Microbial transformation of highly persistent chlorinated pesticides and industrial chemicals

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Stellingen

1. Bij het bezigen van de term 'afbraak' wordt vaak achterwege gelaten of het 'verdwijning', 'transformatie' of 'mineralisatie' betreft, vooral door diegenen die er geld mee willen verdienen.

2. De bewering van Sahu et al. (1990) dat bij de microbiële *mineralisatie* van 3.61 μM γ-*hexa*chloorcyclohexaan *stoichiometrisch* 2.95 μM chloride wordt geproduceerd is (uiteraard) onjuist.
   (Sahu et al., 1990. Degradation of α-, β-, and γ-hexachlorocyclohexane by a soil bacterium under aerobic conditions. Appl. Environ. Microbiol. 56:3620-3622).

3. Door anno 1995 te betogen dat er tot dan toe geen bewijs is voor aërobe afbraak van HCH-isomeren, spreken Sahu en collega's de toonaangevende literatuur van de laatste 15 jaar tegen.
   (Sahu et al. 1995. Mineralization of α-, β-, and γ-isomers of hexachlorocyclohexane by a soil bacterium under aerobic conditions. J. Agric. Food Chem. 43:833-837).

4. De bewering dat door milieuverontreiniging het uitsterven van de mensheid dreigt, gaat voorbij aan populatiedynamica en evolutie.
   (De Volkskrant, 18 mei 1996)

5. Het veranderen van partij-ideologie, ingegeven door tegenvallende verkiezingsresultaten is een goed voorbeeld van machtsverslaving.

6. Perfectionisme leidt soms tot voldoening, maar meestal tot grote frustratie.

7. Voor wie een reductief dechlorerende bacterie isoleert gaan de geldsluizen wijd open.

8. Gedisciplineerde mensen zijn saai.

9. De intensiteit en lengte van de discussie in de kantine na een lezing is recht evenredig met de hoeveelheid chips en pinda's die voorhanden is.

10. Iemand die in Rotterdam gaat wonen riskeert een verlaging van zijn IQ.
    (Phibbs 1996. EPA reassessment finds as much as 20 times less cancer risk from PCBs. Environ. Sci. Technol. 30, 332A-333A).

11. Om de verspilling van kaas te voorkomen, zou de hoogte van een Hollandse kaas moeten worden aangepast aan de hoogte van een Hollandse boterham.

12. Stellingen van meer dan drie regels zijn te lang.

Stellingen behorende bij het proefschrift "Microbial transformation of highly persistent chlorinated pesticides and industrial chemicals" van Peter Middeldorp.

Wageningen, 20 januari 1997

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CHAPTER 1

General Introduction

INTRODUCTION

The release of toxic man-made chemicals into the environment is a reason for concern, both in industrialized and in developing countries. The increasing negative impact of polluted soils on the quality of groundwater and agricultural products, and incidents like PCB fires, accidental exposures like the Yusho and Yu Yeng poisonings, the explosion in Seveso, the exposures associated with Love Canal, the use of chlorinated phenoxy herbicides in Vietnam, and the PBB food chain contamination in Michigan (177), have awakened the world to its toxic waste problem and made soil protection and clean-up a political issue.

Both in unsaturated and saturated zones in the soil, organic pollutants can be transformed by means of biological reactions. From a toxicological point of view, data on the prediction of fate and behavior of organic compounds in soil and sediments were required. Additionally, the potential of soil microorganisms to remove pollutants and to be applied in biological remediation processes was recognized. Due to these needs, research programmes were initiated in many countries, and an enormous amount of knowledge about microbial degradation processes of organic pollutants has been gained.

Biological remediation processes are now widely applied in various systems, like landfarming, *in situ* remediation, on site composting, and "pump-and-treat" techniques. Disappointingly, full scale application of biological soil remediation is mainly limited to (sandy) soils, polluted with easily degradable organics like light mineral oils and volatile monoaromatics. Problems have been encountered with the biodegradation of compounds like polycyclic aromatic hydrocarbons, and highly chlorinated aromatics and aliphatics. The biodegradation potential of aerobic microorganisms and the degradation pathways are by now greatly known, and research has shifted to biochemistry and genetics of pure cultures on one hand, and application of bioremediation techniques on the other hand. In contrast, knowledge about the organic pollutants degrading capacities of anaerobic microorganisms is still scarce and isolation of these bacteria has only started recently.

Another aspect of bioremediation is the creation of the appropriate environmental conditions for the microorganisms to carry out and maintain their degrading activity. Hereby, we can think of parameters like temperature, pH and redox conditions, but also soil organic matter and moisture content, nutrient status and survival aspects.

However, the lack of microbial activity alone does not account for all problems concerning bioremediation of polluted soils. Even when organic pollutants are easily biodegradable and conditions are favorable, degradation often levels off and ceases at lower pollutant concentrations. The main reason is that organic pollutants generally are poorly soluble in water, and therefore tend to interact with the solid phase of the soil (adsorption/absorption, bound residue formation), thus reducing the access of degrading organisms to the pollutant. This causes a so-called "limited bioavailability".

The aforementioned aspects: aerobic and anaerobic biodegradability, bioavailability, and environmental conditions will be dealt with in this thesis. The compounds studied belong to the group of chlorinated pesticides (polychlorinated phenols, hexachlorocyclohexane, hexachlorobenzene) and chlorinated industrial chemicals (polychlorinated biphenyls and benzenes). In this chapter, these compounds will be introduced to the reader in terms of production, toxicity, sources and occurrence in the environment. Furthermore, their microbial degradation reported in the literature will be discussed.

POLYCHLORINATED PHENOLS

Chlorinated phenols (CPs), were produced through chlorination of phenol or hydrolysis of chlorinated benzenes, yielding mainly 2,4,6-trichlorophenol (TCP), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) (152). Around 1980, about 50,000 tons were annually produced in the world (54) and have been used worldwide, mainly as effective and inexpensive fungicides in forest industry to control sapstain fungi in freshly sawn timber and lumber, but also in agriculture as fungicide, herbicide and insecticide in rice fields. At present, use and production of CPs has been banned or discontinued in most countries.

Due to accidents and spills during dip treatment of timber, soils have been locally heavily polluted with CPs (116, 210). Other sources of chlorophenols in the environment are the combustion of organic material in the presence of chloride (e.g. incineration of municipal waste, forest fires), or chlorination of humic water (pulp bleaching industries) (114).

The toxic and pesticidal effect of PCP is based on its capacity to uncouple oxidative phosphorylation and to alter the electrical conductivity of cell membranes (197). Toxicity to waterfleas and guppies was found to increase with the degree of chlorination, with PCP as the most toxic isomer (182). Acute effects, reported for exposed sawmill workers, are skin irritation, headache, and malaise of the respiratory tract (127)

Besides persistence in the environment, also the occurrence of impurities in the technical product such as the highly toxic chlorinated phenoxyphenols, polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans has been a reason for discouraging the use of CPs (115).

Biodegradation of CPs has been observed under both aerobic and anaerobic conditions. Apajalahti and Salkinoja-Salonen isolated a *Rhodococcus chlorophenolicus* (8, 9), recently reclassified as *Mycobacterium chlorophenolicum* (39, 83), which is able to mineralize penta- to dichlorophenols. Also other bacteria, belonging to different geni, are able to degrade CPs (41, 81, 117, 128, 139, 154, 202, 226). The degradation pathway of PCP was reported to occur via an initial monooxygenase attack on the *para* position, forming tetrachlorohydroquinone, which is subsequently dechlorinated to 2,6-dichlorohydroquinone and further mineralized (201). In another pathway PCP is first *ortho* hydroxylated, then dechlorinated completely to 1,2,4-trihydroxybenzene and further mineralized (figure 1.1) (10).

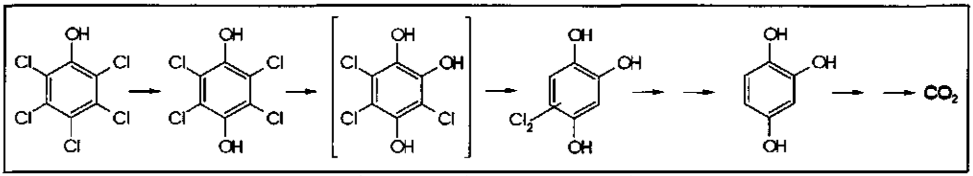


Figure 1.1. Aerobic degradation pathway of PCP by *R. chlorophenolicus* as proposed in reference (10).

Lower chlorinated CPs are known to be monohydroxylated to form a chlorocatechol, which is further degraded by *meta* ring fission and subsequently mineralized (47).

Besides complete mineralization of CPs, also methoxylation of the hydroxyl group (O-methylation) to chlorinated anisoles has been observed (7, 82, 84, 150). This is a co-metabolic process, which is stimulated by the addition of an easily degradable carbon source to the medium (84). The products of O-methylation are generally more lipophilic and have therefore more bioaccumulative and toxic properties than the parent compounds. Their formation should therefore be avoided (151).

Degradation of CPs by various classes of fungi, producing highly reactive exo-enzymes like peroxidase, have been described (120, 121, 122, 130, 142, 161, 176, 190). However, mineralization of CPs by e.g. *Phanerochaete chrysosporium* accounts only for 2% of the initial concentration (122). Additionally, peroxidase has been shown to catalyze the polymerization reaction of CPs to form polychlorinated dibenzodioxins and -furans (156, 205).

Reports on the use of aerobic microorganisms in laboratory scale experiments to enhance the decontamination of soils polluted with CPs, are manifold. Crawford and Mohn (52) reported the removal of PCP from polluted soil by repeated inoculation of a CPs mineralising *Flavobacterium* sp.. Briglia et al. showed that inoculation of at least 10^7 cells of *R. chlorophenolicus* per gram soil was required for a significantly increased rate of mineralization of PCP in two different soil types (40). Inoculation of three sterile PCP contaminated soils with the fungus *P. chrysosporium* led to a removal of up to 98% of the extractable PCP. It was mainly converted to non-volatile products (122). Valo et al. (211) achieved extensive degradation of CPs in large scale composting systems, used to clean up Finnish sawmill soils.

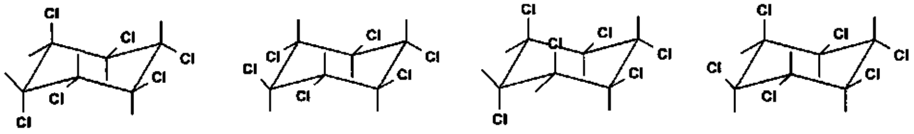
CPs are also degradable under anaerobic conditions. The reductive dechlorination (the mechanism is described later in this chapter) of PCP under anoxic conditions in paddy soil from rice fields was already reported in 1972 (106). Since then, many CPs degrading anaerobic enrichments have been obtained, using sediment, sewage sludge or soil as inoculum. Most studies focus on the degradation of CPs under methanogenic conditions (59, 85, 97, from rice fields was already reported in 1972 (106). Since then, many CPs degrading anaerobic enrichments have been obtained, using sediment, sewage sludge or soil as inoculum. Most studies focus on the degradation of CPs under methanogenic conditions (59, 85, 97, 110, 112, 132). This process generally consists of an initial reductive dechlorination after which the less chlorinated isomer persists (45, 91, 92, 125, 140). However, also further mineralization to CH_4 and CO_2 has been described (38, 59, 78, 102, 103, 112, 141). Mineralization of CPs has also been shown under sulfidogenic conditions (77, 86, 87, 88, 113, 118, 129, 136).

To date, several CPs dechlorinating bacteria have been isolated, which use CPs as a terminal electron acceptor. The 3-chlorobenzoate dechlorinating *Desulfomonile tiedjei* DCB-1 is capable of dechlorination of *meta*-substituted CPs after induction with 3-chlorobenzoate (143). Dechlorination of both *ortho-* and *meta*-substituted CPs was performed by an obligate anaerobe, later identified as *Desulfitobacterium hafniense* (48, 133). Cole et al. (51) isolated a bacterium that dechlorinates 2-chlorophenol. Only the *ortho*-position was dehalogenated, while additional chlorines at other positions decreased or blocked *ortho*-dechlorination. Furthermore, two other CPs dechlorinating *Desulfitobacterium* strains have been isolated (79, 209).

Bioremediation of polluted soils by using anaerobic CPs degrading consortia or pure cultures has not been reported. This is probably due to the relatively successful remediation attempts under aerobic conditions. However, application of anaerobic CPs dechlorinating bacteria in waste water treatment systems is an important subject of study (5, 49, 50, 97, 126).

HEXACHLOROCYCLOHEXANE

1,2,3,4,5,6-Hexachlorocyclohexane (HCH) is an alicyclic chlorinated hydrocarbon, which has been used in large quantities as an insecticide in agriculture. The technical HCH mixture was synthesized by photochemical chlorination of benzene. The product of this reaction is a mixture of different stereo-isomers of HCH, mainly α-HCH (60-80%), β-HCH (5-10%), γ-HCH (10-16%), and δ-HCH (5-10%) (203). They can be identified on the basis of the spatial orientation of the chlorine atoms relative to the carbon ring, which is either axial (perpendicular to the plane of the ring) or equatorial (within the plane of the ring) (Figure 1.2).



α (aaeeee) β (eeeeee) γ (aaaeee) δ (aeeeee)

Figure 1.2. The spatial arrangement of the chlorine atoms in the four most important isomers of hexachlorocyclohexane (HCH).

After DDT was legally restricted for use in many countries, HCH (also incorrectly called benzene hexachloride (BHC)) became important as a substitute for DDT (56). As γ-HCH was found to be by far the most effective insecticide, this isomer was separated from the mixture by crystallization and was sold under the name "lindane". Whereas the use of technical HCH has been banned, lindane is still used in the Netherlands as insecticide for seed preservation, soil treatment during the cropping of e.g. beets, flower bulbs and maize, and in cattle breeding to disinfect sheep, horses and pigs from ectoparasites (196). In India and many other developing countries, technical HCH is still widely used in agriculture (180). In 1983, the world-wide annual production of technical HCH and pure lindane was estimated to be 150,000 and 15,000 tons respectively (196).

The pesticidal action is based on an increased release of neurotransmitters in the synapse (109).γ-HCH is a potent convulsant agent in humans and other mammals (53, 220) as a result of a direct action on the central nervous system (108).

There is sufficient evidence for the carcinogenicity in experimental animals of technical-grade hexachlorocyclohexane and α-hexachlorocyclohexane, and limited evidence for the carcinogenicity of γ-hexachlorocyclohexane and β-hexachlorocyclohexane. Concerning carcinogenicity in humans, the International Agency for Research on Cancer (IARC) concluded that there is no adequate evidence to consider such effects for lindane and other hexachlorocyclohexane isomers (105).

Through the use of technical HCH and γ-HCH as pesticide, and due to inappropriate disposal of waste isomers during lindane production, large amounts of HCH were released into the environment, causing serious soil pollution (61, 98). The occurrence of individual isomers in the environment can also be the result of an isomerization of one isomer to another. Microbial isomerization of γ-HCH to α-HCH by a *Pseudomonas* sp. has been observed both in the laboratory and in aquatic sediments (26, 67), but also abiotic interconversion mechanisms have been described (56).

The four HCH isomers differ in their susceptibility to microbial degradation. α-, and γ-HCH were shown to be readily degradable in the environment under aerobic conditions (13, 32, 62, 63, 111, 123, 124, 175, 218, 225). Numerous bacterial strains, capable of degrading α-, γ-, and δ-HCH have been isolated. Tu (207) found that 71 out of 147 bacterial and fungal soil isolates were capable of growth with γ-HCH as sole carbon source. Senoo and Wada (192, 193) isolated several *Pseudomonas paucimobilis* strains (later reclassified as *Sphingomonas paucimobilis* (221)) from an upland field which had been treated with γ-HCH for over 10 years. Huntjens et al. (104) isolated a *Pseudomonas* strain which was able to mineralize α-, γ-, and δ-HCH. Also a *P. tralucida* strain was isolated on α-HCH, which produced a specific emulsifier to increase the solubility of HCH (11). β-HCH has long been considered to be persistent under aerobic conditions (14, 62, 63). However, recently a *Pseudomonas* sp.

capable of co-metabolic degradation of β-HCH with acetate has been isolated (180). β-HCH was mineralized for ca. 5 % after 10 days of incubation (181). Inoculation of this strain into β -HCH polluted soil did not lead to significant degradation. (179).

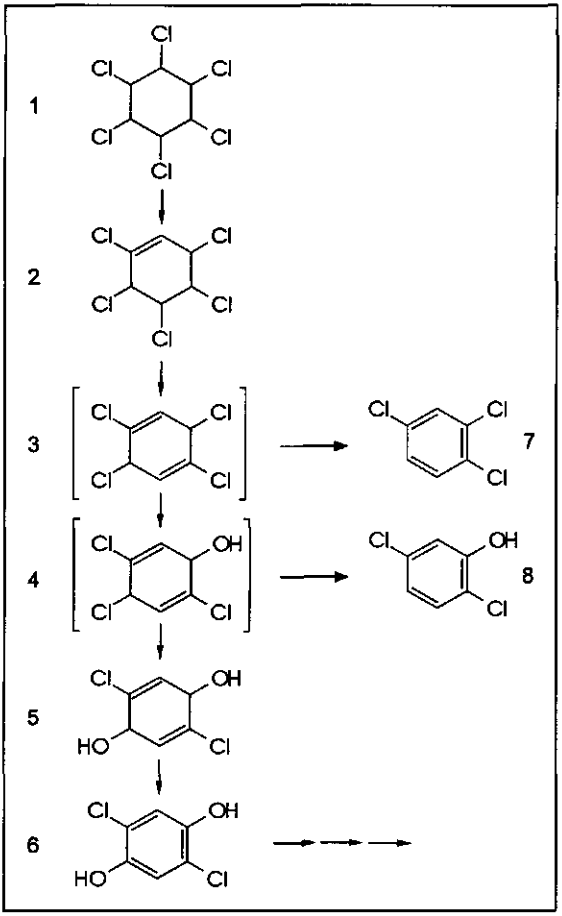


Figure 1.3. Aerobic degradation pathway of γ-HCH as proposed by Nagasawa et al. (147). 1) γ-HCH; 2) γ-pentachlorocyclohexene; 3) 1,3,4,6-tetrachloro-1,4-diene; 4) 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 5) 2,5-dichloro-2,5-cyclohexadiene-1,4-diol; 6) 2,5-dichlorohydroquinone; 7) 1.2.4-trichlorobenzene; 8) 2,5-dichlorophenol.

The aerobic degradation pathway of γ-HCH has been described in detail for a *Sphingomonas paucimobilis* UT26 (147, 148, 149). This pathway involves a dehydrohalogenation to γ-pentachlorocyclohexene and is proposed to proceed further via two subsequent dehydrohalogenations to form 1,2,4-trichlorobenzene, or via a dehydrohalogenation and a hydrolytic dechlorination followed by another dehydrohalogenation yielding 2,5-dichlorophenol. However, the major part of the pentachlorocyclohexene was proposed to be degraded via a dehydrohalogenation and two subsequent hydrolytic dechlorinations followed by another dehydrohalogenation to form 2,5-dichloro-2,5-cyclohexadiene-1,4-diol, which was further mineralized via 2,5-dichlorohydroquinone (Figure 1.3).

Degradation of HCH by fungi is documented poorly. Dehydrochlorination of γ-HCH to γ-pentachlorocyclohexene was shown to occur in aqueous suspensions of fungi (68) and similar results were observed by Tu (207) for several fungal isolates. Bumpus et al. (46) reported partial mineralization of γ-HCH by a *P. chrysosporium*.

Several attempts have been made to decontaminate HCH polluted soil using bacteria. In laboratory experiments, α-HCH was degraded faster when soil slurries were aerated by vigorously stirring instead of shaking. This was explained by breaking up of soil particles through stirring. This increased the accessibility of the HCH, located inside the particles, to the degrading microorganisms (175). Doelman et al. (62) showed that in batches of 60 kg of polluted soil, the concentration of α-HCH decreased from ca. 420 mg/kg to 15 mg/kg in a continuously aerated thick slurry system. At a larger scale (6 m^3 of soil), the concentration of α-HCH soil was reduced from ca. 370 mg/kg to 110 mg/kg in moist soil, and to ca.60 mg/kg in aerated slurry (63). In both studies, β-HCH was not removed. Inoculation of the γ-HCH degrading *S. paucimobilis* SS86 in γ-HCH polluted soil in a small scale experimental upland field resulted in a degradation of more than 95% of the initial concentration (6-9 mg/kg of soil) within 2-6 months, depending on the season (191).

Anaerobic removal of γ-HCH in submerged soils was first shown to occur in 1966 (170). Although under these conditions degradation of all four isomers has been observed, large differences exist in rate and extent. MacRae et al. (131) showed that in submerged soil contaminated with α-, β-, γ-, and δ-HCH (15 mg/kg each), all isomers disappeared within 70 days of incubation. A small amount of the spiked ^{14}C-γ-HCH was recovered as $^{14}CO_2$. In a submerged soil from rice fields containing 250 mg/kg γ- and β-HCH, degradation of ca. 65% and 90% of resp. β- and γ-HCH was observed in three soils within 41 days of incubation. However, in a sandy soil, no degradation was observed. Redox measurements revealed that degradation of β-HCH did not start until E_h decreased to -40 mV, whereas degradation of γ-HCH occurred already before this value was reached (195). Bachmann et al. (14) tested the degradation of α- and β-HCH in soil slurries under different anaerobic conditions. Ca. 380 mg/kg of α-HCH was completely degraded within 100 days under methanogenic conditions. Under nitrate and sulfate reducing conditions little or no degradation was observed. No degradation β-HCH occurred under any of the studied conditions.

Several pure bacterial strains were shown to degrade HCH. Some *Clostridium* strains and representatives of *Bacillaceae* and *Enterobacteriaceae* were capable of converting different HCH isomers under fermentative conditions. Whereas up to 90% of the γ-HCH was degraded by these bacteria, α- and β-HCH were degraded only up to 30% and δ-HCH was hardly (up to 5%) or not degraded (107). Ohisa and Yamaguchi (159) showed that 7 out of 13 tested *Clostridium* strains were able to degrade γ-HCH, but none of the strains was able to transform β- and δ-HCH. From experiments with whole cells and cell extracts of *Clostridium rectum*, the anaerobic degradation of γ-HCH was proposed to proceed via two dihalo-eliminations, first to γ-3,4,5,6-tetrachlorocyclohexene (TeCCH) and then to 5,6-dichlorocyclohexa-1,2-diene (not identified), and a dehydrohalogenation to chlorobenzene as the end product (160). A similar anaerobic degradation pathway of γ-HCH to chlorobenzene was observed after anaerobic incubation of γ-HCH with rat liver cytochrome P450 (31), or with a range of hemoproteins, porphyrins and corrins (135). However, Beland et al. (25) found the production of benzene from γ-HCH via TeCCH after incubating sewage sludge under reducing conditions. The benzene production accounted for only 10% of the original γ-HCH concentration. Also production of both benzene and chlorobenzene in a ratio of ca. 1:20 was observed in rat liver microsomes incubated with γ-HCH (15). Chlorobenzene, but not TeCCH was found to be produced from α-HCH in soil slurries incubated under methanogenic conditions (14). Except for the release of chloride (107), nothing is known about the anaerobic degradation of β-HCH and the transformation pathway of this environmentally important isomer.

The potential large scale application of anaerobic transformation of HCH for soil bioremediation purposes was determined by Doelman et al. (61). They found that in batches of ca. 90 kg of polluted soil, no degradation of any of the HCH isomers occurred at temperatures simulating soil conditions in temperate regions (5-17 °C).

CHLORINATED BENZENES

Chlorinated benzenes (CBs) are a group of twelve isomeric compounds consisting of an aromatic ring, of which each carbon atom may be substituted with a chlorine atom. These highly volatile lipophilic compounds are used as industrial solvents, insect repellents, fungicides, dielectric fluids, odorisers and intermediates in the manufacturing of various chemicals.

Production and emission of CBs showed maxima in the 1960s and 1970s (27). For example, the annual world-wide production of the odoriser and insect repellent 1,4-dichlorobenzene (DCB) in the 1970s was estimated to be 80,000 tons (166).

CBs were shown to have a non-specific narcotic effect on fish (214). Especially higher chlorinated benzenes tend to accumulate in biota (163). Hexachlorobenzene (HCB), used as fungicide in seed coating for cereal crops, caused an outbreak of human porphyria after consumption of HCB treated wheat (185).

As a result of the abundant use of CBs for the various purposes mentioned above, both diffuse and concentrated contamination of soils, groundwater, sediments and biota has been reported (162, 189, 215). Due to the toxicity and bioaccumulative properties of chlorinated benzenes, polluted sites are of major concern and subject to bioremediation studies. Under aerobic conditions, microbial degradation of low concentrations (nM to μM) of chlorinated benzenes with up to three chlorine atoms has been observed during passage of polluted river water through soil (34, 119, 188). Marinucci and Bartha (134) showed degradation of ^{14}C-labelled 1,2,3- and 1,2,4-trichlorobenzene (TCB). However, only 10% of the total substrate added was recovered as CO_2. Many pure bacterial cultures using CBs as sole carbon and energy source have been obtained. Most of these are able to degrade mono- and dichlorobenzenes (55, 90, 164, 172, 187, 200, 226). However, more recently also mineralization of

CBs with up to four chlorine atoms has been observed (183, 212). Interestingly, all mentioned CB mineralising strains belong to the geni *Pseudomonas* or *Alcaligenes*.

Co-metabolic biodegradation was shown by Bouwer and McCarthy (37), who observed bioremoval of trace concentrations of 1,4-DCB in acetate-supported biofilms with an acclimation period of 10 days. Mineralization of chlorobenzene (80-90%) was observed in benzene pregrown cell suspensions of three *Pseudomonas* sp. and one *Nocardia* sp. (89). When di- and trichlorinated benzenes were tested, only the *Pseudomonas* spp. showed mineralization (ca. 10%).

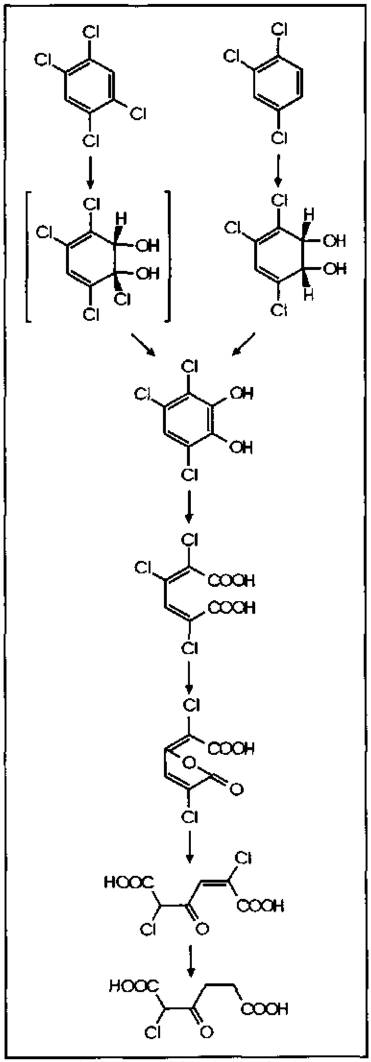


Figure 1.4. Aerobic degradation pathway of 1,2,4-TCB and 1,2,4,5-TeCB as proposed by Sander et al. (183).

The degradation pathway of CBs is generally initiated by a dioxygenase attack on two vicinal chlorine-free carbon atoms in the aromatic ring, thus forming a chlorinated *cis*-cylohexa-3,5-diene (chlorodihydroxydiol), which is further converted to a chlorinated catechol (Figure 1.4) (55, 172, 187). In the case of 1,2,4,5-TeCB degradation as described by Sander et al. (183), a hydrolytic dechlorination is suggested to take place since two vicinal chlorine free carbon atom are not present in this isomer: after dehydrohalogenation of the intermediate chlorinated dihydroxydiol, 2,3,5-trichlorocatechol is produced (Figure 4). Further mineralization of the chlorinated catechol occurs via a ring fission at the *ortho*-position to a chlorinated *cis, cis*-muconic acid, and via several steps to maleylacetate and finally to succinic acid (47, 173). Besides the commonly observed dioxygenase attack, a hydroxylation of the aromatic ring in the case of all TCB isomers by means of a monooxygenase has been reported for a *Pseudomonas* sp. (16). The thus formed chlorinated phenols, were the only observed transformation products.

The capacity of microorganisms to mineralize CBs under aerobic conditions is used in the purification of river water by dune infiltration for the preparation of drinking water (36). Besides this, no large scale operations are known for aerobic bioremediation purposes for with CBs polluted soil or water.

Reductive dechlorination is the only anaerobic transformation process observed for CBs. Fathepure et al. (71) showed reductive dechlorination of HCB in anaerobic sewage sludge within 3 weeks of incubation. HCB was first dechlorinated to pentachlorobenzene (QCB), which was further dechlorinated mainly via 1,2,3,5-tetrachlorobenzene (TeCB) to 1,3,5-TCB. A minor amount of HCB was dechlorinated via 1,2,4,5-TeCB to 1,2,4-TCB and finally to 1,2-, 1,3-, and 1,4-DCB (Figure 1.5).

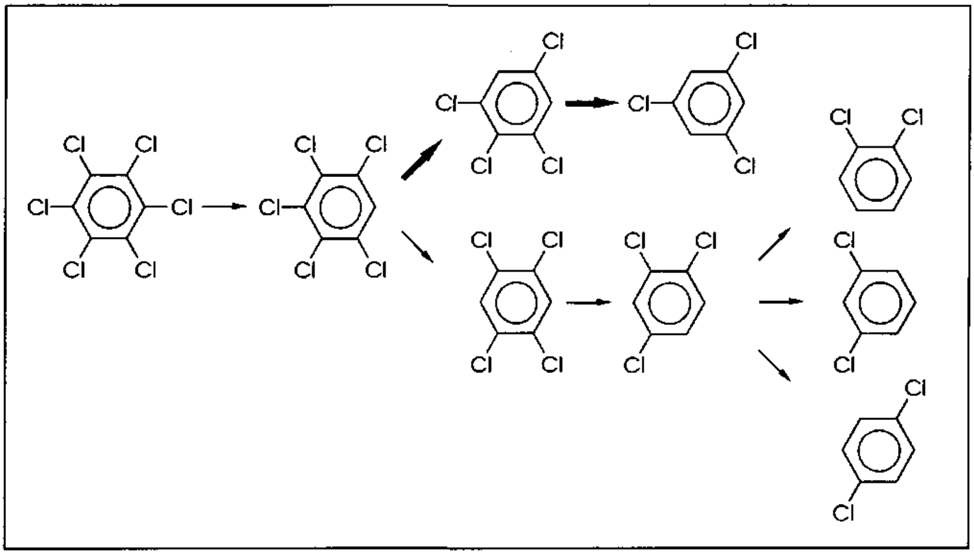


Figure 1.5. Microbial anaerobic reductive dechlorination of HCB as proposed by Fathepure et. al. (71). The bold arrows indicate the main pathway through which more than 90% is dechlorinated.

Dechlorination of all three TCB isomers to chlorobenzene was reported to occur in soil percolation columns under methanogenic conditions (36). In similar systems, almost complete dechlorination of di- through hexachlorobenzenes to DCB and monochlorobenzene was observed (35). From these soil columns, enrichment cultures were obtained which were capable of dechlorinating HCB, QCB, all TeCBs, 1,2,3-TCB and 1,2,4-TCB in the presence of several electron donors (100). When the enrichment on 1,2,3-TCB was incubated with HCB, it showed a similar dechlorination pathway as in Figure 5, with 1,3,5-TCB as the main end product. Another methanogenic enrichment was obtained by enrichment on HCB from a lake sediment (29). This consortium was tested with all CBs, and was shown to exhibit a dechlorination pathway in which only chlorine atoms with two adjacent chlorines were removed. This pathway, which was also reported for HCB in the previously mentioned studies (35, 71, 100), was proposed to be thermodynamically the most favourable. Core samples from that lake sediment which was deposited in 1972, were compared with top-layer sediment samples which were taken in 1972 and stored in frozen condition. This comparison showed that the HCB concentration had decreased, while the 1,3,5-TCB concentration had increased since the time of sedimentation. It indicates that dechlorination occurred *in situ* and via a similar pathway as described for the enrichment culture (30). Ramanand et al. (171) obtained an enrichment culture on a mixture of HCB, QCB and 1,2,4-TCB. They reported a previously unreported dechlorination pathway of HCB, which occurred via QCB, 1,2,3,4-TeCB, which was further dechlorinated either via 1,2,3-TCB and 1,2-TCB, or via 1,2,4-TCB and 1,4-DCB, to chlorobenzene.

Little is known about the microorganisms responsible for the dechlorination of CBs.

Tsuchiya and Yamaha (206) isolated twelve bacterial strains from the intestinal contents of rats, which were able to reductively dechlorinate 1,2,4-TCB to chlorobenzene. Dechlorination mainly took place during the stationary growth phase of the bacteria. Since all DCB isomers were formed as intermediates and only ca. 0.5% of the initial amount of 1,2,4-TCB was dechlorinated, this transformation is probably non-specific and not involved in the main metabolic activity of the bacteria.

In most microcosm studies, dechlorination of CBs was observed under methanogenic conditions. The addition of 2-bromoethanesulphonic acid (BrES) did not inhibit dechlorination of CBs (100), indicating that either acetogenic bacteria or bacteria that can use CBs as exclusive electron acceptor for their metabolism are involved.

Reductive dechlorination of CBs under sulfidogenic conditions was reported for an enrichment culture obtained from an estuarine sediment (28). Dechlorination of tri- and dichlorobenzenes has also been tested in soil columns under denitrifying conditions, but no dechlorination was observed (35).

Reductive dechlorination of CBs yields lower chlorinated benzenes. Chlorobenzene is not dechlorinated and thus persists under anaerobic conditions. Low chlorinated benzenes are considered less toxic, but more mobile in environmental systems than the parent CB. Since aerobic degradation of CBs with more than four chlorine atoms has not been observed, bioremediation should essentially involve anaerobic dechlorination of highly chlorinated CBs as a pretreatment before an aerobic mineralization step can take place.

POLYCHLORINATED BIPHENYLS

Polychlorinated biphenyls (PCBs) are a group of 209 isomers of chlorinated biphenyls. These synthetic chemical compounds, manufactured by the chlorination of biphenyl, are referred to as congeners. Different arrangements of chlorine atoms around the biphenyl ring (Figure 1.6) cause changes in the physical properties and thus their

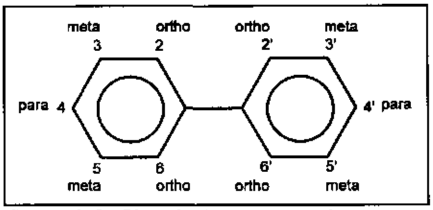


Figure 1.6. Chemical structure of a biphenyl molecule and the numbering system for chlorine substitution.

industrial application. As a result, complex mixtures of individual congeners with different degrees and positions of chlorine substituents have been produced. Since 1927, about 600,000 tons of PCBs have been marketed by Monsanto, the major world manufacturer, and other North American producers. Production has also taken place in e.g. France, Germany, Italy, Japan, and the former Soviet Union. The total world-wide production through 1980 was estimated to be about 1,000,000 tons (69).

The properties that make PCBs applicable for industry is their chemical inertness, thermic stability, resistance to hydrolysis by water, alkali and acids, low flammability and a high dielectric constant. PCBs are virtually insoluble in water, but are soluble in organic solvents. Due these properties, PCBs have been used in diverse industrial applications, such as in hydraulic fluids, heat transfer fluids, dielectric fluids in transformer and capacitors, flame retardants, pesticides, paints, printing inks, dedusting agents and others (1, 69).

The widespread use of PCBs has led to world-wide contamination of the environment through accidents and improper use. PCBs have been shown to be present in almost every compartment of the global ecosystem, such as fish, wildlife, human and animal adipose samples, milk, serum as well as in sediments, and more generally in all aquatic ecosystems (69, 177). Because the production of PCBs is presently banned, the amount entering the environment has decreased. However, continued release from reservoirs (e.g. old refrigerators) and municipal waste incineration appears still inevitable.

The toxicity of PCBs has been studied mostly for commercial mixtures while little is known about the toxicity of individual chlorobiphenyls. PCBs are highly soluble in fats, and their accumulation resides primarily within organs that are rich in lipids. The mechanism of toxic action of PCBs appears to differ for various chlorinated biphenyls (70). Highly chlorinated co-planar (i.e. non-*ortho*-substituted) PCBs are considered as the congeners that contribute significantly to the toxicity of the commercial PCB mixtures. This is due to their spatial resemblance to the highly toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (1, 178). They have been held responsible for common toxic responses such as body weight loss, thymic atrophy, impairment of immune responses, hepatotoxicity and porphyria, chloracne and related dermal lesion, carcinogenesis, teratogenicity and reproductive toxicity (177). PCBs are considered potential carcinogens, based on experiments with Aroclor 1260 which was shown to increase the incidence of hepatic tumors in rats (155). However, there is no conclusive evidence for PCBs related carcinogenicity in humans (12).

The many characteristics that have made PCBs industrially desirable, also make them persistent and accumulatible in the environment. However, under laboratory conditions, aerobic degradation of PCBs has been shown in numerous studies (reviewed in (1, 18, 74)). Utilization of PCBs as sole carbon source by pure cultures is limited to mono- and disubstituted chlorobiphenyls, whereas co-metabolic degradation (mostly with biphenyl as carbon source) has been observed for PCBs with up to seven chlorine atoms (20, 74).

The degradation of PCBs (Figure 1.7) occurs via a dioxygenase attack at carbon positions 2,3 on the less chlorinated ring, followed by dehydrogenation and ring fission at the *meta* position (between carbons nos. 1 and 2), and cleavage to form chlorobenzoic acid (CBA) and a five carbon fragment (75). Most strains do not further metabolise the formed CBA. Although many PCB degrading bacteria have been isolated, their degrading capacity towards PCBs with more than four chlorine atoms is generally limited (23). 2,3-dioxygenase attack of a biphenyl generally requires at least three chlorine-free *ortho*-positions and one chlorine-free *meta*-position next to a free *ortho*-site.

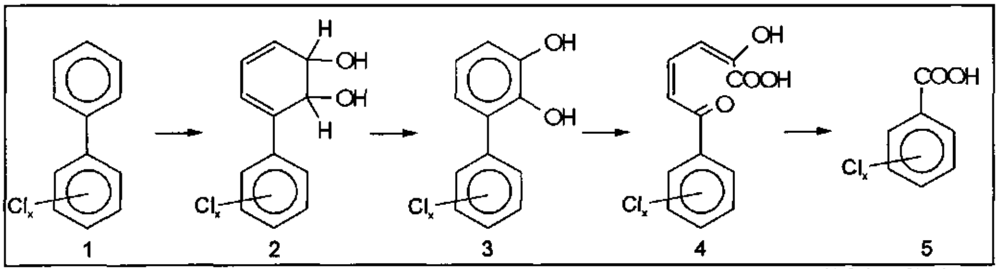


Figure 1.7. Aerobic degradation of PCBs following the 2,3-dioxygenase pathway. 1) PCB; 2) cis-chlorobiphenyl dihydrodiol; 3) 2,3-dihydroxybiphenyl; 4)2-hydroxy-6-oxo-6-phenylhexa-2,4-dioenic acid; 5) chlorobenzoic acid.

Higher chlorination levels may result in steric hindrance of the enzyme (165). However, several bacterial strains (*Pseudomonas* sp. LB400 (33), *Alcaligenes eutrophus* H850 (20), *Corynebacterium* sp. MB1 (24), *Acinetobacter* sp. P6 (75, 76)) were shown to posses the capacity to degrade PCBs with up to seven chlorine atoms. Although these strains possess 2,3-dioxygenase activity, it was suggested that several PCBs, especially those containing a 2,5-chlorophenyl ring, are degraded via a 3,4-dioxygenase attack (20, 23). There are indications that the enzyme specificity of the 2,3-dioxygenase system is essentially equal in all PCB degrading microorganisms. Based on biochemical and genetic data is was speculated that the 2,3- and the 3,4-dioxygenase activity is catalysed by the same enzyme (80).

Both strain LB400 and H850 generate also transient chlorinated acetophenones (CAP) from certain PCBs, by a mechanism that is not yet understood (18, 20). Barton and Crawford (17) described 4-CAP as final product formed by a *Pseudomonas* sp. from 4-chlorobiphenyl. Microorganisms, capable of co-metabolising PCBs, generally tend to accumulate CBAs and/or CAPs as they are unable to grow on these substrates. In addition, CBAs can possibly inhibit PCB degradation (199). Nevertheless, several bacteria are able to completely mineralise PCBs (72, 94, 165, 167), while complete mineralization was also achieved using constructed strains (94, 96, 138, 194) or bacterial consortia consisting of PCB- and of CBA-degraders (3, 4, 73, 95, 167).

Degradation of PCBs by fungi has also been reported (46, 58, 60, 66, 184, 217, 227). A *P. chrysosporium* sp. was shown to mineralize various PCB congeners (46). Dmochewitz et al (60) reported an *Aspergillus niger* to be capable of degrading mixtures of PCBs. Zeddel et al. (227) showed extensive degradation of PCBs with up to six chlorines, by the white rot fungi *Pleurotus ostreatus* and *Trametes versicolor*. In this study, 4,4'-substituted chlorobiphenyls were degraded to a lesser extent, 2,2',4,4',5,5'-hexachlorobiphenyl was not degraded at all, while data on mineralization are not available. However, up to 11% mineralization of 4,4'-dichlorobiphenyl was achieved by a *P. chrysosporium* sp., and 4-chlorobenzoic acid and 4-chlorobenzyl alcohol were identified as metabolites (58). In contrast, there was negligible mineralization of the 3,3',4,4'-tetrachloro- and 2,2',4,4',5,5'-hexachlorobiphenyl. A large fraction of the non-degraded PCBs appeared to be non-specifically adsorbed to the fungal hyphae. A *P. chrysosporium* was also reported to degrade several Aroclors (222).

Aerobic degradation of PCBs was applied by Harkness et al. (93), who stimulated the indigenous degrading microorganisms in Hudson River sediments by aeration and the addition of inorganic nutrients and biphenyl. They reported PCB degradation as indicated by (up to 55%) loss of PCBs and production of chlorobenzoates. Repeated inoculation of the PCB degrading *A. eutrophus* H850 did not enhance degradation, probably due to the poor survival of this bacterium in the sediment.

Anaerobic transformation of PCBs occurs via reductive dechlorination. This process has recently been reviewed by Bedard and Quensen (21), and applies preferentially to higher chlorinated congeners. Microbial reductive dechlorination of PCBs was first suggested to occur *in situ* in Hudson River sediments. Compared to the commercial PCB mixtures (Aroclors) that had been originally released, altered congener distributions of residual PCB contamination were found (42, 43, 44). This was confirmed in laboratory experiments with autoclaved controls (169). Several studies with Aroclors showed that during reductive dechlorination of PCBs, selectively *meta* and *para* substituted chlorines are removed (146, 153, 168, 223, 224), while *ortho* dechlorination appears to be rather exceptional (213, 219). This has two advantages: the less chlorinated PCBs formed after dechlorination, are less toxic, and they are more susceptible to aerobic degradation. For these reasons, it is important to know which PCBs are formed from the reductive dechlorination of commercial mixtures in polluted environments. It appears that different dechlorination preferences are exhibited for the same Aroclor in different sediments, indicating different dechlorinating populations (168). All reported dechlorination patterns have recently been evaluated and classified into seven different "processes". These are mainly based on different dechlorination preferences for different substituted chlorophenyl rings, which would possibly distinguish between different

PCB dechlorinating populations (21). Dolfing et al. (65) suggested a model in which the dechlorination preferences of dechlorinating microorganisms toward single PCB congeners is related to the thermodynamics of the dechlorination reaction. The chlorine that is removed (with H_2 as electron donor) is the one that yields most Gibbs free energy under standard conditions (27). Since *ortho* dechlorination is rarely observed, this reaction is considered to be blocked by e.g. steric hindrance of the enzyme, and has been excluded (although it generally has the highest Gibbs free energy yield). This model has been supported by data from the literature on dechlorination of single PCB congeners, incubated with different sediments (2, 174, 219). However, enrichments have been described which dechlorinate one congener simultaneously at two different chlorines, yielding different amounts of Gibbs free energy. Sokol et al. (198) showed dechlorination of 2,3,4-TCBP to both 2,3- and 2,4-DCBP, while Ye et al. (224) observed dechlorination of 2,3',4-TCBP to both 2,4- and 2,3'-DCBP. Furthermore, thermodynamics do not explain the different dechlorination patterns that have been observed for Aroclor mixtures.

As for CBs, the microbiology of PCB dechlorinating bacteria is poorly understood. Reductive dechlorination of PCBs has mainly been observed under methanogenic conditions. Ye et al. observed that *para* dechlorination of PCBs in Aroclor 1242 was inhibited along with methanogenesis after heat and/or ethanol or BrES treatment, while exclusively para dechlorination was occurring when eubacteria-inhibiting antibiotics were added along with methanogenic substrates to these cultures (223, 224). They concluded that at least anaerobic sporeformers and methanogenic bacteria belong to the physiological groups that are able to dechlorinate PCBs. Although sulfate has been reported to inhibit dechlorination of PCBs (6, 137, 146, 204), dechlorination has been shown to occur also under sulfate reducing conditions (137, 157). It has been suggested that PCB dechlorinating microorganisms use PCBs as electron acceptors and that they may derive energy from their reduction. In field experiments, Bedard et al. (22) successfully stimulated the *in situ* dechlorination of PCBs by the addition of 2,6-dibromobiphenyl, which may be used as electron acceptor for growth by the PCB dechlorinating bacteria. After one year, it appeared that 2,6-dibromobiphenyl significantly stimulated the dechlorination of particularly *meta* chlorines positioned next to other chlorines. Until now, no bacteria, capable of reductive dechlorination of PCBs have been isolated. This is partly due to the complexity of interactions between different types of anaerobic bacteria and the unknown role which the dechlorinating bacteria play herein. Additionally, this complexity is increased by the presence of sediment needed, which introduces undefined (amounts of) carbon sources, vitamins and trace elements to the system. Therefore, cultivation and especially subculturing PCB dechlorinating enrichments has been shown to be laborious.

REDUCTIVE DECHLORINATION

Reductive dechlorination is a dehalogenation process which is commonly involved in the microbial transformation of chlorinated organic compounds under reduced conditions. Three types of reductive dechlorination can be distinguished (Figure 1.8) (216). The first reaction, hydrogenolysis, replaces one chlorine substituent with a hydrogen atom. This process was shown to occur for both alkyl and aryl chlorinated compounds, such as chlorinated ethenes, CPs, CBs and PCBs. The second type is called dihalo-elimination or vicinal reduction. This reaction removes two vicinal chlorine atoms and forms a carbon-carbon double bond. This type of dechlorination has been shown to occur for aliphatic chlorinated compounds, such as chlorinated alkanes and HCH isomers. The third reaction type is a coupling reaction, which involves two chlorinated molecules from which a chlorine radical is released, after which a dimerization follows.

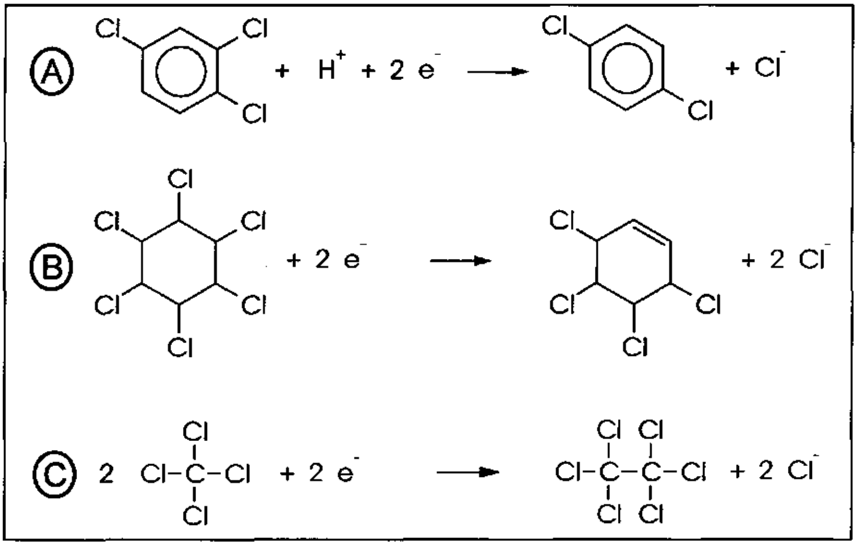


Figure 1.8. Examples of different types of reductive dehalogenation. (A) hydrogenolysis; (B) dihalo-elimination; (C) coupling.

Microbiologically mediated reductive dechlorination has been demonstrated for numerous types of chlorinated organic compounds and was extensively reviewed by Mohn and Tiedje (145). It is the result of the microbial oxidation of an organic or inorganic substrate (electron donor) from which the generated electrons are used to reduce the chlorinated compound (electron acceptor) to a less chlorinated one. The dechlorination may occur as a co-metabolic activity (i.e. no energy for the microbe is derived), but may also be part of a novel respiration. Co-metabolic dechlorination was shown to be performed by numerous different bacteria, ranging from stictly anaerobic to strictly aerobic bacteria (99, 208), and was mostly observed for aliphatic compounds. However, dechlorination of 1,2,4-TCB by *Staphylococcus* *epidermidis* and related strains has been reported, which is probably a co-metabolic reaction (206). Enzyme systems, which are suggested to be involved in this reaction, include iron(II)porphyrins, corrinoids, factor F_{430}, flavoprotein-flavin complexes, and ferredoxins.

Halogenated organic compounds are potentially strong oxidants and may therefore serve as terminal electron acceptors in anaerobic respiration (216). Indeed, anaerobic bacteria which are capable of coupling reductive dechlorination to growth and ATP synthesis have been isolated. Holliger et al. (101) described a tetrachloroethene (PCE) dechlorinating bacterium, which could only use this compound for respiration. Recently, also less restricted PCE dechlorinating bacteria were isolated (79, 186). Indications for ATP synthesis coupled to reductive dechlorination of γ-HCH have been reported (158).

Although reductive dechlorination of many chlorinated organic compounds has abundantly been reported, only few dechlorinating bacteria have been isolated. Aryl dechlorination by a pure culture was first described for *Desulfomonile tiedjei*, which derives energy from the reductive dechlorination of 3-chlorobenzoate (57, 64, 144). Till now, isolated aryl dechlorinating bacteria were shown to dechlorinate only derivatives of chlorinated benzenes and phenols for growth (48, 51, 79, 133, 209). Although different specific dechlorination pathways suggest that dechlorination of CBs and PCBs is enzyme-mediated, it is unknown whether "chlororespiration" of this class of compounds exists in nature. Holliger et al. (100) showed that a 1,2,3-TCB dechlorinating enrichment culture was not inhibited by the addition of the methanogenic inhibitor BrES. However, no acetogenic bacteria, isolated from this culture, were capable of dechlorination. From these results was concluded that dechlorination of 1,2,3-TCB was probably carried out by bacteria that selectively use the 1,2,3-TCB as electron acceptor. An indication of the use of reductive dechlorination for growth was shown for PCBs. Bedard et al. (19) showed that the addition of a selected PCB to a with Aroclor 1260 polluted sediment, stimulated *para*-dechlorination of all PCBs. This was explained by the fact that bacteria, using the energy resulting from the dechlorination of the added PCB for growth, were enriched and therefore dechlorinated other *para*-chlorinated PCBs faster. A similar stimulation of PCB dechlorination was also applied in an *in situ* dechlorination experiment, using bromobiphenyls instead of PCBs (22). In this case, mainly *meta*-chlorines were removed.

Outline of this thesis

The aim of the research presented in this thesis is to gain basic knowledge on the biodegradation of chlorinated pesticides and industrial chemicals and the microorganisms involved. This knowledge is related to a potential application of these processes to the bioremediation of polluted environments.

In Chapter 2, the biodegradation of the environmentally persistent β-isomer of HCH by a bacterial consortium was studied under methanogenic conditions. The degradation pathway of β-HCH, as well as a mass balance was established for this biotransformation. To determine the applicability of this process for bioremediation purposes, degradation of the other HCH isomers (α-, γ-, and δ-) by this consortium was tested.

Chapter 3 deals with the reductive dechlorination of 1,2,4-TCB in a methanogenic consortium (culture 124). Based on the dechlorination of other CBs and also PCBs, different dechlorination preferences which possibly originate from different microbial populations in the consortium, are described.

In Chapter 4, the highly specific reductive dechlorination of CBs and PCBs by another methanogenic consortium (culture 61) is described. By inhibiting growth of different physiological groups of microorganisms in the consortium, information was gained on the involvement of these groups in the reductive dechlorination.

Chapter 5 addresses the reduction of the lag phase before the reductive dechlorination of PCBs starts. The effect of inorganic (Rhine sand) and organic/biological additions on the duration of the lag phase of a PCB mixture and a single congener was studied in culture 61.

Chapter 6 deals with the application of an aerobic PCP degrading bacterium, *R. chlorophenolicus,* in two different soil types to initiate PCP degradation at different initial PCP concentrations. The competition of the inoculated degraders with both indigenous microorganisms and with bacteria competing for the pollutant (methylating bacteria), with or without addition of an easily degradable carbon source, was studied. A chlorine and carbon mass balance of PCP in the soil was made to quantitate PCP losses in the form of non-extractable or soil-bound residues before and after degradation.

Finally, the contents of the previous chapters is summarized and the meaning of the research for potential application in the bioremediation of organic pollutants is discussed (Chapter 7).

REFERENCES

1. **Abramowicz, D. A.** 1990. Aerobic and Anaerobic Biodegradation of PCBs - A Review, p. 241-249. *In* G. G. Steward and I. Russell (ed.), CRC Critical Reviews in Biotechnology, CRC Press, Boca Raton, FL.
2. **Abramowicz, D. A., M. J. Brennan, H. M. Van Dort and E. L. Gallagher.** 1993. Factors influencing the rate of polychlorinated biphenyl dechlorination in Hudson River sediments. Environ. Sci. Technol. **27:**1125-1131.
3. **Adriaens, P. and D. D. Focht.** 1990. Continuous coculture degradation of selected polychlorinated biphenyl congeners by *Acinetobacter* spp. in an aerobic reactor system. Environ. Sci. Technol. **24:**1042-1049.
4. **Adriaens, P., H.-P. E. Kohler, D. Kohler-Staub and D. D. Focht.** 1989. Bacterial dehalogenation of chlorobenzoates and coculture biodegradation of 4,4'-dichlorobiphenyl. Appl. Environ. Microbiol. **55:**887-892.
5. **Ahring, B. K., N. Christiansen, I. Mathrani, H. V. Hendriksen, A. J. L. Macario and E. Conway de Macario.** 1992. Introduction of a *de novo* bioremediation ability, aryl reductive dechlorination, into anaerobic granular sludge by inoculation of sludge with *Desulfomonile tiedjei*. Appl. Environ. Microbiol. **58:**3677-3682.
6. **Alder, A. C., M. M. Häggblom, S. R. Oppenheimer and L. Y. Young.** 1993. Reductive dechlorination of polychlorinated biphenyls in anaerobic sediments. Environmental Science and Technology **27:**530-538.

7. **Allard, A.-S., M. Remberger and A. H. Neilson.** 1987. Bacterial O-methylation of halogen-substituted phenols. Appl. Env. Microb. **53(4)**:839-845.

8. **Apajalahti, J. H. A., P. Kärpänoja and M. S. Salkinoja-Salonen.** 1986. *Rhodococcus chlorophenolicus* sp. nov., a chlorophenol mineralizing actinomycete. Int. J. Syst. Bacteriol. **36**:246-251.

9. **Apajalahti, J. H. A. and M. S. Salkinoja-Salonen.** 1986. Degradation of polychlorinated phenols by *Rhodococcus chlorophenolicus*. Appl. Microbiol. Biotechnol. **25**:62-67.

10. **Apajalahti, J. H. A. and M. S. Salkinoja-Salonen.** 1987. Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced *Rhodococcus chlorophenolicus*. J. Bacteriol. **169**:5125-5130.

11. **Appaiah, K. A. A. and N. G. K. Karanth.** 1991. Insecticide specific emulsifier production by hexachlorocyclohexane utilizing *Pseudomonas tralucida* Ptm+ strain. Biotechnol. Lett. **13**:371-374.

12. **ATSDR** (1993). Update: Toxicological profile for selected PCBs (Aroclor-1260, -1254, -1248, -1242, -1232, -1221, and -1016) No. TP-92/16). Atlanta, GA: US Dept. of Human Health & Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry.

13. **Bachmann, A., W. de Bruin, J. C. Jumelet, H. H. M. Rijnaarts and A. J. B. Zehnder.** 1988. Aerobic biomineralization of alpha-hexachlorocyclohexane in contaminated soil. Appl. Environ. Microbiol. **54**:548-554.

14. **Bachmann, A., P. Walet, W. de Bruin, J. L. M. Huntjens, W. Roelofsen and A. J. B. Zehnder.** 1988. Biodegradation of alpha- and beta-hexachlorocyclohexane in a soil slurry under different redox conditions. Appl. Environ. Microbiol. **54**:143-149.

15. **Baker, M. T., R. M. Nelson and R. A. Van Dyke.** 1985. The formation of chlorobenzene and benzene by the reductive metabolism of lindane of rat liver microsomes. Archives of Biochemistry and Biophysics **236**:506-514.

16. **Ballschmiter, K. and C. Scholz.** 1980. Mikrobielle Abbau von chlorierten Aromaten. Chemosphere **9**:457-467.

17. **Barton, M. R. and R. L. Crawford.** 1988. Novel biotransformations of 4-chlorobiphenyl by a *Pseudomonas* sp. Appl. Environ. Microbiol. **54**:594-595.

18. **Bedard, D. L.** 1990. Bacterial transformation of polychlorinated biphenyls, p. 369-388. *In* D. Kamely , A. Chakrabarty and G. S. Omenn (ed.), Biotechnology and biodegradation, Portfolio Publishing Company, The Woodlands, Tx, USA.

19. **Bedard, D. L., S. C. Bunnell and L. A. Smullen.** 1996. Stimulation of microbial *para*-dechlorination of polychlorinated biphenyls that have persisted in Housatonic River sediment for decades. Environ. Sci. Technol. **30**:687-694.

20. **Bedard, D. L., M. L. Haberl, R. J. May and M. J. Brennan.** 1987. Evidence for novel mechanisms of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53**:1103-1112.

21. **Bedard, D. L. and J. F. Quensen III.** 1995. Microbial reductive dechlorination of PCBs, *In* L. Y. Young and C. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals, Wiley-Liss Division, John Wiley & Sons, Inc., New York.

22. **Bedard, D. L., L.-A. Smullen, K. A. DeWeerd, D. K. Dietrich, G. M. Frame, R. J. May, J. M. Principe, T. O. Rouse, W. A. Fessler and J. S. Nicholson.** 1995. Chemical activation of microbially-mediated PCB dechlorination: A field study. Organohalogen compounds **In press**.

23. **Bedard, D. L., R. Unterman, L. H. Bopp, M. J. Brennan, M. L. Haberl and C. Johnson.** 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51**:761-768.

24. **Bedard, D. L., R. E. Wagner, M. J. Brennan, M. L. Haberl and B. J. J.F.** 1987. Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by Alcaligenes eutrophus H850. Appl. Environ. Microbiol. **53**:1094-1102.

25. **Beland, F. A., S. O. Farwell, A. E. Robocker and R. D. Geer.** 1976. Electrochemical reduction and anaerobic degradation of lindane. J. Agric. Food Chem. **24**:753-756.

26. **Benezet, H. J. and F. Matsumura.** 1973. Isomerization of gamma-BHC to alpha-BHC in the environment. Nature (London) **243**:480-.

27. **Beurskens, J. E. M.** (1995) Microbial transformation of chlorinated aromatics in sediments. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.

28. **Beurskens, J. E. M., J. De Wolf and J. Dolfing.** 1996. Reductive dechlorination of hexachlorobenzene by microorganisms from polluted estuarine sediment. Appl. Environ. Microbiol.

29. **Beurskens, J. E. M., C. G. C. Dekker, H. Van den Heuvel, M. Swart and J. De Wolf.** 1994. Dechlorination of chlorinated benzenes by an anaerobic microbial consortium that selectively mediates the thermodynamic most favorable reactions. Environ. Sci. Technol. **28:**701-706.
30. **Beurskens, J. E. M., G. A. J. Mol, H. L. Barreveld, B. Van Munster and H. J. Winkels.** 1993. Geochronology of priority pollutants in a sedimentation area of the Rhine river. Environ. Toxicol. Chem. **12:**1549-1566.
31. **Beurskens, J. E. M., A. J. M. Stams, A. J. B. Zehnder and A. Bachmann.** 1991. Relative biochemical reactivity of three hexachlorocyclohexane isomers. Ecotoxicol. Environ. Safety **21:**128-136.
32. **Bhuyan, S., S. K. Sahu, T. K. Adhya and N. Sethunathan.** 1992. Accelerated Aerobic Degradation of gamma-Hexachlorocyclohexane in Suspensions of Flooded and Non-Flooded Soils Pretreated with Hexachlorocyclohexane. Biology and Fertility of Soils **12:**279-284.
33. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. **1:**23-29.
34. **Bosma, T. N. P.** (1994) Simulation of subsurface biotransformation. Ph.D.-thesis, Wageningen Agricultural University, The Netherlands.
35. **Bosma, T. N. P., E. M. W. Ballemans, N. K. Hoekstra, R. A. G. Te Welscher, J. G. M. M. Smeenk, G. Schraa and A. J. B. Zehnder.** 1996. Biotransformation of organics in soil columns an in an infiltration area. Groundwater **34:**49-57.
36. **Bosma, T. N. P., J. R. Van der Meer, G. Schraa, M. E. Tros and A. J. B. Zehnder.** 1988. Reductive dechlorination of all trichlorobenzene isomers. FEMS Microbiol. Ecol. **53:**223-229.
37. **Bouwer, E. J.** 1985. Secondary utilization of trace halogenated organic compounds in biofilms. Environ. Prog. **4:**43-45.
38. **Boyd, S. A. and D. R. Shelton.** 1984. Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. Appl. Environ. Microbiol. **47:**272-277.
39. **Briglia, M., R. I. L. Eggen, D. J. Van Elsas and W. M. De Vos.** 1994. Phylogenetic evidence for transfer of pentachlorophenol-mineralizing *Rhodococcus chlorophenolicus* PCP-I-T to the genus *Mycobacterium*. Int. J. Syst. Bacteriol. **44:**494-498.
40. **Briglia, M., P. J. M. Middeldorp and M. S. Salkinoja-Salonen.** 1994. Mineralization performance of *Rhodococcus chlorophenolicus* strain PCP-1 in contaminated soil stimulating on site conditions. Soil Biol. Biochem. **26:**377-385.
41. **Briglia, M., F. A. Rainey, E. Stackebrandt, G. Schraa and M. S. Salkinoja-Salonen.** 1995. *Rhodococcus percolatus* sp. nov. strain MBS1, a bacterium degrading 2,4,6-trichlorophenol. Int. J. Syst. Bacteriol. **46:**23-30.
42. **Brown, J. F., R. E. Wagner, D. L. Bedard, M. J. Brennan, J. C. Carnahan, R. J. May and T. J. Tofflemire.** 1984. PCB transformations in upper Hudson sediments. Northeast. Environ. Sci. **3:**167-179.
43. **Brown Jr., J. F., D. L. Bedard, M. J. Brennan, J. C. Carnahan, H. Feng and R. E. Wagner.** 1987. Polychlorinated biphenyl dechlorination in aquatic sediments. Science **236:**709-712.
44. **Brown Jr., J. F., R. E. Wagner, H. Feng, D. L. Bedard, M. J. Brennan, J. C. Carnahan and R. J. May.** 1987. Environmental dechlorination of PCBs. Environ. Toxicol. Chem. **6:**579-593.
45. **Bryant, F. O., D. D. Hale and J. E. Rogers.** 1991. Regiospecific dechlorination of pentachlorophenol by dichlorophenol-adapted microorganisms in freshwater, anaerobic sediment slurries. Appl. Environ. Microbiol. **57:**2293-2301.
46. **Bumpus, J. A., M. Tien, D. Wright and S. D. Aust.** 1985. Oxidation of persistent environmental pollutants by a white rot fungus. Science **228:**1434-1436.
47. **Chaudhry, G. R. and S. Chapalamadugu.** 1991. Biodegradation of halogenated organic compounds. Microbiol. Rev. **55:**59-79.
48. **Christiansen, N. and B. K. Ahring.** 1996. *Desulfitobacterium hafniense* sp. nov., an anaerobic, reductively dechlorinating bacterium. Int. J. Syst. Bacteriol. **46:**442-448.
49. **Christiansen, N. and B. K. Ahring.** 1996. Introduction of a de novo bioremediation activity into anaerobic granular sludge using the dechlorinating bacterium DCB-2. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology **69:**61-66.
50. **Christiansen, N., H. V. Hendriksen, K. T. Jarvinen and B. K. Ahring.** 1995. Degradation of chlorinated aromatic compounds in UASB reactors. Water Sci. Tech. **31:**249-259.
51. **Cole, J. R., A. L. Cascarelli, W. W. Mohn and J. M. Tiedje.** 1994. Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol. Appl. Environ. Microbiol. **60:**3536-3542.
52. **Crawford, R. L. and W. W. Mohn.** 1985. Microbiological removal of pentachlorophenol from soil using a *Flavobacterium*. Enzyme Microbiol. Technol. **7:**617-620.

53. **Cristofol, R. M. and E. Rodriguezfarre.** 1991. Differential Presynaptic Effects of Hexachlorocyclohexane Isomers on Noradrenaline Release in Cerebral Cortex. Life Sciences **49:**1111-1119.
54. **Crosby, D. G.** 1981. Environmental chemistry of pentachlorophenol. Pure Appl. Chem. **53:**1051-1080.
55. **De Bont, J. A. M., M. J. A. W. Vorage, S. Hartmans and W. J. J. Van den Tweel.** 1986. Microbial degradation of 1,3-dichlorobenzene. Appl. Environ. Microbiol. **52:**677-680.
56. **Deo, P. G., N. G. Karanth and N. G. K. Karanth.** 1994. Biodegradation of hexachlorocyclohexane isomers in soil and food environment. Crit. Rev. Microbiol. **20:**57-78.
57. **DeWeerd, K. A., L. Mandelco, R. S. Tanner, C. R. Woese and J. M. Suflita.** 1990. *Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic dehalogenating, sulfate-reducing bacterium. Arch. Microbiol. **154:**23-30.
58. **Dietrich, D., W. J. Hickey and R. Lamar.** 1995. Degradation of 4,4'-dichlorobiphenyl, 3,3',4,4'-tetrachlorobiphenyl, and 2,2',4,4',5,5'-hexachlorobiphenyl by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **61:**3904-3909.
59. **Dietrich, G. and J. Winter.** 1990. Anaerobic degradation of chlorophenol by an enrichment culture. Appl. Microbiol. Biotechnol. **34:**253-258.
60. **Dmochewitz, S. and K. Ballschmiter.** 1988. Microbial transformation of technical mixtures of polychlorinated biphenyl (PCB) by the fungus *Aspergillus niger*. Chemosphere **17:**111-121.
61. **Doelman, P., L. Haanstra and A. Vos.** 1988. Microbial degradation by the autochtonous soil population of alpha- and beta-HCH under anaerobic field conditions in temperate regions. Chemosphere **17:**481-487.
62. **Doelman, P., L. Haanstra and A. Vos.** 1988. Microbial sanitation of soil with alpha- and beta-HCH under aerobic glasshouse conditions. Chemosphere 17:489-492.
63. **Doelman, P., L. Haanstra and A. Vos.** 1990. Decomposition of alpha- and beta-hexachlorocyclohexane in soil under field conditions in a temperate climate. Soil Biol. Biochem. **22:**629-634.
64. **Dolfing, J.** 1990. Reductive dechlorination of 3-chlorobenzoate is coupled to ATP production and growth in an anaerobic bacterium, strain DCB-1. Arch. Microbiol. **153:**264-266.
65. **Dolfing, J. and J. E. M. Beurskens.** 1995. The microbial logic and environmental significance of reductive dehalogenationed. Plenum Press Div. Plenum Publishing Corp., New York.
66. **Eaton, D. C.** 1985. Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*, a ligninolytic fungus. Enzyme Microb. Technol. 7:194-196.
67. **Engst, R., W. Fritsche, R. Knoll, M. Kujawa, R. M. Macholz and G. Straube.** 1979. Interim results of studies of microbial isomerization of gamma-hexachlorocyclohexane. Bull. Environ. Contam. Toxicol. **22:**699-707.
68. **Engst, R., R. M. Macholz and M. Kujawa.** 1974. Metabolismus des Lindan. Abbau von Lindan durch Schimmelpilzkulturen. Nahrung **18:**737-745.
69. **Facchetti, S.** 1993. Mass spectrometry in the analysis of polychlorinated biphenyls. Mass Spectrometry Reviews **12:**173-203.
70. **Farquhar, G. J. and J. Sykes.** 1981. PCBs behavior in soils environmental Canadaed. Environmental Protection Service, Ottawa, Canada.
71. **Fathepure, B. Z., J. M. Tiedje and S. A. Boyd.** 1988. Reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in anaerobic sewage sludge. Appl. Environ. Microbiol. **54:**327-330.
72. **Fava, F., D. Digioia, S. Cinti, L. Marchetti and G. Quattroni.** 1994. Degradation and Dechlorination of Low-Chlorinated Biphenyls by a Three-Membered Bacterial Co-Culture. Appl. Microbiol. Biotechnol. **41:**117-123.
73. **Fava, F. and L. Marchetti.** 1991. Degradation and mineralization of 3-chlorobiphenyl by a mixed aerobic bacterial culture. Appl. Microbiol. Biotechnol. **36:**240-245.
74. **Furukawa, K.** 1986. Modifications of PCBs by bacteria and other microorganisms, p. 89. *In* J. S. Waid (ed.), PCBs and the environment, CRC Press, Boca Raton, FL.
75. **Furukawa, K., N. Tomizuka and A. Kamibayashi.** 1979. Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. Appl. Environ. Microbiol. **38:**301-310.
76. **Furukawa, K., K. Tonomura and A. Kamibayashi.** 1978. Effect of chlorine substitution on the biodegradability of polychlorinated biphenyls. Appl. Environ. Microbiol. **35:**223-227.
77. **Genthner, B. R. S., W. A. Price II and P. H. Pritchard.** 1989. Anaerobic degradation of chloroaromatic compounds in aquatic sediments under a variety of enrichment conditions. Appl. Environ. Microbiol. **55:**1466-1471.
78. **Genthner, B. R. S., W. A. Price II and P. H. Pritchard.** 1989. Charcterization of anaerobic dechlorinating consortia derived from aquatic sediments. Appl. Environ. Microbiol. **55:**1472-1476.

79. **Gerritse, J., V. Renard, T. M. P. Gomes, P. A. Lawson, M. D. Collins and J. C. Gottschal.** 1996. *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or *ortho*-chlorinated phenols. Arch. Microbiol. **165**:132-140.

80. **Gibson, D. T., D. L. Cruden, J. D. Haddock, G. J. Zylstra and J. M. Brand.** 1993. Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **175**:4561-4564.

81. **Golovleva, L. A., O. E. Zaborina and A. Y. Arinbasarova.** 1993. Degradation of 2,4,6-TCP and a Mixture of Isomeric Chlorophenols by Immobilized *Streptomyces rochei* 303. Appl. Microbiol. Biotechnol. **38**:815-819.

82. **Häggblom, M. M., D. Janke, P. J. M. Middeldorp and M. S. Salkinoja-Salonen.** 1989. O-methylation of chlorinated phenols in the genus *Rhodococcus*. Arch. Microbiol. **152**:6-9.

83. **Häggblom, M. M., L. J. Nohynek, N. J. Palleroni, K. Kronqvist, E. L. Nurmiaho-Lassila, M. S. Salkinoja-Salonen, S. Klatte and R. M. Kroppenstedt.** 1994. Transfer of polychlorophenol-degrading *Rhodococcus chlorophenolicus* (Apajalahti *et al.* 1986) to the genus *Mycobacterium* as *Mycobacterium chlorophenolicum* comb. nov. Int. J. Syst. Bacteriol. **44**:485-493.

84. **Häggblom, M. M., L. J. Nohynek and M. S. Salkinoja-Salonen.** 1988. Degradation and O-methylation of chlorinated phenolic compounds by and *Mycobacterium* strains. Appl. Environ. Microbiol. **54**:3043-3052.

85. **Häggblom, M. M., M. D. Rivera and L. Y. Young.** 1993. Effects of auxiliary carbon sources and electron acceptors on methanogenic degradation of chlorinated phenols. Environ. Toxicol. Chem. **12**:1395-1403.

86. **Häggblom, M. M., M. D. Rivera and L. Y. Young.** 1993. Influence of Alternative Electron Acceptors on the Anaerobic Biodegradability of Chlorinated Phenols and Benzoic Acids. Appl. Environ. Microbiol. **59**:1162-1167.

87. **Häggblom, M. M. and L. Y. Young.** 1990. Chlorophenol degradation coupled to sulfate reduction. Appl. Environ. Microbiol. **56**:3255-3260.

88. **Häggblom, M. M. and L. Y. Young.** 1995. Anaerobic degradation of halogenated phenols by sulfate-reducing consortia. Appl. Environ. Microbiol. **61**:1546-1550.

89. **Haider, K., G. Jagnow, R. Kohnen and S. U. Lim.** 1974. Abbau chlorierter Benzole, Phenole, und Cyclohexan-derivate durch Benzol und Phenol verwertende Bodenbakterien unter aeroben Bedingungen. Arch. Microbiol. **96**:183-200.

90. **Haigler, B. E., S. F. Nishino and J. C. Spain.** 1988. Degradation of 1,2-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **54**:294-301.

91. **Hale, D. D., J. E. Rogers and J. Wiegel.** 1990. Reductive Dechlorination of Dichlorophenols by Nonadapted and Adapted Microbial Communities in Pond Sediments. Microb. Ecol. **20**:185-196.

92. **Hale, D. D., J. E. Rogers and J. Wiegel.** 1991. Environmental factors correlated to dichlorophenol dechlorination in anoxic freshwater sediments. Environ. Toxicol. Chem. **10**:1255-1265.

93. **Harkness, M. R., J. B. Mcdermott, D. A. Abramowicz, J. J. Salvo, W. P. Flanagan, M. L. Stephens, F. J. Mondello, R. J. May, J. H. Lobos, K. M. Carroll, M. J. Brennan, A. A. Bracco, K. M. Fish, G. L. Warner, P. R. Wilson, D. K. Dietrich, D. T. Lin, C. B. Morgan and W. L. Gately.** 1993. In situ stimulation of aerobic PCB biodegradation in Hudson River sediments. Science **259**:503-507.

94. **Havel, J. and W. Reineke.** 1991. Total degradation of various chlorobiphenyls and *in vivo* constructed hybrid pseudomonads. FEMS Microbiol. Lett. **78**:163-170.

95. **Havel, J. and W. Reineke.** 1992. Degradation of Aroclor 1221 and Survival of Strains in Soil Microcosms. Appl. Microbiol. Biotechnol. **38**:129-134.

96. **Havel, J. and W. Reineke.** 1993. Degradation of Aroclor 1221 in soil by a hybrid pseudomonad. FEMS Microbiol. Lett. **108**:211-217.

97. **Hendriksen, H. V., S. Larsen and B. K. Ahring.** 1991. Anaerobic degradation of PCP and phenol in fixed-film reactors - The influence of an additional substrate. Wat. Sci. Technol. **24**:431-436.

98. **Hernandez, L. M., M. A. Fernandez and J. Gonzalez.** 1991. Lindane pollution near an industrial source in northeast Spain. Bull. Environ. Contam. Toxicol. **46**:9-13.

99. **Holliger, C. and G. Schraa.** 1994. Physiological meaning and potential for application of reductive dechlorination by anaerobic bacteria. FEMS Microbiol. Rev. **15**:297-305.

100. **Holliger, C., G. Schraa, A. J. M. Stams and A. J. B. Zehnder.** 1992. Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. Appl. Environ. Microbiol. **58**:1636-1644.

101. **Holliger, C., G. Schraa, A. J. M. Stams and A. J. B. Zehnder.** 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. **59**:2991-2997.

102. **Hrudey, S. E., E. Knettig, S. A. Daignault and P. M. Fedorak.** 1987. Anaerobic biodegradation of monochlorophenols. Environ. Technol. Letters **8**:65-76.
103. **Hrudey, S. E., E. Knettig, P. M. Fedorak and S. A. Daignault.** 1987. Anaerobic Semi-Continuous Culture Biodegradation of Dichlorophenols Containing an Ortho Chlorine. Water Poll. Res. J. Canada **22**:427-436.
104. **Huntjens, J. L. M., W. Brouwer, K. Grobben, O. Jansma, F. Scheffer and A. J. B. Zehnder.** 1988. Degradation of alpha-hexachlorocyclohexane by a bacterium isolated from polluted soil, p. 733-737. *In* K. Wolf, W. J. Van den Brink and F. J. Colon (ed.), Contaminated Soil '88, Kluwer Academic Publishers,
105. **IARC.** 1979. Some halogenated hydrocarbonsed. World Health Organization, Lyon.
106. **Ide, A., Y. Niki, F. Sakomoto, I. Watanabe and H. Watanabe.** 1972. Decomposition of pentachlorophenol in paddy soil. Agr. Biol. Chem. **36**:1937-1944.
107. **Jagnow, G., K. Haider and P.-C. Ellwardt.** 1977. Anaerobic dechlorination and degradation of hexachlorocyclohexane isomers by anaerobic and facultative anaerobic bacteria. Arch. Microbiol. **115**:285-292.
108. **Joy, R. M.** 1976. Convulsive properties of chlorinated hydrocarbon insecticides in the cat central nervous system. Toxicol. Appl. Pharmacol. **35**:95-106.
109. **Joy, R. M.** 1982. Mode of action of lindane, dieldrin and related insecticides in the central nervous system. Neurobehav. Toxicol. Teratol. **4**:813-823.
110. **Juteau, P., R. Beaudet, G. Mcsween, F. Lepine and J. G. Bisaillon.** 1995. Study of the reductive dechlorination of pentachlorophenol by a methanogenic consortium. Can. J. Microbiol. **41**:862-868.
111. **Kaushik, C. P.** 1991. Persistence and metabolism of HCH and DDT in soil under subtropical conditions. Soil Biol. Biochem. **23**:131-134.
112. **Kennes, C., W. M. Wu, L. Bhatnagar and J. G. Zeikus.** 1996. Anaerobic dechlorination and mineralization of pentachlorophenol and 2,4,6-trichlorophenol by methanogenic pentachlorophenol-degrading granules. Appl. Microbiol. Biotechnol. **44**:801-806.
113. **King, G. M.** 1988. Dehalogenation in marine sediments containing natural sources of halophenols. Appl. Environ. Microbiol. **54**:3079-3085.
114. **Kitunen, V. H. and M. S. Salkinoja-Salonen.** 1989. Occurrence of PCDDs and PCDFs in pulp and board products. Chemosphere **19**:721-726.
115. **Kitunen, V. H., R. J. Valo and M. S. Salkinoja-Salonen.** 1985. Analysis of chlorinated phenols, phenoxyphenols and dibenzofurans around wood preserving facilities. Intern. J. Environ. Anal. Chem. **20**:13-28.
116. **Kitunen, V. H., R. J. Valo and M. S. Salkinoja-Salonen.** 1987. Contamination of soil around wood-preserving facilities by polychlorinated aromatic compounds. Environ. Sci. Technol. **21**:96-101.
117. **Kiyohara, H., T. Hatta, Y. Ogawa, T. Kakuda, H. Yokoyama and N. Takizawa.** 1992. Isolation of *Pseudomonas pickettii* strains that degrade 2,4,6-trichlorophenol and their dechlorination of chlorophenols. Appl. Environ. Microbiol. **58**:1276-1283.
118. **Kohring, G.-W., X. Zhang and J. Wiegel.** 1989. Anaerobic dechlorination of 2,4-dichlorophenol in freshwater sediments in the presence of sulfate. Appl. Environ. Microbiol. **55**.
119. **Kuhn, E. P., P. J. Colberg, J. L. Schnoor, O. Wanner, A. J. B. Zehnder and R. P. Schwarzenbach.** 1985. Microbial transformations of substituted benzenes during infiltration of river water to groundwater: Laboratory column studies. Environ. Sci. Technol. **19**:961-968.
120. **Lamar, R. T. and D. M. Dietrich.** 1990. In Situ Depletion of Pentachlorophenol from Contaminated Soil by Phanerochaete spp. Appl. Environ. Microbiol. **56**:3093-3100.
121. **Lamar, R. T., J. W. Evans and J. A. Glaser.** 1993. Solid-Phase Treatment of a Pentachlorophenol-Contaminated Soil Using Lignin-Degrading Fungi. Environ. Sci. Technol. **27**:2566-2571.
122. **Lamar, R. T., J. A. Glaser and T. K. Kirk.** 1990. Fate of pentachlorophenol (PCP) in sterile soils inoculated with the white-rot basidiomycete *Phanerochaete chrysosporium*: mineralization, volatilization and depletion of PCP. Soil Biol. Biochem. **22**:433-440.
123. **Lang, E. and H. Viedt.** 1994. Degradation by and toxicity to bacteria of chlorinated phenols and benzenes, and hexachlorocyclohexane isomers. Microb. Ecol. **28**:53-65.
124. **Lang, E., H. Viedt, J. Egestorff and H. H. Hanert.** 1992. Reaction of the soil microflora after contamination with chlorinated aromatic compounds and HCH. FEMS Microbiol. Ecol. **86**:275-282.
125. **Larsen, S., H. V. Hendriksen and B. K. Ahring.** 1991. Potential for thermophilic (50 C) anaerobic dechlorination of pentachlorophenol in different ecosystems. Appl. Environ. Microbiol. **57**:2085-2090.
126. **Lepisto, R. and J. Rintala.** 1994. The removal of chlorinated phenolic compounds from chlorine bleaching effluents using thermophilic anaerobic processes. Wat. Sci. Technol. **29**:373-380.

127. **Levin, J. O., C. Rappe and C. A. Nilsson.** 1976. Use of chlorophenols as fungicides in sawmills. Scand. J. Work Environ. Health **2**:71-81.

128. **Li, D. Y., J. Eberspacher, B. Wagner, J. Kuntzer and F. Lingens.** 1991. Degradation of 2,4,6-trichlorophenol by *Azotobacter* sp. strain GP1. Appl. Environ. Microbiol. **57**:1920-1928.

129. **Liu, S. M., C. E. Kuo and T. B. Hsu.** 1996. Reductive dechlorination of chlorophenols and pentachlorophenol in anoxic estuarine sediments. Chemosphere **32**:1287-1300.

130. **Logan, B. E., B. C. Alleman, G. L. Amy and R. L. Gilbertson.** 1994. Adsorption and removal of pentachlorophenol by white rot fungi in batch culture. Water Res. **28**:1533-1538.

131. **MacRae, I. C., K. Raghu and T. F. Castro.** 1967. Persistence and biodegradation of four common isomers of benzene hexachloride in submerged soils. J. Agr. Food Chem. **15**:911-914.

132. **Madsen, T. and J. Aamand.** 1992. Anaerobic transformation and toxicity of trichlorophenols in a stable enrichment culture. Appl. Environ. Microbiol. **58**:557-561.

133. **Madsen, T. and D. Licht.** 1992. Isolation and characterization of an anaerobic chlorophenol-transforming bacterium. Appl. Environ. Microbiol. **58**:2874-2878.

134. **Marinucci, A. C. and R. Bartha.** 1979. Biodegradation of 1,2,3- and 1,2,4-trichlorobenzene in soil and liquid enrichment culture. Appl. Environ. Microbiol. **38**:811-817.

135. **Marks, T. S. and A. Maule.** 1992. The Use of Immobilized Porphyrins and Corrins to Dehalogenate Organochlorine Pollutants. Appl. Microbiol. Biotechnol. **38**:413-416.

136. **Masunaga, S., S. Susarla, J. L. Gundersen and Y. Yonezawa.** 1996. Pathway and rate of chlorophenol transformation in anaerobic estuarine sediment. Environ. Sci. Technol. **30**:1253-1260.

137. **May, H. D., A. W. Boyle, W. A. Price II and C. K. Blake.** 1992. Subculturing of a polychlorinated biphenyl-dechlorinating anaerobic enrichment on solid media. Appl. Environ. Microbiol. **58**:4051-4054.

138. **McCullar, M. V., V. Brenner, R. H. Adams and D. D. Focht.** 1994. Construction of a novel polychlorinated biphenyl-degrading bacterium: Utilization of 3,4'-dichlorobiphenyl by *Pseudomonas acidovorans* M3GY. Appl. Environ. Microbiol. **60**:3833-3839.

139. **Menke, B. and H. J. Rehm.** 1992. Degradation of Mixtures of Monochlorophenols and Phenol as Substrates for Free and Immobilized Cells of *Alcaligenes* sp A7-2. Appl. Microbiol. Biotechnol. **37**:655-661.

140. **Mikesell, M. D. and S. A. Boyd.** 1985. Reductive dechlorination of the pesticides 2,4-D, 2,4,5-T, and pentachlorophenol in anaerobic sludges. J. Environ. Qual. **14**:337-340.

141. **Mikesell, M. D. and S. A. Boyd.** 1986. Complete reductive dechlorination and mineralization of pentachlorophenol by anaerobic microorganisms. Appl. Environ. Microbiol. **52**:861-865.

142. **Mileski, G. J., J. A. Bumpus and S. D. Aust.** 1988. Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **54**:2885--2889.

143. **Mohn, W. W. and K. J. Kennedy.** 1992. Reductive dehalogenation of chlorophenols by *Desulfomonile tiedjei* DCB-1. Appl. Environ. Microbiol. **58**:1367-1370.

144. **Mohn, W. W. and J. M. Tiedje.** 1990. Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation. Arch. Microbiol. **153**:267-271.

145. **Mohn, W. W. and J. M. Tiedje.** 1992. Microbial Reductive Dehalogenation. Microbiol. Rev. **56**:482-507.

146. **Morris, P. J., W. W. Mohn, J. F. Quensen, J. M. Tiedje and S. A. Boyd.** 1992. Establishment of a Polychlorinated Biphenyl-Degrading Enrichment Culture with Predominantly *Meta*-dechlorination. Appl. Environ. Microbiol. **58**:3088-3094.

147. **Nagasawa, S., R. Kikuchi and M. Matsuo.** 1993. Indirect Identification of an Unstable Intermediate in gamma-HCH Degradation by *Pseudomonas paucimobilis* UT26. Chemosphere **26**:2279-2288.

148. **Nagasawa, S., R. Kikuchi, Y. Nagata, M. Takagi and M. Matsuo.** 1993. Aerobic mineralization of gamma-HCH by *Pseudomonas paucimobilis* UT26. Chemosphere **26**:1719-1728.

149. **Nagasawa, S., R. Kikuchi, Y. Nagata, M. Takagi and M. Matsuo.** 1993. Stereochemical analysis of gamma-HCH degradation by *Pseudomonas paucimobilis* UT26. Chemosphere **26**:1187-1201.

150. **Neilson, A. H., A.-S. Allard, P.-Å. Hynning, M. Remberger and L. Landner.** 1983. Bacterial methylation of chlorinated phenols and guaiacols: formation of veratroles from guaiacols and high-molecular-weight chlorinated lignin. Appl. Environ. Microbiol. **45**:774-783.

151. **Neilson, A. H., A.-S. Allard, S. Reiland, M. Remberger, A. Tärnholm, T. Viktor and L. Landner.** 1984. Tri- and tetrachloroveratrole, metabolites produced by bacterial O-methylation of tri- and tetrachloroguaiacol: an assessment of their bioconcentration potential and their effects on fish reproduction. Can. J. Fish Aquat. Sci. **41**:1502-1512.

152. **Newman, J. F.** 1978. Pesticides, p. 1-16. *In* I. R. Hill and S. J. L. Wright (ed.), Pesticide microbiology: Microbiological aspects of pesticide behaviour in the environment, Academic Press, London.

153. **Nies, L. and T. M. Vogel.** 1990. Effects of organic substrates on dechlorination of Aroclor 1242 in anaerobic sediments. Appl. Environ. Microbiol. **56:**2612-2617.

154. **Nohynek, L. J., M. M. Häggblom, N. J. Palleroni, K. Kronqvist, E. L. Nurmiaho-Lassila and M. Salkinoja-Salonen.** 1993. Characterization of a *Mycobacterium fortuitum* strain capable of degrading polychlorinated phenolic compounds. Syst. Appl. Microbiol. **16:**126-134.

155. **Norback, D. H. and R. H. Weltman.** 1985. Polychlorinated biphenyl induction of hepatocellular carcinoma in the Sprague-Dawley rat. Environ. Health Perpect. **60:**97-105.

156. **Öberg, L. G. and C. Rappe.** 1992. Biochemical Formation of PCDD/Fs from Chlorophenols. Chemosphere **25:**49-52.

157. **Ofjord, G. D., J. A. Puhakka and J. F. Ferguson.** 1994. Reductive dechlorination of Aroclor 1254 by marine sediment cultures. Environ. Sci. Technol. **28:**2286-2294.

158. **Ohisa, N., N. Kurihara and M. Nakajima.** 1982. ATP synthesis associated with the conversion of hexachlorocyclohexane related compounds. Arch. Microbiol. **131:**330-333.

159. **Ohisa, N. and M. Yamaguchi.** 1979. *Clostridium* species and gamma-BHC degradation in paddy soil. Soil Biol. Biochem. **11:**645-649.

160. **Ohisa, N., M. Yamaguchi and N. Kurihara.** 1980. Lindane degradation by cell-free extracts of *Clostridium rectum*. Arch. Microbiol. **125:**221-225.

161. **Okeke, B. C., J. E. Smith, A. Paterson and I. A. Watson-Craik.** 1993. Aerobic Metabolism of Pentachlorophenol by Spent Sawdust Culture of Shiitake Mushroom (Lentinus edodes) in Soil. Biotechnol. Lett. **15:**1077-1080.

162. **Oliver, B. G. and K. D. Nicol.** 1982. Chlorobenzenes in sediments, water, and selected fish from lakes Superior, Huron, Erie and Ontario. Environ. Sci. Technol. **16:**532-536.

163. **Oliver, B. G. and A. J. Niimi.** 1983. Bioconcentration of chlorobenzenes from water by rainbow trout: correlations with partition coefficients and environmental residues. Environ. Sci. Technol. **17:**287-291.

164. **Oltmanns, R. H., H. G. Rast and W. Reineke.** 1988. Degradation of 1,4-dichlorobenzene by enriched and constructed bacteria. Appl. Microbiol. Biotechnol. **28:**609-616.

165. **Parsons, J. R., D. T. H. Sijm, A. Van Laar and O. Hutzinger.** 1988. Biodegradation of chlorinated biphenyls and benzoic acids by a *Pseudomonas* strain. Appl. Microbiol. Biotechnol. **29:**81-84.

166. **Pearson, C. R.** 1982. Halogenated aromatics, p. 89-116. *In* O. Hutzinger (ed.), The handbook of environmental chemistry, Springer Verlag, New York.

167. **Pettigrew, C. A., A. Breen, C. Corcoran and G. S. Sayler.** 1990. Chlorinated biphenyl mineralisation by individual populations and consortia of freshwater bacteria. Appl. Environ. Microbiol. **56:**2036-2045.

168. **Quensen III, J. F., S. A. Boyd and J. M. Tiedje.** 1990. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. Appl. Environ. Microbiol. **56:**2360-2369.

169. **Quensen III, J. F., J. M. Tiedje and S. A. Boyd.** 1988. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. Science **242:**752-754.

170. **Raghu, K. and I. E. MacRae.** 1966. Biodegradation of the gamma-isomer of BHC in submerged soils. Science **154:**263-264.

171. **Ramanand, K., M. T. Balba and J. Duffy.** 1993. Reductive dehalogenation of chlorinated benzenes and toluenes under methanogenic conditions. Appl. Environ. Microbiol. **59:**3266-3272.

172. **Reineke, W. and H.-J. Knackmuss.** 1984. Microbial metabolism of haloaromatics: Isolation and properties of a chlorobenzene-degrading bacterium. Appl. Environ. Microbiol. **47:**395-402.

173. **Reineke, W. and H.-J. Knackmuss.** 1988. Microbial degradation of haloaromatics. Ann. Rev. Microbiol. **42:**263-287.

174. **Rhee, G. Y., R. C. Sokol, C. M. Bethoney and B. Bush.** 1993. Dechlorination of polychlorinated biphenyls by Hudson River sediment organisms - Specificity to the chlorination pattern of congeners. Environ. Sci. Technol. **27:**1190-1192.

175. **Rijnaarts, H. H. M., A. Bachmann, J. C. Jumelet and A. J. B. Zehnder.** 1990. Effect of desorption and intraparticle mass transfer on the aerobic biomineralization of alpha-hexachlorocyclohexane in a contaminated calcareous soil. Environ. Sci. Technol. **24:**1349-1354.

176. **Roy-Arcand, L. and F. S. Archibald.** 1991. Direct dechlorination of chlorophenolic compounds by laccases from *Trametes (Coriolus) versicolor*. Enzyme Microb. Technol. **13:**194-203.

177. **Safe, S. H.** 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Critical Reviews in Toxicology **21:**51-88.

178. **Safe, S. H.** 1994. Polychlorinated Biphenyls (PCBs) - Environmental Impact, Biochemical and Toxic Responses, and Implications for Risk Assessment. Critical Reviews in Toxicology **24:**87-149.

179. **Sahu, S. K., K. K. Patnaik, S. Bhuyan and N. Sethunathan.** 1993. Degradation of soil-applied isomers of hexachlorocyclohexane by a *Pseudomonas* sp. Soil Biol. Biochem. **25**:387-391.
180. **Sahu, S. K., K. K. Patnaik, M. Sharmila and N. Sethunathan.** 1990. Degradation of alpha-, beta-, and gamma-hexachlorocyclohexane by a soil bacterium under aerobic conditions. Appl. Environ. Microbiol. **56**:3620-3622.
181. **Sahu, S. K., S. S. Patnaik, S. Bhuyan, B. Sreedharan, N. Kurihara, T. K. Adhya and N. Sethunathan.** 1995. Mineralization of a-, g-, and ß-isomers of hexachlorocyclohexane by a soil bacterium under aerobic conditions. J. Agric. Food Chem. **43**:833-837.
182. **Salkinoja-Salonen, M. S., M.-L. Saxelin, J. Pere, T. Jaakkola, J. Saarikoski, R. Hakulinen and O. Koistinen.** 1981. Analysis of toxicity and biodegradability of organic chlorine compounds released into the environment in bleaching effluents of Kraft pulping, p. 1131-1164. *In* L. H. Keith (ed.), Advances in identification and analysis of organic pollutants in water, Ann Arbor Science Publishers, Inc., Ann Arbor, MI.
183. **Sander, P., R. M. Wittich, P. Fortnagel, H. Wilkes and W. Francke.** 1991. Degradation of 1,2,4-trichlorobenzene and 1,2,4,5- tetrachlorobenzene by *Pseudomonas* strains. Appl. Environ. Microbiol. **57**:1430-1440.
184. **Sasek, V., O. Volfova, P. Erbanova, B. R. M. Vyas and M. Matucha.** 1993. Degradation of PCBs by white rot fungi, methylotrophic and hydrocarbon uilizing yeasts and bacteria. Biotechnol. Lett. **15**:521-526.
185. **Schmid, R.** 1960. Cutaneous porphyria in Turkey. N. Engl. J. Med. **263**:397-398.
186. **Scholz-Muramatsu, H., A. Neumann, M. Messmer, E. Moore and G. Diekert.** 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Arch. Microbiol. **163**:48-56.
187. **Schraa, G., M. L. Boone, M. S. M. Jetten, A. R. W. Van Neerven, P. J. Colberg and A. J. B. Zehnder.** 1986. Degradation of 1,4-dichlorobenzene by *Alcaligenes* sp. strain A175. Appl. Environ. Microbiol. **52**:1374-1381.
188. **Schwarzenbach, R. P., W. Giger, E. Hoehn and J. K. Schneider.** 1983. Behavior of organic compounds during infiltration of river water to groundwater. Field sudies. Environ. Sci. Technol. **17**:472-479.
189. **Schwarzenbach, R. P., E. Molnar-Kubica, W. Giger and S. G. Wakeham.** 1979. Distribution, residence time, and fluxes of tetrachloroethylene and 1,4-dichlorobenzene in Lake Zuerich, Switzerland. Environ. Sci. Technol. **13**:1367-1373.
190. **Seigle-Murandi, F., R. Steiman, J. L. Benoit-Guyod and P. Guiraud.** 1993. Fungal Degradation of Pentachlorophenol by *Micromycetes*. J. Biotechnol. **30**:27-35.
191. **Senoo, K., M. Nishiyama and S. Matsumoto.** 1996. Bioremediation of gamma-HCH-polluted field soil by inoculation with an aerobic gamma-HCH-decomposing bacterium (*Sphingomonas paucimobilis* SS86). Soil Sci. Plant Nutr. **42**:11-19.
192. **Senoo, K. and H. Wada.** 1989. Isolation and identification of an aerobic gamma-HCH-decomposing bacterium from soil. Soil Sci. Plant Nutr. **35**:79-87.
193. **Senoo, K. and H. Wada.** 1990. gamma-HCH-Decomposing Ability of Several Strains of *Pseudomonas paucimobilis*. Soil Sci. Plant Nutr. 36:677-678.
194. **Shields, M. S., S. W. Hopper and G. S. Sayler.** 1985. Plasmid mediated mineralisation of 4-chlorobiphenyl. J. Bacteriol. **163**:882-889.
195. **Siddaramappa, R. and N. Sethunathan.** 1975. Persistence of gamma-BHC and beta-BHC in Indian rice soils under flooded conditions. Pesticide Science **6**:395-403.
196. **Slooff, W. and A. J. C. M. Matthijssen** (1988). Integrated criteria document hexachlorocyclohexanes No. 758473011). National Institute of Public Health and Environmemtal Protection, Bilthoven, The Netherlands.
197. **Smejetek, S., K. Hsu and W. H. Perman.** 1976. Electrical conductivity in lipid bilayer membranes induced by pentachlorophenol. Biophys. J. **16**:319-335.
198. **Sokol, R. C., C. M. Bethoney and G. Y. Rhee.** 1994. Effect of hydrogen on the pathway and products of PCB dechlorination. Chemosphere **29**:1735-1742.
199. **Sondossi, M., M. Sylvestre and D. Ahmad.** 1992. Effects of chlorobenzoate transformation on the *Pseudomonas testosteroni* biphenyl and chlorobiphenyl degradation pathway. Appl. Environ. Microbiol. **58**:485-495.
200. **Spain, J. C. and S. F. Nishino.** 1987. Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **53**:1010-1019.
201. **Steiert, J. G. and R. L. Crawford.** 1985. Microbial degradation of chlorinated phenols. Trends in Biotechnology 3(**12**):300-305.

202. **Steiert, J. G., J. J. Pignatello and R. L. Crawford.** 1987. Degradation of Chlorinated Phenols by a Pentachlorophenol-Degrading Bacterium. Appl. Environ. Microbiol. **53**:907-910.

203. **Straube, G.** 1991. Microbial transformation of hexachlorocyclohexane. Zentralblatt Fuer Mikrobiologie **146**:327-338.

204. **Suflita, J. M., S. A. Gibson and R. E. Beeman.** 1988. Anaerobic biotransformations of pollutant chemicals in aquifers. J. Ind. Microbiol. **3**:179-194.

205. **Svenson, A., L.-O. Kjeller and C. Rappe.** 1989. Enzyme-mediated formation of 2,3,7,8-substituted chlorinated dibenzodioxins and dibenzofurans. Environ. Sci. Technol. **23**:900-902.

206. **Tsuchiya, T. and T. Yamaha.** 1984. Reductive dechlorination of 1,2,4-trichlorobenzene by *Staphylococcus epidermidis* isolated from intestinal contents of rats. Agric. Biol. Chem. **48**:1545-1550.

207. **Tu, C. M.** 1976. Utilization and degradation of lindane by soil microorganisms. Arch. Microbiol. **108**:259-263.

208. **Uotila, J. S., V. H. Kitunen, T. Saastamoinen, T. Coote, M. M. Häggblom and M. S. Salkinoja-Salonen.** 1992. Charcterization of aromatic dehalogenases of *Mycobacterium fortuitum* CG-2. J. Bacteriol. **174**:5669-5675.

209. **Utkin, I., C. Woese and J. Wiegel.** 1994. Isolation and characterization of *Desulfitobacterium dehalogenans* gen nov, sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. Int. J. Syst. Bacteriol. **44**:612-619.

210. **Valo, R. J., V. H. Kitunen, M. S. Salkinoja-Salonen and S. Räisänen.** 1984. Chlorinated phenols as contaminants of soil and water in the vicinity of two Finnish sawmills. Chemosphere **13**:835-844.

211. **Valo, R. J. and M. S. Salkinoja-Salonen.** 1986. Bioreclamation of chlorophenol-contaminated soil by composting. Appl. Microbiol. Biotechnol. **25**:68-75.

212. **Van der Meer, J. R., W. Roelofsen, G. Schraa and A. J. B. Zehnder.** 1987. Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 in nonsterile soil columns. FEMS Microbiol. Ecol. **45**:333-341.

213. **Van Dort, H. M. and D. L. Bedard.** 1991. Reductive *ortho-* and meta-dechlorination of a polychlorinated biphenyl congener by anaerobic microorganisms. Appl. Environ. Microbiol. **57**:1576-1578.

214. **Van Leeuwen, C. J., D. M. M. Adema and J. Hermens.** 1990. Quantitative structure-activity relationships for fish early life stage toxicity. Aquat. Toxicol. **16**:321-334.

215. **Van Zoest, R. and G. T. M. Van Eck.** 1993. Historical input and behaviour of hexachlorobenzene, polychlorinated biphenyls and polycyclic aromatic hydrocarbons in 2 dated sediment cores from the Scheldt estuary, SW Netherlands. Marine Chem. **44**:95-103.

216. **Vogel, T. M., C. S. Criddle and P. L. McCarthy.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21**:722-736.

217. **Vyas, B. R. M., V. Sasek, M. Matucha and M. Bubner.** 1994. Degradation of 3,3',4,4'-Tetrachlorobiphenyl by selected white rot fungi. Chemosphere **28**:1127-1134.

218. **Wada, H., K. Senoo and Y. Takai.** 1989. Rapid degradation of gamma-HCH in upland soil after multiple applications. Soil Sci. Plant Nutr. **35**:71-77.

219. **Williams, W. A.** 1994. Microbial reductive dechlorination of trichlorobiphenyls in anaerobic sediment slurries. Environ. Sci. Technol. **28**:630-635.

220. **Woolley, D., L. Zimmer, D. Dodge and K. Swanson.** 1985. Effects of lindane-type insecticides in mammals: unsolved problems. Neurotoxicol. 6:165-192.

221. **Yabuuchi, E., I. Yano, H. Oyaizu, Y. Hashimoto, T. Ezaki and H. Yamamoto.** 1990. Proposals of *Sphingomonas paucimobilis* gen. nov. and com. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. Microbiol. Immunol. **34**:99-119.

222. **Yadav, J. S., J. F. Quensen, J. M. Tiedje and C. A. Reddy.** 1995. Degradation of polychlorinated biphenyl mixtures (Aroclors 1242, 1254, and 1260) by the white rot fungus *Phanerochaete chrysosporium* as evidenced by congener-specific analysis. Appl. Environ. Microbiol. **61**:2560-2565.

223. **Ye, D. Y., J. F. Quensen III, J. M. Tiedje and S. A. Boyd.** 1992. Anaerobic dechlorination of polychlorobiphenyls (Aroclor 1242) by pasteurized and ethanol-treated microorganisms from sediments. Appl. Environ. Microbiol. **58**:1110-1114.

224. **Ye, D. Y., J. F. Quensen, J. M. Tiedje and S. A. Boyd.** 1995. Evidence for *para* dechlorination of polychlorobiphenyls by methanogenic bacteria. Appl. Environ. Microbiol. **61**:2166-2171.

225. **Yule, W. N., M. Chiba and H. B. Morley.** 1967. Fate of insecticide residues. Decomposition of lindane in the soil. J. Agric. Food Chem. **15**:1000-1004.

226. **Zaitsev, G. M., J. S. Uotila, I. V. Tsitko, A. G. Lobanok and M. S. Salkinoja-Salonen.** 1995. Utilization of halogenated benzenes, phenols and benzoates by *Rhodococcus opacus* GM-14. Appl. Environ. Microbiol. **61**:4191-4201.

227. **Zeddel, A., A. Majcherczyk and A. Huttermann.** 1993. Degradation of polychlorinated biphenyls by white-rot fungi *Pleurotus ostreatus* and *Trametes versicolor* in a solid state system. Toxicologic. Environ. Chem. **40**:255-266.

CHAPTER 2

Biotransformation of α-, β-, γ-, and δ-hexachlorocyclohexane under methanogenic conditions

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SUMMARY

During the production of the pesticide lindane (γ-hexachlorocyclohexane, (γ-HCH)), large quantities of by-products, like the α-, β-, and δ-HCH isomers were discarded at dump sites. β-HCH was found to be extremely persistent in the environment under aerobic conditions. We studied the degradation of this isomer under methanogenic conditions in a flow-through column packed with polluted sediment.

β-HCH was completely removed in this system. Chlorobenzene was detected in the effluent as a product. A β-HCH transforming anaerobic enrichment culture was obtained in batch cultures by using the column material as inoculum. δ-2,3,4,5-tetrachlorocyclohexene is proposed as an intermediate during transformation, while benzene and chlorobenzene were formed as stable end products. The enrichment culture was also able to dechlorinate α-HCH at a comparable rate and γ-, and δ-HCH at lower rates. Dechlorination was inhibited by the addition of vancomycin, but not by the addition of bromoethanesulfonic acid. Pasteurization inhibited dechlorination completely. This is the first detailed description of the biodegradation of β-HCH, including intermediate and end product identification, under defined anaerobic conditions.

INTRODUCTION

Lindane (γ-hexachlorocyclohexane) has been widely used as an insecticide for crop protection for over two decades. During the production of γ-hexachlorocyclohexane (γ-HCH), large quantities of by-products, such as the α-, β-, and δ-HCH isomers were discarded at open air dump sites. This has resulted in heavily contaminated soils in e.g. The Netherlands and Spain (7, 12). The four HCH isomers differ in their susceptibility to microbial degradation. A number of laboratory and field studies has shown that the α-, γ-, and δ-isomers are readily mineralized by aerobic microorganisms (1, 15, 28, 31). β-HCH was found to be the most persistent isomer to microbial degradation under aerobic conditions. This may be due to the spatial arrangement of the chlorine atoms in the molecule (5). Only one bacterium, a *Pseudomonas* sp. has been isolated, which is capable of co-metabolic degradation of the β-isomer with acetate under aerobic conditions (29). However, only 5% of the β-HCH was mineralized after 10 days of incubation (30), and inoculation of this strain into freshly with β-HCH polluted soil did not lead to significant degradation (27). One may conclude from the literature that aerobic β-HCH degrading microorganisms do not prevail in contaminated soil. With the use of aerobic bioremediation techniques this isomer may thus persist (2, 7, 8).

Information about biotransformation of HCH isomers under anaerobic conditions is limited and diverse. Although disappearance of all four isomers has been observed under anaerobic conditions (i.e. flooded soil), large differences exist in rate and extent. In submerged soil, contaminated with α-, β-, γ-, and δ-HCH (15 mg/kg each), all isomers disappeared within 70 days of incubation. A small amount of the applied ^{14}C-γ-HCH was recovered as $^{14}CO_2$ (19). In another study, 250 mg β-HCH/kg was removed after 41 days of incubation in submerged soils, provided that the redox potential was below -40 mV (32). Several *Clostridium* strains and representatives of *Bacillaceae* and *Enterobacteriaceae* were capable of converting different HCH isomers under fermentative conditions. Only 20-30% of the initial concentration of β-HCH was degraded (16). Ohisa and Yamaguchi (23) showed that seven out of thirteen tested *Clostridium* strains were able to degrade γ-HCH, but none of the strains was able to transform β- and δ-HCH.

From experiments with whole cells and cell extracts of *Clostridium rectum*, Ohisa et al. (24) proposed the degradation of γ-HCH to proceed via two dihalo-eliminations, first to γ-3,4,5,6-tetrachlorocyclohexene (γ-TeCCH) and then to 5,6-dichlorocyclohexa-1,2-diene (not identified), and a dehydrohalogenation to chlorobenzene as end product. Chlorobenzene, but not TeCCH was found to be produced from α-HCH in soil slurries incubated under methanogenic culture conditions (2). Except for the release of chloride (16), nothing is known about the anaerobic degradation and the transformation pathway of the environmentally important β-isomer.

In this paper we describe the enrichment of an anaerobic microbial consortium that reductively dechlorinates β-HCH to chlorobenzene and benzene. Its ability to convert other isomers of HCH (α, γ, and δ), added separately or simultaneously, and the effect of pasteurization and inhibition of methanogenesis on the dechlorination will be shown.

MATERIALS AND METHODS

Chemicals. α-, β-, and δ-hexachlorocyclohexane (97-99.6% purity) were purchased from Promochem (Wesel, Germany). γ-HCH (99.6% purity), benzene, chlorobenzene, 2-bromoethanesulfonic acid (>98% purity) and analytical grade chloroform were obtained from E.Merck (Amsterdam, The Netherlands). Vancomycin-HCl was from Sigma (Bornem, Belgium).

Column Experiment. A glass column (15 cm x 2.3 cm i.d.) was packed with a mixture of a) sediment from the River Rhine (Wageningen, The Netherlands), b) dredging sludges heavily polluted with oil (Rotterdam and Zierikzee, The Netherlands), c) sediment from Lake Ketelmeer (The Netherlands) containing polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and polychlorinated benzenes, d) HCH polluted soils (Hengelo, The Netherlands), and e) granular sludge from an anaerobic waste water treatment plant (CSM, Breda, The Netherlands). The column was percolated under up-flow conditions at 20°C with anaerobic medium, favouring methanogenic conditions (17). The medium was saturated with β-HCH (about 160 μg/L) by leading it through a small column (5 cm x 1 cm i.d.) containing a mixture of glass beads (0.3 mm) and fine crystals of β-HCH. Figure 2.1 is a schematic representation of the experimental set-up. Samples for analysis were taken from the influent and the effluent of the column with a glass syringe as described previously (6).

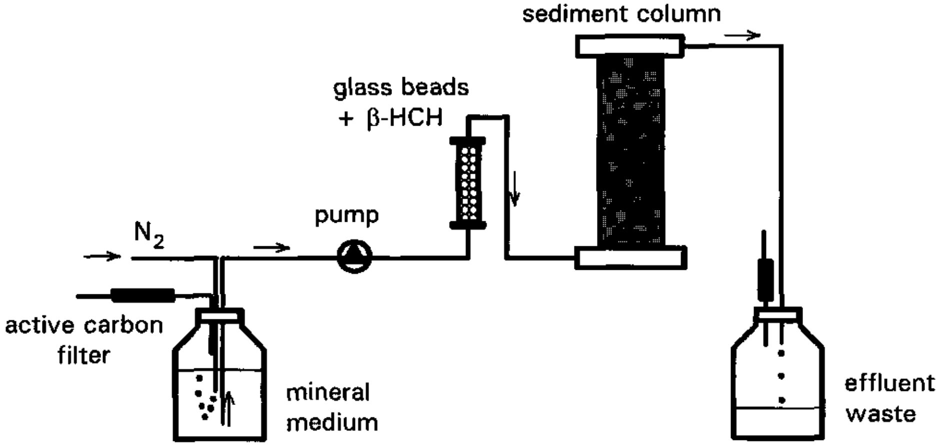

Figure 2.1. Diagram of the percolation column system.

Medium and Culture Conditions. Approximately 0.5 g portions of the column material in which β-HCH was degraded, were transferred into 120 ml serum bottles. These contained 40 ml of anaerobic mineral medium free of chloride, as used by Holliger et al. (13) with slight modification replacing chloride-salts of calcium and magnesium with the corresponding acetate salts (final acetate concentration 2 mM). Sodium lactate was added to a final concentration of 20 mM. β-HCH was supplied in 3 ml of hexadecane (ca. 0.12 mg/ml). The bottles were sealed with viton stoppers (Maag Technic AG, Dübendorf, Switzerland). The gas phase consisted of N_2/CO_2 (80%/20%). Sterile controls were prepared by autoclaving. The bottles were incubated stationary at 30°C in the dark. HCH degradation was followed by measuring the chloride concentration. After four weeks of incubation, 2 ml culture from active batches was transferred into fresh medium, containing both sodium lactate and β-HCH. After four subsequent transfers, batch experiments for detailed analyses were carried out with inoculum from this fourth generation enrichment culture.

Batch Experiments. α-,β-,γ- and δ-HCH were each added to a separate series of reagent tubes with 10 ml of medium containing 20 mM sodium lactate and 1 g of sterile sediment. The latter was found to be necessary to sustain reductive dechlorination over longer periods of time (data not shown). The HCH-isomers were added dissolved in 50 µl acetone (2 mg/mL) yielding a final concentration in the batches of 10 mg/L. Vancomycin (100 mg/L) or 2-bromoethanesulfonic acid (BrES, 5 mM) was added to the series containing β-HCH to inhibit either growth of eubacteria or methanogens. All tubes were inoculated with 1 mL of the above mentioned active dechlorinating culture. One series was pasteurized (80 °C, 20 min.) and a control series was heat sterilized. At different time intervals, one tube of each series was sacrificed to measure HCH, its metabolites, and lactate.

Parallel experiments were carried out in 120 mL serum bottles containing 40 ml of medium, 4 g sterile sediment and lactate (20 mM). They were inoculated with 2 mL of active culture. β-HCH was added in 200 µL of acetone, and samples were taken at different time intervals to follow the HCH-concentration. After β-HCH was depleted in the bottles, a new spike with all four isomers (50 µL, 2 mg/ml acetone of each isomer, final concentration 2.5 mg/L each) was added and HCH concentrations were followed. Sterile controls were heat sterilized.

Sample Preparation and Analyses. For the measurement of chloride release from HCH, 0.4 ml of centrifuged sample (5 min, 13500 rpm) was diluted tenfold in distilled water and analyzed with a Dionex DX-100 HPLC system (Dionex, Breda, The Netherlands), equipped with an ion chromatograph and a conductivity detector (flow 2 ml/min). Samples from influent and effluent of the sediment column were prepared by extracting 3-10 mL medium in 1 mL of chloroform. For the analysis of the 10 mL batch experiments, 2 mL of chloroform was added to each tube for extraction. The chloroform extracts were analyzed on a Hewlett-

Packard 5890 series II gas chromatograph equipped with a mass selective detector (series 5971A), an automatic sampler (series 7673A), and a fused silica analytical column (HP5, 30 m x 0.25 mm i.d.) from Hewlett-Packard (Amsterdam, The Netherlands). Oven temperature conditions for the separation were: 40°C for 6 min, 5°/min to 60°C, 50°/min to 280°C, total run length was 20 min. For the analysis of benzene, a capillary column (25m x 0.32 i.d.) was used with an isothermal elution at 40°C. Data were acquired using either a full scan mode for identification of metabolites, or a selected ion monitoring mode (SIM) for quantification.

The lactate concentration in the batch cultures was determined as follows: samples (1 mL) were centrifuged (15 min, 13500 rpm), diluted twofold with 0.02 M H_2SO_4 and analyzed with an LKB-HPLC system (Pharmacia/LKB, Woerden, The Netherlands) equipped with a high performance (2150) pump and a differential refractometer (2142) detector. The samples (20 μL) were applied to a Polyspher OA HY column (E. Merck), which was equilibrated with 0.01 M H_2SO_4 (flow rate 2 mL/min.). The methane concentration was determined by analyzing an 0.2 mL headspace sample with a Chrompack CP9001 gas chromatograph (Chrompack, Middelburg, The Netherlands), equipped with a molsieve 13X packed column (2 m x 4.3 mm i.d., 60-80 mesh) and a thermal conductivity detector. All quantifications were performed using external standards. Concentrations determined were within a 5% variance.

RESULTS AND DICUSSION

Column experiment. An increasing breakthrough of β-HCH was observed in the first 20 days of the column experiment (Figure 2.2). After the addition of 0.1 mM sodium lactate as electron donor on day 21, a complete disappearance of β-HCH was observed (detection limit for β-HCH is 1 μg/L). β-HCH was not detected in the effluent, even after decreasing the residence time of the medium in the column from ~10 to ~5 hrs.

Analysis of chloroform extracts of influent and effluent revealed that the effluent contained chlorobenzene (CB), as identified using mass spectrometry. The CB was found in quantities of 25-37% of the original input of β-HCH.

Batch Experiments. In the batch cultures with active column material and β-HCH, the chloride concentration increased after five weeks of incubation, indicating dechlorination. In heat-sterilized controls neither transformation of β-HCH nor formation of metabolites was observed (data not shown). The transformation of β-HCH by a fourth generation of this enrichment culture is shown in Figure 2.3. Upon disappearance of β-HCH, an intermediate peak was observed on the chromatogram and measured qualitatively. Mass spectral analysis gave a dissociation pattern with fragments of a parent molecular ion of *m/z* 218 of low intensity. Also, an ion of *m/z* 183, which represents a fragment of the parent compound less one chlorine atom, occurred along with the base peak at *m/z* 147. The latter represents a

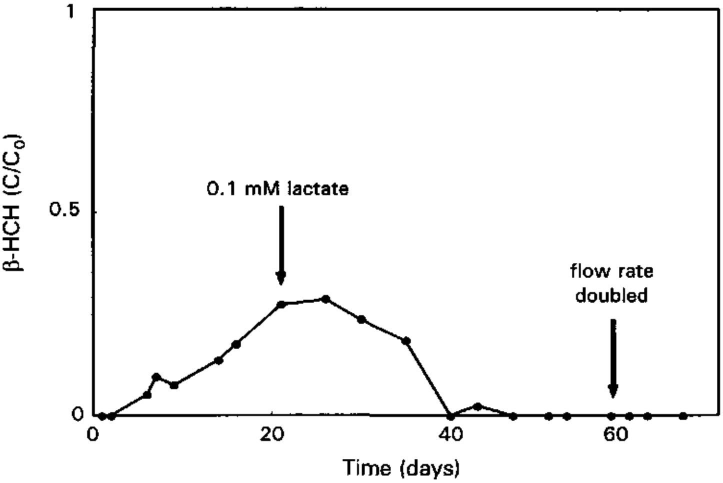

Figure 2.2. Behavior of β-hexachlorocyclohexane (β-HCH) in the methanogenic sediment column. The values on the vertical axis represent the quotient of the effluent and the influent concentration. The influent concentration of β-HCH was 160 μg/L. The initial flow rate was 3 ml/hr and resulted in a hydraulic residence time of ~10 hrs. The flow rate was doubled on day 58.

fragment that has further lost a chlorine and a hydrogen atom. This spectrum is consistent with that of 3,4,5,6-tetrachlorocyclohexene (TeCCH) (16), indicating that β-HCH had undergone a dihalo-elimination. The only isomer that can be formed from β-HCH via a dihalo-elimination, is δ-TeCCH. Since we do not have reference isomers of TeCCH this has not been confirmed. After 49 days of incubation, ca. 67% of the initial β-HCH had been converted to chlorobenzene (CB). A third product that was detected in the medium was benzene. It was found up to a concentration of ca. 4 μM (~19% of the initial β-HCH). We ascribe the missing fraction of ca. 15% to complete the mass balance, to losses of the highly volatile products during sample handling and to variance in the analyses. Both CB and benzene accumulated in the medium.

We propose for our enrichment culture a degradation pathway in which β-HCH is converted through dihalo-elimination to δ-TeCCH, subsequently dechlorinated to dichlorocyclohexadiene (dihalo-elimination), and further converted to both chlorobenzene (dehydrohalogenation) and benzene (dihalo-elimination) (Figure 2.4). Except for the formation of benzene, this pathway is similar to the one proposed by Ohisa et al. (24) for the dechlorination of γ-HCH by a *Clostridium rectum*. The pathway is in contrast with the degradation of γ-HCH by a *Pseudomonas paucimobilis* UT26, in which molecular oxygen is not involved in the first degradation steps. It consists of two subsequent dehydrohalogenations to a tetrachlorohexadiene, followed by two anaerobic hydrolytic dechlorinations and a dehydrogenation to 2,5-dichlorohydroquinone (21).

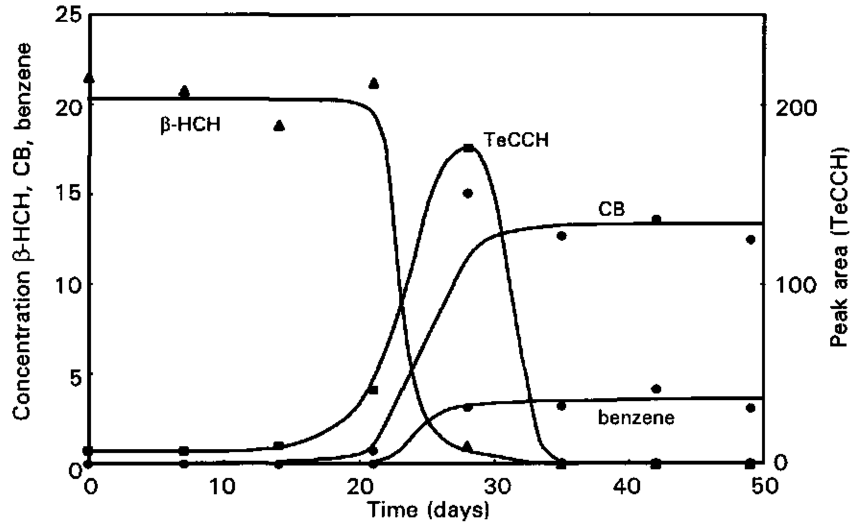

Figure 2.3. Transformation of β-hexachlorocyclohexane (β-HCH) and formation of δ-3,4,5,6-tetrachlorocyclohexene (TeCCH), benzene and chlorobenzene (CB) by a fourth generation of the enrichment culture.

The simultaneous biological formation of CB and benzene from HCH has never been shown, except for a study with rat liver microsomes. Baker et al. (3) incubated ^{14}C -γ-HCH with rat liver microsomes under a N_2 atmosphere and observed the formation of chlorobenzene and benzene in a ratio of ca. 20:1. However, the recovery of total radioactivity was at most ca. 40%. Beland et al. (4) showed that an anaerobic mixed microbial culture dechlorinated γ-HCH via TeCCH to benzene, although the amount of benzene formed accounted for 10% of the input of γ-HCH. The formation of CB was not reported.

To our knowledge, these results are the first detailed description of the quantitative biotransformation of β-HCH under anaerobic conditions, including the identification of the key metabolite, two end products and the determination of their ratio.

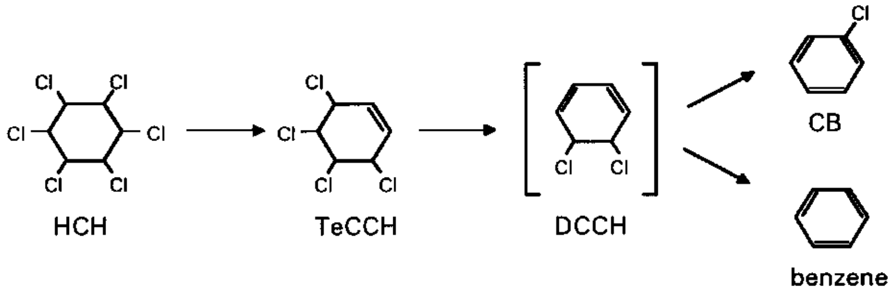

Figure 2.4. Proposed transformation pathway of β-HCH and formation of chlorobenzene and benzene based on our experiments. β-HCH is converted through dihalo-elimination to δ-TeCCH, subsequently dechlorinated to dichlorocyclohexadiene (dihalo-elimination), and further converted to benzene (dihalo-elimination) or chlorobenzene (dehydrohalogenation).

A similar dihalo-elimination, γ-HCH to γ-TeCCH, was shown to occur when γ-HCH was incubated with anaerobic sewage sludge (22) or with a *Clostridium rectum* (24). In these studies, like in ours, a subsequent dihalo-elimination to dichlorocyclohexadiene was proposed, although the product of this reaction was not observed. The proposed intermediate dichlorocyclohexadiene is a chemically labile compound and would immediately be dehydrohalogenated to CB. This reaction does not involve any electron transfer and is expected to occur non-biologically and spontaneously (3). Besides dehydrohalogenation, a further dihalo-elimination of dichlorocyclohexadiene yields benzene.

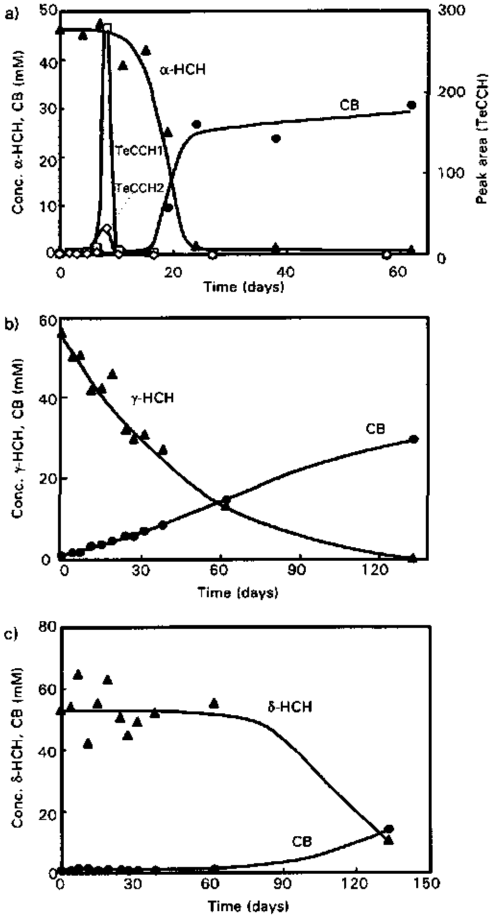

Figure 2.5. Dechlorination of α-, γ- and δ-hexachlorocyclohexane (HCH) added as single compounds to individual batches, inoculated with bacteria from the β-HCH dechlorinating culture. (a) α-HCH, (b) γ-HCH and (c) δ-HCH. TeCCH1 and 2 are different isomers of TeCCH (exact isomers are not known).

Lactate, which acted as the major electron donor, was converted to propionate and acetate, and partly to methane. The addition of 5 mM BrES inhibited the methanogenic activity completely, but this had no effect on the transformation of β-HCH. This indicates that methanogenic bacteria are not involved in the transformation reactions. In the batches treated with vancomycin (100 mg/L), transformation of β-HCH was not observed. Also, no dechlorination of β-HCH was found in the pasteurized batches (data not shown). Therefore, we deduce that non-sporeforming eubacteria are responsible for the dechlorination of β-HCH.

The microorganisms involved in the dehalogenation reactions described here are not known. Although reductive dehalogenation may be the result of a non-specific side reaction performed by non-methanogens (10), it may also be an essential part of the anaerobic respiration system (dehalorespiration) as observed in e.g. *Desulfomonile tiedjei* (chlorobenzoate) (20) or in *Dehalobacter restrictus* (tetrachloroethene) (14).

The same inoculum also dechlorinated α-HCH, at a rate comparable to that of β-HCH (Figure 2.5a). γ-HCH was only slowly transformed, but without lag phase (Figure 2.5b). However, a lag period of at least 60 days occurred before the dechlorination of δ-HCH started (Figure 2.5c). In all cases, CB was detected as an end product. Formation of TeCCH as an intermediate was not observed in the cultures with γ- and δ-HCH. In these and the following experiments we did not analyze for benzene, since at the time of these experiments, the fact that benzene may be formed was still unknown. During the transformation of α-HCH, two different peaks of the intermediate TeCCH were observed on the chromatogram, as deduced from their mass spectrum. Theoretically, four different TeCCH isomers can be formed from α-HCH. Due to the unavailability of the appropriate reference compounds, isomer identification could not be performed. Cultures, pre-incubated with β-HCH were spiked simultaneously with all four isomers upon depletion of the β-HCH (Figure 2.6). α- and γ-HCH were dechlorinated within 15 days without lag phase. Dechlorination of β-HCH did not start until after 5 days, while δ-HCH disappeared slowly and was depleted only after 130 days (data point not shown). The formed CB corresponded to about 60% of the total amount of HCH isomers transformed.

Except for α-HCH, the transformation rates of the singly added HCH isomers are clearly different from those when all four isomers are added simultaneously to cultures pre-incubated with β-HCH. γ-HCH is degraded slowly when added to the culture alone (Figure 2.5b), but is rapidly converted when added together with the other isomers (Figure 2.6). Dechlorination of δ-HCH alone did not occur until after at least 60 days, (Figure 2.5c). This lag period indicates a long adaptation period before the degrading activity is established. It suggests that β-HCH is needed for the induction of the responsible enzymes, but that the enzymes themselves are rather unspecific towards the different HCH isomers.

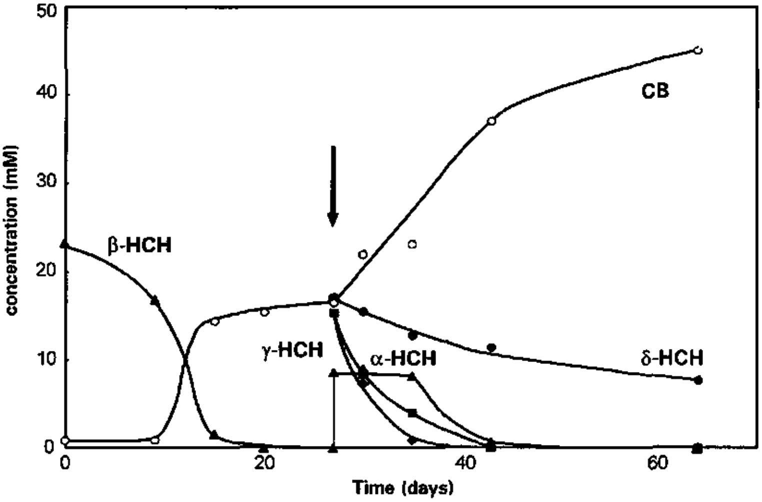

Figure 2.6. Dechlorination of α-, β-, γ- and δ-hexachlorocyclohexane (HCH) added simultaneously to batch cultures pre-incubated with β-HCH. The arrow indicates the addition of the four HCH isomers.

Chlorobenzene and benzene accumulated during the anaerobic transformation of β-HCH. Although we have not analyzed for benzene in the degradation of α-,γ-, and δ-HCH, this formation can also be expected here. The anaerobic mineralization of benzene has been shown in two recent studies (9, 18). However, biotransformation of CB has not yet been reported to occur under anaerobic conditions. Additionally, both benzene and CB are highly volatile compounds. Nevertheless, both benzene and CB are readily mineralized under aerobic conditions as sole carbon and energy source (25, 26) or as co-metabolic substrate (11). Therefore, the results described in this paper provide in addition to a novel biotransformation, good perspectives for the application of a sequential anaerobic/aerobic biological treatment of soils and aquifers polluted with HCH isomers, including β-HCH.

CONCLUSIONS

- β-HCH is reductively dechlorinated by an anaerobic microbial consortium;
- The dechlorination pathway proceeds via δ-3,4,5,6-TeCCH to the end products chlorobenzene and benzene;
- The consortium is able to dechlorinate the most important other isomers of HCH (α, γ, δ) to chlorobenzene at similar (α) or lower rates (γ, δ). Benzene formation was not monitored for;
- Methanogenic bacteria and non-sporeforming eubacteria are not involved in the dechlorination of β-HCH;
- The results provide good perspectives for the application of a sequential anaerobic/aerobic biological treatment of soils and aquifers polluted with HCH isomers, including β-HCH.

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REFERENCES

1. **Bachmann, A., W. de Bruin, J. C. Jumelet, H. H. M. Rijnaarts and A. J. B. Zehnder.** 1988. Aerobic biomineralization of alpha-hexachlorocyclohexane in contaminated soil. Appl. Environ. Microbiol. **54:**548-554.
2. **Bachmann, A., P. Walet, W. de Bruin, J. L. M. Huntjens, W. Roelofsen and A. J. B. Zehnder.** 1988. Biodegradation of alpha- and beta-hexachlorocyclohexane in a soil slurry under different redox conditions. Appl. Environ. Microbiol. **54:**143-149.
3. **Baker, M. T., R. M. Nelson and R. A. Van Dyke.** 1985. The formation of chlorobenzene and benzene by the reductive metabolism of lindane of rat liver microsomes. Archives of Biochemistry and Biophysics **236:**506-514.
4. **Beland, F. A., S. O. Farwell, A. E. Robocker and R. D. Geer.** 1976. Electrochemical reduction and anaerobic degradation of lindane. J. Agric. Food Chem. **24:**753-756.

5. **Beurskens, J. E. M., A. J. M. Stams, A. J. B. Zehnder and A. Bachmann.** 1991. Relative biochemical reactivity of three hexachlorocyclohexane isomers. Ecotoxicol. Environ. Safety **21**:128-136.
6. **Bosma, T. N. P., J. R. Van der Meer, G. Schraa, M. E. Tros and A. J. B. Zehnder.** 1988. Reductive dechlorination of all trichlorobenzene isomers. FEMS Microbiol. Ecol. **53**:223-229.
7. **Doelman, P., L. Haanstra and A. Vos.** 1988. Microbial degradation by the autochtonous soil population of alpha- and beta-HCH under anaerobic field conditions in temperate regions. Chemosphere 17:481-487.
8. **Doelman, P., L. Haanstra and A. Vos.** 1988. Microbial sanitation of soil with alpha- and beta-HCH under aerobic glasshouse conditions. Chemosphere 17:489-492.
9. **Edwards, E. A. and D. Grbic-Galic.** 1992. Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions. Appl. Environ. Microbiol. **58**:2663-2666.
10. **Egli, C., S. Stromeyer, A. M. Cook and T. Leisinger.** 1990. Transformation of tetra- and trichloromethane to CO_2 by anaerobic bacteria is a non-enzymic proces. FEMS Microbiol. Lett. **68**:207-212.
11. **Haider, K., G. Jagnow, R. Kohnen and S. U. Lim.** 1974. Abbau chlorierter Benzole, Phenole, und Cyclohexan-derivate durch Benzol und Phenol verwertende Bodenbakterien unter aeroben Bedingungen. Arch. Microbiol. **96**:183-200.
12. **Hernandez, L. M., M. A. Fernandez and J. Gonzalez.** 1991. Lindane pollution near an industrial source in northeast Spain. Bull. Environ. Contam. Toxicol. **46**:9-13.
13. **Holliger, C., G. Schraa, A. J. M. Stams and A. J. B. Zehnder.** 1992. Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. Appl. Environ. Microbiol. **58**:1636-1644.
14. **Holliger, C., G. Schraa, A. J. M. Stams and A. J. B. Zehnder.** 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. **59**:2991-2997.
15. **Huntjens, J. L. M., W. Brouwer, K. Grobben, O. Jansma, F. Scheffer and A. J. B. Zehnder.** 1988. Degradation of alpha-hexachlorocyclohexane by a bacterium isolated from polluted soil, p. 733-737. *In* K. Wolf , W. J. Van den Brink and F. J. Colon (ed.), Contaminated Soil '88, Kluwer Academic Publishers,
16. **Jagnow, G., K. Haider and P.-C. Ellwardt.** 1977. Anaerobic dechlorination and degradation of hexachlorocyclohexane isomers by anaerobic and facultative anaerobic bacteria. Arch. Microbiol. **115**:285-292.
17. **Langenhoff, A. A. M., G. Schraa and A. J. B. Zehnder.** 1996. Behaviour of toluene, benzene and naphtalene under anaerobic conditions in sediment columns. Biodegradation **7**:267-274.
18. **Lovley, D. R., J. D. Coates, J. C. Woodward and E. J. P. Phillips.** 1995. Benzene oxidation coupled to sulfate reduction. Appl. Environ. Microbiol. **61**:953-958.
19. **MacRae, I. C., K. Raghu and T. F. Castro.** 1967. Persistence and biodegradation of four common isomers of benzene hexachloride in submerged soils. J. Agr. Food Chem. **15**:911-914.
20. **Mohn, W. W. and J. M. Tiedje.** 1990. Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation. Arch. Microbiol. **153**:267-271.
21. **Nagata, Y., R. Ohtomo, K. Miyauchi, M. Fukuda, K. Yano and M. Takagi.** 1994. Cloning and sequencing of a 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase gene involved in the degradation of gamma-hexachlorocyclohexane in *Pseudomonas paucimobilis*. J. Bacteriol. **176**:3117-3125.
22. **Ohisa, N., N. Kurihara and M. Nakajima.** 1982. ATP synthesis associated with the conversion of hexachlorocyclohexane related compounds. Arch. Microbiol. **131**:330-333.
23. **Ohisa, N. and M. Yamaguchi.** 1979. *Clostridium* species and gamma-BHC degradation in paddy soil. Soil Biol. Biochem. **11**:645-649.
24. **Ohisa, N., M. Yamaguchi and N. Kurihara.** 1980. Lindane degradation by cell-free extracts of *Clostridium rectum*. Arch. Microbiol. **125**:221-225.
25. **Pettigrew, C. A., B. E. Haigler and J. C. Spain.** 1991. Simultaneous biodegradation of chlorobenzene and toluene by a *Pseudomonas* strain. Appl. Environ. Microbiol. **57**:157-162.
26. **Reineke, W. and H.-J. Knackmuss.** 1984. Microbial metabolism of haloaromatics: Isolation and properties of a chlorobenzene-degrading bacterium. Appl. Environ. Microbiol. **47**:395-402.
27. **Sahu, S. K., K. K. Patnaik, S. Bhuyan and N. Sethunathan.** 1993. Degradation of soil-applied isomers of hexachlorocyclohexane by a *Pseudomonas* sp. Soil Biol. Biochem. **25**:387-391.
28. **Sahu, S. K., K. K. Patnaik and N. Sethunathan.** 1992. Dehydrochlorination of delta-isomer of hexachlorocyclohexane by a soil bacterium, *Pseudomonas* sp. Bull. Environ. Contam. Toxicol. **48**:265-268.
29. **Sahu, S. K., K. K. Patnaik, M. Sharmila and N. Sethunathan.** 1990. Degradation of alpha-, beta-, and gamma-hexachlorocyclohexane by a soil bacterium under aerobic conditions. Appl. Environ. Microbiol. **56**:3620-3622.

30. **Sahu, S. K., S. S. Patnaik, S. Bhuyan, B. Sreedharan, N. Kurihara, T. K. Adhya and N. Sethunathan.** 1995. Mineralization of a-, g-, and ß-isomers of hexachlorocyclohexane by a soil bacterium under aerobic conditions. J. Agric. Food Chem. **43**:833-837.
31. **Senoo, K. and H. Wada.** 1989. Isolation and identification of an aerobic gamma-HCH-decomposing bacterium from soil. Soil Sci. Plant Nutr. **35**:79-87.
32. **Siddaramappa, R. and N. Sethunathan.** 1975. Persistence of gamma-BHC and beta-BHC in Indian rice soils under flooded conditions. Pesticide Science **6**:395-403.

CHAPTER 3

Enrichment and properties of a 1,2,4-trichlorobenzene dechlorinating methanogenic microbial consortium

Peter J.M. Middeldorp, Johan de Wolf, Alexander J.B. Zehnder and Gosse Schraa

SUMMARY

A methanogenic microbial consortium, capable of reductively dechlorinating 1,2,4-trichlorobenzene (TCB), was enriched from a mixture of polluted sediments. 1,2,4-TCB was dechlorinated via 1,4-dichlorobenzene (DCB) to chlorobenzene (CB). Lactate, which was used as electron donor during the enrichment, was converted via propionate and acetate to methane. Glucose, ethanol, methanol, propionate, acetate and hydrogen were also suitable electron donors for dechlorination, whereas formate was not. The addition of 5%(w/v) sterile Rhine sand was necessary to maintain the dechlorinating activity of the consortium. The addition of 2-bromoethanesulphonic acid (BrES) inhibited methanogenesis completely, but had no effect on the dechlorination of 1,2,4-TCB. The consortium was also able to dechlorinate other chlorinated benzenes via various simultaneous pathways to 1,3,5-TCB, 1,2-DCB, 1,3-DCB or CB as end products. The addition of BrES inhibited several of the simultaneously occurring dechlorination pathways of 1,2,3,4- and 1,2,3,5-tetrachlorobenzene (TeCB) and of pentachlorobenzene (QCB), which resulted in the formation of chlorobenzene as the only final product. Hexachlorobenzene (HCB) and polychlorinated biphenyls (PCBs) were dechlorinated after a lag phase of ca. 15 days, showing a dechlorination pattern, which is different from those observed for lower chlorinated benzenes: only chlorines with two adjacent chlorines were removed. The results show that the consortium possesses at least three distinct dechlorination activities toward chlorinated benzenes and PCBs.

INTRODUCTION

Chlorinated benzenes are widely used industrial chemicals. Their application ranges from solvents, odorizers, insect repellents, fungicides to intermediates in the manufacturing of various chemicals. Through a variety of usages, chlorinated benzenes have found their way into the environment and have so contaminated sediments and aquifers (27). Due to the toxicity and bioaccumulative properties of chlorinated benzenes, polluted sites are of major concern and subject to bioremediation studies (26). Under aerobic conditions, complete microbial mineralization of chlorinated benzenes as sole carbon and energy source with up to four chlorine atoms has been observed, and several bacterial isolates have been obtained and described (8, 12, 14, 19, 21, 22, 23, 25).

In many cases chlorinated benzenes are present in environments in which oxygen is not available (e.g. sediments, deep aquifers). Under these circumstances, reductive dechlorination, yielding lower chlorinated benzenes, was shown in laboratory experiments (3, 6, 10, 13, 15, 20) and proven to occur *in situ* (4). In most studies, reductive dechlorination of chlorinated benzenes was observed under methanogenic conditions, but it also occurred in a marine sediment incubation in the presence of sulfate (1).

The dechlorination patterns of highly chlorinated benzenes observed in most studies, involve the preferential dechlorination of a carbon atom with a chlorine on each adjacent carbon atom (3, 10, 13, 15). Ramanand et al. (20) described the dechlorination of hexachlorobenzene (HCB) and pentachlorobenzene (QCB) in soil slurries via 1,2,3,4-tetrachlorobenzene (TeCB), 1,2,3-TCB and 1,2,4-TCB to 1,2-DCB, 1,4-DCB and chlorobenzene, while 1,2,3,5-TeCB, added as single isomer, was not dechlorinated. A similar dechlorination pathway of HCB has recently been reported for enrichments from German river sediment (18). Several chlorinated benzenes dechlorinating microbial enrichments were able to dechlorinate polychlorinated biphenyls (PCBs) and dibenzodioxins as well. In two recent studies, methanogenic consortia were found capable of dechlorinating PCBs with the same specificity as for their corresponding chlorinated benzenes, i.e. removal of a chlorine between two adjacent chlorines (1, 5, 15).

Microbial consortia which are capable of reductive dechlorination of chlorinated benzenes have a potential for bioremediation of polluted sites, either *in situ* or in bioreactors. A subsequent aerobic treatment may then lead to mineralization of the chlorinated benzenes. However, most existing consortia dechlorinate pollutants like HCB and QCB mainly to 1,3,5-TCB, which is persistent under aerobic conditions. Moreover, the presence of co-pollutants, like PCBs and chlorinated dioxins, in polluted soil requires the use of microbial consortia with a broad dechlorination spectrum. In this chapter, we describe the enrichment and properties of a methanogenic consortium, capable of dechlorinating chlorinated benzenes. We show the capability of this consortium to dechlorinate several PCBs, and the presence of at least three distinct dechlorinating activities in the consortium, which may be carried out by different subpopulations.

MATERIALS AND METHODS

Chemicals. Chlorobenzene (CB), 1,3- and 1,4-dichlorobenzene (DCB), 1,2,4-trichlorobenzene (TCB), 1,2,3,4-tetrachlorobenzene (TeCB), pentachlorobenzene (QCB), 2-bromoethanesulfonic acid (>98% purity), sodium azide and all analytical grade solvents were obtained from E.Merck (Amsterdam, The Netherlands). 1,2-DCB, 1,3,5-TCB, 1,2,3,5- and 1,2,4,5-TeCB, and hexachlorobenzene (HCB) were from Aldrich (Zwijndrecht, The Netherlands). 1,2,3-TCB was from Janssen Chimica ('s-Hertogenbosch, The Netherlands). All PCBs used were from C.N. Schmidt B.V. (Amsterdam, The Netherlands).

Enrichment. Approx. 2 g of a mixture of sediments and sludges containing a) sediment from the River Rhine (Wageningen, The Netherlands), b) dredging sludges heavily polluted with oil (Rotterdam and Zierikzee, The Netherlands), c) sediment from Lake Ketelmeer (The Netherlands) containing polycyclic aromatic hydrocarbons, PCBs, and chlorinated benzenes, and d) granular sludge from an anaerobic waste water treatment plant (CSM, Breda, The Netherlands), was added to 120 mL serum bottles, containing 20 mL of medium with a low chloride concentration (ca. 2 mM) and favouring methanogenic conditions (13). The bottles were sealed with viton stoppers (Maag Technic AG, Dübendorf, Switzerland). The headspace was flushed with N_2/CO_2 (80%/20%), and sodium lactate was added to a final concentration of 20 mM. Pentachlorobenzene (QCB) was supplied in 2 mL of hexadecane (10 mg/mL). After significant chloride release was observed, 4 mL of the active enrichment was transferred into 40 mL of fresh medium, containing sodium lactate (20 mM), 1,2,4-TCB (added in 2 mL, 10 mg/mL hexadecane), and 2 g of Rhine sand. The latter was added to the medium prior to sterilization and was found to be necessary to sustain reductive dechlorination for over several subsequent transfers (data not shown). Controls were treated with 20 mM sodium azide or did not receive lactate. This enrichment was maintained for 11 generations by subculturing 10% of active culture into fresh medium containing lactate, 1,2,4-TCB, and sterile Rhine sand.

Electron donors. Serum bottles with 20 mL of medium, containing 1 g of sterile Rhine sand were inoculated with 2mL of active culture. 1,2,4-TCB was added in hexadecane (0.5 mL, 12 mg/mL). The electron donors added were lactate (20 mM), acetate (30 mM), methanol (50 mM), formate (25 mM), propionate (60 mM), ethanol (25 mM), glucose (10 mM) or hydrogen (80%/20% H_2/N_2 headspace). Controls did not receive any electron donor. The headspace of bottles that received hydrogen, was refreshed with 80%/20% H_2/N_2 after 7, 17, 25 and 32 days. All tests were run in duplicate.

Dechlorination tests. All chlorinated benzenes, except chlorobenzene, were tested. The PCBs tested were 2,5-dichlorobiphenyl (DCBP), 2,3,4- and 2,4,5-trichlorobiphenyl (TCBP), 2,3,4,5-and 2,3',4',5-tetrachlorobiphenyl (TeCBP), 2,3,4,5,6-pentachlorobiphenyl (PCBP), and 2,2',3,4,4',5,5'-heptachlorobiphenyl (HpCBP). A culture of the eleventh generation was used in this experiment. 2 mL of the active enrichment was transferred to 20 mL of medium, containing sodium lactate, 1 g of sterile Rhine sand, and the chlorinated benzene (100 µL, 1 mg/mL acetone solution) or PCB (100 µL, 0.5 mg/L acetone solution) to be tested. All tests were run in duplicate. The cultures were incubated statically at 30°C in the dark.

Determination of temperature optimum. 2 mL of the active enrichment was transferred to serum bottles with 20 mL of medium, containing sodium lactate, 1 g of sterile Rhine sand, and 1,2,4-trichlorobenzene (100 µL 1 mg/mL acetone solution). The bottles were incubated at 4°C, 10°C, 20°C, 30°C, 37°C and 55°C. All temperature tests were run in duplicate.

Analyses. The chloroform extracts were analyzed on a Hewlett-Packard 5890 series II gas chromatograph equipped with a mass selective detector (series 5971A), an automatic sampler (series 7673A), and a fused silica analytical column (HP5, 30 m x 0.25 mm i.d.) from Hewlett-Packard (Amsterdam, The Netherlands). Oven temperature conditions for the separation of the chlorobenzenes were: 60°C for 3 min., 5°/min. to 180°C, total run length 30 minutes. For the separation of the PCBs oven temperatures were 70°C for 2 min., 10°C/min. to 250°C, total run length 25 minutes. Data were acquired using either a full scan mode for identification of metabolites, or a selected ion monitoring mode (SIM) for quantification.

To determine volatile fatty acid concentrations, samples (1 ml) were centrifuged (15 min., 13500 rpm), diluted twofold with 0.02 M H_2SO_4 and analyzed with an LKB-HPLC system (Pharmacia/LKB, Woerden, The Netherlands), equipped with a high performance (2150) pump and a differential refractometer (2142) detector. The samples (20 µL) were applied to a Polyspher OA HY column (E. Merck), which was equilibrated with 0.01 M H_2SO_4 (flow rate 2 ml/min.). The methane concentration was determined by analyzing an 0.2 ml headspace sample with a Chrompack CP9001 gas chromatograph (Chrompack, Middelburg, The Netherlands), equipped with a molsieve 13X packed column (2 m x 4.3 mm i.d., 60-80 mesh) and a thermal conductivity detector. All quantifications were performed using external standards.

Chloride ion concentrations were measured with a Microchlor-o-counter (Marius, Utrecht, The Netherlands), using NaCl as external standard. Prior to analysis, 1 ml sample was acidified with 20 µL conc. H_2SO_4 and purged with nitrogen for 5 min to eliminate sulfide ions, which interfere with the chloride measurement.

RESULTS

Enrichment. A mixture of polluted sediments was incubated with QCB under methanogenic conditions. After 40 days, chloride was produced, indicating dechlorination. Qualitative analysis of the medium showed the presence of 1,2,3,4-TeCB, 1,2,3,5-TeCB, all TCBs, 1,4-DCB and chlorobenzene. After several transfers of this slurry to fresh medium with 1,2,4-TCB, we obtained a methanogenic consortium, which dechlorinates 1,2,4-TCB via mainly 1,4-DCB to CB, with minor amounts of 1,2- and 1,3-DCB accumulating (Figure 3.1).

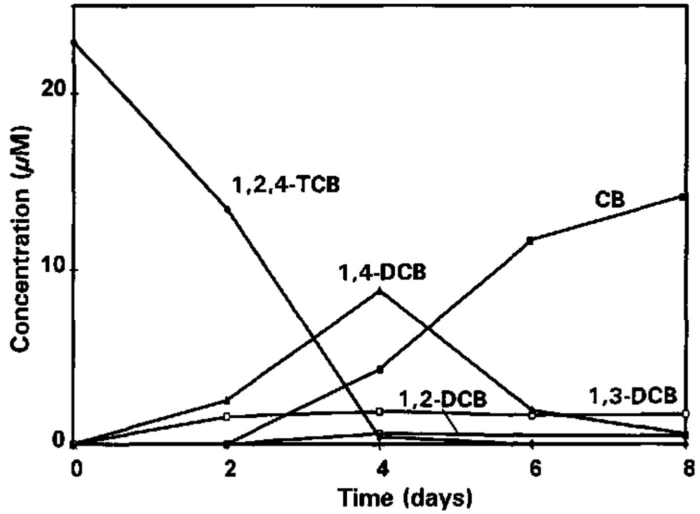

Figure 3.1. Dechlorination of 1,2,4-TCB, added in acetone to a freshly transferred culture.

When 55 µmole of 1,2,4-TCB was added, dissolved in hexadecane, chloride was produced up to 80 µmole/bottle after 40 days of incubation (Figure 3.2A). This corresponds to a conversion of 74% of the added 1,2,4-TCB to CB. No data are available for after 40 days of incubation. Lactate was metabolized to propionate and acetate in a ratio of roughly 2:1. Both propionate and acetate were further converted to methane (Figure 3.2B). When 5 mM of BrES was added, methanogenesis was completely inhibited, and propionate and acetate accumulated. However, dechlorination was not inhibited (data not shown). Sodium azide treated controls did not show dechlorination, lactate conversion or methanogenesis. The culture was subcultured 11 times successfully on 1,2,4-TCB during a period of three years.

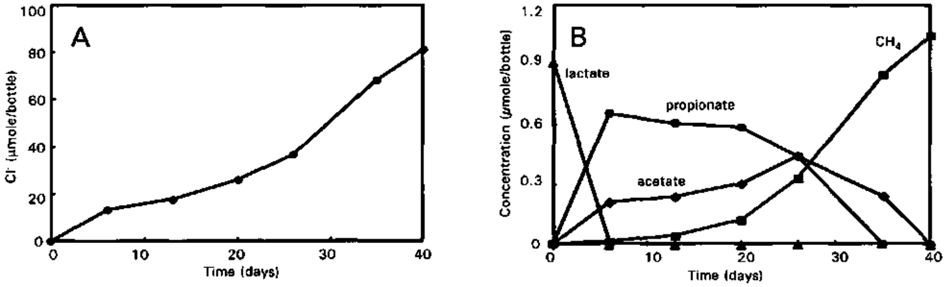

Figure 3.2. Chloride released from the dechlorination of 1,2,4-TCB, added in hexadecane (panel A), and lactate conversion and methane production (panel B).

Electron donors. The consortium was also able to dechlorinate 1,2,4-TCB with lactate, glucose, propionate, ethanol, methanol, hydrogen or acetate as electron donor. The chloride production after 40 days was highest with lactate, glucose, and propionate (35-45 µmole/bottle, with 33 µmole of 1,2,4-TCB added). Formate was the only electron donor tested, which did not support dechlorination of 1,2,4-TCB. In the controls, without electron donor, dechlorination did not take place.

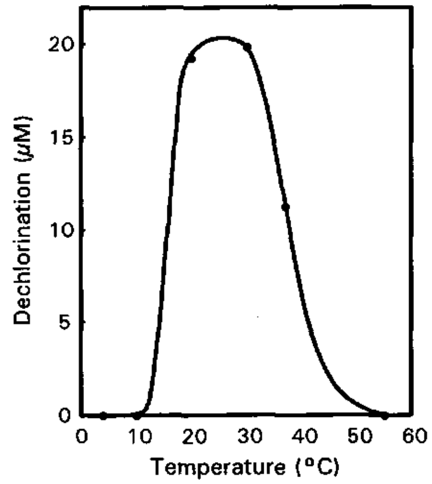

Figure 3.3. Temperature dependence of the reductive dechlorination of 1,2,4-TCB. Dechlorination is expressed as the theoretical chlorine release calculated from the lower chlorinated benzenes formed.

Temperature optimum. Figure 3.3 shows the dechlorination of 1,2,4-TCB by the consortium at different temperatures, as calculated from the dechlorination products formed after 4 days of incubation. At 4°C, 10°C and 55°C, no dechlorination was observed. Dechlorination rates were almost equal at 20, and 30°C, while at 37°C dechlorination was ca. 50% of the maximum rate.

Dechlorination tests. Table 3.1 shows the dechlorinating activity of the consortium after 4 days of incubation with individual chlorinated benzenes and PCBs. 1,4-DCB was dechlorinated to chlorobenzene, while 1,2- and 1,3-DCB were not dechlorinated. 1,2,3-TCB was dechlorinated to both 1,2- and 1,3-DCB, and 1,2,4-TCB was converted via 1,4-DCB to chlorobenzene. 1,3,5-TCB was not dechlorinated. 1,2,4,5-TeCB was dechlorinated via 1,2,4-TCB and 1,4-DCB to CB. Dechlorination of 1,2,3,4-TeCB occurred via both 1,2,3-TCB (ca. 50%) and 1,2,4-TCB (ca. 50%), while 1,2,3,5-TeCB was dechlorinated via mainly 1,2,4-TCB (ca. 95%) and also to

1,3,5-TCB (ca. 5%). QCB was dechlorinated via both 1,2,3,4-TeCB and 1,2,3,5-TeCB, which were further dechlorinated as described above (Figure 3.4).

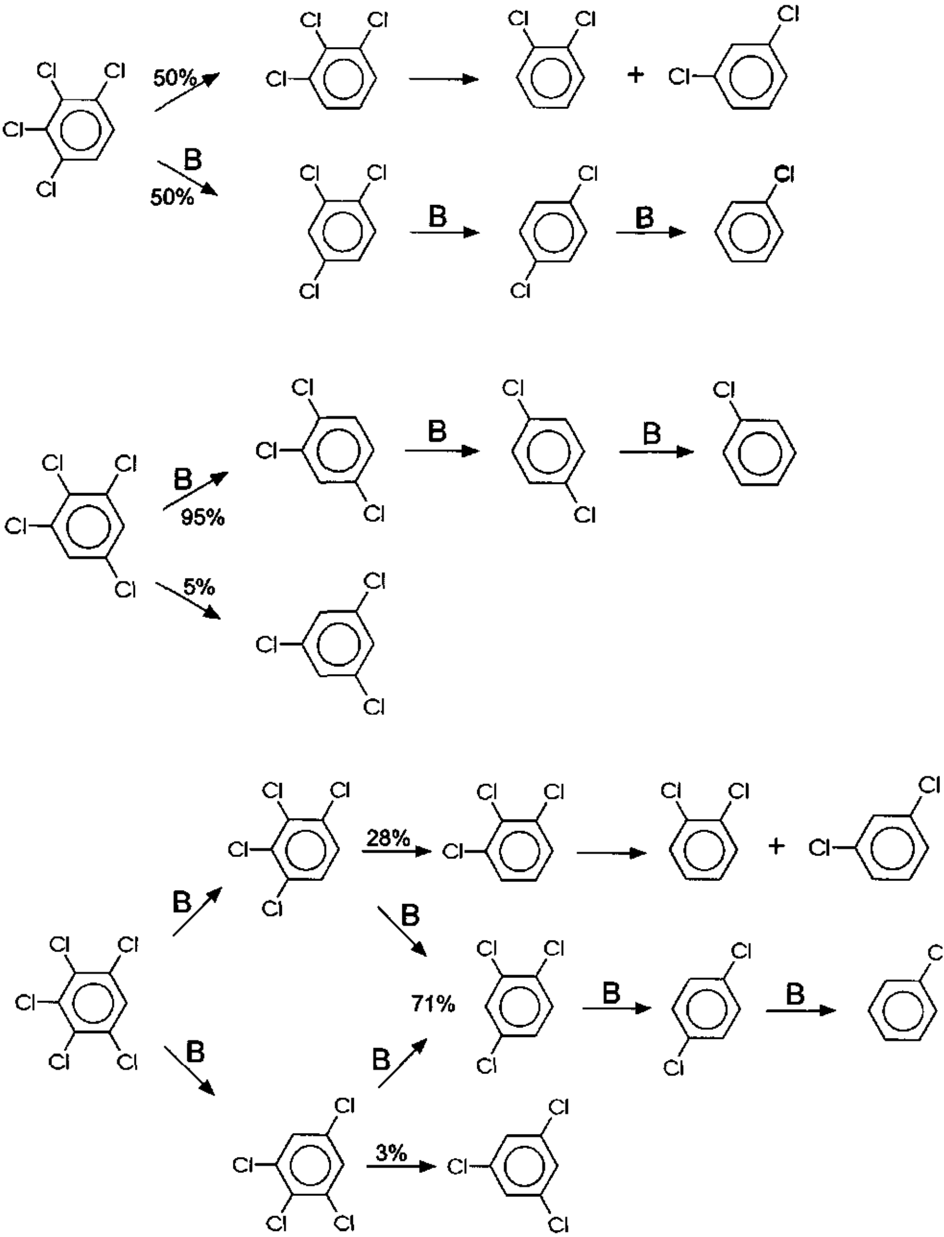

Figure 3.4. Dechlorination pathway of 1,2,3,4-TeCB, 1,2,3,5-TeCB, and QCB as observed after 5 days of incubation. Percentages indicate the fraction of the reactants that follows a distinct dechlorination. Reactions marked with "B" indicate reactions which proceed in the presence of 5 mM of BrES.

The dechlorination of HCB did not start until after 12 days. HCB was dechlorinated to QCB, which was further dechlorinated exclusively via 1,2,3,5-TeCB to 1,3,5-TCB, which accumulated. HCB dechlorination was not inhibited by the addition of BrES.

Figure 3.4 also shows the effect of BrES on the dechlorination pattern of QCB, 1,2,3,4- and 1,2,3,5-TeCB. Generally, the presence of BrES channeled the dechlorination of these isomers via 1,2,4-TCB to CB.

After a lag time of ca. 15 days, the consortium dechlorinated 2,3,4-TCBP to 2,4-DCBP, and 2,3,4,5,6-PCBP to both 2,3,5,6-TeCBP and 2,4,6-TCBP. 2,2',3,4,4',5,5'-HpCBP was dechlorinated to 2,2',3,4',5,5'-hexachlorobiphenyl (HxCBP). BrES had no effect on these dechlorinations. 2,5-DCBP, 2,4,5-TCBP, and 2,3',4',5-TeCBP were not dechlorinated within 60 days of incubation. In all cases, BrES inhibited methanogenesis completely.

Table 3.1. Dechlorination of chlorinated benzenes and PCBs and the observed dechlorination products after 4 days of incubation.

Substrate	Conc. (μM)	Dechlorination[1]	Products
Chlorinated benzenes			
1,2-DCB	34	-	--
1,3-DCB	34	-	--
1,4-DCB	34	+	CB
1,2,3-TCB	28	+	1,2-DCB; 1,3-DCB
1,2,4-TCB	28	+	1,4-DCB; CB
1,3,5-TCB	28	-	--
1,2,3,4-TeCB	23	+	1,2,3-TCB; 1,2,4-TCB; 1,2-DCB; 1,4-DCB; CB
1,2,3,5-TeCB	23	+	1,3,5-TCB; 1,2,4-TCB; 1,4-DCB; CB
1,2,4,5-TeCB	23	+	1,2,4-TCB; 1,4-DCB; CB
QCB	20	+	1,2,3,4-TeCB; 1,2,3,5-TeCB, 1,2,3-TCB; 1,2,4-TCB; 1,3,5-TCB; 1,2-DCB; 1,4-DCB; CB
HCB	18	+[2]	QCB, 1,2,3,5-TeCB, 1,3,5-TCB
Polychlorinated biphenyls			
2,5-DCBP	22	-	--
2,3,4-TCBP	19	+[2]	2,4-DCBP
2,4,5-DCBP	19	-	--
2,3,4,5,-TeCBP	17	+[2]	2,3,5-TCBP
2,3',4',5-TeCBP	17	-	--
2,3,4,5,6-PCBP	15	+[2]	2,3,5,6-TeCBP; 2,4,6-TCBP
2,2',3,4,4',5,5'-HpCBP	13	+[2]	2,2',3,4',5,5'-HxCBP

[1] - less than 0.1% dechlorinated
+ more than 90% dechlorinated

[2] dechlorination observed after 15 days

DISCUSSION

We obtained a stable microbial methanogenic consortium, able to dechlorinate 1,2,4-TCB via 1,4-DCB to CB. The sum of the DCB isomers and CB formed after 8 days, accounted for 79% of the 1,2,4-TCB added at the start of the experiment (Fig.1). We ascribe the remaining fraction to losses of the more volatile products during sample handling and analytical errors (within 10%). Unlike several chlorinated benzenes dechlorinating enrichments, which show lag times of weeks to months before dechlorination starts (10, 16), this culture exhibited a lag time of less than 2 days (Figure 3.1).

Dechlorination of 1,2,4-TCB was observed in the presence of several electron donors, suitable to sustain growth of fermenting, acetogenic and methanogenic bacteria. Possibly, the dechlorinating organisms are able to utilize these compounds or their metabolites as electron donors, with 1,2,4-TCB as electron acceptor. The use of chlorinated aromatics as electron acceptor for growth has been demonstrated for several bacterial strains. *Desulfomonile tiedjei* was shown to couple reductive dechlorination of 3-chlorobenzoate to ATP synthesis (9, 17). Cole et al. (7) isolated a bacterium, capable of growth on acetate with 2-chlorophenol as electron acceptor, and two *Desulfitobacterium* strains were isolated which are able to grow on *ortho*-chlorinated phenols as electron acceptor (11, 24). It cannot be said whether our consortium conserves energy for growth from the reductive dechlorination of 1,2,4-TCB.

Upon BrES addition, methane formation was inhibited, but lactate was still converted to propionate and acetate. Dechlorination of 1,2,4-TCB was not inhibited, which indicates that methanogenic bacteria are probably not involved in the actual dechlorination reaction. A similar finding was reported by Holliger et al. (13), who showed that growth and dechlorination of 1,2,3-TCB in an anaerobic mixed culture was not inhibited by BrES. Negative effects of BrES on the dechlorination of chlorinated benzenes and PCBs have also been shown in various studies. In another methanogenic dechlorinating consortium, obtained in our laboratory, dechlorination of chlorinated benzenes and PCBs was inhibited completely by the addition of BrES. Eubacteria-inhibiting antibiotics added along with methanogenic substrates also inhibited dechlorination in this consortium, indicating an interaction of methanogens and eubacteria (15). Nowak et al. (18) reported a decreased dechlorination rate of several chlorinated benzenes upon the addition of BrES. Ye et al. (28) studied bacteria, eluted from PCB-contaminated sediment, which were capable of dechlorinating 2,3',4'-TCBP both at *meta-* and *para*-positions. The addition of BrES resulted in an initial *meta*-dechlorination to 2,4'-DCBP. In the same consortium, also direct involvement of methanogenic bacteria in the dechlorination of PCBs has been shown. The addition of eubacteria-inhibiting antibiotics along with methanogenic substrates resulted in an exclusive dechlorination at the *para*-position (28).

Our consortium dechlorinates a wide range of other chlorinated benzenes. It exhibits a dechlorination pattern which is different from those previously described (2, 10, 13). The closest resemblance is to the pattern given by Ramanand et al. (20), who obtained a chlorinated benzenes dechlorinating enrichment by incubating sediment slurry with a mixture of HCB, QCB and 1,2,4-TCB under a hydrogen atmosphere. In contrast to our results, that culture did not dechlorinate 1,2,3,5-TeCB, while HCB was dechlorinated to CB.

In our consortium, HCB was not dechlorinated until after a lag time of about 14 days, while the first dechlorination product, QCB, was further dechlorinated via a different pathway than when QCB was incubated directly (Figure 3.4 and Table 3.1). Two distinct dechlorination patterns are expressed by the consortium. Pattern 1 involves the dechlorination of chlorinated benzenes with at least one unsubstituted carbon atom present, e.g. QCB. Dechlorination pattern 2 applies to HCB and PCBs and removes only a chlorine between two adjacent chlorines. Whereas dechlorination which follows the first pattern starts almost immediately upon incubation, the second pattern is expressed after a lag time of about 2 weeks. The specificity of both patterns indicates that these dechlorination reactions are probably enzymatically mediated reactions.

The addition of BrES to the consortium with 1,2,3-TCB, 1,2,3,4-, 1,2,3,5-TeCB and QCB inhibited methane formation completely and reduced the diversity of the respective dechlorination pathways. 1,2,3-TCB was dechlorinated to 1,2-DCB and the other chlorinated benzenes were dechlorinated via 1,2,4-TCB to 1,4-DCB and CB. We conclude that the addition of BrES excludes a dechlorinating population, probably methanogens or a methanogen-dependent group of bacteria. This means that the dechlorinating population that follows pattern 1, consists of two different dechlorinating groups of bacteria, i.e. BrES sensitive and BrES insensitive ones.

Dechlorination pattern 2 was not altered or inhibited by the addition of BrES. In another study, we describe the enrichment of a chlorinated benzenes and PCBs dechlorinating consortium from the same sediment mixture, with a similar dechlorination pattern. In contrast to the consortium we decribe here, that dechlorination was inhibited by the addition of BrES (15).

The amount of 1,2,4-TCB dechlorinated after 4 days of incubation was equal over a temperature range from 20 to 30°C. In contrast, Holliger et al. (13) observed a sharp temperature optimum between 25°C and 30°C for an enrichment culture dechlorinating 1,2,3-TCB.

At 4°C, 10°C and 55°C, no dechlorination of 1,2,4-TCB was observed. The sediments and sludges from which the consortium was obtained, had temperatures of ca. 10°C at the time of collection, and were stored at 4°C. Therefore, we deduce that the enriched bacteria will dechlorinate chlorinated benzenes and PCBs only very slowly in their original environment. This may partly explain the low dechlorination rates of such compounds *in situ* (4).

Most previously described chlorinated benzenes dechlorinating enrichments have been initiated and maintained on HCB (1, 3, 10). This yielded consortia with a relatively limited range of chlorinated benzenes as substrates for dechlorination. Our results and those of Ramanand et al. (20), who used a mixture of HCB, QCB and 1,2,4-TCB, show that enrichments on 1,2,4-TCB may yield consortia with the capacity to dechlorinate more CB isomers, and showing a more extensive dechlorination. This is probably due to the fact that 1,2,4-TCB does not have three chlorines next to each other and therefore will result in the enrichment of dechlorinating bacteria with fewer selective requirements. Our consortium possesses dechlorinating properties towards PCBs similar to those described for other PCB dechlorinating consortia, enriched on chlorinated benzenes. The dechlorination of chlorinated benzenes and PCBs in the consortium is probably carried out by two different groups of microorganisms. Our results, integrated with data from the literature, show that microbial reductive dechlorination of chlorinated benzenes is carried out by a variety of different microorganisms, possessing different properties and dechlorination capacities.

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REFERENCES

1. **Beurskens, J. E. M.** (1995) Microbial transformation of chlorinated aromatics in sediments. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.
2. **Beurskens, J. E. M., C. G. C. Dekker, J. Jonkhoff and L. Pompstra.** 1993. Microbial dechlorination of hexachlorobenzene in a sedimentation area of the Rhine river. Biogeochem. **19:**61-81.
3. **Beurskens, J. E. M., C. G. C. Dekker, H. Van den Heuvel, M. Swart and J. De Wolf.** 1994. Dechlorination of chlorinated benzenes by an anaerobic microbial consortium that selectively mediates the thermodynamic most favorable reactions. Environ. Sci. Technol. **28:**701-706.
4. **Beurskens, J. E. M., G. A. J. Mol, H. L. Barreveld, B. Van Munster and H. J. Winkels.** 1993. Geochronology of priority pollutants in a sedimentation area of the Rhine river. Environ. Toxicol. Chem. **12:**1549-1566.
5. **Beurskens, J. E. M., M. Toussaint, J. De Wolf, J. M. D. Van der Steen, P. C. Slot, L. C. M. Commandeur and J. R. Parsons.** 1995. Dehalogenation of chlorinated dioxins by an anaerobic microbial consortium from sediment. Environ. Toxicol. Chem. **14:**939-943.
6. **Bosma, T. N. P., J. R. Van der Meer, G. Schraa, M. E. Tros and A. J. B. Zehnder.** 1988. Reductive dechlorination of all trichlorobenzene isomers. FEMS Microbiol. Ecol. **53:**223-229.
7. **Cole, J. R., A. L. Cascarelli, W. W. Mohn and J. M. Tiedje.** 1994. Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol. Appl. Environ. Microbiol. **60:**3536-3542.
8. **De Bont, J. A. M., M. J. A. W. Vorage, S. Hartmans and W. J. J. Van den Tweel.** 1986. Microbial degradation of 1,3-dichlorobenzene. Appl. Environ. Microbiol. **52:**677-680.
9. **Dolfing, J.** 1990. Reductive dechlorination of 3-chlorobenzoate is coupled to ATP production and growth in an anaerobic bacterium, strain DCB-1. Arch. Microbiol. **153:**264-266.
10. **Fathepure, B. Z., J. M. Tiedje and S. A. Boyd.** 1988. Reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in anaerobic sewage sludge. Appl. Environ. Microbiol. **54:**327-330.
11. **Gerritse, J., V. Renard, T. M. P. Gomes, P. A. Lawson, M. D. Collins and J. C. Gottschal.** 1996. *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or *ortho*-chlorinated phenols. Arch. Microbiol. **165:**132-140.
12. **Haigler, B. E., S. F. Nishino and J. C. Spain.** 1988. Degradation of 1,2-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **54:**294-301.
13. **Holliger, C., G. Schraa, A. J. M. Stams and A. J. B. Zehnder.** 1992. Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. Appl. Environ. Microbiol. **58:**1636-1644.
14. **Marinucci, A. C. and R. Bartha.** 1979. Biodegradation of 1,2,3- and 1,2,4-trichlorobenzene in soil and liquid enrichment culture. Appl. Environ. Microbiol. **38:**811-817.
15. **Middeldorp, P. J. M., J. De Wolf, A. J. B. Zehnder and G. Schraa.** 1996. Dechlorination of polychlorinated benzenes and biphenyls by a methanogenic consortium. Submitted.
16. **Middeldorp, P. J. M., J. De Wolf, A. J. B. Zehnder and G. Schraa.** 1996. Reduction of the lag phase for microbial reductive dechlorination of polychlorinated biphenyls. Submitted.
17. **Mohn, W. W. and J. M. Tiedje.** 1991. Evidence for chemiosmotic coupling of reductive dechlorination and ATP synthesis in *Desulfomonile tiedjei*. Arch. Microbiol. **157:**1-6.
18. **Nowak, J., N. H. Kirsch, W. Hegemann and H.-J. Stan.** 1996. Total reductive dechlorination of chlorobenzenes to benzene by a methanogenic mixed culture enriched from Saale river sediment. Appl. Microbiol. Biotechnol. **45:**700-709.
19. **Oltmanns, R. H., H. G. Rast and W. Reineke.** 1988. Degradation of 1,4-dichlorobenzene by enriched and constructed bacteria. Appl. Microbiol. Biotechnol. **28:**609-616.
20. **Ramanand, K., M. T. Balba and J. Duffy.** 1993. Reductive dehalogenation of chlorinated benzenes and toluenes under methanogenic conditions. Appl. Environ. Microbiol. **59:**3266-3272.
21. **Sander, P., R. M. Wittich, P. Fortnagel, H. Wilkes and W. Francke.** 1991. Degradation of 1,2,4-trichlorobenzene and 1,2,4,5- tetrachlorobenzene by *Pseudomonas* strains. Appl. Environ. Microbiol. **57:**1430-1440.
22. **Schraa, G., M. L. Boone, M. S. M. Jetten, A. R. W. Van Neerven, P. J. Colberg and A. J. B. Zehnder.** 1986. Degradation of 1,4-dichlorobenzene by *Alcaligenes* sp. strain A175. Appl. Environ. Microbiol. **52:**1374-1381.
23. **Spain, J. C. and S. F. Nishino.** 1987. Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **53:**1010-1019.

24. **Utkin, I., C. Woese and J. Wiegel.** 1994. Isolation and characterization of *Desulfitobacterium dehalogenans* gen nov, sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. Int. J. Syst. Bacteriol. **44**:612-619.
25. **Van der Meer, J. R., W. Roelofsen, G. Schraa and A. J. B. Zehnder.** 1987. Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 in nonsterile soil columns. FEMS Microbiol. Ecol. **45**:333-341.
26. **Van Leeuwen, C. J., D. M. M. Adema and J. Hermens.** 1990. Quantitative structure-activity relationships for fish early life stage toxicity. Aquat. Toxicol. **16**:321-334.
27. **Van Zoest, R. and G. T. M. Van Eck.** 1993. Historical input and behaviour of hexachlorobenzene, polychlorinated biphenyls and polycyclic aromatic hydrocarbons in 2 dated sediment cores from the Scheldt estuary, SW Netherlands. Marine Chem. **44**:95-103.
28. **Ye, D. Y., J. F. Quensen, J. M. Tiedje and S. A. Boyd.** 1995. Evidence for *para* dechlorination of polychlorobiphenyls by methanogenic bacteria. Appl. Environ. Microbiol. **61**:2166-2171.

CHAPTER 4

Dechlorination of polychlorinated benzenes and biphenyls by a methanogenic consortium

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SUMMARY

A 2,3,4,5-tetrachlorobiphenyl dechlorinating microbial methanogenic consortium was studied to gain more knowledge about the microorganisms responsible for the dechlorination. Both the addition of 2-bromoethanesulfonate (an inhibitor of methanogens) and eubacteria-inhibiting antibiotics, along with methanogenic substrates, blocked dechlorination of 2,3,4,5-TeCBP completely. The consortium was tested for its dechlorination capacities toward other polychlorinated biphenyls (PCBs) and benzenes (CBs). It was able to remove chlorines between two adjacent chlorines on the aromatic ring from both CBs and PCBs.

INTRODUCTION

Microbial reductive dechlorination of chlorinated benzenes (CBs) and polychlorinated biphenyls (PCBs) has been ascribed to various physiological groups of microorganisms. Reductive dechlorination has mostly been observed in methanogenic systems. The effect of the addition of the methanogenic inhibitor, 2-bromoethanesulfonic acid (BrES), on the dechlorination of CBs and PCBs is not consistent. Whereas several studies show no effect on the dechlorination (10, 12, 17, 18), also inhibiting effects of BrES on the dechlorination have also been observed (15, 16). It has been suggested that methanogens are involved in certain dechlorination pathways of PCBs (19, 20). Analogous to findings with pure bacterial cultures, which dehalogenate chlorinated compounds like chlorinated benzoates, phenols and ethenes (6, 7, 9, 11, 14), it has been suggested that PCBs can be used as electron acceptors by microorganisms which then derive energy from the dechlorination reactions (2). Bedard et al. (2) found that certain dechlorination reactions were enhanced when 2,6-dibromobiphenyl and 2-bromobiphenyl were added. These may be used as alternate electron acceptors, supporting selective enrichment of the dehalogenating organisms.

Here we describe the dechlorinating capacities of a microbial methanogenic consortium, which was enriched from a mixture of polluted sediments as described elsewhere (13). By using specific inhibitors for different physiological groups of microorganisms, an indication will be given of the involvement of these groups in the reductive dechlorination of CBs and PCBs.

MATERIALS AND METHODS

Chemicals. Chlorobenzene (MCB), 1,3- and 1,4-dichlorobenzene (DCB), 1,2,4-trichlorobenzene (TCB), 1,2,3,4-tetrachlorobenzene (TeCB), pentachlorobenzene (QCB), 2-bromoethanesulfonic acid (>98% purity), and all analytical grade solvents were obtained from E.Merck (Amsterdam, The Netherlands). 1,2-DCB, 1,3,5-TCB, 1,2,3,5- and 1,2,4,5-TeCB, and hexachlorobenzene (HCB) were from Aldrich (Zwijndrecht, The Netherlands). 1,2,3-TCB was from Janssen Chimica ('s-Hertogenbosch, The Netherlands). All single PCB congeners were from C.N. Schmidt B.V. (Amsterdam, The Netherlands). Penicillin G (sodium salt) and D-cycloserin were obtained from Sigma (Zwijndrecht, The Netherlands).

Growth medium and culture conditions for culture 61. Ten mL of the previously described (13) dechlorinating consortium was inoculated in 200 mL of medium favoring methanogenic conditions (10) in 500 mL serum bottles. Lactate (20 mM) served as electron donor and 2,3,4,5-tetrachlorobiphenyl (2,3,4,5-TeCBP) was added from a stock solution of 0.5 mg/mL to a final concentration of 2.5 mg/L. Sand from the Rhine river (5% w/v) was essential for maintenance of dechlorinating activity and was added prior to sterilization of the medium. The bottles were sealed with viton stoppers (Maag Technic AG, Dübendorf, Switzerland) and the headspace was flushed with N_2/CO_2 (80%/20%). This consortium, in the following referred to as "culture 61", was used as inoculum for the following experiments.

Alternative electron donors, bromoethanesulfonic acid (BrES) and antibiotics. 20 mL of medium containing 1 g of sterile Rhine sand and 2,3,4,5-TCBP (100 µL 0.5 mg/mL acetone solution) was inoculated with 2 mL of active culture. BrES was added to a final concentration of 5 mM to batches with sodium lactate (20 mM) as electron donor. Alternative electron donors used were sodium acetate (30 mM), methanol (100 mM) and hydrogen (80% H_2/20% CO_2 headspace). Combined additions of penicillin G (100 mg/L) and D-cycloserin (250 mg/L, added in 0.5 mL 0.1 M phosphate buffer) were applied to batches with H_2 as electron donor.

Dechlorination tests. All chlorinated benzenes, except chlorobenzene, were tested for dechlorination. The PCBs tested were 2,5-DCBP, 2,4,5-TCBP, 2,3,4-TCBP, 2,3',4',5-TeCBP, 2,3,4,5,6-PCBP, and 2,2',3,4,4',5,5'-HpCBP. 2 mL of the active enrichment was transferred to 20 mL of medium, containing sodium lactate (20 mM), 1 g of sterile Rhine sand, and the chlorinated benzene (100 µL 1 mg/mL acetone solution) or PCB (100 µL 0.5 mg/mL acetone solution) to be tested. All tests were run in triplicate. The cultures were incubated statically at 30°C in the dark.

CBs and PCBs analyses

Samples of 1-2 mL were taken from the batch cultures after shaking, and were extracted with 1 mL chloroform in an end-over-end shaker for 24 hrs (10 rpm). The chloroform extracts were analyzed for CBs and PCBs using gas chromatography coupled to a mass selective detector either in a single ion monitoring mode (SIM) for quantification or in a SCAN mode for identification as described in (12, 13). Concentrations were quantified by using external standards.

Bromide analysis. The bromide concentration was determined in the batches which were incubated with BrES, to detect possible debromination. 0.4 mL of a centrifuged sample (15 min., 13500 rpm) was diluted tenfold in distilled water and analyzed with a Dionex DX-100 HPLC system (Dionex, Breda, The Netherlands), equipped with an ion chromatograph and a conductivity detector (flow 2 mL/min.).

RESULTS

Effect of alternative electron donors, BrES and antibiotics on 2,3,4,5-TeCBP dechlorination by culture 61. Besides lactate, also H_2 was found to be a suitable electron donor for dechlorination of 2,3,4,5-TeCBP. H_2 was converted to acetate and methane. In batches with acetate or methanol added as electron donor, dechlorination was not observed.

The addition of 5 mM of BrES to the dechlorinating culture at the time of inoculation, resulted in a complete inhibition of the dechlorination of 2,3,4,5-TeCBP. No release of bromide was observed during incubation, indicating that BrES itself was not debrominated. Propionate and acetate accumulated in the medium and methane was not produced. The addition of BrES to batches with H_2 as electron donor also inhibited dechlorination completely. The addition of the antibiotics penicillin G and D-cycloserin to batches with H_2 as electron donor, resulted in the production of methane, while no dechlorination of 2,3,4,5-TeCBP was observed.

Dechlorination tests. Table 4.1 shows the dechlorinating activity of the consortium after 35 days of incubation with individual chlorinated benzenes and PCBs. Di- and trichlorobenzenes were not dechlorinated, except for 1,2,3-TCB, which was converted to 1,3-DCB. 1,2,3,4-TeCB and 1,2,3,5-TeCB were dechlorinated to 1,2,4-TCB and 1,3,5-TCB, respectively, whereas the consortium showed no activity towards 1,2,4,5-TeCB. QCB was dechlorinated via 1,2,3,5-TeCB to 1,3,5-TCB. HCB was converted to QCB and further dechlorinated to 1,3,5-TCB as the final product.

The consortium dechlorinated 2,3,4-TCBP to 2,4-DCBP, 2,3,4,5,6-PCBP to 2,3,5,6-TeCBP and 2,4,6-TCBP. 2,2',3,4,4',5,5'-HpCBP was dechlorinated to 2,2',3,4',5,5'-HxCBP. 2,5-DCBP, 2,4,5-TCBP, and 2,3',4',5-TeCBP were not dechlorinated after 60 days of incubation.

DISCUSSION

Dechlorination of CBs and PCBs has been ascribed to various physiological groups of microorganisms. The addition of 5 mM of BrES to culture 61 inhibited both dechlorination of 2,3,4,5-TeCBP and methanogenesis completely. Since bromide analysis revealed that the BrES itself was not dehalogenated, we can conclude that the lack of dechlorination was not due to competitive inhibition, but purely due to the inactivation of methanogenic bacteria in the consortium. In several studies, no effect of BrES on the dechlorination of CBs or PCBs was observed (10, 12, 17, 18). However, Nowak et al. (16) found a decreased dechlorination rate of CBs in enrichments from polluted river sediment. In another study it was found predominantly *para*-dechlorination of PCBs was inhibited by the addition of BrES (15). In a study in which heat and ethanol treatments eliminated methanogens and non-sporeforming

Table 4.1. Dechlorination of chlorinated benzenes and polychlorinated biphenyls and the observed dechlorination products.

Substrate	Conc. (μM)	Dechlorination*	Products
Chlorinated benzenes			
1,2-DCB	34	-	--
1,3-DCB	34	-	--
1,4-DCB	34	-	--
1,2,3-TCB	28	+	1,3-DCB
1,2,4-TCB	28	-	--
1,3,5-TCB	28	-	--
1,2,3,4-TeCB	23	+	1,2,4-TCB
1,2,3,5-TeCB	23	+	1,3,5-TCB
1,2,4,5-TeCB	23	-	--
QCB	20	+	1,2,3,5-TeCB, 1,3,5-TCB
HCB	18	+	QCB, 1,2,3,5-TeCB, 1,3,5-TCB
Polychlorinated biphenyls			
2,5-DCBP	22	-	--
2,4,5-DCBP	19	-	--
2,3,4-TCBP	19	+	2,4-DCBP
2,3,4,5,-TeCBP	17	+	2,3,5-TCBP
2,3',4',5-TeCBP	17	-	--
2,3,4,5,6-PCBP	15	+	2,3,5,6-TeCBP, 2,4,6-TCBP
2,2',3,4,4',5,5'-HpCBP	13	+	2,2',3,4',5,5'-HxCBP

* - less than 0.1% dechlorinated
\+ more than 90% dechlorinated

bacteria, a *meta*-preferential dechlorination was maintained (19). Dechlorination of PCBs at the *para* position was found to be inhibited by the addition of BrES (20).

Dechlorination of 2,3,4,5-TeCBP by culture 61 was also inhibited in the presence of penicillin G and D-cycloserin. These antibiotics inhibit growth of eubacteria, but should not affect methanogenic bacteria. Ye et al. (20) showed that the same antibiotics inhibited the dechlorination of 2,3',4'-TCBP to 2,4'-DCBP, but not to 2,3'-DCBP. Dechlorinating enrichments which are inhibited both by BrES and eubacteria-inhibiting antibiotics have not been described. From these results alone, we cannot conclude that dechlorination of 2,3,4,5-TeCBP is performed by either eubacteria or by methanogens in this consortium. Interactions between these groups of microorganisms seem to be essential for the dechlorination to occur. This is supported by the fact that our repeated attempts to isolate a CB or PCB dechlorinating pure culture from this consortium have failed till now.

Culture 61 was very specific in its ability to dechlorinate both chlorinated benzenes (CBs) and polychlorinated biphenyls (PCBs). This specificity was similar to that of an enrichment culture from Lake Ketelmeer (NL) sediment, described by Beurkens et al. (3). Although culture 61 was enriched from a sediment mixture which contained Lake Ketelmeer sediment, it is not identical to that enrichment. Unlike culture 61, the culture of Beurkens et al. did not lose its dechlorinating activity in the absence of Rhine sand. In a previous study (12) we described the ability of another methanogenic consortium which is also capable of dechlorinating CBs and PCBs. The difference in dechlorination pattern and the different lag phases observed for CBs one one hand and PCBs on the other hand, indicated that different groups of microorganisms were responsible for the dechlorination of these different classes of compounds.

The dechlorination selectivity of culture 61 towards CBs and several PCBs was similar. Only chlorines, flanked on both sides by at least one chlorine, were removed (Figure 4.1). Such a typical pattern has been observed before, both for chlorinated benzenes (4, 5, 8, 10) and PCBs (3, 13).

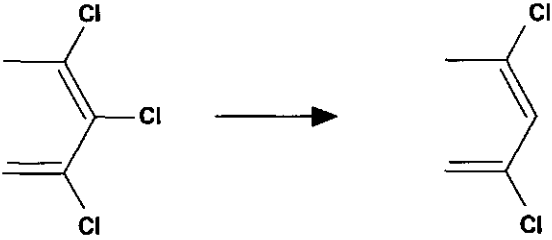

Figure 4.1. Schematic picture of the dechlorination specificity of culture 61.

The dechlorination patterns of PCBs, observed in sediments polluted with technical mixtures (e.g. Aroclors), have been extensively reviewed by Bedard and Quensen (1). Numerous different individual dechlorination "processes" (patterns) were recognized and described. The dechlorination specificity toward single PCB congeners, as observed in our consortium, was not resembling any of these "processes". We therefore propose that the described processes can be further subdivided into "subprocesses", of which the process, observed by us and by Beurskens et al. (3), forms such a subprocess. For example, the dechlorination of 2,3,4,5-chlorinated biphenyls to 2,3,5- chlorinated biphenyls is then a subprocess of the processes designated with the letters H, H', and P (1). The different subprocesses may be carried out by different microbial consortia with their own characteristics.

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REFERENCES

1. **Bedard, D. L. and J. F. Quensen III.** 1995. Microbial reductive dechlorination of PCBs, p. 127-216. *In* L. Y. Young and C. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals, Wiley-Liss Division, John Wiley & Sons, Inc., New York.
2. **Bedard, D. L., L.-A. Smullen, K. A. DeWeerd, D. K. Dietrich, G. M. Frame, R. J. May, J. M. Principe, T. O. Rouse, W. A. Fessler and J. S. Nicholson.** 1995. Chemical activation of microbially-mediated PCB dechlorination: A field study. Organohalogen compounds **24:**23-28.
3. **Beurskens, J. E. M.** (1995) Microbial transformation of chlorinated aromatics in sediments. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.
4. **Beurskens, J. E. M., C. G. C. Dekker, J. Jonkhoff and L. Pompstra.** 1993. Microbial dechlorination of hexachlorobenzene in a sedimentation area of the Rhine river. Biogeochem. **19:**61-81.
5. **Beurskens, J. E. M., C. G. C. Dekker, H. Van den Heuvel, M. Swart and J. De Wolf.** 1994. Dechlorination of chlorinated benzenes by an anaerobic microbial consortium that selectively mediates the thermodynamic most favorable reactions. Environ. Sci. Technol. **28:**701-706.
6. **Cole, J. R., A. L. Cascarelli, W. W. Mohn and J. M. Tiedje.** 1994. Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol. Appl. Environ. Microbiol. **60:**3536-3542.
7. **Dolfing, J.** 1990. Reductive dechlorination of 3-chlorobenzoate is coupled to ATP production and growth in an anaerobic bacterium, strain DCB-1. Arch. Microbiol. **153:**264-266.
8. **Fathepure, B. Z., J. M. Tiedje and S. A. Boyd.** 1988. Reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in anaerobic sewage sludge. Appl. Environ. Microbiol. **54:**327-330.
9. **Gerritse, J., V. Renard, T. M. P. Gomes, P. A. Lawson, M. D. Collins and J. C. Gottschal.** 1996. *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or *ortho*-chlorinated phenols. Arch. Microbiol. **165:**132-140.
10. **Holliger, C., G. Schraa, A. J. M. Stams and A. J. B. Zehnder.** 1992. Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. Appl. Environ. Microbiol. **58:**1636-1644.
11. **Holliger, C., G. Schraa, A. J. M. Stams and A. J. B. Zehnder.** 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. **59:**2991-2997.
12. **Middeldorp, P. J. M., J. De Wolf, A. J. B. Zehnder and G. Schraa.** 1996. Enrichment and properties of a 1,2,4-trichlorobenzene dechlorinating methanogenic microbial consortium. in preparation.
13. **Middeldorp, P. J. M., J. De Wolf, A. J. B. Zehnder and G. Schraa.** 1996. Reduction of the lag phase for microbial reductive dechlorination of polychlorinated biphenyls. In preparation.
14. **Mohn, W. W. and J. M. Tiedje.** 1990. Strain DCB-1 conserves energy for growth from reductive dechlorination coupled to formate oxidation. Arch. Microbiol. **153:**267-271.
15. **Morris, P. J., W. W. Mohn, J. F. Quensen, J. M. Tiedje and S. A. Boyd.** 1992. Establishment of a Polychlorinated Biphenyl-Degrading Enrichment Culture with Predominantly *Meta*-dechlorination. Appl. Environ. Microbiol. **58:**3088-3094.
16. **Nowak, J., N. H. Kirsch, W. Hegemann and H.-J. Stan.** 1996. Total reductive dechlorination of chlorobenzenes to benzene by a methanogenic mixed culture enriched from Saale river sediment. Appl. Microbiol. Biotechnol. **45:**700-709.
17. **Rhee, G. Y., B. Bush, C. M. Bethoney, A. Denucci, H. M. Oh and R. C. Sokol.** 1993. Anaerobic Dechlorination of Aroclor 1242 as Affected by Some Environmental Conditions. Environ. Toxicol. Chem. **12:**1033-1039.
18. **Sokol, R. C., C. M. Bethoney and G. Y. Rhee.** 1994. Effect of hydrogen on the pathway and products of PCB dechlorination. Chemosphere **29:**1735-1742.
19. **Ye, D. Y., J. F. Quensen III, J. M. Tiedje and S. A. Boyd.** 1992. Anaerobic dechlorination of polychlorobiphenyls (Aroclor 1242) by pasteurized and ethanol-treated microorganisms from sediments. Appl. Environ. Microbiol. **58:**1110-1114.
20. **Ye, D. Y., J. F. Quensen, J. M. Tiedje and S. A. Boyd.** 1995. Evidence for *para* dechlorination of polychlorobiphenyls by methanogenic bacteria. Appl. Environ. Microbiol. **61:**2166-2171.

CHAPTER 5

Reduction of the lag phase for microbial reductive dechlorination of polychlorinated biphenyls

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SUMMARY

The lag phase for the microbial reductive dechlorination of polychlorinated biphenyls (PCBs) was significantly reduced by the addition of anaerobic granular sludge (AGS). AGS, either autoclaved or not, reduced the lag phase for dechlorination of 2,3,4,5-tetrachlorobiphenyl (2,3,4,5-TeCBP) by a methanogenic consortium from ca. 3 weeks to 8 days. Also the addition of sterile sand reduced this lag phase, and combining these two additions resulted in a cumulative decrease of the lag phase. In all three cases, the rate of dechlorination was not affected. The addition of vitamin B_{12} had only a slight reducing effect on the lag phase. AGS also reduced the lag phase for the dechlorination of Aroclor 1260 from 75 to 14 days in a microbial enrichment from polluted sediments. The decrease of the lag phase, achieved by the addition of AGS, may be caused by unknown heat-stable factors, originating from microorganisms in the AGS.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are industrial chemicals which have been widely used for various applications, such as flame retardants, hydraulic fluids, plasticizers, and dielectric fluids. Due to accidents and spills, the release of these compounds into the environment has been widespread. They appear to persist in many aquatic sediments and are of concern because they tend to accumulate in biota and, particularly PCBs, are potential carcinogens (2, 17, 27 1651).

In 1984, the occurrence of microbial anaerobic *in situ* dechlorination of highly chlorinated PCBs in Hudson river sediments was suggested (11), and was confirmed by *in vitro* experiments (23). Numerous studies have now shown that reductive dechlorination of PCBs is an ubiquitous process in anaerobic environments, which may lead to the *in situ* decontamination and detoxification of polluted soils (6, 8, 12).

The effectiveness of the dechlorination process is limited by the occurrence of low dechlorination rates, long lag phases before dechlorination starts, and loss of activity towards specific congeners in subcultures. This is partly due to our limited knowledge on the microorganisms involved in the reductive dechlorination process. The occurrence of a long time period, often referred to as “adaptation time” or “lag phase”, before the reductive dechlorination starts, has been observed in a number studies. Abramowicz et al. (1) studied the dechlorination of a mixture of PCBs at different concentrations in Hudson River sediment and found in all cases a lag phase of 4 weeks. A mixture of PCBs was dechlorinated in anaerobic soil slurry cultures after a lag period of 4 weeks of incubations (12). For the dechlorination of individual PCB congeners, lag phases of 2-24 weeks have been reported (21, 29, 31). However, specific studies on the possibilities to overcome this problem have not been published.

In this chapter, we describe the effect of anaerobic granular sludge (AGS) and other amendments on the lag phase for the dechlorination of PCBs. This is studied both in a methanogenic 2,3,4,5-tetrachlorobiphenyl dechlorinating consortium, and in an Aroclor 1260 dechlorinating enrichment from polluted sediments. Mechanisms, which may be responsible for reducing the lag phase are discussed.

MATERIALS AND METHODS

Chemicals. Pentachlorobenzene (QCB), vitamin B_{12} (cyanocobalamin) and all analytical grade solvents were obtained from E.Merck (Amsterdam, The Netherlands). Single PCBs congeners were from C.N. Schmidt B.V. (Amsterdam, The Netherlands). Aroclor 1260 was a kind gift from dr. G.K. Robinson (University of Kent at Canterbury, England).

Enrichment of a dechlorinating methanogenic consortium. Approximately 2 g (wet wt.) of a mixture of sediments and sludges containing a) sediment from the river Rhine (Wageningen, The Netherlands), b) dredging sludges heavily polluted with oil (Rotterdam and Zierikzee, The Netherlands), c) sediment from Lake Ketelmeer (The Netherlands) containing polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and polychlorinated benzenes, and d) sludge from an anaerobic waste water treatment plant (CSM, Breda, The Netherlands), was added to 20 mL of medium favouring methanogenic conditions (16) in 120 mL serum bottles. The bottles were sealed with viton stoppers (Maag Technic AG, Dübendorf, Switzerland). The headspace was flushed with N_2/CO_2 (80%/20%), and sodium lactate was added to a final concentration of 20 mM. Pentachlorobenzene (QCB) was supplied in 2 mL of hexadecane (10 mg/mL).The bottles were statically incubated at 30°C in the dark. The enrichment culture was maintained for 5 subsequent transfers by subculturing 10% of the active culture in fresh medium, containing lactate, QCB and 1 g of sterile Rhine sand per 20 mL of medium. The Rhine sand was boiled (20 min), washed (10 times) with water, dried at 100°C, and added to the medium prior to sterilization. Its addition was found to be necessary to sustain reductive dechlorination over longer periods of time (data not shown). After the fifth transfer (after 6 months), the reductive dechlorination was tested with 2,3,4,5-tetrachlorobiphenyl (TeCBP, IUPAC No. 61) and the enrichment was maintained for more than two years (15 transfers) on this PCB, which was added in 100 µL acetone to a final concentration of 2.5 mg/L. Stock cultures of the fourteenth and fifteenth subculture were used in the following experiments. The consortium will in the following be referred to as "culture 61".

Aroclor 1260 dechlorinating enrichment

Approximately 4 g (wet wt.) of the sediment mixture mentioned before, was added to 40 mL of mineral medium in 120 ml serum bottles. The headspace was flushed with N_2/CO_2 (80%/20%), and sodium lactate was added to a final concentration of 20 mM. Aroclor 1260 was added in 200 µL acetone to a final concentration of circa 70 mg/L total PCBs. After dechlorination was observed, 2.5 mL amounts of the sediment slurry were transferred to fresh medium, containing 20 mM lactate, Aroclor 1260 and 2 g of sterile Rhine sand. Dechlorinating cultures were used as inoculum for the following experiment. 2.5 mL of culture was transferred to fresh medium with lactate, Rhine sand and Aroclor 1260. Half of the tests received 4 g (wet wt.) of anaerobic granular sludge (AGS), obtained from an upflow anaerobic sludge blanket reactor (UASB) which was fed with butyrate, propionate and acetate. The bottles were statically incubated in the dark at 30°C. All tests were run in duplicate.

Influence of various additions on the lag phase. 20 mL of medium, containing sodium lactate and 2,3,4,5-TCBP (100 µL 0.5 mg/mL acetone solution) was inoculated with 2 mL of culture 61. Sterile Rhine sediment (1 g/20 mL) was added to the medium before heat sterilization. Vitamin B_{12} was added to a concentration of 0.93 µM (25 times the regular concentration in the medium). Anaerobic granular sludge (AGS), was crushed by pressing it through needles with decreasing diameter (smallest diameter 0.5 mm) with a small amount of water. Of the obtained suspension, 0.5 mL was added to the medium, and heat sterilized three times within 5 days prior to inoculation with culture 61. All treatments were tested in triplicate.

Reduction potential measurements. Two double-neck 0.5 L bottles were filled with 200 mL anaerobic medium. The headspace was flushed with 80% N_2/20% CO_2, closed on both sides with viton stoppers and autoclaved. After autoclaving, one stopper was replaced with a 300 x 12 mm Hamilton redox electrode with built-in Pt reference electrode (ThIS Scientific, The Hague, The Netherlands), while flushing with sterile N_2/CO_2. After autoclaving, 2,3,4,5-TeCBP (0.9 mL 0.5 mg/mL in acetone) was added and the bottles were inoculated with 9 mL of an active culture. One bottle received 4.9 g (wet wt.) of uncrushed AGS. The bottles were incubated at room temperature (16-22°C). Samples for PCB measurements were taken with a syringe through the viton stopper. Overpressure, due to methane production, was taken away with a syringe.

The redox electrodes were calibrated with a Zobell solution (0.987 g $K_3Fe(CN)_6$ and 1.266 g $K_4Fe(CN)_6$ in 1 L 0.1 M KCl) with E_{ref} = +245 mV. The electrodes were connected to a PHM82 Standard pH meter (Radiometer Copenhagen, The Hague, The Netherlands), and values were read as displayed in the mV mode.

Sample preparation and analyses of PCBs and chlorinated benzenes. Samples of 1-2 mL were taken from the batch cultures after shaking, and were extracted with 1 mL chloroform in an end-over-end shaker for 24 hrs (10 rpm). The chloroform extracts were analyzed on a Hewlett-Packard 5890 series II gas chromatograph equipped with a mass selective detector (series 5971A), an automatic sampler (series 7673A), and a fused silica analytical column (HP5, 30 m x 0.25 mm i.d.) from Hewlett-Packard (Amsterdam, The Netherlands). Oven temperature conditions for the separation of chlorinated benzenes were: 60°C for 3 min., 5°C/min. to 180°C, total run length was 30 minutes. For the separation of the PCBs, oven temperatures were 70°C for 2 min., 10°C/min. to 250°C, total run length was 25 minutes. PCBs in Aroclor 1260 were separated at oven temperatures of 70°C for 1 min., 40°C/min. to 150°C for 10 min., 3.5°C/min. to 300°C, total run length was 60 minutes. Data were acquired using either a full scan mode for identification of metabolites, or a selected ion monitoring mode (SIM) for quantification. Concentrations were quantified by using external standards.

Since 2,3,5-trichlorobiphenyl (2,3,5-TCBP) was not available, concentrations were quantified by comparing the peak areas of 2,3,5-TCBP with those of external standards of 2,4,5-TCBP. The average amount of chlorines per biphenyl molecule was calculated from the peak areas of the different chlorinated biphenyls related to the total area of PCBs.

RESULTS

Enrichment of a dechlorinating methanogenic consortium. A mixture of polluted sediments was incubated with pentachlorobenzene (QCB) under methanogenic conditions. After 40 days, a significant chloride production was observed, indicating dechlorination. Qualitative analysis of the medium showed the presence of 1,2,3,4-and 1,2,3,5-tetrachlorobenzene (TeCB), all trichlorobenzene (TCB) isomers and 1,4-dichlorobenzene. Upon subculturing the sediment to fresh medium with QCB, a more specific dechlorination to 1,2,3,5-TeCB and 1,3,5-TCB was observed. This pathway remained unchanged during the following transfers. From the fourth subculture on, the consortium (referred to as "culture 61") was maintained on 2,3,4,5-tetrachlorobiphenyl (TeCBP), which was dechlorinated to 2,3,5-trichlorobiphenyl (TCBP). Culture 61 was maintained for over 15 subsequent transfers during two years. Upon transfer, the lag phase remained stable at 2-3 weeks before dechlorination started (Figure 5.1).

A second addition of 2,3,4,5-TeCBP to cultures in which this PCB was depleted, resulted in a continuation of the dechlorination after a considerably shorter lag phase: about 6 days (Figure 5.1). The amount of 2,3,5-TCBP recovered was approximately 35% of the amount of 2,3,4,5-TeCBP removed (after 40 days).

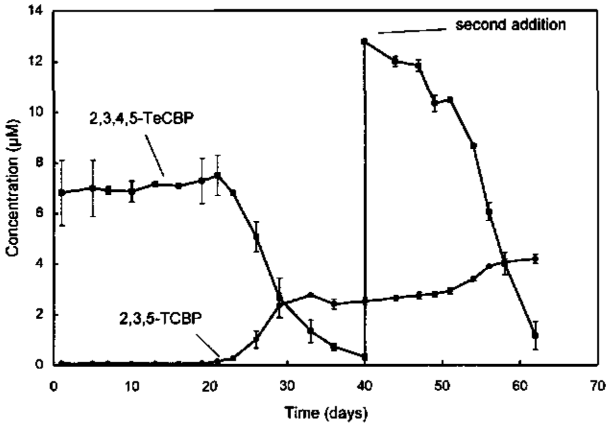

Figure 5.1. Dechlorination of 2,3,4,5-TeCBP to 2,3,5-TCBP as a function of time, and and the effect of a second addition of TeCBP. Error bars indicate the concentrations measured in duplicate tests.

Lactate, which was added as electron donor, was metabolized to propionate and acetate in a ratio of 2:1. Both propionate and acetate were further converted to methane.
The dechlorinating activity of the consortium during the enrichment was only sustained in the presence of Rhine sediment. Omitting the sand resulted in a gradual increase of the lag phase and a decrease of the dechlorinating activity over several transfers.

Influence of various additions on the lag phase. The dechlorination of 2,3,4,5-TeCBP to 2,3,5-TCBP by culture 61 in the presence of sand, AGS or vitamin B_{12} is shown in Figure 5.2. The control, which was inoculated without further additions, showed a lag phase of ca. 20 days followed by an almost complete dechlorination to 2,3,5-TCBP within another 10 days at a fastest rate of about 19 nmoles/day. The addition of sterile Rhine sand reduced the lag phase to about 11 days, while the rate of dechlorination was not significantly increased (25 nmole/day). Vitamin B_{12} (0.93 μM) reduced the lag phase to about 17 days. Increased amounts of vitamin B_{12} (up to 4-fold) did not shorten the lag phase any further (data not shown). Anaerobic granular sludge (AGS) in the medium without culture 61 did not dechlorinate of 2,3,4,5-TeCBP. However, when inoculated, the addition of the AGS resulted in a decrease of the lag phase to ca. 8 days with a susequent dechlorination rate of 25 nmole/day. The addition of AGS, autoclaved or not, to the inoculated batches, in combination with sterile Rhine sand yielded a lag phase of ca. 6 days.

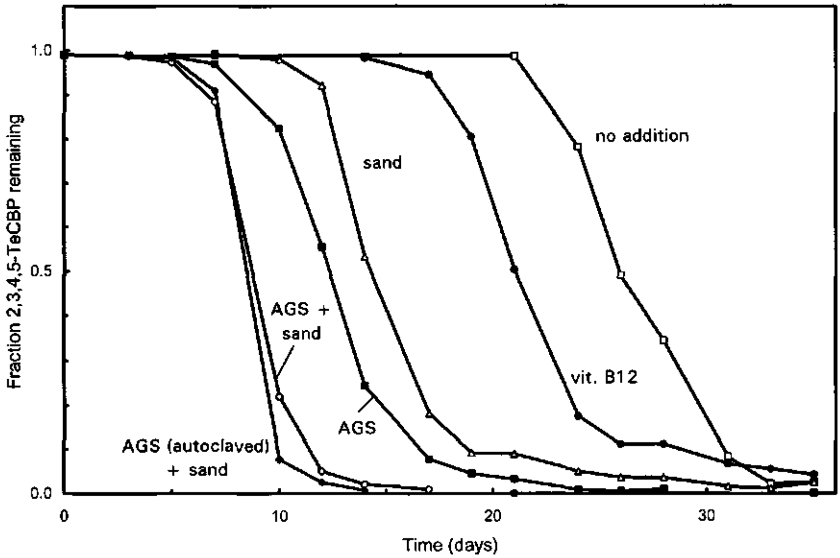

Figure 5.2. The effect of various additions on the dechlorination of 2,3,4,5-TeCBP. The values on the vertical axis represent the area fraction of 2,3,4,5-TeCBP relative to that of the dechlorination product 2,3,5-TCBP. In batches with either AGS or vit. B_{12} added to uninoculated medium, no dechlorination was observed (not shown).

Reduction potential measurements

Dechlorination of 2,3,4,5-TeCB started faster in the presence of AGS (after 10 days) than in its absence (after 35 days). The redox potential in both batches decreased within 20 days from -400 mV to ca. -530 mV, after which it increased slowly to ca. -490 mV after 60 days of incubation. Significant differences in redox potentials during time in the two cultures were not observed (data not shown).

Dechlorination of Aroclor 1260

Dechlorination of Aroclor 1260 in a second subculture of a dechlorinating slurry of polluted sediments was tested in the presence and absence of AGS (Figure 5.3). In the presence of AGS, dechlorination was observed after 14 days, and after 61 days the average number of chlorines per biphenyl had dropped from 6.1 to 4.6. In inoculated controls without AGS dechlorination did not start until after 75 days of incubation. Aroclor 1260 was not dechlorinated with AGS alone (data not shown).

DISCUSSION

A lag phase for the dechlorination of individual PCB congeners has commonly been observed and appears to vary from 2-24 weeks (21, 29, 31). We studied the effect of sand, AGS and vitamin B_{12} on the lag phase and dechlorination rate in a stable microbial methanogenic consortium (culture 61), which was enriched from a mixture of polluted sediments. Culture 61 was maintained on 2,3,4,5-TeCBP, which was dechlorinated to 2,3,5-TCBP, and has been successfully subcultured with this congener at least 14 times. We observed an incomplete recovery of 2,3,4,5-TeCBP as 2,3,5-TCBP: only 35% after 40 days and 21% after 62 days of incubation (Figure 5.1). Part of the missing fraction may be ascribed to a difference between the response factors of 2,3,5-TCBP and 2,4,5-TCBP, which was used as external standard. The difference between response factors of PCB homologs has been reported to vary within a factor 2 (20). Furthermore, due to a higher volatility, part of the formed 2,3,5-TCBP may adsorb irreversibly to glass and stoppers, and may not have been sampled. Similar low recoveries of single PCB congeners after dechlorination have been reported (7, 24, 25). GC/MS analyses of the samples did not indicate that other reactions than dechlorination had taken place. During the enrichment, the dechlorinating activity in the corsortium was only maintained in the presence of sterile Rhine sediment in the medium. Omitting the sediment resulted in a complete loss of dechlorinating activity within three subsequent transfers. Similar effects on the maintenance of enrichment cultures, caused by the addition of Rhine sediment have also been observed for the dechlorination of 1,2,4-TCB (21), hexachlorocyclohexane isomers (22), and for the anaerobic mineralization of toluene (19). Boyle et al. (10) showed that the microbial dechlorination of 2,3,6-TCBP could only be

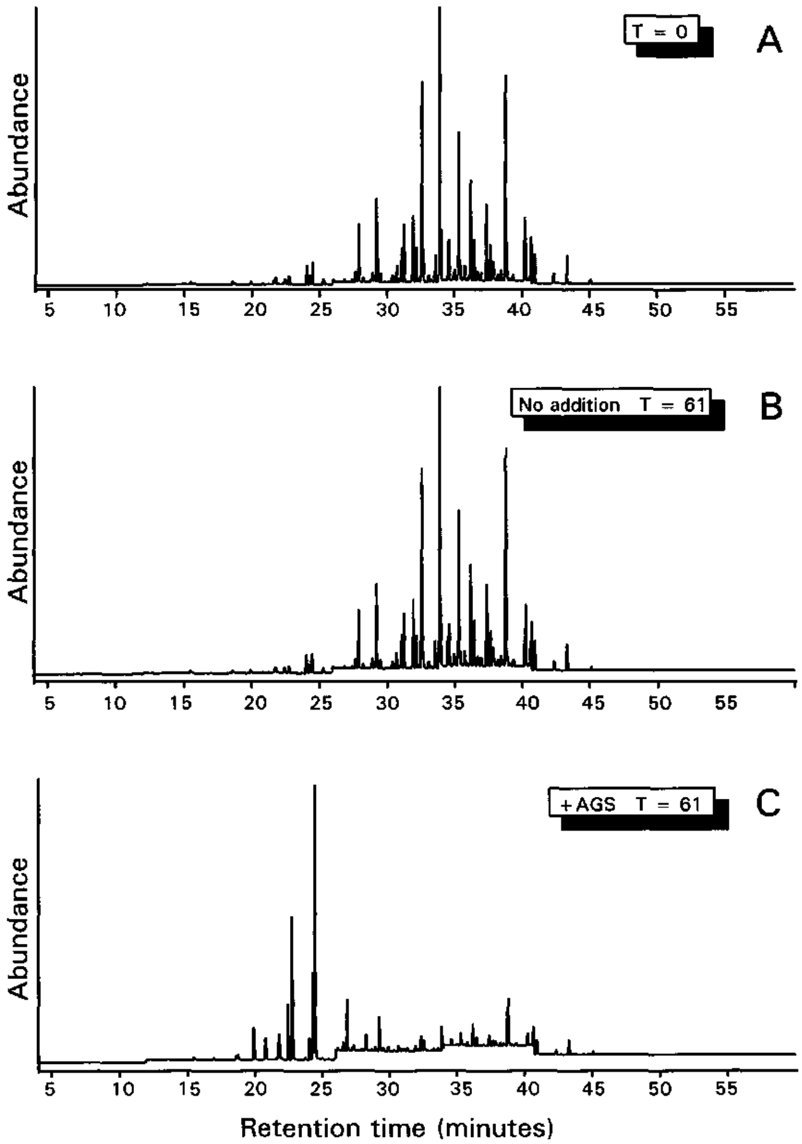

Figure 3. Chromatograms of chloroform extracts of an Aroclor 1260 dechlorinating enrichment after 0 (panel A) and 61 days of incubation (panel B and C). Panel B represents the inoculated controls without anaerobic granular sludge (AGS). Panels C represents the enrichment with AGS.

sustained in a batch culture as long as sterile sediment was repeatedly added to the medium (50% w/w), together with the addition of 2,3,6-TCBP. They also observed an increase in dechlorination rate, after larger amounts of 2,3,6-TCBP were added. Whether this increased rate was due to the sediment additions or to the larger amounts of added TCBP remains unclear.

During the enrichment of culture 61, dechlorination of 2,3,4,5-TeCBP in the presence of Rhine sediment was usually preceded by a lag phase of approximately two weeks (see also Figure 5.2). The mechanism of the reduction of the lag phase for dechlorination of 2,3,4,5-TeCBP by Rhine sand is not known. The Rhine sand that we added to our medium, may contain trace metals, necessary for dechlorination. Abramowicz et al. (1) incubated Hudson River sediment with a mixture of Aroclors in medium, from which the trace metals were removed. This removal did not reduce the lag phase, and only slightly decreased the dechlorination rate. They concluded that Hudson River sediment may have provided the necessary trace metals.

Although the organic carbon content in the Rhine sand we used is very low (<0.05% w/w), it may nevertheless be that the organic matter served as a supply of growth factors for the dechlorinating bacteria. The sand might also improve the contact between the 2,3,4,5-TeCBP and the dechlorinating cells, if these cells tend to settle out or grow on the sand surface.

A second addition of 2,3,4,5-TeCBP to culture 61 after 40 days resulted in dechlorination after a considerable shorter lag phase (6 days) than after the initial addition (21 days), but did not result in an increase in dechlorination rate (Figure 5.1). This indicates that the conditions for dechlorination are not immediately optimal. The concentration of 2.5 mg/L 2,3,4,5-TeCBP used in our experiment is well above the aqueous solubility (10-50 μg/L, (26)). The similar dechlorination rate after the second addition of 2,3,4,5-TeCBP may be explained by the dissolution rate of 2,3,4,5-TeCBP, which is degradation limiting.

The lag phase of 2,3,4,5-TeCBP dechlorination could be dramatically decreased by the addition of AGS. AGS alone was not capable to dechlorinate this PCB. AGS consists of a dense microbial population (10^{10}-10^{12} bacteria/ml (14)) and may therefore contain high amounts of metal cofactors, especially vitamin B_{12}. These are known to act in the reductive dechlorination as electron carriers between electron donors and chlorinated aliphatics and aromatics (3, 4, 9, 13, 15, 18, 28, 30). The addition of vitamin B_{12} to the consortium did also decrease the lag phase, but not to the same extent as the AGS did. In contrast to our results, Abramowicz et al. (1) found that vitamin B_{12} (0.36 μM) appeared to inhibit the dechlorination rate of technical mixtures of PCBs 3-fold. Another explanation would be that, provided that a certain redox potential is required below which dechlorination can take place (5), AGS decreases the redox potential of the medium more rapidly than the inoculum alone, and therefore allows the dechlorination to start sooner. We measured the redox potential in the consortium in the presence and absence of AGS during incubation with 2,3,4,5-TeCB, but found no differences in the redox plots (data not shown).

Autoclaved AGS was equally effective in reducing the lag phase as live AGS. Sorption to the AGS may reduce the aqueous concentration of 2,3,4,5-TeCBP, and may therefore help dechlorinating bacteria to overcome eventual toxicity of this PCB. The amount of organic matter in the AGS exceeds the amount of 2,3,4,5-TeCBP that can be sorbed to it. Therefore, the cumulative effect on the lag phase reduction by the addition of AGS plus sterile Rhine sand, indicates that reduced toxicity due to sorption of 2,3,4,5-TeCBP cannot be the only reason for the shortening of the lag phase.

We suggest that other factors than living organisms are directly responsible for the decrease in lag phase by the addition of AGS. There may be inorganic or organic growth factors, which have to be produced by bacteria in culture 61, but which may already be present in the AGS. Similarly, Abramowicz et al. (1) found that the addition of fluid thioglycolate medium with beef extract (FTMBE) to slurries from Hudson River sediments shortened or eliminated the lag phase before dechlorination of added PCBs, but did not increase the subsequent rate of dechlorination.

Although the mechanism of lag phase reduction by AGS is not well understood, it appears to be also effective on the dechlorination of a technical mixture of PCBs. We show that the addition of AGS to a second subculture of a polluted sediment mixture also decreases the lag phase for dechlorination of Aroclor 1260 from 75 to 14 days. To our knowledge, the only material reported to explicitly reduce the lag phase for dechlorination of PCBs is the FTMBE as mentioned before (1).

The use of undefined additions such as AGS and FTBE illustrates how little is known about the biology of the reductive dechlorination of PCBs. Although the use of AGS as an inexpensive addition to stimulate *in situ* dechlorination of PCBs should be considered, more information may be obtained about the mechanism of dechlorination of PCBs, the microorganisms involved, and the occurrence of lag phases. This may then lead to a more directed stimulation of dechlorinating bacteria in polluted environments.

ACKNOWLEDGMENTS

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REFERENCES

1. **Abramowicz, D. A., M. J. Brennan, H. M. Van Dort and E. L. Gallagher.** 1993. Factors influencing the rate of polychlorinated biphenyl dechlorination in Hudson River sediments. Environ. Sci. Technol. **27:**1125-1131.
2. **Ankley, G. T., P. M. Cook, A. R. Carlson, D. J. Call, J. A. Swenson, H. F. Corcoran and R. A. Hoke.** 1992. Bioaccumulation of PCBs from Sediments by Oligochaetes and Fishes - Comparison of Laboratory and Field Studies. Canadian Journal of Fisheries and Aquatic Sciences **49:**2080-2085.
3. **Assaf-Anid, N., K. F. Hayes and T. M. Vogel.** 1994. Reductive dechlorination of carbon tetrachloride by cobalamin(II) in the presence of dithiothreitol: mechanistic study, effect of redox potential and pH. Environ. Sci. Technol. **28:**246-252.
4. **Becker, J. G. and D. L. Freedman.** 1994. Use of cyanocobalamin to enhance anaerobic biodegradation of chloroform. Environ. Sci. Technol. **28:**1942-1949.
5. **Bedard, D. L. and J. F. Quensen III.** 1995. Microbial reductive dechlorination of PCBs, p. 127-216. *In* L. Y. Young and C. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals, Wiley-Liss Division, John Wiley & Sons, Inc., New York.
6. **Bedard, D. L., L.-A. Smullen, K. A. DeWeerd, D. K. Dietrich, G. M. Frame, R. J. May, J. M. Principe, T. O. Rouse, W. A. Fessler and J. S. Nicholson.** 1995. Chemical activation of microbially-mediated PCB dechlorination: A field study. Organohalogen compounds **24:**23-28.
7. **Beurskens, J. E. M., J. De Wolf, H. Van den Heuvel and J. Dolfing.** 1996. Microbial dechlorination of polychlorinated biphenyls by a hexachlorobenzene acclimated enrichment culture. Environ. Sci. Technol.:Accepted for publication.
8. **Beurskens, J. E. M., G. A. J. Mol, H. L. Barreveld, B. Van Munster and H. J. Winkels.** 1993. Geochronology of priority pollutants in a sedimentation area of the Rhine river. Environ. Toxicol. Chem. **12:**1549-1566.
9. **Bosma, T. N. P., F. H. M. Cottaar, M. A. Posthumus, C. J. Teunis, A. Van Veldhuizen, G. Schraa and A. J. B. Zehnder.** 1994. Comparison of Reductive Dechlorination of Hexachloro-1,3-Butadiene in Rhine Sediment and Model Systems with Hydroxocobalamin. Environ. Sci. Technol. **28:**1124-1128.
10. **Boyle, A. W., C. K. Blake, W. A. Price II and H. D. May.** 1993. Effects of polychlorinated biphenyl congener concentration and sediment supplementation on rates of methanogenesis and 2,3,6-trichlorobiphenyl dechlorination in an anaerobic enrichment. Appl. Environ. Microbiol. **59:**3027-3031.
11. **Brown, J. F., R. E. Wagner, D. L. Bedard, M. J. Brennan, J. C. Carnahan, R. J. May and T. J. Tofflemire.** 1984. PCB transformations in upper Hudson sediments. Northeast. Environ. Sci. **3:**167-179.
12. **Fava, F., S. Cinti and L. Marchetti.** 1993. Dechlorination of Fenclor-54 and of a Synthetic Mixture of Polychlorinated Biphenyls by Anaerobic Microorganisms. Appl. Microbiol. Biotechnol. **38:**808-814.
13. **Gantzer, C. J. and L. P. Wackett.** 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. Environ. Sci. Technol. 25:715-722.
14. **Grotenhuis, J. T. C., M. Smit, C. M. Plugge, X. Yuansheng, A. A. M. Van Lammeren, A. J. M. Stams and A. J. B. Zehnder.** 1991. Bacteriological composition and structure of granular sludge adapted to different substrates. Appl. Environ. Microbiol. **57:**1942-1949.
15. **Hashsham, S. A., R. Scholze and D. L. Freedman.** 1995. Cobalamin-enhanced anaerobic biotransformation of carbon tetrachloride. Environ. Sci. Technol. **29:**2856-2863.
16. **Holliger, C., G. Schraa, A. J. M. Stams and A. J. B. Zehnder.** 1992. Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. Appl. Environ. Microbiol. **58:**1636-1644.
17. **Hong, C. S., B. Bush, J. Xiao and H. C. Qiao.** 1993. Toxic Potential of Non-Ortho and Mono-Ortho Coplanar Polychlorinated Biphenyls in Aroclors(R), Seals, and Humans. Archives of Environmental Contamination and Toxicology **25:**118-123.
18. **Kuhler, R. J., G. A. Santo, T. R. Caudill, E. A. Betterton and R. G. Arnold.** 1993. Photoreductive Dehalogenation of Bromoform with Titanium Dioxide Cobalt Macrocycle Hybrid Catalysts. Environ. Sci. Technol. **27:**2104-2111.
19. **Langenhoff, A. A. M., D. L. Brouwers-Ceiler, J. H. L. Engelberting, J. J. Quist, J. G. P. N. Wolkenfelt, A. J. B. Zehnder and G. Schraa.** 1996. Microbial reduction of manganese coupled to toluene oxidation. FEMS Microbiol. Ecol. **submitted.**
20. **Martelli, G. P., M. G. Castelli and R. Fanelli.** 1981. Biomed. Mass Spectrom. **8:**347.
21. **Middeldorp, P. J. M., J. De Wolf, A. J. B. Zehnder and G. Schraa.** 1996. Enrichment and properties of a 1,2,4-trichlorobenzene dechlorinating methanogenic microbial consortium. in preparation.

22. **Middeldorp, P. J. M., M. Jaspers, A. J. B. Zehnder and G. Schraa.** 1996. Biotransformation of alpha-, beta-, gamma-, and delta-hexachlorocyclohexane under methanogenic conditions. Environ. Sci. Technol. **30:**2345-2349.
23. **Quensen III, J. F., J. M. Tiedje and S. A. Boyd.** 1988. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. Science **242:**752-754.
24. **Rhee, G. Y., R. C. Sokol, C. M. Bethoney and B. Bush.** 1993. Dechlorination of polychlorinated biphenyls by Hudson River sediment organisms - Specificity to the chlorination pattern of congeners. Environ. Sci. Technol. **27:**1190-1192.
25. **Rhee, G. Y., R. C. Sokol, C. M. Bethoney and B. Bush.** 1993. A long-term study of anaerobic dechlorination of PCB congeners by sediment microorganisms - Pathways and mass balance. Environ. Toxicol. Chem. **12:**1829-1834.
26. **Shiu, W. Y. and D. A. Mackay.** 1986. A critical review of aqueous solubilities, vapor pressures, Henry's Law constants, and octanol-water partition coefficients of the polychlorinated biphenyls. J. Phys. Chem. Ref. Data **15:**911-929.
27. **Silberhorn, E. M., H. P. Glauert and L. W. Robertson.** 1990. Carcinogenity of polyhalogenated biphenyls: PCBs and PBBs. Crit. Rev. Toxicol. **20:**439-496.
28. **Ukrainczyk, L., M. Chibwe, T. J. Pinnavaia and S. A. Boyd.** 1995. Reductive dechlorination of carbon tetrachloride in water catalyzed by mineral supported biomimetic cobalt macrocycles. Environ. Sci. Technol. **29:**439-445.
29. **Van Dort, H. M. and D. L. Bedard.** 1991. Reductive *ortho-* and meta-dechlorination of a polychlorinated biphenyl congener by anaerobic microorganisms. Appl. Environ. Microbiol. **57:**1576-1578.
30. **Wackett, L. P. and C. A. Schanke.** 1992. Mechanisms of reductive dehalogenation by transition metal cofactors found in anaerobic bacteria, p. 329-356. *In* H. Sigel and A. Sigel (ed.), Metal ions in biological systems, Marcel Dekker, Inc., New York, Basel, Hong Kong.
31. **Williams, W. A.** 1994. Microbial reductive dechlorination of trichlorobiphenyls in anaerobic sediment slurries. Environ. Sci. Technol. **28:**630-635.

CHAPTER 6

Biodegradation of pentachlorophenol in natural soil by inoculated *Rhodococcus chlorophenolicus.*

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SUMMARY

Rhodococcus chlorophenolicus PCP-1, a mineralizer of polychlorinated phenols, was inoculated into natural sandy loam and peaty soils with pentachlorophenol (PCP) at concentrations usually found at lightly and heavily polluted industrial sites (30 to 600 mg PCP/kg). A single inoculum of 10^5 to 10^8 cells/g of peat soil and as little as 500 cells in sandy soil initiated mineralization of ^{14}C-PCP. The mineralization rates were 130 to 250 mg of PCP mineralized per kg soil in 4 months in the heavily (600 mg/kg) polluted soils and 13 to 18 mg/kg in the lightly (30 mg/kg) polluted soils. There were no detectable PCP mineralizing organisms in the soils prior to inoculation, and also there was no significant adaptation of the indigenous microbial population to degrade PCP during 4 months observation in the uninoculated soils. The inoculum-induced mineralization continued for longer than 4 months after a single inoculation. Uninoculated, lightly polluted soils (30 mg PCP/kg) also showed loss of PCP, but some of this reappeared as pentachloroanisole and other organic chlorine compounds (EOX). Such products did not accumulate in the *R. chlorophenolicus*-inoculated soils, where instead EOX was mineralized 90 to 98%. *R. chlorophenolicus* mineralized PCP unhindered by the substrate competition offered by the PCP-methylating bacteria indigenously occurring in the soils or by simultaneously inoculated O-methylating *R. rhodochrous.*

INTRODUCTION

When a large area of subsurface soil is polluted by a hazardous organic chemical, there are few options for clean-up other than to wait for the development of bioremediation methods (27, 35). It is generally thought that the environmental recalcitrance of many chemicals is explained by the scarcity of microbes capable of degrading the chemical in that environment. Consequently, the problem should be solved by introducing the appropriate degrader microbes into such environments. For many pollutants, such microbes have been found and their degradation pathways are known. However, successful introduction of microbes into soil has proved to be all but simple (3, 36), and few long-term successes have been reported. Also the experience with microbial inoculants in agriculture (30, 31) has shown the difficulty of establishing introduced microbial inocula in natural soil. The published reports on successful bioremediation of oil contamination in soil, for instance, has relied so far on the indigenous oil degrading microbes, although genetically constructed strains that were far more effective oil degraders under laboratory conditions have been available for some time (10, 32).

Introduced populations of several different bacterial genera were reported to decline one or more orders of magnitude per week in natural loamy soil (1, 2). The main reason for the decline of the bacterial inocula is thought to be predation by protozoa (1, 2, 14), although such factors as competition for nutrients by the indigenous population and stress caused by changes in moisture and temperature may also play a role. It is clear that gaps in knowledge on the ecological competitiveness required from the microbes - apart from possessing the enzymes for the biodegrading metabolic pathway - form the main obstacle to transformation of toxic chemical degrading microbes into useful tools of environmental biotechnology.

We have previously demonstrated biodegradation of polychlorinated phenols in nonsterile soil and groundwater catalyzed by bacterial inoculants (28, 29, 37, 38, 40). The bacteria capable of this were actinomycetes, members of the genus *Rhodococcus* and *Mycobacterium* (5, 20). The bacteria were effective at high pollutant concentration, prevailing at heavily polluted industrial sites (22, 23, 39). We also demonstrated bioremediation of polychlorophenol polluted soils on a field scale (28, 29, 40). In this paper, we describe in more detail the mineralization of pentachlorophenol in natural soil by *Rhodococcus chlorophenolicus* inoculants. We show the results with different amounts of inoculum, amendment with organic substrate, and with a competing inoculum, *Rhodococcus rhodochrous*, a known chlorophenol O-methylating bacterium (18, 19).

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used: PCP, pentachlorophenol; PCA, pentachloroanisol; ^{14}C-U-PCP, ^{14}C uniformly labelled pentachlorophenol; d.wt, dry weight;

DSM, Deutsche Sammlung für Mikroorganismen; PU, polyurethane; dH_2O, distilled water; MPN, most probable number; SIM, single ion monitoring; EOX, extractable organic halogen; GC-MS, gas chromatography-mass spectrometry; GC-ECD, gas chromatography-electron capture detector.

Soil characteristics. Two types of forest soil, an organic and a sandy loam, were collected from the experimental forestry farm Kettula, Suomusjärvi, Finland. The area of collection had neither been treated with any pesticide nor fertilized for the past 40 years. Plant debris and stones were removed and the soils were sieved (16 mm diameter) in their natural moisture. The soils were stored at 4°C until used. For dry weight determination, soil was dried at 105°C. For the experiments, the soils were used in their natural moisture (ca. 55% of water saturation).

The pH of the soils was measured from a 1:5 (w:w) mixture of soil (d.wt.) in either distilled water (dH_2O) or 1 M KCl after shaking for 1 hour.

Soil organic matter was determined after combustion for 16 hours at 375°C and organic carbon content was calculated as described in (11).

The metal contents were determined as follows: 20 ml of 50%/50% HNO_3/dH_2O was added to 1 or 5 g of dry soil, the slurry autoclaved for 30 minutes at 110°C and diluted with 80 ml of dH_2O. The clear supernatant was analyzed with a Spectroscan VI Spectrometer (Beckman) plasma emission analyzer. Characteristics of the soils are given in Table 6.1.

Table 6.1. Characteristics of the soils used in the experiments

	Soil 1	Soil 2
Classification	Sandy loam	Peaty
Dry weight (%)	92	38
Organic carbon (%)	0.7	14.7
pH(H_2O)	5.1	6.0
pH(KCl)	4.6	4.7
EOX (mg Cl/kg d.wt.)[a]	<0.1	<0.1
Inorganic elements (mg/kg d.wt.)		
Mg	1170	2400
Mn	120	550
Fe	6400	4300
Ca	420	9300
Al	10600	3900
Si	3300	7100

[a] Extractable organic halogen

Media and chemicals. Pentachlorophenol (PCP, 97% purity), tetra-,tri- and dichlorophenols were from E. Merck (Darmstadt, Germany), 2,4,6-tribromophenol and hexadecane (>98% purity) were from Fluka Chemie AG (Buchs, Switzerland). 2,4,6-tribromoanisole (tribromomethoxybenzene), penta-, tetra-, tri- and dichloroanisoles were all synthesized from their corresponding phenol and dimethylsulphate as described in (41). Uniformly ^{14}C-labelled pentachlorophenol (2.4 x 10^{10} Bq/mmol, radiochemical purity 96%) was from Pathfinder Laboratories Inc. (St. Louis, MO, USA). Liquid scintillator was from Kontron Analytic (Zürich, Switzerland). Amberlite XAD-2 resin (20-50 mesh) used for chloroanisole trapping was from Fluka Chemie AG (Buchs, Switzerland). Distillery waste (DW)(wheat) was obtained from Alko Ltd. Koskenkorva Distilleries (Koskenkorva, Finland). All solvents used for chemical analysis of the soils and the resins were from Rathburn Chemicals Ltd. (Walkerburn, Scotland) and were of glass-distilled grade.

Inocula. The bacterial strains used were (a) the polychlorophenol degrading *Rhodococcus chlorophenolicus* PCP-1 (DSM 43826) type strain isolated by Apajalahti and Salkinoja-Salonen (7) and taxonomically described by Apajalahti et al. (5), and (b) *Rhodococcus rhodochrous* (DSM 43241) type strain which is a chlorophenol O-methylating actinomycete (18). The bacteria were grown (28°C on a gyrotory shaker, 140 rpm, in the dark) in 300 ml of DSM-65 medium, (containing per liter: 4 g of glucose; 4 g of yeast extract; 4 g of malt extract, pH 7.2) for one week in the presence of 25 g d.wt. of sterilized polyurethane (PU) as cell adsorbent (PUR 90/16, Bayer, Leverkusen, Germany). The PU was prewashed with dH_2O and presterilized in DSM-65 medium for 10 min at 110°C. The *R. chlorophenolicus* cultures were induced to degrade chlorophenol by adding PCP to the culture in a daily increasing amount up to a concentration of 50 μM. The PU with cells adsorbed was harvested by Büchner filtration and used to inoculate soil. To achieve lower cell densities in PU, fully grown, induced cultures of *R. chlorophenolicus* were diluted in DSM-65 medium to an appropriate cell density and then incubated for 2 hours at 28°C in the presence of sterile PU. The PU was then harvested and inoculated into the soil as described above or the cells were detached and counted (see below).

Experimental setup. Amounts of 50 g w.wt. of the soils were placed in bottles of 500 mL and mixed with inoculants and carbon source as explained in Table 6.2A. PCP was added either to a concentration of ca. 630 mg/kg or 30 mg/kg (d.wt.) (stock solutions of 30 g/L and 1.5 g/L in 0.15 M NaOH, respectively). The NaOH thus added had no effect on soil pH. Five bottles of each type of soil at each concentration of PCP were immediately stored at -20°C for measurement of time zero concentration.

The distillery waste, added to some bottles, was mixed as dry powder (non-sterilized) to 0.5 g/50 g moist soil.

The inoculants were *Rhodococci*, adsorbed to 0.5 g PU (d.wt.)/50 g moist soil, corresponding to an inoculum size as stated in Table 6.2B. Controls were run with sterilized PU.

Five replicates of each treatment (Table 6.2) were prepared to allow for sacrificing the whole content of one bottle each time of sampling. ^{14}C-PCP was spiked (5000 dpm) to one of each treatment replicate, to which no DW was added, to monitor for evolution of $^{14}CO_2$, trapped in 0.5 mL of 1 M NaOH solution in a glass cup anchored to the stopper of the flask. The flasks with added DW were not tested for mineralization of ^{14}C-PCP because, after immediate hermetical closure of the bottles, conditions would turn anoxic due to consumption of oxygen due to the microbial degradation of the DW. All of the replicate bottles with added DW were therefore closed with sterile cotton plugs for the first 3 days of incubation and then closed with a teflon lined cap. Loss of moisture from the soil, determined by weight loss of the bottles, was corrected for by addition of sterilized dH_2O. The remaining replicate bottles (without DW or ^{14}C-PCP) were immediately hermetically closed with a teflon lined cap.

Table 6.2. Inoculants and carbon sources introduced to the soils.

A. Dist.Waste[a]	Inoculum[b]	PCP-conc. (mg/kg d.wt.)
-	no inoculation	630
-	no inoculation	30
+	no inoculation	630
+	no inoculation	30
-	*R. rhodochrous*	630
-	*R. rhodochrous*	30
+	*R. rhodochrous*	630
+	*R. rhodochrous*	30
-	*R. chlorophenolicus*	630
-	*R. chlorophenolicus*	30
+	*R. chlorophenolicus*	630
+	*R. chlorophenolicus*	30
-	*R. rhod. + R. chloroph.*	630
-	*R. rhod. + R. chloroph.*	30
+	*R. rhod. + R. chloroph.*	630
+	*R. rhod. + R. chloroph.*	30

B. Inoculum	Inoculum size[c] (cells/g d.wt.)	PCP-conc. (mg/kg d.wt.)
R. chlorophenolicus	0	630
R. chlorophenolicus	0	30
R. chlorophenolicus	5×10^2	630
R. chlorophenolicus	5×10^2	30
R. chlorophenolicus	8×10^4	630
R. chlorophenolicus	8×10^4	30
R. chlorophenolicus	1×10^8	630
R. chlorophenolicus	1×10^8	30

[a] +, present; -, absent; added as 0.5 g nonsterile powder per 50 g soil (w.wt.)

[b] Inoculum = 1×10^8 cells/g moist soil (microscopic count)

[c] Determined by viable count

The bottles were incubated static in the dark at 22 ±2°C, the production of $^{14}CO_2$ was measured in duplicate by adding 4 mL of liquid scintillator to 0.25 mL of the 1 M NaOH collected from the glass cups, and analyzed in a LKB Wallac 1215 Rackbeta liquid scintillation counter (LKB Stockholm, Sweden). Representative samples were checked for ^{14}C-labelled products other than CO_2 as follows: ca. 100 mg of Na_2CO_3 was added, after which the samples were acidified to drive out all CO_2. Then the samples were neutralized with 10 M NaOH, measured for radioactivity and compared with a blank.

Cell counts. The amount of PCP-mineralizing bacteria in the soils was determined using a radioisotopic MPN-technique. 5 g of soil were shaken with 45 mL of minimal salts medium (33), and serially diluted in a five-fold, three-replicate dilution series. Replication dilutions were supplemented with 3000 dpm of ^{14}C-labelled PCP and nonlabelled PCP to a concentration of 4 ppm. Degradation was interpreted as positive if a minimum of 10% of the added radioactivity was recovered as $^{14}CO_2$ after 3 weeks of incubation at 28°C. Final MPN was calculated as described in (24). Cell densities of the PU-inoculants were estimated by shaking 1 g of PU in 40 mL minimal salts medium on a mechanical shaker for 20 min. The cells released in the medium were counted using a Bürker-Türk counting chamber or by viable count on agarized DSM-65.

Chlorophenol and chloroanisol extraction and analysis. Two internal standards were added to each sample, 2,4,6-tribromophenol to a concentration of 500 mg/kg or 20 mg/kg d.wt. and 2,4,6-tribromoanisol to a concentration of 5 mg/kg d.wt. Acetone (100 mL) and 6 M HCl (1 mL) were then added (the pH of the slurry ranged from 1 to 2), sonicated for 10 min (Bransonic 32 bath sonicator) and shaken for 20 minutes. After the soil had settled, 20 mL of the supernatant was transferred to a 100 mL flask with a teflon lined cap, and made alkaline (pH 9-11) with a 40% KOH solution and 20 mL of an acetylation buffer (91 mL 0.1 M aqueous NaOH + 500 mL 0.05 M aqueous $NaHCO_3$; pH 9.9). One mL of acetic anhydride was added and the flask shaken vigorously and left to stand for 12 hours. The mixture was then extracted with 5 mL of pentane, whereafter it was cleared from debris by centrifugation at 1000 rpm for 10 min.

Pentachlorophenol was quantified using a Hewlett-Packard 5890A gas chromatograph equipped with a Nordibond SE-30 fused silica capillary column (25 m x 0.2 mm internal diameter, 0.25 µm film) and a ^{63}Ni electron capture detector. Di-, tri- and tetrachlorophenols and -chloroanisoles were identified and quantified by using a GC-MS analysis in SIM mode. As PCA and 2,4,6-tribromophenol have virtually the same retention time, in case of extensive O-methylation of PCP to PCA, the GC-MS SIM method was also used to correct the PCP-concentrations found by GC-ECD. Chlorocatechols and -guaiacols (2-methoxyphenols) and other possible metabolites were searched for using GC-MS scan mode as described in

Häggblom et al. (17). The following temperature program was used: 1 min at 50°C, with a rate of 10°C/min up to 260°C. Chlorophenol and chloroanisol concentrations were calculated using the response factors of authentic model compounds and corrected for recovery of the internal standards.

EOX (extractable organic halogen) measurements in soil. Fifty g of fresh soil was sonicated in a 250 mL glass stoppered conical flask with 100 mL of acetone for 10 min in a bath sonicator and then shaken for 10 minutes on a gyrotory shaker at 270 rpm. Hexane (100 mL) was then added and the flasks were shaken again for 10 minutes. One hundred mL of the extract was transferred into a 500 mL separation funnel, 0.5 mL of 5 M aqueous HNO_3 was added, and the mixture was washed twice with 200 mL of dH_2O. A 2 mL amount of the hexane phase was evaporated under a N_2 stream and the residue redissolved in 500 μL of hexane plus 50 μL of hexadecane. The total halogen content of this extract was measured with the EOX-mode of a microcoulometer analyzer (Euroglas B.V., Delft, The Netherlands) (4). The system was calibrated with a standard solution of PCP in hexane.

To determine the PCP-Cl and PCA-Cl, 0.5 mL of the EOX extract was evaporated under a stream of N_2, the residue redissolved in 1 mL of acetone and internal standards were added. Then 2 mL of acetylation buffer and 0.1 mL of acetic anhydride were added, whereafter the mixture was extracted with 0.5 mL of pentane. PCP and PCA in the extract were quantified as described above.

RESULTS

Mineralization of ^{14}C-U-PCP. Figures 6.1 and 6.2 show the results of mineralization of ^{14}C-U-PCP at two concentrations in natural sandy loam and peaty soil, collected from pristine forest area and unexposed for the past 40 years to any known pollutant apart from air pollution. The soils contained no indigenous PCP-mineralizing bacteria at day zero (< 10 cells/g soil). Evaporated ^{14}C-labelled products other than CO_2 trapped in the NaOH solution were less than 1% of the $^{14}CO_2$ found in the samples. In the peaty soils without inoculum, approximately 6% was mineralized into CO_2 at the PCP-concentration of 630 mg/kg d.wt. PCP and 13% at the PCP-concentration of 30 mg PCP/kg d.wt. within 4 months (Figure 6.1). In the sandy soils, less than 5% of the ^{14}C-PCP was mineralized in 4 months time (Figure 6.2). Except for the peaty soil at input concentration of 30 mg/kg PCP, these figures are only slightly in excess to the radiochemical purity of the ^{14}C-U-PCP used (96%) and considering also the experimental error (1-2%) cannot be interpreted as proof of mineralization. These figures show also that there was no clear adaptation of the natural soil microflora to mineralize PCP within the time of 4 months.

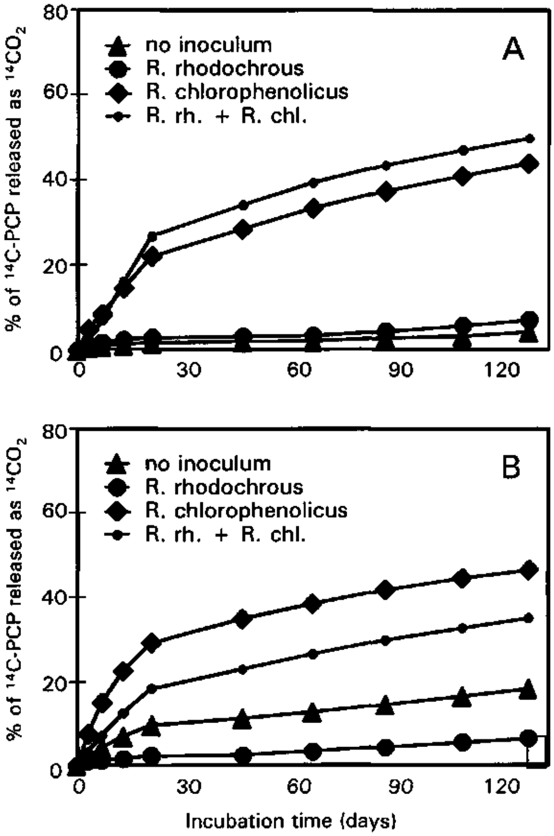

Figure 6.1. Mineralization of ^{14}C-PCP in the peat soil with different inocula. The input concentration of PCP is in (A): 630 mg/kg soil (d.wt.) and in (B): 30 mg/kg soil (d.wt.). Inoculum size: 1 x 10^8 cells/g moist soil.

Inoculation of polychlorophenol degrader *Rhodococcus chlorophenolicus* PCP-1 into the same soils (ca. 10^8 cells/g soil) resulted in an immediate onset of the mineralization of ^{14}C-PCP. The strong enhancement of the mineralization remained visible over the period of 4 months. The percentage of mineralization in soils with an input concentration of 630 mg PCP/kg d.wt. was lower than in soils with 30 mg PCP/kg d.wt. However, the absolute amount mineralized in 4 months time in the soils with an input of 630 mg/kg of PCP was ten-fold higher (150 to 250 mg of PCP mineralized per kg dry soil) than in soil with 30 mg of PCP/kg (13 to 18 mg/kg mineralized). This shows that the activity of the degrading bacteria was not inhibited by a concentration of 630 mg PCP/kg d.wt.

Simultaneous introduction of an organism competing for the same substrate (PCP) (18), i.e. polychlorophenol O-methylating *Rhodococcus rhodochrous*, had little effect on the rate of mineralization by *R. chlorophenolicus* (Figures 6.1 and 6.2).

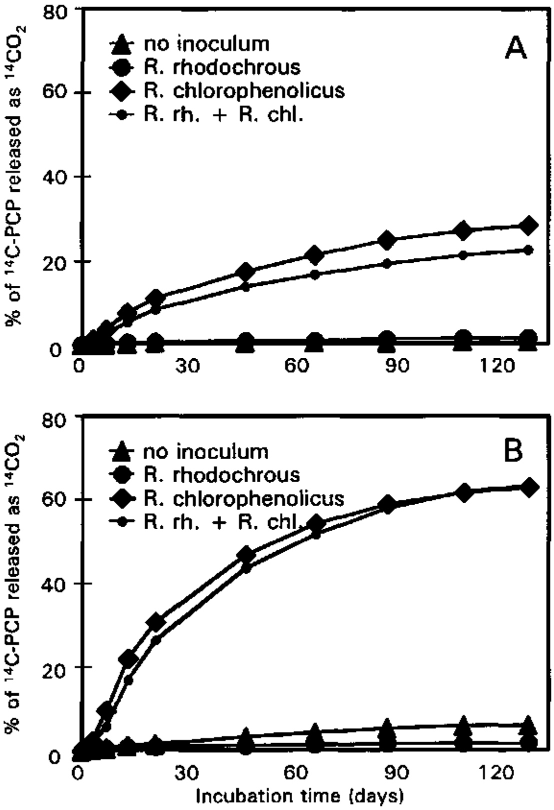

Figure 6.2. Mineralization of ^{14}C-PCP in the sandy loam soil with different inocula. The input concentration of PCP is in (A): 630 mg/kg soil (d.wt.) and in (B): 30 mg/kg soil (d.wt.). Inoculum size: 1 x 10^8 cells/g moist soil.

Removal of PCP from soil as measured by GC. Concentrations of pentachlorophenol in bottles replicating the conditions used in ^{14}C-U-PCP mineralization experiments (Figures 6.1 and 6.2). Figure 6.3 shows that the concentration of PCP decreased in peaty soil over time. Up to 20% of 630 mg PCP/kg d.s and 50% of 30 mg of PCP/kg d.wt. disappeared in the samples that were not inoculated (Figure 6.3). In the sandy soil, no such spontaneous decrease was found (Figure 6.4). No mono-, di-, tri- or tetrachlorophenols were detected at concentrations higher than 0.5% of the input concentration of PCP, so these were not significant products of PCP metabolism in the soils.

Figures 6.3 and 6.4 show further that in the presence of *R. chlorophenolicus* the concentration of PCP in the soils decreased dramatically. It can be seen that the amount of PCP that had disappeared in 129 days from the soils with an input 630 mg PCP/kg d.wt., corresponded to the amounts of ^{14}C-PCP mineralized (Figures 6.1 and 6.2). However, of the input of 30 mg PCP/kg d.wt. more PCP disappeared than the mineralization data in Figures 6.1 and 6.2 give reason to expect.

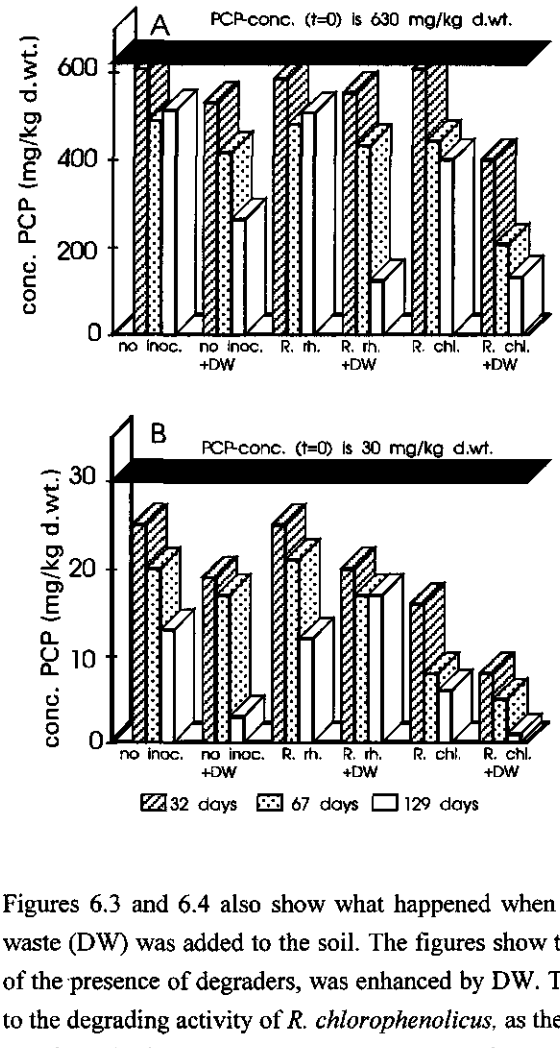

Figure 6.3. Concentrations of PCP found in the peat soil after application of different inocula and an additional carbon source (DW). The black horizontal bar indicates the soil concentration of PCP on day 0 which was: (A): 630 mg/kg soil (d.wt.) and (B): 30 mg/kg soil (d.wt.). R.rh. = *R. rhodochrous*; R.chl. = *R. chlorophenolicus*. Inoculum size: 1 x 10^8 cells/gram moist soil.

Figures 6.3 and 6.4 also show what happened when an additional carbon source, distillery waste (DW) was added to the soil. The figures show that disappearance of PCP, independent of the presence of degraders, was enhanced by DW. This disappearance of PCP was additive to the degrading activity of *R. chlorophenolicus*, as the removal of PCP in the inoculated soils was faster in the presence than in the absence of DW.

O-methylation and other transformations of PCP. It is known that bacteria and fungi capable of O-methylating chlorophenols are ubiquitous in the environment. We therefore analyzed the soils for the possible presence of mono-, di-, tri-, tetra- and pentachloroanisoles (methoxybenzenes). The results of GC-MS analyses revealed that except for pentachloroanisole no other of the above named anisoles were detected (detection limit is 0.1 mg/kg d.wt.). The results show for the sandy soil at input concentration of 30 mg PCP/kg d.wt., when it was not inoculated with *R. chlorophenolicus* and DW was added, O-methylation could explain almost stoichiometrically the disappearance of PCP after 32 days. The thus formed PCA disappeared after longer incubation times. In the peaty soil at input concentration of 30 mg PCP/kg d.wt., O-methylation enhanced by the addition of DW was observed (data not shown). Experiments using XAD-2 columns and a headspace air refreshment system with similarly prepared flasks showed that there was no significant volatilization of chlorophenols or chloroanisoles during the experiment (data not shown).

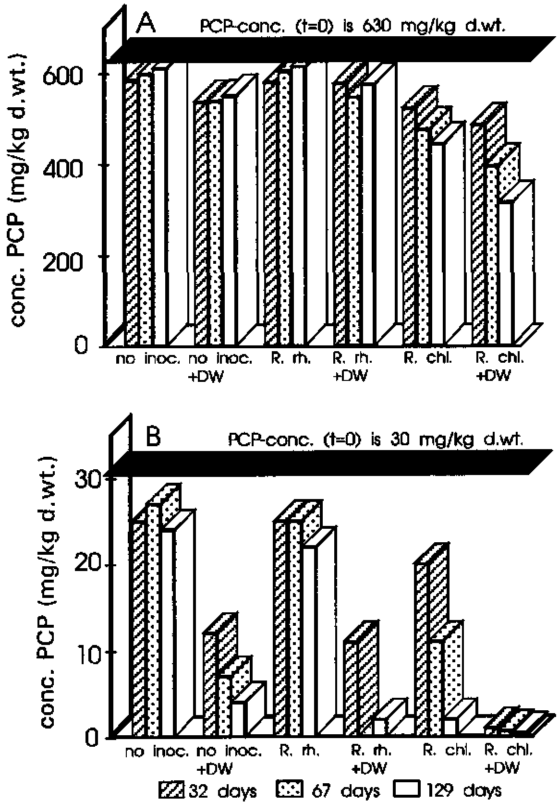

Figure 6.4. Concentrations of PCP found in the sandy loam soil after application of different inocula and an additional carbon source (DW). The black horizontal bar indicates the soil concentration of PCP on day 0 which was: (A): 630 mg/kg soil (d.wt.) and (B): 30 mg/kg soil (d.wt.). Abbreviations and inocula as in Figure 6.3.

In soils inoculated with *R. chlorophenolicus* the amount of PCA was small or below the detection limit of 0.1 mg/kg d.wt. At an initial concentration of 630 ppm PCP, the O-methylation was less than 2% in both soils, probably indicating toxicity of PCP to O-methylating microorganisms. The external introduction of methylaters (10^8 *R. rhodochrous* cells/g) did not enhance the formation of PCA (data not shown). This shows that the natural microflora of the soils included the organisms responsible of the observed O-methylation of PCP.

To trace the fate of the disappeared PCP, the amount of extractable organic halogen (EOX) was measured at time zero and at the end of the experiment in the soils with an input of 30 mg PCP/kg d.wt. and DW. Table 6.3 shows that the amount of organic halogen in both soils had decreased from the input concentration of 23 mg of organic Cl to 8.9 mg Cl/kg d.wt. in the peat soil and 1.5 mg Cl/kg in the sandy soil within 130 days. The inoculation of *R. chlorophenolicus* resulted in disappearance of EOX until values of 2.3 mg Cl/kg d.wt. in the peaty soil and 0.4 mg/kg d.wt. in the sandy soil within the same time, which means mineralization of organically bound chlorine (PCP) to 90% and 98%, respectively. Table 6.3 also shows that the amount of EOX measured in the soils did not completely correspond to the amount of PCP-Cl plus PCA-Cl analyzed in the same extract. The discrepancy between the EOX values and the PCP-Cl + PCA-Cl values expressed in mg Cl/kg was not large but in all cases the EOX value was the highest. This and the fact that this difference was neglegible on day 0 indicate that a small amount of PCP has been transformed into compounds not identified by gas chromatography.

Table 6.3. Concentrations of extractable organic halogen (EOX) measured after 130 days of incubation in soils with 30 mg PCP/kg soil (d.w.), corresponding to an EOX value of 23 mg Cl/kg soil (d.w.) and with distillery waste (0.5 g/50 g moist soil).

		Chlorine conc.(mg/kg d.wt.) measured as:		
Soil type	Inoculum	EOX:	PCP-Cl	PCA-Cl
Peat	day 0	23	23	0
	no inoculum	8.9	4.9	1.2
	R. rhodochrous	5.2	2.5	0.5
	R. chlorophenolicus	2.3	0.7	0.1
	R. rhod.+ R. chlor.	2	0.6	0.2
Sand	day 0	23	23	0
	no inoculum	1.5	0.9	0.4
	R. rhodochrous	1.3	0.7	0.4
	R. chlorophenolicus	0.4	0.1	<0.06
	R. rhod.+ R. chlor.	n.d.[a]	n.d.	n.d.

[a] n.d. = not determined

The effect of inoculum size introduced to soil. The results described above showed that mineralization (degradation) of PCP can be induced in natural soil by an external inoculum. A large inoculum was used in experiments represented in Figures 6.1 to 6.4. Figures 6.5 and 6.6 show results obtained with smaller inocula of *R. chlorophenolicus*. It can be noticed that in peaty soils an inoculum of 5 x 10^2 cells/g moist soil made no difference compared to the uninoculated controls within 70 days. 8 x 10^4 cells/g moist soil and higher introduced to soil with 630 mg PCP/kg d.wt. improved mineralization with 15%. At the PCP concentration of 30 mg/kg d.wt., only the largest inoculum (1 x 10^8 cells/g) resulted in significant (10%) increase of mineralization, compared to the control in 70 days (Figure 6.5B). In sandy soil, however, already the inoculum of 5 x 10^2 degrading cells/g induced the mineralization at both 30 and 630 mg PCP/kg d.wt. input concentrations. When introduced to soil with 30 ppm of PCP the inocula of 5 x 10^2 and 8 x 10^4 cells/g soil were similarly effective, while the largest inoculum (10^8 cells/g) doubled the mineralization rate. Inoculated in sandy soil with 630 mg PCP/kg d.wt. the inocula of ca. 10^5 and 10^8 cells/g soil enhanced mineralization equally (Figure 6.6B).

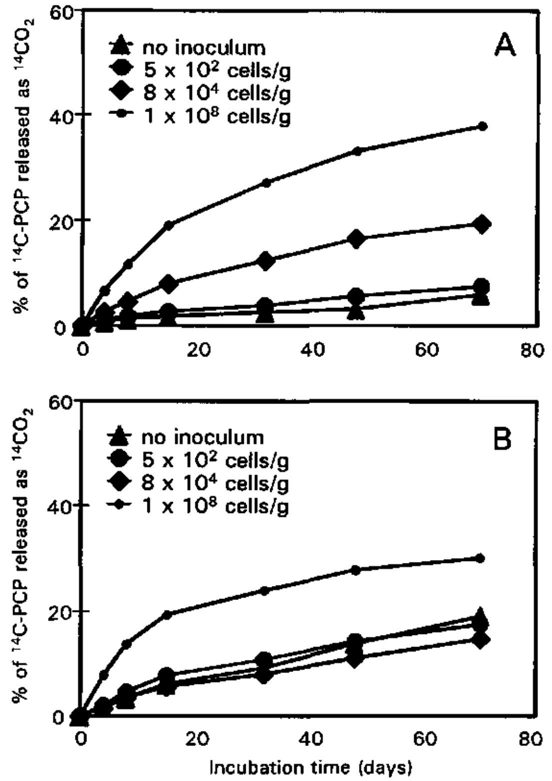

Figure 6.5. Mineralization of ^{14}C-PCP in the peat soil induced by different amounts of *R. chlorophenolicus* PCP-1. The input concentrations of PCP were: (A): 630 mg/kg soil (d.wt.) and (B): 30 mg/kg soil (d.wt.). The inoculum sizes are indicated as the number of *R. chlorophenolicus* cells per g soil (w.wt.)

DISCUSSION

The presence of heavy pollution (100 to 10000 mg/kg soil) by mainly 2,4,6-tri-, 2,3,4,6-tetra- and pentachlorophenol (PCP) in the subsurface, even at sites that were abandoned for 10 or more years ago (22, 23, 39), shows that indigenous soil microbes were not able to degrade these chemicals and that microbial adaptation has not taken place to any useful extent during the past 50 years. Similar situations have been reported in North America (25).

We tested soils that were polluted for decades, and found bacteria capable of mineralizing ^{14}C-PCP up to population titers of 10^4/g of soil, clearly above the background level of agricultural soil (10^2/g soil) or pristine soil (<10/g soil) [this study; Salkinoja-Salonen, 1989 #1822; Valo, 1986 #218]. It thus seems that even if chlorophenol mineralizing bacteria were present, there numbers were too low or the environmental conditions were not suitable for degradation to occur, since the pollution persists.

Because of the high concentrations of the toxic chemicals in soil, abandoned require restoration before the site is safe for other uses. We reported earlier on the use of microbes to bioremediate such soils and groundwater, both in the laboratory and in the field (29, 38, 40). The *Rhodococcus chlorophenolicus* PCP-1 (type strain) (5, 6, 7), other strains of the same species, and *Mycobacterium* sp. (20) proved to be effective in mineralizing many different chlorophenols both in axenic culture and when inoculated in polluted soil containing 30 to 9000 mg of polychlorinated phenols per kg soil (28, 29).

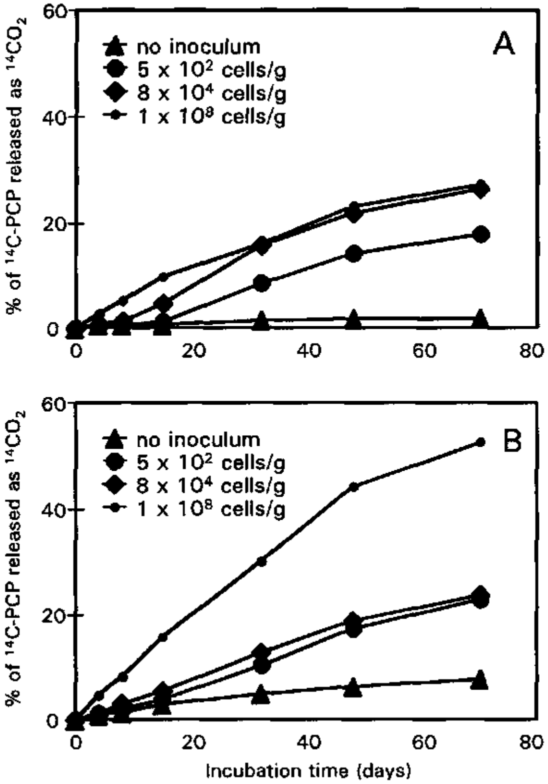

Figure 6.6. Mineralization of ^{14}C-PCP in the sandy loam soil induced by different amounts of *R. chlorophenolicus* PCP-1. The input concentrations of PCP were: (A): 630 mg/kg soil (d.wt.) and in (B): 30 mg/kg soil (d.wt.). Inocula as in Figure 6.5.

The present results show that *R. chlorophenolicus* effectively induced mineralization of pentachlorophenol in pristine soils, whereas prior to introduction of the inoculant, no PCP mineralizing activity was detected (<10 mineralizing bacteria/g soil). The results show further that the mineralization continued for at least 4 months after a single inoculation. The critical inoculum size was lower in sandy soil than in peaty soil. This may be explained by the presence of a competing autochtonous soil population, which is more numerous in peaty soil.

PCP was converted into CO_2 more efficiently at high soil concentrations of PCP than at low concentrations (Figures 6.1-6.4). This may reflect toxicity of PCP at high concentrations toward fungi and bacteria that transform PCP rather than mineralize it. Therefore, when the transforming reactions were not competing for the chlorophenol substrate (>30 mg PCP/kg), efficiency of mineralization of PCP into CO_2 was improved. We measured the number of degrading bacteria by the MPN-method and found that their numbers remained virtually unchanged over 7 months after inoculation (13). We found earlier that these introduced PCP mineralizing microbes persisted under field conditions in virtually the same numbers (10^6/g soil) over 4 years, even after the concentration of chlorophenols in the remediated soil had dropped below 1 mg/kg (29, 40). Eleven months after inoculation, we reisolated from one soil a degrader bacterium which was morphologically and physiologically similar to the inoculated R. chlorophenolicus (A. McBain & M. Salkinoja-Salonen, unpublished results). We are presently working on an immunoprobe method to study this question in more detail.

It has also been shown that *Mycobacterium* (21), *Arthrobacter* (16), and *Corynebacterium* (1, 2), like *Rhodococcus* (this Study), activated biodegradation after being introduced into soil and survived dessication and starvation with little loss of viability.

Our results show that the mineralization averaged 16 pg PCP per day and per added *R. chlorophenolicus* cell over a 4-month period (Figures 6.5 and 6.6). The rate was faster in heavily polluted soil (600 mg/kg) than in the lightly polluted soil (30 mg/kg). This is approximately 1000-fold higher than the rate Heitkamp and Cerniglia (21) reported for a *Mycobacterium*-induced mineralization of pyrene in microcosms. Crawford and Mohn (15) described results from which a mineralization rate of 0.2 pg of PCP per added *Flavobacterium* cell per day can be calculated during the first 10 days after inoculation. However, they found an equal rate of mineralization (40% of 100 mg/kg soil) in both inoculated and uninoculated soils after 30 days.

The results also show that in some cases PCP disappeared but was not accounted for by mineralization (^{14}C-CO_2 evolution). This was observed especially at low (30 mg/kg) pollutant concentration. The major part of the PCP that had disappeared from the gas chromatogram, reemerged in the first instance as another peak, pentachloroanisol (PCA), which was later partially transformed into some other form of solvent extractable (hexane) organically bound chlorine (EOX).

Fungal enzymes, like ligninases, peroxidases, or laccases have been described to oxidize also phenols into dimeric or polymeric material (12). Such conversion may occur in soil. The environmental safety of the potential condensation products has not been studied. The fact that at least fungal laccase has been shown to convert 2,4,5-trichlorophenol into 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (34) stresses the need for careful product analysis in cases where the xenobiotic chemical is not mineralized during the process of bioremediation.

Chlorinated methoxybenzenes are environmentally undesirable, as they are highly lipophilic and thus have the potential to bioaccumulate (18). PCDDs are highly toxic and lipophilic. The biotransformation reaction that channeled considerable portions of PCP into PCA was observed in the uniculated controls when DW was added and also in those inoculated with the

O-methylating *R. rhodochrous*, but only in trace amounts (<3mg/kg) when PCP mineralizing bacteria (*R. chlorophenolicus*) were present. Such a 'side reaction' was less significant (<2%) in the soils with high PCP concentration (600 mg/kg), probably as a result of toxic inhibition of the microbes involved.

The degradation pathway of PCP by *R. chlorophenolicus* PCP-1 is known in detail (7, 8, 9). This bacterium does not methylate chlorophenols, although many other *Rhodococci* do (18, 19). According to the present results and literature reports (26), O-methylating microbes are also found in natural soil. These observations indicate potential danger in uncontrolled dilution of polluted soils with nonpolluted or differently polluted soils (e.g. after transport to landfills) or in amendment of polluted soils with organic matter with the aim of 'stimulating' biological activity, as is widely practiced in commercial bioremediation based on the activity of indigenous microflora.

In conclusion, this example of microbiological clean-up of pentachlorophenol-contaminated soil has shown that bioremediation can effectively be accomplished using an external inoculum. Use of defined inocula is safe because the intermediate and final degradation products are known. Stimulation of natural soil microflora by addition of nutrients, fertilizer, oxygen, etc. to react with the pollutant may lead to unpredictable products [Alexander, 1988 #1831; this study].

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REFERENCES

1. **Acea, M. J. and M. Alexander.** 1988. Growth and survival of bacteria introduced into carbon-amended soil. Soil Biol. Biochem. **20**:703-709.
2. **Acea, M. J., C. R. Moore and M. Alexander.** 1988. Survival and growth of bacteria introduced into soil. Soil Biol. Biochem. **20(4)**:509-515.
3. **Alexander, M.** 1984. Ecological constraints of genetic engineering, p. 151-168. *In* G. Omenn and A. Hollaender (ed.), Genetic control of environmental pollutants, Plenum Press, New York.
4. **Anonymous** (1984). German standard method for the determination of extractable organic halogen (EOX) No. DIN 38409 (In German)).
5. **Apajalahti, J. H. A., P. Kärpänoja and M. S. Salkinoja-Salonen.** 1986. *Rhodococcus chlorophenolicus* sp. nov., a chlorophenol mineralizing actinomycete. Int. J. Syst. Bacteriol. **36**:246-251.

6. **Apajalahti, J. H. A. and M. S. Salkinoja-Salonen.** 1984. Absorption of pentachlorophenol (PCP) by bark chips and its role in microbial PCP degradation. Microb. Ecol. **10**:359-367.
7. **Apajalahti, J. H. A. and M. S. Salkinoja-Salonen.** 1986. Degradation of polychlorinated phenols by *Rhodococcus chlorophenolicus*. Appl. Microbiol. Biotechnol. **25**:62-67.
8. **Apajalahti, J. H. A. and M. S. Salkinoja-Salonen.** 1987. Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced *Rhodococcus chlorophenolicus*. J. Bacteriol. **169**:5125-5130.
9. **Apajalahti, J. H. A. and M. S. Salkinoja-Salonen.** 1987. Dechlorination and *para*-hydroxylation of polychlorinated phenols by *Rhodococcus chlorophenolicus*. J. Bacteriol. **169**:675-681.
10. **Atlas, R. M.** 1988. Biodegradation of hydrocarbons in the environment, p. 211-222. *In* G. Omenn (ed.), Environmental biotechnology, Plenum Press, New York.
11. **Ball, D. F.** 1964. Loss-on-ignition as an estimate of organic matter and organic carbon in noncalcareous soils. J. Soil Sci. **15**:84-92.
12. **Bollag, J.-M. and S.-Y. Liu.** 1985. Copolymerization of halogenated phenols and syringic acid. Pestic. Biochem. Physiol. **23**:261-272.
13. **Briglia, M., E.-L. Nurmiaho-Lassila, G. Vallini and M. S. Salkinoja-Salonen.** 1990. The survival of the pentachlorophenol-degrading *Rhodococcus chlorophenolicus* PCP-1 and *Flavobacterium* sp. in natural soil. Biodegradation **1**:273-281.
14. **Casida, L. E.** 1992. Competitive Ability and Survival in Soil of *Pseudomonas* Strain 679-2, a Dominant, Nonobligate Bacterial Predator of Bacteria. Appl. Environ. Microbiol. **58**:32-37.
15. **Crawford, R. L. and W. W. Mohn.** 1985. Microbiological removal of pentachlorophenol from soil using a *Flavobacterium*. Enzyme Microbiol. Technol. **7**:617-620.
16. **Edgehill, R. U. and R. K. Finn.** 1983. Microbial treatment of soil to remove pentachlorophenol. Appl. Environ. Microbiol. **45**:1122-1125.
17. **Häggblom, M. M., J. H. A. Apajalahti and M. S. Salkinoja-Salonen.** 1988. O-methylation of chlorinated *para*-hydroquinones by *Rhodococcus chlorophenolicus*. Appl. Environ. Microbiol. **54**:1818-1824.
18. **Häggblom, M. M., D. Janke, P. J. M. Middeldorp and M. S. Salkinoja-Salonen.** 1989. O-methylation of chlorinated phenols in the genus *Rhodococcus*. Arch. Microbiol. **152**:6-9.
19. **Häggblom, M. M., D. Janke and M. S. Salkinoja-Salonen.** 1989. Transformation of chlorinated phenolic compounds in the genus *Rhodococcus*. Microb. Ecol. **18**:147-159.
20. **Häggblom, M. M., L. J. Nohynek and M. S. Salkinoja-Salonen.** 1988. Degradation and O-methylation of chlorinated phenolic compounds by and *Mycobacterium* strains. Appl. Environ. Microbiol. **54**:3043-3052.
21. **Heitkamp, M. A. and C. E. Cerniglia.** 1989. Polycyclic aromatic hydrocarbon degradation by a Mycobacterium sp. in microcosms containing sediment and water from a pristine ecosystem. Appl. Environ. Microbiol. **55(8)**:1968-1973.
22. **Kitunen, V. H., R. J. Valo and M. S. Salkinoja-Salonen.** 1985. Analysis of chlorinated phenols, phenoxyphenols and dibenzofurans around wood preserving facilities. Intern. J. Environ. Anal. Chem. **20**:13-28.
23. **Kitunen, V. H., R. J. Valo and M. S. Salkinoja-Salonen.** 1987. Contamination of soil around wood-preserving facilities by polychlorinated aromatic compounds. Environ. Sci. Technol. **21**:96-101.
24. **Koch, A. L.** 1981. Growth measurement, p. 179-192. *In* P. Gerhard , R. Murray , R. Costilow , E. Nester , W. Wood , N. Krieg and G. B. Philips (ed.), Manual of methods for general microbiology, American Society for Microbiology, Washington DC.
25. **Krahn, P. K., J. A. Shrimpton and R. D. Glue** (1987). Assessment of storm water related chlorophenol releases from wood protection facilities in British Columbia No. 87-14). Environment Canada Regional Program Report.
26. **Murthy, N. B. K., D. D. Kaufman and G. F. Fries.** 1979. Degradation of PCP in aerobic and anaerobic soil. J. Environ. Sci. Health. Part B **14**:1-14.
27. **Nicholas, R. B.** 1987. Biotechnology in hazardous waste disposal: an unfulfilled promise. ASM News **53**:138-142.
28. **Salkinoja-Salonen, M. S., J. H. A. Apajalahti, V. H. Kitunen and M. M. Häggblom.** 1987. Bacterial and abiotic degradation of chlorinated phenols and related compounds occurring in forest industry wastes, p. 75-80. *In* Water pollution report 6: behaviour of organic micropollutants in biological waste water treatment, Published by the Commision of the European Communities,
29. **Salkinoja-Salonen, M. S., P. J. M. Middeldorp, M. Briglia, R. J. Valo, M. M. Häggblom, A. McBain and J. H. A. Apajalahti.** 1989. Clean-up of old industrial sites, p. 347-365. *In* D. Kamely , A. Chakrabarty and G. Omenn (ed.), Advances in applied biotechnology, Gulf Publishing Company, Houston.

30. **Schippers, B., A. W. Bakker and P. A. H. M. Bakker.** 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Ann. Rev. Phytopathol. **25**:339-358.

31. **Schroth, M. N. and J. G. Hancock.** 1981. Selected topics in biological control. Ann. Rev. Microbiol. **35**:453-476.

32. **Shapiro, J. A., D. J. Owen, M. Kok and G. Eggink.** 1984. *Pseudomonas* hydrocarbon oxidation, p. 151-168. *In* G. Omenn and A. Hollaender (ed.), Genetic control of environmental pollutants, Plenum Press, New York.

33. **Sundman, V.** 1964. A description of some ligninolytic soil bacteria and their ability to oxidize simple phenolic compounds. J. Gen. Microbiol. **36**:171-183.

34. **Svenson, A., L.-O. Kjeller and C. Rappe.** 1989. Enzyme-mediated formation of 2,3,7,8-substituted chlorinated dibenzodioxins and dibenzofurans. Environ. Sci. Technol. **23**:900-902.

35. **Thomas, J. M. and C. H. Ward.** 1989. *In situ* biorestoration of organic contaminants in the subsurface. Environ. Sci. Technol. **23**:760-766.

36. **Unterman, R., D. L. Bedard, M. J. Brennan, L. H. Bopp, F. J. Mondello, R. E. Brooks, D. P. Mobley, J. B. McDermott, C. C. Schwartz and D. K. Dietrich.** 1988. Biological approaches for polychlorinated biphenyl degradation, p. 381-384. *In* G. Omenn (ed.), Environmental biotechnology, Plenum Press, New York.

37. **Valo, R. J., J. H. A. Apajalahti and M. S. Salkinoja-Salonen.** 1985. Studies on the physiology of microbial degradation of pentachlorophenol. Appl. Microbiol. Biotechnol. **21**:313-319.

38. **Valo, R. J., M. M. Häggblom and M. S. Salkinoja-Salonen.** 1990. Bioremediation of chlorophenol containing simulated groundwater by immobilized bacteria. Water Res. **24**:253-258.

39. **Valo, R. J., V. H. Kitunen, M. S. Salkinoja-Salonen and S. Räisänen.** 1984. Chlorinated phenols as contaminants of soil and water in the vicinity of two Finnish sawmills. Chemosphere **13**:835-844.

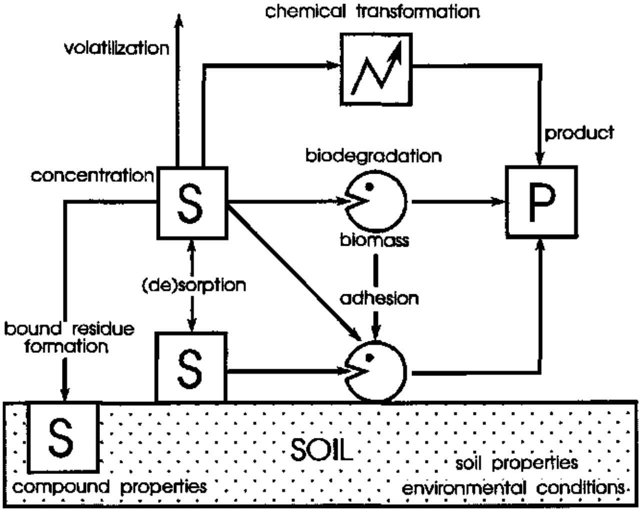
40. **Valo, R. J. and M. S. Salkinoja-Salonen.** 1986. Bioreclamation of chlorophenol-contaminated soil by composting. Appl. Microbiol. Biotechnol. **25**:68-75.

41. **Vogel, A. I.** 1956. Practical organic chemistry, 3rd enlarged ed. Longman, London.

CHAPTER 7

Summary and discussion

Organic pollutants can be transformed, both in unsaturated and saturated areas of the soil, by means of biologically mediated reactions. The potential of soil microorganisms to clean up polluted soils is enormous. However, soil systems are highly heterogeneous with respect to the spatial distribution of substrates, nutrients and microorganisms, and also with respect to various phases and interfaces (e.g. water, air, minerals, organic matter). To come to the development of appropriate soil bioremediation techniques, comprehensive knowledge is required about the biological and physico-chemical processes and parameters that determine the rate, the nature and the degree of degradation of organic compounds within a certain time scale in soil. The most important processes and parameters that influence the behavior of pollutants in soil are shown in Figure 7.1.



R. v/d Berg, RIVM

Figure 7.1. Schematic overview of the most important processes determining the fate and behaviour of organic pollutants in soil.

Several aspects, which may arise when assessing the application of microorganisms in soil remediation techniques, have been studied in this thesis.

A major part of this thesis concerns the environmental conditions, that are essential for biodegradation. The biodegradability of a pollutant depends not only on the molecular structure of this pollutant, but also on the occurrence of the appropriate environmental conditions. The biodegradation of many environmental pollutants has been studied primarily under aerobic conditions, since these conditions are prevailing in polluted locations and easy to maintain. However, the anaerobic degradation of pollutants forms a relatively new potential of interesting reactions, which may be used for bioremediation purposes.

For instance, β-hexachlorocyclohexane (β-HCH), a waste product from the production of the pesticide lindane, is considered extremely recalcitrant under aerobic conditions (3, 21, 22).

This may be due to the spatial arrangement of the chlorine atoms in this HCH isomer (11). Disappearance of β-HCH under anaerobic conditions has been reported, but product identifications and mass balances were lacking in these studies (32, 38, 60). In Chapter 2, the biodegradation of β-HCH under anaerobic conditions is described. It is shown that β-HCH can be dechlorinated under methanogenic conditions. The observed dechlorination products of β-HCH, benzene and chlorobenzene, have individually been found for the anaerobic biodegradation of γ-HCH (5, 45), but they were never reported to be formed simultaneously from any HCH isomer by microorganisms.

The dechlorination products of β-HCH, benzene and chlorobenzene, accumulate under the conditions studied. The formation of these products, which are potentially toxic, introduces a new problem since chlorobenzene is recalcitrant under methanogenic conditions and anaerobic benzene degradation has been observed in only a few cases (24, 35, 36). However, many studies have shown that these compounds are degradable in the presence of molecular oxygen (28, 47, 50). Therefore a bioremediation process in which subsequently anaerobic and aerobic conditions are created would lead to a total mineralization of β-HCH.

Such a sequential change from anaerobic to aerobic conditions also applies to the decontamination of soils polluted with polychlorinated benzenes (CBs) and biphenyls (PCBs). These compounds, when substituted with five or more chlorine atoms, generally persist under aerobic conditions. However, anaerobic reductive dechlorination of these compounds has abundantly been shown. The products of this dechlorination process are important for several reasons. From a toxicological point of view, because *ortho*-chlorinated PCBs are considered 10-100 times less toxic than non *ortho*-chlorinated ones (coplanar PCBs) (53), but also from a biotechnological point of view, since the dechlorination products are the substrates in an eventually following aerobic treatment. Microbial reductive dechlorination of PCBs is mainly limited to *meta*- and *para*-dechlorination, and therefore reduces the toxicity of the PCBs. Dechlorination of CBs and PCBs also increases the water solubility and therefore the mobility of these compounds in the soil. This may be advantageous in the case of an *ex situ* bioremediation. However, since this process has been shown to occur in undisturbed polluted sediment (10) and will also occur in biological soil treatments *in situ*, one should prevent the pollutant to move away from the polluted site.

The dechlorination of CBs and PCBs is carried out by different groups of microorganisms, originating from different locations (8, 9, 26, 27, 31, 41, 44, 48, 49, 51). Chapter 3 describes the dechlorination of these compounds in a methanogenic consortium, enriched from a mixture of polluted sediments on 1,2,4-trichlorobenzene (TCB). CBs appear to be extensively dechlorinated by this consortium, with 1,2- and 1,3-dichlorobenzene (DCB) as well as chlorobenzene as the final products. This is in contrast with the performance of previously reported enrichments, which have been obtained mainly using hexa- and pentachlorobenzene as substrates for dechlorination (9, 26, 31). Such cultures show a lesser extent of dechlorination and produce 1,3,5-TCB or 1,3-TCB as final products, which are problematic compounds for an eventually following aerobic mineralization. Chapter 3 also shows that, whereas mono- through pentachlorobenzenes are dechlorinated within 2 days, hexachlorobenzene (HCB) and several PCBs are dechlorinated after a lag phase of about two weeks. Dechlorination of the latter compounds occurs via a different pathway, in which only chlorines with two adjacent chlorines are removed. Chapter 3 also shows that dechlorination of highly chlorinated benzenes can be directed to the formation of chlorobenzene as the only product by the addition of 2-bromoethanesulfonic acid (BrES), a methanogenic inhibitor. These observations show that different dechlorinating activities are present in the consortium. They are probably carried out by different physiological groups of microorganisms. In contrast, Chapter 4 describes a different methanogenic consortium which has the capacity to dechlorinate both CBs and PCBs with the same specificity toward chlorine substution pattern of these compounds. This and the fact that similar lag phases for the dechlorination of CBs and PCBs were observed, shows that the dechlorination of these compounds may be carried out by the same type of microorganisms.

Chapter 5 applies to the environmental conditions which are necessary for a fast dechlorination of PCBs. Anaerobic reductive dechlorination of PCBs is a slow process, in which lag periods of weeks to months are commonly observed before dechlorination starts (1, 27, 63, 68). Also the maintenance and subcultivation of PCB dechlorinating microbial consortia are extremely difficult. The optimal environmental conditions for the dechlorination process are not known. This is partly due to our lack of knowledge about the physiology of microbial reductive dehalogenation in general and about dechlorination of PCBs in particular. In Chapter 5, the lag phase of the reductive dechlorination of 2,3,4,5-tetrachlorobiphenyl (TeCBP), performed by the consortium described in Chapter 4, was reduced by means of different types of additions to the medium. Sterile Rhine sand appears to shorten the lag phase and to be essential for the maintenance of the consortium. A similar positive effect of sand was also observed in other dechlorinating consortia (Chapter 2 and 3 and ref. (20)) and for the degradation of toluene in a manganese reducing consortium (34).

The mechanism of the reduction of the lag phase by Rhine sand is not known. The Rhine sand that we added to our medium, may contain additional trace metals, necessary for dechlorination. Although the organic carbon content in the Rhine sand we used is very low (<0.05% w/w), another explanation may be that the organic matter served as a supply of growth factors, like vitamins, for the dechlorinating bacteria. Sorption of 2,3,4,5-TeCBP to the sand might also improve the contact between the PCBs and the dechlorinating cells, if these cells tend to settle out or grow on the sand surface (adhesion).

The addition of anaerobic granular sludge (AGS), either autoclaved or not, also reduced the lag phase before dechlorination of 2,3,4,5-TeCBP drastically. The decrease of the lag phase, achieved by the addition of AGS, may be caused by factors, originating from microorganisms in the AGS. However, the addition of the redox mediator vitamin B_{12}, which is abundantly present in AGS, had little effect on the lag phase. Reduction of the lag phase for PCB dechlorination was also observed by Abramowicz et al. (1) who added fluid thioglycolate medium with beef extract (FTMBE) to polluted Hudson River sediments. In fact, the use of undefined additions such as AGS and FTMBE illustrates how little is known about the mechanism of reductive dechlorination of PCBs. It is of practical importance that a relatively cheap and easily available material such as AGS also reduces the lag phase for dechlorination in a methanogenic enrichment, which is capable of dechlorinating the complex PCB mixture Aroclor 1260 (Chapter 5). It may be worth trying to introduce AGS into methanogenic river sediments polluted with PCBs, to stimulate *in situ* dechlorination.

Another aspect one should consider when taking biodegradation into practice is the availability of the pollutant for biodegradation. This is illustrated in Chapter 6, which deals with the biodegradation of pentachlorophenol (PCP) in different soil types by inoculated *Rhodococcus chlorophenolicus*. After an initially high mineralization rate of the PCP in the inoculated soils, this rate decreased during time and levelled off, leaving behind a residual amount of PCP. Such a non-degraded residue has been observed in many soil biodegradation studies and is referred to as a fraction that is not available for biotransformation, i.e. not bioavailable. Limited bioavailability is considered as one of the most important problems impeding the development of successful bioremediation techniques.

The rate at which microorganisms can degrade pollutants during bioremediation depends on the intrinsic metabolic activity of the cell, and on the transfer of the pollutant to the cell. Thus, bioavailability of a pollutant is determined by the rate of mass transfer, relative to the activity of the microbial cells. As bacteria generally degrade pollutants to concentrations which are much lower than the residual concentrations observed in soils (58, 59, 62), it appears that mass transfer is the limiting factor for further degradation. This was confirmed by Bosma et al. (19) who quantified bioavailability by modelling the effect of both mass transfer and the intrinsic activity of the microbial cells. Bioavailability of a pollutant is controlled by a number of physico-chemical processes, such as sorption, diffusion and dissolution (46, 56, 57). Especially in old polluted sites, part of the pollutants appears to be inaccessible to degrading bacteria. This was shown for a soil contaminated with chlorophenols for over 40 years, in which bioremediation through composting resulted in a residual concentration of about 40 mg/kg. No further degradation was observed when the soil was inoculated with PCP mineralizing bacteria. However, ^{14}C-labelled PCP which was freshly added to this soil led immediately to a complete mineralization of the added PCP (54). Similar results were reported for two soils contaminated with polycyclic aromatic hydrocarbons (67) and for the reductive dechlorination of hexachlorobenzene in sediment (9).

Rijnaarts et al. (52) showed that biodegradation rates of α-hexachlorocyclohexane in soil could be increased by breaking up the soil particles.

Such observations indicate a reduced bioavailability due to a so-called "ageing" of the pollutant in the soil. This may be caused by different processes. The first process is a slow diffusion of the pollutant into the deeper and smaller pores of the soil particles and absorption into organic matter (4, 25, 29, 30, 69) as visualized in Figure 7.2. The pollutant can only reach the degrading microorganisms - which are not small enough to penetrate into the deeper pores - by means of *diffusion*.

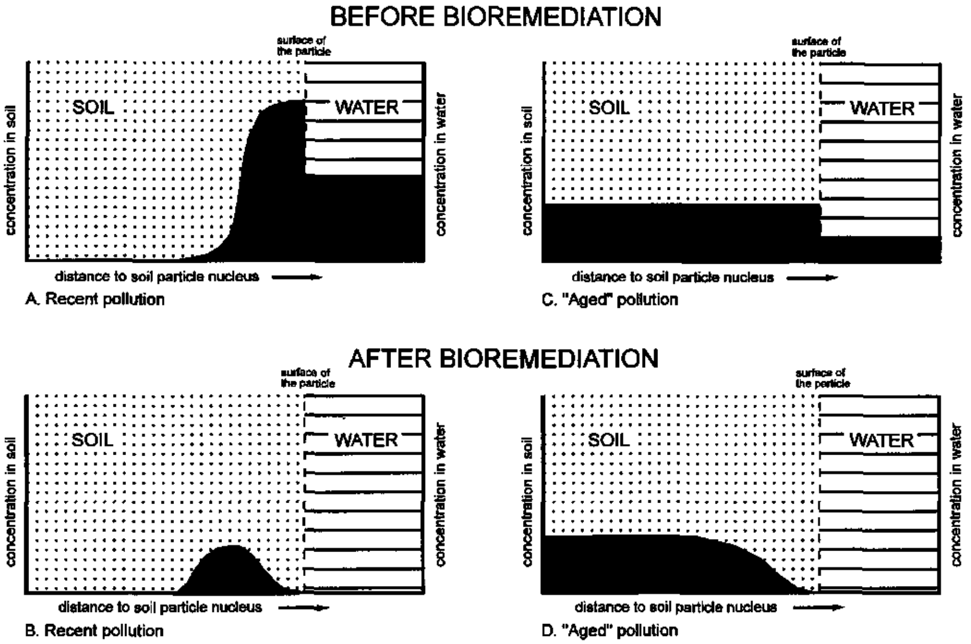


Figure 7.2. Schematic visualization of the distribution of a pollutant in a soil particle and surrounding liquid (23). The vertical scales are different in a way that the soil concentration >> water concentration.

A second process is the occurrence of the pollution as solid particles or as non-aqueous phase liquids (NAPLs) with surrounding semi-rigid films, from which mass transfer into the water phase is limited (37, 64, 65). Here, bioavailability is controlled by the actual dissolution rate. The third process is bound residue formation and involves the incorporation of pollutants into humic compounds via oxidative coupling reactions. This process especially applies to naphtolic compounds, chlorinated phenols, benzoic acids and anilines, due to their similarity to natural organic compounds (6, 13, 14, 15). Oxidative coupling can be catalyzed by inorganic materials, such as sesquioxides, clay minerals, oxides and oxohydroxides of iron, silica, and allophane (33, 55, 66, 70), but also by peroxidases and phenol monooxygenases

(39). Bound residue formation alters the original chemical and biological activity of the pollutants, which therefore become less available, less toxic and less mobile in the soil system (12). Chapter 6 shows that PCP, freshly added to a sandy and a peaty soil, can be partly recovered as extractable organic halogen (EOX) after 4 months of incubation. However, a chloride mass balance reveals that in the peaty soil, not all measured EOX can be ascribed to PCP, its transformation product pentachloroanisole, or any other possible low molecular weight organic chlorinated compound (MW < 512). This indicates that bound residue formation may have taken place in a time period of 4 months.

The use of the oxidative enzymes has been proposed as a method to immobilize organic pollutants into the humic material of the soil by stimulating bound residue formation (7, 16, 17, 18). However, uncontrollable release and distribution of the pollutant cannot be guaranteed. Macromolecular components of dissolved organic matter may form colloids which serve as carriers facilitating the transport of contaminants that bind to the organic matter (40). Furthermore, fungal oxidative enzymes have also been shown to catalyze unwanted reactions, like the formation of highly toxic chlorinated dibenzodioxins from coupling reactions with chlorinated phenols as substrate (42, 43, 61).

It can be concluded that bioavailability is more a physico-chemical problem rather than a microbiological problem. Solutions have therefore to be sought in physico-chemical techniques to increase the transport of the pollutants to the degrading bacteria. These techniques may comprise pulverizing soil particles, increasing the water content of the soil (slurry systems), heating the soil, and using detergents to increase solubility and/or desorption.

In this thesis, the ever increasing capability of microorganisms to degrade chlorinated pesticides and industrial chemicals is demonstrated, provided that the appropriate environmental conditions are created. Despite this enormous potential, the limited bioavailability of pollutants in old contaminated sites and strict soil quality standards, largely hamper the application of microorganisms in soil remediation techniques. The mentioned techniques to increase the availability of pollutants have economical or environmental drawbacks. Alternative remediation techniques like incineration or solvent-extraction yields soils without any biological activity, which' destiny is mainly to be used as raw material for building purposes.

On the other hand, one should ask what the consequences can be of the slow release of the residual amount of a pollutant from a soil that has been biologically treated (2). (Eco)toxicological assessment of such soil may serve the development of guidelines for the urgency to remove residual pollutants from such soils. Such an approach would stimulate the use of *in situ* bioremediation techniques, which eventually prevents polluted soil from being excavated. Of course, a continuous monitoring of the soil quality and an appropriate groundwater management of run-off water are essential.

REFERENCES

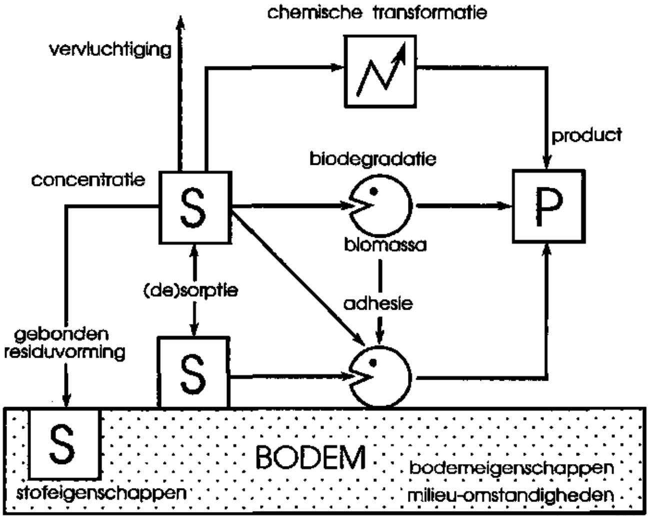
1. **Abramowicz, D. A., M. J. Brennan, H. M. Van Dort and E. L. Gallagher.** 1993. Factors influencing the rate of polychlorinated biphenyl dechlorination in Hudson River sediments. Environ. Sci. Technol. **27:**1125-1131.
2. **Alexander, M.** 1995. How toxic are toxic chemicals in soil? Environ. Sci. Technol. **29:**2713-2717.
3. **Bachmann, A., P. Walet, W. de Bruin, J. L. M. Huntjens, W. Roelofsen and A. J. B. Zehnder.** 1988. Biodegradation of alpha- and beta-hexachlorocyclohexane in a soil slurry under different redox conditions. Appl. Environ. Microbiol. **54:**143-149.
4. **Ball, W. P. and P. V. Roberts.** 1991. Long-term sorption of halogenated organic chemicals by aquifer material. 2. Intraparticle diffusion. Environ. Sci. Technol. **25:**1237-1249.
5. **Beland, F. A., S. O. Farwell, A. E. Robocker and R. D. Geer.** 1976. Electrochemical reduction and anaerobic degradation of lindane. J. Agric. Food Chem. **24:**753-756.
6. **Berry, D. F. and S. A. Boyd.** 1984. Oxidative coupling of phenols and anilines by peroxidase: Structure-activity relationships. Soil Sci. Soc. Am. J. **48:**565-569.
7. **Berry, D. F. and S. A. Boyd.** 1985. Decontamination of soil through enhanced formation of bound residues. Environ. Sci. Technol. **19:**1132-1133.
8. **Beurskens, J. E. M.** (1995) Microbial transformation of chlorinated aromatics in sediments. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.
9. **Beurskens, J. E. M., C. G. C. Dekker, J. Jonkhoff and L. Pompstra.** 1993. Microbial dechlorination of hexachlorobenzene in a sedimentation area of the Rhine river. Biogeochem. **19:**61-81.
10. **Beurskens, J. E. M., G. A. J. Mol, H. L. Barreveld, B. Van Munster and H. J. Winkels.** 1993. Geochronology of priority pollutants in a sedimentation area of the Rhine river. Environ. Toxicol. Chem. **12:**1549-1566.
11. **Beurskens, J. E. M., A. J. M. Stams, A. J. B. Zehnder and A. Bachmann.** 1991. Relative biochemical reactivity of three hexachlorocyclohexane isomers. Ecotoxicol. Environ. Safety **21:**128-136.
12. **Bollag, J.-M.** 1991. Enzymatic Binding of Pesticide Degradation Products to Soil Organic Matter and Their Possible Release. Pesticide Transformation Products **459:**122-132.
13. **Bollag, J.-M. and S.-Y. Liu.** 1985. Copolymerization of halogenated phenols and syringic acid. Pestic. Biochem. Physiol. **23:**261-272.
14. **Bollag, J.-M., S.-Y. Liu and R. D. Minard.** 1980. Cross-coupling of phenolic humus constituents and 2,4-dichlorophenol. Soil Sci. Am. J. **44:**52-56.
15. **Bollag, J.-M. and M. J. Loll.** 1983. Incorporation of xenobiotics into soil humus. Experientia **39:**1221-1231.
16. **Bollag, J.-M., K. H. Shuttleworth and D. H. Anderson.** 1988. Laccase-mediated detoxification of phenolic compounds. Appl. Environ. Microbiol. **54:**3068-3091.
17. **Bollag, J. M.** 1992. Decontaminating Soil with Enzymes. Environmental Science and Technology **26:**1876-1881.
18. **Bollag, J. M. and C. Myers.** 1992. Detoxification of Aquatic and Terrestrial Sites Through Binding of Pollutants to Humic Substances. Sci. Total Environ. **118:**357-366.
19. **Bosma, T. N. P., P. J. M. Middeldorp, G. Schraa and A. J. B. Zehnder.** 1996. Bioavailability: A limiting factor in the bioremediation of polluted soil. Environ. Sci. Technol.:In press.
20. **Boyle, A. W., C. K. Blake, W. A. Price II and H. D. May.** 1993. Effects of polychlorinated biphenyl congener concentration and sediment supplementation on rates of methanogenesis and 2,3,6-trichlorobiphenyl dechlorination in an anaerobic enrichment. Appl. Environ. Microbiol. **59:**3027-3031.
21. **Doelman, P., L. Haanstra and A. Vos.** 1988. Microbial degradation by the autochtonous soil population of alpha- and beta-HCH under anaerobic field conditions in temperate regions. Chemosphere **17:**481-487.
22. **Doelman, P., L. Haanstra and A. Vos.** 1988. Microbial sanitation of soil with alpha- and beta-HCH under aerobic glasshouse conditions. Chemosphere 17:489-492.
23. **Douben, P. E. T. and J. Harmsen.** 1991. Saneren: doen of niet doen? Bodem **1:**157-160.

24. **Edwards, E. A. and D. Grbic-Galic.** 1992. Complete mineralization of benzeen by aquifer microorganisms under strictly anaerobic conditions. Appl. Environ. Microbiol. **58:**2663-2666.
25. **Farrell, J. and M. Reinhard.** 1994. Desorption of Halogenated Organics from Model Solids, Sediments, and Soil Under Unsaturated Conditions .2. Kinetics. Environ. Sci. Technol. **28:**63-72.
26. **Fathepure, B. Z., J. M. Tiedje and S. A. Boyd.** 1988. Reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in anaerobic sewage sludge. Appl. Environ. Microbiol. **54:**327-330.
27. **Fava, F., S. Cinti and L. Marchetti.** 1993. Dechlorination of Fenclor-54 and of a Synthetic Mixture of Polychlorinated Biphenyls by Anaerobic Microorganisms. Appl. Microbiol. Biotechnol. **38:**808-814.
28. **Haider, K., G. Jagnow, R. Kohnen and S. U. Lim.** 1974. Abbau chlorierter Benzole, Phenole, und Cyclohexan-derivate durch Benzol und Phenol verwertende Bodenbakterien unter aeroben Bedingungen. Arch. Microbiol. **96:**183-200.
29. **Harms, H. and A. J. B. Zehnder.** 1994. Influence of substrate diffusion on degradation of dibenzofuran and 3-chlorodibenzofuran by attached and suspended bacteria. Appl. Environ. Microbiol. **60:**2736-2745.
30. **Hatzinger, P. B. and M. Alexander.** 1995. Effect of aging of chemicals in soil on their biodegradability and extractability. Environ. Sci. Technol. **29:**537-545.
31. **Holliger, C., G. Schraa, A. J. M. Stams and A. J. B. Zehnder.** 1992. Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. Appl. Environ. Microbiol. **58:**1636-1644.
32. **Jagnow, G., K. Haider and P.-C. Ellwardt.** 1977. Anaerobic dechlorination and degradation of hexachlorocyclohexane isomers by anaerobic and facultative anaerobic bacteria. Arch. Microbiol. **115:**285-292.
33. **Kyuma, K. and K. Kamaguchi.** 1964. Oxidative changes of polyphenols as influenced by allophane. Soil Sci. Soc. Am. J. **28:**371-374.
34. **Langenhoff, A. A. M., D. L. Brouwers-Ceiler, J. H. L. Engelberting, J. J. Quist, J. G. P. N. Wolkenfelt, A. J. B. Zehnder and G. Schraa.** 1996. Microbial reduction of manganese coupled to toluene oxidation. FEMS Microbiol. Ecol. **submitted.**
35. **Lovley, D. R., J. D. Coates, J. C. Woodward and E. J. P. Phillips.** 1995. Benzene oxidation coupled to sulfate reduction. Appl. Environ. Microbiol. **61:**953-958.
36. **Lovley, D. R., J. C. Woodward and F. H. Chapelle.** 1996. Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. Appl. Environ. Microbiol. **62:**288-291.
37. **Luthy, R. G., A. Ramaswami, S. Ghoshal and W. Merkel.** 1993. Environ. Sci. Technol. 27:2914-2918.
38. **MacRae, I. C., K. Raghu and T. F. Castro.** 1967. Persistence and biodegradation of four common isomers of benzene hexachloride in submerged soils. J. Agr. Food Chem. **15:**911-914.
39. **Martin, J. P. and K. Haider.** 1971. Microbial activity in relationship to soil humus formation. Soil Sci. **111:**54-63.
40. **McCarthy, J. F. and J. M. Zachara.** 1989. Subsurface transport of contaminants. Environ. Sci. Technol. **23:**496-502.
41. **Nowak, J., N. H. Kirsch, W. Hegemann and H.-J. Stan.** 1996. Total reductive dechlorination of chlorobenzenes to benzene by a methanogenic mixed culture enriched from Saale river sediment. Appl. Microbiol. Biotechnol. **45:**700-709.
42. **Öberg, L. G., B. Glas, S. E. Swanson, C. Rappe and K. G. Paul.** 1990. Peroxidase-catalyzed oxidation of chlorophenols to polychlorinated dibenzo-*para*-dioxins and dibenzofurans. Arch. Environ. Contam. Toxicol. **19:**930-938.
43. **Öberg, L. G. and C. Rappe.** 1992. Biochemical Formation of PCDD/Fs from Chlorophenols. Chemosphere **25:**49-52.
44. **Ofjord, G. D., J. A. Puhakka and J. F. Ferguson.** 1994. Reductive dechlorination of Aroclor 1254 by marine sediment cultures. Environ. Sci. Technol. **28:**2286-2294.
45. **Ohisa, N., M. Yamaguchi and N. Kurihara.** 1980. Lindane degradation by cell-free extracts of *Clostridium rectum*. Arch. Microbiol. **125:**221-225.
46. **Peters, C. A. and R. G. Luthy.** 1994. Environ. Sci. Technol. **28:**1331-1340.
47. **Pettigrew, C. A., B. E. Haigler and J. C. Spain.** 1991. Simultaneous biodegradation of chlorobenzene and toluene by a *Pseudomonas* strain. Appl. Environ. Microbiol. **57:**157-162.
48. **Quensen III, J. F., J. M. Tiedje and S. A. Boyd.** 1988. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. Science **242:**752-754.
49. **Ramanand, K., M. T. Balba and J. Duffy.** 1993. Reductive dehalogenation of chlorinated benzenes and toluenes under methanogenic conditions. Appl. Environ. Microbiol. **59:**3266-3272.
50. **Reineke, W. and H.-J. Knackmuss.** 1984. Microbial metabolism of haloaromatics: Isolation and properties of a chlorobenzene-degrading bacterium. Appl. Environ. Microbiol. **47:**395-402.

51. **Rhee, G. Y., B. Bush, C. M. Bethoney, A. Denucci, H. M. Oh and R. C. Sokol.** 1993. Reductive Dechlorination of Aroclor 1242 in Anaerobic Sediments: Pattern, Rate and Concentration Dependence. Environ. Toxicol. Chem. **12**:1025-1032.
52. **Rijnaarts, H. H. M., A. Bachmann, J. C. Jumelet and A. J. B. Zehnder.** 1990. Effect of desorption and intraparticle mass transfer on the aerobic biomineralization of alpha-hexachlorocyclohexane in a contaminated calcareous soil. Environ. Sci. Technol. **24**:1349-1354.
53. **Safe, S. H.** 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Critical Reviews in Toxicology **21**:51-88.
54. **Salkinoja-Salonen, M. S., P. J. M. Middeldorp, J. K. Jokela, J. S. Uotila, M. Briglia, E. Kostyal, J. Puhakka and I. Nevalainen.** 1993. Clean-up of chemically polluted soil and ground water using biocatalytes, p. 61-77. *In* J.-M. Punning and J. Hult (ed.), Human impact on environment - Pilot study in Baltic, Institute of Ecology, Estonian Academy of Sciences, Tallinn, Estonia.
55. **Scheffer, F., B. Meyer and E. A. Niederbudde.** 1959. Huminstoffbildung unter katalytischer Einwirkung natürlich Vorkommender Eisenverbindungen im Modellversuch. Z. Pflanzenernährung Bodenkd. **87**:26-44.
56. **Scow, K. M. and M. Alexander.** 1992. Effect of diffusion on the kinetics of biodegradation - Experimental results with synthetic aggregates. Soil Sci. Soc. Am. J. **56**:128-134.
57. **Scow, K. M. and J. Hutson.** 1992. Effect of diffusion and sorption on the kinetics of biodegradation - Theoretical considerations. Soil Sci. Soc. Am. J. **56**:119-127.
58. **Shishido, M. and M. Toda.** 1996. Apparent zero-order kinetics of phenol biodegradation by substrate-inhibited microbes at low substrate concentrations. Biotechnol. Bioeng. **50**:709-717.
59. **Shuttleworth, K. L. and C. E. Cerniglia.** 1996. Bacterial degradation of low concentrations of phenanthrene and inhibition by naphthalene. Microb. Ecol. **31**:305-317.
60. **Siddaramappa, R. and N. Sethunathan.** 1975. Persistence of gamma-BHC and beta-BHC in Indian rice soils under flooded conditions. Pesticide Science **6**:395-403.
61. **Svenson, A., L.-O. Kjeller and C. Rappe.** 1989. Enzyme-mediated formation of 2,3,7,8-substituted chlorinated dibenzodioxins and dibenzofurans. Environ. Sci. Technol. **23**:900-902.
62. **Tros, M., G. Schraa and A. J. B. Zehnder.** 1996. Transformation of low concentrations of 3-chlorobenzoate by *Pseudomonas* sp. strain B13: Kinetics and residual concentrations. Appl. Environ. Microbiol. **62**:437-442.
63. **Van Dort, H. M. and D. L. Bedard.** 1991. Reductive *ortho*- and meta-dechlorination of a polychlorinated biphenyl congener by anaerobic microorganisms. Appl. Environ. Microbiol. **57**:1576-1578.
64. **Volkering, F., A. M. Breure, A. Sterkenburg and J. G. Van Andel.** 1992. Microbial Degradation of Polycyclic Aromatic Hydrocarbons - Effect of Substrate Availability on Bacterial Growth Kinetics. Appl. Microbiol. Biotechnol. **36**:548-552.
65. **Volkering, F., A. M. Breure and J. G. Van Andel.** 1993. Effect of Micro-Organisms on the Bioavailability and Biodegradation of Crystalline Naphthalene. Appl. Microbiol. Biotechnol. **40**:535-540.
66. **Wang, T. S. C., P. M. Huang, C.-H. Chou and J.-H. Chen.** 1986. The role of soil minerals in the abiotic polymerization of phenolic compounds and formation of humic substances, p. 251-281. *In* P. M. Huang and M. Schnitzer (ed.), Interactions of soil minerals with natural organics and microbes, SSSA Inc., Madison, Wisconsin, USA.
67. **Weissenfels, W. D., H. J. Klewer and J. Langhoff.** 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles - Influence on biodegradability and biotoxicity. Appl. Microbiol. Biotechnol. **36**:689-696.
68. **Williams, W. A.** 1994. Microbial reductive dechlorination of trichlorobiphenyls in anaerobic sediment slurries. Environ. Sci. Technol. **28**:630-635.
69. **Wu, S. and P. M. Gschwend.** 1986. Sorption kinetics of hydrophobic organic compounds to natural sediments and soils. Environ.Sci. Technol. **20**:717-725.
70. **Ziechmann, W.** 1959. Die Darstellung von Huminsäuren im heterogenen System mit neutraler Reaktion. Z. Pflanzenernährung, Düng. **84**:155-159.

SAMENVATTING

Bodems verontreinigd met gechloreerde pesticiden en industriële chemicalien is een veel voorkomend probleem zowel in Nederland als daarbuiten. Zulke chemicaliën zijn vaak schadelijk voor de gezondheid van mens en dier, waardoor deze bodems niet meer gebruikt mogen worden voor bv. landbouw, woningbouw, recreatie, etc. De talloze ontdekte bacteriën die zulke verontreinigingen kunnen afbreken tot ongevaarlijke produkten, vormen een machtig potentieel om zulke verontreinigde bodems op een elegante manier te reinigen. Niettemin moet er een aantal problemen worden opgelost voordat een dergelijke biologische bodemreiniging kans van slagen heeft. Deze afbrekende bacteriën zijn vaak al aanwezig in verontreinigde grond, maar zij doen hun afbrekende werk niet of nauwelijks. Dit heeft voor een groot deel te maken met het feit dat de omstandigheden in een bodem (zgn. milieu-omstandigheden) niet zo gunstig zijn als in het laboratorium, waar deze afbraak wel is aangetoond. Een bodem is een complex geheel (bodemdeeltjes, water, lucht) met een grote chemische en fysische heterogeniteit. De processen die belangrijk zijn voor het lot van een verontreiniging en de afbrekende bacteriën in de bodem, zijn afgebeeld in Figuur 1.



R. v/d Berg, RIVM

Figuur 1. Processen die een rol spelen bij biologische afbraak van een organische verontreiniging (S) in de bodem.

Dit proefschrift behandelt een aantal van de problemen die men tegen kan komen wanneer men de mogelijkheden van het gebruik van bacteriën voor het opruimen van organische verontreinigingen bestudeert.

De afbreekbaarheid van chemicaliën wordt niet alleen bepaald door hun molekuulstructuur, maar ook door de heersende milieu-omstandigheden, zoals temperatuur, pH, en de

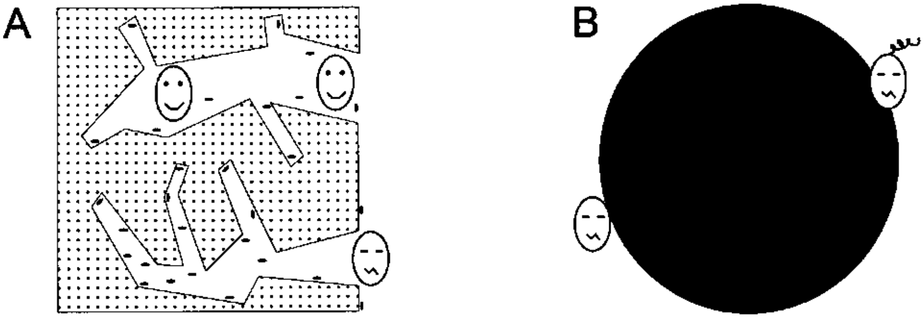
aanwezigheid van zuurstof. Dichtbij het oppervlak in de bodem heersen zuurstofrijke omstandigheden (aërobe condities), terwijl dieper in de bodem en speciaal in het grondwater zuurstofloze omstandigheden (anaërobe condities) voorkomen. Vaak kunnen de bacteriën die onder de ene conditie leven, zich niet handhaven onder de andere. Door hun fysiologische verschillen kunnen aërobe bacteriën andere chemicaliën afbreken dan anaërobe bacteriën, of dezelfde stoffen, maar op een andere manier.
Het onderzoek naar de biologische afbraak van organische verontreinigingen heeft zich in het begin geconcentreerd op aërobe bacteriën, aangezien zuurstofrijke condities veel voorkomen in verontreinigde bodems, en deze bacteriën relatief gemakkelijk te bestuderen zijn. Gebleken is echter dat ook anaërobe bacteriën grote capaciteiten hebben voor het afbreken van zulke verontreinigingen. Een voorbeeld hiervan is de afbraak van β-hexachloorcyclohexaan (β-HCH), een afvalproduct bij de productie van het pesticide 'lindaan' (γ-HCH). Momenteel zijn grote hoeveelheden grond in Nederland met deze stof verontreinigd. Voorheen werd β-HCH gezien als een zeer persistente verontreiniging onder aërobe condities. Verdwijning van deze stof is wel beschreven onder anaërobe condities, maar er is niet aangetoond dat alle β-HCH dat verdween, ook teruggevonden werd als andere producten. In Hoofdstuk 2 wordt aangetoond dat β-HCH in een mengcultuur van anaërobe bacteriën kan worden afgebroken tot de stoffen benzeen en chloorbenzeen. Deze bacteriën zijn tevens in staat ook andere belangrijke isomeren van HCH (α-, γ-, en δ-HCH) om te zetten. De producten, benzeen en chloorbenzeen, zijn nog steeds giftig en moeten dus verder worden afgebroken. Dit blijkt moeilijk te gaan onder anaërobe, maar wel onder aërobe condities. Daarom zou in principe een bodemreinigingsmethode moeten worden ontwikkeld waarin eerst anaërobe en vervolgens aërobe condities heersen. Op deze manier zouden alle belangrijke HCH isomeren tot onschadelijke producten (koolzuurgas en water) kunnen worden afgebroken.

Een dergelijke opeenvolging van anaërobe en aërobe condities kan ook toegepast worden voor de eliminatie van andere hooggechloreerde industriële chemicaliën zoals gechloreerde benzenen en bifenylen (CB's en PCB's). Deze stoffen zijn onder aërobe omstandigheden niet afbreekbaar omdat er teveel chlooratomen in het molekuul zitten. Wel bestaan er anaërobe bacteriën die een aantal chlooratomen in deze molekulen vervangen door waterstof (dechlorering). Hierdoor worden deze stoffen laaggechloreerd, zijn minder giftig en tevens kunnen zij dan wel worden afgebroken door aërobe bacteriën. Een nadeel is echter wel dat de gedechloreerde benzenen en bifenylen ook minder aan bodemdeeltjes adsorberen en zich in de grond gaan verspreiden, waardoor eventueel drinkwaterputten met deze stoffen 'besmet' kunnen worden. Het dechloringsproces verloopt in de natuur slechts langzaam en zou gestimuleerd moeten worden. De benodigde kennis van dit proces en de bacteriën die erbij betrokken zijn, is echter niet erg groot. Daarom werd de dechlorering van CB's en PCB's door een anaërobe mengcultuur bestudeerd (Hoofdstuk 3). Het bleek dat deze anaërobe bacteriën in staat waren om binnen een week de meeste hooggechloreerde benzenen te dechloreren tot vooral chloorbenzeen. De toevoeging van BrES (2-broomethaansulfonzuur), een stof die methaanvormende bacteriën remt, had tot gevolg dat de dechloreringsroute minder gecompliceerd werd, en alleen chloorbenzeen als eindproduct werd gevormd. De dechlorering van hexachloorbenzeen en verschillende PCB's begon echter pas na twee weken (aanpassingsfase) en verliep volgens een ander dechloreringspatroon waarin alleen chlooratomen die twee naburige chlooratomen hebben, werden verwijderd. BrES had geen invloed op de dechlorering. Uit deze resultaten kan men concluderen dat tenminste drie verschillende dechlorerende bacteriepopulaties aktief zijn in deze mengcultuur, waarvan één uit methaanvormers bestaat of van methaanvormers afhankelijk is.

Een andere anaërobe mengcultuur wordt beschreven in Hoofdstuk 4. Deze kan ook CB's en PCB's dechloreren. Echter, aangezien de CB's en PCB's alle op dezelfde manier worden gedechloreerd en de aanpassingsfase in alle gevallen gelijk is, kan men concluderen dat er geen sprake is van een combinatie van verschillende populaties zoals in Hoofdstuk 3. Ook blijkt dat zowel methaanvormende als zuurvormende bacteriën in deze mengcultuur bij het dechloreringsproces betrokken zijn.

Zoals eerder opgemerkt, wordt de dechlorering van CB's en PCB's vaak gekenmerkt door een aanpassingsfase die weken tot maanden kan duren, voordat de dechlorering begint. Aangezien ook in de bodemreiniging tijd geld is, is het zaak deze aanpassingsfase zo kort mogelijk te maken of te houden. In Hoofdstuk 5 wordt de aanpassingsfase van een anaërobe 2,3,4,5-tetrachloorbifenyl dechlorerende mengcultuur bestudeerd. Het blijkt dat de aanpassingsfase voor deze dechlorering aanzienlijk kan worden verkort (van drie weken naar een week) door de toevoeging van zand uit de Rijn of anaëroob korrelslib uit een waterzuiveringsinstallatie. De combinatie van deze twee toevoegingen levert een nog kortere aanpassingsfase op. Hoewel we niet precies weten waarom deze toevoegingen een verkorting van de aanpassingsfase teweegbrengen, blijken deze ook werkzaam te zijn bij de dechlorering van een mengsel van PCB's zoals die bij bodemverontreiniging voorkomen. Hier werd de aanpassingsfase verkort van 75 dagen naar 14 dagen.

Een laatste aspect van biologische bodemreiniging wordt besproken in Hoofdstuk 6. Hier wordt de pentachloorfenol (PCP, fungicide) afbrekende bacteriestam *Rhodococcus chlorophenolicus* toegevoegd aan met PCP-verontreinigde grond. Het blijkt dat deze bacterie de in de grond aanwezige verontreiniging in het begin vrij snel afbreekt, maar dat het laatste restje PCP maar heel langzaam of helemaal niet wordt afgebroken. Dit is niet het gevolg van het doodgaan van de bacteriën, maar van het feit dat de PCP niet meer 'beschikbaar' is voor de bacteriën. Dit verschijnsel werd reeds eerder beschreven en kan op een aantal manieren veroorzaakt worden. Allereerst kunnen verontreinigingen door biologische of chemische processen worden ingebouwd in humus. Hierdoor wordt de chemische structuur van de verontreiniging veranderd (het maakt nu deel uit van een veel groter molekuul) waardoor bacteriën de verontreiniging niet meer kunnen afbreken. Dit proces heet 'gebonden residuvorming'. Hier is eigenlijk niets aan te doen. Sommige mensen stellen zelfs voor om dit proces te stimuleren, waardoor alle verontreiniging in de humus gaat zitten en dus niet meer giftig is. Helaas blijkt dat bij gebonden residuvorming de molekulen van de verontreiniging ook met elkaar kunnen worden verbonden waardoor uiterst giftige dioxinen kunnen ontstaan. Daar moet dus nog heel wat uitgezocht worden. Ook kunnen verontreinigingen, naarmate zij langer in de bodem zitten, aan bodemdeeltjes gaan 'plakken' of dieper in de bodemdeeltjes doordringen. Ze kunnen in zulke kleine poriën terechtkomen dat de bacteriën er niet meer bij kunnen (Figuur 2A). Dit proces heet 'door diffusie gelimiteerde sorptie'. Om de verontreiniging beter bereikbaar te maken, kunnen de bodemdeeltjes verpulverd worden, waardoor er een groter buitenoppervlak van de bodemdeeltjes wordt gecreëerd. Dit is echter een kostbare zaak. Een laatste oorzaak van 'onbeschikbaarheid' is dat verontreinigingen slecht in water oplossen en kleine druppeltjes gaan vormen (bijv. olie). Aangezien de afbrekende bacteriën in het omringende water zitten kunnen zij alleen maar van de buitenkant 'eten' (Figuur 2B). Hier geldt: hoe groter de druppels, hoe minder groot het relatieve oppervlak en dus hoe langzamer de afbraak. Detergentia (zeep) kunnen de druppels verkleinen en dus de afbraak versnellen. Maar dan zit je weer met al die zeep in de bodem.



Figuur 2. Schematische voorstelling van twee mogelijke manieren van 'onbeschikbare' verontreiniging. (A) Een bodemdeeltje met poriën en afbrekende bacteriën. (B) Een druppel verontreiniging met bacteriën in het omringende water.

In Nederland en vele andere landen wordt gesteld dat bij bodemreiniging de laatste restjes verontreiniging uit de bodem worden verwijderd. Kan dit niet op een biologische manier, dan wordt de grond afgegraven en verbrand of chemisch behandeld. Een dergelijk reinigingsmethode is niet wenselijk aangezien dit een 'dode' grond oplevert die meestal alleen nog kan worden gebruikt als bouwmateriaal. Echter, we kunnen het ook op een andere manier bekijken. Waarom zouden we die laatste restjes verontreiniging nog uit een biologisch gereinigde grond willen verwijderen als zelfs bacteriën er niet meer bij kunnen? Natuurlijk moeten we goed uitkijken en blijven controleren dat die verontreiniging niet op een later tijdstip door een of andere verandering van omstandigheden toch vrijkomt, maar we zouden de grond toch alvast voor relatief risicoloze toepassingen (bijv. golfbanen, industrieterreinen) kunnen gebruiken.

PUBLICATIONS

Häggblom M.M., Janke D., Middeldorp P.J.M. and Salkinoja-Salonen M.S. (1989). O-methylation of chlorinated phenols in the genus *Rhodococcus*. Arch. Microbiol. 152:6-9.

Salkinoja-Salonen M.S., Middeldorp P.J.M., Briglia M., Valo R.J., Häggblom M.M., McBain A. and Apajalahti J.H.A. (1989). Cleanup of old industrial sites. *In*: Advances in Applied Biotechnology, Vol. 4. Ed. by D.Kamely, A.Chakrabarty and G.Omenn, Gulf publishing Company Houston (Tx) USA, pp.347-365.

Middeldorp P.J.M., Briglia M. and Salkinoja-Salonen M.S. (1990). Biodegradation of pentachlorophenol in natural soil by inoculated *Rhodococcus chlorophenolicus*. Microbial Ecology, 20:123-139.

Briglia M., Middeldorp P.J.M., and Salkinoja-Salonen M.S.S. (1994). Mineralization performance of *Rhodococcus chlorophenolicus* strain PCP-1 in contaminated soil simulating on site conditions. Soil Biology and Biochemistry, 26:377-385.

Middeldorp P.J.M. and Schraa G. (1994). The fate of organic pollutants in soils and sediments and the development of biological soil remediation techniques - An evaluation of quality, scientific significance and policy relevance. The Netherlands Integrated Soil Research Programme, Wageningen. The Netherlands Integrated Soil Research Programme Reports; vol 1, 37 pp.

Middeldorp P.J.M., Jaspers M., Zehnder A.J.B. and Schraa G. (1996).Biotransformation of α-, β-, γ-, and δ-hexachlorocyclohexane under methanogenic culture conditions. Environmental Science & Technology, 30: 2345-2349.

Bosma T.N.P., Middeldorp P.J.M., Schraa G. and Zehnder A.J.B. (1996). Mass transfer limitation of biotransformation: Quantifying bioavailability. Environ. Sci. Technol., in press.

Middeldorp, P.J.M., Schraa, G. (1997). Bioremediation of contaminated soils. *In*: Microbial interactions in agriculture and forestry. Subba Rao, N.S. and Dommergues, Y.R. (eds.). In press.

Middeldorp P.J.M., De Wolf J., Zehnder A.J.B., Schraa G. Reduction of the lag phase for dechlorination of polychlorinated biphenyls. Submitted.

Middeldorp P.J.M., De Wolf J., Zehnder A.J.B., Schraa G. Enrichment and properties of a 1,2,4-trichlorobenzene dechlorinating methanogenic consortium. Submitted.

Middeldorp P.J.M., De Wolf J., Zehnder A.J.B., Schraa G. Specificity of dechlorination of chlorinated benzenes and biphenyls by a microbial methanogenic consortium. Submitted.

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CURRICULUM VITAE

Petrus Johannes Maria Middeldorp werd op 10 maart 1964 geboren in Den Haag, en kreeg als roepnaam Peter-Jan. Hij groeide op in Den Haag, waar hij op het Edith Stein College in 1982 het eindexamen VWO behaalde. Na korte twijfel of hij niet iets in de muziek zou gaan doen, werd toch besloten om naar de Landbouwuniversiteit te Wageningen te gaan om iets voor de bescherming van het milieu te kunnen betekenen. In Wageningen zag hij ook zijn kans schoon om de helft van zijn dubbele naam kwijt te raken. Na een half jaar sloeg de twijfel weer even toe en veranderde hij binnen Wageningen van studierichting en haalde zijn propaedeuse in de Humane Voeding. De rest van de studie werd toch weer in de studierichting Milieuhygiëne gevolgd met als specialisaties Bodemkwaliteitsbeheer en Gezondheidsleer. Zijn stage deed hij zes maanden bij de Universiteit van Helsinki waar onderzoek op het gebied van de microbiologische afbraak van chloorfenolen werd gedaan. Dit onderzoek (en dat niet alleen) trok hem zozeer dat hij na zijn buluitreiking in 1989 terugging naar Finland om daar als gastonderzoeker voor anderhalf jaar te werken.

Eind 1990 had hij het in Finland wel gezien en ging hij terug naar Wageningen om bij de vakgroep Microbiologie promotieonderzoek te beginnen. Een stuk of wat werkcontracten (variërend van twee weken tot drie jaar) en vijf en een half jaar later had hij gewerkt aan de afbreekbaarheid van een nieuw geheim pesticide, de aerobe en anaerobe afbraak van HCH en de reductieve dechlorering van chloorbenzenen en PCBs. Tussendoor werkte hij ook nog een jaar aan een zgn. "topping-up" project, een soort evaluatieproject van onderzoek gefinancieerd door het Speerpuntprogramma Bodemonderzoek.

De resultaten van een deel van het onderzoek uitgevoerd in Finland en in Wageningen staan in dit proefschrift opgetekend.