APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1995, p. 3185–3188 Vol. 61, No. 8
0099-2240/95/$04.00+0

Degradation of *p*-Xylene by a Denitrifying Enrichment Culture

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Received 21 March 1995/Accepted 13 May 1995

Microbial cultures enriched from a diesel fuel-contaminated aquifer were able to grow on *p*-xylene under denitrifying conditions. The oxidation of *p*-xylene to CO_2 was coupled to the reduction of NO_3^-. The enrichment cultures also grew on toluene and *m*-xylene, but they did not degrade benzene, ethylbenzene, and *o*-xylene.

In mineral oil-contaminated soils and aquifers, benzene, toluene, ethylbenzene, *p*-xylene, *m*-xylene, and *o*-xylene are often of major concern because of their high water solubility and toxicity. Under aerobic conditions, monoaromatic hydrocarbons are rapidly mineralized (4, 15, 20), and the initial metabolic step is catalyzed by oxygenases (15). At contaminated sites, however, O_2 is usually depleted (4, 8, 23, 32, 36) and oxygenases are inactive. Nevertheless, within the last decade, the anaerobic degradation of monoaromatic hydrocarbons in the field has been postulated (2, 7, 23, 28) and laboratory experiments demonstrated anaerobic degradation in aquifer columns (20, 37), microcosm experiments (17, 19, 22, 24, 25, 36), and enrichment cultures (10, 11, 13, 16, 34). Pure cultures growing on toluene, ethylbenzene, and *m*-xylene in the absence of O_2 were isolated (9, 12, 14, 21, 26, 27). Under denitrifying conditions, degradation of benzene and that of *p*-xylene and *o*-xylene have been observed only in aquifer columns (20) and in microcosms (19, 24, 25). These reports do not include carbon mass and electron balances, and a coupling of substrate removal with growth is not demonstrated.

We now report the growth of enrichment cultures on *p*-xylene under denitrifying conditions and provide carbon mass and electron balances. Furthermore, the ability of these enrichment cultures to grow on other monoaromatic compounds was investigated.

Source of inoculum. A diesel fuel-contaminated aquifer in Switzerland was bioremediated in situ from 1989 to 1994 by adding O_2, NO_3^-, NH_4^+, and PO_4^{3-} through a circulation system to stimulate the activity of the indigenous microorganisms (18). Samples from the contaminated zone were taken in November 1992 from a depth of 4.1 m, sieved, and used to study the degradation of diesel fuel in laboratory aquifer columns, microcosms (6), and enrichment cultures. In denitrifying microcosms the addition of monoaromatic as well as aliphatic hydrocarbons stimulated NO_3^- reduction. When a mixture of benzene, toluene, *p*-xylene, *m*-xylene, and *o*-xylene was applied, only benzene persisted after an incubation time of 250 days (6).

In this study, denitrifying enrichment cultures were prepared by incubating 10 g of sieved aquifer material with 50 ml of growth medium supplemented with 0.15 mM *p*-xylene in 117-ml serum bottles. The liquid phase was transferred monthly into fresh medium (10% inoculum), and eventually, enrichment cultures which no longer contained aquifer solids were obtained.

Growth medium. Basal medium (35) supplemented with 1.4 mM Na_2SO_4 and KNO_3 at various concentrations was autoclaved and cooled under an N_2-CO_2 atmosphere (98:2, vol/vol). The following components were added from sterile stock solutions: 1 ml of nonchelated trace element mixture SL10 per liter (35), 1 ml of selenite-tungstate solution per liter (33), 0.5 ml of 7-vitamin solution per liter (33), 1 M $NaHCO_3$ solution (15 ml/liter). The pH was adjusted to between 7.2 and 7.4 with 1 M HCl. Aliquots were transferred to sterile serum bottles, purged with N_2-CO_2, and sealed with butyl rubber stoppers. Monoaromatic hydrocarbon substrates were added as pure liquids by microsyringe. Polar compounds, such as alcohols, aldehydes, and acids, were added as aqueous stock solutions. Parts of the substrates sorbed to the butyl rubber stoppers, and if not stated otherwise, concentrations indicated in the text refer to actual concentrations measured in the medium after equilibration. The incubations were performed under denitrifying conditions on a rotary shaker (100 rpm) at 28°C. Samples were taken with plastic syringes that were aseptically flushed with N_2 prior to use.

Analytical procedures. Monoaromatic hydrocarbons were measured by extracting 0.6 ml of medium with an equal amount of pentane. One microliter of the extract was injected into a Carlo Erba GC 8000 gas chromatograph equipped with a flame ionization detector (Fisons Instruments, Rodano, Italy) and a 2-m glass column (diameter, 3 mm) packed with 5% SP-1200 and 5% Bentone 34 on Supelcoport 100/120 mesh (Supelco Inc., Bellefonte, Pa.). The carrier gas was N_2, and the oven temperature was held at 70°C for 2 min and then increased to 100°C at a rate of 15°C/min. Polar compounds were analyzed by high-pressure liquid chromatography as previously described (29).

NO_3^- concentrations were determined by a Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC column. The flow rate of the eluent (1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$) was 2 ml/min. NO_2^- concentrations were determined by a colorimetric method (1), and N_2O concentrations were determined by gas chromatography (13).

Growth was measured as the increase in optical density of the culture at 546 nm. The carbon content of the cells was estimated indirectly by quantifying the cellular protein (5).

Dissolved inorganic carbon was calculated from measured pH and alkalinity. The latter was measured by potentiometric titration, using Gran Plots for graphical determination of the end point (31).

Metabolism of *p*-xylene. After inoculation, the enrichment cultures grew on *p*-xylene as the sole source of carbon and energy (Fig. 1A and B), and the degradation of *p*-xylene was

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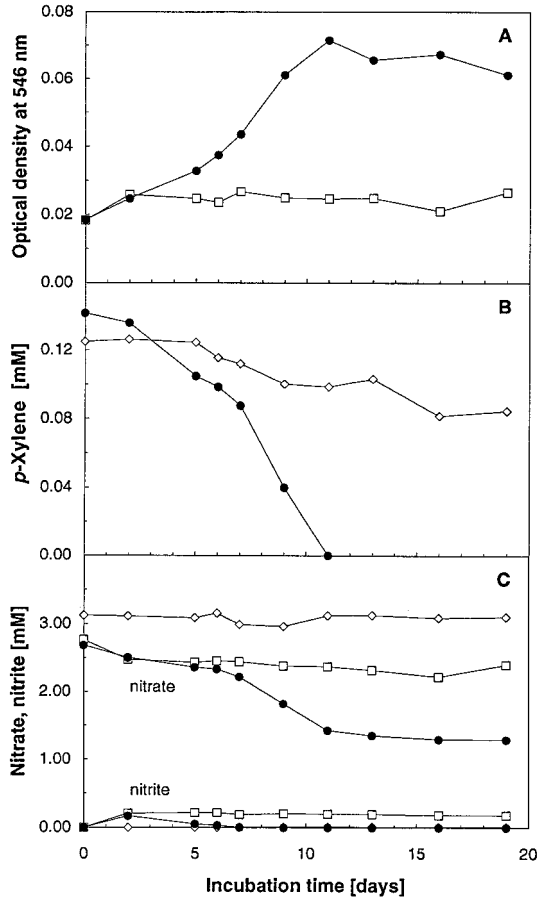

FIG. 1. Optical density at 546 nm (A) and concentrations of *p*-xylene (B) and NO_3^- (nitrate) and NO_2^- (nitrite) (C) in an enrichment culture growing in the presence (●) and absence (□) of *p*-xylene. ◇, sterile control. The data are mean values from three independent cultures. Relative standard deviations were highest (around 25%) between days 5 and 9 because of different lag phases of the three cultures.

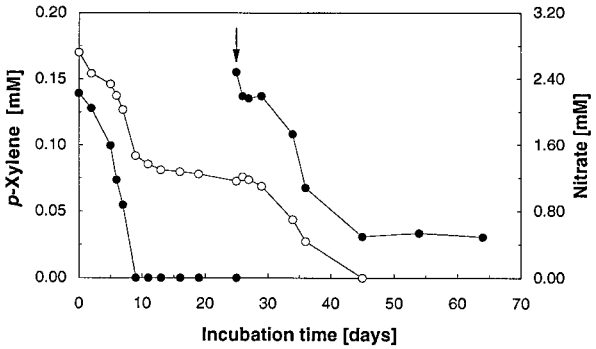

FIG. 2. *p*-Xylene oxidation (●) and NO_3^- (nitrate) reduction (○) in an enrichment culture. ↓, additional *p*-xylene was added at day 25.

coupled with the reduction of NO_3^- (Fig. 1C). NO_2^- accumulated only transiently (Fig. 1C), and N_2O was not detectable at all (data not shown). Therefore, we assumed N_2 to be the major product of NO_3^- reduction. An enrichment culture which was incubated in the absence of *p*-xylene showed only growth (Fig. 1A) and NO_3^- reduction (Fig. 1C) in the first 2 days of incubation, which was probably due to the degradation of carbon substrates other than *p*-xylene. A sterile control showed only a slow decrease of the *p*-xylene concentration, which was due to sorption (Fig. 1B), but neither mineralization of *p*-xylene nor consumption of NO_3^- (Fig. 1C).

Carbon mass and electron balances. A carbon mass balance was determined by considering the carbon content of substrates and products. After an incubation time of 11 days, *p*-xylene was completely degraded (Fig. 1B), and 10.9 mg of dissolved inorganic carbon per liter (corresponding to 0.91 mM carbon) and 4.0 mg of cellular protein per liter were produced. Assuming a 50% protein content and a molar composition of $C_4H_9O_2N$ (3), 0.31 mM biomass carbon was produced. A total production of 1.22 mM carbon is equivalent to 0.15 mM *p*-xylene, which corresponds well to the concentration of 0.14 mM *p*-xylene initially found in the medium (Fig. 1B). An electron balance was determined by considering that a complete mineralization of 1 mM *p*-xylene to CO_2 liberates 42 mM electrons and by comparing rates of *p*-xylene oxidation and NO_3^- reduction between days 7 and 9 of incubation, when NO_2^- concentrations were zero. During this period the *p*-xylene consumption rate was 0.024 mM/day (Fig. 1B), representing an electron release rate of 1.0 mM/day if all *p*-xylene had been mineralized to CO_2. The NO_3^- consumption rate was 0.199 mM/day (Fig. 1C), which reflected a rate of electron transfer from NO_3^- to N_2 of 1.0 mM/day. One also has to consider that some *p*-xylene is continuously desorbed from the stopper and degraded (probably <0.007 mM/day) and that some biomass is produced (about 0.014 mM/day). The two processes, however, have a minor effect on the overall electron balance.

Degradation of *p*-xylene and reduction of NO_3^-. The data shown in Fig. 1 are mean values from three independent enrichment cultures. One of these cultures was further incubated to investigate the coupling of *p*-xylene oxidation and NO_3^- reduction (Fig. 2). Between days 9 and 25, after the depletion of *p*-xylene, NO_3^- concentration nearly reached a plateau (Fig. 2). Upon further addition of *p*-xylene at day 25, NO_3^- reduction resumed. After the depletion of NO_3^- at day 45, *p*-xylene degradation stopped. To exclude the possibility that even traces of O_2 might be involved in the oxidation of *p*-xylene, several control experiments were performed in an anaerobic glove box under an atmosphere of N_2. These experiments yielded the same results as those presented in Fig. 2, which indicated that *p*-xylene oxidation depended solely on NO_3^- reduction.

Ability to degrade other monoaromatic compounds. When a mixture of the monoaromatic hydrocarbons benzene, toluene, ethylbenzene, *p*-xylene, *m*-xylene, and *o*-xylene was added to enrichment cultures, toluene and *m*-xylene were preferentially utilized prior to the degradation of *p*-xylene (Fig. 3). Benzene, ethylbenzene, and *o*-xylene persisted within 37 days of incubation, and the slight decrease in concentrations was probably due to sorption to the butyl rubber stopper (Fig. 3).

Enrichment cultures were also tested for the ability to grow on monoaromatic substrates other than *p*-xylene as the sole source of carbon and energy. The cultures were spiked with 0.25 to 0.35 mM substrates like monoaromatic hydrocarbons, potential intermediates of monoaromatic hydrocarbon degradation, and toluene analogs with an alternate substituent in the *para* position. Within 21 days, growth, complete conversion of the substrate, and a reduction of >1.5 mM NO_3^- were observed for cultures containing toluene, benzoate, *p*-xylene, *p*-methylbenzaldehyde, *p*-methylbenzoate, *m*-xylene, *m*-methylbenzoate, *p*-ethyltoluene, and *p*-cresol. In contrast, no substrate conversion and no NO_3^- consumption were observed in cul-

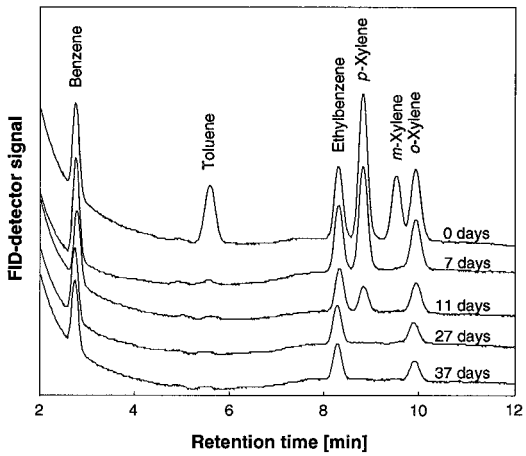

FIG. 3. Gas chromatograms of samples periodically taken from an enrichment culture growing on a mixture of benzene (initial concentration, 0.047 mM), toluene (0.048 mM), ethylbenzene (0.040 mM), *p*-xylene (0.086 mM), *m*-xylene (0.041 mM), and *o*-xylene (0.044 mM). FID, flame ionization detector.

tures containing benzene, ethylbenzene, *o*-xylene, *o*-methylbenzoate, *o*-ethyltoluene, *p-tert*-butyltoluene, *p*-fluorotoluene, *p*-chlorotoluene, *p*-aminotoluene, *p*-nitrotoluene, and the three trimethylbenzene isomers. Control experiments in which these substrates were incubated together with *p*-xylene were set up. All of these cultures demonstrated a complete degradation of *p*-xylene, indicating that the persistence of these substrates was not due to toxic or inhibitory effects.

In enrichment cultures containing *p*-fluorotoluene and *p*-xylene, both substrates were completely degraded within 10 days and up to 0.015 mM *p*-fluorobenzoate was transiently accumulated (Fig. 4). A conversion of *p*-fluorotoluene to *p*-fluorobenzoate by pure cultures of denitrifying toluene degraders was also observed by Seyfried et al. (30), which suggested that oxidation of the *methyl* substituent might be the initial step in denitrifying toluene metabolism. It is likely that *p*-xylene degradation is also initiated by the oxidation of the *methyl* substituent to an aldehyde and, eventually, to a carboxy group. This assumption is supported by the finding that the enrichment cultures described in this study were able to grow on *p*-methylbenzaldehyde and *p*-methylbenzoate. Studies with pure cultures are necessary to further examine the metabolic pathways of *p*-xylene.

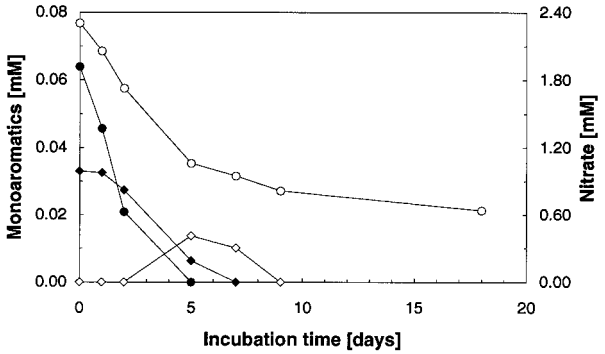

FIG. 4. NO_3^- (nitrate) (○), *p*-fluorotoluene (◆), and *p*-xylene (●) consumption and transient accumulation of *p*-fluorobenzoate (◇) by an enrichment culture growing on *p*-fluorotoluene and *p*-xylene.

This work was supported by the Swiss National Science Foundation (Priority Programme Environment).

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