REVIEW ARTICLE

Nitrate Reducing and Denitrifying Bacteria in Oil / Brine Contaminated Soils

Samer M. AbuBakr

Department of Biological and Forensic Sciences, Fayetteville State University, Fayetteville, NC 28301, USA

Abstract

Soil microorganisms are a fundamental part of biogeochemical cycling of nitrogen. Nitrate reducing bacteria and denitrifying bacteria are diverse groups of bacteria involved in nitrogen transformations in soils. Current research has shown that soil denitrification releases nitrogen from the soil ecosystem to the atmosphere. As a result, the balance between denitrification and N-fixation can determine the biologically available nitrogen for soils. In fact, nitrogen deficiency in soil is a common limiting factor for plant growth and productivity. Nitrate reduction and denitrification could be affected by some environmental factors (e.g. oxygen levels) and could also be affected by various contaminants such as crude oil and brine as they may alter the abundance and species composition of nitrate reducing and denitrifying bacteria. On the other hand, different environmental factors (e.g., oxygen and humic substances) affect the degradation of petroleum hydrocarbons in soil by soil microorganisms.

Introduction

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 soils, in which some microorganisms (e.g. denitrifying bacteria) use nitrate or nitrite as alternative electron acceptors. Multiple 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 denitrifiers at the site (Enwall et al., 2010). Since denitrification releases mineralized nitrogen from 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 (Shim and Yang, 1999; Lee et al., 2002), and 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). Also, it was suggested that N_2O production activity

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* Corresponding author: sabubakr@uncfsu.edu

could be sensitive to heavy metal pollution (Liu et al., 2016). 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. 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). In this review, I summarize the role nitrate reducing and denitrifying bacteria play in biogeochemical cycling of nitrogen in soils, discuss some factors that affect their abundance and their species composition, and highlight the necessity of investigating communities of denitrifying bacteria using an approach based on the enzymes responsible for key steps in denitrification pathway rather than 16S rRNA gene since closely related species vary in their ability to denitrify and denitrification is common among phylogenetically unrelated microbial groups.

Biogeochemical Cycling of Nitrogen

Nitrogen is an abundant element, with 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). Nitrate (NO_3^-) or ammonium ions (NH_4^+) are the main nitrogen forms absorbed by plants from the soil. Nitrogen cycles 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 (Figure 1). This latter process is the traditionally defined pathway of denitrification (Braker et al., 2000).

Physiology, Taxonomic, and Distribution Patterns of Nitrate Reducing (NR) / Denitrifying (DN) 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 (Philippot, 2005). 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
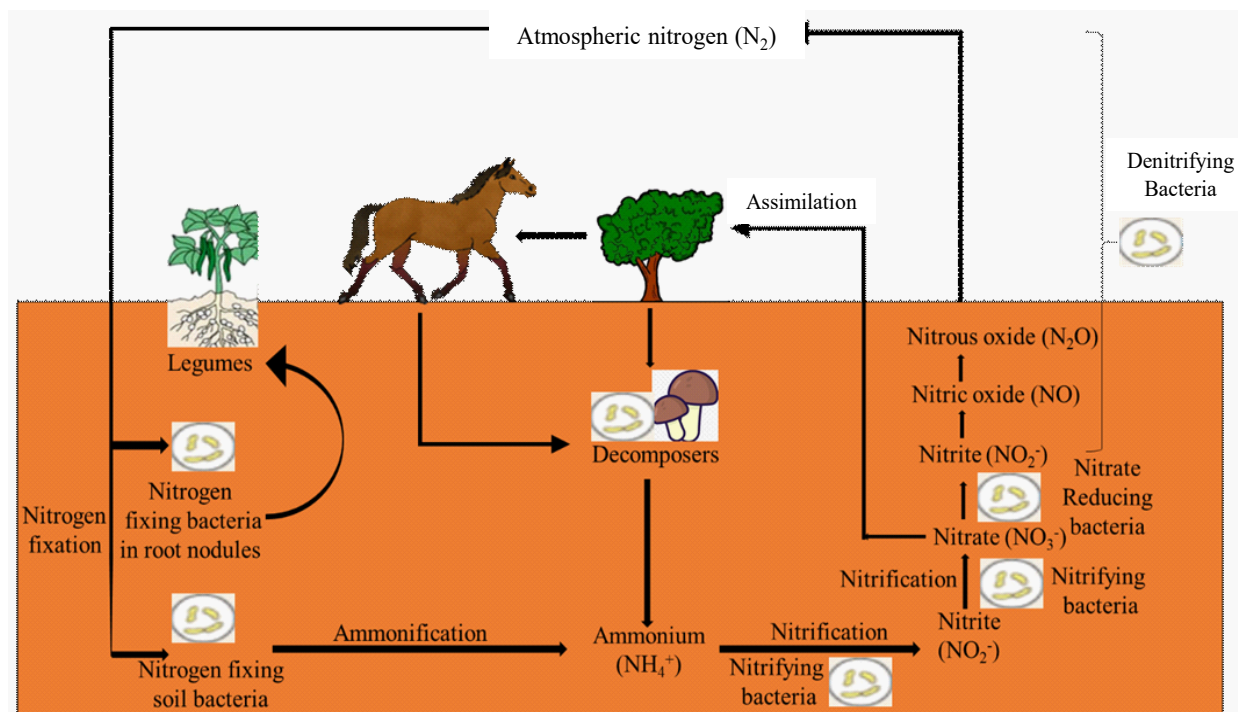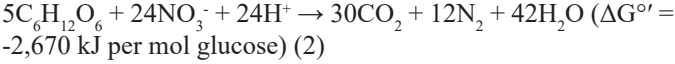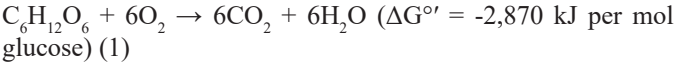

Figure 1. The Nitrogen Cycle.

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).

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

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

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

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 (Table 1). 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 d1 and is encoded by nirS (Zumft, 1997). 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 33942T. 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). 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). It was shown that some bacterial strains demonstrated nitrite reduction under strict anaerobic conditions, but not in the presence of oxygen (AbuBakr and Duncan, 2015). Also, the diversity of NR bacteria in a waste water

Table 1. Denitrification steps in bacteria.

Step No.	Step	Catalyzed by (Enzyme)	Encoded by (Operon/Gene)	Reference
1	Nitrate to nitrite	Membrane bound nitrate reductase	*narGHJI*	Kandeler et al., 2006
		Periplasmic nitrate reductase	*napABC*	
2	Nitrite to nitric oxide	Nitrite reductase (contains copper)	*nirK*	Zumft, 1997
		Nitrite reductase (contains cytochromes (hemes) *c* and *d*)	*nirS*	
3	Nitric oxide to nitrous oxide	Nitric oxide reductase small subunit	*norC*	Braker and Tiedje, 2003
		Nitric oxide reductase large subunit	*norB*	
4	Nitrous oxide to dinitrogen gas	Nitrous oxide reductase	*nosZ*	Philippot, 2002
			nosR	
			nosDFYL	

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). Moreover, previous studies identified soil moisture and surface hydrology as important factors for supporting high denitrification activity (Groffman and Tiedje, 1989; Hunter et al., 2008). However, it was suggested that factors other than levels of soil moisture controlled the % NR and DN bacteria since 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 (AbuBakr et al., 2019).

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 (Shim and Yang, 1999; Lee et al., 2002). 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. 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). 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). 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). Some nitrate-reducing organisms also reduced hydroquinones within humic acids (HA) (Lovley et al., 1999; Coates et al., 2002). This reduction of HA by nitrate reducing bacteria may make hydrocarbons more accessible to this group of bacteria. It was illustrated that 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 (AbuBakr et al., 2008).

Nitrogen as a Limiting Nutrient in the Tallgrass Prairie

Nitrogen limitation is an important regulator of plant growth (Aber et al., 1997; Shaver et al., 2001; LeBauer and Treseder, 2008). 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 (Friend, 1991; Reich et al., 1995; 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 (Aber et al., 1997; Shaver et al., 2001; LeBauer and Treseder, 2008). 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. (2007) 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., 2007). A different study on the fate of the applied NH4 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 burning 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.

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