The Effect of Oxygen Supply on Nitrite Reduction by Tallgrass Prairie Soil Bacteria

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Abstract: Nitrite reduction, catalyzed by nitrite reductase, is a key step in the denitrification pathway because it catalyzes the reduction of soluble nitrite (NO_2^-) into nitric oxide gas (NO). The production of nitric oxide gas therefore decreases the amount of nitrogen in soils. Other studies have investigated the effect of oxygen on denitrification in a few specific microorganisms (e.g. *Aquaspirillum magnetotacticum*, *Thiosphaera pantotropha*, and *Paracoccus denitrificans*). In this study, we tested the effect of oxygen on nitrite reduction in seven strains representing 5 different genera obtained from Tallgrass Prairie soil. The strains were chosen based on positive detection of at least one functional gene in the denitrification pathway along with positive results for nitrate (NO_3^-) and/or nitrite reduction after growth in nitrate broth in a microtiter plate assay. Under these conditions which did not totally exclude oxygen, three strains were able to reduce nitrite while four strains did not reduce nitrite. All seven strains were retested for the production/consumption of nitrite under strictly anaerobic conditions using nitrate as the electron acceptor. In addition, the strains were screened for genes encoding the copper nitrite reductase (*nirK*) and the cytochrome cd_1-nitrite reductase (*nirS*) and other functional markers of denitrification pathway, namely nitric oxide reductase gene (*norB*) and nitrous oxide (N_2O) reductase gene (*nosZ*) by PCR amplification using specific primers. Our results show that under strict anaerobic conditions, two additional strains demonstrated nitrite reduction. Although none of the molecular markers showed perfect correlation with the ability to reduce nitrite, *nirS*/*nirK*, commonly used to screen environmental samples for denitrifying bacteria, was detected in only two of the five strains shown in this study to reduce nitrite. More nitrite reducing strains were correctly identified when both the *cnorB* and *nosZ* primer sets were used. *©2015 Oklahoma Academy of Science*

Introduction

Nitrite reductase is a significant enzyme in the denitrification pathway as it converts nitrite, a toxic intermediate, to nitric oxide, the first gaseous product of denitrification. Primers designed to detect *nirS* nitrite reductase gene (Braker et al. 1998) and *nirK* nitrite reductase gene (Qiu et al. 2004) have often been used to detect the presence of denitrifying (DN) bacteria. Molecular detection of denitrification genes has become the norm (Oakley et al., 2007; Dandie et al., 2008) while studies evaluating the phylogenetic range of DN species detected by molecular means have lagged. Even more critically, there are few recent studies of the environmental conditions under which denitrification enzymes are active, and those focus on a relatively narrow

group of microorganisms (Saleh-Lakha et al., 2009a; Saleh-Lakha et al., 2009b). This study contributes to a better understanding of the effect of environmental conditions on the activity of denitrification enzymes by determining the effect of oxygen on the ability of a group of strains to reduce nitrite, and also evaluates how well molecular detection of various denitrification genes serves to identify denitrifying bacteria.

To express the trait of denitrification, microorganisms must have the genetic capacity (e.g. denitrification genes) that are transcribed and translated into the denitrification enzymes, and those enzymes must be active under the given environmental conditions. Denitrification is known to be an environmentally-regulated process with respect to oxygen supply, the presence and nature of an N oxide, and possibly additional external factors such as metal ions (Zumft, 1997). Various studies have investigated the effect of oxygen on denitrification in specific microorganisms. It was thought that synthesis of denitrifying enzymes only occurs when nitrate is present and oxygen is absent and that oxygen is a repressor of enzyme synthesis in many denitrifyers (Payne, 1973). However, it was shown that little or no repression of denitrifying enzymes was caused by oxygen in nitrifying activated sludge systems (Simpkin and Boyle, 1988). Moreover, several studies showed the production of nitrogen-containing gases under aerobic conditions. For example, *Aquaspirillum magnetotacticum*, a bacterium that has an obligate requirement for oxygen, was shown to reduce nitrate to nitrous oxide and dinitrogen in the presence of low levels (0.2-1.0%) of oxygen (Bazylinski and Balkemore, 1983). Also, the presence of nitrate reductase and the production of nitrogen-containing gases from nitrate by *Thiosphaera pantotropha* were demonstrated at dissolved O_2 concentrations of up to 90% air saturation (Robertson and Kuenen 1983; 1984a). In addition, the consumption of NO_3^- and production of nitrogen-containing gases by the facultative anaerobes *Pseudomonas aeruginosa* and *Paracoccus denitrificans* in the presence of relatively high concentrations of dissolved O_2 (150 µM or 195 µM, respectively) provides strong additional proof that denitrification may not strictly require anaerobic conditions. Indeed, with anaerobically grown *P. aeruginosa* washed cell suspension, not only were N_2 and N_2O produced after the addition of O_2, but also their production increased (Davies et al., 1989). Furthermore, several nitrifying strains were found to denitrify via nitrite in the presence of oxygen which indicates that they have an oxygen-tolerant denitrification system (Matsuzaka et al., 2003). However, the effect of oxygen on denitrification has not been systematically examined simultaneously on a wide variety of strains.

Many of the strains in this study were obtained from tallgrass prairie soils contaminated by spills of crude oil or oil field brine (Sublette et al., 2005; Sublette et al., 2007). In fact, oxygen levels decrease as oil contaminants are degraded (Duncan et al., 1999; Ehrenreich et al., 2000). This decrease in oxygen levels may give an advantage to facultative anaerobes such as DN bacteria and strict anaerobes over strict aerobes. If then the rate of nitrate reduction/ denitrification increases, nitrogen will be lost from soil. One objective of this study was to compare the ability of DN bacteria to reduce nitrite in a microtiter plate system containing nitrate broth (e.g. microtiter plate assay) versus inoculation and growth under strictly anaerobic conditions. Since oxygen is known to regulate denitrification (Zumft, 1997), and since oxygen levels decrease when oil contaminants are degraded, we hypothesized that the low oxygen levels also increase the loss of nitrogenous compounds due to the creation of conditions more favorable to denitrification.

Seven strains were chosen for investigation of the effect of oxygen levels on nitrite reduction. These seven strains reduced nitrate and/or nitrite using the microtiter plate assay. Also, they all were shown by PCR amplification with primers specific for functional genes in the denitrification pathway and sequencing of the PCR product to possess one or more functional genes in the denitrification pathway. These seven strains represented 5 different genera, namely: *Pseudomonas* strains I-1 and I-65, *Ensifer* strain I-4, *Achromobacter* strains I-5 and I-49, *Aeromonas* strain I-6, and *Enterobacter* strain I-25.

Sites Description

The sampling sites used in this work are located in the Tallgrass Prairie Preserve (TPP) in Osage County, Oklahoma. Nitrate reducing and DN bacteria were isolated from a total of 2 contaminated sites and 2 uncontaminated sites. The contaminated sites were contaminated with crude oil or with a mixture of oil/brine (e.g. salt water) due to accidental spills as described previously (Sublette et al., 2005; Sublette et al., 2007; Duncan et al., 1998; Duncan et al., 1999).

Methods

Sampling soils for the seven strains

The seven strains were obtained from tallgrass prairie soils. Strains I-1, I-4, I-5, and I-6 were isolated from G7 (brine contaminated) in July 21, 2005. Strain I-25 was sampled from LF (oil contaminated) in August 2, 2005. Strain I-49 was sampled from G7P (prairie, not contaminated) in July 21, 2005. And finally, strain I-65 was isolated from LFP (prairie, not contaminated) in August 2, 2005. In brief, soils were homogenized, serially diluted and added to microtiter plates containing nitrate broth (Difco), and after two weeks of incubation at room temperature (23-25°C), all wells were examined for evidence of growth (turbidity) and tested for nitrate reduction and nitrite reduction using Griess reagents (Smibert and Krieg, 1994). Based on positive scoring for growth and nitrate or nitrite reduction in the microtiter plates, nutrient agar plates (Nutrient Agar, Difco, Detroit, MI) were inoculated with 25 µL from microtiter plate wells, chosen randomly from 10^{-1} to 10^{-4} dilutions that scored positive for growth and nitrate reduction or nitrite reduction. The nutrient agar plates were incubated at 30°C. A colony chosen from a plate was streaked at least three times in succession from a single colony to a fresh plate in order to obtain pure cultures. All seven strains were retested using the microtiter plate assay to confirm the ability to reduce nitrate or nitrite once purified. In addition, the following strains of known phenotype were included as controls: *P. aeruginosa* ATCC 27853 (Denitifyer), *P. putida* pG7 (not a nitrate reducer), *S. maltophilia* ATCC 13637 (Nitrate reducer), and uninoculated wells containing sterile nitrate broth.

DNA extraction from isolates

A single colony was used to inoculate 1-3 mL nutrient broth, and grown overnight at 30°C. The culture was pelleted by centrifugation, then DNA was extracted using a commercial DNA extraction kit, FastDNA®SPIN Kit (QBIOgene, Solon, Ohio, USA).

Molecular detection of 16S rRNA gene sequences and denitrification pathway functional genes

Universal bacterial primers 27F/1492R (Wilson et al., 1990) for 16S rRNA gene were used to amplify ~1400 bp. 16S rRNA gene was amplified from the seven strains. In addition, the seven strains were screened for the following functional markers: *nirS*, *nirK*, *cnorB*, *qnorB*, and *nosZ* using the primers listed in Table 1. 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. 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 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, 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 four minutes (exception: 5 minutes

Table 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
nirS 1F	CCTAYTGGCCGCCRCART	*Pseudomonas stutzeri* ZoBell	763-780	890	Touchdown	1
nirS 6R	CGTTGAACTTRCCGGT	(X56813)	1638-1653		56-51˚C	
nirK F	TCATGGTCCTGCCGCGYGACGG	*Alcaligenes faecalis*	1319-1337	329	Touchdown	2
nirK R	GAACTTGCCGGTNGCCCAGAC	(D13155)	1668-1648		63-53˚C	
cnorB 2F	GACAAGNNNTACTGGTGGT	*Pseudomonas denitrificans* Pd1222	553-571	389	Touchdown	3
cnorB 6R	GAANCCCCANACNCCNGC	(U28078)	942-925		57-52.5˚C	
qnorB 2F	GGNCAYCARGGNTAYGA	*Ralstonia eutropha* H16	1204-1220	637	Touchdown	3
qnorB 7R	GGNGGRTTDATCADGAANCC	(AF002661)	1841-1822		57-52.5˚C	
nosZ-F-1181	CGCTGTTCITCGACAGYCAG	*Pseudomonas stutzeri*	1463-1482	680	56˚C	4
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. Braker et al. (1998).

2. Qiu et al. (2004).

3. Braker and Tiedje (2003).

4. Rich et al. (2003).

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 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). Three strains were obtained from ATCC for use as positive controls for PCR detection of denitrification functional genes. *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*. The PCR products were visualized using agarose gel electrophoresis technique.

DNA sequencing and analysis

PCR products were prepared for sequencing using ExoSAP-IT (Bell, 2008). Sequence chromatograms were analyzed using Sequencher (Windows version 4.2; Gene Codes Corp., Ann Arbor, MI). The sequences of the 16S rRNA gene and denitrifying functional genes were compared with the Genbank nt/nr 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).

Nitrate reduction/denitrification under strictly anaerobic conditions

The ability of seven strains to reduce nitrate/nitrite under strict anaerobic conditions was tested. All seven strains reduced nitrate/nitrite using the microtiter plate assay and contained sequences with high similarity to one or more of the denitrifying functional genes. The chosen strains were: *Pseudomonas* strains I-1 and I-65; *Ensifer* strain I-4; *Achromobacter* strain I-5; *Aeromonas* strain I-6; *Enterobacter* strain I-25; and *Achromobacter* strain I-49; along with the following control strains: *S. maltophilia* ATCC

13637 (nitrate reducer), *P. aeruginosa* ATCC 27853 (denitrifyer), *P. putida* F1 ATCC 17485 (neither nitrate reducer nor denitrifyer). The strains and the controls were streaked on Nutrient agar plates overnight followed by transferring a loopful into 15 mL nutrient broth under aerobic conditions. After incubating overnight at 30˚C with shaking, 15 mL was transferred to sterile anaerobic 125-mL serum bottles followed by changing the gas phase using N_2/CO_2 gas. Each bottle was used as an inoculum (1.5 mL/bottle) to inoculate 5 serum bottles containing 50 mL minimal medium prepared anaerobically. This medium contains 1g NaCl, 0.5g NH_4Cl, 0.3g KH_2PO_4, 0.4g $MgCl_2.6H_2O$, 0.15g $CaCl_2.2H_2O$, 1g of 10 mM KNO_3, 5.0g yeast extract, and 10 mL RST metals (Tanner, 1997) per liter. After adjusting the pH to 7.2, the medium was boiled and gassed with N_2/CO_2 in an ice bath followed by the addition of 3g/L $NaHCO_3$. Finally, aliquots of 15 mL/serum bottle were prepared and autoclaved for sterilization.

Two out of the 5 bottles were autoclaved and used as sterile controls in the experiment. Bottles were incubated at room temperature (23-25˚C). Samples (0.2 mL) were taken and frozen at time zero and subsequent time points for two weeks. Optical density (O.D.) readings at 600 nm were recorded to monitor the growth of those strains. Nitrate and nitrite concentrations were measured by ion chromatography (model DX500 fitted with an AS-4A column; Dionex Corp., Sunnyvale, CA). Subsequently, second and third transfers of 1.5 mL to fresh bottles containing 50 mL minimal medium prepared anaerobically were performed to obtain well-established anaerobic cultures. O.D readings and sampling for Ion chromatography were performed for all transfers. Results are reported for the third transfer only in order to ensure adaption to anaerobic conditions had occurred.

Results

Summary of 16S rRNA phylogeny

Based on the 16S rRNA sequence analyses, the seven strains included four γ-proteobacteria belonging to the genera *Pseudomonas*, *Aeromonas*, or *Enterobacte*r, one α-proteobacterium (*Ensifer*), and two β-proteobacteria (*Achromobacter*) at the 99% threshold of similarity (RDP Classifier, Wang et al., 2007) (Table 2).

Molecular detection of denitrifying functional genes

nirS/*nirK* genes were detected in three strains (Table 2). The PCR primers successfully amplified 890-bp *nirS* fragments from *Pseudomonas* strain I-1. Sequences were 96% similar to *nirS* from *Pseudomonas migulae* (DQ518189). Also, 514-bp fragments from *nirK* were successfully amplified from *Enterobacter* strain I-3 and *Achromobacter* strain I-49. The sequences obtained matched 77% to *nirK* from *Rhizobium* sp.R-24654 (AM230814) and 90% to *nirK* from *Alcaligenes* sp. STC1 (AB046603), respectively.

A PCR product with highest similarity to the c*norB* gene sequence was obtained from the *Pseudomonas* strain I-1 and was 75% similar to *cnorB* from *P. stutzeri* (AY957389). In addition, the *cnorB* sequence from *Pseudomonas* strain I-65 was 86% similar to *cnorB* from *P. fluorescens* (AF197467). *Ensifer* strain I-4 *cnorB* sequence showed 93% similarity to *cnorB* from *Achromobacter cycloclastes* (AJ298324).

Primers specific for nitrous oxide reductase (Rich et al., 2003) were successful in amplifying a *nosZ* fragment from *Pseudomonas* strain I-1. The sequence was 95% similar to *nosZ* from *Pseudomonas* sp. PD 22 (DQ377794). *Pseudomonas* strain I-65 *nosZ* sequence was 94% similar to *nosZ* from *P. fluorescens* (AF197468). In addition, *Aeromonas* strain I-6 *nosZ* sequence was 84% similar to *nosZ* from *Pseudomonas stutzeri* A1501 (CP000304). Finally, the *nosZ* PCR product from *Achromobacter* strain I-5 was 93% similar to *nosZ* sequence from *Achromobacter* sp. PD 25 (DQ377797) while *Achromobacter* strain I-49 *nosZ* sequence was 91% similar to *nosZ* sequence from *A. xylosoxidans* (AY072227). In summary, *nirS*/*nirK* was amplified from three strains, *cnorB* from three strains, and *nosZ* from five strains.

Nitrate reduction/denitrification physiology

Table 2: Nitrite reduction and detected denitrification functional genes.

Group #	Strain #	Genus [% similarity]* (Class)	Nitrite reduced[1]	Nitrite reduced[2]	*nirS*/*nirK*	*cnorB*	*nosZ*
1	1	*Pseudomonas* [100%] (γ)	Yes	Yes	*nirS*	Yes	Yes
	65	*Pseudomonas* [100%] (γ)	Yes	Yes	No	Yes	Yes
	4	*Ensifer* [99%] (α)	Yes	Yes	No	Yes	No
2	6	*Aeromonas* [100%] (γ)	No	No	No	No	Yes
	25	*Enterobacter* [99%] (γ)	No	No	*nirK*	No	No
3	5	*Achromobacter* [99%] (β)	No	Yes	No	No	Yes
	49	*Achromobacter* [100%] (β)	No	Yes	*nirK*	No	Yes

*Thresold level of similarity as determined by The Ribosomal Database Project (RDS) Classifier program.
NR: nitrate reduction.
DN: nitrite reduction.
[1]not strictly anaerobic, microtiter plate assay.
[2]strictly anaerobic.
γ = γ-proteobacteria.
α = α-proteobacteria.
β = β-proteobacteria.
nirS: cytochrome cd1-nitrite reductase.
nirK: copper nitrite reductase.
cnorB: nitric oxide reductase gene.
nosZ: nitrous oxide reductase gene.

A. Microtiter plate assay: initially aerobic

In the microtiter plate assay, bacteria were inoculated into nitrate broth and incubated for two weeks. Cells are exposed to the ambient level of oxygen in the microtiter plate wells at the beginning of the experiment and the microtiter plate was presumed to become anaerobic as the microorganism grew and consumed oxygen. Although oxygen level was not monitored, the control microorganisms gave the expected results regarding nitrate/nitrite reduction which suggests that the concentration of oxygen decreased over time. Under these conditions, *Aeromonas* strain I-6, *Enterobacter* strain I-25, and *Achromobacter* strains I-5 and I-49 reduced nitrate to nitrite. However, *Pseudomonas* strains I-1 and I-65; and *Ensifer* strain I-4 were able to reduce nitrate and nitrite under the same conditions, suggesting the ability to perform nitrite reduction (Table 2).

B. Strictly anaerobic conditions

The seven strains and the control strains *S. maltophilia* ATCC 13637 (nitrate reducer), *P. aeruginosa* ATCC 27853 (denitrifyer), and *P. putida* F1 ATCC 17485 (neither nitrate reducer nor denitrifyer) were tested for the ability to

reduce nitrate/nitrite under strict anaerobic conditions. Based on the results of nitrate/nitrite reduction under both conditions (e.g. microtiter plate assay and strictly anaerobic conditions), the seven strains were clustered into three groups.

Group 1

Three strains (e.g. strains I-1, I-65, and I-4) behaved similarly to denitrifying bacterium *P. aeruginosa* ATCC 27853. When inoculated with *P. aeruginosa* ATCC 27853, all nitrate was depleted after one day of incubation and there was no significant nitrite accumulation at any time during 14 days of incubation (data not shown). *Pseudomonas* strain I-1 (*nirS*$^+$, *norB*$^+$, and *nosZ*$^+$) was able to reduce nitrite when grown in nitrate broth in the microtiter plate assay. It also showed its ability to reduce nitrite when grown under strictly anaerobic conditions. Nitrate was completely depleted after three days and there was no significant accumulation of nitrite when *Pseudomonas* strain I-1 was grown under nitrate reducing conditions. The largest detectable amount of nitrite was around 0.25 mM after one day of incubation (Figure 1.a). *Pseudomonas* strain I-65 (*cnorB*$^+$, *nosZ*$^+$) and *Ensifer* strain I-4 (*cnorB*$^+$) were able to reduce nitrate and nitrite under nitrate reducing conditions. The same results were obtained from microtiter plate assay. *Pseudomonas* strain I-65 was a very fast nitrite reducer, similar to the denitrifyer control *P. aeruginosa* ATCC 27853. After one day of incubation, all nitrate was depleted and there was no significant nitrite accumulation (Figure 1.b). On the other hand, *Ensifer* strain I-4, there was no significant loss of nitrate or accumulation of nitrite after seven days of incubation. However, after 14 days of incubation, all nitrate was depleted and there was no significant accumulation of nitrite (Figure. 1.c).

Group 2

Two strains (e.g. strains I-6 and I-25) showed a loss of nitrate and an accumulation of nitrite. In *Aeromonas* strain I-6 (*nosZ*$^+$), after 14 days of incubation, nitrate was depleted to around 3.5 mM and nitrite was accumulated to the same value (e.g. 3.5 mM). These two values remained almost the same even after 53 days of incubation (Figure 1.d). This strain also reduced nitrate to nitrite in the microtiter plate assay. *Enterobacter* strain I-25 (*nirK*$^+$) was able to reduce nitrate to nitrite under strictly anaerobic conditions (Figure 1.e). After three days, all nitrate was depleted and nitrite was accumulated to around 7.0 mM and remained around this value after 14 days (*Enterobacter* strain I-25, Figure 1.e). In comparison, the nitrate reducer control strain (*S. maltophilia* ATCC 13637), depleted all nitrate and accumulated nitrite to around 8.3 mM in four days (data not shown). *Enterobacter* strain I-25 also reduced nitrate to nitrite when the microtiter plate assay was used (Table 2).

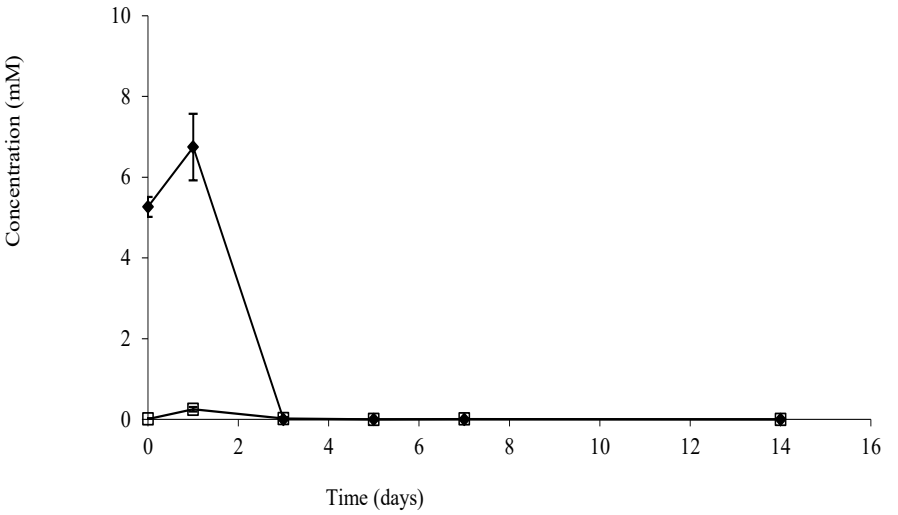
Group 3

Finally, based on the microtiter plate assay, *Achromobacter* strains I-5 and I-49 had the ability to reduce nitrate to nitrite, but did not reduce nitrite. However, under strictly anaerobic conditions they were able to reduce both nitrate and nitrite. In *Achromobacter* strain I-5 (*nosZ*$^+$), after one day of incubation, nitrate was depleted to around 1.4 mM and nitrite accumulated to around 4.8 mM. After three days of incubation, almost all nitrate and nitrite were depleted (Figure 1.f). Also, in *Achromobacter* strain I-49 (*nirK*$^+$, *nosZ*$^+$), after one day of incubation, nitrate was depleted to around 2.9 mM and nitrite was accumulated to around 5.0 mM. After three days of incubation, almost all nitrate was depleted and nitrite was depleted to around 2.8 mM. Nitrate and nitrite were completely depleted after five days of incubation (Figure 1.g). The negative control *P. putida* pG7 did not show any significant depletion of nitrate or accumulation of nitrite even after 14 days of incubation (data not shown).

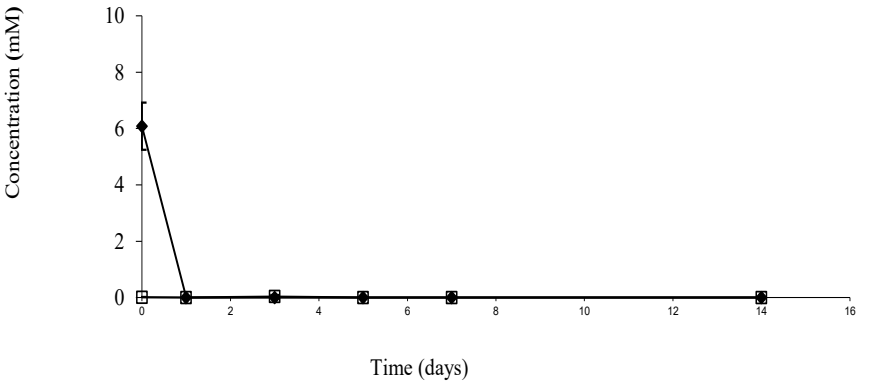
Discussion

The effect of oxygen on nitrite reduction

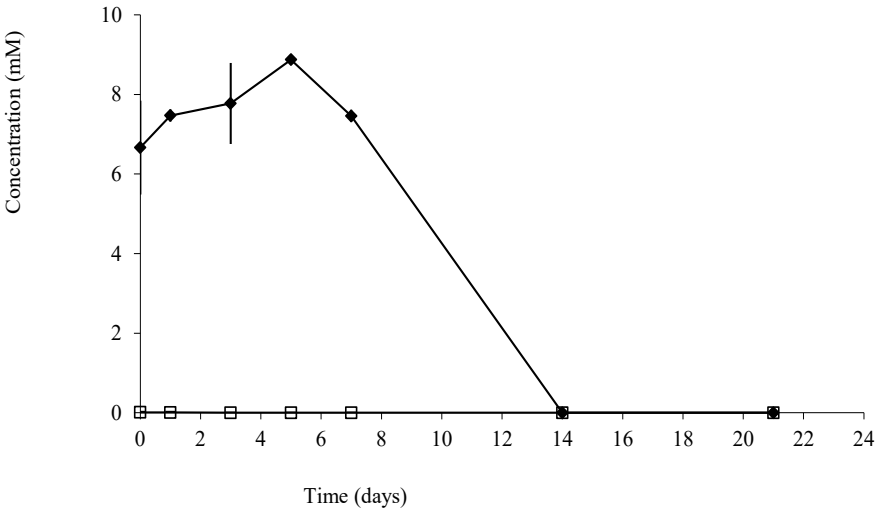
This study compared the effect of oxygen on the production/loss of nitrite in seven strains from five different genera of bacteria. We found that nitrate/nitrite reduction in five strains was relatively insensitive to oxygen. However, nitrite reduction in two strains of *Achromobacter* only occurred under strictly anaerobic conditions. We conclude that oxygen levels are



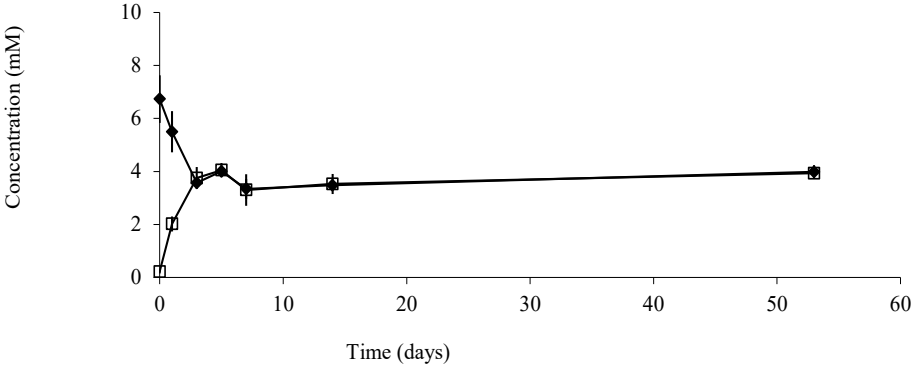
1.a. *Pseudomonas* strain I-1.



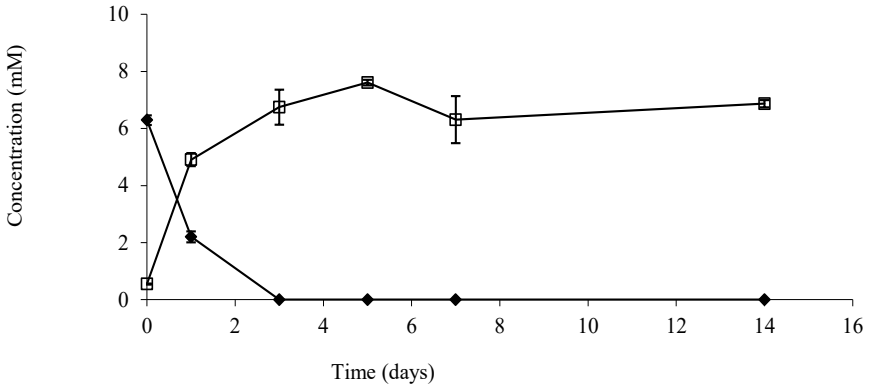
1.b. *Pseudomonas* strain I-65.



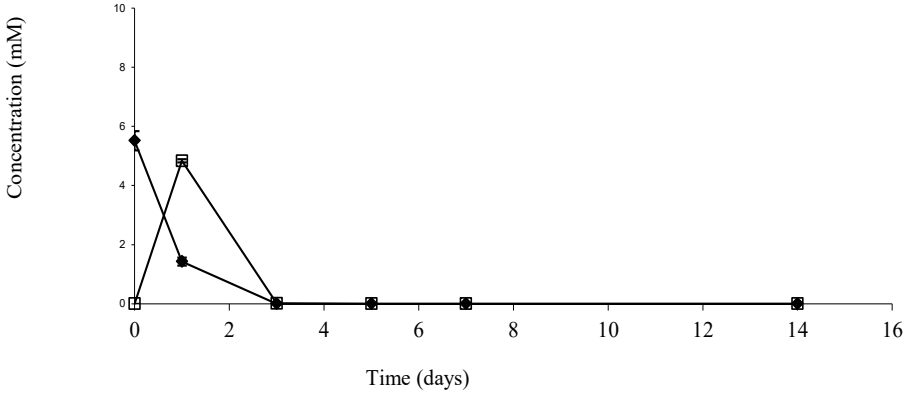
1.c. *Ensifer* strain I-4.



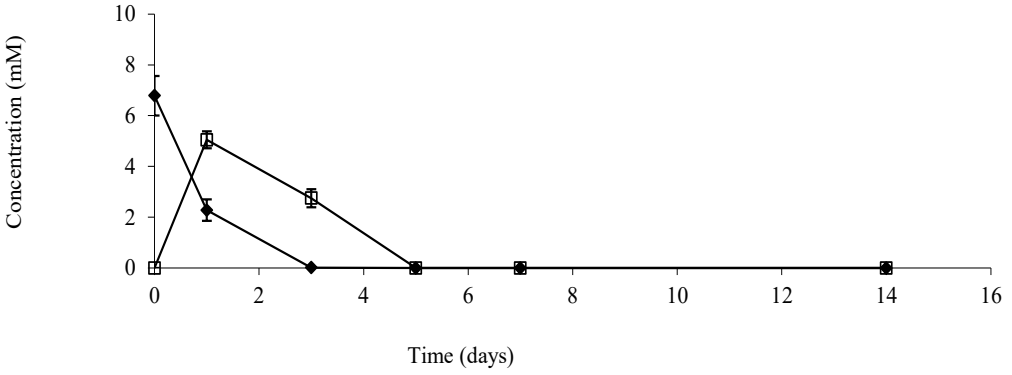
1.d. *Aeromonas* strain I-6.



1.e. *Enterobacter* strain I-25.



1.f. *Achromobacter* strain I-5.



1. g. *Achromobacter* strain I-49.

Figure 1. Nitrate or nitrate/nitrite reduction under strictly anaerobic conditions for seven strains. Time in days is plotted on the X axis and nitrate (diamonds)/nitrite (hollow squares) concentration in micromolar is plotted on the Y axis. Each data point represent the average of 3 data points. The bars represent one standard deviation. 1.a. *Pseudomonas* strain I-1; 1.b. *Pseudomonas* strain I-65; 1.c. *Ensifer* strain I-4; 1.d. *Aeromonas* strain I-6; 1.e: *Enterobacter* strain I-25; 1.f: *Achromobacter* strain I-5; 1.g: *Achromobacter* strain I-49.

important in the expression of nitrite reduction genes in some strains or genera of bacteria commonly found in Tallgrass Prairie soil.

Based on the effect of oxygen on nitrite reduction, we can categorize the seven strains into three groups. Group 1 includes *Pseudomonas* strains I-1 and I-65, and *Ensifer* strain I-4. This group showed no effect of the two test regimens on nitrite reduction, nitrite reduction appeared to be relatively oxygen-insensitive. Group 2 includes *Aeromonas* strain I-6 and *Enterobacter* strain I-25. This group, which reduced nitrate but did not reduce nitrite under either oxygen regimen, showed that there was no effect of strict oxygen exclusion on nitrite reduction. Finally, Group 3 includes *Achromobacter* strains I-5 and I-49. Nitrite reduction in this group appears to be oxygen sensitive because nitrite was consumed only under strictly anaerobic conditions.

Group 1: Nitrite reduction under both microtiter plate assay and strict anaerobic conditions

Strain I-1 (99% similar to 16S rRNA sequence from *Pseudomonas* sp. MT14, accession number DQ647192) showed its ability to reduce nitrate and nitrite when microtiter plate assay was used and it reduced nitrite under strictly anaerobic conditions which may indicate that the nitrite reductase of some *Pseudomonas* strains is not sensitive to oxygen. In support, it was shown that *Pseudomonas* sp. strain K50 produced N_2 even under oxic conditions indicating that it is an O_2 resistant, aerobic denitrifyer (Takaya et al., 2003). Also, oxygen was shown to have no effect on nitrite production/loss in *Pseudomonas* strain I-65 (99% similar to the 16S rRNA sequence of *Pseudomonas* sp. strain MTQ15, accession number HQ143608) that reduced nitrate/nitrite when microtiter plate assay was used and under strictly anaerobic conditions. However, not all *Pseudomonas* strains are capable of denitrification or even nitrate reduction. For example, *Pseudomonas* sp. S-JS-8 (FJ529034) does not reduce nitrate (Huang et al., 2010) while *Pseudomonas* sp. ONBA-17 (DQ079062) reduces nitrate to nitrite under aerobic conditions (Fang-Bo et al., 2006).

Ensifer strain I- 4 (100% similar to the 16S rRNA sequence of *Ensifer* sp. TA12-B, accession

number HM219616) reduced nitrate/nitrite when microtiter plate assay was used and under strictly anaerobic conditions which indicates some degree of oxygen tolerance on nitrite reduction in this strain. In fact, *Ensifer adhaerens strains* 2FB8 and 4FB6 ***were shown*** to utilize different halobenzoates under aerobic and denitrifying conditions, but the metabolic pathways were not investigated (Song et al., 2000).

Group 2: No nitrite reduction under either oxygen level

Strain I-6 (99% similar to the 16S rRNA sequence of *Aeromonas punctata* strain JA11, accession number GU205200) and strain I-25 (99% similar to the 16S rRNA sequence of *Enterobacter cloacae* strain P04, HM854373) were able to reduce nitrate when the microtiter plate was assayed but nitrite was not consumed. The same results were obtained for these two strains under strictly anaerobic conditions. However, strain I-6 (*nosZ*$^{+}$) and strain I-25 (*nirK*$^{+}$) each possesses a sequence highly similar to one denitrification gene.

Group 3: Nitrite reduction only under strict anaerobic conditions

Oxygen was shown to play a role in the production/loss of nitrite in *Achromobacter* strains I-5 and I-49. Strain I-5 (99% similar to the 16S rRNA sequence of *Achromobacter xylosoxidans*, accession number AF531768) possesses *nosZ* and strain I- 49 (99% similar to the 16S rRNA sequence of *Achromobacter* sp. MMD19, accession number JN546225) possesses *nirK* and *nosZ*. In fact, *A. xylosoxidans* strains are known to vary in their ability to reduce nitrate. Tatum divided *A. xylosoxidans* based on its ability to reduce nitrate into two groups: one group reduces nitrate to nitrite while the other reduces nitrate to nitrogen gas (Tatum et al., 1974). Also, Shigeta found the same results where in different strains of *A. xylosoxidans* varied in their ability to reduce nitrate. Some *A. xylosoxidans* strains reduced nitrate to nitrogen gas, whereas other strains reduced nitrate to nitrite only (Shigeta et al., 1983). On the other hand, *Achromobacter insolitus* strain LMG 6003 (NR_025685) reduces nitrate but does not reduce nitrite and does not denitrify (Coenye et al., 2003). Our results showed that both *Achromobacter* strain I-5 and strain I-49 have the ability to reduce nitrate when using the microtiter plate assay. However, under strictly anaerobic conditions, *Achromobacter* strain I-5 and strain I-49 were able to reduce both nitrate and nitrite. These results are consistent with our hypothesis that in certain groups, the production/loss of nitrite is regulated by oxygen supply which may indicate that certain denitrifying genes are expressed only under strictly anaerobic conditions. This regulation may be important in the transition from aerobic to anaerobic conditions, such as produced in the bioremediation of contaminated soils. In fact, a consortium that contained *A. xylosoxidans* from weathered, oil-contaminated soil was shown to degrade hydrocarbons in oxygen-limited environments (Medina-Moreno et al., 2005).

Stres et al. (2008) showed that some environmental factors (e.g. absence of plant cover, changes in water content and temperature) played a minor role in shaping bacterial and denitrifyers community structures but significantly altered their activity. *Achromobacter* strain I-5 was isolated from the brine contaminated G7 site which was periodically watered to enhance remediation. *Achromobacter* strain I-49 was obtained from the uncontaminated G7P site which depended on natural rainfall. Apparently, *Achromobacter* strains could be isolated from both the relatively more aerobic uncontaminated prairie as well as from intermittently anaerobic soil, but may be able to reduce nitrite only when the soil is flooded producing oxygen deficient soil conditions.

The power of molecular detection in identifying denitrifying bacteria

We detected nitrite reductase genes (e.g. *nirS*$^{+}$ or *nirK*$^{+}$) in only two out of the five strains that showed nitrite reduction under strictly anaerobic conditions. However, when we used *cnorB* and *nosZ* primers to detect these two functional genes, the additional three nitrite reducing strains (*nirS*$^{-}$ and *nirK*$^{-}$) were identified as denitrifying bacteria by at least one of these two denitrifying genes (e.g. *cnorB*$^{+}$ and/or *nosZ*$^{+}$). These results indicate that the more denitrifying genes we

target in the denitrification pathway using the PCR-approach, the higher chance in identifying denitrifying bacteria since nitrite reductase genes may not be sufficient by themselves to identify denitrifying bacteria. In fact, another study (Hallin and Lindgren, 1999) showed that two sets of PCR primers were used to amplify *nirS* and *nirK* from nitrite reducing bacteria where in some cases only one of these two sets of primers was able to amplify the expected fragment of the corresponding gene. However, in some other cases, neither of these two primer sets was able to detect the nitrite reductase genes.

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