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NOTES

Roles of Bacterial Attachment and Spontaneous Partitioning in the Biodegradation of Naphthalene Initially Present in Nonaqueous-Phase Liquids

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The mineralization by an *Arthrobacter* sp. of naphthalene initially dissolved in di(2-ethylhexyl)phthalate exhibited a slow phase followed by a rapid phase. Triton X-100, which inhibited cell attachment, prevented the onset of the second phase. Triton X-100 increased the extent of mineralization of naphthalene initially present in 2,2,4,4,6,8,8-heptamethylnonane. Cells attached to the interface mineralized the aromatic hydrocarbon at a rate four times higher than the rate of partitioning in the absence of microorganisms, but this microbial activity was markedly reduced by Triton X-100. We suggest that utilization of naphthalene originally present in nonaqueous-phase liquids may involve a partitioning-limited initial stage carried out by bacteria freely suspended in the aqueous phase and a subsequent, more rapid stage effected by bacteria present directly at the nonaqueous-liquid–water interface.

Hydrophobic organic pollutants are often present at waste disposal sites in nonaqueous-phase liquids (NAPLs), from which the rate of partitioning to water is frequently low. Hence, NAPLs serve as reservoirs for such toxicants. It is not certain whether the biodegradation of hydrophobic organic compounds present in NAPLs is similarly limited by the rate of abiotic partitioning of the pollutant from NAPL to water or whether microorganisms have some mechanism to degrade pollutants at rates higher than the rate of spontaneous partitioning.

In a previous study in this laboratory, it was shown that the rates of biodegradation of phenanthrene in NAPLs were frequently higher than the rate of partitioning from nonaqueous to aqueous phases in the absence of biodegrading microorganisms (2). However, it was not established how microorganisms were acquiring the substrate under these conditions. Hence, a study was conducted not only to compare rates of biodegradation and partitioning but also to establish possible mechanisms by which microorganisms acquire substrates that are present in NAPLs.

A strain of an *Arthrobacter* sp. capable of using naphthalene but not 2,2,4,4,6,8,8-heptamethylnonane or di(2-ethylhexyl) phthalate (DEHP) as a sole carbon and energy source was grown as described by Efroymson and Alexander (3). To measure mineralization, duplicate 40-ml portions of the bacterial suspension were added to 60-ml screw-cap bottles and a glass, open-ended tube (65 mm long; 8-mm internal diameter) was introduced into each bottle. Fifty microliters of DEHP or heptamethylnonane containing 40,000 to 60,000 dpm of [1,4,5,8-^{14}C]naphthalene (4.5 mCi/mmol, radiochemical purity of 97.1%) and sufficient unlabeled substrate to give the desired

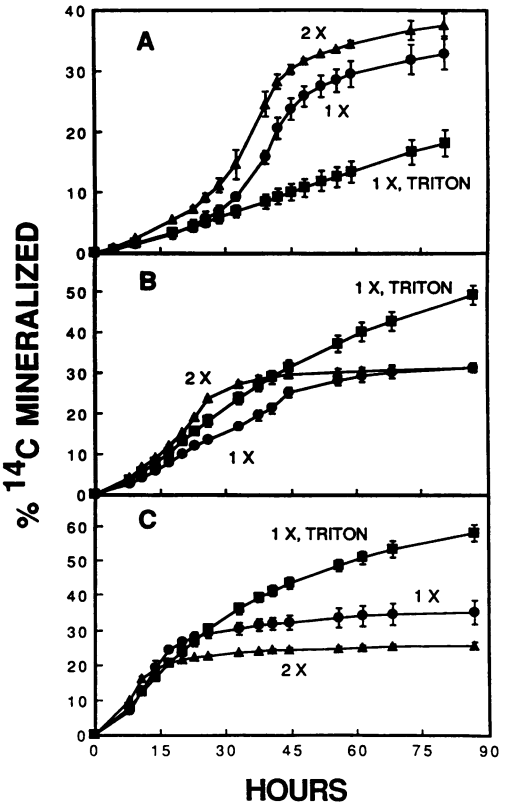

FIG. 1. Effect of NAPL-water interfacial area and Triton X-100 on mineralization by an *Arthrobacter* sp. of naphthalene initially at 1.0 mg/ml of DEHP (A), 1.0 mg/ml of heptamethylnonane (B), or 0.1 mg/ml of heptamethylnonane (C). The inoculum sizes were 3.1×10^8 and 2.4×10^6 cells per ml of water when the NAPL was DEHP and heptamethylnonane, respectively. The relative surface areas are designated 1× and 2×.

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TABLE 1. Rates of partitioning and mineralization of naphthalene initially dissolved in DEHP or heptamethylnonane

Naphthalene concn (mg/ml of NAPL)	NAPL	Treatment	Partitioning rate (ng/ml/h)	Mineralization rate (ng/ml/h)	Mineralization conditions[a]	
					Inoculum (cells per ml, 10^6)	Time[b] (h)
1.0	DEHP	Single surface	3.10	3.03[c]	310	0–28.5 (7)
				13.21[d]	310	28.5–45 (5)
1.0	DEHP	Double surface	4.95[e]	4.47[c]	310	0–25.5 (6)
				14.45[d]	310	25.5–42 (5)
1.0	DEHP	Single surface + Triton	3.26[f]	2.80[c]	310	0–80.5 (17)
1.0	HMN[g]	Single surface	8.27	6.79[c]	0.82	12–33.5 (6)
				12.18[d]	0.82	33.5–45 (2)
				7.62[c]	2.4	8–26 (7)
				12.05[d]	2.4	40–44.5 (2)
				9.34[c]	24	8–23 (6)
				12.07[d]	24	23–33 (3)
1.0	HMN	Double surface	13.83[e]	11.80[c]	2.4	11–20 (4)
				17.86[d]	2.4	20–26 (3)
1.0	HMN	Single surface + Triton	10.20[h]	10.69[c]	0.82	9–22 (4)
				10.43[c]	2.4	11–23 (5)
0.1	HMN	Single surface	0.80	0.75[c]	0.82	3–12 (4)
				2.35[d]	0.82	15–27 (3)
				1.78[d]	2.4	0–17 (5)
				2.64[d]	24	8–11 (2)
0.1	HMN	Double surface	1.33[e]	1.85[d]	2.4	8–14 (3)
0.1	HMN	Single surface + Triton	1.06[i]	1.00[c]	0.82	3–12 (4)
				1.48[d]	0.82	12–29.5 (6)
				1.48[d]	2.4	8–27 (7)

[a] Conditions in the period during which mineralization was measured.
[b] The interval during which the rate of mineralization was calculated. The values in parentheses are the number of points used for calculation of the rate.
[c] Not statistically different from partitioning rate at $P = 0.05$.
[d] Statistically greater than partitioning rate at $P = 0.05$.
[e] Statistically greater than partitioning rate with a single surface at $P = 0.05$.
[f] Not statistically different from partitioning rate without Triton at $P = 0.05$.
[g] HMN, heptamethylnonane.
[h] Statistically greater than partitioning rate without Triton at $P = 0.10$.
[i] Statistically greater than partitioning rate without Triton at $P = 0.05$.

concentration were added to the surface of the aqueous phase inside the tube. When the interfacial area was increased twofold, 25 μl of the same solution of substrate in NAPL was added to each of two tubes. A test tube (10 by 75 mm) containing 2.0 ml of 0.5 M NaOH was also introduced upright into the bottle. Because of the relatively small headspace above the water (about 15 ml), naphthalene loss by volatilization was small. The bottles were closed with Teflon-lined screw caps and incubated at 22 ± 3°C on a rotary shaker operating at 85 rpm. Naphthalene mineralization was determined as described previously (3).

The mineralization of 1.0 mg of naphthalene per ml of DEHP is shown in Fig. 1A. Mineralization was different when the NAPL-water interfacial area was changed or when Triton X-100 was added to give 0.1% in the aqueous phase. Doubling the interfacial area increased the initial rate and the extent of mineralization. However, the maximum rates were not statistically different ($P = 0.05$). Triton X-100 (0.1%) did not affect the initial phase of mineralization, but the subsequent increase in rate of transformation did not occur if the medium contained the surfactant. In the presence of Triton X-100, mineralization was linear ($r = 0.999$) during the entire test period. The rate of mineralization of 1.0 mg of naphthalene per ml of heptamethylnonane was also affected by the interfacial area (Fig. 1B). The mineralization rate was increased by doubling the interfacial area, although the amounts of naphthalene mineralized were identical. Triton X-100 (0.1%) stimulated the rate of biodegradation of naphthalene initially dissolved in heptamethylnonane ($P = 0.05$), and the surfactant also increased the extent of mineralization. When the naphthalene concentration in heptamethylnonane was reduced 10-fold, its mineralization was still rapid, but doubling the interfacial area reduced the amount of substrate mineralized (Fig. 1C). As with the higher naphthalene concentration, Triton X-100 increased the amount of substrate mineralized but not the rate of biodegradation.

The mass transfer of naphthalene from the NAPL to water was measured to determine whether the rate of partitioning of the substrate to the aqueous phase is the rate-limiting factor for biodegradation. The methods previously described were used to measure naphthalene concentrations in the aqueous phase at different intervals after the addition of the NAPL to uninoculated bottles, with each partitioning rate usually involving measurements of naphthalene concentration at 10 time

points. The concentrations increased linearly with time in the first 15 h ($r > 0.96$ in all cases). The measured rates of partitioning are shown in Table 1. These rates for the abiotic process are compared with the initial and maximum mineralization rates. The amount of substrate mineralized was calculated from the quantity of CO_2 produced. The partitioning of naphthalene initially dissolved at two concentrations in DEHP or heptamethylnonane was enhanced by doubling the interfacial area. The addition of 0.1% Triton X-100 had no effect on the rate of partitioning of naphthalene from DEHP, but it increased somewhat the rate of partitioning of 1.0 and 0.1 mg of naphthalene per ml of heptamethylnonane. During the initial phase of biodegradation of either concentration of naphthalene provided in DEHP or heptamethylnonane, the mineralization rates were not significantly different from the measured partitioning rates. However, except when the surfactant was present, the mineralization rates increased with time and the later rates were higher than the measured rates of partitioning. Hence, during these latter stages of the transformation, the organisms must have acquired the substrate by a mechanism other than one relying on spontaneous partitioning from the NAPL to the aqueous phase.

Bacterial growth at the interface of the NAPL and water was observed macroscopically, especially when DEHP was used. After the culture was centrifuged for 10 min at 10,000 × *g* to remove bacteria suspended in the aqueous phase, the NAPL-water interface was microscopically observed and found to be coated with a continuous film of cells and extracellular polymeric substances.

The possible role of the adhering cells was investigated by measuring mineralization by bacteria attached to DEHP. Naphthalene was initially present to give a concentration of 1.0 mg/ml of DEHP. The method involved removing the NAPL from the culture and freeing it of most of the suspended cells. This was accomplished by removing the tube containing the NAPL drop and the underlying aqueous phase (approximately 2 ml) from the bottle. Before removal, the tube was closed at the upper end with a cork, which left an air phase (height, ca. 10 mm) above the NAPL. Most of the bacteria not attached to the NAPL were removed from the tube by injecting 5 ml of fresh medium 10 times through the cork and the NAPL with a hypodermic syringe fitted with an 18-gauge needle. The tip of the needle was inside the aqueous phase to a depth of about 10 mm below the NAPL surface. The washing solution was free of visible turbidity. The tube was then introduced into a second bottle containing 38 ml of sterile medium and an NaOH trap. After the cork and the needle were removed, the bottle was closed and naphthalene mineralization was measured. After the NAPL was transferred to fresh medium, mineralization proceeded without a detectable acclimation period (Fig. 2). The maximum activity of the attached cells (13.03 ± 3.14 ng/ml/h) was four times greater than the partitioning rate. In the presence of 0.1% Triton X-100 but under the same conditions otherwise, the mineralization rate was 1.04 ± 0.63 ng/ml/h, which was less than 30% of the partitioning rate.

The results may be explained by postulating the existence of two physiologically different populations. One population consists of bacteria suspended in the aqueous phase whose activity is limited by the partitioning of the substrate to water, and they bring about the initial phase of biodegradation. The second population consists of bacteria growing at the NAPL-water interface, and these bacteria can transform the substrate faster than it appears in the bulk aqueous phase. The precise mechanism by which attached cells acquire their substrates from the NAPL is unknown, but it may involve hydrophobic

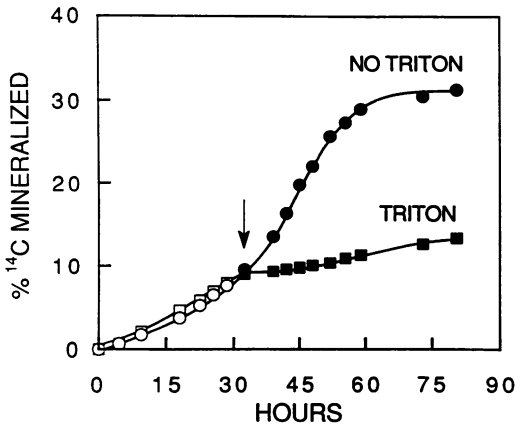

FIG. 2. Mineralization of naphthalene by *Arthrobacter* sp. cells attached to the NAPL in the presence and absence of Triton X-100. The arrow indicates the time when the NAPL was removed from the culture, washed, and transferred to fresh medium. Open symbols represent mineralization before NAPL removal. The initial population density was 3.1×10^8 cells per ml of water.

constituents of the membrane or surfactants associated with the cell surface that facilitate transfer of the molecules into the cell. However, bacteria at the interface are at a microsite in the aqueous phase at which the concentration of substrate is higher than in the bulk water phase. The gradient of substrate concentrations is thus sharper near the cells than at some distance away; this means that the partitioning rate in the microenvironment surrounding the microorganisms may be different from that which was measured.

Triton X-100 inhibits attachment of *Arthrobacter* sp. cells to heptamethylnonane (3). Our results show that Triton X-100 can influence the mineralization of the same substrate by this strain in different ways. With DEHP as the NAPL, the surfactant depressed the degradation, but it stimulated mineralization when the substrate was dissolved in heptamethylnonane. The dissimilar outcomes may be a consequence of the balance between two effects, namely, an increase in the partitioning rate on the one hand and an inhibition of cell attachment on the other.

Efroymson and Alexander (3) found that attachment of this strain of *Arthrobacter* to the NAPL-water interface was necessary for the degradation of hexadecane dissolved in heptamethylnonane, but because of the low water solubility of hexadecane, its partitioning to water could not be detected. Microbial activity at the interface of biphasic aqueous-organic systems has also been observed in the degradation of aromatic and aliphatic hydrocarbons (4–6) and chlorinated and nonchlorinated organic compounds (1). Our results extend those findings and indicate that bacteria at the interface can degrade organic compounds at rates higher than rates of abiotic partitioning.

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REFERENCES

1. **Ascon-Cabrera, M., and J.-M. Lebeault.** 1993. Selection of xenobiotic-degrading microorganisms in a biphasic aqueous-organic system. Appl. Environ. Microbiol. **59:**1717–1724.
2. **Efroymson, R. A.** 1993. Biodegradation of hydrophobic chemicals in nonaqueous phase liquids (NAPLs). Ph.D. thesis. Cornell University, Ithaca, N.Y.

3. **Efroymson, R. A., and M. Alexander.** 1991. Biodegradation by an *Arthrobacter* species of hydrocarbons partitioned into an organic solvent. Appl. Environ. Microbiol. **57:**1441–1447.
4. **Goswami, P., and H. D. Singh.** 1991. Different modes of hydrocarbon uptake by two *Pseudomonas* species. Biotechnol. Bioeng. **37:**1–11.
5. **Rosenberg, M., and E. Rosenberg.** 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. **148:**51–57.
6. **Wodzinski, R. S., and D. Larocca.** 1977. Bacterial growth kinetics on diphenylmethane and naphthalene-heptamethylnonane mixtures. Appl. Environ. Microbiol. **33:**660–665.