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Utilization of Alkylbenzenes during Anaerobic Growth of Pure Cultures of Denitrifying Bacteria on Crude Oil

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Four pure cultures of denitrifying bacteria, which had previously been isolated on defined alkylbenzenes, were capable of anaerobic growth with crude oil as the only source of organic substrates. Chemical analyses after growth revealed that the known growth substrates toluene, ethylbenzene, and *m*-xylene were selectively consumed from the oil. *o*-Xylene and *p*-xylene, which as pure compounds did not support growth, were consumed to a lesser extent.

Benzene, toluene, ethylbenzene, and xylenes (together abbreviated as BTEX) are natural constituents of crude oil (21, 24). Leakage from oil pipelines (26) and underground fuel tanks (25) may result in contamination of soils and deeper horizons. Even though the equilibrium partitioning of BTEX between oil and water is largely on the side of the hydrophobic phase, BTEX exhibit a certain water solubility that is higher than that of other oil hydrocarbons (5). Due to their solubility and toxicity (3, 13), BTEX belong to the groundwater contaminants of major concern. The frequent occurrence of anoxic conditions in subsurface waters invokes an interest in bacteria that may degrade BTEX without molecular oxygen. Among BTEX, toluene has been studied most intensively as a substrate, mostly in anaerobic pure cultures of denitrifying (1, 4, 10, 11, 17, 22), iron(III)-reducing (15), and sulfate-reducing (16) bacteria. Two denitrifiers isolated with toluene also used *m*-xylene (4, 11). Anaerobic degradation of benzene (6, 14), *o*-xylene (7, 8, 20), and *p*-xylene (12) was shown in enriched bacterial communities but not in pure cultures. Recently, we reported two new denitrifying bacteria, strains EbN1 and PbN1, that utilized ethylbenzene and propylbenzene, respectively; in the same study, two other strains (ToN1 and mXyN1) were isolated on toluene and *m*-xylene, respectively (17). The four isolates were the first cultures of denitrifying bacteria shown to grow anaerobically with crude oil as the sole source of organic substrates. Specific consumption of toluene from oil was demonstrated with one strain, ToN1. Here, we provide a more detailed study on the growth of all four strains on crude oil and the resulting, strain-specific depletion of alkylbenzenes.

MATERIALS AND METHODS

Bacterial strains and maintenance. The alkylbenzene-degrading, denitrifying strains EbN1, PbN1, ToN1, and mXyN1 have been subcultured in our laboratory since their isolation (17). Stock cultures of the four strains were maintained in chemically defined medium with toluene (strain ToN1), *m*-xylene (strain mXyN1), ethylbenzene (strain EbN1), and propylbenzene (strain PbN1) as the only organic substrates; these substrates were dissolved in heptamethylnonane as an inert carrier phase (17). To guarantee strictly anoxic conditions, freshly prepared sodium ascorbate (4 mM) was added as a reductant. In media with ascorbate and nitrate but without addition of an organic substrate, neither growth nor nitrate reduction was observed, as in medium without ascorbate and organic substrate. This showed that ascorbate did not serve as a growth substrate.

Growth experiments with crude oil. For growth experiments with crude oil, the same defined medium with ascorbate as a reductant was used as for maintenance of bacterial strains. North Sea crude oil from an oil tank was kindly provided by J. Fischer, Wilhelmshaven, Germany. Oil was deaerated, autoclaved, and stored in a special 300-ml flask (Fig. 1). The applied procedure of deaeration and autoclaving did not cause a measurable change in the hydrocarbon composition of the crude oil, as shown by gas chromatographic analyses of the untreated and the anoxic, sterile oil. Flat glass bottles (500 ml) were provided with 400 ml of medium and 6 to 15 ml of anoxic, sterile crude oil. Each bottle was sealed anoxically under an atmosphere of N_2-CO_2 (90:10 [vol/vol]) with a black rubber stopper that was fixed by means of a screw cap with an open top. The inocula (5% [vol/vol]) were injected through the rubber stoppers with sterile N_2-flushed syringes. During inoculation and incubation, the stoppered orifices of the bottles were always kept below the surface level of the medium. In this way, adsorption of oil in direct contact with the stoppers was avoided. The cultures were incubated horizontally on a rotary shaker (70 rpm) at 28°C (16).

Unlike a recently described, alkane-degrading, sulfate-reducing bacterium (20), the presently examined denitrifiers grew homogeneously in the medium; they neither attached to nor emulsified the oil. Samples from the aqueous phase were withdrawn from the inverted bottles through the stoppers with sterile, N_2-flushed syringes. Microscopically, cells but no oil droplets were detectable in the samples. Hence, growth could be monitored by measuring the optical density (OD); the OD (1-cm path) was determined at 660 nm (UV-1202 spectrophotometer; Shimadzu Europe, Duisburg, Germany). Quantification of cell mass with strain EbN1 (17) revealed that an OD of 0.1 corresponded to 31 mg of cell dry mass per liter.

Analytical determinations. Nitrate and nitrite were measured by high-performance liquid chromatography as previously described (17). The detection limit of nitrate and nitrite was 10 μM.

Crude oil was separated by medium-pressure liquid chromatography into an aromatic and an aliphatic fraction (19). The oil fractions were analyzed by gas chromatography and mass spectrometry as previously described (20). The applied method of fractionation and sample processing did not allow an absolute quantification of the particular aromatic hydrocarbons in the oil. Changes of alkylbenzenes were determined relative to naphthalene (20). Naphthalene yielded a prominent peak (outside the range of the chromatograms shown in Fig. 3) and belonged to those aromatic hydrocarbons which exhibited the same ratios of peak areas in all samples. Hence, naphthalene was considered an oil constituent that was not consumed under the given conditions. From each growth experiment, three oil samples were analyzed; they always yielded the same consumption pattern. The isomers of xylene were separated with a type 3700 gas chromatograph (Varian, Darmstadt, Germany) on an FFAP fused silica capillary column (50 m; internal diameter, 0.2 mm; coating, 0.22 μm). The temperature program was run from 70°C (5-min isotherm) to 200°C at 3°C/min. Oil analyses were kindly performed by H. Wilkes and H. Willsch, Jülich, Germany.

RESULTS AND DISCUSSION

When strains ToN1, mXyN1, EbN1, and PbN1 were transferred from media with toluene, *m*-xylene, ethylbenzene, or *n*-propylbenzene, respectively, to medium with crude oil, anaerobic growth started within 4 days. Because nutritional characteristics had been previously determined (17), all four strains were expected to grow on crude oil at the expense of alkylbenzenes. Furthermore, the pattern of alkylbenzene utilization from crude oil should reflect the strain-specific capacities determined with single compounds (17).

To verify that each strain demonstrated a specific alkylben-

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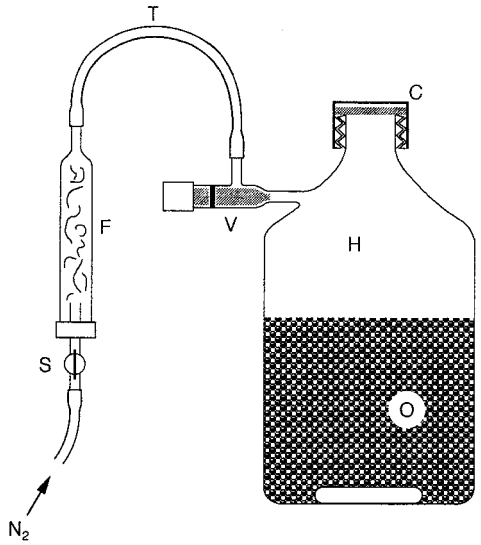

FIG. 1. Special flask for storage of anoxic, sterile crude oil (O). The cotton filter (F) with flexible tubings (T) was connected to a supply of N_2. The bottle could be completely sealed by means of a vacuum stopcock (V) with a Teflon stem and a screw cap (C) with a Teflon-coated rubber disk. Hence, the crude oil and its vapor in the closed system were only exposed to glass and Teflon, respectively. In this way, loss of volatile crude oil constituents due to evaporation through or adsorption to rubber during autoclaving and storage could be avoided. After addition of crude oil, the headspace (H) was briefly (1 to 2 min) flushed with N_2. Then the oil was magnetically stirred in the closed bottle under N_2 for a long period (6 h). Thereafter, the headspace was again briefly flushed with N_2 while stirring was turned off. This deoxygenation procedure was repeated four times. The anoxic crude oil was sterilized by autoclaving under the anoxic atmosphere in the tightly closed bottle. When the cooled bottle was opened in order to take a sample with a pipette preflushed with N_2, a gentle stream of N_2 prevented access of air. The time during which the bottle had to be open was kept as brief as possible to prevent loss of volatile compounds; furthermore, the oil was never agitated while the bottle cap was unscrewed. For storage, the bottle, together with the filter and the closed stopcock (S), was disconnected from the gas supply.

zene utilization pattern as predicted, a defined oil-to-nitrate ratio which allowed for both detectable depletion of the alkylbenzene and measurement of growth curves was determined. At a very high oil-to-nitrate ratio, alkylbenzene depletion might be difficult to detect, whereas at a very low ratio growth would be marginal. Parallel growth cultures of each strain with various amounts of oil revealed that the maximum cell density with 10 mM nitrate was reached by strain ToN1 with around 21 ml (the initially reported value of 13 ml [17] had to be revised) and by strains mXyN1 and EbN1 with around 14 ml of crude oil per liter of medium. Growth curves with these amounts of oil in subsequent experiments for quantitative analyses are shown in Fig. 2. Strain PbN1 grew only poorly on crude oil; growth always ceased at an OD of about 0.04 (data not shown), even if more oil was added than for the other strains. With the same inoculum size, growth of all four strains on crude oil took approximately twice as long as on the pure alkylbenzenes. As already observed with pure substrates (17), strain ToN1 exhibited the fastest growth of the four isolates on oil. Strain ToN1 was the only one that did not produce detectable concentrations of nitrite. No growth occurred in nitrate-free controls. In sterile controls, the nitrate concentration stayed constant and no nitrite formation was observed.

Growth resulted in strain-specific changes in the gas chromatographic pattern of the aromatic oil fraction compared with the original oil and oil from nitrate-free or cell-free controls. Comparison of original oil with that from the control experiments did not reveal detectable abiotic loss of hydrocarbons due to unspecific adsorption by the stopper. The results obtained with the strains that grew well, ToN1, mXyN1, and EbN1, are shown in Fig. 3. The aliphatic fraction of the crude oil always remained unchanged (data not shown). The utilization of alkylbenzenes from crude oil by strains ToN1, mXyN1, and EbN1 was in agreement with their substrate spectra determined with pure compounds (17). Whereas strain ToN1 utilized only toluene, strains mXyN1 and EbN1 utilized *m*-xylene and ethylbenzene, respectively, in addition to toluene from the crude oil.

Strains ToN1 and mXyN1 completely consumed the electron acceptor (Fig. 2A and B) and left only a minor part ($\leq$10%) of the utilizable alkylbenzenes. This showed that growth was limited by nitrate and that the electron acceptor-to-donor ratio was close to a stoichiometric balance. The final ODs (at 660 nm) reached by strains ToN1 and mXyN1 after complete reduction of 10 mM nitrate were around 0.3 and thus somewhat lower than the ODs (0.44 and 0.35, respectively) measured with pure hydrocarbons and the same nitrate concentration. One may speculate that energy conservation or cell synthesis was slightly affected by oil components other than the utilized alkylbenzenes. Strain EbN1 seemed to be somewhat

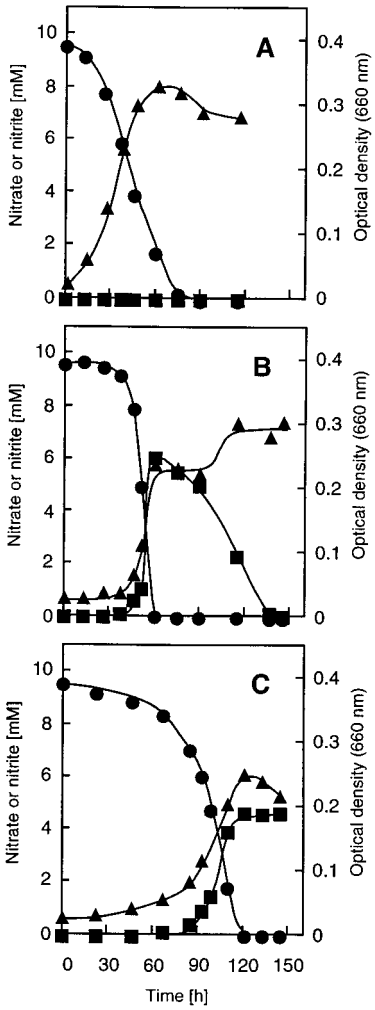

FIG. 2. ODs at 660 nm of cells (▲) and concentrations of nitrate (●) and nitrite (■) during anaerobic growth of strains ToN1 (A), mXyN1 (B), and EbN1 (C) with crude oil as the only source of organic substrates.

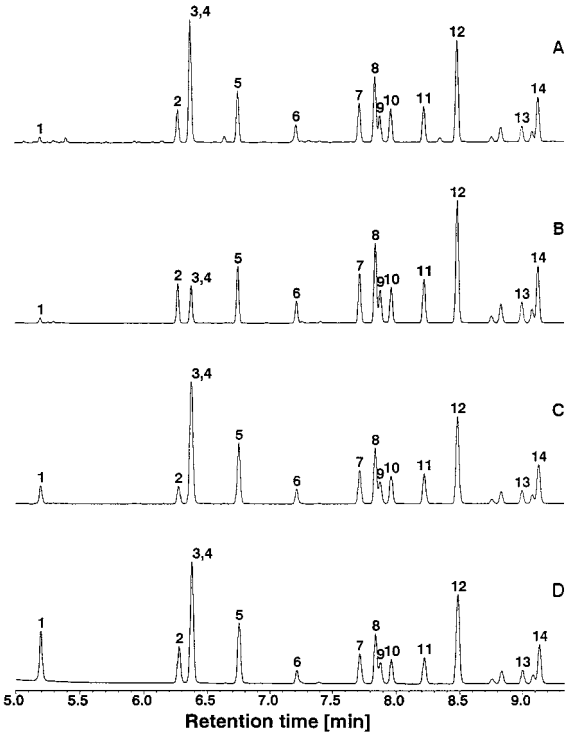

FIG. 3. Gas chromatograms of the aromatic hydrocarbon fraction of crude oil after anaerobic growth of denitrifying strains ToN1 (A), mXyN1 (B), and EbN1 (C). In addition, a chromatogram of the anoxic, sterile original oil is shown in panel D. Samples for oil analyses were taken at the end of the experiments depicted in Fig. 2. Structural assignment of the indicated peaks is based on comparison of their gas chromatographic and mass spectrometric characteristics with those of authentic standards: 1, toluene; 2, ethylbenzene; 3,4, *p*-xylene and *m*-xylene (not separated); 5, *o*-xylene; 6, isopropylbenzene; 7, propylbenzene; 8, *m*-ethyltoluene; 9, *p*-ethyltoluene; 10, 1,3,5-trimethylbenzene; 11, *o*-ethyltoluene; 12, 1,2,4-trimethylbenzene; 13, *m*-isopropyltoluene; 14, 1,2,3-trimethyltoluene. The separation of *m*-xylene and *p*-xylene (not shown) was achieved as described in Materials and Methods.

less effective than strains ToN1 and mXyN1 with respect to utilization of alkylbenzenes from oil. Neither the electron acceptor (Fig. 2C) nor the degradable alkylbenzenes (Fig. 3C) clearly limited growth of strain EbN1. Growth ceased at an OD of around 0.25, and nitrite and a significant part of the toluene and ethylbenzene (around 35 and 50%, respectively) were left. Addition of more nitrate or more oil did not lead to higher cell densities (data not shown). With the poorly growing strain PbN1, only utilization of ethylbenzene from crude oil was detectable (data not shown), although in growth tests with pure substances this bacterium grew on ethylbenzene as well as on propylbenzene; strain PbN1 is unable to utilize toluene (17). The inability of strain PbN1 to utilize propylbenzene from crude oil may be due to the lower water solubility of this compound in comparison to ethylbenzene (5) and to ineffective substrate consumption at low concentrations. When added as a pure compound (2% [vol/vol]) to a carrier phase, propylbenzene was not completely consumed in the presence of excess nitrate within an incubation time of 10 days. Also, on pure alkylbenzenes strain PbN1 always exhibited the slowest growth of the four isolates (17). In the growth cultures of strain mXyN1 on crude oil, the content of *o*-xylene and *p*-xylene also decreased substantially (by approximately 30 and 50%, respectively, in comparison to oil from a nitrate-free control). Their partial consumption may be a result of a cometabolic conversion to dead-end products such as succinate or fumarate adducts of *o*-xylene, or *p*-methylbenzoate, as observed in pure cultures of other anaerobic toluene-degrading bacteria (2, 9, 18, 23).

To determine if polar (oxygen-containing) compounds which are naturally present in oil (24) and which are more water soluble than hydrocarbons also contributed to growth, medium without inoculum was shaken under anoxic conditions for 1 week with crude oil. Then, only the aqueous phase was transferred to anoxic culture tubes and inoculated with strains ToN1, mXyN1, and EbN1. However, no visible growth occurred in the separated aqueous phase, whereas cells did grow in parallel tubes to which oil was added again. This supported the assumption that the growth substrates were indeed aromatic hydrocarbons with a partitioning between medium and oil highly favoring the hydrophobic phase.

In summary, strains ToN1, mXyN1, EbN1, and PbN1 are the first pure cultures of denitrifying bacteria demonstrated to grow anaerobically on crude oil by selective utilization of alkylbenzenes. To our knowledge, the ability to consume alkylbenzenes from oil under anoxic conditions was only shown before with an enrichment culture of marine sulfate-reducing bacteria (20). The method of oil analysis applied in this study allowed determination of relative but not of absolute hydrocarbon concentrations. Nevertheless, the amounts of hydrocarbons consumed from the added oil can be estimated from nitrate consumption. Equations for the oxidation of hydrocarbons with nitrate are derived from this general equation: $C_mH_n+[(4m+n)/5]NO_3^-+[(n-m)/5]H^+ \rightarrow mHCO_3^-+[(4m+n)/10]\ N_2 + [3(n-m)/5]\ H_2O$. It is calculated from the particular equations that reduction of 1 mmol of NO_3^- to N_2 (equivalent to a transport of 5 mmol of electrons to nitrate) allows oxidation of approximately 12.8 mg of toluene or 12.6 mg of xylene or ethylbenzene; this is approximately 15 μl for any of these hydrocarbons oxidized per mmol of nitrate reduced. Assuming that strains ToN1 and mXyN1 oxidized only alkylbenzenes from oil, the consumed compounds amount to 0.7 and 1.1% (vol/vol), respectively, of the added oil (21 and 14 ml, respectively, per liter of medium or 10 mmol of nitrate). The consumption of the respective alkylbenzenes by these strains was ≥90%. The calculated values are thus in agreement with literature values (24), according to which the concentration of toluene or xylenes in oil can be around 1%. In the culture of strain EbN1, a residual nitrite concentration of 4.5 mM remained from 10 mM nitrate; the consumption of alkylbenzenes from the oil in this culture was, therefore, 0.8% (vol/vol). The strain consumed around 65% of the toluene and 50% of the ethylbenzene.

Anaerobic degradative capacities such as those studied here with four denitrifying strains contribute to our understanding of the potentials and limits of anaerobic bacteria in decontamination of polluted anoxic underground aquifers. On the one hand, the compounds utilized by the investigated strains belong to the most water-soluble oil hydrocarbons and hence to the main potential groundwater pollutants at sites contaminated with oil or gasoline. On the other hand, it has to be kept in mind that even by a combined action of anaerobes with the degradative capacities of the four isolates the aromatic compounds oxidized from oil under anoxic conditions would represent only a small fraction of the total oil.

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REFERENCES

1. **Altenschmidt, U., and G. Fuchs.** 1991. Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch. Microbiol. **156:** 152–158.
2. **Biegert, T., and G. Fuchs.** 1995. Anaerobic oxidation of toluene (analogues) to benzoate (analogues) by whole cells and by cell extracts of a denitrifying *Thauera* sp. Arch. Microbiol. **163:**407–417.
3. **Dean, B. J.** 1985. Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. Mutat. Res. **154:**153–181.
4. **Dolfing, J., J. Zeyer, P. Binder-Eicher, and R. P. Schwarzenbach.** 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. **154:**336–341.
5. **Dunn, W. J., III, J. H. Block, and R. S. Pearlman.** 1986. Partition coefficient, determination and estimation. Pergamon Press, New York.
6. **Edwards, E. A., and D. Grbić-Galić.** 1992. Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. Appl. Environ. Microbiol. **58:**2663–2666.
7. **Edwards, E. A., and D. Grbić-Galić.** 1994. Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl. Environ. Microbiol. **60:** 313–322.
8. **Edwards, E. A., L. E. Wills, M. Reinhard, and D. Grbić-Galić.** 1992. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Appl. Environ. Microbiol. **58:**794–800.
9. **Evans, P. J., W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young.** 1992. Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial step of toluene mineralization. Appl. Environ. Microbiol. **58:**496–501.
10. **Evans, P. J., D. T. Mang, K. S. Kim, and L. Y. Young.** 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. **57:**1139–1145.
11. **Fries, M. R., J. Zhou, J. Chee-Sanford, and J. M. Tiedje.** 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. **60:**2802–2810.
12. **Häner, A., P. Höhener, and J. Zeyer.** 1995. Degradation of *p*-xylene by a denitrifying enrichment culture. Appl. Environ. Microbiol. **61:**3185–3188.
13. **Koch, R., and B. O. Wagner.** 1989. Umweltchemikalien: physikalisch-chemische Daten, Toxizitäten, Grenz- und Richtwerte, Umweltverhalten. VCH Verlagsgesellschaft, Weinheim, Germany.
14. **Lovley, D. R., J. D. Coates, J. C. Woodward, and E. J. P. Phillips.** 1995. Benzene oxidation coupled to sulfate reduction. Appl. Environ. Microbiol. **61:**953–958.
15. **Lovley, D. R., and D. J. Lonergan.** 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl. Environ. Microbiol. **56:**1858–1864.
16. **Rabus, R., R. Nordhaus, W. Ludwig, and F. Widdel.** 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. **59:**1444–1451.
17. **Rabus, R., and F. Widdel.** 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. **163:**96–103.
18. **Rabus, R., and F. Widdel.** 1995. Conversion studies with substrate analogues of toluene in a sulfate-reducing bacterium, strain Tol2. Arch. Microbiol. **164:** 448–451.
19. **Radke, M., H. Willsch, and D. H. Welte.** 1980. Preparative hydrocarbon group type determination by automated medium pressure liquid chromatography. Anal. Chem. **52:**406–411.
20. **Rueter, P., R. Rabus, H. Wilkes, F. Aeckersberg, F. A. Rainey, H. W. Jannasch, and F. Widdel.** 1994. Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. Nature (London) **372:**455–458.
21. **Schmidt, K. H., and I. Romey.** 1981. Kohle, Erdöl, Erdgas—Chemie und Technik. Vogel-Verlag, Würzburg, Germany.
22. **Schocher, R. J., B. Seyfried, F. Vazquez, and J. Zeyer.** 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. **157:**7–12.
23. **Seyfried, B., G. Glod, R. Schocher, A. Tschech, and J. Zeyer.** 1994. Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl. Environ. Microbiol. **60:**4047–4052.
24. **Tissot, B. P., and D. H. Welte.** 1984. Petroleum formation and occurrence, 2nd ed. Springer-Verlag, New York.
25. **U.S. Environmental Protection Agency.** 1986. Underground motor fuel storage tanks: a national survey. Publication NTIS PB 86-216512. U.S. Environmental Protection Agency, Washington, D.C.
26. **Vanlooke, R., R. De Borger, J. P. Voets, and W. Verstraete.** 1975. Soil and groundwater contamination by oil spills; problems and remedies. Int. J. Environ. Studies **8:**99–111.