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RESEARCH NOTE

RELATIONSHIP BETWEEN THE CONCENTRATION OF DENITRIFIERS AND *PSEUDOMONAS* spp. IN SOILS: IMPLICATIONS FOR BTX BIOREMEDIATION

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Abstract—Aquifer microcosms were used to investigate the effect of stimulating denitrification on microbial population shifts and BTX degradation potential. Selective pressure for facultative denitrifiers was applied to a treatment set by feeding acetate and nitrate, and cycling electron acceptor conditions twice between aerobic and denitrifying stages. A second (control) set degraded the same amount of acetate under aerobic conditions. The resulting concentrations of total heterotrophs were not significantly different between the two sets. Nevertheless, the concentrations of denitrifiers, *Pseudomonas* spp., and BTX degraders were significantly higher in the cycled microcosms than in the aerobic controls. The predominant isolates from the cycled microcosms were fluorescent *Pseudomonas* species that are known to degrade BTX. Following the complete removal of acetate, cycled microcosms also showed higher aerobic BTX degradation activity. These results suggest that nitrate addition to oxygen-limited aquifers might enhance BTX bioremediation not only by supplementing the electron acceptor pool as is widely accepted, but also by fostering favorable changes in the composition of the microbial consortium. Specifically, denitrifying conditions could have the ancillary benefit of fortuitously selecting for *Pseudomonas* spp. that can degrade BTX. This syllogism is supported by a survey of international soils (from France, Denmark, Brazil and Iowa, USA), which showed a correlation between the concentration of denitrifers and *Pseudomonas* spp.
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Key words—BTX, aquifer restoration, denitrification, microbial ecology

INTRODUCTION

In situ bioremediation, the enhancement of microbial activity to degrade environmental pollutants within aquifers, shows great promise as an approach to hazardous waste management (Lee *et al.*, 1988). Successful bioremediation requires overcoming site-specific limitations to natural degradative processes, such as the common insufficient supply of appropriate electron acceptors. In this regard, nitrate addition has been proven cost-effective for supplementing the electron acceptor pool and enhancing the degradation of benzene, toluene and xylenes (BTX) in oxygen-limited aquifers (Hutchins *et al.*, 1991; Lemon *et al.*, 1989; Sheehan *et al.*, 1988; Werner, 1985). Nevertheless, the effect of nitrate addition on microbial ecology and aerobic BTX degradation potential are not fully understood. A better understanding of how such anthropogenic changes in aquifer chemistry affect microbial population shifts is desirable for the rational development of bioremediation.

In anoxic aquifers, the presence of nitrate should provide a competitive advantage to facultative denitrifiers because nitrate is thermodynamically a more favorable electron acceptor than other chemical species associated with anaerobic respiration (e.g., SO_4^{2-} and CO_2). Although denitrifiers typically comprise only 0.1–5% of the total culturable population in soils, they are ubiquitous (Tiedje, 1988), and an increase in their concentration is probably beneficial for *in situ* BTX bioremediation. This rationale is deduced from two facts: (1) the most commonly isolated denitrifying bacteria from soils and aquifers are pseudomonads (Gamble *et al.*, 1977; Knowles, 1999; Sugahara *et al.*, 1986; Terai, 1979; Tiedje, 1988); and (2) pseudomonads coincidentally tend to have a broad catabolic specificity and are generally capable of degrading BTX (Gibson *et al.*, 1990; Mikesell *et al.*, 1993; Palleroni, 1984; Stanier *et al.*, 1966; Williams and Sayers, 1994). Indeed, recent surveys of microbial communities from BTX contaminated aquifers found that the prevalent

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species were *Pseudomonas* (Palleroni *et al.*, 1973; Ridgeway *et al.*, 1990).

The genus *Pseudomonas* has recently been subjected to taxonomic revision, and is now restricted to the species of Group I as defined by ribosomal RNA–DNA re-association experiments of Palleroni *et al.* (1973). Nevertheless, most of its members still can denitrify and are among the most catabolically versatile of gram-negative bacteria (Palleroni, 1995). This paper addresses the syllogistic hypothesis that denitrifying conditions could provide a competitive advantage for *Pseudomonas* spp. that can degrade BTX and thus, could fortuitously select for BTX degraders.

MATERIALS AND METHODS

Experimental approach

Two sets of batch microcosms were used to study the effect of stimulating denitrification on the phenotypic composition and BTX degradation activity of an indigenous microbial consortium. One (treatment) set was subjected to cyclic aerobic and denitrifying conditions to exert selective pressure for facultative denitrifiers. The other set was kept aerobic and served as a control. Both sets were prepared in triplicate with 200 ml of basal mineral medium and 20 g of sand in 250-ml serum bottles. The basal medium was prepared to provide inorganic nutrients for microbial growth (e.g., NH_4^+, trace metals) and to buffer aqueous solutions at pH 7, as described elsewhere (Alvarez and Vogel, 1991). The alluvial sand ($f_{oc} = 0.001$) used as seed was obtained from a quarry site near Iowa City, IA, and had no known previous BTX exposure.

All microcosms were fed acetate at an initial concentration of approximately 100 mg/l. Acetate was chosen because it cannot be fermented, and it has been proven as an effective electron donor for denitrification (Blaszczyk *et al.*, 1980; McCarty *et al.*, 1969). The treatment set microcosms were cycled twice between aerobic and denitrifying stages. During the first enrichment stage, denitrifying conditions were stimulated by adding nitrate in stoichiometric amounts to oxidize the acetate and then purging the microcosms with N_2 gas for 30 min to remove molecular oxygen. An aerobic enrichment stage was started following the complete removal of acetate. Microcosms were sparged continuously with compressed air during this aerobic enrichment stage. This cycle, which lasted about 4 days, was then repeated (Fig. 1). The control microcosms were also fed nitrate (100 mg/l), but were kept aerobic during all four phases by continuous sparging with compressed air. At the beginning of each enrichment stage, all microcosms had degraded acetate below detection limits (1 mg/l) and were again fed acetate at about 100 mg/l. Thus, both microcosm sets received and degraded the same amount of acetate throughout the experiment. Following this enrichment, the concentration of total (culturable) heterotrophs, denitrifiers, *Pseudomonas* spp., and BTX degraders were measured in both sets. Whether changes were significant was evaluated using Student's *t*-test at the 95% confidence level (Kleinbaum *et al.*, 1988). In order to investigate whether stimulation of denitrification had an effect on potential BTX degradation activity, both microcosm sets were subsequently aerated and spiked with benzene, toluene and *o*-xylene (5–7 mg/l each), and BTX degradation patterns were compared. Prior to BTX addition, the nitrate concentration was adjusted to 100 mg/l to equalize the carry over of nitrate in both sets. This was necessary because nitrate had been removed in the cycled set, but not in the aerobic controls.

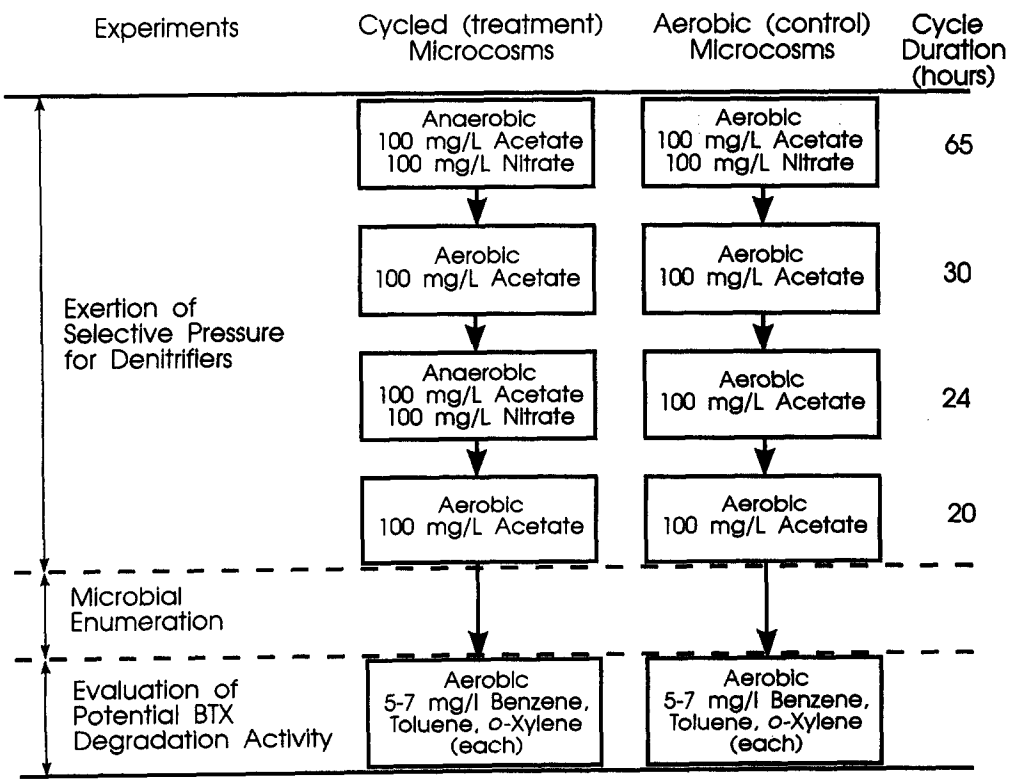

Fig. 1. Treatment of aquifer microcosms.

Table 1. Soils used to investigate the correlation between the concentration of denitrifiers and *Pseudomonas* spp.

Sample ID	Soil description
Brazil # 1	Silt Loam: Light brown–rust brown, sandy silt, trace clay, micaceous; collected near Florianópolis from area that received effluent from septic tanks at a depth of 3–4 ft. Soil organic carbon was 0.7% and soil nitrate ws 2 mg-N/g of soil.
Brazil # 2	Sandy Loam: Brown, sandy silt, trace gravel, trace organics, slightly mottled, musty odor; collected near Florianópolis from forested area at a depth of 3–4 ft. Soil organic carbon was 1.3% and soil nitrate was 5 mg-N/g of soil.
Denmark # 1	Loam: Dark brown to gray brown, sandy silt, trace clay, some organics; collected near Lingby from corn field at a depth of 0.5 ft. Soil organic carbon was 3.9% and soil nitrate was 36 mg-N/g of soil.
Denmark # 2	Loam: Dark brown to gray brown, sandy silt, trace clay, some organics; collected near Lingby from forested area at a depth of 0.5 ft. Soil organic carbon was 3.0% and soil nitrate was 16 mg-N/g of soil.
France # 1	Loess Silt: Light brown, silt, trace clay, trace organics, micaceous; collected near Nancy from agricultural field at a depth of 1–2 ft. Soil organic carbon was 4.5% and soil nitrate was 23 mg-N/g of soil.
France # 2	Loess Silt: Light brown, silt, trace clay, trace organics, micaceous; collected near Nancy from forested area at a depth of 1–2 ft. Soil organic carbon was 5.8% and soil nitrate was 16 mg-N/g of soil.
Iowa # 1	Silt Clay Loam: Rust brown and brown gray, mottled, clayey silt, some sand, trace organics; collected near Amana from forested area at a depth of 1–2 ft. Soil organic carbon was 1.1% and soil nitrate was 4.7 mg-N/g of soil.
Iowa # 2	Silt Loam: Black to dark gray, clayey silt, trace sand, some organics; collected near Amana from root zone of a poplar tree at a depth of 3–4 ft. Soil organic carbon was 2.2% and soil nitrate was 3.7 mg-N/g of soil.

A separate study was conducted to investigate whether the concentration of denitrifiers and *Pseudomonas* spp. in soils are correlated. This complementary study is related to the proposed hypothesis as it directly addresses the first premise of the syllogism. While numerous studies have shown that *Pseudomonas* spp. are the most commonly isolated denitrifying bacteria from soils and aquifers, a direct correlation between the concentration of *Pseudomonas* spp. and denitrifiers has not been established. That these concentrations are correlated in nature is not known *a priori* because not all denitrifiers are *Pseudomonas* and several *Pseudomonas* spp. cannot denitrify (Palleroni, 1984; Zumft, 1992). A survey of international soils was conducted to investigate this correlation. Two different soil samples were collected from uncontaminated sites at each of the following locations: Florianópolis, Brazil; Lingby, Denmark; Nancy, France; and Amana, Iowa, USA (Table 1). Samples were collected with sterilized tools and stored at 4°C until use to minimize changes in the microbial consortium prior to microbial enumerations.

Microbial enumeration procedures

Microcosms were emptied into a Warring blender and microorganisms were detached from soil particles by mixing vigorously for 1 min as described by Webster *et al.* (1985). The slurry was allowed to settle and serial dilutions of the supernatant were prepared for microbial enumerations.

Total (culturable) heterotrophs and *Pseudomonas* spp. were enumerated using viable plate counts of appropriate dilutions of the microcosm extract. Bacto® tryptic soy agar (15 g/l) (Difco Laboratories, Detroit, MI) was used to grow and count total heterotrophic colonies. For *Pseudomonas* spp., the growth medium was selective, and consisted of Bacto® Pseudomonas Isolation agar (45 g/l) and glycerol (20 ml/l).

Denitrifier and BTX degrader concentrations were determined using a most probable number (MPN) method. All incubations were prepared in 10 ml Hewlett Packard headspace auto-sampler vials containing 4 ml of culture medium plus 1 ml of inoculum from appropriate dilutions of the microcosm extract. Ten-fold dilutions were prepared with ten replicates per dilution. For the enumeration of denitrifiers, the culture medium consisted of 8 g/l of Bacto® Tryptic Soy Broth and 0.5 g/l of KNO_3 in basal mineral medium; the 5-ml headspace containing 1 ml of acetylene (C_2H_2). The reduction of greater than 20% of the added nitrate to N_2O in the presence of acetylene was taken as a positive indication of the presence of denitrifiers (Focht and Joseph, 1973). For the enumeration of BTX degraders, the culture medium contained approximately 5 mg/l (each) of benzene, toluene and *o*-xylene in basal mineral medium; the 5-ml headspace contained air. All vials were sealed with a Teflon®-lined rubber septum (West Company, Lionville, PA), crimped with an aluminium cap, and incubated in the dark for two weeks at 25°C. The removal of any of the three added BTX compounds from viable (aerobic) incubations but not from sterile (autoclaved) controls was taken as a positive indication of the presence of BTX degraders.

Predominant microbial species were isolated from the cycled microcosms and identified using a Biolog Micro Station™ bacterial identification system.

Chemical analyses

Nitrate was analyzed in a Dionex 4500i ion chromatograph using an AS4A ion exchange column for separation followed by chemical suppression and conductivity detection. The concentration of N_2O was measured by headspace injection into a Hewlett Packard 5890 Series II gas chromatograph (GC) fitted with a molecular sieve and thermal conductivity detector. BTX were analyzed with a Hewlett Packard 5890 Series II GC equipped with a Hewlett Packard 19395A headspace autosampler and flame-ionization and photoionization detectors in series. Separation was achieved using a J&W Scientific DB-WAX column. Acetate was analyzed with a Hewlett Packard 5890 GC equipped with a flame-ionization detector. Separation was achieved with a Supelco 1%SP-1000 60/80 Carbopack B column. A YSI 5300 biological oxygen monitor, equipped with an Instech microchamber and an oxygen micro probe (Instech Instruments, Plymouth Meeting, PA) was used to verify that sufficient dissolved oxygen was present in aerobic incubations and that anoxic conditions prevailed during denitrifying stages.

RESULTS

Microbial population shifts

The initial (culturable) heterotrophic concentration was about 10^7 colony forming units per gram of soil (cfu/g-soil). Following degradation of about 400 mg/l of acetate, the concentration of total heterotrophs were not significantly different between the cycled (treatment) and aerobic (control) microcosms. Total heterotrophs grew to about 10^{11} cfu/g-soil in all microcosms. Nevertheless, the concentrations of denitrifiers, *Pseudomonas* spp., and BTX degraders were significantly higher ($p < 0.02$) in the cycled microcosms than in the aerobic controls (Table 2).

Table 2. Initial and final microbial concentrations in aquifer microcosms (average ± one standard deviation from triplicate microcosms)

Treatment	Denitrifiers (MPN/g-soil)	*Pseudomonas* spp. (cfu/g-soil)*	BTX Degraders (MPN/g-soil)
Initial concentrations	$1.3 \pm 0.9 \times 10^5$	$8.8 \pm 2.1 \times 10^2$	$7.9 \pm 6.1 \times 10^2$
Aerobic (control) set	$7.8 \pm 8.1 \times 10^7$	$1.7 \pm 1.0 \times 10^7$	$3.3 \pm 1.6 \times 10^6$
Cycled (treatment) set	$1.3 \pm 0.8 \times 10^{10}$	$3.5 \pm 0.5 \times 10^{10}$	$9.8 \pm 3.0 \times 10^6$

*Viable plate counts are given as colony forming units (cfu).

BTX degradation activity

Once acetate had been removed below detection limits (1 mg/l), both microcosm sets were aerated and spiked with benzene, toluene and *o*-xylene. BTX removal in viable but not in sterile (autoclaved) microcosms provided evidence of biodegradation. All BTX compounds were degraded (aerobically) faster in the cycled set than in the aerobic control microcosms (Fig. 2). The time required for BTX concentrations to drop below detection levels was also shorter in the cycled set.

Survey of international soils

A microbial survey of international soils was conducted to investigate whether the concentrations of denitrifiers and *Pseudomonas* spp. in soils were correlated. A plot of *Pseudomonas* spp. versus denitrifiers concentrations was developed to linearize the data. The regression yielded a correlation coefficient (r^2) of 0.64 (Fig. 3).

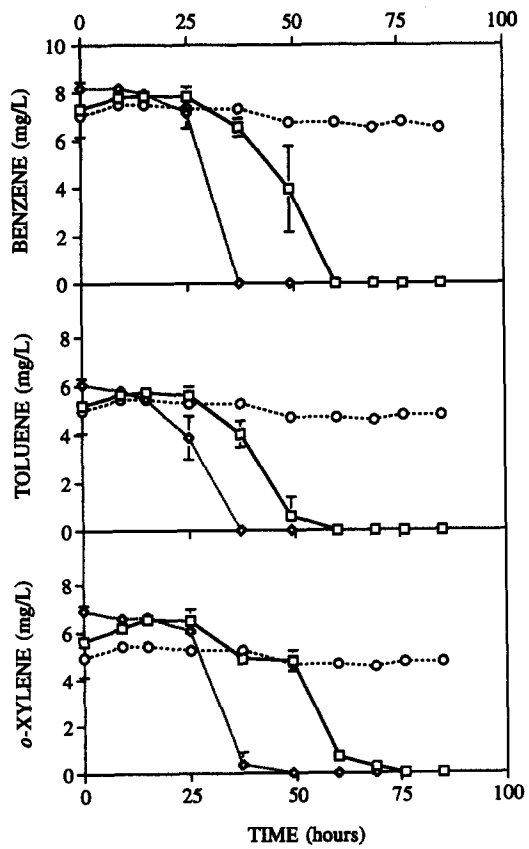

Fig. 2. Benzene, toluene and *o*-xylene degradation in batch aquifer microcosms. Graph depicts time course BTX concentrations in the cycled set (◇); aerobic set (□); and sterile controls (○). Error bars represent ± 1 SD from the average of triplicate incubations. Error bars smaller than symbols are not depicted.

DISCUSSION

The stimulation of denitrifying conditions in aquifer microcosms significantly increased the concentration of denitrifiers and *Pseudomonas* spp. relative to the aerobic controls (Table 2). The concurrent increase in both concentrations is consistent with previous studies that found the majority of denitrifying isolates from soils and aquifers to be *Pseudomonas* spp. (Gamble *et al.*, 1977; Knowles, 1982; Sugahara *et al.*, 1986; Terai, 1979; Tiedje, 1988). The large increase in the concentration of *Pseudomonas* in the cycled (treatment) set may have been due to a combination of the competitive advantage that several *Pseudomonas* spp. have under denitrifying conditions and the use of acetate as electron donor. Harder and Dijkhuizen (1982) found that organic acids, such as acetate, are generally better substrates for *Pseudomonas* than sugars and allow for higher growth rates. Nevertheless, the aerobic (control) set degraded the same amount of acetate and had a similar concentration of total heterotrophs but did not exhibit such a large increase in *Pseudomonas* spp. concentration. This indicates that denitrifying conditions were a major factor in the selection for *Pseudomonas* spp.

The most abundant isolates from cycled microcosms were fluorescent *Pseudomonas*, and were identified as *P. aeruginosa*, *P. putida* and *P. fluorescens*. Many strains of each of these species can respire with nitrate and can degrade BTX (Gibson *et al.*, 1990; Mikesell *et al.*, 1993; Palleroni, 1984). Thus, it is not surprising that the concentration of BTX degraders was higher in cycled microcosms than in the aerobic controls (Table 2). Although the relative increase in BTX degrader concentration was statistically significant ($p < 0.02$), it was not commensurate with the large increase measured in *Pseudomonas* and denitrifier concentrations. The lack of preferential proliferation of BTX degraders is likely due to repression of BTX degradation activity by acetate (Duetz *et al.*, 1994; Worsey and Williams, 1975). Nevertheless, once acetate was removed, a higher BTX degradation activity was observed in the cycled set than in the aerobic control microcosms

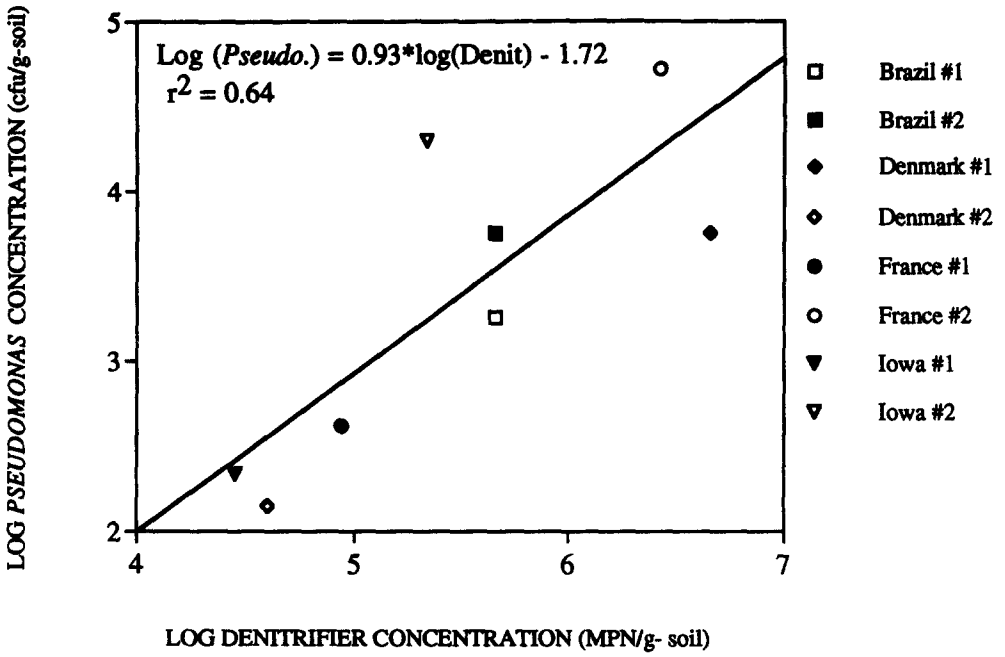

Fig. 3. Correlation of denitrifier and *Pseudomonas* spp. concentrations in various soils.

(Fig. 2). Apparently, this increase in activity (e.g., shorter lag times) was greater than would be expected based on the enumeration of BTX-degraders alone.

Recent studies suggest that co-respiration of nitrate and oxygen is not uncommon in environments which are rich in reduced carbon or are subjected to fluctuating oxygen availability (Carter *et al.*, 1995). Nevertheless, nitrate was not removed in aerobic (control) microcosms or in cycled microcosms during aerobic stages. This indicates that aerobic denitrification was not a major factor in this experiment. Ammonium chloride (100 mg/l) had been added to all microcosms to deter the use of nitrate as a nitrogen source for anabolism.

The onset of biodegradation was faster in the cycled set than in the aerobic control microcosms for all three BTX compounds (Fig. 2). The shorter lag times are consistent with the higher initial concentration of BTX degraders (Alvarez *et al.*, 1994). Faster BTX degradation kinetics may also be due to a larger fraction of BTX degraders belonging to the genus *Pseudomonas*. Bacteria belonging to this genus are gram-negative and are also *r*-strategists (Atlas and Bartha, 1993). As *r*-strategists, through their rapid growth rate, *Pseudomonas* can take over and dominate environments where resources are abundant, such as microcosms spiked with BTX. As gram-negative, *Pseudomonas* may have a physiological advantage for protection against potential BTX toxicity because the lipopolysaccharide of the outer membrane reduces the BTX concentration in or near the cytoplasmic membrane and cytoplasm (Nickens and Hegeman, 1989). Regardless of the reasons why *Pseudomonas* might predominate in BTX contaminated sites, their presence seems to be beneficial for BTX bioremediation. While it cannot be inferred that the predominant *Pseudomonas* isolates from the treated (cycled) microcosms are the same bacteria that would predominate in a BTX-contaminated aquifer, or that a BTX-acclimated culture would behave similarly, the microcosm experiments indirectly support our hypothesis. That is, nitrate addition to oxygen-limited aquifers could enhance BTX bioremediation not only by supplementing the electron acceptor pool as shown elsewhere (Alvarez *et al.*, 1994; Alvarez and Vogel, 1995; Hutchins *et al.*, 1991; Lemon *et al.*, 1989; Sheehan *et al.*, 1988; Werner, 1985), but also by fostering favorable microbial population shifts. Specifically, nitrate addition to oxygen-limited, hydrocarbon-contaminated aquifers could have the ancillary benefit of fortuitously selecting for *Pseudomonas* spp. that can degrade BTX.

The first premise of this syllogism is supported by the survey of international soils. A good correlation ($r^2 = 0.64$) was found between the log of denitrifier and *Pseudomonas* concentrations (Fig. 3). The regression line slope was greater than zero at the 99% confidence level, indicating that the increase in *Pseudomonas* spp. concentration with denitrifier concentration is statistically significant. In addition, based on the 99% confidence interval, the slope was not statistically different than one, indicating that these concentrations may be correlated by a direct linear relationship.

In conclusion, converging lines of evidence suggest that an increase in the concentration of facultative denitrifiers could result in a coincidental increase in the concentration of microorganisms that are

desirable for BTX bioremediation. Such a population shift could potentially enhance BTX degradation rates and decrease the duration (and cost) of bioremediation. This work also suggests that investigating the effectiveness of cycling nitrate and oxygen addition to oxygen-limited BTX plumes could be a fruitful avenue of field research. This approach, which could be applicable to sites where continuous oxygen delivery is technically or economically unfeasible, could result in an ancillary increase in potential BTX degradation activity.

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