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Degradation of Trimethylbenzene Isomers by an Enrichment Culture under N_2O-Reducing Conditions

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A microbial culture enriched from a diesel fuel-contaminated aquifer was able to grow on 1,3,5-trimethylbenzene (1,3,5-TMB) and 1,2,4-TMB under N_2O-reducing conditions, but it did not degrade 1,2,3-TMB. The oxidation of 1,3,5-TMB to CO_2 was coupled to the production of biomass and the reduction of N_2O. N_2O was used to avoid toxic effects caused by NO_2^- accumulation during growth with NO_3^- as the electron acceptor. In addition to 1,3,5-TMB and 1,2,4-TMB, the culture degraded toluene, *m*-xylene, *p*-xylene, 3-ethyltoluene, and 4-ethyltoluene.

In mineral oil-contaminated soils and aquifers, monoaromatic hydrocarbons are often of major concern due to their high water solubility and toxicity. Under aerobic conditions, these compounds are rapidly mineralized (7, 25, 26). Monoaromatic hydrocarbons, such as benzene, toluene, ethylbenzene, and xylene isomers, are also known to be degraded in microbial cultures under NO_3^--reducing (11, 15–17, 20, 23, 24, 33, 35, 43), Fe(III)-reducing (28, 29), sulfate-reducing (5, 10, 12, 14, 19, 27, 32, 34), or methanogenic (13, 18, 40, 42) conditions. However, only limited data are available on the anaerobic degradation of monoaromatic hydrocarbons like *n*-propylbenzene, isopropylbenzene, trimethylbenzene (TMB) isomers, and ethyltoluene isomers, which represent a prominent portion of the water-soluble fraction of mineral oil (25). A pure culture growing on *n*-propylbenzene under NO_3^--reducing conditions has been isolated (33), but no anaerobic cultures which degrade isopropylbenzene, TMB isomers, or ethyltoluene isomers have been reported. Field experiments and studies in microcosms filled with aquifer material suggested that an anaerobic degradation of 1,3,5-TMB (30, 37) and 1,2,4-TMB (1, 23, 24) takes place, but solid proofs have been lacking. We now report the growth of an enrichment culture on 1,3,5-TMB and 1,2,4-TMB under N_2O-reducing conditions and provide carbon mass and electron balances for the degradation of 1,3,5-TMB.

Source of inoculum. A diesel fuel-contaminated aquifer in Menziken, Switzerland, was bioremediated in situ from 1989 to 1994 by supplementing the groundwater with O_2, NO_3^-, NH_4^+, and PO_4^{3-} to stimulate the activity of the indigenous microorganisms (9, 22). 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 (21), microcosms (9), and enrichment cultures (20).

Enrichment culture. In this study, O_2-free cultures on TMB isomers were enriched by incubating 25 g of sieved aquifer material with 50 ml of growth medium in 117-ml serum bottles sealed with butyl rubber stoppers. The growth medium was supplemented with 5 mM NO_3^- and 2 ml of 2,2,4,4,6,8,8-heptamethylnonane containing 4% (vol/vol) of an equimolar mixture of the three TMB isomers. Heptamethylnonane served as an inert carrier for TMB to avoid toxic effects (32). The cultures were incubated on a rotary shaker (120 rpm) at 25°C. After an incubation time of about 35 days, the rate of nitrate reduction increased with respect to controls containing only heptamethylnonane. This increase was accompanied by the growth of microorganisms in the medium. After an incubation time of 57 days, the liquid phase was transferred into fresh medium containing 2 mM NO_3^- (20% inoculum). These cultures received only one specific TMB isomer diluted in 1 ml of heptamethylnonane (2% [vol/vol]). The cultures grew without a lag phase on 1,3,5-TMB and 1,2,4-TMB with NO_3^- as the electron acceptor, but no culture grew on 1,2,3-TMB. NO_3^- was reduced only to NO_2^-, which increasingly inhibited further growth on these substrates. Therefore, additional enrichments in which NO_3^- was replaced by N_2O as the electron acceptor were performed. Eventually, an enrichment culture growing on both 1,3,5-TMB and 1,2,4-TMB was obtained.

Further subcultivations and metabolic studies were performed in serum bottles with a total volume of 1.1 liters and a liquid volume of 0.3 liter. TMB isomers were added as pure liquids by microsyringe.

Growth medium. Basal medium (41) supplemented with 1.4 mM Na_2SO_4 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 (41) per liter, 1 ml of selenite-tungstate solution (39) per liter, seven-vitamin solution (39), and 1 M $NaHCO_3$ solution (15 ml/liter). The pH was adjusted to 7.25 with 1 M HCl. Aliquots were transferred to sterile serum bottles, purged with N_2-CO_2, and sealed with butyl rubber stoppers. After inoculation, the bottles were purged with N_2-CO_2 for an additional 15 min and with N_2O for 1 min, and an overpressure of 0.2 bar N_2O was supplied. Complete absence of O_2 was confirmed by gas chromatography (9). The incubations were performed under N_2O-reducing conditions on a rotary shaker (120 rpm) at 25°C. Samples were taken with plastic syringes that were aseptically flushed with N_2 prior to use. In incubations using NO_3^- as the electron acceptor, C_2H_2 (10% of the headspace) was applied to inhibit the reduction of N_2O to N_2 (38).

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Chemicals. 2,2,4,4,6,8,8-Heptamethylnonane was supplied by Sigma Chemie (Buchs, Switzerland). Diesel fuel was from an Esso gas station (Schlieren, Switzerland). H_2, N_2-CO_2 (98:2 [vol/vol]), N_2O, and C_2H_2 were obtained from Pan Gas (Schlieren, Switzerland). All other chemicals were purchased from either Fluka AG (Buchs, Switzerland) or Merck ABS (Dietikon, Switzerland) at the highest purity available.

Analytical procedures. Growth was determined by measuring the optical density of the culture at 546 nm (OD_{546}). The carbon content of the cells was estimated indirectly by quantifying the cellular protein (8) and by assuming a molar composition of $C_4H_9O_2N$ (6) and a 50% protein content.

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^- was determined by a colorimetric method (2). O_2, CO_2, and N_2O were measured by gas chromatography (9). Dissolved inorganic carbon was calculated from measured pH and alkalinity values (36). The latter was quantified by potentiometric titration with Gran Plots for graphic determination of the end point (36).

Quantification of TMB isomers. TMB isomers were measured by extracting 0.5 ml of medium with 1.0 ml of pentane. To determine TMB isomers, 1 μl of the extract was injected automatically 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 maintained at 100°C for 15 min. Since 1,3,5-TMB and 1,2,4-TMB have the same retention time on this packed column system, capillary gas chromatography had to be employed for the separation of the mixture of all three TMB isomers. There, 2 μl of the extract was injected into a Fisons HRGC Mega II gas chromatograph equipped with a flame ionization detector (Fisons Instruments) and a DB-5 fused silica capillary column (length, 30 m; diameter, 0.32 mm) with a (5% phenyl)-methylpolysiloxane (0.25-μm film thickness) bonded phase (J&W Scientific, Folsom, Calif.). Splitless injection with H_2 as the carrier gas was employed. The column temperature was maintained at 70°C for 10 min. For the analysis of the water-soluble fraction of diesel fuel, 50 ml of the medium was extracted with 1 ml of pentane. Two microliters of the extract was injected into the capillary gas chromatograph. The oven temperature was maintained at 40°C for 2 min and then increased to 250°C at a rate of 3°C/min.

All chromatographic data were processed with Chrom-Card for Windows (Fisons Instruments).

Degradation of 1,3,5-TMB and reduction of NO_3^-. The enrichment culture was incubated with 0.14 mM 1,3,5-TMB and 0.5 mM NO_3^- as the electron acceptor in the presence of C_2H_2. About two-thirds of the NO_3^- was reduced to NO_2^-, and only one-third was further reduced to N_2O within 30 h. NO_3^- that was added to the culture after 31 h was not reduced. The accumulation of NO_2^- increasingly inhibited the growth of the culture. Although excellent electron balances can be performed by using NO_3^- as the electron acceptor, all further studies were performed with N_2O as the electron acceptor to avoid toxic effects caused by NO_2^-.

Degradation of TMB isomers under N_2O-reducing conditions. A medium which contained all three TMB isomers (0.08 mM each) was inoculated with an aliquot of an enrichment culture pregrown on 1,3,5-TMB under N_2O-reducing conditions. After inoculation, 1,3,5-TMB and 1,2,4-TMB were degraded in the presence of N_2O at equal rates without any lag phase. The concentrations dropped below the detection limit within about 20 h (Fig. 1A). In contrast, the concentration of 1,2,3-TMB in this culture (Fig. 1A) and the concentrations of all three isomers in a control culture which was inhibited by adding 20 mM NaN_3 (Fig. 1B) were reduced by only about one-third within 97 h. This reduction was due to adsorption to the butyl rubber stoppers. The same degradation pattern was observed when an inoculum of an enrichment culture pregrown on 1,2,4-TMB was used (results not shown).

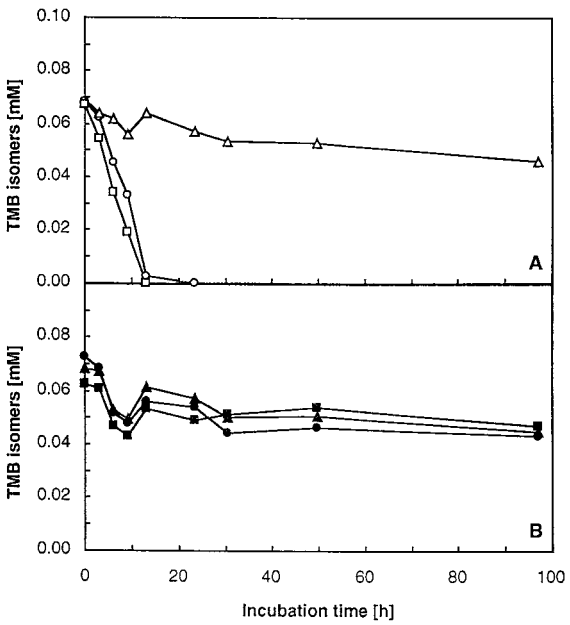

FIG. 1. (A) Concentrations of 1,3,5-TMB (○), 1,2,4-TMB (□), and 1,2,3-TMB (△) in enrichment cultures growing in the presence of N_2O. (B) Concentrations of 1,3,5-TMB (●), 1,2,4-TMB (■), and 1,2,3-TMB (▲) in enrichment cultures incubated in the presence of N_2O and 20 mM NaN_3. The data in Fig. 1A represent the mean of two independent replicates. The deviation of the data in the two replicates was always <5%.

Since a microbial degradation of 1,2,3-TMB was never observed and an elimination of this compound was due only to sorption, 1,2,3-TMB was used as a standard. In the following metabolic studies which focused predominantly on 1,3,5-TMB degradation, substrate concentrations are indicated as the ratio between the substrate 1,3,5-TMB and the standard 1,2,3-TMB to allow distinction between sorption and biodegradation.

Growth on 1,3,5-TMB under N_2O-reducing conditions. The enrichment culture grew on 1,3,5-TMB as the sole source of carbon and energy (Fig. 2A and B). A control which received no substrate showed no growth (Fig. 2B). A control which was inhibited by adding 20 mM NaN_3 showed no substrate removal (Fig. 2A) and no growth (Fig. 2B). Cultures (two replicates) and the inhibited control received 0.14 mM 1,3,5-TMB (besides 0.14 mM 1,2,3-TMB), and N_2O was applied in excess.

One of the two replicates was supplemented with 0.14 mM 1,3,5-TMB after the depletion of the substrate at time 45.5 h (Fig. 2A). At this time the OD_{546} almost reached a plateau (Fig. 2B). Upon addition of 1,3,5-TMB at time 45.5 h, growth resumed. After the depletion of 1,3,5-TMB at time 69.5 h, growth stopped again (Fig. 2B). These results indicated that growth and 1,3,5-TMB degradation were coupled.

Carbon mass and electron balances. To determine carbon mass and electron balances of the 1,3,5-TMB metabolism, degradation studies were performed under a N_2O-N_2-CO_2 (20/

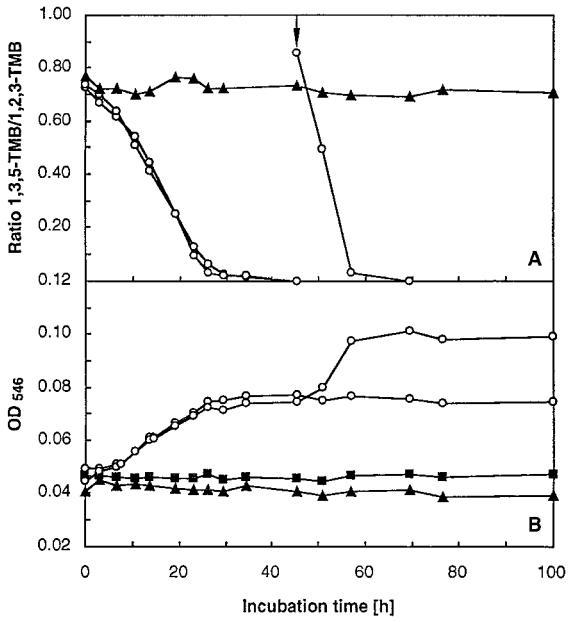

FIG. 2. Concentrations of 1,3,5-TMB (A) and OD_{546} (B) in enrichment cultures growing in the presence of 1,3,5-TMB (○) with N_2O as the electron acceptor (two replicates). A poisoned control (20 mM NaN_3) incubated with 1,3,5-TMB showed no growth and no substrate removal (▲). A control incubated without substrate showed no growth (■). Additional 1,3,5-TMB was added at time 45.5 h to one replicate (arrow).

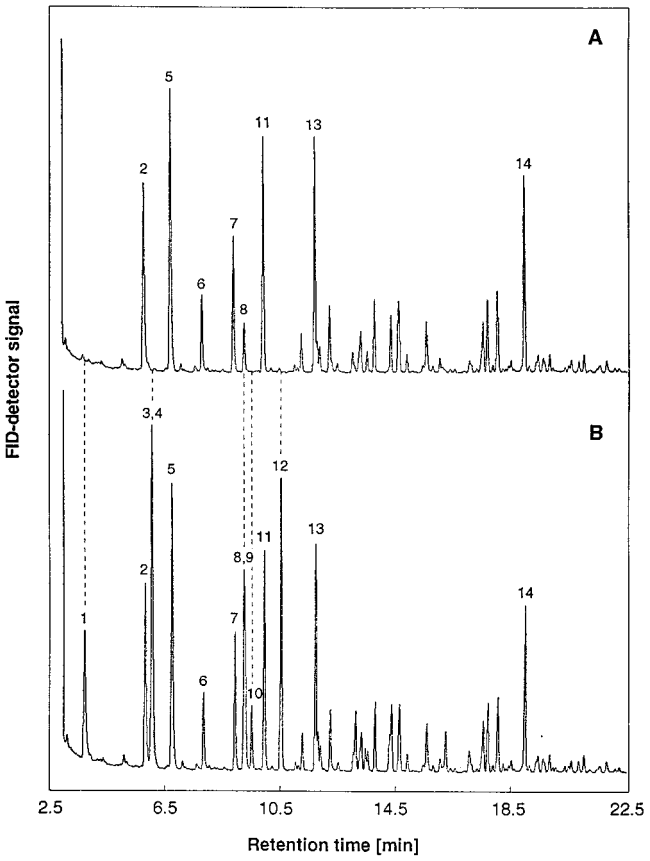

FIG. 3. Gas chromatograms of the water-soluble fraction of diesel fuel (0.2 ml) incubated for 20 days in a viable (0.3 liter) enrichment culture (A) and a (0.3 liter) enrichment culture poisoned with 20 mM NaN_3 (B). 1, toluene; 2, ethylbenzene; 3, *p*-xylene; 4, *m*-xylene; 5, *o*-xylene; 6, isopropylbenzene; 7, *n*-propylbenzene; 8, 4-ethyltoluene; 9, 3-ethyltoluene; 10, 1,3,5-TMB; 11, 2-ethyltoluene; 12, 1,2,4-TMB; 13, 1,2,3-TMB; 14, naphthalene. FID, flame ionization.

78.4/1.6 [vol/vol/vol]) atmosphere. The concentration and oxidation state of carbon substrates and products as well as the N_2O reduction were quantified. The effect of the inoculum on the carbon mass and electron balance was determined by incubating a control without 1,3,5-TMB. After an incubation time of 72 h, 0.36 mM 1,3,5-TMB initially supplied was completely degraded. A total of 5.64 mM net N_2O was reduced, and 1.97 mM net total inorganic carbon and 8.53 mg of net cellular protein $liter^{-1}$, corresponding to 0.17 mM net biomass, were produced. The reduction of 5.64 mM N_2O to N_2 consumes 11.28 mM electrons, which would require the complete mineralization of 0.24 mM 1,3,5-TMB. This mineralization would yield 2.12 mM CO_2, which corresponds well with the 1.97 mM total inorganic carbon measured. Complete assimilation of the remaining 0.12 mM 1,3,5-TMB would theoretically yield 0.27 mM biomass. The discrepancy between the theoretical (0.27 mM) and experimental (0.17 mM) values may be due to some substrate which remained adsorbed to the butyl rubber stopper but may also be due to inappropriate assumptions about the protein content and molar composition of biomass and/or due to the production of soluble metabolites which have not been assessed.

Ability to degrade other monoaromatic compounds. The enrichment culture was also tested for the ability to degrade monoaromatic substrates other than the TMB isomers. The culture (0.3 liters) was spiked with 0.2 ml of diesel fuel and incubated. After 20 days, the free phase was discarded and the aqueous phase was extracted and analyzed. The gas chromatograms presented in Fig. 3 show the remaining water-soluble fraction of diesel fuel of a viable enrichment culture (Fig. 3A) and of a culture inhibited by the addition of 20 mM NaN_3 (Fig. 3B). To detect benzene and to differentiate between *p*-xylene and *m*-xylene and between 4-ethyltoluene and 3-ethyltoluene, the packed column gas chromatographic analysis was employed as described above. The analysis revealed that toluene, *p*-xylene, *m*-xylene, 3-ethyltoluene, 1,3,5-TMB, and 1,2,4-TMB were completely, and 4-ethyltoluene was partially, degraded. In contrast, benzene, ethylbenzene, *o*-xylene, isopropylbenzene, *n*-propylbenzene, 2-ethyltoluene, and 1,2,3-TMB were not metabolized.

The selective degradation pattern recorded in Fig. 3 may have two causes. On the one hand, it is very likely that the culture which was enriched from the aquifer material by providing TMB as the sole source of carbon and energy has lost the ability to degrade a broad range of monoaromatic hydrocarbons. On the other hand, it is possible that only compounds with a certain chemical structure can be degraded under denitrifying conditions. With the exception of ethylbenzene and *n*-propylbenzene, which can be degraded by denitrifying pure cultures (33), the results presented in Fig. 3 are in good agreement with the data reported in the literature. Growth of enrichment cultures or pure cultures on monoaromatic hydrocarbons under either NO_3^-- or N_2O-reducing conditions has been demonstrated only for monoaromatic compounds carrying at least one alkyl substituent with no neighboring substituent in the *ortho* position, such as toluene (11, 15, 17, 33, 35), ethylbenzene (33), *p*-xylene (20), *m*-xylene (11, 16, 17, 33, 35), or *n*-propylbenzene (33). Benzene (31) and *o*-xylene (23, 24, 26, 31) removal was observed only in microcosm and aquifer column studies or, in the case of *o*-xylene, in the presence of

toluene (3, 15). Our results obtained with the ethyltoluene and TMB isomers support this hypothesis.

This hypothesis cannot be extended to iron-reducing, sulfate-reducing, and/or methanogenic conditions, under which compounds such as benzene, *o*-xylene, or 2-ethyltoluene can be degraded (10, 12–14, 27, 29, 34). Several redox regimens were employed to extend the range of degradable monoaromatic hydrocarbons at Seal Beach, Calif. (4). These observations suggest that a spatial and temporal variation of active treatment (e.g., addition of nitrate) and intrinsic processes (mainly iron-reducing, sulfate-reducing, and methanogenic processes) may enhance the overall efficiency of an in situ bioremediation of mineral oil-contaminated sites.

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