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# Rapid Microbial Mineralization of Toluene and 1,3-Dimethylbenzene in the Absence of Molecular Oxygen

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**Up to 0.4 mM 1,3-dimethylbenzene (*m*-xylene) was rapidly mineralized in a laboratory aquifer column operated in the absence of molecular oxygen with nitrate as an electron acceptor. Under continuous flow conditions, the degradation rate constant (pseudo-first order) was $>0.45\ h^{-1}$. Based on a carbon mass balance with [ring-$^{14}$C]*m*-xylene and a calculation of the electron balance, *m*-xylene was shown to be quantitatively (80%) oxidized to $CO_2$ with a concomitant reduction of nitrate. The mineralization of *m*-xylene in the column also took place after reducing the redox potential, E′, of the inflowing medium with sulfide to $<-0.11$ V. Microorganisms adapted to growth on *m*-xylene were also able to degrade toluene under denitrifying conditions. These results suggest that aromatic hydrocarbons present in anoxic environments such as lake sediments, sludge digestors, and groundwater infiltration zones from landfills and polluted rivers are not necessarily persistent but may be mineralized in the absence of molecular oxygen.**

The infiltration of water from landfills (7, 21) or contaminated rivers (20, 23) into groundwater aquifers is usually accompanied by an aerobic microbial mineralization of the organic constituents of water. Degradation of high concentrations of organic compounds, however, may lead to a rapid depletion of the dissolved molecular oxygen and to an eventual decrease in the redox potential in the aquifer (3, 7, 28, 29). Aerobic microorganisms which predominate at high redox potentials (2) [$E_0'(O_2/H_2O) = +0.8$ V (28)] are replaced by denitrifying (16), sulfate-reducing (20), or even methanogenic [$E' < -0.3$ V (13)] populations (3, 7, 21, 30). The redox potential also largely determines the metabolic diversity of the microorganisms in the aquifer. Some pollutants, such as chlorinated benzenes, are metabolized only under aerobic conditions, while the mineralization of most halogenated aliphatic compounds requires an anaerobic environment (3, 29). Aromatic hydrocarbons such as benzene, toluene, and xylenes are ubiquitous pollutants (7, 19, 20, 21), and they are rapidly degraded under aerobic conditions (3, 9, 18). In anaerobic microbial cultures from lake sediments and sewage sludge (12, 22), however, they have been found to be very recalcitrant. Nevertheless, studies conducted in sulfate reducing and methanogenic aquifers (20, 21; J. F. Rees, B. H. Wilson, and J. T. Wilson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, Q5, p. 258) and, in particular, results of experiments in our laboratory with denitrifying aquifer columns (18) suggest that metabolism of toluene and xylenes in the absence of molecular oxygen may be possible. In those studies, however, solid evidence of anaerobic mineralization was not obtained, since the toluene and xylene concentrations used were too low (<5.5 μM) to allow reliable electron and carbon mass balances, no degradation products were identified, and traces of oxygen may have been present. In this paper, we demonstrate that *m*-xylene and toluene are completely mineralized under denitrifying conditions in the absence of molecular oxygen.

A continuous-flow system for a denitrifying laboratory aquifer column was constructed which allowed the continuous replacement of oxygen in the medium by nitrogen in a gas exchange chamber (Fig. 1). This chamber consisted of a gas-tight flask (160 ml) permanently flushed (1 ml/min) with $N_2$ (99.995% pure), containing 2.0 m of silicone tube (wall thickness, 1.0 mm; inner diameter, 1.0 mm) through which the medium flowed. The silicone tube is highly permeable to gases, and the dissolved oxygen in the medium was replaced by nitrogen. The efficiency of the gas exchange in the 2.0-m tube was determined by using a Clark-type oxygen electrode, and the values are shown in Fig. 1 as a function of the

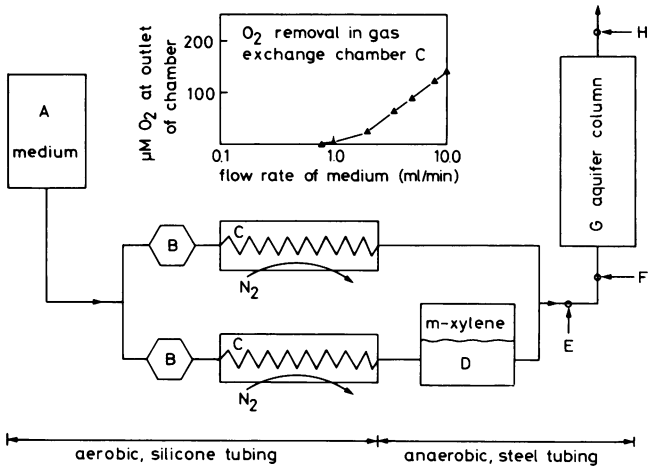


FIG. 1. Experimental set-up for studying *m*-xylene degradation under continuous-flow conditions in the absence of molecular oxygen. (A) Basal medium consisting of (millimoles per liter of $H_2O$) $KH_2PO_4$, 2.4; $Na_2HPO_4$, 8.9; $Mg_2SO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.3; and $NH_4Cl$, 0.2; plus 3.35 mg of trace elements (as described previously [33]) per liter (pH 7.5). Unless indicated otherwise, the basal medium was supplemented with 5 mM $NaNO_3$. (B) Peristaltic pump with individually adjustable pumping rates. (C) Gas exchange chamber. (D) Gas-tight flask (350 ml) containing oxygen-free medium and about 30 ml of *m*-xylene (top layer). The medium was stirred gently to ensure saturation with *m*-xylene. The *m*-xylene in the flask had to be replenished only after several months of operation. (E) Position at which [ring-$^{14}$C]*m*-xylene could be injected or at which a flask with chemically reduced media could be inserted. (F) Sampling port inlet. (G) Laboratory aquifer column. (H) Sampling port outlet.

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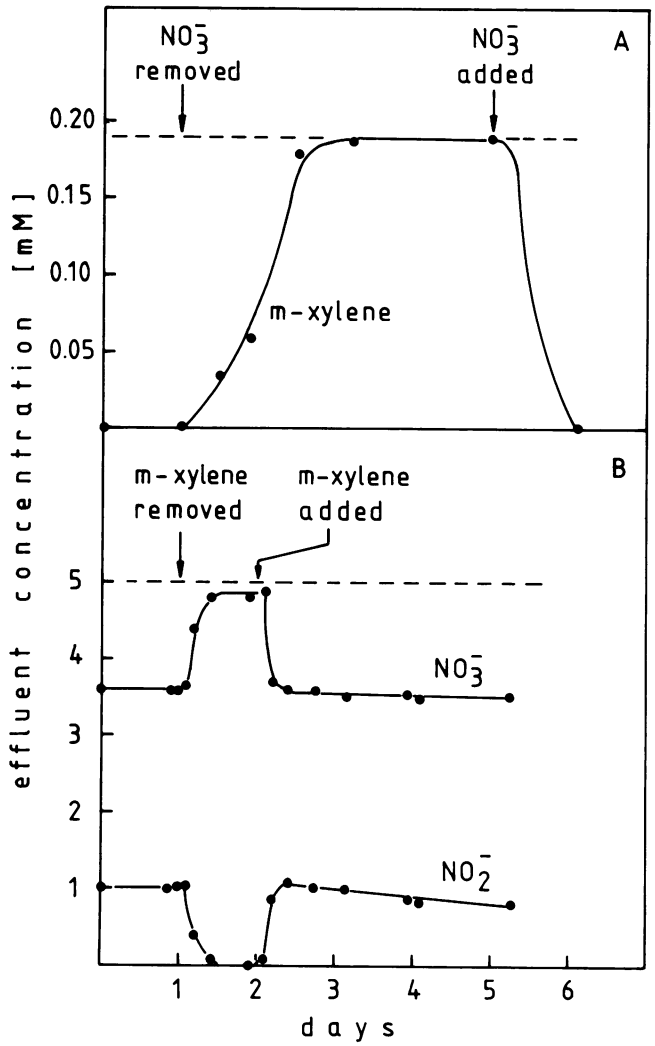


FIG. 2. Coupling of *m*-xylene degradation with $NO_3^-$ reduction in the aquifer column. The inflowing basal medium was supplemented with 0.19 mM *m*-xylene and 5 mM $NaNO_3$ (dashed lines). (A) Response of *m*-xylene degradation to the removal of $NO_3^-$; (B) response of $NO_3^-$ reduction to the removal of *m*-xylene.

medium flow rate. At flow rates below 0.8 ml/min, the residual concentrations of oxygen were below the detection limit (1 μM $O_2$). Calculations based on both the experimental conditions (flow rates of <0.8 ml/min) and the purity of the nitrogen used in the gas exchange chamber confirmed the measured values. Reintroduction of oxygen after its removal was prevented by using stainless steel tubing and glass vessels. After the removal of oxygen, one part of the medium was saturated with *m*-xylene (saturation, 1.5 mM [27]) by flowing through an airtight flask which contained a layer of liquid *m*-xylene. The other part of the medium was not supplemented with *m*-xylene. Therefore, any desired *m*-xylene concentration at the column inlet could be established by adjusting the ratios of the two pumping rates. The laboratory aquifer column consisted of a glass cylinder (length, 21.5 cm; inner diameter, 4 cm) filled with 30% aquifer material from the interface of a river-groundwater infiltration site (23) and 70% expanded slate grain (diameter, 2 mm). The column was maintained at 30°C with a water jacket. The average flow velocity of the medium in the

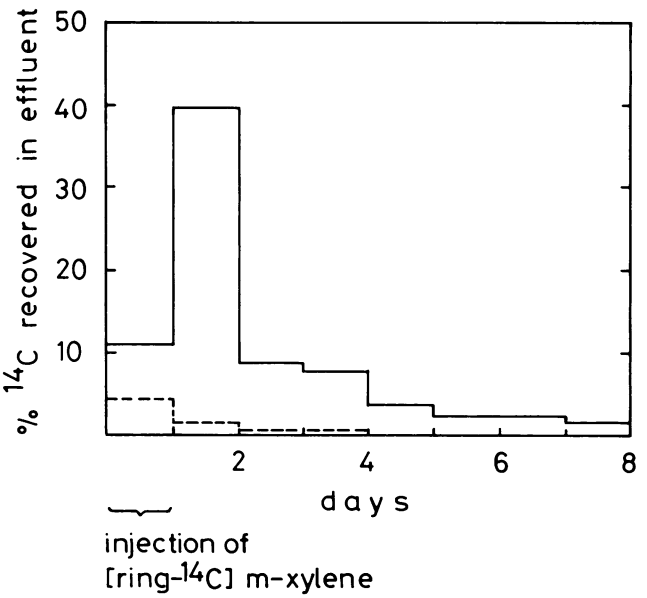


FIG. 3. Analysis of column effluent after addition of 0.44 μCi of [ring-$^{14}$C]*m*-xylene to a continuous flow of 0.3 mM *m*-xylene. The [ring-$^{14}$C]*m*-xylene (Amersham International plc; specific activity, 93 mCi/mmol; radiochemical purity according to the supplier, 99%) was injected over a 24-h period at position E of Fig. 1, and effluent samples were collected and analyzed daily. Symbols: ——, $^{14}CO_2$ (total, 80%); – – –, acid extractable $^{14}$C (total, 6%).

column was 2.6 cm/h, corresponding to a flow rate of 0.25 ml/min.

The aquifer column was inoculated with denitrifying river sediment which had been continuously fed with 4.5 μM *m*-xylene for several months (18). The initial *m*-xylene concentration in the inflowing medium was adjusted to 0.04 mM. After 3 weeks of continuous operation, *m*-xylene was no longer detectable in the column effluent. Analysis was performed by high-pressure liquid chromatography (Perkin-Elmer series 4; C-18 column; water-methanol as solvent), and the detection limit for *m*-xylene was 0.01 mM. The influent concentration of *m*-xylene was subsequently increased stepwise during the next few months, and degradation was periodically assessed. As much as 0.4 mM *m*-xylene was rapidly (pseudo-first-order rate constant, >0.45 $h^{-1}$) and completely metabolized in the column. The microbial population in the aquifer column apparently grew with *m*-xylene as the sole source of carbon and energy. The degradation was coupled with the reduction of nitrate to nitrite (both were analyzed quantitatively as reported previously [18]) and some unidentified gaseous nitrogen compounds. As observed in our previous column studies at low substrate concentrations (18), *m*-xylene oxidation completely stopped upon removal of nitrate, and reduction of nitrate ceased upon removal of *m*-xylene (Fig. 2).

To quantify the degradation products, a carbon mass balance of *m*-xylene in the column was made by using [ring-$^{14}$C]*m*-xylene as a substrate (Fig. 3). The column effluent was collected in gas-tight vessels containing 100 ml of 0.05 M $H_2SO_4$. The $^{14}$C extractable with ethyl acetate and the $^{14}$C evolved as $^{14}CO_2$ from these acidified samples were quantified daily as described previously (34). Over an 8-day period, a total of 6% of the added radioactivity was recovered as acid-extractable metabolites and some 80% was evolved as $^{14}CO_2$ (Fig. 3). The nonextractable metabolites

TABLE 1. Degradation of *m*-xylene in the presence of nitrate and sulfide[a]

| Sampling position in column | Concn (mM) of the following substrate or product | | | |
|---|---|---|---|---|
| | *m*-Xylene | $NO_3^-$ | $NO_2^-$ | $H_2S$ |
| Inlet | 0.31 | 8.3 | <0.02 | 0.5 |
| Outlet | <0.01 | 3.3 | 1.3 | <0.01 |

[a] Positions A to D in the continous flow system shown in Fig. 1 were replaced by an airtight 6-liter flask inserted at position E and containing the chemically reduced medium. The medium was pumped through the column at a flow rate of 0.25 ml/min and column temperature of 30°C. The data shown represent steady-state conditions after 1 week of operation. The standard deviations for nitrate and nitrite concentrations were <0.1 mM. The ammonium concentration in the effluent consistently equalled that in the influent.

amounted to a total of 3%. About 11% of the injected $^{14}C$ could not be recovered in the effluent within 8 days and was probably incorporated into biomass attached to the aquifer particles. The mineralization of [ring-$^{14}C$]*m*-xylene was also investigated at elevated temperatures. At 37°C, less than 20% of the injected radioactive carbon was evolved as $^{14}CO_2$ over an 8-day period, which strongly suggests that *m*-xylene mineralization is a biological process.

Complete removal of oxygen from the medium is a crucial step in the establishment of anaerobic conditions (13, 14). For the experimental set-up shown in Fig. 1, we found that there was less than 1 μM $O_2$ in the inflowing medium. These traces of oxygen could not have been involved in the stoichiometric turnover of high concentrations (>0.1 mM) of *m*-xylene and would not have prevented denitrification (16). Nevertheless, we attempted to determine the degradation of *m*-xylene not only in the complete absence of molecular oxygen but also with a low redox potential in the inflowing medium; therefore, the system for the continuous removal of oxygen was replaced, and chemically reduced medium was pumped through the column (Table 1). Classical anaerobic techniques (13, 14) were used to reduce the redox potential. The basal medium was first autoclaved then cooled under a nitrogen atmosphere to replace any oxygen. The medium was then supplemented with 0.31 mM *m*-xylene, 8.3 mM sodium nitrate, 0.5 mM sodium sulfide (neutralized to pH 7.5), and 4 μM resazurin. The redox indicator resazurin was colorless in the medium, indicating an E′ of $<-0.11$ V (14). By using the Nernst equation, this redox potential was calculated to correspond to a theoretical oxygen concentration of <1 pM $O_2$ (13, 14).

Under these conditions, *m*-xylene was totally degraded in the presence of nitrate (Table 1), and its mineralization to $CO_2$ was confirmed by using [ring-$^{14}C$]*m*-xylene. $H_2S$ was also oxidized in the column, probably owing to the presence of denitrifying sulfide-oxidizing bacteria (17, 28). An electron balance may be calculated by considering the stoichiometry of all of the electron donors (*m*-xylene and $H_2S$) and electron acceptors ($NO_3^-$) involved in the reaction. The oxidation of 0.31 mM *m*-xylene and 0.5 mM $H_2S$ to $CO_2$ and $SO_4^{2-}$, respectively, yields 17.0 mM electrons. A total of 5 mM $NO_3^-$ was reduced to 1.3 mM $NO_2^-$ and 3.7 mM unidentified gaseous metabolites of the denitrification (NO, $N_2O$, or $N_2$). The reduction of 5 mM $NO_3^-$ to 1.3 mM $NO_2^-$ and NO, $N_2O$, or $N_2$ as the sole gaseous product would require 13.7, 17.4, or 21.1 mM electrons, respectively. $N_2O$ is likely to be the only gaseous product since a strong inhibition of $N_2O$ reductase in the presence of $H_2S$, and, thus, an accumulation of $N_2O$, has been reported previously (16, 25). This electron balance strongly suggests that *m*-xylene was totally degraded under denitrifying conditions in the complete absence of molecular oxygen.

The microorganisms adapted to mineralize *m*-xylene in the column were also able to utilize 0.25 mM toluene as the sole source of carbon and energy under denitrifying conditions. The metabolism started within 24 h of the replacement of *m*-xylene with toluene and was coupled with a reduction of nitrate. A carbon balance with [ring-$^{14}C$]toluene revealed that more than 75% of the injected radioactive carbon was evolved as $^{14}CO_2$ over an 8-day incubation period.

The microbial metabolism of aromatic compounds under anaerobic conditions (denitrifying, sulfate reducing, methanogenic) is fundamentally different from degradation under aerobic conditions. In the presence of molecular oxygen, compounds such as benzoates, phenols, anilines, and aromatic hydrocarbons are initially oxygenated by mono- or dioxygenases and converted to catechols, which are then amenable to ring cleavage and subsequent mineralization (9). In the absence of molecular oxygen, oxygenases are inactive, and only those aromatic compounds with oxygen-containing functional groups (phenols and benzoates) are reportedly mineralized (5, 8, 11, 30). In all cases studied, the aromatic ring is first reduced to a substituted cyclohexane before hydrolytic ring cleavage (8, 30, 31). In some instances, a modification or a removal of substituents (e.g., dehydroxylation and demethoxylation of lignin derivatives [6, 10, 15, 26] or dechlorination of chlorophenols [4] and chlorobenzoates [24]) preceeds reduction of the ring. Unlike phenols and benzoates, however, aromatic hydrocarbons have no such activating substituent groups which facilitate hydration of the ring. Whether the anaerobic degradation of *m*-xylene reported in this paper involves an initial reduction of the ring, an oxidation of the ring to a substituted phenol, or perhaps an oxidation of the methyl group to an alcohol is presently unknown. Recent studies by Grbić-Galić (personal communication), in which traces of phenols and cresols were detected in methanogenic cultures growing in the presence of benzene and toluene, respectively, indicate an oxidation of the ring. It also remains to be investigated whether the primary reaction is catalyzed by an enzymatic system similar to that involved in the anaerobic metabolism of methane (1, 32) or unsaturated linear hydrocarbons (22).

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