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# Test of Polyaromatic Hydrocarbon Degradation by Nitrate-reducing Microorganisms Isolated from Tallgrass Prairie Soils

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**Abstract:** Soils are frequently contaminated with hydrocarbons such as polyaromatic hydrocarbons (PAH). This contamination inhibits the growth of some microorganisms in the contaminated soils. However, the contamination may select for microorganisms capable of hydrocarbon degradation under aerobic or anaerobic conditions. Forty five strains of bacteria isolated from tallgrass prairie soil samples were screened for the ability to grow with naphthalene as sole carbon source under aerobic conditions by culturing on minimal medium containing naphthalene as the sole carbon source. Our results showed that none of the 45 strains were able to grow on naphthalene under these conditions. Three out of the 45 strains, e.g. one strain each classified as *Ensifer*, *Stenotrophomonas*, or *Serratia*, were tested for the ability to degrade naphthalene under nitrate reducing conditions. All three strains were facultative anaerobes and showed the physiology of nitrate or nitrate/nitrite reduction when grown under nitrate-reducing conditions in medium containing yeast extract. Two strains (e.g. *Stenotrophomonas* and *Serratia*) were tested for the ability to grow on naphthalene, gasoline, or benzene under nitrate-reducing conditions. The *Serratia* grew poorly on the hydrocarbons, but *Stenotrophomonas* reached its highest O.D. values on naphthalene. However, upon re-testing a well-washed cell suspension of the *Stenotrophomonas*, no loss of naphthalene was found when grown under nitrate-reducing conditions. Although it might be expected that crude-oil contamination would select for PAH degraders, we did not find the nitrate reducing/nitrite reducing isolates to be capable of naphthalene-degradation under aerobic or nitrate-reducing conditions. ©2015 Oklahoma Academy of Science

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## Introduction

Petroleum contamination results regularly from leaks and accidental spills during production, transportation, and storage of petroleum and petroleum products. Since accumulation of pollutants in animals and in plant tissue may cause death or mutations,

soil contaminated with hydrocarbons causes extensive damage of local systems (Alvarez and Vogel, 1991). Polyaromatic hydrocarbons (PAHs) have been found to show toxic and hazardous properties (Mihelcic and Luthy, 1988). The Environmental Protection Agency has listed 16 PAH compounds, including naphthalene, as priority pollutants to be monitored in industrial effluents (Mihelcic and Luthy, 1988).

Naphthalene and its methylated derivatives are among the most toxic compounds found in the water-soluble fraction of petroleum (Heitkamp et al., 1987). In fact, several physicochemical and biological factors affect the degradation of petroleum hydrocarbons in soil such as: 1- the number and types of microbial species present, 2- the nature, amount, and bioavailability of contaminants, 3- the existing environmental conditions for microbial degradation (e.g., oxygen, temperature, pH, and nutrient levels), 4- soil characteristics (e.g., organic matter and soil aggregates) (MacNaughton et al., 1999; Röling et al., 2002; and Smith et al., 2008). Even worse, some high molecular weight PAHs, such as fluoranthene and benzo[*a*]pyrene (BaP), may not be degraded at all (Atlas and Bragg, 2009).

PAHs can be biodegraded in the presence of oxygen (Ni Chadhain et al., 2006), or under anaerobic condition such as nitrate reducing (e.g. NR) (Al-Bashir et al., 1990), sulfate-reducing (Coates et al., 1997; Davidova et al. 2007), or methanogenic (Godsy et al., 1992) conditions. Microbial degradation of naphthalene has been investigated and it is well documented in the literature (Al-Bashir et al., 1990; Ensley et al., 1982; Mihelcic and Luthy, 1988; Rockne et al., 2000). However, most of the studies were performed under aerobic conditions. In the classical pathway of naphthalene degradation under aerobic conditions, naphthalene dioxygenase (NDO), the multicomponent enzyme system, initiates the metabolism of low-molecular-weight PAHs (Ensley et al., 1982). The classical pathway of naphthalene degradation is via dihydroxylation and cleavage of the first ring and removal of the resulting aliphatic side chain to produce salicylate (2-hydroxybenzoate). Salicylate is then converted by the action of salicylate 1-hydroxylase to catechol (1,2-dihydroxybenzene). Finally, catechol undergoes extradiol cleavage via the same route used for a wide range of other aromatic compounds such as toluene, phenol, and xylenes (Zhou et al., 2002).

Some initial chemical transformations of naphthalene might take place even if strictly aerobic naphthalene degraders are killed

by the initial spill of crude oil. Enzymatic activity has been shown to persist in soil for several weeks (Renella et al., 2007; Stursova and Sinsabaugh, 2008), therefore lysed cells could possibly be a source of NDO or similar oxygenase enzymes under natural conditions. Purified NDO was shown to transform the model substrates naphthalene and indole in the presence of humic acids when incubated for 2 weeks at room temperature which might indicate that NDO maintains some enzymatic activity over days (AbuBakr et al., 2008).

Although the emphasis has been on naphthalene degradation under aerobic conditions, there are some studies about the microbial degradation of naphthalene under anaerobic conditions. Mihelcic and Luthy showed the first evidence of microbial degradation of unsubstituted PAH compounds under denitrifying conditions (Mihelcic and Luthy, 1988). Indeed, denitrifying conditions could play an important role in the decomposition of PAHs in nature, and naphthalene biodegradation rates obtained under denitrifying conditions have been shown to be in the same range as those obtained under aerobic conditions (Al-Bashir et al., 1990). Rockne et al showed that naphthalene was mineralized to carbon dioxide under nitrate reducing (NR) conditions in pure cultures, and that the ability to degrade naphthalene under NR conditions was found in a wide diversity of  $\gamma$ -Proteobacteria including *Pseudomonas* and *Vibrio* species (Rockne et al., 2000).

Previous studies showed that NR and denitrifying (DN) bacteria are abundant in the Tallgrass Prairie Preserve (TPP), which has experienced hydrocarbon contamination from surface spills of crude oil and/or salt water (brine) (Sublette et al, 2007b). Remediation treatments commonly included the application of nitrate-containing fertilizer. Higher levels of culturable aerobic naphthalene degrading bacteria were found in oil contaminated sites the first year of treatment with nitrogen compared to the uncontaminated prairie. However, the naphthalene-degrading bacteria were not identified (Duncan et al., 1999). Since NR bacteria, DN bacteria, and hydrocarbons are

abundant in the TPP, we hypothesized that many of the NR/DN bacteria from TPP have the ability to degrade hydrocarbons under NR/DN conditions. However, as facultative anaerobes, nitrate reducers could degrade PAH under aerobic or NR conditions, if the strains possessed the necessary degradation genes. Therefore, this study also tested the ability of 45 strains to degrade naphthalene under aerobic conditions. The 45 strains (Table 1) included the strains that were confirmed to possess the nitrate reducing (NR) or nitrite reducing (DN) phenotype and were chosen to be representative of the 16S rRNA phylogenetic diversity plus all the remaining *Stenotrophomonas* and *Pseudomonas* strains from the total set of 75 strains. The 75 strains were isolated from the same sites described in the Materials and Methods and by the same isolation procedure as a part of a wider effort to characterize species composition of culturable nitrate reducing/denitrifying bacteria isolated from contaminated and uncontaminated tallgrass prairie soil. Strains of *Stenotrophomonas* and *Pseudomonas* were chosen because they have been shown in previous studies to be hydrocarbon degraders (Gibson et al., 1974; Jung and Kafkewitz, 1996; Jeong et al., 2006; Lee et al., 2002; Shim and Yang, 1999).

## Methods

### Sites Description

The sampling sites used in this work are located in the Tallgrass Prairie Preserve (TPP) in Osage County, Oklahoma. Briefly, strains were obtained from 5 contaminated (e.g. G5, G7, LF, J6F, and J6NF) and 4 uncontaminated (e.g. G5P, G7P, LFP, and J6P) sites. More than 90% of the crude oil in TPP was aliphatic compounds, and less than 10% were aromatic compounds such as polyaromatic hydrocarbons (PAH) (e.g. naphthalene) (Sublette et al., 2007a).

### Sampling soils, isolating bacteria and testing their ability to reduce nitrate/nitrite

Soils were sampled from all 9 sites and serially diluted and scored for growth and nitrate/nitrite reduction. Briefly, soils were homogenized, serially diluted and added to microtiter plates

containing nitrate broth (Difco), after 2 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<sup>-1</sup> to 10<sup>-4</sup> dilutions that scored positive for growth and nitrate reduction or nitrite reduction. The nutrient agar plates were incubated at 30°C. Pure strains were obtained from the microtiter plate wells containing nitrate broth followed by at least three rounds of streaking for single colonies on nutrient agar plates. The purified strains were tested on their ability to reduce nitrate or nitrite after incubation into nitrate broth in microtiter plates and the presence of nitrate/nitrite assayed using Griess reagents (Smibert and Krieg, 1994). In addition, the following strains of known phenotype were included as controls: *P. aeruginosa* ATCC 27853 (Denitrifier), *P. putida* pG7 (not a nitrate reducer), *S. maltophilia* ATCC 13637 (Nitrate reducer), and uninoculated wells containing sterile nitrate broth.

API 20 NE strips (bioMérieux, France) were used to perform different biochemical tests on a subset of three strains, including nitrate/nitrite reduction and glucose utilization.

### DNA extraction from strains

Genomic DNA was isolated from cells using a bead beating method. Briefly, each of the 45 strains was streaked on nutrient agar plate incubated one to three days at 30°C until good colony growth was observed, then a single colony from each plate was used to inoculate 5 mL nutrient broth and incubated at 30°C until turbid growth was seen. A sufficient volume of culture (1 to 2 ml) was centrifuged at 6000xg for 10 minutes to provide a pellet size of approximately 10<sup>9</sup> bacteria. Pellets were resuspended in sterile isotonic saline (0.85%) to give a maximum suspension volume of 200 µL. A commercially available DNA extraction kit, FastDNA®SPIN Kit (QBIogene, Solon, OH,

Table 1. Characteristics of the 45 strains used in this study.

Strain #*	Isolation site characteristics	RDP Classifier**	NR/DN
I-1	Brine	<i>Pseudomonas</i> [100%] ( $\gamma$ )	DN
I-2	Brine	<i>Pseudomonas</i> [100%]( $\gamma$ )	DN
I-3	Brine	<i>Pseudomonas</i> [100%] ( $\gamma$ )	NR
I-4	Brine	<i>Ensifer</i> [99%] ( $\alpha$ )	DN
I-5	Brine	<i>Achromobacter</i> [99%] ( $\beta$ )	NR
I-6	Brine	<i>Aeromonas</i> [100%] ( $\gamma$ )	NR
I-10	Brine	<i>Phenylobacterium</i> [100%] ( $\alpha$ )	NR
I-11	Brine	<i>Stenotrophomonas</i> [100%] ( $\gamma$ )	NR
I-12	Brine	<i>Pseudomonas</i> [95%] ( $\gamma$ )	NR
I-13	Brine	<i>Stenotrophomonas</i> [100%] ( $\gamma$ )	NR
I-18	Brine	<i>Stenotrophomonas</i> [100%] ( $\gamma$ )	NR
I-21	Oil	<i>Stenotrophomonas</i> [100%] ( $\gamma$ )	NR
I-23	Oil	<i>Stenotrophomonas</i> [100%] ( $\gamma$ )	NR
I-24	Oil	<i>Serratia</i> [100%] ( $\gamma$ )	NR
I-25	Oil	<i>Enterobacter</i> [99%] ( $\gamma$ )	NR
I-26	Oil	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	None
I-27	Oil	<i>Stenotrophomonas</i> [100%] ( $\gamma$ )	None
I-28	Oil	<i>Stenotrophomonas</i> [100%] ( $\gamma$ )	None
I-30	Oil	<i>Pseudomonas</i> [100%] ( $\gamma$ )	NR
I-34	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR
I-35	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR
I-36	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR
I-37	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR
I-38	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR
I-39	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR
I-40	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR
I-41	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR
I-42	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR
I-49	Prairie	<i>Achromobacter</i> [100%] ( $\beta$ )	NR
I-50	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	None

Strain #*	Isolation site characteristics	RDP Classifier**	NR/DN
I-51	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	None
I-52	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	None
I-53	Prairie	<i>Brevibacillus</i> [100%] (Bacilli)	NR
I-57	Prairie	<i>Stenotrophomonas</i> [100%] ( $\gamma$ )	DN
I-61	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR
I-62	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	None
I-63	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	None
I-64	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	None
I-65	Prairie	<i>Pseudomonas</i> [100%]( $\gamma$ )	DN
I-67	Prairie	<i>Pseudomonas</i> [100%]( $\gamma$ )	NR
I-68	Prairie	<i>Pseudomonas</i> [100%]( $\gamma$ )	NR
I-70	Prairie	<i>Acinetobacter</i> [100%] ( $\gamma$ )	NR
I-71	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	None
I-73	Prairie	<i>Pseudomonas</i> [100%]( $\gamma$ )	NR
I-74	Prairie	<i>Stenotrophomonas</i> [100%]( $\gamma$ )	NR

Strains were tested for nitrate/nitrite loss using the microtiter plate assay.

NR: Nitrate Reduction using microtiter plate assay.

DN: Nitrite reduction/denitrification using microtiter plate assay.

None: No reduction of nitrate or nitrite using microtiter plate assay.

\*Strain #: Represents the designated numbers from the original set of the 75 strains.

\*\*RDP Classifier: Ribosomal Database Project Classifier.

**Table 2. The distribution of 45 nitrate reducing bacteria. (NR), nitrite reducing bacteria (DN), and the strains that do not reduce nitrate/nitrite (None) along with the distribution of *Pseudomonas* and *Stenotrophomonas* among the three different soil sites.**

Site	# NR	# DN	#None	# <i>Pseudomonas</i>	# <i>Stenotrophomonas</i>
Brine	8	3	0	4	3
Oil	5	0	3	1	5
Uncontaminated	17	2	7	4	19

USA), was used to extract DNA from the cell suspensions by following the manufacturer's directions. The protocol combined mechanical (e.g., bead-beating) and chemical means to lyse the cells. Extracted DNA was stored at  $-20^{\circ}\text{C}$ . Controls for contamination included processing one reagent control to which sterile water rather than cells was added.

### **Molecular detection of 16S rRNA gene**

Universal bacterial primers for 16S rRNA were used to amplify an approximately 1400 bp fragment. Extracted DNA was used as the template in a polymerase chain reaction (PCR) using primer pair 27F/1492R (Wilson et al., 1990). The PCR products were visualized using agarose gel electrophoresis technique.

### **DNA sequencing and analysis**

PCR products were prepared for sequencing using ExoSAP-IT (USBiochemicals) to eliminate unincorporated primers and dNTPs so that they cannot interfere with downstream sequencing reactions (Bell, 2008). Sequencing was performed by the Oklahoma Medical Research Foundation (OMRF) DNA Sequencing facility by using an ABI 3730 capillary sequencer with primer pair 27F/1492R. Sequence chromatograms were analyzed to produce a consensus sequence, and compared to the GenBank nt/nr database BLASTN searches (Basic Local Alignment Search Tool) (Altschul et al., 1990).

### **Testing the ability to degrade naphthalene under aerobic conditions**

To test the ability of the 45 strains to degrade naphthalene under aerobic conditions, mineral salt medium plates without any carbon source were prepared (Youssef et al., 2004). Each of the 45 strains was streaked on a mineral salt medium plate and naphthalene crystals were added to the plate lids. The plates were incubated at room temperature for 2 weeks in a closed plastic container. *Pseudomonas putida* pG7, which contains the NAH7 naphthalene-degradation plasmid (Dunn and Gunsalus, 1973), was used as a positive control. Each of the 45 strains was also streaked on mineral salt medium plus glucose (0.2%) to test the ability of

each of the 45 strains to grow on the mineral salt medium with a commonly used carbon source.

### **Molecular detection of aerobic naphthalene degradation functional genes**

Three strains were further examined, namely: strain I-4 (*Ensifer*), strain I-21 (*Stenotrophomonas*), and strain I-24 (*Serratia*) (Table 1). For the chosen three strains, PCR was used to amplify a portion of the *nahAc* gene which encodes the large subunit of the iron-sulfur protein component of naphthalene dioxygenase, the initial enzyme in the aerobic naphthalene catabolic pathway (Simon et al., 1993). The primer pair used was nahAcl(5'-G1TTGCAGCTATCACGGCTGGGGCTTCGGC-3') corresponding to nucleotides 794 to 823 of the *nahAC* sequence, and nahAc3(5'-TTTCGACAATGGCGTAGGTCCAGACCTCGGT-3') corresponding to nucleotides 1495 to 1466 (Kurkel et al., 1988). The amplicon size with the primer pair nahAcl/nahAc3 is 701-bp (Herrick et al., 1993). DNA extracted from *Pseudomonas putida* G7 containing the NAH7 plasmid was used as a positive control.

### **Nitrate depletion/nitrite accumulation stoichiometry of cell suspensions**

Strains *Ensifer* I-4, *Stenotrophomonas* I-21, and *Serratia* I-24 which had shown their ability to reduce nitrate/nitrite after growth in nitrate broth in a microtiter plate were chosen to test their ability to reduce nitrate/nitrite under NR conditions. Nitrate broth medium (5.0 g  $\text{NaNO}_3/\text{L}$  plus 8.0g Nutrient broth/L) was prepared under strictly anaerobic conditions in which it was boiled and gassed with  $\text{N}_2/\text{CO}_2$  in an ice bath. Each of the three chosen strains was cultivated under NR (e.g. strictly anaerobic) conditions. The cultures were routinely grown in 125-mL serum bottles containing 50 mL of Nitrate broth medium. Two bottles of a well established culture (three transfers) from each strain were used to inoculate, anaerobically, two 1-L bottles each containing 500 mL nitrate broth. After overnight incubation at room temperature, the cells were anaerobically collected in the late exponential phase by centrifugation ( $10,000 \times g$ , 15 min,  $6^{\circ}\text{C}$ ) in sealed bottles and washed once

in anoxic 25 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.2). The washed cells were resuspended in 20 mL of the same buffer and divided into two aliquots. Each aliquot was centrifuged again and suspended in MOPS buffer (pH 7.2) and normalized to similar O.D.<sub>600</sub> (~0.36). Glucose (20 mM) was provided as a substrate and the reactions were initiated with nitrate (5mM). Samples taken at various time points were filter-sterilized immediately and nitrate/nitrite concentrations were monitored by Ion Chromatography (model DX500 fitted with an AS-4A column; Dionex Corp., Sunnyvale, CA) as described in Caldwell et al. (1998).

### **Growth on hydrocarbons under NR conditions**

Growth (e.g. increase in optical density "O.D." at 600 nm) on hydrocarbons under NR conditions was tested using strains that showed the ability to reduce nitrate to nitrite under NR conditions. The tested strains were grown in 125-mL serum bottles containing 50 mL of the mineral medium (Widdel and Bak, 1992). One liter of this medium contains 1g NaCl, 0.5g NH<sub>4</sub>Cl, 0.3g KH<sub>2</sub>PO<sub>4</sub>, 0.4g MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.15g CaCl<sub>2</sub>·2H<sub>2</sub>O, 1g of 10 mM KNO<sub>3</sub>, 5.0g yeast extract, and 10 mL RST metals (Tanner, 1997). Well-established cultures were used as inocula. The following substrates were tested: Gasoline (2 µl/50 mL medium), Naphthalene (10 mg/mL in 2,2,4,4,6,8,8-heptamethyl-nonane (HMN)), Benzene (0.225 mM), and Glucose (20 mM). Glucose was shown to be a good substrate for all three strains based on the API 20 NE strips (bioMérieux) results. Unamended substrate (no glucose or hydrocarbons) was used as a control and autoclaved cultures were used as negative controls. Cell densities, which were used to indicate growth and to infer substrate utilization, were determined by O.D. readings at 600 nm. Samples were taken at various time points and filter-sterilized immediately to be used in the following steps.

### **Nitrate depletion/nitrite accumulation when grown on a hydrocarbon substrate**

Nitrate/nitrite concentrations in the cultures of the strains that showed good growth on a hydrocarbon were monitored by Ion

Chromatography (model DX500 fitted with an AS-4A column; Dionex Corp., Sunnyvale, CA) as described (Caldwell et al., 1998).

### **Monitoring naphthalene depletion using Gas Chromatography**

Naphthalene depletion was monitored under nitrate-reducing conditions in the strain that showed nitrate depletion/nitrite accumulation when naphthalene was the carbon source. Mineral medium was prepared under NR conditions. Five milliliters of cell suspension were used to inoculate 40 mL mineral medium plus 3 mL overlay of naphthalene (5 mg/mL) dissolved in HMN. Five milliliters autoclaved inoculum, as a sterile control, was used to inoculate 40 mL minimal medium plus 3 mL overlay of naphthalene (5 mg/mL) in HMN. For unamended controls, 5 mL inoculum was used to inoculate 45 mL minimal medium. Blanks containing 45 mL minimal medium were overlaid with 3 mL naphthalene (5 mg/mL) in HMN. All samples were prepared in triplicates. The concentration of naphthalene in the incubations was measured by periodic sampling of the HMN overlay and injection of a small portion (1 µL) directly into an Agilent Technologies 5973 Network Gas Chromatograph (GC) equipped with a HP5-MS column (30 m x 0.25 mm, 0.25µm film) and a mass selective (MS) detector. The oven temperature was held at 100°C for 2 min, then increased at 5°C per min to 160°C and then raised at a rate 20°C per minute to 220°C. The injector temperature was 250°C. Helium was used as a carrier gas at a flow rate of 1.2 mL/min.

A parallel experiment was conducted in which the wash medium was made from the mineral medium without nitrate and without yeast extract to avoid any traces of yeast extract in the cell suspension. Briefly, after 5 transfers, 18 bottles (50 mL of anaerobic culture/bottle) were pooled under anaerobic conditions in 2 centrifuge bottles. The bottles were centrifuged for 20 min at 6000 xg at 6°C, subsequently centrifuged in smaller bottles for 10 min at 10000 xg at 6°C. The collected cells were washed twice with a mineral medium minus yeast extract and minus nitrate. Cell pellets were resuspended in 60 mL of wash medium. The following mixtures

were prepared: mixtures 1 and 2 (replicates) contained 10 mL of the cell suspension plus 3 mL of naphthalene (0.5 mg/ mL in HMN) and 0.5 mL of KNO<sub>3</sub> (20.2 mg/ mL), mixture 3 contained 10 mL of the cell suspension plus 3 mL of naphthalene (0.5 mg/ mL in HMN) (e.g. “No nitrate”), mixture 4 contained 10 mL of the cell suspension plus 0.5 mL of KNO<sub>3</sub> (20.2 mg/ mL), (e.g. “No naphthalene”), mixture 5 contained 10 mL of sterile (e.g. autoclaved) cell suspension plus 3 mL of naphthalene (0.5 mg/ mL) and 0.5 mL of KNO<sub>3</sub> (20.2 mg/ mL), mixture 6 contained 10 mL of the cell suspension plus 1 mL of glucose (20 mM) and 0.5 mL of KNO<sub>3</sub> (20.2 mg/ mL), mixtures 7 and 8 (replicates) contained 10 mL of wash medium plus 3 mL of naphthalene (0.5 mg/ mL) and 0.5 mL of KNO<sub>3</sub> (20.2 mg/ mL). Samples were taken for both Ion Chromatography (nitrate depletion and nitrite accumulation) and Gas Chromatography (naphthalene concentration) at 0, 5, and 10 days. A naphthalene standard curve was made using naphthalene concentrations at 0.5, 1.0, 3.0, and 5.0 mg/mL in HMN.

## Results

### Characteristics of the 45 strains

Out of a total of 75 strains obtained, 45 strains were chosen that included a subgroup representing the range of taxonomic diversity plus all the remaining *Stenotrophomonas* and *Pseudomonas* strains based on their 16S rRNA gene sequence as determined by the Ribosomal Database Project (RDP) Classifier (Wang et al., 2007; Cole et al, 2009). Most of the 45 strains were members of the genus *Stenotrophomonas* (e.g. 61%). The genus *Pseudomonas* represented 20% of the 45 strains. Proportionately more strains from these genera were chosen because previous studies have shown *Stenotrophomonas* and *Pseudomonas* to be hydrocarbon degraders (Gibson et al., 1974; Jung and Kafkewitz, 1996; Jeong et al., 2006; Lee et al., 2002; Shim and Yang, 1999). Other genera represented lower percentages (Table 1).

The majority (67%) of the 45 strains were nitrate reducers, according to colorimetric

detection of nitrate/nitrite by Griess reagents after inoculation into nitrate broth and growth in microtiter plates (Smibert and Krieg, 1994). On the other hand, 22% did not show any nitrate/nitrite reduction while 11% reduced both nitrate and nitrite (Table 2).

### Testing the ability to degrade naphthalene under aerobic conditions

None of the 45 strains grew on mineral salt medium plus naphthalene under aerobic conditions, although the positive control (e.g. *P. putida* G7) grew well. However, all 45 strains grew on mineral salt medium plus glucose. These results indicate that the 45 strains did not utilize naphthalene under these aerobic conditions.

### Molecular detection of aerobic naphthalene degradation functional genes

Three strains (*Ensifer* I-4, *Stenotrophomonas* I-21, and *Serratia* I-24) were tested using specific PCR primers for the presence of one functional gene of aerobic naphthalene degradation. Our PCR results showed that we did not detect the naphthalene dioxygenase gene in any of the three strains when we used nahAcl/nahAc3 primer pair. However, we obtained amplicons from all three strains. The amplicons obtained from *Ensifer* strain I-4 and *Serratia* strain I-24 were larger in size than the expected nahAc size (701 bp) but after sequencing, *Ensifer* strain I-4 and *Serratia* strain I-24 sequences were not significantly similar to nahAc of *P. putida* G7 or to any sequence in the GenBank database. The amplicon from *Serratia* strain I-21 was similar in size to the nahAc. But, after sequencing, the top two matches (93%) were similar to succinate dehydrogenase, a flavoprotein subunit in *Burkholderia* sp. JV3 (CP002986), and to a dehydrogenase, a flavoprotein subunit in *S. maltophilia* R5S1-3 (CP001111).

### Nitrate depletion/nitrite accumulation under NR conditions

Since none of the 45 strains utilized naphthalene under aerobic conditions, a subset of three strains, namely *Ensifer* strain I-4, its 16S rRNA gene sequence was 100% similar to that of *Ensifer* sp. TA12-B (HM219616),



*Stenotrophomonas* strain I-21, its 16S rRNA gene sequence was 100% similar to that of *Stenotrophomonas maltophilia* strain YNA104-1 (JN867123), and *Serratia* strain I-24, its 16S rRNA gene sequence was 99% similar to that of *Serratia marcescens* strain NBRC 3736 (AB680122) was chosen to test their ability to degrade different hydrocarbons (HC) including naphthalene under NR conditions. Before testing their ability to degrade hydrocarbons, we tested the ability of the three strains to reduce nitrate/nitrite under NR conditions with yeast extract as their carbon source. Ion chromatography was used to study nitrate reduction under NR conditions. After providing cell suspension of *Serratia* I-24 with 5 mM nitrate, nitrate was depleted down to 2.17 mM and nitrite was accumulated up to 2.17 mM after 120 minutes of incubation at room temperature (23-25°C) under NR conditions (Figure 1.a). After further incubation for 24 hours nitrate was completely depleted (e.g. 0 mM) and nitrite was accumulated up to 1.3 mM (data not shown). However, in *Ensifer* strain I-4, the 5mM nitrate was depleted completely after 36 hours but nitrite did not accumulate to any significant level. The highest level nitrite reached was 0.15 mM after 36 hours then reduced to 0 mM on hour 39 (Figure 1.b). Finally, in *Stenotrophomonas* strain I-21, the 5mM nitrate was almost depleted completely (e.g. 0.01 mM) after 7 hours and the nitrite accumulated up to 3.8 mM after 9 hours. Subsequently, nitrite started to decrease slowly until it reached 3 mM after 22 hours (Figure 1.c).

Based on the depletion of nitrate and accumulation of nitrite, *Serratia* strain I-24 and *Stenotrophomonas* strain I-21 were confirmed as nitrate reducers. However, *Ensifer* strain I-4 did not show any significant accumulation of nitrite during sampling points although all nitrate was depleted after 36 hours suggesting *Ensifer* reduced nitrite and therefore might be a denitrifier.

### **Growth on hydrocarbons under NR conditions**

The two strains that showed nitrate reduction under NR conditions (e.g. *Stenotrophomonas* strain I-21 and *Serratia* strain I-24) were tested for growth in the presence of different hydrocarbons,

namely gasoline, benzene, or naphthalene, under NR conditions. *Serratia* strain I-24 showed little growth on any of the hydrocarbons used, though it grew well on glucose. On glucose, the O.D. reached its highest value (0.611) after 2 days (Figure 2.a). Naphthalene was the best substrate among the hydrocarbons with an O.D. of 0.26 after 20 days (Figure 2.a). In contrast, *Stenotrophomonas* strain I-21 grew best on naphthalene reaching even higher densities than those on glucose. Its O.D. on naphthalene reached 0.202 after 41 days, while the highest O.D. on glucose was 0.069 after 13 days of incubation (Figure 2.b). Since this study focused on hydrocarbon degradation, and since *Serratia* strain I-24 did not show good growth on any of the hydrocarbon used as substrates, *Serratia* strain I-24 was eliminated from further tests.

### **Nitrate depletion/nitrite accumulation in *Stenotrophomonas* strain I-21 when grown on naphthalene**

Glucose, naphthalene, and unamended medium (e. g. no added carbon source) were used in monitoring nitrate depletion/nitrite accumulation in *Stenotrophomonas* strain I-21. Although well-established cultures (5 transfers) were used in this study, there was still nitrate reduction/nitrite accumulation in the unamended medium samples (Figure 3) which indicates an unknown carbon source was utilized at the expense of nitrate. In fact, extracellular materials were visible inside the culture bottles which could serve as an alternative carbon source. We hypothesized that *Stenotrophomonas* strain I-21 degraded naphthalene under NR conditions. To test this hypothesis, naphthalene depletion in *Stenotrophomonas* strain I-21 was monitored using Gas Chromatography to follow the loss of naphthalene at the expense of nitrate. An alternative hypothesis was that extracellular materials or media components might serve as substrates under NR conditions. To test this hypothesis, we monitored nitrate loss and nitrite accumulation in unamended substrate samples using cells washed in medium without nitrate or yeast extract.

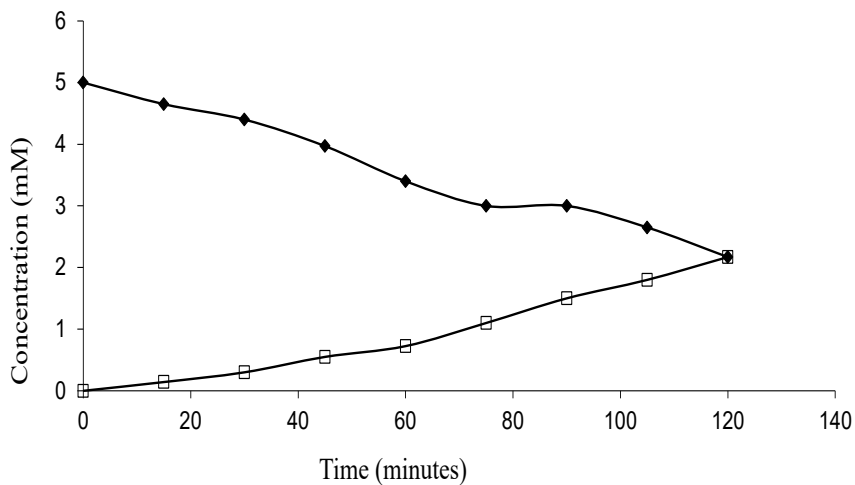


Figure 1.a. Nitrate/Nitrite Stoichiometry of *Serratia* strain I-24.

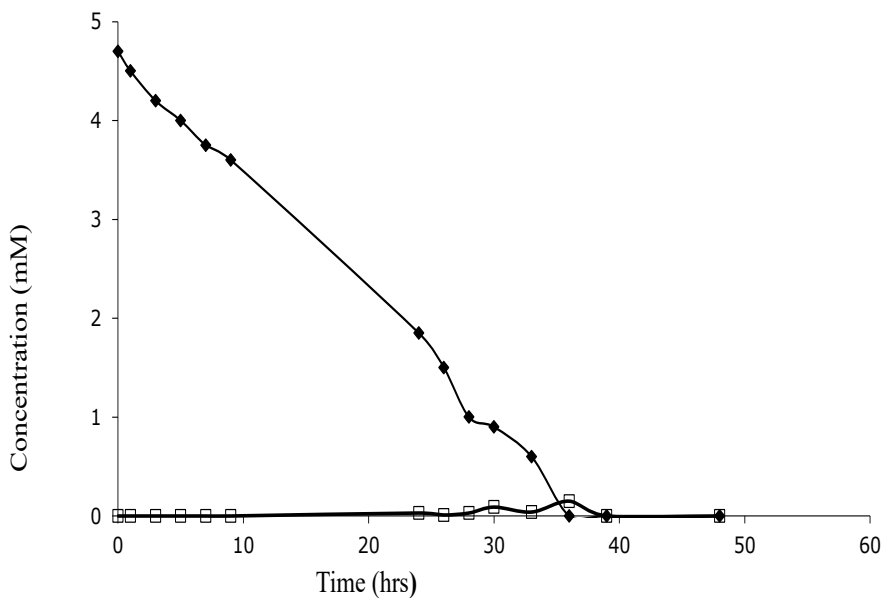


Figure 1.b. Nitrate/Nitrite Stoichiometry of *Ensifer* strain I-4.

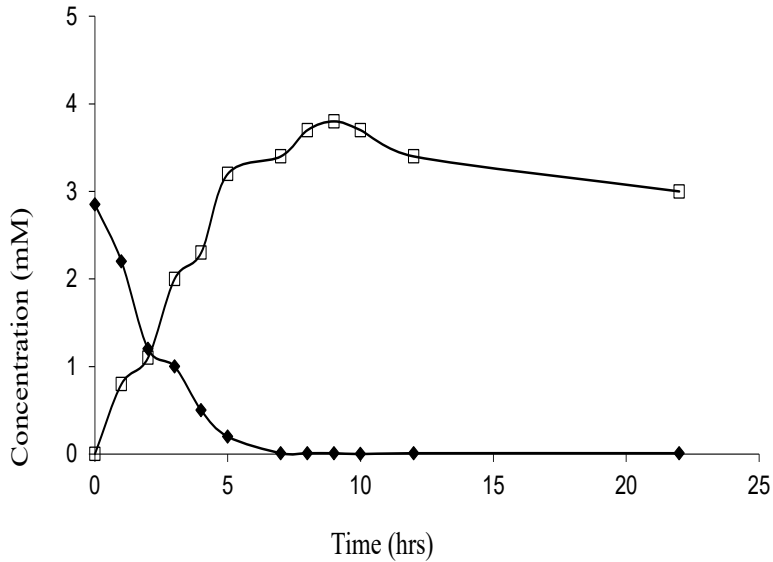


Figure 1.c. Nitrate/Nitrite Stoichiometry of *Stenotrophomonas* strain I-21.

**Figure 1. Nitrate depletion and nitrite accumulation.** A cell suspension was obtained after an overnight incubation of a well-established culture (three transfers) for three strains grown under strictly anaerobic conditions. Glucose (20 mM) was provided as a substrate and the reactions were initiated with 5 mM nitrate. Two replicates for each strain were pooled together to obtain one cell suspension for each strain. Time in minutes is plotted on the X axis and the nitrate (diamonds)/nitrite (hollow squares) concentration in micromolars is plotted on the Y axis. a. *Serratia* strain I-24. b. *Ensifer* strain I-4. c. *Stenotrophomonas* strain I-21.

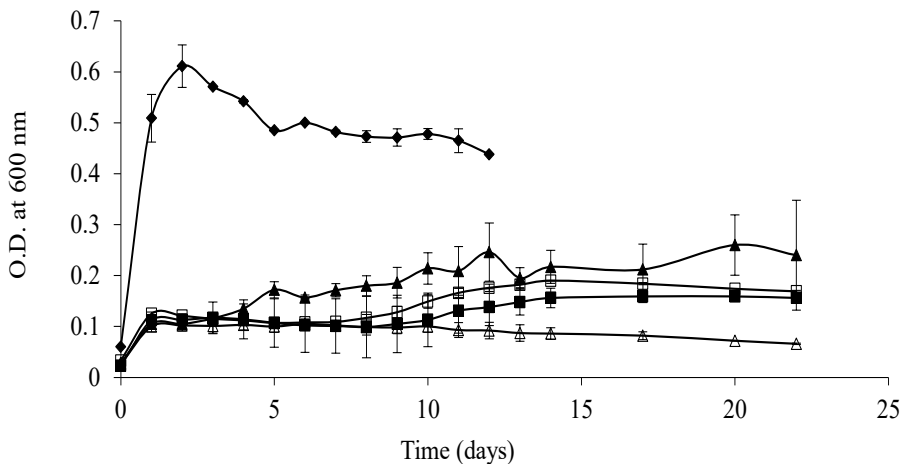


Figure 2.a. Growth of *Serratia* strain I-24 on various substrates under NR conditions.

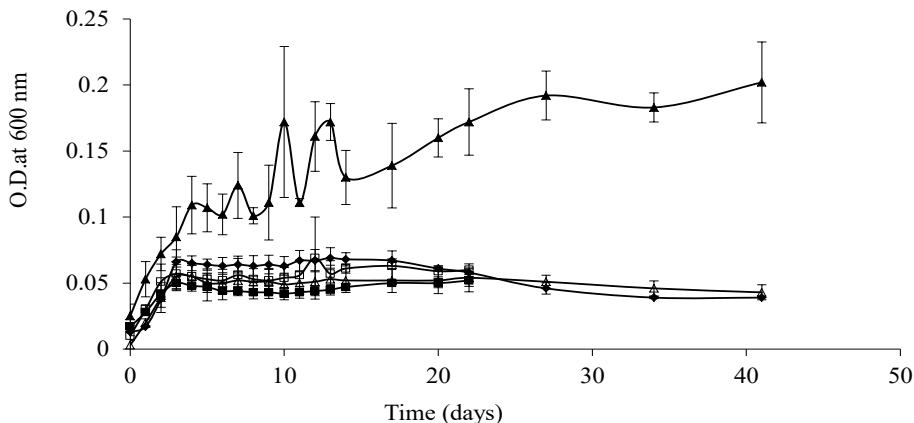


Figure 2.b. Growth of *Stenotrophomonas* strain I-21 on various substrates under NR conditions.

Figure 2. Growth curve on different substrates: Glucose (20 mM) (diamonds, average of 2 samples), naphthalene (10 mg/mL in 2,2,4,4,6,8,8-heptamethyl-nonane (HMN)) (filled triangles, average of 3), benzene (0.225 mM) (hollow squares, average of 2), gasoline (2 µl/50 mL medium) (filled squares, average of 3), and unamended (hollow triangles, average of 3). Time in days is plotted on the X axis and the O.D. at 600 nm is plotted on the Y axis. a. *Serratia* strain I-24. b. *Stenotrophomonas* strain I-21.

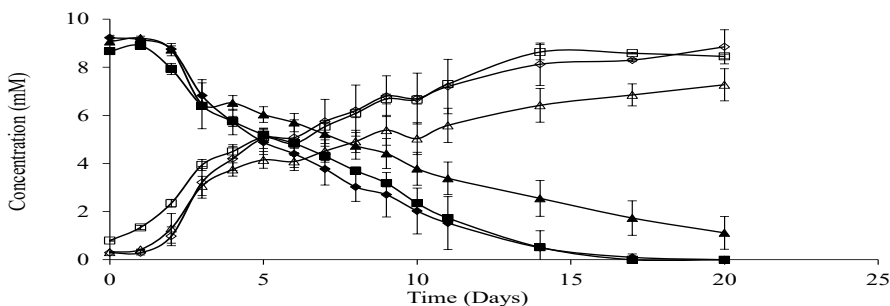


Figure 3. Nitrate depletion/nitrite accumulation by *Stenotrophomonas* strain I-21 on various substrates.

Figure 3. Nitrate depletion/nitrite accumulation by *Stenotrophomonas* strain I-21 on various substrates: Glucose (20 mM) (average of 2 samples). Nitrate as filled diamonds and nitrite as hollow diamonds, naphthalene (10 mg/mL in 2,2,4,4,6,8,8-heptamethyl-nonane (HMN)) (average of 3). Nitrate as filled squares and nitrite as hollow squares, unamended (average of 3). Nitrate as filled triangles and nitrite as hollow triangles. Time in days is plotted on the X axis and the nitrate/nitrite concentrations (µM) is plotted on the Y axis.

**Table 3. Stoichiometry of naphthalene loss coupled to nitrate reduction by *Stenotrophomonas* strain I-21.**

Naphthalene mineralization:  $C_{10}H_8 + 24NO_3^- \rightarrow 10CO_2 + 24 NO_2 + 4H_2O$

Nitrate loss (mM)	6.2 ± 0.05
Total naphthalene (mM)	1.5 ± 0.05
Naphthalene consumed (mM)	0.15
Theoretical consumed naphthalene (mM)	0.25
% of expected	60%

After four weeks of incubation; initial naphthalene level was 5 mg/mL.

$NO_3^-$  consumed from the unamended was 5 mM ± 0.2.

### Monitoring naphthalene depletion in *Stenotrophomonas* strain I-21 using Gas Chromatography

Naphthalene loss at the expense of nitrate in *Stenotrophomonas* strain I-21 was monitored using Gas Chromatography. The actual naphthalene consumed was estimated to be 0.15 mM (Table 3). However, the theoretical naphthalene consumed based on the naphthalene mineralization equation ( $C_{10}H_8 + 24NO_3^- \rightarrow 10CO_2 + 24 NO_2 + 4H_2O$ ) (Rockne et al., 2000) should have been 0.25 mM, based on the loss of nitrate. So, the actual naphthalene lost is 60% of the theoretical. This means that there was more nitrate reduced than naphthalene depleted. The excess of the reduced nitrate might drive the degradation of media components or the extracellular products that were produced by *Stenotrophomonas* strain I-21. This hypothesis was supported by the fact that 5 mM nitrate was consumed even in the unamended substrate culture (Table 3).

To avoid having traces of media components such as yeast extract even after 5 transfers, the same experiment above was repeated with the following changes. The wash medium was made this time from the mineral medium without nitrate and without yeast extract. Almost 50% of nitrate was reduced in the first five days in samples 1 and 2, containing cells, naphthalene, and nitrate (Table 4). However, the GC results showed that no loss of naphthalene during the course of the experiment. So, it appears that there was some other electron donor, perhaps extracellular

materials and/or medium components drove the reduction of nitrate to nitrite. Samples 7 and 8, which did not contain viable cells, showed a similar pattern, suggesting abiotic loss of nitrate. The existence of an electron donor other than naphthalene was confirmed because nitrate was lost from sample 4 (with nitrate without naphthalene) for the first five days. Between days 5 and 10 there was no more loss of nitrate, nitrate remained almost the same from day 5 to day 10. This means that the electron donor was not sufficient to support nitrate reduction for more than 5 days. Sample 6 that contained glucose continued nitrate depletion until nitrate was completely utilized which means that microorganisms consumed their substrates at the expense of nitrate. We are unable to account for some of the initial differences in nitrate level and the loss shown on days 5 and 10 in control samples 7 and 8 (e.g. without cells).

## Discussion

### PAH degradation under aerobic conditions

None of the 45 strains tested were able to grow in mineral medium containing naphthalene as the sole carbon source under aerobic conditions. Thirty six of the strains were *Stenotrophomonas* or *Pseudomonas*, both genera are known to contain many strains with the ability to degrade different hydrocarbons (Gibson et al., 1974; Jung and Kafkewitz, 1996; Jeong et al., 2006; Lee et al., 2002; Shim and Yang, 1999). For example, complete mineralization of aromatic compounds

under aerobic conditions was performed by *Stenotrophomonas* HPC383 (Verma et al., 2011). Different microorganisms may use different pathways for hydrocarbon degradation (Fuchs et al., 2011). PAH degradation under aerobic conditions by gram-positive bacteria has not been studied as extensively as that in gram-negative bacteria under the same conditions. Recent studies documented genetic and biochemical analysis of PAH aerobic degradation by *Rhodococcus*, *Mycobacterium*, *Terrabacter*, and *Nocardioides* (Boyd et al., 1997; Brezna et al., 2003; Khan et al., 2001; and , Nojiri et al., 2001). Although one of our 75 strains is *Rhodococcus*, we did not test its ability to utilize naphthalene under aerobic or NR conditions.

### Detecting naphthalene aerobic degradation genes

The *nahAc* gene coding for a subunit of naphthalene dioxygenase was not detected in any of the three tested strains using the primer pair nahAcl/nahAc3. Although it may be that the primers did not amplify the *nahAc* gene in our strains if they had a variant *nahAc* sequence, it is more likely that these genes were absent, given the lack of growth on naphthalene vapors under aerobic conditions. Polyaromatic hydrocarbon degradation pathways are well studied in *Pseudomonas* (Bosch et al., 1999; Simon et al., 1993). The best studied PAH dioxygenase is naphthalene dioxygenase from *Pseudomonas putida* NCIB 9816-4 (Kauppi et al., 1998; Lee et al., 1997; Parales et al., 2000), encoded by the NAH plasmid pDTG1 (Dennis et al., 2004). The *nah* genes have been found in a broad variety of bacteria such as *Pseudomonas*, *Burkholderia*, and *Ralstonia* and have been found in strains originating from many different geographic locations (Ahn et al., 1999; Ferrero et al., 2002; Lloyd-Jones et al., 1999, Tuomi et al., 2004; Widada et al., 2002).

Indeed, as more PAH-degrading bacteria have been isolated and characterized, it has become obvious that *Pseudomonas* and the *nah*-like genes represent only a fraction of the PAH degradation picture. However, surveys of PAH degradation potential frequently rely on *nah*-based primers or probes to assess biodegradation

potential in the environment (Ahn et al., 1999; Stapleton et al., 1998; Wilson et al., 1999).

The *phn* genes, possessing activity similar to that of the *nah* genes, are more distantly related PAH degradation genes and have also been studied. *Burkholderia* sp. strain RP007 that contains the *phn* genes was isolated from a PAH-contaminated site based on its ability to degrade phenanthrene (Laurie and Lloyd-Jones., 1999). It was shown that *Pseudomonas*-type *nah* genes are not always dominant in the environment and that the *phn*-type genes can have a greater ecological significance than the *nah*-like genotype (Laurie and Lloyd-Jones, 2000). Although we did not test the presence of *phn* genes in the three strains, again the lack of growth on naphthalene under aerobic conditions is a more comprehensive test than detection using specific primers.

### Degradation of polyaromatic hydrocarbons under nitrate-reducing conditions

We acknowledge that our results do not represent an extensive study of the anaerobic degradation of polyaromatic hydrocarbons (see Fuchs et al., 2011 for a review). Nevertheless, none of the tested strains (e.g. *Stenotrophomonas* strain I-21 and *Serratia* strain I-24) degraded the tested hydrocarbons under NR conditions. However, nitrate depletion and nitrite accumulation were shown in *Stenotrophomonas* strain I-21 and *Serratia* strain I-24 under NR conditions. Strains of *Stenotrophomonas maltophilia* (Kaparullina et al., 2009) and *Serratia marcescens* (Borsodi et al., 2010) were previously shown to have the ability to reduce nitrate. In addition, the ability of *Ensifer* strain I-4 to reduce nitrite suggests its ability to denitrify. In fact, it was shown that two *Ensifer adhaerens* strains were able to utilize different substrates such as benzoate, 3-hydroxybenzoate, and 2-fluorobenzoate under aerobic and DN conditions (Song et al., 2000). The ability of *Ensifer* strain I-4 to degrade hydrocarbons under NR conditions was not studied.

To the best of our knowledge, strains of *S. marcescens* have never been shown to degrade hydrocarbons which is consistent with our

**Table 4. Naphthalene and nitrate levels over time in cultures of *Stenotrophomonas* strain I-21 grown without yeast extract.**

Day	Day 0		Day 5		Day10	
	Naph (mg/mL)	Nitrate (mM)	Naph (mg/mL)	Nitrate (mM)	Naph (mg/mL)	Nitrate (mM)
1 <sup>a</sup>	0.62	18.6	0.6	8.7	0.64	7.7
2 <sup>a</sup>	0.58	19	0.61	8.8	0.59	7.7
3 <sup>b</sup>	0.66	0	0.57	0	0.56	0
4 <sup>c</sup>	ND <sup>g</sup>	14.6	ND	9	ND	7.8
5 <sup>d</sup>	0.65	11.3	0.47	11.2	0.6	10.5
6 <sup>e</sup>	ND	2.9	ND	3.4	ND	0.49
7 <sup>f</sup>	0.58	22.3	0.4	9.8	0.59	9
8 <sup>f</sup>	0.63	19.3	0.37	9.9	0.61	9.1

<sup>a</sup> Cell suspension + naphthalene + KNO<sub>3</sub>.

<sup>b</sup> No nitrate: cell suspension + naphthalene.

<sup>c</sup> No naphthalene: cell suspension + KNO<sub>3</sub>.

<sup>d</sup> Sterile cell suspension + naphthalene + KNO<sub>3</sub>.

<sup>e</sup> Cell suspension + glucose + KNO<sub>3</sub>.

<sup>f</sup> Wash medium + naphthalene + KNO<sub>3</sub>.

<sup>g</sup> ND: Not determined because sample did not contain naphthalene.

findings. However, the ability of *S. marcescens* to degrade different contaminants such as the organophosphate insecticide, diazinon (O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate) was shown before (Abo-Amer, 2011). On the other hand, *S. maltophilia* strains have been isolated from oil-polluted soils (Duarte et al., 2001) which may indicate the ability to degrade hydrocarbons. However, our final Gas Chromatography results showed that our *Stenotrophomonas* strain I-21 was not able to mineralize naphthalene under NR conditions and that the reduction of nitrate in our previous experiments might have been driven by the degradation of extracellular materials observed in the culture bottles. Therefore, one must be cautious before stating a microorganism's ability to mineralize hydrocarbons based on its isolation from hydrocarbon-contaminated sites. Also, our GC results (Table 4) showed that measuring naphthalene loss is a necessary complement to measuring nitrate loss since our results showed around 50% loss of nitrate in the control samples 7 and 8 (e.g. no cell suspension), while documenting

no loss of naphthalene occurred (Table 4).

### Loss of plasmid-borne naphthalene degradation genes

It is possible that at least some of the strains were capable of naphthalene degradation but lost the ability during the process of isolation. Pathways for aerobic degradation are often borne on conjugative plasmids (Basta et al., 2004). In a previous study, a plasmid-borne naphthalene degradation phenotype was transferred from *P. putida* CSV86 to *S. maltophilia* CSV89 by conjugation. However, the transferred property was lost completely when transconjugants were grown on glucose or on a medium that contained tryptone, yeast extract, and NaCl. This loss indicates that the degradation property was not stable and it was lost when more favorable substrate was available (Basu and Phale, 2008). In other cases, a transferred plasmid (e.g. NAH7) was shown to be rapidly selected to become more stable (Duncan et al., 1995). We did not make any attempt to screen for plasmids in our strains, or test for growth on naphthalene immediately upon isolation of the strains.

## Response of soil microbial communities to contamination by crude oil

The structure of microbial communities in soil changes due to changes in environmental conditions such as oil contamination that can cause drastic effects on microbial diversity in soil. The supposed shifts in community structure caused by selective pressure (e.g. oil) are important for the catabolism of soil pollutants (Powell et al., 2006). In fact, the degradation of PAH from geographically diverse soils suggests that this degradation is associated with distinct genera, notably *Sphingomonas* (formerly *Pseudomonas*) and *Burkholderia*, independent of geographic location (Mueller et al., 1997). A different study showed a shift in microbial community structure from alpha- and betaproteobacteria to gammaproteobacteria when a microbial community was exposed to a mixture of aromatic hydrocarbons (Stoffels et al., 1998). Other factors that alter the catabolism of soil pollutants and possibly select for NR/DN bacteria are the application of fertilizer containing nitrogenous compounds. In fact, fertilization increased hydrocarbon degradation in both anaerobic and aerobic soils, especially anaerobic soils. This increase of hydrocarbon degradation in the anaerobic soils corresponded with a shift in the denitrifier community composition and an increased abundance of denitrifiers and benzoyl-CoA reductase (Powell et al., 2006). However, in our study we found no evidence that PAH degradation was performed by culturable NR/DN bacteria from the Tallgrass Prairie sites.

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Received August 14, 2015 Accepted October 29, 2015