I. General aspects

Microorganisms and xenobiotic compounds

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1. Xenobiotics as pollutants

Before man started large scale industrial activities, the concentrations of the organic chemicals on the surface of this planet remained more or less constant with biosynthesis and biodegradation being held in equilibrium by the integrated activities of plants, animals and microbes. Today we are faced with certain industrial chemicals that do not readily participate in the global cycles of carbon, nitrogen or sulfur[19]. Such compounds cause problems of disposal and may, if they escape containment, lead to adverse effects on the environment. Chemicals exhibiting transitory or permanent accumulation have been termed 'pollutants' or 'environmental pollutants', expressions which stress their undesirable effects on the environment. In relation to the total volume of organic compounds involved in the carbon cycle the formation of chemical pollutants by human activities is modest: the turnover of organic matter in the carbon cycle by photosynthesis and biodegradation amounts to approximately $2 \cdot 10^{11}$ t per year as compared to world oil production with approximately $3 \cdot 10^{9}$ t per year and to the synthesis of organic chemicals, pollutants and others, with $2 \cdot 10^{8}$ t per year[34,40]. To assess the pollution potential of a particular compound one thus has to consider not only the quantity in which it is released into the environment but also its chemical and toxicological properties. A chemical's *structure* is of primary importance in determining whether it is accumulated or not, while its *concentration* and its *toxicity* determine the environmental impact of accumulation.

Structure. Since mineralization or complete biodegradation of organic chemicals in natural ecosystems is primarily due to microorganisms[2], any structural feature of a chemical precluding or retarding its attack by microbes will lead to accumulation in the environment, i.e. to persistence or recalcitrance of the compound. Recalcitrance of a molecule may be caused by insolubility as in the case of synthetic polymers like polystyrene, polyethylene and polyvinylchloride[25] or it may be due to novel chemical structures, to which microorganisms have not been exposed in evolutionary history. In the latter case the compound is a xenobiotic[40]. The challenge to the evolutionary potential of microbes presented by xenobiotics has facinated microbiologists and has stimulated basic research on experimental enzyme evolution[17,37,57,75]. Studies performed with pure cultures using known segments of microbial metabolism as model systems, have given insight into the genetics and the biochemistry of enzyme acquisition and thereby have provided guidelines for adaptation experiments with xenobiotics. It has indeed been possible, using continuous culture techniques, to select bacteria capable of degrading previously nondegradable xenobiotics. Successful adaptations in the utilization of recalcitrant xenobiotics have been performed with 2,2-dichloropropionate[64], naphthalyl-2-sulphonic acid[13], 2,4,5-trichlorophenoxyacetic acid[44], and with the azo dyes 1-(4′-carboxyphenylazo)-2-naphthol and 1-(4′-carboxyphenylazo)-4-naphthol[49,77]. Since these adaptations required extended periods of cultivation in continuous culture (up to 2 years in some cases) they were performed with mixed cultures under non-sterile conditions. The systems were thus extremely complex to analyze and the molecular events having led to the novel pathways had to remain obscure in most cases. The strongly selective environment of continuous cultures thus has proven effective for obtaining bacteria with novel biodegradative capacities. However, the relative contributions to the evolutionary process of novel genetic information generated by mutation and of preexisting genetic information put together by gene transfer are hard to assess.

Concentration. Hutzinger[40] pointed out that the term 'xenobiotic compound' should not be reserved for compounds with structural features foreign to life but should be used for all 'compounds that are released in any compartment of the environment by the action of man and thereby occur in a concentration in this or another compartment of the environment that is higher than 'natural''. In view of the difficulties often encountered in determining whether a particular compound is exclusively of anthropogenic origin and thereby may represent a xenobiotic in the narrow sense, this wide definition of 'xenobiotic compounds' is justified. Heavy metals brought into soil by sewage sludge or oil that contaminates ground-water are thus examples of natural compounds which have become xenobiotics by increases in concentration above natural levels. The definition of xenobiotics given above implies that any deviation from the natural chemical composition of the ecosphere deserves attention and should be carefully monitored for possible adverse effects on man and/or the environment.

Toxicity. Since about 65,000 chemicals are believed to be in everyday use[56], monitoring the environment for

increased levels of all or even a sizeable proportion of the commercially produced chemicals is not feasible. In order to effectively control pollution, potentially hazardous chemicals have to be identified and their concentration-limit standards in the environment have to be specified. Such as task involves an interdisciplinary approach by toxicologists, epidemiologists, analytical chemists, microbiologists and ecologists. Obviously, toxicity, carcinogenicity and mutagenicity are the most important criteria in evaluating the harmful effects of pollutants. The efforts of the U.S. Environmental Protection Agency in identifying quantitatively significant and potentially dangerous pollutants originally led to the 'Toxic Pollutant List', which contained 65 compounds and classes of compounds[43]. It comprised thousands of individual chemicals and proved too voluminous as a guideline for chemical analyses to be performed on environmental samples and industrial effluents. The Toxic Pollutant List was therefore reduced to the 'EPA List of Priority Pollutants', which encompasses 114 organic compounds plus cyanide, asbestos and 13 metals[43]. A summary of the organic compounds in this list of 129 priority pollutants is presented in table 1.

The significance of priority pollutants in practice was confirmed by a survey of more than 3000 samples of industrial effluents originating in a wide variety of industries. About half of the compounds on the EPA list occurred with sufficient frequency in industrial or combined effluents to warrant broadscale control measures[60]. Microbial treatment is important in the array of treatment technologies available to control priority pollutants and other problem compounds. Since no technology is uniformly applicable to all of the pollutants, specific solutions have to be sought for the waste and pollution problems of particular branches of industry. Microbiological aspects of waste treatment in the chemical industry are discussed in the contribution by Ghisalba[30]. Other applications of microbes in pollution control such as their use in the treatment of effluents from the pulp industry[29] and the removal of metals from industrial effluents by microbes[45,55] are not covered in the present review but have been discussed elsewhere in pertinent articles.

2. *Entry of pollutants into the environment*

Environmental pollutants are defined as chemicals of natural or synthetic origin that are released by man's activity into the environment where they have an undesirable effect on the environment or on man via the environment[40]. The producers of chemicals are often made solely responsible for the entry of chemicals into the environment. This is not justified since the responsibility for the proper disposal of chemicals is handed over with the products to a wide array of consumers. However, to make this shift in responsibilities workable, the producer of hazardous chemicals has to supply, together with his products, instructions for their safe disposal to the consumer. The draft of a Swiss environmental law therefore includes a paragraph specifying the obligation of producers of chemicals to instruct the users accordingly[11]. The dispersion of chemicals on their way to the user often makes their release into the environment inconspicuous and difficult to control. Figure 1 illustrates the major pathways by which chemicals are released from their containment under human control into the ecosphere.

A first route leads directly from the consumer to the environment (pathway 1, fig. 1). It is taken by products such as pesticides and aerosol propellants whose utilization is coupled with their release into soil, water or air. Other chemicals such as certain solvents or 1,2-dichloroethane and 1,2-dibromoethane which are present in gasoline as lead scavenging agents[3] escape containment by the consumer because of their volatility. Finally, a great variety of pollutants are deliberately released into the environment by illegal dumping. It is hard to conceive that biological technologies will manage to check the spread of pollution for which the consumer is responsible. One suggestion to this end, the use of pesticide detoxifying enzymes in agricultural practice, is discussed in the article by Johnson and Talbot[41]. A decrease, however, of pollution caused by product users will be achieved through consumer education and by banning the use of toxic or potentially toxic chemicals.

Other major entry points of pollutants into the environment are effluents from municipal or industrial waste treatment plants and, quantitatively less important, exhausts from waste incineration facilities (pathways 2 and 3, fig. 1). Pollution arising from biological waste treatment plants is due to organic chemicals that are recalcitrant, volatile or insoluble and thus escape degradation in conventional systems. Furthermore, the toxicity of an organic compound may

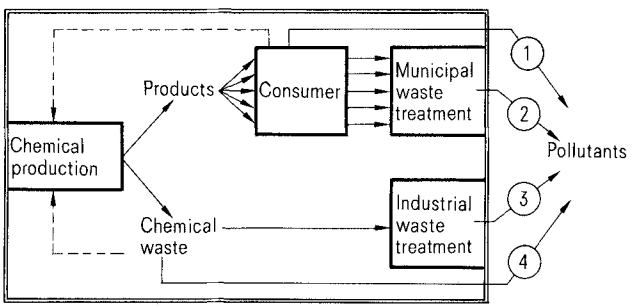

Figure 1. Release of organic chemicals into the environment. *1* Chemicals whose use leads to their entry into the environment, e.g. aerosol propellants, pesticides, fertilizers. *2* Chemicals entering the environment in the effluents of municipal sewage treatment systems, e.g. hard detergents, solvents. *3* Chemicals resistant to biological degradation in industrial waste treatment systems, e.g. chlorobenzenes, aminonaphthol sulfonic acids. *4* Direct discharge, losses, spills and accidents leading to the entry of chemicals from production sites into the environment.

interfere with the proper functioning of the biological treatment and lead to discharge of pollutants. Some organic compounds adsorb during the treatment process onto soil particles or sludge. They do not appear in the effluent but cause problems in sludge disposal and sludge application to land. The problems arising with the treatment of some organic chemicals in conventional biological systems are illustrated by a recent survey on 96 organic compounds from the EPA list of priority compounds (compare table 1)[60,71]: in biodegradability tests performed with these compounds at the 5 mg/l level, using 3 successive subcultures with domestic sewage as the primary inoculum, 50% of the compounds tested were completely, 30% insignificantly or not at all, and 20% partially degraded. One compound exhibited toxic effects and 20% of the compounds tested were concentrated on sludge by factors of 5 to 170.

Since conventional biological treatment procedures are inadequate for the removal of many of the potentially dangerous pollutants, specific technologies for their treatment have to be developed. Industrial waste streams are more suitable for the application of specialized technologies than municipal sewage which may contain low and varying concentrations of a multitude of recalcitrant chemicals originating from household products. The composition of industrial waste waters is usually known and can be controlled. It is also possible to provide individual treatment for the different waste streams of a plant and to prevent the formation of waste mixtures with difficult treatment characteristics. As illustrated in the articles by Finn[26] and by Ghisalba and Küenzi[31,32], microbiology is expected to make significant contributions to the development of new technologies for industrial waste treatment. The controlled degradation of specific problem compounds using specialized microbial cultures and the improvement of waste treatment plants by the addition of adapted microbial strains are promising applications that need to be developed in the future. Technologies based on microbial processes, however, will be in competition with physico-chemical and chemical processes (adsorption, flocculation, chemical oxidation and other transformations, incineration) for the removal of recalcitrant chemicals. These latter processes are usually less specific and need less time to be developed than their biological counterparts. Some of the physico-chemical technologies are readily available while the use of microbial processes will lead to considerable investments for research and development. On the other hand, biological processes consume less energy than physico-chemical processes making them competitive under certain conditions.

Table 1. Organic priority pollutants according to the U.S. Environmental Protection Agency[43]

Chemical class	Number of compounds
Aliphatics	3
Halogenated aliphatics	31
Nitrosamines	3
Aromatics	14
Chloroaromatics (including TCDD)[a]	16
Polychlorinated biphenyls (PCB's)	7
Nitroaromatics	7
Polynuclear aromatic hydrocarbons	16
Pesticides and metabolites (including DDT)[b]	17

[a] TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; [b] DDT = 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane.

Accidents, spills during transportation and leakage from waste disposal sites may lead to the direct entry of untreated chemicals into the environment (pathway 4, fig. 1). The risks to human health presented by dumping sites and their contribution to pollution of air and groundwater are important issues that are under intensive investigation. Potential contributions of microbiology in diminishing the hazards and the damage caused by the discharge of chemicals through these pathways are discussed by Finn[26] and by Johnson and Talbot[41].

3. Microbiology and the pollution problem

The two major applications of microbiological research that are anticipated to become important for pollution control have already been mentioned. They are, excepting improvements to conventional waste treatment plants, the use of specific bioreactors for one family of chemicals, sited between the chemical reactor vessel or the formulating plant and the waste treatment plant; and, preparations of microbial cells or microbial enzymes to treat point sources of contamination in the environment.

Another segment of microbiological research is concerned with the fate of xenobiotics in nature. This is an area of great importance for assessing the environmental impact of chemicals. The scheme presented in figure 2 gives an overview of the types of reactions a xenobiotic can undergo in the environment. Total absence of degradation of a chemical in nature (fig. 2) has not been demonstrated so far, and even TCDD, one of the most persistent chemicals known, has been shown to be metabolized at a low rate by microbial cultures[62]. Indeed, as pointed out by Alexander[2], microorganisms are of primary importance for the changes in the structures of xenobiotics introduced into soil or water.

The various types of microbial transformations of xenobiotics listed in figure 2 have been discussed by Bollag[9]. Complete *mineralization* of a compound is the most desirable of these processes. It generates carbon and energy for microbial growth and leads to the disappearance of the xenobiotic compound. *Cometabolism*[39] is a process by which microorganisms, in the obligate presence of a growth substrate, transform a non-growth substrate. Although cometabolizing organisms do not derive benefits from the meta-

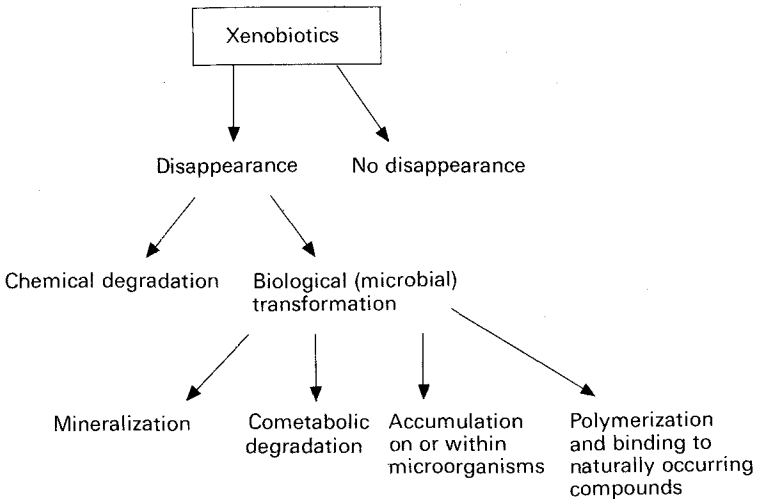

Figure 2. Fate of xenobiotics in the environment.

bolism of non-growth substrates, cometabolism is thought to play a significant role in the degradation of xenobiotics in nature. As pointed out by Harder[35] and by Dalton and Stirling[20] this assumption is difficult to test. Cometabolic transformations in the environment do not necessarily result in the complete oxidation of xenobiotics but may lead to the accumulation of transformation products with increased or decreased toxicity as compared to the original compound[2]. Cellular *accumulation* represents a further type of interaction of microorganisms with xenobiotics. This process which may be the result of active uptake or of adsorption phenomena has usually adverse effects. It may lead to bioconcentration of hazardous chemicals and to their entry into the food chain[8,51]. Microorganisms can favor and microbial enzymes can catalyze the *binding of xenobiotics to soil humus.* The formation, the properties and the implications of xenobiotic-humus complexes are reviewed in the article by Bollag and Loll[10]. Under some conditions the xenobiotics bound to humic substances are persistent while other conditions lead to their release from soil. The question whether or not humus-bound xenobiotics represent a danger to health, and other questions on the complex behavior of soil-bound residues have to be resolved by further research.

To provide microbial strains exhibiting improved biodegradation capacities is one of the most challenging fields of microbiological research related to pollution control. Strains with degradative capacities for heretofore persistent compounds have to be enriched from nature or generated in the laboratory by continuous culture techniques as described in the article of Cook et al.[18]. Strains with improved degradation rates or with a widened range of degradative ability may be constructed by in vivo or in vitro genetic manipulation. Such an approach requires extensive knowledge on the biochemistry of the microbial pathway under investigation. Information on the rate limiting steps in the degradative pathways, on the substrate specificities of the relevant enzymes and on the types of regulatory mechanisms involved in gene expression are prerequisites for a rational approach in strain construction. Examples of this type of research are given in the review by Motosugi and Soda[58] which summarizes the knowledge that has accumulated on the biochemistry of dehalogenation reactions.

For strain constructions to become feasible, biochemical research has to be complemented by studies on the genetics of degradative pathways. The development of genetic techniques for *Pseudomonas* described by Haas[33] provides the tools for manipulating genes among members of the one bacterial genus that exhibits the most varied biodegradative capacities. Considering the large amount of information neccessary before rational and reproducible experiments in strain construction can be performed, it is not surprising that this approach, with the exception of the examples discussed by Haas[33], has not yet been applied on a wide scale in biodegradation reseach. Compared to enrichment from nature and to strain development in the chemostat, assembling degradative pathways from different bacterial strains in one cell by genetic manipulation is a laborious technique by which to obtain organisms with novel degradative capacities. It will find its application in cases where a precise strategy can be formulated and where obstacles to degradation, as for example the intracellular accumulation of dead-end metabolites from problem compounds[47,50], cannot be overcome by cocultivation of strains with different degradative pathways.

4. Microorganisms and halogenated aliphatic hydrocarbons: A case study

The general aspects of the interactions between microorganisms and xenobiotics outlined so far will now be illustrated with a particular class of chemicals,

the halogenated aliphatic hydrocarbons. They represent one of the most important categories of industrial chemicals with respect to production volumes, use categories, dispersion in the environment, toxicological effects and population exposure. The environmental behavior of C1- and C2-halocarbons has recently been reviewed by Pearson[61].

Production, use and distribution in the environment

There are about 150 different halogenated aliphatics in use including chlorinated, brominated and fluorinated compounds. The annual production volumes of the 10 leading compounds of this class are listed in table 2. They are very significant as illustrated by the fact that 1,2-dichloroethane ranked 6th among the organic chemicals produced in the U.S. in 1981[52]. Also listed in table 2 is the input of the commercially important halocarbons into the environment. It is estimated that for those compounds that are mainly used as solvents in a dispersed manner, the entire production capacity is needed to replace losses to the environment. For compounds that serve primarily as intermediates in chemical syntheses, losses into the environment are minimized and represent only a fraction of the production.

Most halogenated aliphatic hydrocarbons found in the environment are thought to be of direct industrial origin. In the case of chloromethane, natural sources such as forest fires and decomposition of seaweeds[54] release 10–100 times more of the compound than manufacture by the chemical industry[23] (compare table 2). Trihalogenated methanes such as chloroform, bromoform and others are found in drinking water. They represent a special case in that they are formed in concentrations up to 50 µg/l by the action of chlorine on naturally occurring organic compounds during the chlorination process[6]. Epidemiological studies suggest, but do not prove, a causal relation between the presence of chlorinated organic contaminants in drinking water and an increase in cancer incidence[7].

The major halocarbons listed in table 2 are used as chemical intermediates or as industrial solvents mainly for degreasing of metal parts, for dry-cleaning of textiles and in the extraction of fats in food processing. Other applications include their use as aerosol propellants (dichloromethane), as lead scavengers in leaded gasoline (1,2-dichloroethane and 1,2-dibromoethane) and as pesticidal fumigants for fruits and vegetables (1,2-dibromoethane)[73] or grain (carbon tetrachloride). The alkyl halides bromoethane, 1,2-dibromoethane, 1,2-dibromo-3-chloropropane and cis- and trans-1,3-dichloropropene are employed as nematocidal soil fumigants[15].

Halogenated aliphatics enter the environment by direct volatilization into the atmosphere from production and use sites or in effluents into which they have been discharged. The major route for their degradation is thought to be by photoinduced tropospheric hydroxyl ion attack[61]. Abiotic destruction of the primary C1- and C2-halocarbons in water is insignificant. The compounds are therefore detected almost anywhere in the environment. In a recent study conducted in the Federal Republic of Germany on the contamination by halogenated aliphatic hydrocarbons, the compounds were found in surface waters (10–100 µg/l, mainly trichloromethane), in drinking water (~ 20 µg/l, mainly trichloromethane), in the air of an urban urea (> 10 µg/m^3, predominantly tetrachloroethane) and in various non-processed (~ 1 µg/kg) and processed (> 10 µg/kg) foods[6]. Since most of the compounds are proven or suspected carcinogens (compare table 2), such data raise toxicological concern. Although it is not now possible to determine the extent to which exposure to halogenated aliphatic hydrocarbons will influence future cancer rates[28], it is clear that human exposure to these agents has to be minimized.

Microbial metabolism

Do microorganisms contribute to the elimination of haloalkanes and haloalkenes from the environment and could they possibly be used in controlled processes for the disposal of these problem com-

Table 2. Production, uses and toxicity of chlorinated aliphatic hydrocarbons

Compound	Synonym	World production[a] (10^6 t/year)	Estimated release into environment[a] (10^6 t/year)	Major uses	Suspected or proven carcinogenicity and/or mutagenicity (reference number)	Swiss MAK-values[b] (ppm)
Chloromethane	Methyl chloride	0.4	5.0	Intermediate	–	50
Dichloromethane	Methylene chloride	0.5	0.5	Solvent	+ (42)	100
Trichloromethane	Chloroform	0.25	0.02	Intermediate, solvent	+ (27)	10
Tetrachloromethane	Carbon tetrachloride	1.0	0.05	Intermediate, solvent	+ (27)	10
Chloroethane	Ethyl chloride	0.4	0.015	Solvent, intermediate	–	1000
1,2-Dichloroethane	Ethylene dichloride	13.0	1.2	Intermediate	+ (3, 21)	20
1,1,1-Trichloroethane	α-Trimethyl chloroform	0.6	0.6	Solvent	–	200
Chloroethene	Vinyl chloride	10.0	0.2	Intermediate in PVC production	+ (5)	2
Trichloroethene	Trichloroethylene	0.6	0.6	Solvent	+ (38, 72)	50
Tetrachloroethene	Perchloroethylene	1.1	1.1	Solvent	–	100

[a] Data from Pearson[61]; [b] Swiss threshold limit values for 40 h/week exposure[63].

pounds? The information available on the metabolism of halocarbons by microbes is summarized in table 3. It is evident that few microbiologists have focused attention on this class of compounds as compared for example to the halogenated fatty acids whose dehalogenation by microbial enzymes is well studied[58]. One reason for this may lie in the fact that halocarbons, because of their volatility and their toxicity for microorganisms, are difficult to handle as microbial growth substrates.

Two principal types of experiments on the microbial metabolism of halogenated aliphatics have been performed, each of them addressing a different environmental question. One approach consisted of setting up soil enrichments and isolating pure cultures utilizing a single halocarbon as the sole source of carbon and energy. This strategy has worked for the few compounds listed as being mineralized in table 3. It has yielded pure bacterial strains, mainly pseudomonads which grow on g/l concentrations of the toxic substrates and exhibit doubling times of 6–10 h on these compounds[14,22,59,67]. These isolates are used for determining the degradative pathways of halocarbons. Such bacteria may also be of interest for the development of specific treatment processes for industrial effluents containing dichloromethane, 1,2-dichloroethane or vinylchloride.

There are also observations which suggest that dichloromethane- and 1,2-dichloroethane-degrading bacteria are actively removing halocarbon contaminants from ground-water. In several cases of ground-water pollution due to mixtures of chlorinated aliphatics (not including vinylchloride) in Germany, the migration of the pollutants in the aquifer was monitored. It was noticed that the leachate plumes of dichloromethane and of 1,2-dichloroethane were consistently smaller than the leachate fields of other halocarbons like chloroform, 1,1,1-trichloroethane, trichloroethene, tetrachloroethene[4,24]. It now is important to prove that the disappearance of the 2 solvents from groundwater was due to biodegradation. If dichloromethane and 1,2-dichloroethane really are degraded in ground-water, they should be given preference for industrial application over other, non-biodegradable chlorinated aliphatic hydrocarbons.

In the 2nd appraoch to study the interaction of microbes with halocarbons, natural microbial communities which previously had not been exposed to halogenated aliphatics were confronted with a range of these xenobiotics. Experiments of this type were guided by the need of understanding the fate of chemicals which are either deliberately released as soil fumigants[16] or, due to inadequate disposal and accidental spills, appear as contaminants of groundwater[12] (table 3).

Since readily utilizable carbon sources were offered to these cultures together with halocarbons, there existed no selective pressure for growth on the halogenated compounds. They thus were transformed at much slower rates than the halogenated aliphatics serving as growth substrates. It is evident from table 3 that a wider range of halogenated substrates was attacked under anaerobic or semi-anaerobic conditions using mixed cultures than under the more stringent pure culture conditions. Because of the toxicity of halocarbons to methanogenic bacteria[74] their concentration in the anaerobic system[12] had to be kept very low, i.e. at 100 µg/l. Thus, while being less efficient than pure culture systems, the mixed culture systems proved more versatile. Furthermore, the anaerobic system prevented volatilization of the added xenobiotics. An understanding of the conditions required for the transformations of halocarbons in mixed cultures under methanogenic conditions may lead to anaero-

Table 3. Microbial metabolism of halogenated aliphatic hydrocarbons

Substrate		Product(s) detected	Organism(s)	Reference number
A) Mineralization				
Dichloromethane	CH_2Cl_2	Cl^-, biomass	*Pseudomonas* sp. *Hyphomicrobium* sp.	14 68
1,2-Dichloroethane	CH_2ClCH_2Cl	Cl^-, biomass	*Pseudomonas* sp.	69
Chloroethene	CH_2CHCl	–	–	22
1,9-Dichlorononane	$CH_2Cl(CH_2)_7CH_2Cl$	Cl^-, biomass	*Pseudomonas* sp.	59
1-Chloroheptane	$CH_3(CH_2)_5CH_2Cl$	Cl^-		
B) Transformation				
Chloromethane	CH_3Cl, CH_3Br	CHOH	*Methylococcus capsulatus*	65, 66
Trichloromethane	$CHCl_3$	CO_2	Methanogenic consortium, anaerobic conditions	12
Tetrachloromethane	CCl_4	CO_2		
1,2-Dichloroethane	CH_2ClCH_2Cl	CO_2		
Tetrachloroethene	CCl_2CCl_2	CCl_2CHCl, CO_2		
1,1,2,2-Tetrachloroethane	$CHCl_2CHCl_2$	$CHCl_2CH_2Cl$, CO_2		
1,2-Dibromoethane	CH_2BrCH_2Br	CH_2CH_2, Br^-	Mixed cultures of soil bacteria, static incubation	16
2,3-Dibromobutane	$CH_3CHBrCHBrCH_3$	$CH_3CHCHCH_3$, Br^-		
1,2-Dibromo-3-chloropropane	$CH_2BrCHBrCH_2Cl$	$CH_3CH_2CH_2OH$, Br^-		

bic processes for the treatment of waste water containing a range of chlorinated aliphatic hydrocarbons[48,76]. Irrespective of whether a halogenated aliphatic compound is used as a carbon source or as a cometabolite, there are 3 general kinds of initial microbial transformation it may undergo: nucleophilic substitution, oxidation, reductive dehalogenation. Examples of each type of reaction can be found among the microbial activities listed in table 3:

Nucleophilic substitution was observed in the bacterial metabolism of dichloromethane. Facultative methylotrophic bacteria utilizing this xenobiotic compound are readily isolated from soil[14,67]. They are also present in waste water treatment plants as shown by the fact that dichloromethane was mineralized by activated sludge that had been acclimated to the compound[46]. Our studies on a dichloromethane-utilizing *Hyphomicrobium* sp. have shown that a single, strongly inducible enzyme was responsible for the conversion of dichloromethane to formaldehyde[68]. This enzyme, a glutathione S-transferase, catalyzed the nucleophilic displacement of chloride yielding S-chloromethyl glutathione as an intermediate. The intermediate was assumed to undergo non-enzymatic hydrolysis to yield S-hydroxymethyl glutathione which decomposes to formaldehyde and reduced glutathione (fig. 3). An analogous conversion of dichloromethane to formaldehyde has been described in rat liver cytosol[1]. There are, however, no reports on other glutathione-dependent microbial dehalogenations. Microbial enzymes catalyzing the direct displacement of halogen on haloalkanes with a hydroxyl group from water have not been described.

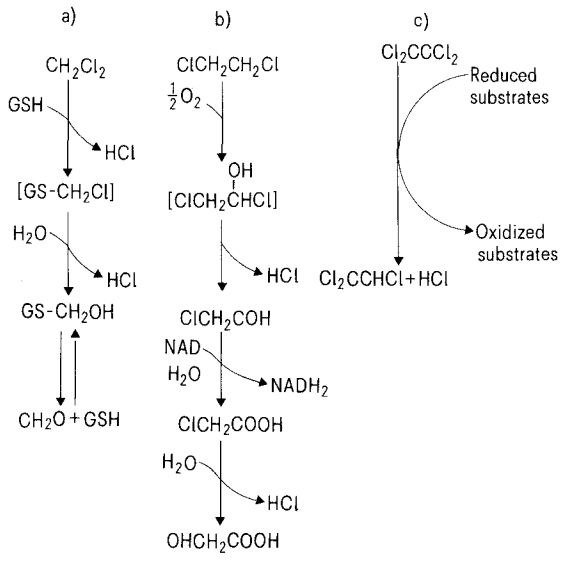

Figure 3. Microbial dehalogenation of chlorinated aliphatic hydrocarbons. Examples of the 3 types of dehalogenation reactions for aliphatic hydrocarbons reported in the literature are presented. *a* Nucleophilic substitution: Dehalogenation of dichloromethane by glutathione S-transferase from *Hyphomicrobium* DM2[68]. *b* Oxidative attack: Hypothetical scheme for the degradation of 1,2-dichloromethane by an unidentified soil bacterium[69]. *c* Reductive dehalogenation: Formation of trichloroethane from tetrachloroethene by a methanogenic consortium[12].

The oxidative attack on halomethanes has been observed with resting cells of *Methanococcus capsulatus* which converted chloromethane[65] and bromomethane[66] to formaldehyde. It was shown that the oxidation was catalyzed by methane mono-oxygenase. Although formaldehyde is an intermediate of carbon assimilation in this organism, the halomethanes were not utilized as growth substrates. This was thought to be due to the toxicity of the halide ions generated during the conversion of the halomethanes to formaldehyde.

An oxidative step is also presumed to initiate the degradation of 1,2-dichloroethane[69]. Since the first step in the degradative pathway (fig. 3b), the oxidative conversion of the substrate to 2-chloroacetaldehyde has not been proven to occur, the scheme presented in figure 3b remains hypothetical.

Reductive dehalogenation is probably responsible for the anaerobic transformation of chlorinated C1- and C2-hydrocarbons (table 3) and for the conversion under conditions that might be termed 'semi-anaerobic' of the brominated alkanes listed in table 3. The process of reductive dehalogenation is thought to involve the transfer of electrons from reduced organic material by microorganisms or by biological catalysts like flavins, porphyrins or cytochromes to halogenated substrates (fig. 3c). The process is reported to occur only in environments with a redox potential (Eh) below −350 mV[48,51]. Reductive dehalogenations on the alkyl structures of the chlorinated pesticides DDT and lindane are well known[51], and a recent report describes the reductive dehalogenation of a number of chlorobenzoates[70]. With the exception of the reductive dehalogenation of 3-chloropropionic acid by resting cells of *Clostridium kluyveri*[36], the experiments on anaerobic dehalogenations of halogenated aliphatics have been performed with mixed cultures. The organisms or the enzyme systems responsible for the anaerobic transformation of these halogenated xenobiotics are thus not known. Since anaerobic microbial communities seem to offer degradative abilities that are not found with aerobic microbes[70] (compare table 3), it will be an exciting task to explore the biochemistry of anaerobic bioconversions and to evaluate their potential in biodegradation.

1 Ahmed, A.E., Kubic, V.L., Stevens, L., and Anders, M.W., Halogenated methanes: metabolism and toxicity. Fedn Proc. *39* (1980) 3150–3155.
2 Alexander, M., Biodegradation of chemicals of environmental concern. Science *211* (1981) 132–138.
3 Ames, B.N., Infante, P., and Reitz, R., Ethylene dichloride: A potential health risk? Banbury report 5, 350 pp. Cold Spring Harbor Laboratory 1980.
4 Baldauf, G., Der Fall Grenzach – Beispiel einer Grundwasserverschmutzung mit umweltrelevanten Stoffen, in: Halogenkohlenwasserstoffe in Grundwässern. DVGW-Schriftenreihe. Wasser Nr. 29, Frankfurt 1981.

5 Bartsch, H., and Montesano, R., Mutagenic and carcinogenic effects of vinyl chloride. Mutation Res. *32* (1975) 93–114.
6 Bauer, U., Belastung des Menschen durch Schadstoffe in der Umwelt. Untersuchungen über leicht flüchtige organische Halogenverbindungen in Wasser, Luft, Lebensmitteln und im menschlichen Gewebe. III. Mitteilung: Untersuchungsergebnisse. Zbl. Bakt. Hyg. I. Abt. Orig. B *174* (1981) 200–237.
7 Bauer, U., Belastung des Menschen durch Schadstoffe in der Umwelt. Untersuchungen über leicht flüchtige organische Halogenverbindungen in Wasser, Luft, Lebensmitteln und im menschlichen Gewebe. IV. Mitteilung: Bilanzierung der Belastung des Menschen durch Organohalogenverbindungen aus der Umwelt. Zbl. Bakt. Hyg. I. Abt. Orig. B *174* (1981) 556–583.
8 Baughman, G.L., and Paris, D.F., Microbial bioconcentration of organic pollutants from aquatic systems – a critical review. CRC Crit. Rev. Microbiol. *8* (1981) 205–228.
9 Bollag, J.M., Transformation of xenobiotics by microbial activity, in: Microbial degradation of pollutants in marine environments, pp. 19–27. Eds A.W. Bourquin and P.E. Pritchard. U.S. Environmental Protection Agency, Gulf Breeze 1979.
10 Bollag, J.M., and Loll, M.J., Incorporation of xenobiotics into soil humus. Experientia *39* (1983) 1221–1231.
11 Botschaft zu einem Bundesgesetz über den Umweltschutz. Art. 24, 1979.
12 Bouwer, E.J., and McCarty, P., Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl. environ. Microbiol. *45* (1983) 1286–1294.
13 Brilon, C., Beckmann, W., Hellwig, M., and Knackmuss, H.J., Enrichment and isolation of naphthalenesulfonic acid-utilizing pseudomonads. Appl. environ. Microbiol. *42* (1981) 39–43.
14 Brunner, W., Staub, D., and Leisinger, Th., Bacterial degradation of dichloromethane. Appl. environ. Microbiol. *40* (1980) 950–958.
15 Castro, C.E., Biodehalogenation. Environ. Health Perspect. *21* (1977) 279–283.
16 Castro, C.E., and Belser, N.O., Biodehalogenation. Reductive dehalogenation of the biocides ethylene dibromide, 1,2-dibromo-3-chloropropane, and 2,3-dibromobutane in soil. Environ. Sci. Technol. *2* (1968) 779–783.
17 Clarke, P.H., Experiments in microbial evolution: new enzymes, new metabolic activities. Proc. R. Soc. Lond. B *207* (1980) 385–404.
18 Cook, A.M., Grossenbacher, H., and Hütter, R., Isolation and cultivation of microbes with biodegradative potential. Experientia *39* (1983) 1191–1198.
19 Dagley, S., Microbial catabolism, the carbon cycle and environmental pollution. Naturwissenschaften *65* (1978) 85–95.
20 Dalton, H., and Stirling, I.D., Co-metabolism. Phil. Trans R. Soc. Lond. B *297* (1982) 481–496.
21 Davidson, I.W.F., Sumner, D.D., and Parker, J.C., Ethylene dichloride: a review of its metabolism, mutagenic and carcinogenic potential. Drug chem. Toxic. *5* (1982) 319–388.
22 De Bont, J.A.M., personal communication.
23 Edwards, P.R., Campbell, I., and Milne, G.S., The impact of chloromethanes on the environment. Part 2: Methyl chloride and methylene chloride. Chem. Ind., Lond. (1982) 619–622.
24 Engesser, W., personal communication.
25 Faber, M.D., Microbial degradation of recalcitrant compounds and synthetic aromatic polymers. Enzyme Microb. Technol. *1* (1979) 226–232.
26 Finn, R.K., Use of specialized microbial strains in the treatment of industrial waste and in soil decontamination. Experientia *39* (1983) 1231–1236.
27 Fishbein, L., Industrial mutagens and potential mutagens. I. Halogenated aliphatic derivatives. Mutation Res. *32* (1976) 267–308.
28 Fishbein, L., Overview of mutagens and carcinogens in the environment, in: 3rd Int. Conf. environ. Mutagens. Eds T. Sugimura, S. Kondo and H. Takebe. Tokyo 1981.
29 Gasche, U.P., The pulp industry's wastes and effluents, in: Microbial degradation of xenobiotics and recalcitrant compounds, pp. 53–63. Eds T. Leisinger, A.M. Cook, R. Hütter and J. Nüesch. Academic Press, New York 1981.
30 Ghisalba, O., Chemical wastes and their biodegradation – an overview. Experientia *39* (1983) 1247–1257.
31 Ghisalba, O., and Küenzi, M., Biodegradation and utilization of monomethyl sulfate by specialized methylotrophs. Experientia *39* (1983) 1257–1263.
32 Ghisalba, O., and Küenzi, M., Biodegradation and utilization of quarternary alkylammonium compounds by specialized methylotrophs. Experientia *39* (1983) 1264–1271.
33 Haas, D., Genetic aspects of biodegradation by pseudomonads. Experientia *39* (1983) 1199–1213.
34 Hall, D.O., Solar energy through biology: fuels from biomass. Experientia *38* (1982) 3–10.
35 Harder, W., Enrichment and characterization of degrading organisms, in: Microbial degradation of xenobiotics and recalcitrant compounds, pp. 77–96. Eds T. Leisinger, A.M. Cook, R. Hütter and J. Nüesch. Academic Press, New York 1981.
36 Hashimoto, H., and Simon, H., Reduktive Dehydrohalogenierung β-halogenierter Fettsäuren und stereoselektive Hydrierung α-halogenierter, α,β-ungesättigter Fettsäuren durch *Clostridium kluyveri*. Angew. Chem. *87* (1975) 111–112.
37 Hegeman, G.D., Acquisition of new metabolic capabilities: what we know and some questions that remain,: in Genetics of Industrial Microorganisms, pp. 263–267. Eds O.K. Sebek and A.I. Laskin. Washington D.C. 1979.
38 Henschler, D., Prüfung von Trichloräthylen auf carcinogene Wirkung bei inhalatorischer Aufnahme und Mechanismen der Bioaktivierung im Stoffwechsel. Bundesministerium für Forschung und Technologie, Forschungsbericht HA 82-007 (1982).
39 Horvath, R.S., Microbial co-metabolism and the degradation of organic compounds in nature. Bact. Rev. *36* (1972) 146–155.
40 Hutzinger, O., and Veerkamp, W., Xenobiotics with pollution potenial, in: Microbial degradation of xenobiotics and recalcitrant compounds, pp. 3–45. Eds T. Leisinger, A.M. Cook, R. Hütter and J. Nüesch. Academic Press, New York 1981.
41 Johnson, L.M., and Talbot, H.W., Detoxification of pesticides by microbial enzymes. Experientia *39* (1983) 1236–1246.
42 Jongen, W.M.F., Alink, G.M., and Koeman, J.H., Mutagenic effect of dichloromethane. Mutation Res. *56* (1978) 245–248.
43 Keith, L.H., and Telliard, W.A., Priority pollutants, a perspective view. Environ. Sci. Technol. *13* (1979) 416–423.
44 Kellogg, S.T., Chatterjee, D.K., and Chakrabarty, A.M., Plasmid-assisted molecular breeding: New technique for enhanced biodegradation of persistent toxic chemicals. Science *214* (1981) 1133–1135.
45 Kelly, D.P., Norris, P.R., and Brierley, C.L., Microbiological methods for the extraction and recovery of metals. Symp. Soc. Gen. Microbiol. *29* (1979) 263–308.
46 Klečka, G.M., Fate and effects of methylene chloride in activated sludge. Appl. environ. Microbiol. *44* (1982) 701–707.
47 Knackmuss, H.J., Degradation of halogenated and sulfonated hydrocarbons, in: Microbial degradation of xenobiotics and recalcitrant compounds, pp. 189–212. Eds T. Leisinger, A.M. Cook, R. Hütter and J. Nüesch. Academic Press, New York 1981.
48 Kobayashi, H., and Rittmann, B.E., Microbial removal of hazardous organic compounds. Environ. Sci. Technol. *16* (1982) 170–183.
49 Kulla, H., Aerobic bacterial degradation of azo dyes, in: Microbial degradation of xenobiotics and recalcitrant compounds, pp. 387–399. Eds T. Leisinger, A.M. Cook, R. Hütter and J. Nüesch. Academic Press, New York 1981.
50 Kulla, H.G., Klausener, F., Meyer, U., Lüdeke, B., and Leisinger, T., Interference of aromatic sulfo groups in the microbial degradation of the azo dyes Orange I and Orange II. Archs Microbiol. *135* (1983) 1–7.
51 Lal, R., and Saxena, D.M., Accumulation, metabolism, and effects of organochlorine insecticides on microorganisms. Microbiol. Rev. *46* (1982) 95–127.
52 Layman, P.L., Big-volume chemicals' output fell again in 1981. Chem. Engng News *60* (1981) 10–16.
53 Leisinger, T., Cook, A.M., Hütter, R., and Nüesch, J., eds, Microbial degradation of xenobiotics and recalcitrant compounds, 415 pp. Academic Press, London, New York 1981.
54 Lovelock, J.E., Natural halocarbons in the air and in the sea. Nature *256* (1975) 193–194.
55 Lundgren, D.G., and Malouf, E.E., Microbial extraction and concentration of metals. Adv. Biotechnol. Proc. *1* (1983) 223–237.
56 Maugh, T.H., Chemicals: how many are there? Science *199* (1978) 162.

57 Mortlock, R.P., Metabolic acquisitions through laboratory selection. A. Rev. Microbiol. *36* (1982) 259–284.
58 Motosugi, K., and Soda, K., Microbial degradation of synthetic organochlorine compounds. Experientia *39* (1983) 1214–1220.
59 Omori, T., and Alexander, M., Bacterial and spontaneous dehalogenation of organic compounds. Appl. environ. Microbiol. *35* (1978) 512–516.
60 Patterson, J.W., and Kodukala, P.S., Biodegradation of... Chem. Engng Progr, April (1981) 48–55.
61 Pearson, C.R., C1- and C2 halocarbons, in: The handbook of environmental chemistry, vol. 3, p. 69. Ed. O. Hutzinger. Springer Verlag, Berlin 1982.
62 Philippi, M., Schmid, J., Wipf, H.K., and Hütter, R., A. microbial metabolite of TCDD. Experientia *38* (1982) 659–661.
63 Schweizerische Unfall-Versicherungs-Anstalt (SUVA). MAK-Werte (1983).
64 Senior, E., Bull, A.T., and Slater, J.H., Enzyme evolution in a microbial community growing on the herbicide Dalapon. Nature *263* (1976) 476–479.
65 Stirling, D.I., and Dalton, H., The fortuitous oxidation and cometabolism of various carbon compounds by whole-cell suspensions of *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. *5* (1979) 315–318.
66 Stirling, D.I., and Dalton, H., Oxidation of dimethyl ether, methyl formate and bromoethane by *Methylococcus capsulatus* (Bath). J. gen. Microbiol. *116* (1980) 277–283.
67 Stucki, G., Brunner, W., Staub, D., and Leisinger, T., Microbial degradation of chlorinated C1- and C2 hydrocarbons, in: Microbial degradation of xenobiotics and recalcitrant compounds, pp. 131–137. Eds T. Leisinger, A.M. Cook, R. Hütter and J. Nüesch. Academic Press, New York 1981.
68 Stucki, G., Gälli, R., Ebersold, H.R., and Leisinger, T., Dehalogenation of dichloromethane by cell extracts of *Hyphomicrobium* DM2. Archs Microbiol. *130* (1981) 366–371.
69 Stucki, G., Krebser, U., and Leisinger, T., Bacterial growth on 1,2-dichloroethane. Experientia *39* (1983) 1271–1273.
70 Suflita, J.M., Horowitz, A., Shelton, D.R., and Tiedje, J.M., Dehalogenation: A novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science *218* (1982) 1115–1117.
71 Tabak, H.H., Quave, S.A., Mashui, Ch.I., and Barth, E.F., Biodegradability studies with organic priority pollutant compounds. J. Wat. Pollut. Control Fed. *53* (1981) 1503–1519.
72 Triebig, G., Neue Aspekte zur Beurteilung einer Einwirkung von Halogenkohlenwasserstoff-Lösemitteln am Arbeitsplatz. Eine Literaturübersicht. Zbl. Bakt. Hyg. I.Abt. Orig. B *173* (1981) 29–44.
73 Walsh, J., Spotlight on pest reflects on pesticide. Science *215* (1982) 1592–1596.
74 Wolfe, R.S., Microbial formation of methane. Adv. Microb. Physiol. *6* (1971) 107–146.
75 Wu, T.T., Experimental evolution in bacteria. CRC Crit. Rev. Microbiol. *6* (1978) 33–51.
76 Yang, J., Speece, R.E., Parkin, G.F., Gossett, J., and Kocher, W., The response of methane fermentation to cyanide and chloroform. Prog. Water Tech. *12* (1980) 977–989.
77 Zimmermann, Th., Kulla, H.G., and Leisinger, T., Properties of purified Orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF 46. Eur. J. Biochem. *129* (1982) 197–203.

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Isolation and cultivation of microbes with biodegradative potential

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1. Introduction

Microorganisms play a crucial role in the natural cycles of elements such as carbon, nitrogen, phosphorus and sulfur[54]. The carbon cycle, for example, refers to the largely photosynthetic fixation of CO_2, which is converted by e.g. plants, herbivores and carnivores into a multitude of complex molecules that are then degraded to CO_2 by fungi and bacteria, and this mineralization is termed biodegradation[54].

The development of enzymes for biodegradation is presumed to be by evolution during the 3×10^9 years that microorganisms have been in the biosphere[54], but this cannot be tested by examination of a fossil record (in contrast to e.g. the evolution of plant or vertebrate morphology) because of the paucity of the known record and the limited structure/function correlation in microorganisms. However, evolution of bacteria can be tested and demonstrated readily in the laboratory (due to their rapid rates of multiplication) and sets of extensive experiments have been done with biodegradative enzymes[13]. Long-term evolution in nature is seen as an on-going process which is presumed to occur not only vertically within a direct descendant pedigree but also horizontally through exchange of genetic information amongst otherwise independent vertical lines of development[4].

Man's purposeful application of biodegradation to clean up his wastes began in the middle of the last century[51] and was developed to a recognizable and functional sewage works early in this century[43]. Sewage works are usually capable of degrading a very wide range of naturally-occurring and synthetic (non-natural, alien or xenobiotic[35]) compounds[65]. But 'hard' detergents, which were not significantly degraded in sewage works and which then foamed in rivers and streams[11], raised public concern. Thus political pressure developed to eliminate these and other *recalcitrant* compounds, compounds that were not degraded in conventional sewage works.

Recalcitrance, however, is not a property like a molecular weight, which has a fixed numerical value; rather, it has become a label to indicate that a