centration of the air contaminant for a given value of air changes per hour by the volume of the space of interest. Typically, the air infiltration rate for U.S. residences is between 0.5 and 1.5 air changes per hour (*3*). In rooms without perfect mixing the actual exposures could be increased, while furnishings that are present may act as a sink for the contaminants, thereby reducing the levels of exposure.

Figure 1 shows that the use of convective and radiant kerosene heaters can result in concentrations of SO_2 and NO_2 in excess of the relevant ambient air quality standards, and in levels of CO_2 in excess of the guidelines set by the American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE) and of the occupational health standard for CO_2, even under the conditions of larger room volume and higher infiltration rates. Concentrations of CO may be of concern if a radiant kerosene heater is used in a small room with a moderate ventilation rate. The concentrations of air contaminants shown in Fig. 1 would be added to the concentrations of the contaminants in the infiltration air.

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7. Supported by PHS grant ES-00354.

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Dehalogenation: A Novel Pathway for the Anaerobic Biodegradation of Haloaromatic Compounds

Abstract. *Microorganisms of lake sediment and sewage sludge anaerobically metabolized halobenzoates by a novel pathway. The primary degradative event was loss of the aryl halide without the alteration of the aromatic ring. Dehalogenation required strict anaerobic conditions and depended on the halogen and position, but not the number of halogen substituents. A stable methanogenic bacterial consortium was enriched from sludge and found capable of dehalogenating and often mineralizing a variety of halobenzoates to CH_4 and CO_2. The results suggest that reductive dehalogenation of aromatics could be important in removal of some chlorinated xenobiotics from the environment.*

Halogenated aromatic compounds are pollutants of major concern because they often enter the environment in substantial quantities, are toxic and resistant to degradation, and accumulate in sediments and biota (*1*). Although these compounds are recognized as persistent contaminants, satisfactory methods of degradation are still needed. The biodegradation potential of anaerobic microorganisms in habitats like sediments, sludges, ground waters, and landfills has largely been ignored. We report here a novel pathway for the reductive dehalogenation of aromatic substrates by the anaerobic microflora from these environments.

Halogenated benzoates were chosen as model substrates because they are used as herbicides (Amiben, 2,3,6-trichlorobenzoic acid, and dicamba) and are degradation products of other xenobiotic materials such as pentachlorobenzyl alcohol, Bidisin, and the polychlorinated biphenyls (*2*). There is also a wide range of benzoates with different substituents available which allowed us to characterize the specificity and rates of metabolism.

Anaerobic sediment or sewage sludges were transferred as 100 ml slurries to serum bottles (160 ml) with the use of strict anaerobic techniques (*3*). Substrates (0.2 to 0.8 m*M*) were injected by syringe into bottles; incubation was in the dark at 20°C for sediment and 37°C for the sludges. Parent substrate depletion, the appearance of intermediates, and the net amount of gaseous end products were determined by high-performance liquid chromatography (HPLC) and gas liquid chromatography (GLC) methods (*4*).

Initial screening for complete substrate mineralization to CH_4 and CO_2 indicated that both sediment and sludge possessed the ability to anaerobically metabolize halobenzoates. The organisms responsible for halobenzoate degradation were enriched from sludge by their ability to grow on 3-chlorobenzoate in a mineral salts medium (*3*). The specificity of this dehalogenating bacterial consortium was assayed by measuring substrate depletion after a 4-week incubation period.

The ability of sediment microflora and the enriched sewage consortium to metabolize halobenzoate substrates is summarized in Table 1. Microbes from both habitats degraded mono-, di-, and trihalogenated aromatic compounds. Significant halobenzoate metabolism occurred immediately in the enriched consortium, whereas sediment and sludges often exhibited a lag period before biodegradation proceeded. However, once acclimated to halobenzoate degradation, both sediment and sludge metabolized subsequent substrate additions without a lag.

On the basis of sequence and identity of the intermediates and final products observed (*5*), the primary degradative event for halobenzoates in anoxic environments was the removal of the aryl halides from the aromatic ring (Table 1). This reaction did not occur in sterile sediment, sludge, or enrichment culture, or in acclimated sediment incubated aerobically.

We conclude that dehalogenations of this type are biologically catalyzed and occur only in anaerobic habitats. Anaerobic dechlorination of compounds like DDT and lindane are well known (*6*), but the displaced chlorines are on alkyl rather than aromatic structures. The only previous reports of anaerobic dechlorination of an aromatic ring are for pentachlorophenol and Techlofthalam, which are degraded to a mixture of partially dehalogenated intermediates (*7*). These dehalogenations occur in anaerobic soil but have not been well characterized. In contrast, aerobic metabolism of aromatic compounds can be characterized by (i) direct replacement of the halogen by a hydroxyl group (*8*), (ii) the occasional nonenzymatic loss during NIH shifts (*9*), and (iii) removal of the halogen from the alkyl moiety after cleavage of the ring, which is the most frequently cited case (*10*).

A variety of halogens can be removed from the aromatic ring by dehalogenation (Table 1). Bromo- and iodo- substituents are degraded after a shorter lag time than their chloro- or fluoro- counterparts, which suggests that the Br and I species are more readily dehalogenated. In addition, meta halogens are more susceptible to microbial attack when com-

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pared with the ortho or para isomers (Table 1). This appears to be the case for both single and multiple halogenated substrates.

In previous aerobic studies, persistence is positively correlated with the number of halogens (*11*). This is not apparent for anaerobic metabolism where additional chloro-substituents did not restrict dechlorination at the meta position. Reports on the position of halogens affecting aerobic degradation vary. As noted by Bollag (12), meta-substituted aromatic compounds tend to be more recalcitrant. For halobenzoates, meta-substituted substrates generally seem to be more persistent in aerobic habitats though examples of either ortho-, meta-, or para-substituted halobenzoates being the most persistent of an isomeric series have been noted (*10, 13*). In contrast, our anaerobic results indicate the preferential utilization of meta-substituted halogens.

A stable bacterial consortium was enriched from sludge by its ability to use 3-chlorobenzoate as a sole energy and carbon source. As judged by morphology and autofluorescense, the enrichment consisted of both chemolithotrophic and heterotrophic methanogens, as well as three unidentified nonmotile Gram-negative rods. This consortium has been maintained on 3-chlorobenzoate for more than 2 years with no apparent changes in bacterial composition and ability to dehalogenate aromatic substrates.

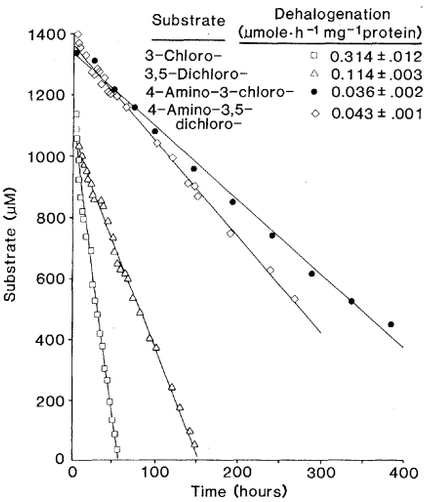
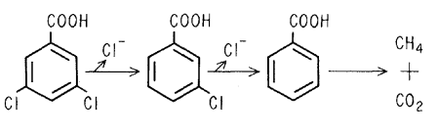


Fig. 1. Zero-order dehalogenation rates exhibited by an enriched methanogenic consortium for several halobenzoates.

The dehalogenation rates for several substrates by the enriched consortium at 37°C (Fig. 1) differed by about an order of magnitude among the various substrates. The enrichment degraded 3-chlorobenzoate relatively rapidly, whereas when exposed to two meta ring substituents (3,5-dichlorobenzoate), the dehalogenation rate decreased by about two-thirds. The introduction of an aryl amino group at the para position to either 3-chlorobenzoate or 3,5-dichlorobenzoate also reduced the relative dehalogenation rate to 11.5 and 13.7 percent, respectively, of that found for the growth substrate, 3-chlorobenzoate.

These data demonstrate the biodegradation potential of haloaromatic substances by anaerobic microbial communities. It remains to be seen whether our observations on the reductive metabolism of halogenated benzoates can be extended to other types of anaerobic habitats and to different classes of aromatic compounds. Considering the many halogenated xenobiotic compounds that are manufactured, more information is needed on this novel bioconversion and on the enzymology and ecology of the requisite microorganisms.

Table 1. Representative pathway and examples of benzoates examined for reductive dehalogenation.



Substrate	Intermediates	End products
Benzoate		CH_4, CO_2
	Monohalogen	
2 or 4-Iodo-		CH_4, CO_2
3-Iodo	Benzoate	CH_4, CO_2
2 or 4-Bromo-	Benzoate	CH_4, CO_2
3-Bromo-	Benzoate	CH_4, CO_2
2 or 4-Chloro-	None	None
3-Chloro-	Benzoate	CH_4, CO_2
3 or 4-Fluoro-	None	None
4-Amino-3-Chloro-		4-Aminobenzoate (C)*
	Dihalogen	
2,4-Dichloro-	None	None
2,5-Dichloro-		2-Chlorobenzoate (C)*
2,6-Dichloro-	None	None
3,4-Dichloro-		4-Chlorobenzoate
3,5-Dichloro-	3-Chlorobenzoate; benzoate	CH_4, CO_2
4-Amino-3,5-dichloro		4-Amino-3-chlorobenzoate (S)*
	4-Amino-3-chlorobenzoate (C)*	4-Aminobenzoate (C)*
	Trihalogen	
2,3,6-Trichloro-		2,6-Dichlorobenzoate

*These compounds were either found as the only metabolic end products after incubation with sediment (S) microorganisms or the enriched bacterial consortium (C).

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3. Pelagic sediment was collected by Eckman dredge from a hypereutrophic lake and mixed with filter-sterilized hypolimnion water inside an anaerobic glovebox (69 mg · ml^{-1}; dry weight). Sludge from municipal digestors was diluted to 10 percent with a sterile anaerobic mineral salts medium [A. J. B. Zehnder and K. Wuhrman, *Arch. Microbiol.* **111**, 199 (1977)].
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Rhodamine-123 Selectively Reduces Clonal Growth of Carcinoma Cells in vitro

Abstract. *Rhodamine-123, a cationic laser dye, markedly reduced the clonal growth of carcinoma cells but had little effect on nontumorigenic epithelial cells in vitro. This selective inhibitory effect of Rhodamine-123 on some carcinomas is unusual since known anticancer drugs, such as arabinosyl cytosine and methotrexate, have not been shown to exhibit such selectivity in vitro.*

The fluorescent dye Rhodamine-123 (Rh-123) selectively accumulates in the mitochondria of living cells (*1*). The specific accumulation of this dye appears to depend on its cationic and permeant properties and on the high electric potential (inside negative) across the mitochondrial membrane (*2, 3*). Although all living cells we have examined thus far accumulate Rh-123 in their mitochondria, cell types differ in their ability to retain Rh-123 in dye-free medium (*3, 4*). We found that most carcinoma cells retain Rh-123 for 2 to 5 days when they are incubated in dye-free medium, whereas nontumorigenic epithelial cells and tumorigenic or nontumorigenic cells of fibroblastic, neural, or hematopoietic origin release the dye within 1 to 16 hours (*4*). It may be possible to exploit the difference in Rh-123 retention between carcinoma cells and other cell types for cancer chemotherapy (*5*). In the study described herein, we compared the effects of Rh-123 treatment on the clonal growth of carcinoma cells and nontumorigenic epithelial cells in vitro.

For these experiments we used MB 49 cells, a mouse bladder epithelial line transformed by 7,12-dimethylbenz[*a*]anthracene (DMBA) (*6*). These cells are highly tumorigenic and retain a significant amount of Rh-123 in their mitochondria for 4 days when they are incubated in dye-free medium. In contrast, primary cultures of normal bladder epithelial cells lose Rh-123 fluorescence within 2 hours (*4*). To determine whether this difference in retention results in greater inhibition of clonal growth of MB 49 cells than of normal mouse bladder epithelial cells, we treated these cells grown in vitro with Rh-123 and assayed their colony-forming ability. Exposure to Rh-123 (10 μg/ml) for 24 hours had a minimal effect on the colony-forming units (CFU) of normal mouse bladder epithelial cells (92 percent of control) (Fig. 1A), but markedly reduced the CFU of MB 49 cells (4 percent of control). The effect of Rh-123 on MB 49 cells depended on the concentration and duration of exposure. Even 6 hours of exposure to 10 μg of Rh-123 per milliliter reduced the CFU to 45 percent of control, whereas such treatment had no significant effect on the CFU of normal bladder epithelial cells.

We then compared the reductions in CFU of EJ cells (a human bladder carcinoma line), MB 49 cells, and normal mouse bladder epithelial cells that had been exposed for 24 hours or continuously to different concentrations of Rh-123 during the 2-week period of clonal cell growth (Fig. 1, B and C). Continuous exposure of normal mouse bladder epithelial cells to Rh-123 (10 μg/ml) had only a small effect on CFU. However, both EJ and MB 49 cells were susceptible to the inhibitory effects of Rh-123. Colony formation in these cells was reduced to 50 percent of control after 24 hours of exposure to 2 to 5 μg of Rh-123

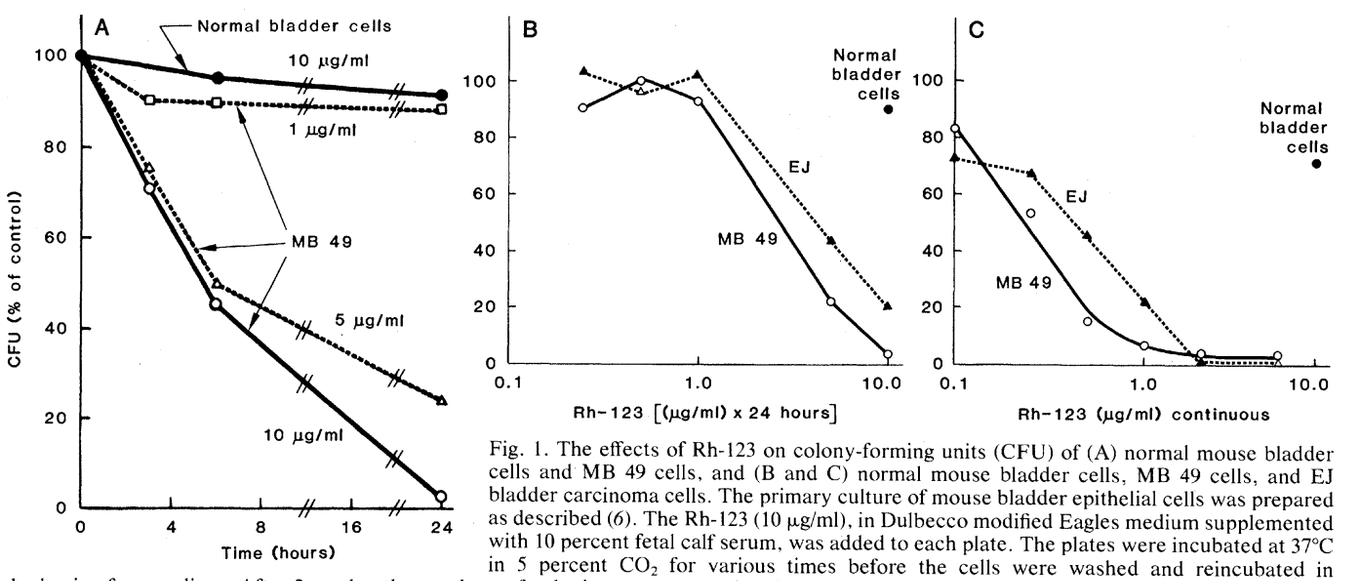

Fig. 1. The effects of Rh-123 on colony-forming units (CFU) of (A) normal mouse bladder cells and MB 49 cells, and (B and C) normal mouse bladder cells, MB 49 cells, and EJ bladder carcinoma cells. The primary culture of mouse bladder epithelial cells was prepared as described (*6*). The Rh-123 (10 μg/ml), in Dulbecco modified Eagles medium supplemented with 10 percent fetal calf serum, was added to each plate. The plates were incubated at 37°C in 5 percent CO_2 for various times before the cells were washed and reincubated in rhodamine-free medium. After 2 weeks, the numbers of colonies were counted and the results were expressed as percentages of control, with control plates normalized to 100 percent CFU. The standard error for triplicate samples was 3 to 5 percent. The MB 49 cells (a mouse bladder epithelial cell line transformed with DMBA) and EJ cells (human bladder carcinoma cell line) were plated and treated with different concentrations of Rh-123 for various times before they were washed and reincubated in fresh medium. The CFU (percentage of control) was determined as described above. The standard error for duplicate samples was 5 percent.

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