**							**NR**

	(JQ917793)		**[100%] (Actino)**	**(100%)**							
30	**J6N-FA10C (JQ917794)**	**Oil**	***Pseudomonas* [100%] (γ)**	**AB646255 (100%)**							**NR**
31	J6N-FB11B (JQ917795)	Oil	*Enterobacter* [92%] (γ)	JF431271 (99%)		+					NR
32	J6N-FB11C (JQ917796)	Oil	*Enterobacter* [100%] (γ)	JF346895 (99%)		+					NR
33	G5PA8B1 (JQ917797)	Prairie	*Lysinibacillus* [100%] (Bacilli)	JQ446580 (99%)							NR
34	**G5PA8B2 (JQ917798)**	**Prairie**	***Stenotrophomonas* [100%] (γ)**	**JN987861 (99%)**		+					**NR**
35	**G5PA9A1 (JQ917799)**	**Prairie**	***Stenotrophomonas* [100%] (γ)**	**JN987861 (99%)**		+					**NR**
36	**G5PA9A2 (JQ917800)**	**Prairie**	***Stenotrophomonas* [100%] (γ)**	**FJ404810 (99%)**							**NR**
37	**G5PA9B (JQ917801)**	**Prairie**	***Stenotrophomonas* [100%] (γ)**	**JN987861 (99%)**							**NR**
38	**G5PA10A1 (JQ917802)**	**Prairie**	***Stenotrophomonas* [100%] (γ)**	**JN987861 (99%)**		+					**NR**
39	**G5PA10A2 (JQ917803)**	**Prairie**	***Stenotrophomonas* [100%] (γ)**	**JN987861 (99%)**		+					**NR**
40	**G5PA10B (JQ917804)**	**Prairie**	***Stenotrophomonas* [100%] (γ)**	**JN987861 (99%)**		+					**NR**
41	**G5PB12A1 (JQ917805)**	**Prairie**	***Stenotrophomonas* [100%] (γ)**	**JN987861 (99%)**		+					**NR**
42	**G5PC11 (JQ917806)**	**Prairie**	***Stenotrophomonas* [100%] (γ)**	**JN987861 (99%)**		+					**NR**
43	J6PA9A (JQ917807)	Prairie	*Achromobacter* [100%] (β)	EU340142 (99%)							NR
44	J6PA9B2 (JQ917808)	Prairie	*Lysinibacillus* [100%] (Bacilli)	AB662958 (99%)							None
45	J6PA12A	Prairie	*Achromobacter*	HQ619222							NR

	(JQ917809)		[100%] (β)	(96%)							
46	J6PB8A (JQ917810)	Prairie	*Brevibacillus* [100%] (Bacilli)	AB112720 (99%)							NR
47	J6PB8B (JQ917811)	Prairie	*Brevibacillus* [100%] (Bacilli)	AB112720 (99%)							None
48	J6PC12A (JQ917812)	Prairie	*Rhodococcus* [100%] (Actino)	AY436807 (98%)							None
49	**G7P1A3A1 (JQ917813)**	**Prairie**	***Achromobacter* [100%] (β)**	**JN546225 (99%)**	+	+		+		+	**NR**
50	G7P1A3A2 (JQ917814)	Prairie	*Stenotrophomonas* [100%] (γ)	FJ976090 (99%)							None
51	G7P1A3B1 (JQ917815)	Prairie	*Stenotrophomonas* [100%] (γ)	FJ976090 (99%)							None
52	G7P1A3B2 (JQ917816)	Prairie	*Stenotrophomonas* [100%] (γ)	FJ976090 (99%)							None
53	**G7P1C4 (JQ917817)**	**Prairie**	***Brevibacillus* [100%] (**Bacilli**)**	**DQ371289 (99%)**							**NR**
54	G7P1B2 (JQ917818)	Prairie	*Brevibacillus* [100%] (Bacilli)	FR686596 (99%)							NR
55	G7P2C6A (JQ917819)	Prairie	*Acinetobacter* [100%] (γ)	JQ031270 (99%)							None
56	G7P2C6B (JQ917820)	Prairie	*Acinetobacter* [100%] (γ)	JQ031270 (99%)							NR
57	G7P2A3A (JQ917821)	Prairie	*Stenotrophomonas* [100%] (γ)	AY259519 (99%)							DN
58	G7P2A3B (JQ917822)	Prairie	*Acinetobacter* [100%] (γ)	JQ031270 (98%)							None
59	G7P2B3A (JQ917823)	Prairie	*Brevibacillus* [100%] (Bacilli)	AB112720 (99%)							NR
60	G7P2B3B (JQ917824)	Prairie	*Bacillus* [100%] (Bacilli)	JF460746 (99%)		+					None
61	**LFP1A9A1**	**Prairie**	***Stenotrophomonas***	**FJ493144**							**NR**

	(JQ917825)		**[100%] (γ)**	**(100%)**							
62	LFP1A9A2 (JQ917826)	Prairie	*Stenotrophomonas* [100%] (γ)	FJ493144 (99%)							None
63	LFP1A9B1 (JQ917827)	Prairie	*Stenotrophomonas* [100%] (γ)	GU564359 (99%)							None
64	LFP1A9B2 (JQ917828)	Prairie	*Stenotrophomonas* [100%] (γ)	AJ293470 (99%)							None
65	**LFP1B10A (JQ917829)**	**Prairie**	***Pseudomonas* [100%] (γ)**	**HQ143608 (99%)**		+			+	+	**DN**
66	LFP1B10B (JQ917830)	Prairie	*Burkholderia* [100%] (β)	AB508895 (99%)							None
67	**LFP1C12A (JQ917831)**	**Prairie**	***Pseudomonas* [100%] (γ)**	**FJ392835 (99%)**	+						**NR**
68	**LFP1C12B (JQ917832)**	**Prairie**	***Pseudomonas* [100%] (γ)**	**AF468453 (99%)**	+						**NR**
69	LFP2A8A2 (JQ917833)	Prairie	*Ensifer* [100%] (α)	JF432095 (100%)	+						DN
70	**LFP2A8B (JQ917834)**	**Prairie**	***Acinetobacter* [100%] (γ)**	**JQ031270 (100%)**							**NR**
71	LFP2B10A (JQ917835)	Prairie	*Stenotrophomonas* [100%] (γ)	EU741084 (99%)							None
72	LFP2B10B1 (JQ917836)	Prairie	*Acinetobacter* [100%] (γ)	JQ031270 (99%)							None
73	**LFP2C11 (JQ917837)**	**Prairie**	***Pseudomonas* [100%] (γ)**	**AF468453 (99%)**	+						**NR**
74	LFP2A8A1 (JQ917838)	Prairie	*Stenotrophomonas* [100%] (γ)	JN987861 (99%)							NR
75	**LFP2B10B2 (JQ917839)**	**Prairie**	***Kocuria* [100%] (Actino)**	**JN644483 (99%)**							**NR**

Isolate name: internal description of strain.

*Thresold level of similarity as determined by The Ribosomal Database Project (RDS) Classifier program.

NR: nitrate reduction.

DN: nitrite reduction/denitrification.

None: no reduction of nitrate or nitrite.

**The 32 selected strains are indicated in bold letters. The quinol-oxidizing single-subunit class (qnorB) gene (Table 1) was not detected in any of the 32 selected strains tested.

napA: periplasmic nitrate reductase.

narG: membrane-bound nitrate reductase.

nirS: cytochrome cd1-nitrite reductase.

nirK: copper nitrite reductase.

cnorB: cytochrome bc-type complex nitric oxide reductase gene.

nosZ: nitrous oxide reductase gene.

Appendix H. The distribution of OTUs among different sites.

OTU# (16S rRNA genus)	G5B	G5P	G7B	G7P	J6O	J6P	LFO	LFP	Total
1 (*Stenotrophomonas*)	1*	8	0	3	3	0	0	5	20
2 (*Enterobacter*)	0	0	0	0	2	0	1	0	3
3 (*Pseudomonas*)	0	0	0	0	0	0	0	3	3
4 (*Burkholderia*)	0	0	0	0	0	0	0	1	1
5 (*Stenotrophomonas*)	2	1	0	0	0	0	1	1	5
6 (*Rhodococcus*)	0	0	0	0	0	1	0	0	1
7 (*Brevibacillus*)	3	0	0	3	0	2	0	0	8
8 (*Lysinibacillus*)	0	0	0	0	0	1	0	0	1
9 (*Achromobacter*)	0	0	0	0	0	1	0	0	1
10 (*Acinetobacter*)	0	0	0	2	0	0	0	0	2
11 (*Pseudomonas*)	0	0	1	0	1	0	0	0	2
12 (*Arthrobacter*)	0	0	1	0	0	0	0	0	1
13 (*Chryseobacterium*)	0	0	0	0	0	0	1	0	1
14 (*Paenibacillus*)	1	0	0	0	0	0	0	0	1
15 (*Pseudomonas*)	1	0	0	0	0	0	0	0	1
16 (*Lysinibacillus*)	0	1	0	0	0	0	0	0	1
17 (*Bacillus*)	2	0	0	1	0	0	0	0	3
18 (*Stenotrophomonas*)	0	0	0	1	0	0	0	0	1
19 (*Pseudomonas*)	0	0	2	0	0	0	0	1	3
20 (*Stenotrophomonas*)	0	0	0	0	0	0	1	0	1
21 (*Sinorhizobium*/*Ensifer*)	0	0	1	0	0	0	0	1	2
22 (*Bosea*)	0	0	2	0	0	0	0	0	2
23 (*Achromobacter*)	0	0	1	1	0	1	0	0	3
24 (*Serratia*)	0	0	0	0	0	0	1	0	1
25 (*Microbacterium*)	0	0	0	0	1	0	0	0	1
26 (*Acinetobacter*)	0	0	0	1	0	0	0	2	3
27 (*Kocuria*)	0	0	0	0	0	0	0	1	1
28 (*Aeromonas*)	0	0	1	0	0	0	0	0	1
29 (*Brevundimonas*)	0	0	1	0	0	0	0	0	1
Total # strains	10	10	10	12	7	6	5	15	75

G5B: G5, brine contaminated.

G5P: G5, prairie uncontaminated.

G7B: G7, brine contaminated.

G7P: G7, prairie uncontaminated

J6O: J6, oil contaminated.

J6P J6, prairie uncontaminated

LFO: LF, oil contaminated.

LFP: LF, prairie uncontaminated

*Number of strains.

Appendix I. Isolates with *napA* and *narG* gene sequences.

Strain # (16S rRNA affiliation genus)	Soil Type	*napA*[*]	*narG*[*]	NR and DN Physiology[**]
I-1 (*Pseudomonas*)	Brine	Yes	Yes	DN
I-2 (*Pseudomonas*)	Brine	Yes	Yes	DN
I-6 (*Pseudomonas*)	Brine	Yes	No	NR
I-12 (*Pseudomonas*)	Brine	Yes	No	NR
I-5 (*Achromobacter*)	Brine	No	Yes	NR
I-7 (*Bosea*)	Brine	Yes	No	None
I-8 (*Bosea*)	Brine	Yes	No	None
I-18 (*Stenotrophomonas*)	Brine	No	Yes	NR
I-24 (*Serratia*)	Oil	No	Yes	NR
I-25 (*Enterobacter*)	Oil	No	Yes	NR
I-31 (*Enterobacter*)	Oil	No	Yes	NR
I-32 (*Enterobacter*)	Oil	No	Yes	NR
I-28 (*Stenotrophomonas*)	Oil	No	Yes	None
I-34 (*Stenotrophomonas*)	Prairie	No	Yes	NR
I-35 (*Stenotrophomonas*)	Prairie	No	Yes	NR
I-38 (*Stenotrophomonas*)	Prairie	No	Yes	NR
I-39 (*Stenotrophomonas*)	Prairie	No	Yes	NR
I-40 (*Stenotrophomonas*)	Prairie	No	Yes	NR
I-41 (*Stenotrophomonas*)	Prairie	No	Yes	NR
I-42 (*Stenotrophomonas*)	Prairie	No	Yes	NR
I-49 (*Achromobacter*)	Prairie	Yes	Yes	NR
I-60 (*Bacillus*)	Prairie	No	Yes	None
I-65 (*Pseudomonas*)	Prairie	No	Yes	DN
I-67 (*Pseudomonas*)	Prairie	Yes	No	NR
I-68 (*Pseudomonas*)	Prairie	Yes	No	NR
I-73 (*Pseudomonas*)	Prairie	Yes	No	NR
I-69 (*Sinorhizobium*)	Prairie	Yes	No	DN

NR: Nitrate Reduction.

DN: Nitrite reduction/denitrification.

None: No reduction of nitrate or nitrite.

[*]Yes; *napA* and *narG* were detected using specific primers. Sequence analysis confirmed the closest match to *napA*/*narG*.

[**]As detected after incubation in nitrate broth.

napA: periplasmic nitrate reductase.

narG: membrane-bound nitrate reductase.

Appendix J. Summary of nitrate reducing/denitrification genes from isolates.

Isolate #	*napA*/ Accession #	*narG*/ Accession #	*nirS*/ Accession #	*nirK*/ Accession #	*cnorB*/ Accession #	*nosZ*/ Accession #
1	*Pseudomonas fluorescens* F113 CP003150, 90% *	*Pseudomonas fluorescens* U71398, 95%	*Pseudomonas migulae* PD 1 DQ518189, 96%		*Pseudomonas fluorescens* AJ507356, 90%	*Pseudomonas* sp. PD 22 DQ377794, 96%
2	*Pseudomonas fluorescens* F113 CP003150, 91%	*Pseudomonas fluorescens* U71398, 95%	*Pseudomonas migulae* PD 1 DQ518189, 97%		*Pseudomonas fluorescens* AJ507356, 96%	*Pseudomonas* sp. PD 22 DQ377794, 96%
4					*Achromobacter cycloclastes* AJ298324, 95%	
5		*Achromobacter xylosoxidans* A8 CP002287, 89%				*Achromobacter xylosoxidans* A8 CP002287, 91%
6	*Aeromonas salmonicida* subsp. *salmonicida* A449, CP000644, 92%					*Pseudomonas stutzeri* DSM 4166 CP002622, 85%
7	*Starkeya novella* DSM 506 CP002026, 89%					
8	*Starkeya novella* DSM 506 CP002026, 89%					
12	*Pseudomonas aeruginosa* PA7 CP000744, 93%					
18		*Stenotrophomonas maltophilia* K279a AM743169, 98%				
24		*Serratia proteamaculans* 568 CP000826, 88%				
25		*Enterobacter cloacae*		*Rhizobium*		

		subsp. *cloacae* NCTC 9394, FP929040, 92%		sp.R-24654 AM230814, 78%		
28		*Bacillus thuringiensis* serovar *chinensis* CT-43 CP001907, 96%				
31		*Enterobacter cloacae* EcWSU1 CP002886, 97%				
32		*Enterobacter cloacae* EcWSU1 CP002886, 98%				
34		*Stenotrophomonas maltophilia* K279a AM743169, 97%				
35		*Stenotrophomonas maltophilia* K279a AM743169, 98%				
38		*Stenotrophomonas maltophilia* K279a AM743169, 97%				
39		*Stenotrophomonas maltophilia* K279a AM743169, 98%				
40		*Stenotrophomonas maltophilia* K279a AM743169, 97%				
41		*Stenotrophomonas maltophilia* K279a AM743169, 98%				
42		*Stenotrophomonas maltophilia* K279a AM743169, 98%				
49	*Achromobacter xylosoxidans* A8 CP002287, 92%	*Achromobacter xylosoxidans* A8 CP002287, 95%		*Achromobacter xylosoxidans* A8 CP002287, 93%		*Achromobacter xylosoxidans* A8 CP002287, 94%

60		*Bacillus thuringiensis* serovar *chinensis* CT-43 CP001907, 96%				
65		*Pseudomonas brassicacearum* subsp. *brassicacearum* NFM421, CP002585, 96%			*Pseudomonas fluorescens* AF197467, 87%	*Pseudomonas brassicacearum* subsp. *brassicacearum* NFM421, CP002585, 96%
67	*Pseudomonas fluorescens* F113 CP003150, 90%					
68	*Pseudomonas fluorescens* F113 CP003150, 90%					
69	*Sinorhizobium fredii* HH103 HE616890, 89%					
73	*Pseudomonas fluorescens* F113 CP003150, 90%					

NR: nitrate reduction

DN: nitrite reduction/denitrification

None: no reduction of nitrate or nitrite.

*% similarity of isolate sequence to that of closest GenBank match.

napA: periplasmic nitrate reductase.

narG: membrane-bound nitrate reductase.

nirS: cytochrome cd1-nitrite reductase.

nirK: copper nitrite reductase.

cnorB: cytochrome bc-type complex nitric oxide reductase gene.

nosZ: nitrous oxide reductase gene.