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DEGRADATION OF BTEX AND THEIR AEROBIC METABOLITES BY INDIGENOUS MICROORGANISMS UNDER NITRATE REDUCING CONDITIONS

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ABSTRACT

Batch incubations, seeded with four different aquifer materials, were used to survey the catabolic capacity of indigenous microorganisms under nitrate reducing conditions. Benzene, toluene, ethylbenzene, xylenes (BTEX), and selected potential metabolites of their incomplete aerobic degradation, were tested as substrates for nitrate-based respiration. Toluene and its potential aerobic metabolites, benzoate, protocatechuate, 3-methylcatechol, 4-methylcatechol, succinate, and adipate were degraded in strictly anoxic ($O_2 < 0.1$ mg/l) nitrate reducing incubations. Toluene degradation was directly coupled to nitrate reduction. *Ortho*-xylene removal was toluene dependent. *Meta-* and *para*-xylenes were degraded in nitrate reducing enrichments from only one of the four aquifer samples. Benzene, ethylbenzene, catechol and gentisate were not degraded within up to four months in any of the incubations, even though nitrate reduction occurred. Anaerobic benzene degradation was not observed. Incubations receiving nitrate as an adjunct electron acceptor to oxygen degraded significantly more benzene than incubations amended with only oxygen, although benzene was only degraded until the dissolved oxygen was depleted. Possibly, more oxygen was available to degrade benzene when nitrate was added because denitrifiers utilizing nitrate as terminal electron acceptor oxidized benzoate, which had been added to increase the biochemical oxygen demand of the system. Benzoate oxidation with nitrate apparently spared oxygen for benzene degradation.

KEYWORDS

Aquifer; benzene; biodegradation; BTEX metabolites ; ethylbenzene; nitrate-based bioremediation; toluene; xylene.

INTRODUCTION

Addition of nitrate to increase the electron acceptor pool for bioremediation of hydrocarbon-contaminated aquifers might serve as a cost-effective groundwater cleanup method. Field evidence of the beneficial addition of nitrate for enhancing *in situ* biodegradation of benzene, toluene, ethylbenzene and *ortho-*, *meta-* and *para*-xylenes (BTEX) has been gathered in Canada (Lemon *et al.*, 1989), Germany (Werner, 1985), and USA (Hutchins *et al.*, 1991a; Sheehan *et al.*, 1988). Nevertheless, the success of nitrate in enhancing *in situ* BTEX degradation could vary considerably among different bioremediation projects, depending on the degree to which site-specific ecological, hydrogeological, or physicochemical conditions permit or inhibit

different mechanisms that influence the overall cleanup process. The efficacy of nitrate as a supplemental electron acceptor depends, in part, on the occurrence of indigenous microorganisms capable of degrading the target BTEX under denitrifying conditions. In addition, aromatic and aliphatic metabolites of incomplete BTEX degradation have been shown to accumulate in oxygen-limited aquifers (Cozzarelli *et al.*, 1990). Since hydrocarbon metabolites are potentially toxic (Belkin *et al.*, 1993; Burback *et al.*, 1993; Shelton *et al.*, 1993), the ability of indigenous microorganisms to further degrade aerobic BTEX metabolites under denitrifying conditions is also important to the ultimate success of nitrate-based bioremediation.

Several potential metabolites of aerobic BTEX degradation have been shown to degrade under denitrifying conditions, as indicated by the speckled circles in Figure 1. Most of these degradation studies, however, have used denitrifiers from biological reactors (e.g., Hu and Shieh, 1986; Jensen *et al.*, 1989; Jørgensen *et al.*, 1990; Tschech and Fuchs, 1987), top soil (e.g., Bakker, 1977; Evans *et al.*, 1991b; Oshima, 1965; Taylor *et al.*, 1970) or surface water bodies (e.g., Bossert and Young, 1986; Häggblom *et al.*, 1990). Several potential toluene metabolites, such as gentisate (Pathway F, Figure 1) and protocatechuate (Pathway C, Figure 1), have not been reported as substrates for indigenous aquifer denitrifiers. Other potential toluene metabolites, such as 3-methylcatechol (Pathways B, D and E, Figure 1) and 4-methylcatechol (Pathway B, Figure 1), which are also potential metabolites of *m*-xylene (Davey and Gibson, 1974) and *p*-xylene (Gibson *et al.*, 1974), respectively, have not been reported as substrates for any denitrifying consortia. Therefore, the capacity of indigenous aquifer denitrifiers to degrade all potential BTEX metabolites is relatively unknown.

Benzene is the most toxic of the BTEX compounds, and its removal under nitrate reducing conditions is critical to the success of nitrate-based bioremediation. Unlike alkylbenzenes, however, benzene degradation under denitrifying conditions is not commonly reported. Many studies have found benzene to be recalcitrant under strictly anoxic denitrifying conditions (e.g., Ball *et al.*, 1991; Evans *et al.*, 1991a; Flyvberg *et al.*, 1991; Hutchins *et al.*, 1991b; Kuhn *et al.*, 1988). Yet, benzene was reported to degrade in denitrifying mixed cultures isolated from wastewater treatment plants (Jensen *et al.*, 1989), aquifer microcosms (Major *et al.*, 1988), and column reactors packed with aquifer material (Anid *et al.*, 1993; Reinhard *et al.*, 1991). In some of these latter studies, the possibility of oxygen intrusion cannot be discounted on the basis of available information concerning the experimental design, although it is unlikely that enough oxygen entered to completely mineralize all the benzene. In such cases, molecular oxygen might have been used by the microbial consortia as a co-substrate during initial benzene biotransformations, which might have produced suitable substrates for nitrate-based respiration. This notion suggests that nitrate addition to oxygen limited aquifers could enhance benzene mineralization.

This study assesses the likelihood of encountering aquifer microorganisms capable of degrading BTEX and their potential aerobic metabolites under strictly anoxic, nitrate-reducing conditions. Potential benefits of nitrate-based respiration in benzene bioremediation are also addressed.

METHODOLOGY

Experimental Approach. Anoxic enrichments, seeded with samples from 4 different aquifers, were used to survey the catabolic capacity of indigenous microorganisms under nitrate reducing conditions. Three aquifer samples (designated A, B, and C) were collected beneath BTEX-contaminated gas plants in Michigan, U.S.A. A fourth sample (D) was collected from the vadose zone of a pristine, sandy aquifer. A sterilized split-spoon sampler and plastic coring tubes were used for sample collection. A hydrometer texture analysis indicated that all 4 aquifer materials were composed primarily of sand (>95%), with clay (~3%) and silt (~2%) making up the difference. The organic carbon content was less than 1% in all cases. The aquifer C sample was obtained downgradient of a regularly fertilized corn field, where groundwater nitrate concentrations of the order of 4 mg/l NO_3^- were measured. Nitrate was not detected in aquifers A, B, or D. Aquifer slurries were prepared by adding 100 grams of aquifer material to 500 ml of autoclaved basal mineral medium (buffered at pH 7). The medium, which has been described elsewhere (Alvarez and Vogel, 1991), contained ammonium chloride (100 mg/l) as nitrogen source to deter the use of nitrate for anabolic requirements. The slurries were deoxygenated (dissolved oxygen ca. 0.5 mg/l) by purging with oxygen-free nitrogen gas for 30 min, and were incubated at 23 ± 2 °C inside a Coy anaerobic chamber ($O_2 < 10^{-6}$ atm).

Nitrate-based respiration was induced by the addition of yeast extract (50 mg/l) and nitrate (100 mg/l as NO_3^-). The degradation of yeast extract reduced the dissolved oxygen (DO) concentration below the detection limit of 0.1 mg/l and increased the number of denitrifiers for more rapid biodegradation assays (Alvarez *et al.*, 1994). These assays were conducted in serum bottles (capped with Mininert valves) which were seeded with 1-ml of liquid from the anoxic slurries. Aquifer material was excluded from the enrichments to minimize potential sorption of hydrophobic substrates. Potential substrates for nitrate-based respiration were added, and their concentrations were monitored over time to compare lag periods and biodegradation patterns in different enrichments. Nitrate and nitrite concentrations were also monitored to assess nitrate reduction.

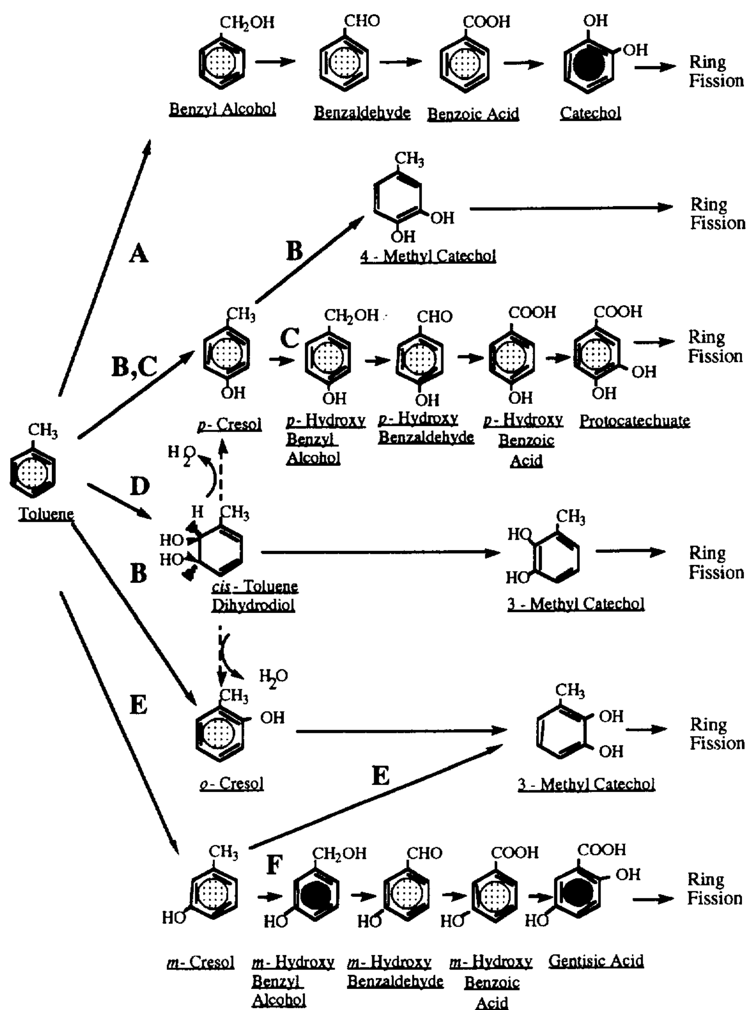

Fig. 1. Potential metabolites from aerobic toluene degradation. Pathways that occur in (A) *P. putida* PaW1 (Kukor and Olsen, 1989) and *P. putida* mt-2 (Williams and Murray, 1974); (B) *P. cepacia* G4 (Shields *et al.*, 1989); (C) *P. mendocina* KR (Whited and Gibson, 1991); (D) *P. putida* F1 (Gibson, *et al.* 1968); and (E) *P. pickettii* PK01 (Kaphammer *et al.*, 1990); and (F) gentisate pathway (Bayly and Barbour, 1984). Compounds with speckled circles have been reported to degrade under denitrifying conditions (although not necessarily by indigenous aquifer microorganisms), whereas those with filled-in circles have been reported to be recalcitrant. Compounds without circles have not been reported as substrates for nitrate-based respiration.

In all cases, biodegradation was statistically assessed using t-tests (at the 95% confidence level) to compare residual substrate concentrations in triplicate incubations to autoclaved (control) replicates. Non-sterile controls were also prepared with deoxygenated, nitrate-free medium.

BTEX Degradation Assays. All six BTEX compounds were added simultaneously to 60 ml anoxic enrichments (at approximately 30 mg of total BTEX per litre) to incorporate potential substrate interactions during their microbial degradation. Nitrate was added at 180 to 250 mg/l (as NO_3^-). Some incubations initially contained nitrite due to nitrate reduction when microbes were grown on yeast extract. Initial BTEX, nitrate and nitrite concentrations are given in Table 1. One additional aquifer slurry was prepared to demonstrate an unambiguous connection between toluene degradation and nitrate reduction. Eight grams of material from aquifer A were added to a 120 ml serum bottle. The bottle was filled with deoxygenated basal mineral medium, sealed with Teflon-lined caps and aluminium crimps, and incubated in the anaerobic chamber. Nitrate-based respiration was induced by the addition of nitrate (200 mg/l as NO_3^-) and yeast extract (100 mg/l) before the first addition of toluene (20 mg/l).

BTEX Metabolite Degradation Assays. Similar to the BTEX degradation assays, potential aromatic metabolites of aerobic BTEX catabolism were added to 250 ml anoxic enrichments that had been seeded with the corresponding supernatant of four different aquifer slurries. Unlike the BTEX degradation assays, nitrate-based respiration had been previously induced by adding benzoate (60 mg/l) along with yeast extract (50 mg/l) and nitrate (100 mg/l as NO_3^-) to the aquifer slurries. Catechol, protocatechuate, gentisate, 3-methylcatechol, and 4-methylcatechol were added concurrently with nitrate at the concentrations listed in Table 2. Potential aliphatic BTEX metabolites, adipate and succinate, were tested separately in aquifer slurries that were not previously fed other substrates. Aquifer samples (100 g) were amended with 500 ml of deoxygenated mineral medium containing nitrate (400 mg/l as NO_3^-) and either adipate (100 mg/l) or succinate (200 mg/l).

Benzene Degradation with Nitrate as Adjunct Electron Acceptor. The extent of benzene degradation in aerobic, oxygen-limited batch incubations was compared with and without nitrate amendment. A nitrate reducing enrichment, seeded with pristine aquifer material (D), was prepared as described previously. The anoxic (i.e., DO < 0.1 mg/l) supernatant of this enrichment was used to prepare triplicate incubations for three electron-acceptor treatment sets: (i) oxygen alone, (ii) nitrate alone, and (iii) oxygen and nitrate. Incubations that were initially aerobic were prepared outside the anaerobic chamber. Approximately 500 ml of the anoxic supernatant were sparged for one hour with air and transferred to (six) 60 ml serum bottles. A 10 ml headspace filled with air was provided for additional oxygen supply. Three of these six aerobic (i.e., 8.9 mg/l DO) incubations were amended with nitrate (130 mg/l as NO_3^-). Six similar anoxic incubations were prepared inside the anaerobic chamber. Three of these incubations were amended with nitrate (130 mg/l as NO_3^-), and the other three were used as controls for electron acceptor amendment. All 12 incubations were capped with Mininert valves and amended with benzene (32 ± 1 mg/l). Benzoate (50 1 mg/l) was also added to increase the biochemical oxygen demand (BOD) of the system. The BOD of the added substrates was higher than the oxygen available in the six aerobic incubations, including 1 ml of O_2 (at 1 atm) that was subsequently added to each aerobic incubation during the experiment.

Analytical Procedures. BTEX were analyzed in a Hewlett Packard 5890 gas chromatograph equipped with an HP 19395A headspace auto-sampler, a 30-m Megabore HP-5 column, and a flame ionization detector. The limit of detection for each BTEX compound was 0.01 mg/l. Catechol, gentisate, 3-methylcatechol, 4-methylcatechol, and protocatechuate were analyzed by gradient-elution liquid chromatography using a Dionex 450 ion chromatograph equipped with a Bondclone 10 C_{18} column and a UV detector (set at 215 nm). The mobile phase was a mixture of methanol (14–27% of the eluant) and phosphoric acid (15 mM). The limit of detection was 0.1 mg/l for each compound. Adipate, benzoate, nitrate, nitrite, and succinate were analyzed isocratically in the Dionex 4500i chromatograph using an AS4A ion exchange column and a conductivity detector. The mobile phase used was an aqueous carbonate solution (1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$). The limit of detection was approximately 1 mg/l for each compound. A biological oxygen monitor (YSI 530), equipped with a microchamber and an oxygen microprobe, was used to ensure that anoxic conditions prevailed in the incubations. The limit of detection for dissolved oxygen was 0.1 mg/l.

RESULTS

BTEX Degradation Assays. Toluene was tested as substrate for nitrate-based respiration in an anoxic aquifer slurry that had been previously fed yeast extract (100 mg/l). The concurrent removal of toluene and nitrate, and the transient appearance of nitrite were observed. This synchronous phenomenon was reproduced by subsequent additions of toluene and nitrate (Figure 2). Toluene and nitrate removal appeared to be coupled. There was no toluene degradation in the absence of nitrate (Points 9 to 10, Figure 2(a)), and no nitrate removal was observed in the absence of toluene (Points 12 to 13, Figure 2(b)).

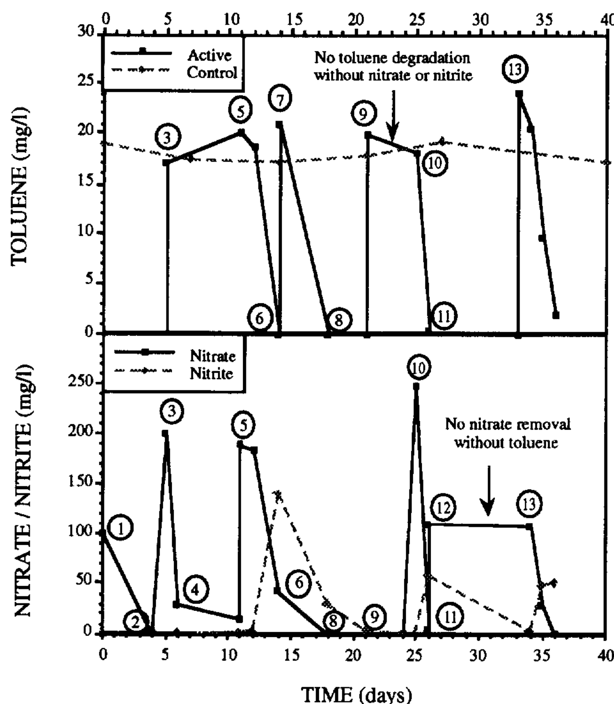

Fig. 2. Toluene degradation coupled to nitrate reduction in anoxic enrichment from Aquifer A. Numbers in circles are points of reference. The toluene control was a viable, nitrate-free, anoxic incubation.

Toluene was also degraded in triplicate enrichments from three BTEX contaminated aquifers (A, B and C) and in two out of three enrichments from pristine aquifer material (D) that had been fed all six BTEX compounds (Table 1). BTEX-amended enrichments from aquifer A experienced a lag period of 27 days before the onset of toluene degradation, and then toluene was completely removed after 40 days. A replicate set of enrichments from aquifer A received toluene alone to compare its degradation pattern with and without the presence of other BTEX compounds. These enrichments degraded toluene from 28 mg/l to less than 0.01 mg/l within 7 days (data not shown). BTEX amended enrichments from aquifer B degraded toluene below detection levels within 23 days, following an 11 day lag period. Enrichments from aquifer C degraded toluene faster; no toluene was detected after 10 days. These enrichments were also the only ones to degrade *meta*-xylene and *para*-xylene, which were removed below 0.01 mg/l within 45 days (Figure 3). The two enrichments from pristine aquifer material (D) that degraded toluene exhibited the longest lag period (40 days) (Table 1). In all cases, BTEX removal coincided with nitrate reduction.

Ortho-xylene was partially removed while toluene was being degraded, and its concentration remained constant once toluene had been removed. This trend, which is illustrated in Figure 3(d), was observed in all four sets of incubations (Table 1). *Meta*- and *para*-xylenes were not removed in enrichments from aquifers A, B, or D. Benzene and ethylbenzene were not removed in any of the BTEX-amended enrichments during

the 77-day incubation period. Benzene was also recalcitrant when fed alone (10 mg/l) or with toluene (10 mg/l) to strictly anoxic nitrate reducing enrichments that were incubated for up to 4 months (data not shown).

TABLE 1. BTEX Degradation in Nitrate Reducing Incubations from Different Aquifer Materials

	Aquifer A (BTEX Contaminated)		Aquifer B (BTEX Contaminated)		Aquifer C (BTEX Contaminated)		Aquifer D (Pristine)	
Compound	Lag Period (days)[a]	Initial (and Final) concentrations (mg/l)[b]	Lag Period (days)[a]	Initial (and Final) Concentrations (mg/l)[b]	Lag Period (days)[a]	Initial (and Final) Concentrations (mg/l)[b]	Lag Period (days)[a]	Initial (and Final) Concentrations (mg/l)[b]
Benzene	ND[c]	19.6 ± 0.3 (19.2 ± 0.8)	ND[c]	11.2 ± 0.7 (11.0 ± 0.5)	ND[c]	11.0 ± 0.5 (10.3 ± 0.4)	ND[c]	11.4 ± 0.;2 (10.9 ± 0.4)
Toluene	27	19.2 ± 0.8 (< 0.01)	11	9.8 ± 0.8 (< 0.01)	< 10	10.1 ± 0.5 (< 0.01)	40	10.1 ± 0.1 (3.4 ± 5.8)[d]
Ethylbenzene	ND[c]	3.7 ± 0.1 (3.5 ± 0.3)	ND[c]	1.7 ± 0.2 (1.5 ± 0.1)	ND[c]	1.7 ± 0.2 (1.4 ± 0.2)	ND[c]	1.7 ± 0.1 (1.5 ± 0.2)
o-Xylene	27	3.8 ± 0.1 (1.8 ± 0.2)[e]	11	1.7 ± 0.2 (1.1 ± 0.1)[e]	< 10	1.8 ± 0.1 (0.9 ± 0.1)[e]	40	1.8 ± 0.1 (1.0 ± 0.4)[d,e]
m-Xylene	ND[c]	9.3 ± 0.3 (9.1 ± 0.6)	ND[c]	4.5 ± 0.6 (4.1 ± 0.3)	< 10	4.8 ± 0.3 (< 0.01)	ND[c]	4.7 ± 0.4 (4.2 ± 0.3)
p-Xylene	ND[c]	3.7 ± 0.1 (3.4 ± 0.3)	ND[c]	1.7 ± 0.2 (1.7 ± 0.1)	< 10	1.8 ± 0.2 (< 0.01)	ND[c]	1.8 ± 0.1 (1.5 ± 0.3)
Nitrate (as NO_3^-)	27	250 ± 1 (116 ± 2)	11	183 ± 2 (122 ± 1)	< 10	176 ± 1 (82 ± 3)	40	161 ± 3 (135 ± 19)
Nitrite (as NO_2^-)	27	8 ± 1 (107 ± 11)	11	59 ± 1 (98 ± 1)	< 10	20 ± 1 (56 ± 3)	40	58 ± 1 (22 ± 3)

Notes: a) All enrichments were fed the six BTEX compounds concurrently and incubated for 11 weeks.
b) All concentrations represent the mean (± standard deviation) of triplicate incubations.
Final concentrations are corrected for abiotic losses in sterile controls, which were about 5%.
c) ND: Not determined because degradation was not significant at the 95% confidence level.
d) Only two out of three incubations from Aquifer D degraded toluene and cometabolized *o*-xylene.
e) *o*-Xylene was partially removed while toluene was being degraded, and then its concentration remained constant once toluene had been removed.

BTEX Metabolites Degradation Assays. Benzoate, which was fed at 60 mg/l to induce nitrate-based respiration, was readily removed in all four anoxic aquifer slurries. Lag periods for benzoate degradation were approximately 3 days for slurries from aquifers A, B and C, and 8 days for the slurry from pristine aquifer material (D). No benzoate was detected in any of the anoxic slurries after 2 weeks. Benzoate degradation coincided with nitrate removal and nitrite appearance, and no benzoate removal was observed in non-sterile, nitrate-free, anoxic incubations during the 2-week incubation period. The potential aliphatic BTEX metabolites adipate and succinate, which were tested separately as substrates for nitrate-based respiration, were also readily removed in nitrate reducing slurries from all four aquifer materials. In all cases, adipate was removed from 100 mg/l to less than 1 mg/l within one week, while succinate was removed from 200 mg/l to less than 1 mg/l within 2 weeks.

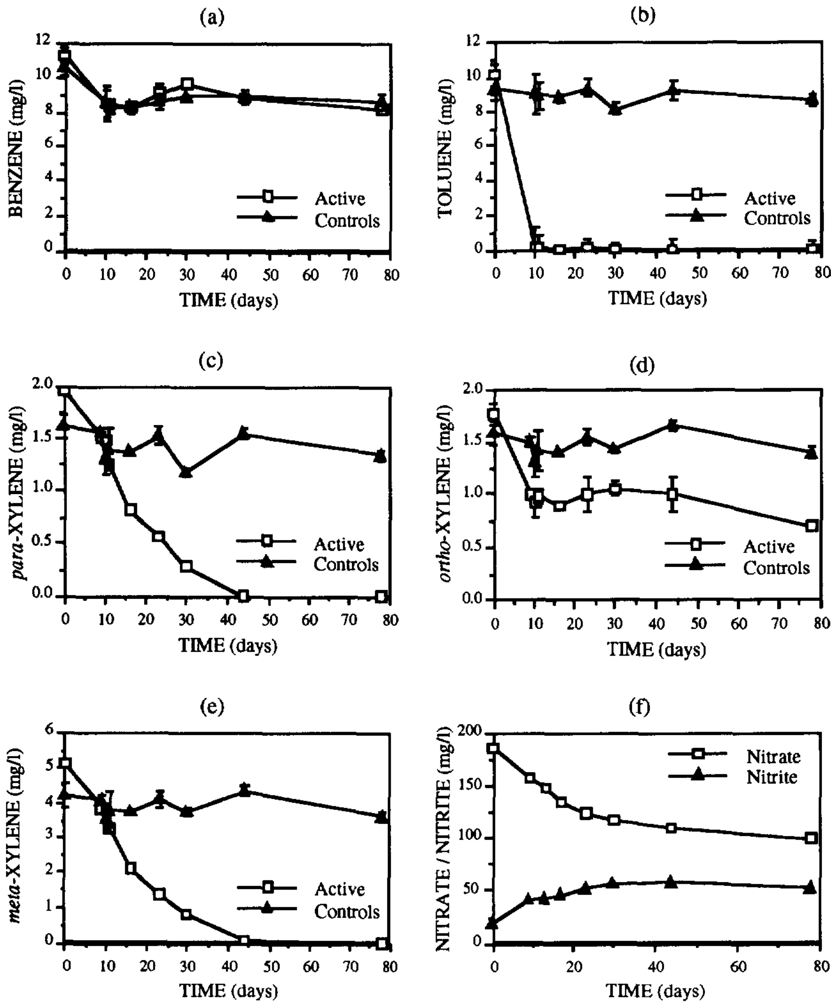

Fig. 3. Concurrent BTEX degradation in denitrifying incubations from Aquifer C. Concentrations of (a) benzene, (b) toluene, (c) *p*-xylene, (d) *o*-xylene, (e) *m*-xylene, and (f) nitrate and nitrite are depicted as time-course plots. Active incubations were amended with nitrate. Controls were autoclaved. Error bars represent standard deviation from triplicate incubations. Error bars smaller than symbols are not depicted.

Nitrate reducing enrichments from aquifer A degraded 3-methylcatechol, 4-methylcatechol, and protocatechuate concurrently (Table 2). All three compounds were removed below 0.1 mg/l after a 4 week lag period. Enrichments from aquifer B also degraded protocatechuate below detection limits after a similar lag period. However, 3-methylcatechol and 4-methylcatechol were not completely removed, even though their degradation started after a shorter lag (4 days). Their residual concentrations averaged 63% an 47% of the original concentrations, respectively. Enrichments from aquifer C removed protocatechuate below 0.1 mg/l within 22 days following a 4 day lag, while 4-methylcatechol was removed faster (within 10 days). However, 3-methylcatechol was not significantly removed. Enrichments from pristine aquifer material (D) removed 3-methylcatechol (39% removal) and 4-methylcatechol (43% removal) following a 9 day lag.

Protocatechuate, however, was not significantly removed. In all cases, the degradation of potential aromatic BTEX metabolites coincided with nitrate reduction.

TABLE 2. Degradation of Potential Aromatic Btex Metabolites In Nitrate Reducing Incubations From Different Aquifer Materials

	Aquifer A (BTEX Contaminated)		Aquifer B (BTEX Contaminated)		Aquifer C (BTEX Contaminated)		Aquifer D (Pristine)	
Compound	Lag Period (days)[a]	Initial (and Final) Concentration (mg/l)[b]	Lag Period (days)[a]	Initial (and Final) Concentrations (mg/l)[b]	Lag Period (days)[a]	Initial (and Final) Concentrations (mg/l)[b]	Lag Period (days)[a]	Initial (and Final) Concentrations (mg/l)[b]
Catechol	ND[c]	14.7 ± 0.3 14.7 ± 0.2	ND[c]	8.2 ± 0.1 (8.0 ± 0.2)	ND[c]	9.6 ± 0.1 (9.7 ± 0.1)	ND[c]	8.5 ± 0.2 (8.2 ± 0.2)
Gentisate	NA[d]	NA[d]	ND[c]	8.4 ± 0.2 (8.2 ± 0.1)	ND[c]	10.4 ± 0.2 (10.2 ± 0.1)	ND[c]	9.3 ± 0.2 (8.9 ± 0.3)
3-Methylcatechol	28	13.6 ± 0.2 (< 0.1)	4	8.2 ± 0.2 (5.2 ± 0.1)	ND[c]	8.5 ± 0.2 (8.0 ± 0.4)	9	8.5 ± 0.1 (5.2 ± 0.1)
4-Methylcatechol	28	16.8 ± 0.2 (< 0.1)	4	7.8 ± 0.3 (3.7 ± 0.1)	4	8.3 ± 1.5 (< 0.1)	9	7.7 ± 0.1 (4.4 ± 0.1)
Protocatechuate	28	14.0 ± 0.5 (< 0.1)	23	8.4 ± 0.1 (< 0.1)	4	9.8 ± 0.1 (< 0.1)	ND[c]	8.7 ± 0.1 (8.6 ± 0.1)
Nitrate (as NO_3^-)	28	249 ± 1 (3 ± 1)	4	167 ± 1 (52 ± 1)	4	166 ± 1 (53 ± 2)	9	100 ± 1 (67 ± 1)
Nitrite (as NO_2^-)	28	7 ± 1 (140 ± 1)	4	17 ± 1 (< 1)	4	16 ± 1 (< 1)	9	56 ± 1 (< 1)

Notes: a) All enrichments were fed the potential aromatic BTEX metabolites concurrently and incubated for 11 weeks.
b) All concentrations represent the mean (± standard deviation) of triplicate incubations.
Final concentrations are corrected for abiotic losses in sterile controls, which were about 5%.
c) ND: Not determined because degradation was not significant at the 95% confidence level.
d) NA: Not applicable because compound was not added.

Catechol was not significantly removed in any of the above incubations, or in nitrate-reducing enrichments receiving catechol alone (10 mg/l) or catechol (10 mg/l) with toluene (10 mg/l). The latter enrichments were incubated for up to 4 months (data not shown). Similarly, gentisate was recalcitrant in all the tested enrichments (Table 2).

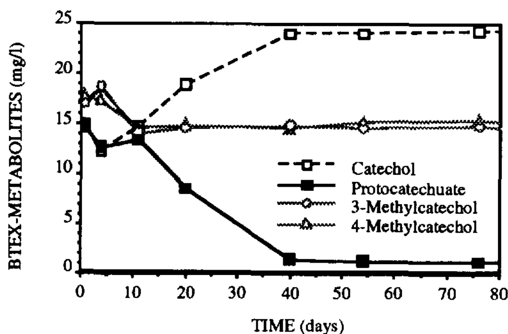

Fig. 4. Apparent decarboxylation of protocatechuate to catechol, and persistence of 3-methylcatechol and 4-methylcatechol in a nitrate-free, viable anoxic incubation from Aquifer A. Nitrate-reducing replicates degraded protocatechuate, 3-methylcatechol, and 4-methylcatechol, but not catechol (Table 2).

Protocatechuate was removed in anoxic, nitrate-free viable controls (Figure 4), but not in autoclaved controls. Unlike the nitrate-amended enrichments, the removal of protocatechuate in all three viable controls (15 mg/l) coincided with the appearance of an equimolar (97 μM) amount of catechol (i.e., 10.6 ± 0.5 mg/l).

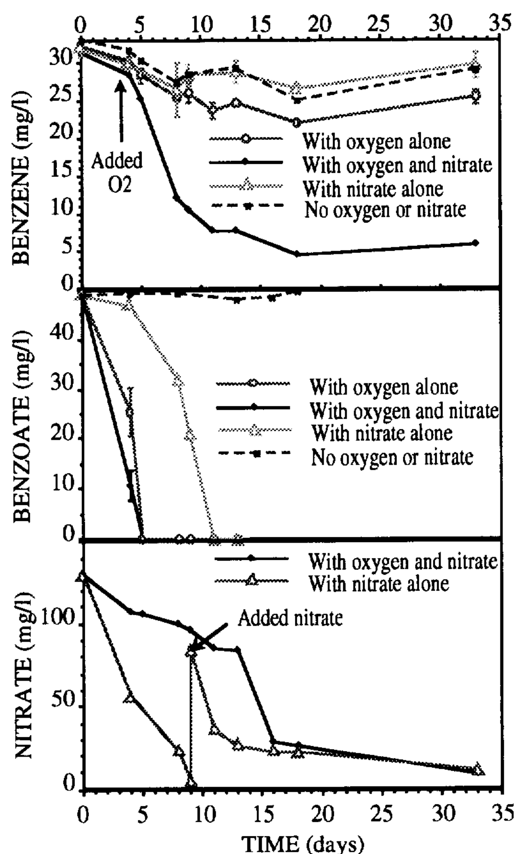

Fig. 5. Concurrent benzene and benzoate degradation by facultative denitrifiers under different electron acceptor amendments. Concentrations of (a) benzene, (b) benzoate and (c) nitrate are depicted versus time. Nitrate addition enhanced benzene degradation, although no benzene was degraded after the dissolved oxygen was depleted. Error bars represent one standard deviation from the mean of triplicate incubations. Error bars smaller than symbols are not depicted.

Effect of Nitrate on Benzene Degradation in the Presence of Benzoate. Benzene was not significantly removed in anoxic enrichments (< 0.1 mg/l DO), whether or not nitrate was present (Figure 5). Residual benzene concentrations after one month of incubation were 29.7 ± 1.7 mg/l in the nitrate-amended set and 29.0 ± 2.8 mg/l the nitrate-free set. The difference between these values is not statistically significant. Benzoate, which was added to increase the BOD, was not degraded in nitrate-free, anoxic enrichments, but was removed from 50 to less than 1 mg/l within 11 days in nitrate-amended replicates. The degradation of benzoate in these incubations coincided with the depletion of nitrate. More nitrate was added after nine days, and it continued to be removed until benzoate had been depleted (Figure 5(c)).

Benzene was degraded, however, in aerobic (oxygen limited) enrichments. These incubations received additional oxygen (1 ml of O_2 gas at 1 atm) after 4 days of incubation because the original dissolved oxygen had been depleted. Only benzoate had been degraded up to that point. Nitrate-free, aerobic enrichments subsequently degraded approximately 7 mg/l of benzene within the following week and completely removed

the residual benzoate. Enrichments receiving both oxygen and nitrate degraded benzoate faster than replicates amended with either oxygen or nitrate alone, and removed benzene to a larger extent. Residual benzene concentrations were 16.4, 22.3, and 6 mg/l, which are significantly lower (at the 95% confidence level) than residual benzene concentrations in the set amended with oxygen alone (25.4 ± 0.9 mg/l). No benzene removal was observed in any enrichment after the dissolved oxygen had been depleted (i.e., < 0.1 mg/l DO). A proportional relationship was observed between the amounts of nitrate and benzene removed in incubations amended with both oxygen and nitrate. The smallest amount of nitrate removal (63.7 mg/l as NO_3^-) was observed in the enrichment with highest residual benzene concentration (22.3 mg/l), while the largest nitrate removal (121 mg/l as NO_3^-, depicted in Figure 5(c)) corresponds to the enrichment with the lowest residual benzene concentration (6 mg/l, depicted in Figure 5(b)). No significant nitrite appearance was observed in this experiment.

DISCUSSION

In this study, biodegradation refers to substrate removal as a result of microbial action. No attempts were made to analyze for the metabolites or end products of oxidation and nitrate reduction. Regardless of the transformation products, toluene, which is the most commonly reported BTEX compound to degrade under denitrifying conditions, was degraded in anoxic enrichments from all four aquifer materials. Toluene removal and nitrate reduction were coupled (Figure 2), which suggests that nitrate-based respiration was responsible. While toluene was rapidly degraded when fed alone to enrichments from aquifer A, its degradation was significantly delayed in replicates amended with other BTEX compounds. Apparently, the tested BTEX concentration (30 mg-total BTEX per litre) was inhibitory to these microbial consortia. This observation is consistent with reports of similar BTEX concentrations inhibiting the basal denitrification rate in aquifer slurries (Hutchins *et al.*, 1991b). The threshold for BTEX toxicity under nitrate reducing conditions was not investigated in this study, although toluene concentrations as high as 80 mg/l have been degraded by pre-acclimated, denitrifying mixed cultures (Alvarez *et al.*, 1994), and up to 200 mg/l total BTEX have been degraded in aerobic aquifer slurries (Alvarez *et al.*, 1991). These BTEX concentrations are substantially higher than those commonly measured in fuel contaminated aquifers, which suggests that BTEX toxicity to microorganisms should not preclude their degradation *in situ.*

Microbial communities pre-exposed to xenobiotics often can respond faster to xenobiotic degradation than previously unexposed microbial consortia (Spain and Van Veld, 1983). This may explain why toluene persisted longer in anoxic enrichments from pristine aquifer material (D) compared with similar enrichments from BTEX-contaminated aquifers A, B, and C (Table 1). In addition, enrichments from aquifer C removed toluene faster than similar enrichments from the other two BTEX contaminated aquifers and were the only ones to degrade *m*- and *p*-xylenes under nitrate reducing conditions. Aquifer C samples were the only ones subjected to sustained nitrate exposure *in situ.* This probably facilitated the establishment of a microbial consortium with broader catabolic capacity under nitrate-reducing conditions. In fact, a separate study using samples from aquifers A, B, and C found higher denitrifying activity in aquifer C (Mikesell *et al.*, 1991).

Ethylbenzene was not degraded in any of the denitrifying enrichments tested in this work. Whether its recalcitrance was due to a lack of denitrifiers capable of degrading ethylbenzene, or to preferential utilization of other substrates (e.g., toluene), or to inhibition of ethylbenzene degraders by other BTEX compounds, was not determined in this study. Ethylbenzene has been reported to degrade under denitrifying conditions (e.g., Hutchins *et al.*, 1991b), although its anaerobic degradation commonly occurs after toluene and xylenes degradation (Edwards and Grbic-Galic), 1992).

The toluene-dependent removal of *o*-xylene was observed in enrichments from all four different aquifer materials (Table 1). This suggests that *o*-xylene was cometabolized with toluene serving as the primary substrate. Several other studies with mixed cultures under denitrifying conditions have also shown partial degradation of *o*-xylene concomitant with toluene degradation (Evans *et al.*, 1991a; Hutchins, 1991; Jensen *et al.*, 1990). Recently, a denitrifying pure culture growing on toluene was shown to cometabolize *o*-xylene, which did not serve as a source of carbon and was transformed into dead-end metabolites (Evans *et al.*,

1992). Apparently, cometabolism of *o*-xylene by toluene degraders is a common substrate interaction under nitrate-reducing conditions.

Benzene, which is commonly reported to be recalcitrant under strictly anoxic denitrifying conditions, was not degraded in any of the anoxic incubations tested in this work. The recalcitrance of benzene, but not alkylbenzenes, under nitrate reducing conditions is probably due to the relationship between the chemical structure of these compounds and the functioning catabolic pathways. Unlike aerobic pathways that use molecular oxygen as a co-substrate in initial oxidative biotransformations, the initial catabolic step under anoxic denitrifying conditions might involve nucleophilic attack with water (Altenschmidt and Fuchs, 1992; Vogel and Grbic-Galic, 1986) or acetyl-CoA (Evans *et al.*, 1992). While alkylbenzenes have electrophilic (methyl) substituents which facilitate such nucleophilic attacks, benzene does not and is recalcitrant under strictly anoxic nitrate reducing conditions. Although anaerobic benzene mineralization can occur under sulfidogenic (Edwards and Grbic-Galic, 1992) and methanogenic (Grbic-Galic and Vogel, 1987; Wilson *et al.*, 1986) conditions, such biotransformations are probably initiated by ring reduction. This notion is supported by the detection of cyclohexene in a methanogenic consortium that had been fed benzene (Grbic-Galic and Vogel, 1987). Reduction of the benzene ring probably requires strong reducing conditions, such as those encountered under methanogenic and sulfidogenic conditions, but possibly not under denitrifying conditions. The fact that no microorganism or mixed culture has been isolated that could degrade or cometabolize benzene under strictly anoxic denitrifying conditions supports the notion that benzene might not serve as a substrate for strictly anoxic nitrate-based respiration.

Benzene might, however, be degraded under hypoxic denitrifying conditions (i.e., if O_2 is present in concentrations that do not avert denitrification). Conceptually, O_2 could be used by facultative denitrifiers as a co-substrate in initial oxidative biotransformations that yield intermediate benzene metabolites. Some of these metabolites could, in turn, serve as substrates for nitrate-based respiration. However, catechol, which is a common metabolite from aerobic benzene degradation, was recalcitrant in all the anoxic enrichments tested in this work and in other studies (e.g., Hutchins, 1991; Taylor *et al.*, 1970; Tschech and Fuchs, 1987; Zeyer *et al.*, 1990). The recalcitrance of catechol under denitrifying conditions implies that sufficient molecular oxygen might be required for fission of the benzene ring (i.e., 2 moles of O_2 per mole of benzene) if nitrate-based respiration is to aid directly in the ultimate mineralization of benzene.

Nitrate addition did significantly increase the extent of benzene degradation in oxygen limited enrichments that had been grown under nitrate reducing conditions on benzoate (Figure 5). Apparently, nitrate-based respiration had a beneficial indirect effect. This is suggested by the proportionality between the amounts of nitrate and benzene removed. Enrichments removing more nitrate probably degraded a larger proportion of benzoate, which had been added to increase the BOD, using nitrate rather than oxygen as an electron acceptor. This would have resulted in the utilization of a larger fraction of the available oxygen to degrade benzene. Enrichments amended with both nitrate and oxygen apparently reduced nitrate aerobically (initial DO > 8 mg/l). The fact that these microbes had been grown anaerobically, and thus probably had an active nitrate reductase system at the start of the experiment, facilitated the occurrence of aerobic nitrate reduction. Nitrate removal was faster after the dissolved oxygen was depleted (Figure 5(c)), probably because of reduced competition for electrons from the respiratory chain. More nitrate was used in incubations amended with nitrate alone than in incubations amended with both oxygen and nitrate because the latter had a larger potential electron acceptor pool, and the nitrate demand was supplemented with oxygen.

Microorganisms capable of further degrading potential metabolites of incomplete aerobic alkylbenzene mineralization under anoxic conditions were encountered in all four of the aquifer materials tested (Table 3). Benzoate, adipate and succinate, were readily degraded in enrichments from all four aquifer materials. Protocatechuate, which had not been previously tested as a substrate for denitrifiers indigenous to aquifers, was degraded in nitrate-reducing enrichments from the three BTEX contaminated aquifers but not in enrichments from pristine aquifer material. 3-Methylcatechol and 4-methylcatechol, which had not been previously tested as substrates for nitrate-based respiration, were degraded in enrichments from both BTEX contaminated and pristine aquifer materials. The degradation of all of these compounds coincided with nitrate reduction, which supports the notion that nitrate-based respiration was responsible.

Potential aromatic BTEX metabolites tend to be chemically less stable than the original monoaromatic hydrocarbon whose resonance energy is reduced by oxidative biotransformations. One may expect such "activated" aromatic metabolites to be more amenable substrates for nitrate-based respiration than the original BTEX compounds. However, while toluene was readily degraded here under nitrate reducing conditions, its potential aerobic metabolites catechol and gentisate were not. This indicates that ecological factors, such as the presence of suitable microorganisms and their catabolic diversity, play a significant role in the degradability of aromatic compounds. Such ecological factors are probably responsible for apparent dichotomies, such as the degradation of protocatechuate and the recalcitrance of 3-methylcatechol in enrichments from aquifer C, and vice-versa in enrichments from aquifer D (Table 3).

TABLE 3. Summary of Catabolic Capacity Of Anoxic, Nitrate-Reducing Enrichments

Compound	Aquifer A (BTEX Contaminated)	Aquifer B (BTEX Contaminated)	Aquifer C (BTEX Contaminated)	Aquifer D (Pristine)
BTEX				
Benzene	-	-	-	-
Toluene	+	+	+	+
Ethylbenzene	-	-	-	-
o-Xylene	±	±	±	±
m-Xylene	-	-	+	-
p-Xylene	-	-	+	-
Aromatic BTEX Metabolites				
Benzoate	+	+	+	+
Catechol	-	-	-	-
Gentisate	-	-	-	-
3-Methylcatechol	+	+	-	+
4-Methylcatechol	+	+	+	+
Protocatechuate	+	+	+	-
Aliphatic BTEX Metabolites				
Adipate	+	+	+	+
Succinate	+	+	+	+

Key: (+) Degraded; (-) Not degraded; (±) Partially degraded cometabolically, with toluene as primary substrate

The removal of protocatechuate in viable, anoxic, nitrate-free controls (Figure 4) was synchronous to the appearance of an equimolar amount of catechol. This phenomenon is probably due to the decarboxylation of protocatechuate (i.e., 3,4-dihydroxybenzoic acid) to form catechol, which has been reported to occur in microbial incubations seeded with isolates from rat intestines (Scheline, 1966). Such decarboxylation, which was not observed in nitrate reducing incubations or in autoclaved controls, is probably a microbial-mediated, electrophilic aromatic substitution in which a proton replaces the carboxyl substituent. Protocatechuate has a hydroxyl substituent in the *para* position to the substitution site, which facilitates such electrophilic aromatic substitution (Scheline, 1966). Gentisate (i.e., 2,5-dihydroxybenzoic acid), on the other hand, is an isomer of protocatechuate that has no hydroxyl substituent in the *para* position to the substitution site, and was not decarboxylated.

In summary, this study suggests that microorganisms capable of degrading some alkylbenzenes and metabolites of incomplete aerobic BTEX degradation under strictly anoxic, nitrate-reducing conditions might be common in aquifers. These results also support the notion that *in situ* benzene degradation could be enhanced by nitrate addition to oxygen limited aquifers, particularly when the available oxygen is exceeded by the BOD of other occurring substrates that could be readily degraded under nitrate reducing conditions.

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