
**Review of scientific literature on microbial
dechlorination and chlorination of
key chlorinated compounds**

1st Quarterly Report

1st Quarter Year 2001

Report prepared for EUROCHLOR

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ACRONYMS

CB	Monochlorobenzene
CF	Chloroform
CM	Chloromethane
CT	Carbon tetrachloride
DBF	Dibenzofuran
1,2-DCA	Dichloroethane
DCB	Dichlorobenzenes
1,1-DCE	1,1-Dichloroethene
1,2-DCE	1,2-Dichloroethene
<i>Cis</i>-DCE	<i>Cis</i> -1,2-dichloroethene
<i>Trans</i>-DCE	<i>Trans</i> -1,2-dichloroethene
DCM	Dichloromethane
E-acceptor	Electron acceptor
E-donor	Electron donor
ETH	Ethene
HCB	Hexachlorobenzene
HCH	Hexachlorohexane
NAPL	Nonaqueous phase liquid
PCBs	Polychlorinated biphenyls
PCE	Tetrachloroethylene
TCB	Trichlorobenzene
TCE	Trichlorethylene
TeCB	Tetrachlorobenzene
TCFE	Trichlorofluoroethene
VC	Vinyl chloride

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1. INTRODUCTION

This report presents a review of scientific literature published during the first three months of 2001 on the microbial chlorination and dechlorination of the following compounds: vinyl chloride, dichloroethane, chloroform, dichloromethane, hexachlorobenzene, chlorobenzene, 1,2,4-1,2,3-1,3,5-trichlorobenzene, hexachlorobutadiene, octachlorostyrene, dioxins and chlorinated furans. In addition, reports regarding the microbial chlorination of compounds structurally related to those listed above were also reviewed. Selected reports published at the end of the year 2000 are also summarized here.

2. SUMMARY OF MOST IMPORTANT DEVELOPMENTS

Microbial dechlorination

- ❖ Anaerobic degradation of vinyl chloride (VC):
 - Reductive cometabolism of VC by a pure culture (*Dehalococcoides ethenogenes*) actively respiring PCE with H₂ as electron donating substrate (Maymo-Gatell *et al.*, 2001).
 - Anaerobic oxidation of VC by acetogenic bacteria in methanogenic consortia (Bradley and Chapelle, 2000).

-
- ❖ Dechlorination of carbon tetrachloride (CT) by extracellular metabolite:
 - Cu(II): pyridine-2,6-bis(thiocarboxylic acid) (PDTC), a natural chelator metabolite from *Pseudomonas stutzeri* KC, caused a one-electron reduction of CT (Lewis *et al.*, 2001).
 - ❖ First report of anaerobic halorespiration for chlorinated benzenes (Adrian *et al.*, 2000b). Halorespiration = energy yielding metabolism with a chlorinated compound as electron acceptor, previously only known for PCE, TCE, chlorobenzoates and chlorophenols.
 - ❖ First report of aerobic degradation of hexachlorobenzene using a genetically engineered *Pseudomonas putida* (Jones *et al.*, 2001).
 - ❖ Half-life of dioxins in lake sediments estimated to be is 35 years (Masunaga *et al.*, 2001).

Microbial chlorination

- ❖ Review article on bacterial chlorination emphasizing a novel FADH₂-dependent halogenase enzymes for halometabolite formation (Van Pee, 2001).
- ❖ Measurement of emission rate of chloroform from forest soil showed the highest rates nearby wood degrading areas or humus layers up to 1 µg CHCl₃ m⁻² h⁻¹ (Hoekstra *et al.*, 2001)

3. MICROBIAL DECHLORINATION

3.a. General reviews

The molecular characterization and detection of halorespiring bacteria was reviewed by Smidt *et al.* (2000). Halorespiration is a process in which halogenated compounds act as terminal electron acceptors (*e-acceptor*) in anaerobic respiration. The first bacterial species capable of coupling the reductive dehalogenation of a chlorinated compound (i.e., 2-chlorobenzoate) to energy conservation and, hence to microbial growth, was discovered in 1990. Since then, an increasing number of bacteria has been isolated that can utilize various chlorinated aromatic and aliphatic compounds like chlorophenols and PCE as terminal e-acceptors. These microorganisms have a great potential for the bioremediation of polluted anoxic environments, because their dehalogenating activity is several orders of magnitude higher than those observed for co-metabolic reductive dehalogenation.

The biodegradation of chlorinated ethenes by aerobic bacteria was reviewed in a paper by Van Hylckama Vlieg and Janssen (2001), which will be discussed in the next section.

3.b. Microbial dechlorination

Vinyl chloride (VC) and other chlorinated ethenes

Vinyl chloride (VC) is often detected as an important metabolite of the microbial dechlorination of tetrachloroethylene (PCE) and trichloroethylene (TCE) and, therefore, relevant literature on the biodegradation of these two chlorinated solvents will also be summarized here. Similarly, selected publications on the biotransformation of *cis*-1,2-dichloroethene (*cis*-DCE) will also be reviewed. *Cis*-DCE is a byproduct from the dehalogenation of different chlorinated ethenes that is often detected together with VC in contaminated sites or microcosms.

Chemical and physical properties of chlorinated ethenes that are critical in determining their toxicological characteristics and recalcitrance to biodegradation were reviewed by Van Hylckama Vlieg and Janssen (2001). The paper also presents a review on the aerobic degradation of halogenated aliphatics. Chlorinated ethenes, with the only exception of VC, can not be used as a growth substrate by aerobic bacteria. Aerobic cooxidation of chlorinated ethenes is known for TCE, DCE and VC. Cooxidation of chlorinated ethenes is caused by accidental oxidation of monooxygenase or dioxygenase involved in the degradation of the primary substrate (e.g., toluene, phenol, methane, etc.). The cooxidation results in the formation of very reactive chlorinated epoxyethanes that are highly toxic to the aerobic bacteria involved in the cooxidation. The authors discuss the potential role of product toxicity, arising from the epoxydes, on the observation that no aerobic bacteria are as yet known capable of utilizing chlorinated ethenes, other than VC, as growth substrate. A substantial amount of the research on the cometabolic transformations of chlorinated ethenes has been carried out with methylotrophs. Research on the transformation of chlorinated ethenes by methylotrophic bacteria, and applications of these microorganisms in organochlorine bioremediation are reviewed. The last part of the review focuses on bacterial enzymes capable of metabolizing epoxides, as well as on glutathione S-transferases and other enzymes involved in the detoxification of epoxides in mammals. The authors conclude that the reactions required for the detoxification of epoxyethanes and for the utilization of chlorinated ethenes exist in bacteria or higher organisms, and that aerobic microorganisms capable of growing on such compounds may be isolated from nature or engineered by recombinant techniques. Aerobic organisms that can use these halogenated aliphatics as a growth substrate could have important applications in bioremediation,

particularly in the removal of chlorinated ethenes bearing less chlorine substituents that are often poorly degraded under anaerobic conditions.

Tetrachloroethylene (PCE) and trichloroethylene (TCE). Anaerobic microbial reductive dechlorination of PCE and TCE contamination in groundwater, resulting in natural attenuation of the chlorinated hydrocarbons was reported by Lollar *et al.*, (2001). Degradation products detected included *cis*-DCE, VC, and ethene (ETH). Similarly, Fennell *et al.*, (2001) observed dechlorination of TCE to VC and ETH in microcosm studies, while *cis*-DCE and VC were found to accumulate at several locations in contaminated groundwater. In a related laboratory study, reductive dechlorination of PCE and TCE by the anaerobic consortium KB-1 consortium resulted in complete conversion to ETH (Slater *et al.*, 2001). Co-metabolic dechlorination of PCE and TCE by unadapted granular methanogenic sludge was also reported, although degradation rates were low (Van Eekert *et al.*, 2001). Removal of one chlorine atom from PCE by reductive hydrogenolysis was observed, while TCE that was converted to substantial amounts of VC. 1,1-DCE was an important intermediate in the dechlorination by unadapted methanogenic sludge, previously this intermediate was not commonly observed.

The biotransformation of TCE under various electron acceptor (e-acceptor) conditions was investigated by Boopathy and Peters (2001) in experiments using enrichment cultures obtained from anaerobic digester sludge. TCE was biotransformed under sulfate reducing, methanogenic, nitrate reducing, iron reducing, and fermenting conditions. The fastest removal of TCE (100% removal in 9 days) was observed under mixed e-acceptor conditions, followed in order by methanogenic, fermenting, iron reducing, sulfate reducing, and nitrate reducing conditions. Under mixed e-conditions, the TCE was converted to ETH, which was further metabolized. Under sulfate and nitrate reducing conditions, the main metabolites were *cis*- and *trans*-DCE. Under methanogenic, iron reducing, and fermenting conditions, *cis*- and *trans*-DCE and ETH were produced from TCE metabolism.

Bioremediation of PCE in a residual nonaqueous phase liquid (NAPL) source zone was investigated (Cope and Hughes, 2001). Experiments were conducted in anoxic upflow columns containing glass beads contaminated with a residual NAPL consisting of tridecane and labeled ¹⁴C-PCE. Three levels of electron donating substrate (pyruvate) were evaluated. PCE was sequentially reduced to TCE, *cis*-DCE, and VC without ETH formation in the columns operated at the lower electron donor levels (25 and 100 mM pyruvate). Biological dechlorination resulted in an increase in total PCE mobilization from NAPL, up to a factor of 16 over dissolution. The system fed the highest pyruvate levels (250 mM) lost dechlorinating activity early in the experiment.

In a recent review article on the aerobic degradation of chloroethenes, Van Hylckama Vlieg and Janssen (2001) reported previously unpublished results showing that their attempts to obtain aerobic microorganisms capable to utilize 1,2-DCE or TCE as a growth substrate (e.g. without cosubstrate) failed. Enrichment was attempted in aerobic biofilm reactors run for over 1 year on a feed containing low concentrations of *cis*- or *trans*-DCE, the oxygenase-requiring substrates methane and toluene, and possible degradation intermediates such as chloroacetate, chloroacetaldehyde, and glyoxylate, among others.

The aerobic degradation of mixtures of chlorinated aliphatics by cloned toluene-*o*-xylene monooxygenase (ToMO) and toluene *o*-monooxygenase (TOM) in resting cells was investigated by Shim and Wood (2000). The compounds considered included TCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, VC, and CF, individually as well as in various mixtures. ToMO oxidized all of these individual compounds well, whereas TOM did not degrade VC significantly (16-fold less) and degraded *cis*- and *trans*-DCE less well (3.7- and 2.4-fold, respectively). For mixtures of these chlorinated aliphatics, ToMO was again more robust than TOM. In mixtures of these chlorinated compounds, degradation was found to occur simultaneously rather than sequentially, and the mineralization of many of these compounds could be confirmed through detection of chloride ions.

The potential of a hybrid biological reactor, combining trickling filter (TF) and activated sludge process (ASP), to treat wastewater containing TCE was investigated by Misra and Gupta (2001). TCE was removed in the TF reactor via cometabolism using sodium acetate as the substrate. Substrate to TCE ratios of 100:1 were found to be optimum, and higher ratios led to decrease in TCE removal, presumably due to enzyme competition and product inhibition. Under optimized conditions, the total TCE removal efficiency was 99.99% (TCE conc. up to 100 mg/l). Biological degradation and volatilization losses accounted for 81.5 and 18.5% of the TCE removed, respectively. TCE dechlorination products were not monitored in this study. It is not clear from the paper if TCE degradation involved cooxidation or reductive dechlorination in anaerobic microniches.

Vinyl chloride (VC) and *cis*-dichloroethene (*cis*-DCE). VC and *cis*-DCE often accumulate in contaminated aquifers in which the solvents such as PCE and TCE undergo reductive dechlorination as confirmed in recent studies (Cope and Hughes, 2001; Fennell *et al.*, 2001; Lollar *et al.*, 2001). While incomplete dechlorination of TCE and PCE is often observed, it is well known that at some sites reductive dechlorination proceeds to ETH (Fennell *et al.*, 2001) (Freedman *et al.*, 2001). Dechlorination of PCE, TCE, *cis*-DCE and VC by a methanogenic consortium has also been reported to result in complete conversion to ETH (Slater *et al.*, 2001). In another study, reductive

dechlorination of TCE in microcosms studies resulted in the production of VC and ETH (Fennell *et al.*, 2001).

The reasons for the incomplete dechlorination of chlorinated ethenes are only partially understood. It appears that organisms that can dechlorinate PCE and TCE are diverse, and microorganisms with different degradative abilities may be present at different contaminated sites. Organisms that dechlorinate past *cis*-DCE seem to be nutritionally fastidious and show slow growth (Maymo-Gatell *et al.*, 2001). Difficulties utilizing *cis*-DCE by a strain of *Dehalococcoides ethenogenes* have been related to toxicity by chloroform impurities in the commercial source of *cis*-DCE (Maymo-Gatell *et al.*, 2001). E-acceptor conditions were also reported to determine the major metabolites formed upon anaerobic biotransformation of TCE (Boopathy and Peters, 2001). Under sulfate and nitrate reducing conditions, mainly *cis*- and *trans*-DCE were produced. Under methanogenic, iron reducing, and fermenting conditions, ETH was also a metabolite from TCE degradation.

New evidence for the anaerobic degradation of lower chlorinated hydrocarbons has been found with VC. Vinyl chloride was cometabolized to ETH by a halorespiring bacterium, *Dehalococcoides ethenogenes*, actively respiring PCE with H₂ as e-donor (Maymo-Gatell *et al.*, 2001). Presently, strain 195 is the only isolated organism known to reductively dechlorinate chloroethenes further than DCE. A new pathway involving anaerobic cooxidation rather than reduction has been discovered for vinyl chloride. This compound was found to be oxidized by acetogenic bacteria in a methanogenic consortium yielding acetate which was anaerobically converted to methane and CO₂ (Bradley and Chapelle, 2000). In a different study, VC, *cis*-DCE, *trans*-DCE and 1,1-DCE were dechlorinated by a methanogenic consortium with methanol as the primary substrate (Van Eekert *et al.*, 2001). Reductive hydrogenolysis with loss of one chlorine atom was the main dechlorination pathway. The observed degradation rates for these chloroethenes were very slow, with the exception of 1,1-DCE.

Freedman *et al.* (2001) evaluated the role of methane and ETH as primary substrates for aerobic biodegradation of VC. Ethene enhanced the aerobic degradation of VC in laboratory experiments, suggesting that this hydrocarbon may play an important role during in situ natural attenuation of VC under aerobic conditions. Methane inhibited ETH metabolism, which apparently caused a decrease in VC biodegradation when methane was present with ethene. *Cis*- and *trans*-DCE were readily consumed by a mixed VC-degrading culture, although addition of VC as the primary substrate was needed to sustain biodegradation. Based on these results, the authors hypothesized that organisms capable of using VC as a sole substrate may play an active role in aerobic natural attenuation of DCEs.

Methane, ethane, and ETH are relevant to the natural attenuation of chlorinated hydrocarbons because these compounds are found at many sites as end products of anaerobic degradation. Verce and Freedman (2001) modeled the batch kinetics of VC oxidation by *Pseudomonas* sp. strain EA1 in the presence and absence of ethane. The strain was found to use ethane but not VC as a growth substrate, and it caused complete dechlorination of VC in the presence and absence of ethane. When grown on ethane, strain EA1 cometabolized VC at a rate somewhat slower than other microbes grown on other primary substrates, but with a comparatively low half-saturation coefficient. When fed both ethane and VC, the rate of VC use by EA1 doubled, even though consumption of ethane was almost completely inhibited until VC was consumed. The mechanism by which a growth substrate can increase the rate of cometabolite use, without itself being consumed, is not yet known. It has been hypothesized that such increase could be related to an increase in the availability of active reaction sites caused by the presence of the growth substrate, even without measurable primary substrate utilization. Ethene inhibited cometabolism of VC and metabolism of ethane; however, in mixtures containing all three compounds, more VC was degraded and at a faster rate compared to VC plus ethene. Based on these results, the authors conclude that ethane-enhanced biodegradation of VC may contribute to VC removal at the aerobic fringe of groundwater plumes undergoing reductive dechlorination.

Carbon tetrachloride (CT)

In the current year, a new reaction pathway was proposed to explain the formation of end products during defined chemical reactions between CT and either the bacterial transition metal chelator pyridine-2,6-bis(thiocarboxylic acid) (PDTC) from *Pseudomonas stutzeri* KC or from the bacterial culture (Lewis *et al.*, 2001). PDTC was recently identified as the extracellular agent responsible for CCl₄ dechlorination activity in strain KC. Previously, microbial dehalogenation of CT generally involves a net two-electron reduction yielding hydrogenolytic products (i.e., replacement of one chlorine atom by one hydrogen atom). The newly described dechlorination mechanism was shown to involve the one-electron reduction of CCl₄ by Cu(II):(PDTC) complex. With added reductants, an increased turnover was seen along with increased chloroform production. The new pathway includes one-electron reduction of CCl₄ by Cu(II):(PDTC) complex, condensation of trichloromethyl and thiyl radicals, and hydrolysis of a labile thioester intermediate. PDTC reacted stoichiometrically rather than catalytically without added reducing equivalents.

Amonette *et al.* (2000) demonstrated that CT dechlorination in Fe(III)-reducing environments may indirectly result from the enzymatic or chemical reduction of Fe(III)-bearing

minerals such as goethite. Biogenic Fe(II) formed by the enzymatic reduction of goethite by the Fe(III)-reducing bacterium *Shewanella alga* dechlorinated CT to chloroform (83-90% conversion).

Dichloromethane (DCM)

Three publications were retrieved that described, respectively, a biodegradation study with DCM, the characterization of DCM dehalogenase genes from methylotrophic bacteria, and the taxonomic characterization of several DCM-utilizing methylotrophic bacteria.

Degradation of DCM by two isolates, *Flavimonas* sp. strain P3310 and *Chryseobacterium* sp. strain G31, was investigated by Ikatsu *et al.* (2000). Upon acclimatization, the strains could dechlorinate DCM at high concentrations of 3 g L⁻¹. DCM-degrading activity was also detected in the cell-free extract and the culture-supernatant.

DCM dehalogenase/glutathione S-transferase allows methylotrophic bacteria to grow with DCM. So far, the sequence of the *dcmA* gene encoding DCM dehalogenase has been determined for *Methylobacterium dichloromethanicum* DM4 and *Methylophilus* sp. DM11. In a recent study, DCM dehalogenase genes closely related to that of strain DM4 were amplified by PCR and cloned from total DNA from 14 different DCM-degrading strains, enrichment cultures and sludge samples from wastewater treatment plants (Vuilleumier *et al.*, 2001). Representative DCM dehalogenase variants were also investigated, revealing minor differences between the properties of DCM dehalogenases related to that from strain DM4.

The taxonomic status of recently characterized DCM-utilizing methylotrophic bacteria (*Methylophilus leisingerii*, *Methylorhabdus multivorans* DM13, *Paracoccus methylutens* DM12) was confirmed by phylogenetic analysis (Tourova *et al.*, 2001). In addition, several novel strains of methylotrophic bacteria capable of DCM degradation were divided into three genotypic groups, whose status is still to be confirmed by phenotypic analysis.

Dichloroethane (1,2-DCA)

Complete mineralization of 1,2-DCA to CO₂ and chloride by the aerobic bacterium *Xanthobacter autotrophicus* was confirmed in a recent study (Hunkeler and Aravena, 2000). The report also provided evidence of substantial carbon isotope fractionation among substrate, inorganic carbon, and biomass during the mineralization of 1,2-DCA, as will be discussed later in this report.

Chloromethane (CM)

Harper dedicated one section of his review of recent developments in the study of the global CM cycle (Harper, 2000) to discuss the microbial degradation of this compound under aerobic and

anaerobic conditions. The review considers the main isolated microorganisms capable of CM degradation, and discusses the key enzyme systems and pathways involved in CM biotransformation.

Two additional publications were retrieved that focused on, respectively, the characterization of recently isolated CM-utilizing methylotrophic bacteria, and the comparison of methyl halide-specific genes in gram-negative bacteria. Two CM-utilizing facultatively methylotrophic bacteria, *Hyphomicrobium chloromethanicum* sp. nov. and *Methylobacterium chloromethanicum* sp. nov., isolated from soil at a petrochemical factory were isolated and characterized (McDonald *et al.*, 2001). *H. chloromethanicum* CM2(T) is an aerobic methylotrophic bacteria that can grow with CM as the sole C and energy source. Methyl halide-utilizing genes in the methyl bromide-utilizing bacterial strain IMB-1 were identified (Woodall *et al.*, 2001). Comparison of *cmu* gene clusters from strain IMB-1 and other halomethane-degrading bacteria suggested a high degree of conservation of methyl halide-specific genes in gram-negative bacteria.

Chlorobenzenes

Mono-chlorobenzene (CB) and dichlorobenzenes (DCB): Effective removal of CB (74-92 mg L⁻¹) and 1,2-dichlorophenol from a synthetic effluent by a method combining ultrasonic treatment and subsequent aerobic biotreatment was reported (Tiehm *et al.*, 2001). CB was largely degraded in the ultrasound reactor (100% CB removal and 90% AOX elimination in 60 min). The remaining COD was significantly removed in the biological step.

Baltzis *et al.* (2001) derived a mathematical model for describing the removal of mixed VOC vapors in biotrickling filters (BTFs). Without using any fitted parameter, the model was found capable of predicting experimentally obtained removal rates of CB and ortho-DCB with less than 10% error in the majority of cases. Removal rates obtained with BTFs were one to more than two orders of magnitude higher than those obtained with conventional biofilters in a previous study. This was attributed to the larger active specific biofilm surface area in BTFs, and improved moisture control.

Trichlorobenzenes (TCB) and tetrachlorobenzenes (TeCB): Adrian *et al.* (2000a) investigated the reductive dechlorination of 1,2,3- and 1,2,4-TCB by an anaerobic mixed bacterial culture. Growth and dechlorination occurred in a medium containing formate or H₂, acetate, and TCB; but high dechlorinating activities was also achieved when only H₂ and TCB were supplied. This indicated that reductive dechlorination of TCB was the primary energy conserving process. Neither acetogenesis nor methanogenesis was detected in the culture. The growth of dechlorinating bacteria was strictly limited by the amount of TCB supplied. In addition, the dechlorinating activity

could be maintained only in the presence of TCB. Vitamin B12, a cofactor also known to mediate the dechlorination of chloroethenes in vitro, significantly stimulated the dechlorinating activity.

The isolation of the oxygen-sensitive strain CBDB1, a pure culture capable of reductive dechlorination of chlorobenzenes, was reported by Adrian *et al.* (2000b). The strain was able to stoichiometrically dechlorinate 1,2,3-TCB, 1,2,4-TCB, 1,2,3,4-tetrachlorobenzene (TeCB), 1,2,3,5-TeCB and 1,2,4,5-TeCB to dichlorobenzenes or 1,3,5-TCB. The presence of chlorobenzene as an e-acceptor and H₂ as e-donor was found to be essential for growth, and indicates that the strain meets its energy needs by a dehalorespiratory process. Strain CBDB1 is the only isolate described to date that uses reductive dechlorination of chlorobenzenes for energy conservation. Analysis of the 16S ribosomal RNA gene sequence of different dehalogenating bacteria including strain CBDB1, several as yet uncultivated strains obtained from the same TCB-dechlorinating mixed culture enrichment as CBDB1, and *Dehalococcoides ethenogenes*, showed that these strains form a new branch phylogenetically distant from all other dechlorinating bacteria. *D. ethenogenes* is the only known isolate to dechlorinate PCE to the non-toxic product ETH.

Genes encoding reductive dehalogenases, the key enzymes of chlororespiration, were detected in different anaerobic consortia capable of dechlorinating TCB and 1,2-dichloropropane, supporting the hypothesis that reductive dechlorination of TCB and 1,2-dichloropropane occurs via a respiratory pathway (Von Wintzingerode *et al.*, 2001).

TCB has been detected as an intermediate in the microbial degradation of γ -hexachlorocyclohexane (γ -HCH). Biotransformation of γ -HCH by *Sphingomonas paucimobilis*, a reaction catalyzed by HCH-dehydrochlorinase (LinA), results in the formation of 1,2,4-trichlorobenzene (1,2,4-TCB) via γ -1,3,4,5,6-pentachlorocyclohexene (γ -PCCH). Recently, Trantírek *et al.* (2001) have proposed a mechanism for the enzymatic biotransformation of γ -HCH to 1,2,4-TCB by LinA consisting of two 1,2-anti conformationally dependent dehydrochlorinations followed by 1,4-anti dehydrochlorination.

Hexachlorobenzene (HCB): Development of anaerobic HCB-dechlorinating mixed cultures using nonionic polysorbate surfactants (Tween 60, 61 or 65) as a carbon source was investigated by Yeh and Pavlostathis (2001). Transition from glucose to Tween surfactants (400 mg L⁻¹ after 1 year) as the e-donor did not affect the rate, extent, and pathway of HCB transformation. Based on these results, the authors state that use of anaerobically degradable Tween surfactants as the carbon and electron source for microbial reductive dechlorination appears to be feasible. These surfactants can be used to increase the bioavailability of subsurface polychlorinated contaminants.

Yague *et al.*, (2001) investigated the degradation of HCB and other organochlorine compounds (γ -HCH, pp'DDE, PCB28, and PCB153) during food processing, more specifically,

during yogurt preparation. A mixture of the investigated compounds was highly inhibitory to the selected yogurt culture organisms (*Lactobacillus delbrueckii* subsp. *bulgaricus* CECT-4005 and *Streptococcus salivarius* subsp. *thermophilicus* CECT-801) at concentrations of 0.5 $\mu\text{g mL}^{-1}$. The lactic microorganisms did not cause any significant degradation of a mixture of the organochlorine contaminants in a nutrient broth.

Polychlorinated benzenes are recalcitrant environmental pollutants primarily because they are resistant to attack by dioxygenases commonly used by micro-organisms for the biodegradation of aromatic compounds. Novel biodegradation systems by genetic engineering of the haem mono-oxygenase CYP101 (cytochrome P450cam) from *Pseudomonas putida* that could convert the inert polychlorinated benzenes to chlorophenols, which are then readily degraded by natural pathways (Jones *et al.*, 2001). Mutation of the cytochrome P450cam increased up to 100-fold the rate of oxidation of pentachlorobenzene and HCB to pentachlorophenol. Additional mutations, resulted in a further 10-fold increase in activity with most substrates and decreased the NADH-turnover rates.

Dioxins and furans

Three publications were retrieved, two of them related to the microbial degradation of dioxins and chlorinated furans and one focusing on the characterization of a dioxin-utilizing bacterial strain.

Ishiguro *et al.* (2000) investigated the aerobic biodegradation of dibenzofuran (DBF) and dioxins by a *Pseudomonas aeruginosa* and a *Xanthomonas maltophilia* strain. *Ps. aeruginosa*, which had a high growth rate with DBF as C source, showed high growth rates in the presence of such dioxins as dibenzo-p-dioxin. Under the same conditions, medium growth was observed with 1-chlorodibenzo-p-dioxin, 2-chlorodibenzo-p-dioxin and 2,8-dichlorodibenzofuran, low growth with 2,6-dichlorodibenzo-p-dioxin and 1,2,3,4-tetrachlorodibenzo-p-dioxin and no growth with octachlorodibenzofuran. The growth rate of *Ps. aeruginosa* using dioxins as carbon source decreased with the increase in the degree of chlorine substitution. The degradation activity of *Ps. aeruginosa* was high for DBF, dibenzo-p-dioxin and 1-chlorodibenzo-p-dioxin and low for 2,8-dichlorodibenzofuran, 3,6-dichlorodibenzofuran, 2-chlorodibenzo-p-dioxin and 2,6-dichlorodibenzo-p-dioxin. These results suggested that there is a correlation between growth and the ability to biodegrade dioxins. Noteworthy was the fact that the efficiency in the biodegradation of 1,2,3,4-tetrachlorodibenzo-p-dioxin was comparatively high although the growth rate on this dioxin was low. Hydroxydibenzofuran, 2-hydroxy-3-allyl-benzofuran and 2-carboxyvinylloxy phenyl acetic acid were identified as products of DBF biodegradation by *Ps. aeruginosa*, suggesting a possible biodegradation pathway for dioxins.

The historical trends of sources, behavior, and mass balance of dioxins in Lake Shinji Basin (Japan) were estimated by statistical analyses of the detailed congener-specific data of a dated sediment core (Masunaga *et al.*, 2001). On the basis of the trend of deposition in sediments, dioxins in the lake soil were estimated to be lost at a rate of about 2%/yr or a half-life of about 35 yr. The possible contribution of the various mechanisms to dioxin losses was not considered in the paper.

Finally, *Sphingomonas* sp. strain RW1(T), a potent metabolizer of dibenzo-p-dioxin and its relatives isolated from the Elbe river, was assigned to a new species, *Sphingomonas wittichii* sp. nov. based on phylogenetic analysis (Yabuuchi *et al.*, 2001).

Chloroform, hexachlorobutadiene and octachlorostyrene

In the first quarter of 2001, no publications regarding the microbial degradation of chloroform, hexachlorobutadiene and octachlorostyrene were found.

Polychlorinated biphenyls (PCBs)

Reviewing the microbial degradation of PCBs is out of the scope of this report, therefore retrieved publications on this topic will be introduced briefly. Several publications addressed the genetics of microbial degradation of PCBs (Seah *et al.*, 2001; Springael *et al.*, 2001). Furthermore, a number of reports investigated the degradation of PCBs by aerobic bacteria and white-rot fungi. Two bacterial strains capable of aerobic growth on ortho-substituted dichlorobiphenyls as sole C and energy sources were isolated (Kim and Flynn, 2001). A *Rhodococcus* recombinant strain developed for the degradation of products from the anaerobic dechlorination of PCBs was able to grow in medium containing 4-chlorobenzoate (4-CBA) and 4-chlorobiphenyl (4-CB) as the only C source, and could remove 4-CB from non-sterile soil (Rodrigues *et al.*, 2001). Biodegradation studies with lactic bacteria showed no degradation of PCB28 nor PCB153 in a mixture with other organochlorine compounds (Yague *et al.*, 2001). Several strains of the white-rot fungus *Pleurotus ostreatus* were found to cause significant removal of a low chlorinated PCB commercial mixture (Delor 103) in contaminated soil (40% in 2 months) (Kubatova *et al.*, 2001). In another study, the effects of the surfactant soya lecithin on the aerobic biodegradation of PCBs in an artificially contaminated soil were studied (Fava and Di, 2001). Finally, determination of enantiomeric ratios (ERs) was found to be a useful tool to characterize processes implicated in the biotransformation of chiral PCBs atropisomers in aquatic bed sediments (Wong *et al.*, 2001). Of the 209 PCB congeners, 78 are atropisomers (i.e., compounds axially chiral in their nonplanar conformations). Chiral PCBs are generally released to the environment as a racemic mixture. Biotransformation processes display selective degradation and, thus they can affect the ER of a chiral compound (i.e., the ratio of the

concentrations of the enantiomers). The enantiomeric composition of eight PCBs atropisomers in aquatic sediment samples collected at different locations was used to confirm the occurrence of reductive dechlorination, and to characterize distinct biotransformation processes based on their enantioselectivity.

3.c. In vitro degradation of chlorinated compounds

In vitro dehalogenation of PCE by cell-free extracts of *Clostridium bifermentans* DPH-1 was reported by Chang *et al.* (2001). PCE degradation was stimulated by addition of a variety of e-donors, ethanol, methyl viologen and glucose being the most effective, and by addition of the cofactor NADH. Dehalogenase activity was inhibited by cyanide and by propyl iodide in the presence of the reducing agent titanium citrate. The dehalogenase caused also dechlorination of TCE, *cis*-DCE, *trans*-DCE, 1,1-dichloroethylene, 1,2-dichloroethane, and 1,1,2-trichloroethane. The highest rate of degradation of the chlorinated compounds was achieved with PCE, and this compound was mainly degraded via TCE to *cis*-DCE.

Dechlorinating enzymes. Haloalkane dehalogenases are enzymes capable of replacing a halide in an organic substrate by a hydroxyl group. These enzymes have been isolated from polluted industrial sites from organisms capable of growing on haloalkanes. Pieters *et al.* (2001) provided the first examples of enantioselectivity of haloalkane dehalogenases. The chiral recognition of was low but enantio-discrimination could be improved after structural optimization of the substrate. The dehalogenases used were obtained from *Xanthobacter autotrophicus* strain capable of 1,2-dichloroethane degradation and a chlorobutane-degrading *Rhodococcus rhodochromus* strain. In a different study, the crystal structure of the haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26 (LinB) was described (Marek *et al.*, 2000). LinB is involved in the degradation of the important environmental pollutant γ -HCH. The enzyme also hydrolyzes a broad range of halogenated cyclic and aliphatic compounds (Marvanova *et al.*, 2001).

3.d. New tools to assess the biodegradation of chlorinated compounds

Carbon isotopic enrichment

The potential of stable carbon isotopic analysis to assess the biodegradation of chlorinated ethenes in contaminated groundwater was discussed in several (Bloom *et al.*, 2000; Lollar *et al.*, 2001;

Slater *et al.*, 2001). These studies confirm that significant isotopic shifts can occur during the anaerobic degradation of PCE, TCE, *cis*-DCE, and VC. During microbial reductive dechlorination of chlorinated hydrocarbons, the light (^{12}C) versus heavy isotope (^{13}C) bonds are preferentially degraded, resulting in isotopic enrichment of the residual contaminant in ^{13}C . Comparison of all reported enrichment factors for reductive dechlorination of the chlorinated ethenes shows significant variation between experiments. Despite this variability, these results demonstrate that carbon isotopic analysis can provide qualitative evidence of the occurrence and relative extent of microbial reductive dechlorination of the chlorinated ethenes.

Substantial carbon isotope fractionation also occurs among substrate, inorganic carbon, and biomass during aerobic mineralization of 1,2-dichloroethane (1,2-DCA) by *Xanthobacter autotrophicus* (Hunkeler and Aravena, 2000). In this study, a mechanistic model was developed to link the fractionation factor to the rate constants of the first catabolic enzyme. Based on the model, it was concluded that the strong enrichment of ^{13}C in 1,2-DCA arises because the first irreversible step of the initial enzymatic transformation of 1,2-DCA consists of an SN2 nucleophilic substitution. SN2 reactions are accompanied by a large kinetic isotope effect.

Co-injection of surrogate compounds

The potential for determining TCE transformation rates in groundwater by measuring the transformation rate of its fluorinated surrogate, trichlorofluoroethene (TCFE) was confirmed by Hageman *et al.* (2001). TCFE, dichlorofluoroethene (DCFE), and TCE were transported similarly to each other. In the shallow water-bearing zone, TCFE was reductively dechlorinated to *cis*-DCFE, *trans*-DCFE, and (E)-1-chloro-2-fluoroethene (CFE), while co-injected TCE was concurrently transformed to *cis*-DCE, *trans*-DCE, 1,1-DCE, and a trace amount of chloroethene. With added formate and the injected TCFE concentration being a factor of 20 higher than that of TCE, the TCFE transformation rate ranged from 0.053 to 0.30 mol L⁻¹ d⁻¹, while that of TCE ranged from 0.009 to 0.012 mol L⁻¹ d⁻¹. Without added formate, the TCFE transformation rate decreased to 0.036 mol L⁻¹ d⁻¹. In the deeper water-bearing zone, TCFE transformation occurred only after a lag time of 55 days with added formate. No TCFE transformation occurred in groundwater that had not previously been exposed to TCE.

Molecular probing

Polymerase chain reaction using primers targeted to unique regions of *Dehalococcoides ethenogenes* 16S rDNA, combined with microcosm studies, and site data was shown to provide a relatively quick and facile method for investigating spatial distributions of dechlorinators on-site

(Fennell *et al.*, 2001). Using these tools, the authors demonstrated that *Dehalococcoides* and dechlorination activity were similarly, heterogeneously distributed in microcosms constructed using material from different contaminated locations.

Several publications report on the development of gene probes to detect microorganisms capable of degrading halogenated compounds. Molecular methods recently developed for the detection of halo-respiring bacteria, and for monitoring their reductive dehalogenation potential and activity (Smidt *et al.*, 2000, 2001) could facilitate the design and operation of in situ bioremediation processes. Furthermore, functional gene probes to detect halomethane-degrading bacteria were developed using the CM utilization (*cmu*) gene cluster from *Hyphomicrobium chloromethanicum* strain CM2(T) (McAnulla *et al.*, 2001).

Enzyme characterization

A colorimetric (pH indicator dye-based) microplate assay useful for the biochemical characterization of haloalkane dehalogenase enzymes was optimized and validated for use with the enzyme LinB (Marvanova *et al.*, 2001). Since haloalkane dehalogenases are enzymes exhibiting broad specificity, a multivariate experimental design was used to select the most representative substrates for experimental testing. A set of 34 substrates was selected out of a database consisting of 194 halogenated compounds and used to test the applicability of the assays for the systematic characterization of mutant LinB enzymes.

Modeling

Öman (2001) developed a model to predict the emissions from landfills of 12 different organic compounds, including α -HCH, PCE, DCM, tetrachloromethane, 1,2-dichloroethane, and 1,4-dichlorobenzene, among others. The fate of each compound was theoretically estimated from literature data on sorption, dissociation, evaporation, and transformation. These processes were described by the octanol/water coefficients, the acid dissociation constants, the Henry's law constants, and the potential of the compounds to be biologically transformed. A good correlation was found between the emissions measured in pilot-scale landfill and the theoretically evaluated fate.

4. MICROBIAL CHLORINATION

4.a. General reviews

A review on the microbial biosynthesis of halometabolites, currently in press, will be published in *Archives of Microbiology* this year (Van Pee, 2001). The article focuses on the enzyme-catalyzed reactions responsible for the incorporation of halogen atoms into organic compounds, and discusses the mechanisms and possible roles played by different types of halogenating enzymes. Haloperoxidases were long thought to be implicated in halometabolite formation, however, these enzymes lack substrate specificity and regioselectivity, and their involvement in the in vivo formation of halometabolites has never been demonstrated. Recently, a different class of halogenating enzymes, FADH₂-dependent halogenase (FDH), has been shown to be implicated in halometabolite formation. FDHs show substrate specificity and regioselectivity, and their genes have been detected in many halometabolite-producing bacteria, suggesting that this type of halogenating enzymes constitutes the major source for aromatic halometabolite formation in bacteria and possibly also in other organisms. Halogenase involvement in the biosynthesis of aliphatic metabolites has not been demonstrated to date. Future detection of new halogenating enzymes is anticipated by the authors.

A very comprehensive review of the principal natural sources and sinks of atmospheric CH₃Cl, both biological and abiotic was published by Harper (2000). In the paper, the environmental concentrations and the total annual global input of CH₃Cl to the atmosphere are compared with those of other volatile halocarbons of both natural and anthropogenic origin, and the significance of CH₃Cl in chlorine-catalysed ozone destruction in the stratosphere is considered. Abiotic as well as biological sinks and sources of CM are reviewed in great detail. Finally, the mechanisms of CH₃Cl biosynthesis, and the possible metabolic roles of the compound in the producing organism are discussed, and recent findings concerning the biochemistry of microbial degradation of the halocarbon are described. In the conclusions, the author emphasizes the recently recognized importance of natural terrestrial sources, most of them as yet unidentified, to global CM emissions (an estimated 2.5 million ton CH₃Cl y⁻¹). However, the CM flux from biological sources may be greater than estimated to date. CM global budget calculations assume that CM sinks are entirely abiotic. Biological sinks are expected to be of considerable importance because soil microorganisms capable of utilizing CM as a carbon and energy source appear to be ubiquitous.

4.b. Microbial chlorination in soils

Chloromethanes: Hoekstra *et al.* (2001) published a detailed work focusing on the emission of chloroform (CF) by forest soils with emphasis on CF emission fluxes from soil into the atmosphere. In the introduction, the authors present a brief review of the main natural and anthropogenic sources of CHCl_3 . Based on earlier work, the authors state that CHCl_3 emissions from soil are likely caused by natural formation, probably through the chloroperoxidase-catalysed chlorination of organic matter, and through de novo biosynthesis by basidiomycetous fungi by a mechanism as yet to be identified. Emission fluxes were measured using enclosures and calculated from the observed concentration gradients in soil air and atmospheric air. Wood-degrading areas and soils with a humic layer were found to emit up to $1000 \text{ ng CHCl}_3 \text{ m}^{-2} \text{ h}^{-1}$ and seem to be larger CF sources than the other areas of study. Rather unexpectedly, emission of 1,1,1-trichloroethane, CT and PCE was detected in one sampling site, likely from natural sources. A good agreement was found between the fluxes using enclosures and those derived from the concentration gradients in soil air and atmospheric air. These flux values could be used as input in the calculation of the global mass balance of CF. However, the area sampled was limited and the authors warn that extrapolation to global scale is not justified. The general conclusion is that additional studies are still required to determine whether soils in temperate zones contribute significantly to the atmospheric CHCl_3 load.

Redeker *et al.* (2000) investigated the emissions of methyl halides (methyl chloride, methyl bromide, and methyl iodide) from rice fields. Factors found to influence methyl halide emissions included stage of rice growth, soil organic content, halide concentrations, and field-water management. Unplanted flooded fields emit as much methyl chloride as planted, flooded fields (approx. 3.0×10^{-5} to $4.0 \times 10^{-5} \text{ g m}^{-2} \text{ d}^{-1}$), which suggests a different mechanisms for methyl chloride generation within the rice field. Nonetheless, the mechanisms of halomethane production in rice fields are poorly understood, and it is still uncertain whether emitted methyl halides are produced in the soils or in the plants themselves. The relative average methyl halide fluxes were not proportional to the concentrations of iodide, bromide and chloride concentrations in the soil. The ratio of the average fluxes observed for methyl chloride to methyl bromide to methyl iodide was 8:2:100. Based on these data, global fluxes from worldwide rice growing estimated for methyl chloride, methyl bromide and methyl iodide were 5.8, 1.3, and 71 Gg/year, respectively. These data would imply that rice production is responsible for approximately 1% of atmospheric of methyl bromide and 4% of atmospheric methyl iodide. The authors indicate that these global estimates are only an approximation and they should be adjusted with new data from other years, locations, etc.

An novel abiotic mechanism for the formation of CH_3Cl and other halomethanes has recently been proposed (Figure 1), that involves natural oxidation processes during degradation of organic matter (Keppler *et al.*, 2000). In soils and sediments, halide ions can be alkylated during the oxidation of organic matter by an e-acceptor such as Fe(III) . Sunlight or microbial mediation is not required for these reactions. When the available halide ion is chloride, the reaction products are CH_3Cl , $\text{C}_2\text{H}_5\text{Cl}$, $\text{C}_3\text{H}_7\text{Cl}$ and $\text{C}_4\text{H}_9\text{Cl}$. The corresponding alkyl bromides or alkyl iodides are produced when bromide or iodide is present. Soils rich in organic matter were observed to release CH_3Cl at rates of up to $100 \text{ pmol g}^{-1} \text{ h}^{-1}$ when incubated in the presence of Fe^{3+} and Cl^- ion. Additionally, in the presence of Fe(III) and halide ion with pH in the range 3 to 5, methoxylated phenols such as guaiacol underwent oxidation to quinones, with concomitant formation of the corresponding monohalomethane. Methoxylated phenols are monomeric constituents of many humic substances, therefore, such abiotic processes could make a significant contribution to the budget of CH_3Cl and other halomethanes. However, further studies will be necessary to assess the extent to which this reaction occurs under field conditions.

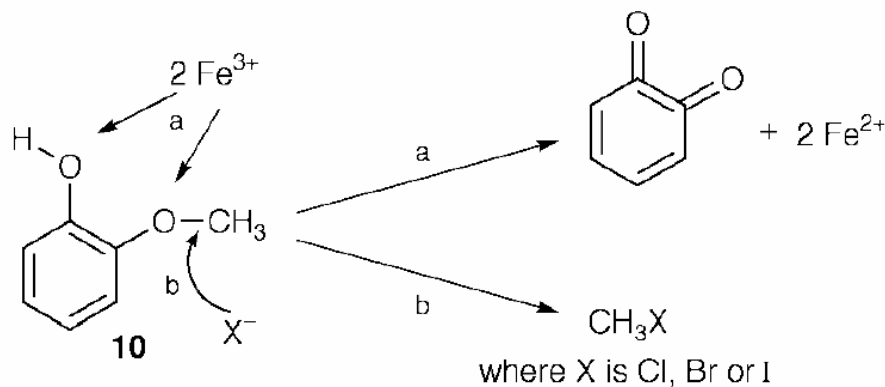


Figure 1 Postulated mechanism for formation of halomethanes from methoxylated phenols.

Other chlorinated organic compounds: Johansson *et al.* (2001) investigated the influence of nitrogen (N) on the non-volatile organic and inorganic chlorine in Swedish spruce forest soil. Total concentrations of chlorine, organic chlorine (TOX) and inorganic chlorine were estimated in samples of forest soils, and changes were observed after the addition of N in incubated samples. All of the investigated samples contained both inorganic and organic chlorine, and the TOX was 2-4 times larger than that of inorganic chlorine (Min-Max TOX 70-1070; median TOX 214 in $\mu\text{g Cl}^- \text{ g}^{-1} \text{ d.w.}$). The results suggest that the TOX in mature spruce forest soils with a moderate chloride

deposition in the temperate region is larger than the amount of inorganic chlorine. The results of the N incubation indicate that addition of ammonium nitrate causes a net decrease in the TOX and a net increase in inorganic chloride concentration. However, the variability in the results of the incubation experiments was very large. The authors state that the observed change appear to result from dechlorination of the organic matter present in the water leachable fraction, but they did not investigate or discussed possible mechanisms.

Two dichloroanisoles (3,5-dichloro-4-methoxybenzaldehyde and 3,5-dichloro-4-methoxybenzyl alcohol) from the white-rot fungus *Psathyrella scobinacea* isolated from liquid cultures were characterized by Taha (2000). These compounds are well known from other fungi.

4.c. Chlorination by marine organisms

Numerous publications report the isolation and characterization of halogenated metabolites from marine organisms. With some interesting exceptions, the chemical structures of most of the identified compounds are not related to that of the chlorinated compounds in our priority list.

Chloromethanes: Baker *et al.* (2001) investigated the production rates of a range of low molecular weight halogenated organics in cultures of five temperate species of macroalgae collected from the north coast of Norfolk, England. Compounds studied included CH₃Br, the chlorinated organics CH₃Cl, CH₂Cl₂ and CHCl₃, and the iodinated organics CH₃I, C₂H₅I, and CH₂ClI. Measurements of a wider range of halocarbon concentrations in an isolated rockpool and in air over the seaweed bed were also conducted to evaluate the local impact of the seaweeds on halocarbon concentrations in the natural environment. Estimates for the global emissions of some of the key halogenated compounds from macroalgae were derived. In general macrophytes appear not to be globally significant producers of the particular halocarbons studied. In coastal regions, however, the impact on local atmospheric composition and chemistry could be greater. Roose and Brinkman (2000) determined the levels of different volatile organic compounds in various marine organisms from the southern North Sea. These authors found that chloroform levels were generally higher than those of the other chlorinated hydrocarbons (i.e., 1,1-DCE, CF, TC, 1,2-DCE, 1,1,1-trichloroethane, TCE and PCE). It was suggested that this could be the result of higher CF concentrations in the water due to biogenic production by marine algae, although anthropogenic production of during chlorination of water was hypothesized to be a CF source of considerably greater significance.

Other chlorinated organic compounds. Faulkner (2001) reviewed the marine natural products literature for 1999. Earlier reports published in the same journal (*Nat. Products Reports*) cover the

period from 1977 to December 1998. Many of the reported secondary metabolites were halogenated compounds, mainly bromo-derivatives. But several chlorinated metabolites (alkaloids, antibiotics, terpenes, aromatics, etc.) were also detected in phytoplakton and marine microorganisms, brown and red algae, and tunicates (ascidians). Three new chlorinated steroids, yonarasterols G, H and I, were also isolated from the Okinawan soft coral, *Clavularia viridis* (Iwashima *et al.*, 2001). Holler *et al.* (2000) reported the production of a chlorinated heterocyclic metabolite by a fungi isolated from a marine sponge.

Numerous studies report the isolation of new brominated metabolites in marine organisms that are out of the scope of this review. Nonetheless, we would like to highlight the isolation of two new tetrabromodibenzo-p-dioxins, spongiadioxins A (1) and B (2) (Figure 2) from an Australian marine sponge *Dysidea dendyi* (Utkina *et al.*, 2001).



Figure 2. Two new polybrominated dibenzo-p-dioxins, spongiadioxins A (left) and B (right), from the sponge *Dysidea dendyi*. R = H (Utkina *et al.*, 2001)

5. MISCELLANEOUS

An interesting article dealing with the global emissions of hexachlorobenzene (HCB) was published by Bailey (2001). Although no data was presented on the microbial (de)chlorination of HCB, this publication is listed here because of its general relevance. Information from a variety of sources was collected to obtain a global estimate of HCB emissions in the mid 1990s. In this study, no single overwhelming source of HCB was identified. The best estimates of global HCB emissions from different categories of sources were as follows: pesticides application - 6500 kg/yr; manufacturing - 9500 kg/yr; combustion - 7000 kg/yr, includes 500 kg from biomass burning. This adds up to total current HCB emissions of approximately 23000 kg/yr with an estimated range 12000-92000 kg/yr. A substantial portion of HCB measured in the atmosphere is thought to come from volatilization of

"old" HCB on the soil from past contamination along with unidentified sources. Based on their findings, the authors hypothesized that the long-term trend of HCB emissions and environmental concentrations are expected to decrease over a period of decades toward an equilibrium concentration. Several sources of uncertainty in HCB emissions are listed, the main one being the lack of information on potential sources in developing countries.

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7. ANNEX

Literature citations and (available) abstracts.