

The Effect of Environmental Factors on the Abundance of Culturable Nitrate Reducing/Denitrifying Bacteria from Contaminated and Uncontaminated Tallgrass Prairie Soil

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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 study, the strength of the association of the abundance of NR and DN bacteria with various environmental factors is estimated using multivariate statistical analysis. 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 sites had been subjected to remediation shortly after contamination and also more recently (e.g. current remediation treatments). 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 (>10⁶/g soil) from all sites were obtained from samples with a broad range of soil moisture (from 10-30% water/g soil) regardless of the source (e.g. site) of the isolates. The abundance of NR and DN bacteria from the contaminated sites was not less than that from the uncontaminated sites. 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 current levels of soil moisture controlled the % NR and DN bacteria. Current remediation treatments of contaminated sites sometimes, but not consis-

tently, were associated with greater abundance of NR and DN bacteria (2-way ANOVA). Therefore, no long-term effect of contamination on the abundance of NR and DN bacteria was shown nor were current remediation treatments effective in stimulating the numbers of NR and DN bacteria.

Keywords: Nitrate reducing bacteria. Denitrifying bacteria, Soil moisture, Crude oil and brine contamination, Nitrogen.

Introduction

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 (Murray et al., 1995; Martin et al., 1988; Harms et al., 2009).

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

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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 increase 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). 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, 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, we 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). 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 can lead to a 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 contained ammonium nitrate (NH₄NO₃) (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, we 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) we 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, we 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.

Materials and Methods

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). Briefly, the contaminated sites were contaminated during 1996-2000 but still contained

Table 1. Values for TPH, Na⁺, Cl⁻, and NO₃⁻ near MPN sampling dates.**Table 1a.** Total petroleum hydrocarbons (TPH).

Site	TPH ^a		
	Sampling Date	# Samples	(mg/kg) (SD ^b)
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)
G7	Spring 2005	12	10941 (1068)
LF	Spring 2005	34	14321 (3163)

^aTotal Petroleum Hydrocarbons. ^bStandard 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 1b. Brine components and NO₃⁻.

Site	Sampling Date	# Samples	Na ⁺ (mg/kg) (SD)	Cl ⁻ (mg/kg) (SD)	NO ₃ ⁻ (mg/kg) (SD)
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
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)

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: NO₃⁻ 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. N/A: not applicable. Data from Sublette et al. 2005, 2007, and unpublished data.

low levels of TPH and Na⁺ and Cl⁻ (Tables 1.a and 1.b) when sampled for the current study. Sites G7 and LF underwent a further round of treatment immediately prior to the course of this research as described in the Materials and Methods. 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.

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 resulting in a spill of approximately 11 m³ of dewatered crude oil. The initial Total Petroleum Hydrocarbon (TPH) con-

centration (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 (NH₄NO₃), diphosphorus pentoxide (P₂O₅), and potassium oxide (K₂O), and tilling. J6 was divided into two sections, fertilizer was applied by tilling in four increments throughout the 2 year-period of remediation on J6-F, while J6-NF was tilled in parallel with J6-F 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, with sodium ranging from 350-600 mg/kg soil (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.

During the period of 2005-2006, treatments were performed on the G7 and LF sites in order to further decrease the contami-

nation 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 30 cm 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 30 cm diameter PVC pipe enclosure with slots drilled into it for drainage. Each 30 cm 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 15 cm of soil, and the hay was placed on the soil surfaces. Each block contained 4 repetitions 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).

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 10 cm 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 autoclaved 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 for more detailed information).

Nitrate broth medium for detection of NR and DN bacteria consisted of 2.5 g of NaNO₃ 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 N₂ gas (Atlas, 1993). Control strains were used to confirm that the test condi-

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

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, 2.00 g 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, N₂O, or N₂, 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/g soil (dry weight).

To test whether the presence of soil interferes with the ability to detect the reduction of nitrate/nitrite in the microtiter plate test format, the four *Pseudomonas* control strains of known phenotype previously described (see Materials and Methods) 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. 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.

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 (Wollum, 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. 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 NO₃⁻ in contaminated-treated (G5, G7, LF, J6-F, and J6-NF) and prairie (G5P, G7P, LFP, and J6P) sites (Duncan et al., 1999; Sublette et al., 2005; Sublette et al., 2007a).

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 log₁₀ 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 1 to show the levels of TPH, Na⁺, Cl⁻, and NO₃⁻ 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 we 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 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 and samples taken in June 2003. Fertilizer was applied in April 2001 to G5 and samples taken in April/June 2003. Nitrate levels varied in different sites (Table 1.b). When we 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 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 denitrifiers 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 and parallel uncontaminated sites (eg. J6P, G5P) that were not treated during the period of study and were sampled on the same dates. Table 2 shows the % soil moisture [= (g water/g wet soil) x 100] in the J6 and G5 soils sampled in March 2005 (Figure 1, One-way ANOVA: F (4, 9) = 89.988, p = 0.000). The uncontaminated prairie samples bounded the range of % soil moisture, with G5P being the lowest (13.6%) and J6P the highest (21%). 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 1).

Estimated viable counts of either heterotroph or NR/DN bacteria varied little with the % soil moisture (Table 2, Figure 1), with the exception of J6-F. It was estimated there were 1.07 x 10⁷ culturable heterotrophic bacteria/g soil in G5P (13.6% soil

Table 2. Most Probable Numbers (MPNs) of NR and DN bacteria, heterotrophic bacteria, %NR and DN bacteria, and % soil moisture.

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

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 wet weight for G7 and LF, N=the value listed. % Soil moisture = (g water/g wet soil) x 100. Soil moisture was calculated from 2 soil samples per sample. SD: Standard deviation. NR and DN: Nitrate reducing and denitrifying bacteria.

moisture) and 1.78 x 10⁷ culturable heterotrophic bacteria/g soil in J6P (21% soil moisture) but 4.72 x 10⁷/g soil in J6-F (17.5% soil moisture). Estimates of DN/NR bacteria were about one quarter to one third that of the heterotrophic bacteria (Table 2), again with the exception of J6-F, in which estimates of DN/NR bacteria were about 70% that of the heterotrophic bacteria. .

Soil Moisture Association with NR and DN Bacteria: G7 and LF Sites

Figure 2 plots average and standard deviation of % soil moisture values in G7 and LF sampled at different seasons during the experiment. Bars with the same numbers form homogeneous sets (Duncan's multiple range test). Our moisture results showed that highest % soil moisture values were obtained in July 2005 (G7, Fig. 2a) and in August 2005 (LF, Fig. 2b). Note that the uncontaminated samples (G7P, LFP) were drier than the samples from the contaminated sites collected at the same time since the contaminated sites were watered throughout the study. Figures 2.a and 2.b also show that in spite of attempts to control water content, G7 generally was wetter than LF. Multiple samples were obtained from each site for every sampling date, which allowed us to more closely investigate the association between MPN values and soil moisture. Repeatedly, for each site and sampling date, our moisture versus MPN results in G7 and LF sites showed that the slope of the regression line was very nearly zero indicating similar MPN values over a fairly broad range of moisture levels (Figures 3a and 3b, Table 2). In addition, the abundance of heterotrophic and DN/NR bacteria did not show a consistent pattern of association—the bivariate correlation between the number of heterotrophic and DN/NR in a particular sample ranged from -0.49 to +0.79 (Table 3).

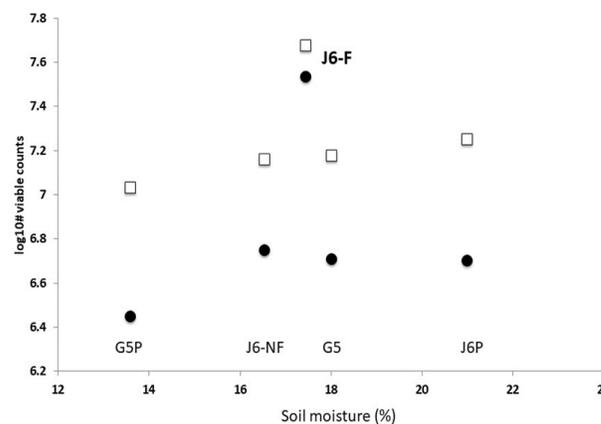


Figure 1. Abundance of heterotrophic and NR/DN bacteria with respect to soil moisture (sites G5, J6). The log₁₀ # viable counts (MPN) for heterotrophic (open squares) and NR/DN (black circles) are plotted on the Y axis versus the % soil moisture (% water/g soil wet wt) for samples taken from the G5 and J6 sites March 2005. See Tables 1 and 2 for additional information about the sites. N=1 for MPN values and N=2 (averaged) for each soil moisture value.

Comparing MPN for G7 and LF Across all Sample Dates

G7 MPN values (Table 2) for heterotrophic bacteria, NR and DN bacteria, and %NR and DN bacteria for all sample dates of G7 and G7P were analyzed together. The abundance of heterotrophic bacteria with respect to G7 and G7P soils sampled on different dates varied significantly (One-way ANOVA: F (4,103) = 11.292, p = 0.000). Duncan's multiple range test, a post-hoc test which groups together homogeneous subsets of

Table 3. Bivariate correlation between log₁₀# heterotrophic and DN and NR bacteria.

Site / Date	Correlation
G7/July2005	0.17
G7/October 2005	0.05
G7/June 2006	0.63
G7P/June 2006	-0.46
LF/August 2005	0.22
LF/October 2005	-0.49
LF/June 2006	0.79
LFP/June 2006	0.74

Table 4. % Coefficient of variation (CV) of NR and DN and heterotrophic bacteria in G7, G7P, LF, and LFP sites.

Site	Sample Date	Mean (SD) log ₁₀ NR and DN	% CV log ₁₀ NR and DN	Mean (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)	6	6.5 (0.19)	3

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

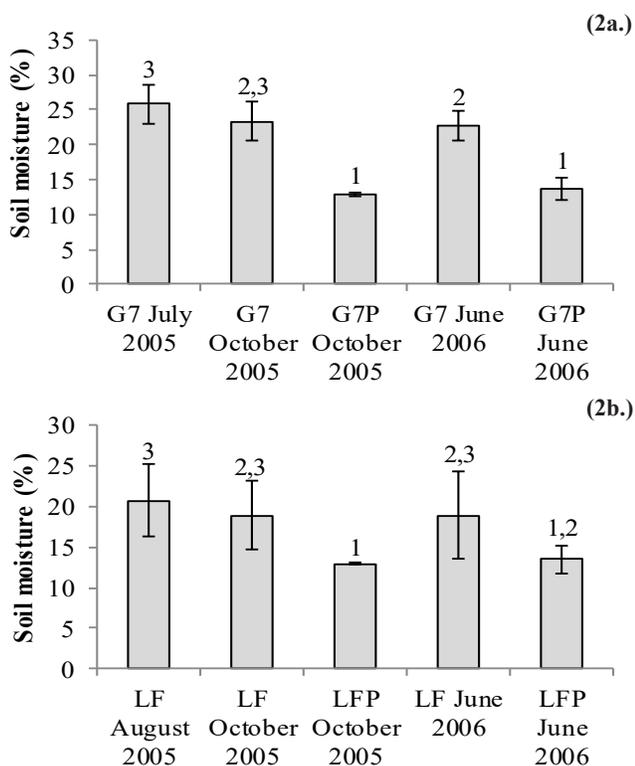


Figure 2. Moisture % in G7 and LF sites. G7 July 2005, G7 October 2005, G7 June 2006, LF August 2005, LF October 2005, and LF June 2006: N=34. G7P June 2006 and LFP June 2006: N=4. G7P October 2005 and LFP October 2005: N=2. Bars indicate average values. Error bars indicate ± 1 standard deviation. (2.a.) 1, 2, 3: Homogenous subsets (Duncan's multiple range test). One-way ANOVA: F (4,103) = 31.341, p = 0.000. (2.b.) 1, 2, 3: Homogenous subsets (Duncan's multiple range test). One-way ANOVA: F (4,103) = 3.420, p = 0.011.

(2a.) the data, grouped together G7P (June 2006) and G7 (July 2005) as having the lowest abundance of heterotrophic bacteria, G7 (July 2005), G7P (October 2005), and G7 (October 2005) as the group with the next to the lowest abundance, and G7P (October 2005), G7 (October 2005), and G7 (June 2006) as the subset with the highest abundance of heterotrophic bacteria. The log₁₀ #NR and DN bacteria varied significantly among the sites, with G7 (July 2005), G7P (October 2005, 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. 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. For LF samples, the log₁₀ #heterotrophic bacteria value in LF October 2005 was significantly higher than that in the remaining samples (One-way ANOVA (Heterotrophs: LF, LFP): F (4,102) = 10.923, p = 0.000). However, the log₁₀ #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. 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.

Coefficient of Variation (CV)

The % coefficient of variation (CV) was calculated (Table 4) for the G7 and LF sites in order to estimate whether subsamples within a sample were more variable for numbers of DN/NR bacteria than for heterotrophs. Values of the CV for log₁₀ # NR

Table 5. Two-way ANOVA: Summary of significant 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

Significant treatment effects are defined as $p < 0.050$.

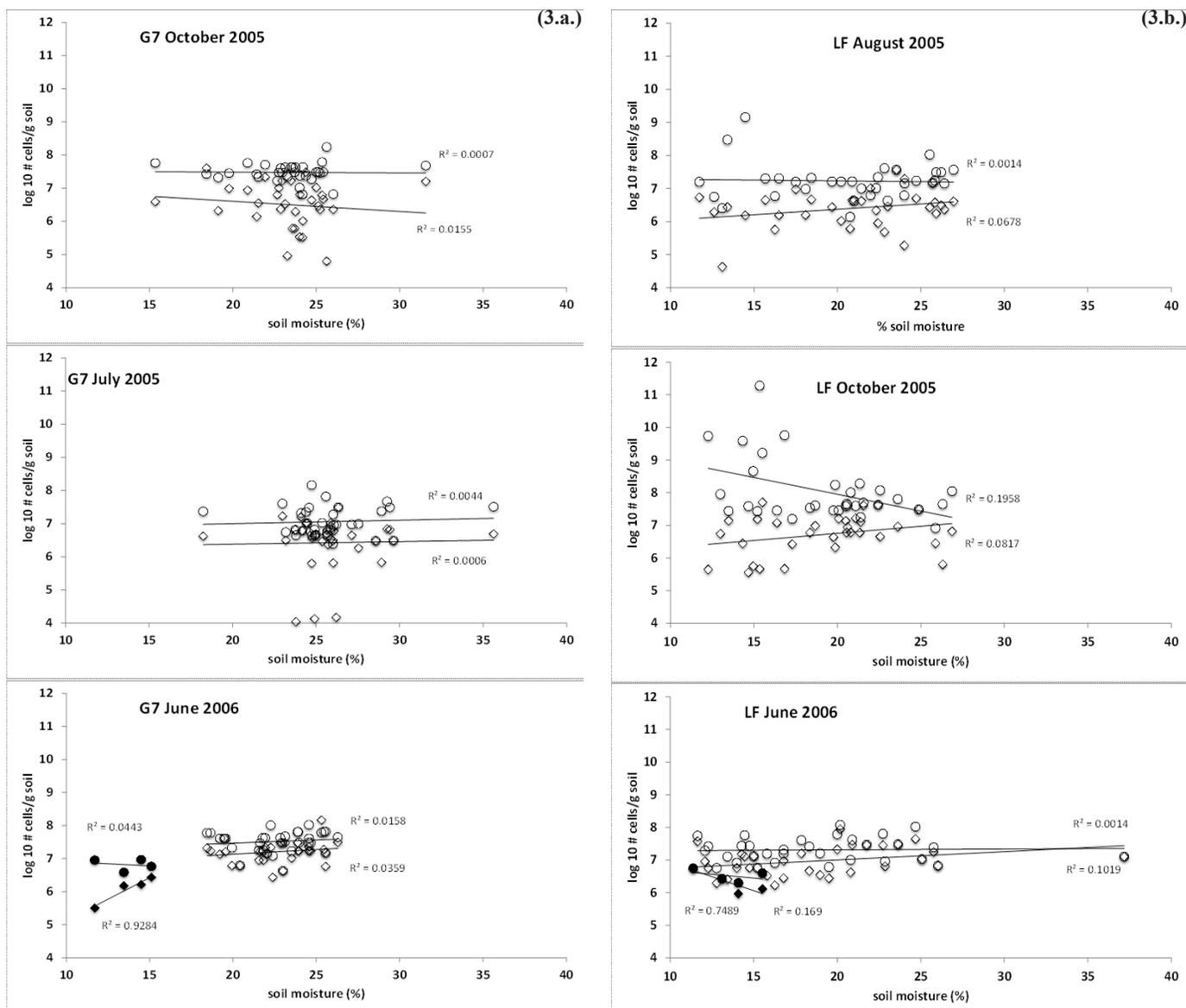


Figure 3. The relation between soil moisture and the log₁₀ number of heterotrophic bacteria or log₁₀ number of NR and DN bacteria in G7 or LF on different dates. Circles: heterotrophic bacteria. Diamonds: NR and DN bacteria. Open shapes: soils from contaminated sites (N= 33 or 34). Filled shapes: soils from uncontaminated prairie sites (N= 2 or 4). R₂ values for linear trend lines fitted to the data are shown on the figures. (3.a.) G7 samples; (3.b.) LF samples.

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 \log_{10} # heterotrophic bacteria for 2 sample dates for G7, the same for June 2006, higher for 2 sample dates for LF and lower for LF October 2005. This indicates that there was proportionally more variation in abundance of NR and DN in LF and G7 than 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, the same for one sample and lower for one sample (LFP October 2005).

Two-way ANOVA: Effect of Hay and/or Fertilizer on Bacterial Numbers in G7 and LF

Hay and/or fertilizer were applied May 2005 to the soil in selected enclosures in the G7 and LF sites (Materials and Methods, "Restoration Phase"). Two-way ANOVA calculations were made to determine if there was a significant effect of applying hay and nitrate-containing fertilizer amendments on the abundance or proportion of NR and DN bacteria. Our multivariate analyses (e.g. 2-Way ANOVA) showed the effect ($p=0.038$) of hay and fertilizer together on the relative abundance of NR and DN (%NR and DN) bacteria sampled from G7 in June 2006 (Table 5). 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 was the no hay plus fertilizer treatment (38.3%) (data not shown). Enclosures with fertilizer addition had higher %NR and DN bacteria sampled from LF site in October 2005 ($p=0.044$) (Table 5) where %NR and DN was 13.9% with no fertilizer added and was 34.0% in enclosures when the fertilizer was added (data not shown). The opposite was true for June 2006, the greatest relative abundance of NR and DN bacteria sampled from LF site 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 5) (data not shown). 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 nor on the abundance of heterotrophic bacteria for any sample date.

Discussion

This study investigated the effect of the long-term (5-10 years) residual TPH and brine contamination on the abundance of NR and DN bacteria. Our results suggested that the residual TPH and brine levels were not associated with differences in abundance of NR and DN bacteria beyond that predicted by soil moisture levels in most cases. Our J6 study sites (TPH contaminated) sampled at an earlier stage after contamination showed that nitrogen mineralization rates in a contaminated non-fertilized soil (J6-NF) were significantly lower than that in a contaminated fertilized soil (J6-F, 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 but MPNs were not performed. Nitrogen mineralization was not measured in the current study, but our 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. Indeed, there was a higher % and greater number of NR and DN bacteria in J6-F than in J6NF or G5 sites (Table 2, Figure 1). Although MPN values for heterotrophs and NR and DN bacteria from the LF and G7 sites 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 current levels of 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, 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 within individual enclosures, we do not know the degree of variation at a smaller scale within enclosures.

The G7 and LF sites were irrigated resulting in increased soil moisture 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 contaminants are degraded. For example, the biodegradation of PAHs has been studied under both aerobic and anaerobic conditions. The biotransformation of pollutants by microbial communities has been observed under anaerobic conditions (Coates et al., 1996a, 1996b). Our results showed that although moisture levels varied (range 11.6-37.2%) 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 ($29.76\% \pm 3.75$), organic matter, and available nitrogen increased denitrifier populations (Peralta et al., 2010). In addition, it was shown that phylogenetic composition of the archaeal and bacterial community and nitrogen cy-

cling 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).

Support for the effect of environmental parameters such as nitrate 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. Other comparisons are suggestive (e.g. higher net NR and DN bacteria and %NR and DN bacteria in G5 than in G5P March 2005) but measurements showing a higher nitrate level in G5 than in G5P were obtained in 2003, none were made in March 2005. The amount of nitrate in soil (Tiedje, 1988) and soil moisture (Xia et al., 2004) are well-known to be 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 this study, nitrate was applied only one time, the easily degraded hydrocarbon may have been already depleted, and moisture levels were generally low. Therefore, the stimulation efforts may not have been successful since not all factors required for denitrification were provided simultaneously. In addition, we are 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, our MPN results show that brine-contaminated sites did not have a lower abundance of NR and DN bacteria. A previous study showed a decrease in the range of organisms that degrade most low-molecular-weight components of mineral oil with increasing salinity (Kleinstuber et al., 2006).

This study provides 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). We 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, our results showed that sites that were contaminated 5-10 years previously by crude oil and/or brine did not have a lower abundance of NR and DN bacteria, therefore, the biological potential for denitrification was present despite residual contamination.

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