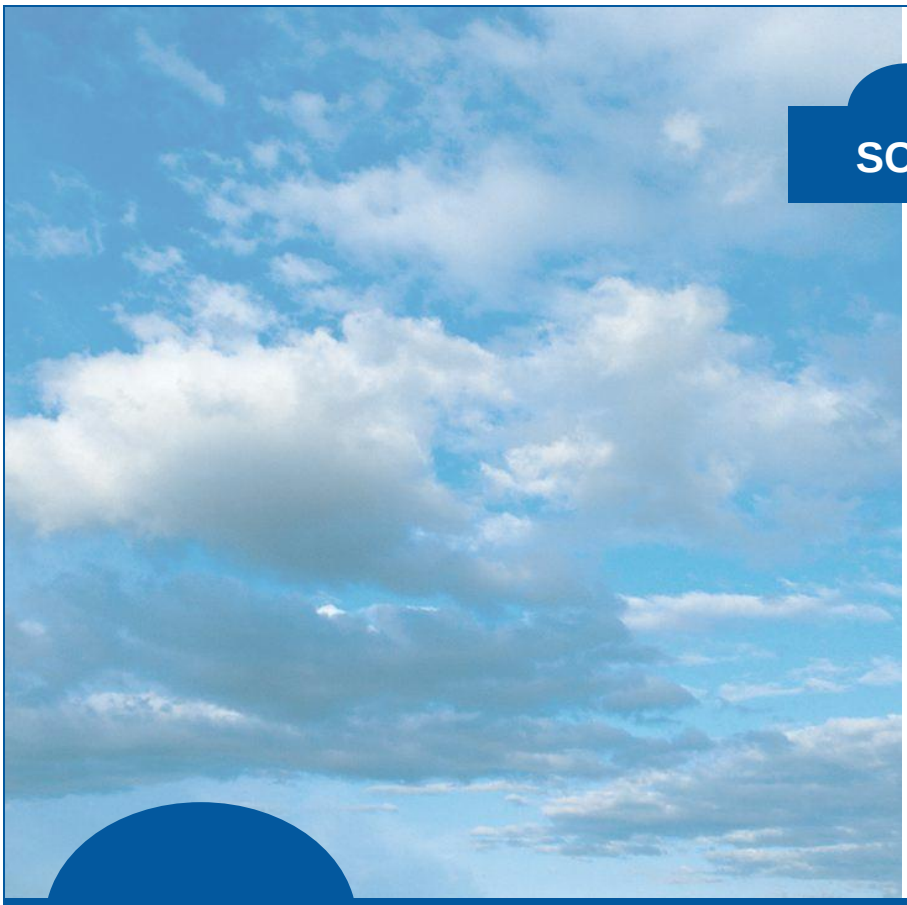

SCIENCE DOSSIER

Biodegradability of chlorinated aromatic compounds

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Foreword

The Environmental Working Group (EWG) is a science group of Euro Chlor, which represents the European chlor-alkali industry. Objectives of the group are to identify both natural and anthropogenic sources of chlorinated substances, study their fate, gather information on the mechanisms of formation and degradation in the environment, and achieve a better knowledge of the persistence of such substances and communicate these findings to a wide audience in order to promote science-based decision making. The EWG often uses external specialists to assist in developing reports that review the state of existing knowledge of the different aspects mentioned.

Dr. Jim Field is a Full Professor at the Department of Chemical & Environmental Engineering, University of Arizona, where he has been a member of the faculty since 2001. One of his main research topics is on the degradation of halogenated compounds in anaerobic environments. Dr. Field is also known for his research on the natural production of organohalogens by fungi. He has been working closely with Euro Chlor for six years, conducting comprehensive literature studies on the biodehalogenation and biodegradation of chlorinated chemicals in the environment. Dr. Reyes Sierra-Alvarez also works at the University of Arizona as an Associate Professor and has 16 years research experience in environmental biotechnology. Her current research includes
work on the biotransformation of chemicals in the environment. Dr Sierra-Alvarez has a particular interest in the microbially catalysed transformation of metals and hazardous organic pollutants, bioremediation, biological treatment of industrial wastewaters and biotechnology for environmental benign manufacturing.

In *Biodegradability of chlorinated aromatics* Field and Sierra-Alvarez demonstrate the capacity of micro-organisms to degrade various chlorinated aromatics (chlorophenols, chlorobenzoates, polychlorinated biphenyls, chlorobenzenes and chlorinated dioxins). This Science Dossier provides compelling evidence for the biodegradation of compounds in each of these categories under a variety of physiological and redox conditions. Many microorganisms gain energy and benefit from the biodegradation by utilizing the chlorinated aromatic compound as an electron donor or as an electron acceptor. If utilized as an electron donor and carbon source, the chlorinated aromatic compounds are oxidized. If utilized as an electron acceptor, the chlorinated aromatic compounds become reductively dehalogenated in a process known as haloresipiration. Biodegradation can also result from cometabolism in which case the chlorinated aromatics are transformed while microorganisms are utilizing other substrates to gain energy. Common strategies responsible for the enzymatic attack of chlorinated aromatics have been identified. This Science Dossier has elucidated general patterns of biodegradability. Chlorinated aromatic compounds with oxygen containing functional groups behaved distinctly from aromatics lacking these groups. The degree of chlorination dramatically affects the biodegradability potential under different redox conditions of aromatic compounds lacking free oxygen groups. Higher chlorinated congeners are only susceptible to anaerobic biotransformation; whereas lower congeners are only susceptible to aerobic biodegradation. On the other hand, aromatics with oxygen containing functional groups are generally fully biodegraded under either anaerobic or aerobic conditions regardless of the degree of chlorination.

Summary

Chlorinated aromatic compounds originate from natural and anthropogenic sources. Biodegradation is an important process dictating their fate in the environment. This report presents a comprehensive literature review on the biodegradability of chlorophenols (CP), chlorobenzoates (CBc), chlorobenzenes (CB), polychlorinated benzenes (PCB) and chlorinated dioxins (CDD) by microorganisms in aerobic and anaerobic environments. Compelling evidence was provided for the biodegradation of compounds in each of the categories under a variety of physiological and redox conditions.

Many microorganisms gain energy and benefit from the biodegradation by utilizing the chlorinated aromatic compound as an electron donor or as an electron acceptor. If utilized as an electron donor and carbon source, the chlorinated aromatic compounds are oxidized to CO_2 and chloride. If utilized as an electron acceptor, the chlorinated aromatic compounds become reductively dehalogenated in a process known as halorespiration, resulting in the accumulation of lower chlorinated congeners. Biodegradation can also result from cometabolism in which case the chlorinated aromatics are transformed while microorganisms are utilizing other substrates to gain energy. Common strategies responsible for the enzymatic attack of chlorinated aromatics have been identified. Under aerobic conditions microorganisms utilize three main mechanisms of initiating the degradation. These include oxygenases (insertion of oxygen from O_2 into the compound), hydrolytic dehalogenases (replacement of a Cl-group with a hydroxyl group) and glutathione S-transferases (replacement Cl-group with the sulfhydryl group of glutathione). The predominant mechanism of initiating degradation of chloroaromatics under anaerobic conditions is via reductive hydrogenolysis in which a Cl-group is replaced by a hydrogen atom.

This review has elucidated general patterns of biodegradability. Chlorinated aromatic compounds with oxygen containing functional groups (CP and CBc) behaved distinctly from aromatics lacking these groups (CB, PCB, CDD). The degree of chlorination dramatically affects the biodegradability potential under different redox conditions of CB, PCB and CDD. Higher chlorinated congeners are only susceptible to anaerobic biotransformation; whereas lower congeners are only susceptible to aerobic biodegradation. Complete biodegradation of the higher chlorinated congeners requires a sequence of anaerobic and aerobic conditions. On the other hand, CP and CBc are potentially fully biodegraded under either anaerobic or aerobic conditions regardless of their degree of chlorination. The environmental half lives of biodegradation are as low as several days to several months for CP and CBc; whereas 7 to 16 years are implicated for higher chlorinated congeners of CB and PCB based on geochronological evidence from sediment cores.

Taken as a whole, the evidence in the literature suggests that chlorinated aromatic compounds are subject to biodegradation in the environment as part of the natural chlorine cycle. Therefore, risk assessment models should account for biodegradation as a major mechanism in the natural attenuation of chlorinated aromatics.

1 Chlorinated Benzoates

1.1. Introduction

Chlorinated benzoates include isomers of chlorobenzoate (CBc), dichlorobenzoate (DCBc) and trichlorobenzoate (TCBc). Chlorinated benzoates enter the environment through their use as herbicides (*eg* 2,3,6-TCBc and dicamba (2-methoxy-3,6-dichlorobenzoate)) (Horvath, 1971) or as metabolites of other halogenated compounds such as the fungicide, pentachlorobenzyl alcohol (Ishida, 1972), via aerobic degradation of polychlorinated biphenyls (Seeger et al., 1997; Flanagan and May, 1993a; Bedard, 2003), or as an intermediate in the anaerobic degradation of 2-chlorophenol (Becker et al., 1999). The natural formation of 2,4-DCBc and 2,5-DCBc was demonstrated in bog samples, possibly via a reaction involving chloroperoxidase (Niedan and Scholer, 1997). Only a few review articles have been written on the biodegradation of chlorinated benzoates (Haggblom, 1992; Vrana et al., 1995).

1.2. Biodegradation of chlorobenzoates

1.2.1. Degradation of chlorobenzoates in the environment

Ample evidence is available indicating biodegradation of chlorinated benzoates in the environment under aerobic as well as anaerobic conditions. With respect to aerobic degradation in soils, the largest body of evidence has been obtained for 3-Cbc. 3-CBc incubated aerobically with soil suspensions at concentrations ranging from 16 to 500 mg l^{-1} was depleted in time periods ranging from 7 to 32 days (Alexander and Lustigman, 1966; Haller, 1978; Ramirez-Saad et al., 2000; Gentry et al., 2001). When incubated directly in soil, [^{14}C]3-CBc was mineralized by 59% in 10 weeks (Haider et al., 1974). In several studies, initial enrichments of indigenous 3-CBc degraders yielded strains of *Burkholderia* (Ramirez-Saad et al., 2000; Gentry et al., 2001; Fulthorpe et al., 1998). However, an extensive study on the occurrence of 3-CBc mineralizing bacteria in soils from pristine environments indicated a broad distribution of 3-CBc degraders world-wide and a large biodiversity was found among the 3-CBc degrading-isolates (Fulthorpe et al., 1996, 1998). Enrichment cultures from the soil samples mineralized [^{14}C]3-CBc to $^{14}CO_2$ at ranges ranging from 1 to 7% per day with an initial 3-CBc concentration of 50 mg l^{-1} (Fulthorpe et al., 1996). Generally, the genotypes of 3-CBc degrading strains obtained from the enrichments were unique (endemic) to the geographical region from which they were isolated (Fulthorpe et al., 1998). Accelerated degradation of 3-CBc in the rhizosphere was also noted and attributed to cometabolism of the chlorinated compound during root exudate degradation (Haby and Crowley, 1996)

Evidence for aerobic biodegradation of 2-CBc, 4-CBc and dicamba in soil is also available. Soil suspensions degraded 16 to 782 mg l^{-1} of 2-CBc within 7 to 14 days (Haller, 1978; Baggi and Zangrossi, 1999; Wang et al., 2004). Likewise, soils suspensions degraded 25 to 782 mg l^{-1} of 4-CBc within 14 to 64 days (Alexander and Lustigman, 1966; Baggi and Zangrossi, 1999). The stoichiometric release of chloride from 2-CBc and 4-CBc was demonstrated during incubations with a soil slurry (Baggi and Zangrossi, 1999). Additionally, dicamba was shown to be degraded in soil (Ferrer et al., 1986).

Aside from soil, aerobic degradation of chlorinated benzoates has been shown to occur in lake water and sewage. 236-TCBc was depleted in lake water but not in heat-killed controls, suggesting its biodegradation (Horvath, 1972). Cometabolism of 236-TCBc was demonstrated by stimulating the rate extent of the herbicide degradation through the addition of benzoate. Raw sewage readily degraded 100 mg l^{-1} of 2-CBc, 3-CBc, 4-CBc and 3,4-DCBc after 6 to 12 days (Digeronimo et al., 1979). On the other hand, primary

sludge degraded 16 mg l^{-1} of 3-CBc within 14 days but did not degraded 2-CBc, 3-CBc nor 2,5-DCBc within 25 days (Haller, 1978).

Evidence for anaerobic degradation of chlorinated benzoates in the environment is based on studies conducted with aquifer, freshwater and estuarine sediments. 3-CBc is consistently degraded. In various experiments in which 3-CBc (16 to 125 mg l^{-1}) was incubated for the first time with freshwater river, pond lake or marsh sediments, biodegradation has been observed to occur after 2.5 to 8 months (Horowitz et al., 1983; Gibson and Suflita, 1986; Genthner et al., 1989a; Haggblom et al., 1993; vanderWoude et al., 1996). Degradation of 3-CBc required from 3 to 4 months or 6 to 7 months in similar experiments conducted with anaerobic aquifer sediments (Gibson and Suflita, 1986; Kazumi et al., 1995a; Townsend et al., 1997) or with estuarine or salt marsh sediments (Genthner et al., 1989a; Haggblom et al., 1993); respectively. Benzoate has been detected as an intermediate of 3-CBc degradation in anaerobic sediments (Horowitz et al., 1983; Gibson and Suflita, 1986).

On the other hand, 2-CBc is seldom degraded anaerobically in unadapted sediments. In experiments with freshwater or saltwater sediments, 2-CBc is generally not anaerobically degraded in incubation periods of one year (Horowitz et al., 1983; Genthner et al., 1989a; Haggblom et al., 1993; vanderWoude et al., 1996). Likewise, no 2-CBc degradation occurred in iron-reducing aquifer sediments after a half year. 2-CBc degradation only was observed in sediments from environments impacted by industrial effluent, in which case degradation occurred after 2 to 8 months (Genthner et al., 1989a; Haggblom et al., 1993).

4-CBc was persistent to degradation under methanogenic conditions in some sediment samples (Horowitz et al., 1983; Gibson and Suflita, 1986; Genthner et al., 1989a; Haggblom et al., 1993); whereas it was degraded in others sediment samples after prolonged incubations of 2.5 to 12 months (Genthner et al., 1989a; vanderWoude et al., 1996). Several sediment samples only degraded 4-CBc after time periods exceeding 1 to 6 months when incubated with NO_3^- (Genthner et al., 1989a; Haggblom et al., 1993).

Reductive dechlorination of dichlorinated and trichlorinated benzoates is commonly observed in anaerobic sediments. The dechlorination of 3,4-DCBc to 3-CBc and 4-CBc as well as the dechlorination of 3,5-DCBc to 3-CBc was observed in pond and aquifer sediments (Gibson and Suflita, 1986). In lake sediments, 3,5-dichloro-4-aminobenzoate(35DCABc) was reductively dechlorinated to 3-chloro-4-aminobenzoate and, likewise, 3,5-DCBc was converted to 3-CBc after 2 to 3 weeks (Horowitz et al., 1983). The same sediments dechlorinated 2,3,6-TCBc to 2,6-DCBc after 12 to 52 weeks. A large variety of DCBc and TCBc isomers were dechlorinated by freshwater marsh sediments (vanderWoude et al., 1996). Some of the conversions observed were as follows with the acclimation period indicated in the parenthesis: 2,3,5-TCBc $\rightarrow$ 3,5-DCBc (23 d); 2,3,6-TCBc $\rightarrow$ 2,5-DCBc (48 d); 3,5-DCBc $\rightarrow$ 3-CBc (84 d); 2,5-DCBc $\rightarrow$ 3-CBc (92 d); 2,3-DCBc $\rightarrow$ 3-CBc (40 d); and 2,4-DCBc $\rightarrow$ 4-CBc (130 d).

1.2.2. Degradation of chlorobenzoates in engineered systems

Chlorinated benzoates are degraded in engineered systems under both aerobic and anaerobic conditions. Table 1.1 (annex) summarizes the performance of several aerobic bioreactor studies towards the removal of different isomers of chlorobenzoates. Volumetric conversion rates varied from 28 to 10868 g $m^{-3}_{reactor}$ d^{-1}. The highest rates were obtained with *Pseudomonas* sp B13 strains utilizing 3-CBc as a sole carbon and energy source (Muller et al., 1996; Tros et al., 1996a). Strain B13 FR1 SN45P was genetically engineered to metabolize 4-CBc at a high rate in addition to 3-CBc (Muller et al., 1996). In some of the bioreactor studies, chlorobenzoates were cometabolized with glucose, acetate or benzoate as the primary substrate. The highest cometabolic degradation rates were achieved in a sequencing batch reactor with activated sludge as the initial inoculum (Wilderer et al., 1991). In many of the studies listed in Table 1.1 (annex), mineralization of chlorobenzoates during treatment was demonstrated by stoichiometric recoveries of inorganic chloride (Wilderer et al., 1991; Muller et al., 1996; Peys et al., 1997; Urgun-Demirtas et al., 2003).

Table 1.2 (annex) summarizes the performance of several anaerobic bioreactor studies towards the removal of different chlorinated benzoate congeners. Volumetric conversion rates ranged from 28 to 499 mg $l^{-1}_{reactor}$ d^{-1}. The highest rate was achieved in by a microbial consortium utilizing 3-CBc as a sole source of carbon and energy which was immobilized in an anaerobic filter after a prolonged period of enrichment (Fathepure and Tiedje, 1994). High rates were also achieved in a denitrifying bioreactor which utilized 3-CBc as the electron donor (Bae et al., 2004). 3-CBc elimination in the reactor accounted for the electron equivalents of the nitrate removed, confirming that 3-CBc utilization was linked to denitrification. In two other studies, 3-CBc was supplied to bioreactors as the electron donor, but only after an initial enrichment with other electron donating substrates. The chlorinated benzoates were degraded as electron acceptors in the remainder of the studies listed in Table 1.2 (annex) and as such were supplied with electron donating substrates to promote reductive dechlorination. In two studies, the 3-CBc-halorespiring bacterium, *Desulfononile tiedje*, was bioaugmented into the bioreactors (Ahring et al., 1992; El Fantroussi et al., 1999). One of the studies indicated that successful degradation of 3-CBc in an upward-flow anaerobic sludge blanket (UASB) could only be obtained with the bioaugmentation (Ahring et al., 1992). However, in another UASB study, enrichment of the sludge for two months yielded a halorespiring population that could convert 3-CBc without the need for bioaugmentation (Sawayama et al., 2002a, b). Evidence for chlorinated benzoate dechlorination in anaerobic bioreactors is based on the release of chloride or the formation of dechlorinated metabolites such as 2,5-DCBc from 2,3,6-TCBc (Gerritse and Gottschal, 1992). Partially dechlorinated metabolites from the anaerobic conversion of polychlorinated benzoates are completely mineralized in coupled anaerobic-aerobic bioreactors, involving the introduction of air into anaerobic bioreactors which allows for the formation of aerobic conditions adjacent to anaerobic microniches (Gerritse and Gottschal, 1992; Ascon and Lebeault, 1999). Under these conditions, aerobic bacteria were shown to mineralize 2,5-DCBc, which otherwise was persistent under anaerobic conditions (Gerritse and Gottschal, 1992). The initial dechlorination of 2,3,6-TCBc under anaerobic conditions was required since 2,3,6-TCBc resisted degradation under aerobic conditions.

Bioremediation of chlorobenzoates in soils has been investigated in a few studies. Addition of strains specialized in chlorobenzoate degradation was shown to shorten the lag phase prior to the degradation of 3-CBc (Pertsova et al., 1984; Gentry et al., 2001), 4-CBc (Baggi and Zangrossi, 1999) or 2-CBc (Wang et al., 2004) compared to controls with only indigenous microflora. For example, the addition of *Comamonas testosteroni* to a soil containing 1000 mg kg^{-1} 3-CBc enabled complete elimination of 3-CBc within 14 days; whereas only 40% degradation occurred in 28 days in uninoculated controls (Gentry et al., 2001). The addition of the genetically engineered *Pseudomonas putida* strain GN2 containing a plasmid encoding a gene for 2-CBc oxidation, *cbdA*, allowed for the complete removal of 2-CBc (500 mg kg^{-1}) from three different soils within 5 days; whereas uninoculated soils either were unable to degrade 2-CBc or required long lag times (Wang et al., 2004). Degradation of chlorobenzoates was also stimulated by plants in combination with specialized bacterial strains (Siciliano and Germida, 1998). Plant-bacteria associations were shown to degrade 74% of 3-CBc (583 mg kg^{-1}); 56% of 2,3-DCBc (125 mg kg^{-1}) and 46% of 2,5-DCBc (211 mg kg^{-1}) in soil (Siciliano and Germida, 1998).

1.3. Microbiology and biochemistry of chlorinated benzoate biodegradation

Chlorinated benzoates are subject to both aerobic and anaerobic metabolism. Under aerobic conditions, lower chlorinated benzoates can serve as sole electron and carbon sources supporting growth. Additionally, there are several examples in which chlorobenzoates are aerobically cometabolized. Under anaerobic conditions, chlorinated benzoates are subject to reductive dechlorination when suitable electron-donating substrates are available. Several halorespiring bacteria are known which can use chlorobenzoates as electron acceptors to support growth. Lower chlorinated benzoates are also used as a carbon and energy source by anaerobic bacteria.

1.3.1. Aerobic bacterial cometabolism of chlorinated benzoates

Table 1.3 (annex) summarizes literature data on the aerobic cometabolism of chlorinated benzoates. The most widely used primary substrate to support chlorinated benzoate cometabolism is the non-halogenated analogue, benzoate. The literature contains examples of cometabolism of each monochlorinated benzoate as well as cometabolism of several dichlorobenzoates and 2,3,6-TCBc. Aside from benzoate, several other aromatic substrates supported cometabolism such as 4-hydroxybenzoate, *o*-anisate, toluate and benzene. In two examples, either 2-CBc or 4-CBc was utilized as primary substrates to support the cometabolism of other chlorinated benzoates. Glucose also served as a primary substrate in the cometabolism of monochlorinated benzoates. 3-Chlorocatechol (3CC) and 4-chlorocatechol (4CC) are common products of monochlorobenzoate cooxidation. In a similar fashion, 3,5-dichlorocatechol (35DCC) was observed as a product in the cometabolism of 2,3,6-TCBc. Aside from chlorocatechols, chlorinated hydroxybenzoates were sometimes observed as products such as 5-chloro-2,3-dihydroxybenzoic acid (5C23DHBc) from 3CBc cometabolism or 3-chloro-4-hydroxybenzoic acid (3C4HBc) from 3,4-DCBc cometabolism.

1.3.2. Aerobic bacterial growth on chlorinated benzoates as a sole source of carbon and energy

A large variety of bacteria are known which can utilize lower chlorinated benzoates as a sole source of carbon and energy under aerobic conditions. Table 1.4 provides a large listing of aerobic bacterial strains from taxonomically diverse genera attesting to the biodiversity of 3-CBc and 4-CBc utilizing strains. The large biodiversity was also evidenced in a comprehensive screening of 3-CBc-utilizing isolates recovered world-wide from pristine soils (Fulthorpe et al., 1998). Of the 150 isolates, 48 genotypes could be distinguished based on analysis of 16s rRNA genes. The biodiversity is lower in the case of 2-CBc as witnessed by the lower number of strains isolated from only a few genera (Table 1.4). In addition to monochlorinated benzoates, several bacterial strains have been isolated that can grow on 2,3-DCBc, 2,4-DCBc, 2,5-DCBc, 3,4-DCBc, 3,5-DCBc, and 2,3,5-DCBc (Table 1.4).

Evidence for the mineralization of chlorinated benzoates by aerobic bacteria utilizing the compounds as growth substrate is often been based on the stoichiometric release of chloride. Chloride release has been reported for 2-CBc (Zaitsev and Karasevich Yu, 1984; Fetzner et al., 1989a; Sylvestre et al., 1989), 3-CBc (Johnston et al., 1972; Hartmann et al., 1979; Focht and Shelton, 1987; Sahasrabudhe et al., 1988; Hernandez et al., 1991), 4-CBc (Hartmann et al., 1979; Reineke and Knackmuss, 1980; Karasevich and Zaitsev, 1984; Shimao et al., 1989; Hernandez et al., 1991; Chae and Kim, 1997; Kim and Picardal, 2000), 2,5-DCBc (Miguez et al., 1990) and 3,5-DCBc (Hartmann et al., 1979; Reineke and Knackmuss, 1980). Direct measurement of bacterial growth linked to the utilization of the chlorobenzoate provides additional evidence which has been observed in the case of 2-CBc (Sylvestre et al., 1989), 3-CBc (Hartmann et al., 1979; Focht and Shelton, 1987; Hernandez et al., 1991; Muller et al., 1996; Krooneman et al., 1998), 4-CBc (Shimao et al., 1989; Muller et al., 1996; Kim and Picardal, 2000; Yi et al., 2000; Rodrigues et al., 2001), 2,5-DCBc (Miguez et al., 1990) and 3,5-DCBc (Hartmann et al., 1979). Lastly, evidence of biodegradation is also based on the conversion of carbon in chlorobenzoates to CO_2. [^{14}C]3-CBc was converted by 59% to $^{14}CO_2$ in 60 h by *Pseudomonas alcaligenes* C-O when incubated in soil (Focht and Shelton, 1987). In the same study, carbon balances in liquid culture indicated the mineralization of 3-CBc by 75%, with the remainder of the carbon recovered as cell biomass.

An overview of aerobic 3-CBc biodegradation pathways are provided in Figure 1.1. The most commonly reported pathways are initiated by dioxygenases yielding chlorocatechols via intermediate formation of diols. The chlorocatechols are *ortho*-cleaved to yield chloromuconic acids (Schmidt et al., 1980; Haggblom, 1992; Muller et al., 1996). In this pathway, the chlorine group is released as the chloromuconic acids are lactonized to form either *cis* or *trans* 4-carboxymethylenebut-2-ene-1,4-olides. Some microorganisms catalyze the *meta* cleavage of chlorocatechols. The *meta* cleavage of 3-chlorocatechol forms a toxic

intermediate, 2-hydroxy-6-chlorocarbonyl-mucconate, that inactivates the 2,3-dioxygenase responsible for the rupture of the chlorocetchol (Bartels et al., 1984; Mars et al., 1997). O n the other hand, the *meta* cleavage of 4-chlorocatechol results in the formation of 5-chloro-2-hydroxymuconic semialdehyde, which is oxidized to 5-chloro-2-hydroxymuconic acid that is metabolized further to chloroacetate and acetate (McCullar et al., 1994; Arensdorf and Focht, 1995). There are also several aerobic pathways of 3-CBc degradation which do not involve chlorocatechols as intermediates.

Table 1.4. Aerobic bacterial strains capable of growing on chlorinated benzoates as a sole source of carbon and energy.

Bacterial Strain	Congener	References
Alcaligenes denitrificans BRI 3010	2-CBc	(Miguez et al., 1990)
Alcaligenes denitrificans BRI 6011	2-CBc	(Miguez et al., 1990)
Pseudomonas sp CPE2	2-CBc	(Fava et al., 1993; 1996a)
Pseudomonas aeruginosa JB2	2-CBc	(Hickey & Focht, 1990)
Pseudomonas aeruginosa sp 142	2-CBc	(Corbella et al., 2001)
Pseudomonas sp. P111 [A]	2-CBc	(Hernandez et al., 1991)
Pseudomonas cepacia KZ2	2-CBc	(Zaitsev et al., 1991)
Pseudomonas cepacia 2CBS	2-CBc	(Fetzner et al., 1989a; 1989b)
Pseudomonas cepacia	2-CBc	(Zaitsev &Karasevich, 1984)
Pseudomonas sp. B-300	2-CBc	(Sylvestre et al., 1989)
Acinetobacter calcoaceticus	3-CBc	(Zaitsev and Baskunov, 1985)
Alcaligenes sp. BR60 [Po]	3-CBc	(Nakatsu et al., 1991)
Alcaligenes sp. L6	3-CBc	(Krooneman et al., 1996)
Alcaligenes sp. CPE3	3-CBc	(Fava et al., 1993)
Bordetella sp.	3-CBc	(Krooneman et al., 2000)
Burkholderia sp. AZ101	3-CBc	(Gentry et al., 2001)
Pseudomonas sp.	3-CBc	(Johnston et al., 1972)
Comamonas sp.	3-CBc	(Krooneman et al., 2000)
Pseudomonas putida DP4	3-CBc	(Saini & Kahlon, 1998)
Pseudomonas aeruginosa JB2	3-CBc	(Hickey and Focht, 1990)
Pseudomonas aeruginosa	3-CBc	(Krooneman et al., 2000)
Pseudomonas alcaligenes C-0	3-CBc	(Focht & Shelton, 1987)
Pseudomonas sp. B13	3-CBc	(Dorn et al., 1974)
Pseudomonas sp. WR912 [A]	3-CBc	(Hartmann et al., 1979)
Pseudomonas sp. P111 [A]	3-CBc	(Hernandez et al., 1991; Brenner et al., 1993)
Ralstonia eutropha JMP134	3-CBc	(Pieper et al., 1993; Perez-Pantoja et al., 2000)
Ralstonia eutropha JMP134(pJP4)	3-CBc	(Trefault et al., 2002; Perez-Pantoja et al., 2003)
Xanthomonas maltophilia	3-CBc	(Krooneman et al., 2000)
Acinetobacter sp. 4CB1 [Ph]	4-CBc	(Adriaens et al., 1989)
Alcaligenes sp. AL3007 [Ph]	4-CBc	(Zhang et al., 1997)
Alcaligenes sp. BR60 [Po]	4-CBc	(Wyndham et al., 1988; Nakatsu et al., 1991)
Alcaligenes sp. CPE3	4-CBc	(Fava et al., 1993)
Alcaligenes denitrificans NTB-1 [Ph]	4-CBc	(Vandentweel et al., 1987)
Arthrobacter globiformis KZT1 [Ph]	4-CBc	(Zaitsev et al., 1991)
Arthrobacter sp. TM-1 [Ph]	4-CBc	(Marks et al., 1984)
Arthrobacter sp. SU [Ph]	4-CBc	(Ruisinger et al., 1976)
Corynebacterium sepedonicum KZ-4 [Ph]	4-CBc	(Zaitsev & Karasevich, 1985; Romanov &Hausinger, 1996)

Continued on next page

Table 1.4 *(Continued).* Aerobic bacterial strains capable of growing on chlorinated benzoates as a sole source of carbon and energy.

Bacterial Strain	Congener	Reference
Micrococcus sp. HR4 [Ph]	4-CBc	(Yi et al., 2000)
Micrococcus luteus	4-CBc	(Yi et al., 2000)
Micrococcus halobius	4-CBc	(Yi et al., 2000)
Nocardia sp.CBS2	4-CBc	(Klages & Lingens, 1979)
Oerskovia sp. HR3	4-CBc	(Yi et al., 2000)
Pseudomonas sp. S-47 [A]	4-CBc	(Seo et al., 1997)
Pseudomonas sp. DJ-12 [Ph]	4-CBc	(Chae & Kim, 1997)
Pseudomonas sp. P111 [A]	4-CBc	(Hernandez et al., 1991; Brenner et al., 1993)
Pseudomonas sp. WR912 [A]	4-CBc	(Hartmann et al., 1979)
Pseudomonas sp. B13 WR241	4-CBc	(Reineke & Knackmuss, 1980)
Pseudomonas sp. CBS3 [Ph]	4-CBc	(Keil et al., 1981; Loffler & Muller, 1991)
Pseudomonas cepacia P166 [A]	4-CBc	(Arensdorf & Focht, 1995)
Rhodococcus sp. strain RHA1 pRHD34 [Ph]	4-CBc	(Rodrigues et al., 2001)
Sphingomonas paucimobilis BPSI-3 [A]	4-CBc	(Davison et al., 1999)
Alcaligenes denitrificans BRI 3010	23-DCBc	(Miguez et al., 1990)
Alcaligenes denitrificans BRI 6011	23-DCBc	(Miguez et al., 1990)
Pseudomonas sp. P111 [A]	23-DCBc	(Hernandez et al., 1991)
Pseudomonas aeruginosa JB2	23-DCBc	(Hickey & Focht, 1990)
Alcaligenes denitrificans NTB-1 [Ph]	24-DCBc	(Vandentweel et al., 1987)
Alcaligenes denitrificans BRI 6011	24-DCBc	(Miguez et al., 1990)
Corynebacterium sepedonicum KZ-4 [Ph]	24-DCBc	(Zaitsev & Karasevich, 1985; Romanov & Hausinger, 1996)
Pseudomonas aeruginosa sp 142	24-DCBc	(Corbella et al., 2001)
Pseudomonas aeruginosa JB2	25-DCBc	(Hickey and Focht, 1990)
Pseudomonas sp CPE2	25-DCBc	(Fava et al., 1993; 1996a)
Alcaligenes sp. CPE3	34-DCBc	(Fava et al., 1993)
Pseudomonas sp. P111 (P111D)	35-DCBc	(Brenner et al., 1993)
Pseudomonas sp. WR912	35-DCBc	(Hartmann et al., 1979)
Pseudomonas sp. B13 WR941	35-DCBc	(Reineke & Knackmuss, 1980)
Pseudomonas aeruginosa JB2	235-TCBc	(Hickey & Focht, 1990)
Pseudomonas sp. P111 [A]	235-TCBc	(Hernandez et al., 1991)

[A] Dioxygenase attack, dechlorination after ring cleavage.
[Po] Dioxygenase attack, dechlorination prior to ring cleavage.
[Ph] Degradation via 4-chlorobenzoyl-conenzyme A dehalogenase (replaces Cl-group with hydroxyl group).

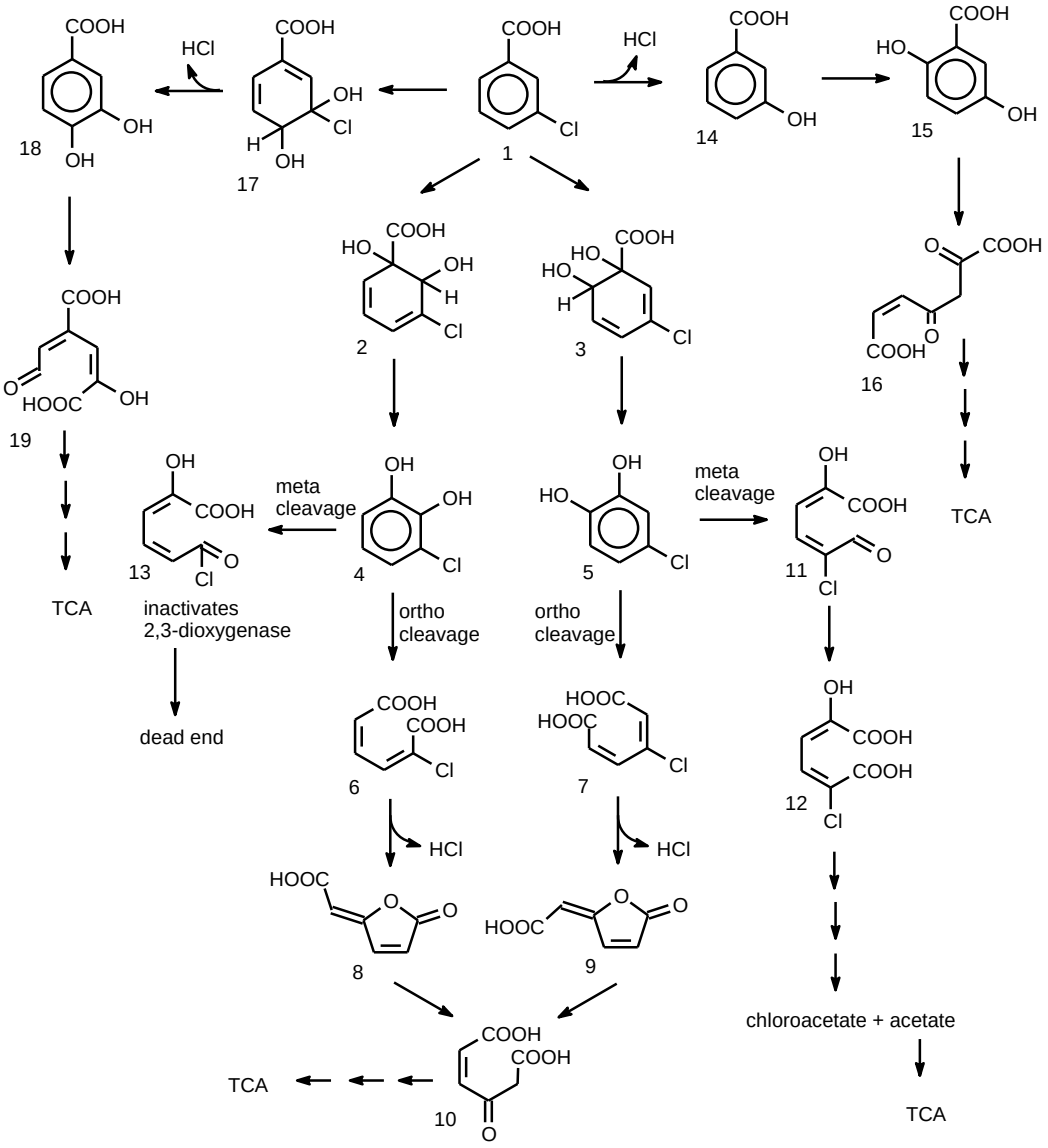

Figure 1.1. *An overview of aerobic 3-CBc biodegradation pathways (see text for literature references). Compounds: 1) 3-chlorobenzoic acid; 2) 3-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid; 3) 5-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid; 4) 3-chlorocatechol; 5) 4-chlorocatechol; 6) 2-chloro-cis,cis-muconic acid; 7) 3-chloro-cis,cis-muconic acid; 8) cis-4-carboxymethylenebut-2-ene-1,4-olide; 9) trans-4-carboxymethylenebut-2-ene-1,4-olide; 10) maleylacetic acid; 11) 5-chloro-2-hydroxymuconic semialdehyde; 12) 5-chloro-2-hydroxymuconic acid; 13) 2-hydroxy-6-chlorocarbonyl-mucconate; 14) 3-hydroxybenzoic acid; 15) gentisic acid; 16) maleylpyruvate; 17) 1-carboxy-3-chloro-3,4-dihydroxycylohexa-1,5-diene; 18) protocatechuic acid; 19) 2-hydroxy-4-carboxymuconic semialdehyde.*

One pathway results in the replacement of the chlorine group by a hydroxyl group to form 3-hydroxybenzoate (Johnston et al., 1972; Krooneman et al., 1996). Another pathway involves the conversion of 3-CBc to protocatechuate (Nakatsu and Wyndham, 1993; man et al., 1996; 2000).

Bacteria responsible for the aerobic biodegradation of 4-CBc utilize either a utilize a pathway similar to that described above involving dioxygenases and the intermediate formation of chlorocatechols (Reineke and Knackmuss, 1980; Haggblom, 1992) or they utilize a unique pathway involving the hydrolytic replacement of the chlorine group to form

4-hydroxybenzoate (Vandentweel et al., 1987; Scholten et al., 1991; Kobayashi et al., 1997; Chae et al., 2000). The majority of 4-CBc degrading bacteria isolated from agricultural soils utilized the hydrolytic pathway; whereas, only one isolate out of twenty utilized the pathway via chlorocatechol (Yi et al., 2000). The hydrolytic pathway used by aerobic bacteria to remove chlorine from 4-CBc involves three enzymes steps as shown in Figure 1.2. 4-CBc is first ligased together with coenzyme A through the formation of a thioester. The adduct, 4-chlorobenzoyl coenzyme A, is dehalogenated by a hydrolytic dehalogenase and, subsequently, the thioester is hydrolyzed to liberate the coenzyme A (Babbitt et al., 1992; Groenewegen et al., 1992; Loffler et al., 1995; Chae et al., 2000; Zhou et al., 2004).

1.3.3. Anaerobic utilization of chlorinated benzoates as a sole source of carbon and energy

Under anaerobic conditions chlorinated benzoates are utilized by both consortia of microorganisms as well as pure cultures to provide energy and/or carbon to support microbial growth. A number of studies have described enrichment cultures which convert 3-chlorobenzoate to CO_2, CH_4 and Cl^- with intermediate formation of benzoate (Suflita et al., 1982; Genthner et al., 1989b; Townsend et al., 1997; Becker et al., 2005, 2006). The pathway described in these studies is shown in Figure 1.3. The measured methane yields from the mineralization of 3-CBc range from 93 to 122% of the theoretical production based on added 3-Cbc, indicating a stoichiometric conversion (Genthner et al., 1989b; Townsend et al., 1997; Becker et al., 2006). Enrichment cultures developed with 3-chlorbenzoate have the capacity to dechlorinate chlorine groups in the *meta* position, consequently 3,5-dichlorobenzoate (with both chlorine groups in the *meta* position) is also completely mineralized (Suflita et al., 1982).

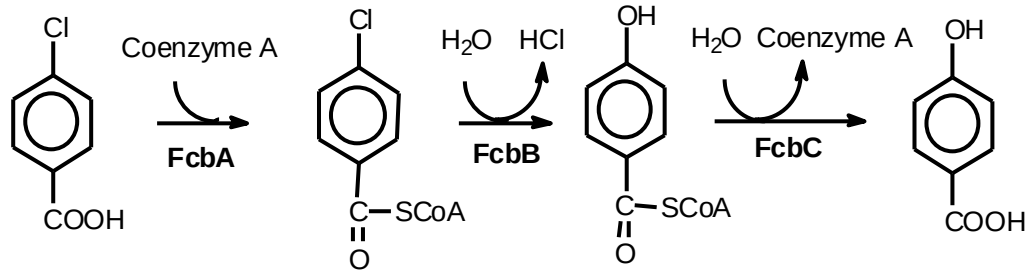

Figure 1.2. *The conversion of 4-CBc to 4-hydroxybenzoate by hydrolytic dechlorination. FcbA = 4-chlorobenzoate-coenzyme A ligase; FcbB = 4-chlorobenzoyl-coenzyme A dehalogenase; FcbB (Ferrer et al., 1986; Chae et al., 2000).*

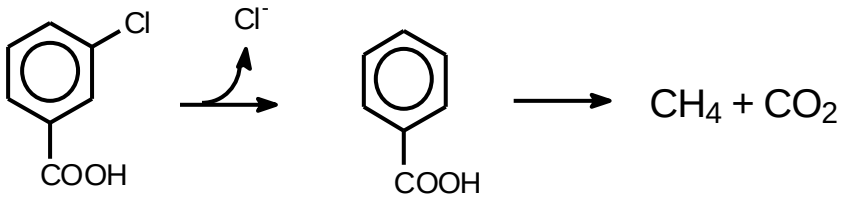

Figure 1.3. *The pathway of anaerobic 3-chlorobenzoate mineralization in methanogenic enrichment cultures (Suflita et al., 1982).*

Thermophilic enrichment cultures have been shown to mineralize 3-CBc at 75°C (Maloney et al., 1997). In addition, to 3-CBc, there is at least one example of a 2-CBc enrichment which completely converts 2-CBc to methane with intermediate formation of benzoate (Genthner et al., 1989b).

Chlorobenzoates are also degraded with alternative electron acceptors under anaerobic conditions. The mineralization of 3-chlorobenzoate under denitrifying, sulfate reducing and iron reducing conditions was inferred from the loss of 3-chlorobenzoate coupled stoichiometrically to NO_3^- loss, SO_4^{2-} loss or Fe^{2+} production (with added iron oxides); respectively, in an enrichment culture derived from the river Nile sediments (Kazumi et al., 1995b). Enrichment cultures from river Nile sediments that could mineralize the other two isomers of chlorobenzoates (2-CBc and 4-CBc) could only be established under denitrifying conditions (Kazumi et al., 1995b). Subsequent work resulted in the isolation of a pure bacteria cultures *Thauera chlorobenzoica* and *Thauera aromatica* that can grow on 3-CBc at the expense of denitrification resulting in the stoichiometric release of Cl^- (Haggblom and Young, 1999; Song et al., 2000; 2001). Additionally, a highly enriched culture was obtained that degraded 4-CBc under denitrifying conditions which contained two bacteria, one closely related to *Thauera aromatica* and another that was closely related to the genera *Limnobacter* and *Ralstonia* (Song et al., 2002). A 3-CBc degrading denitrifying enrichment culture cultivated in a bioreactor was shown to also degrade 4-CBc under denitrifying conditions (Bae et al., 2004).

In several studies the anoxic phototrophic bacterium, *Rhodopseudomonas palustris*, was shown to degrade 3-CBc completely to CO_2 and cell biomass in the presence of light and absence of oxygen as long as benzoic acid was present (Kamal and Wyndham, 1990; Oda et al., 2001; 2004). *Rhodopseudomonas palustris* could be acclimatized to degrade 3-CBc as the sole source of carbon (Oda et al., 2001; 2004). The pathway of 3-CBc degradation in *Rhodopseudomonas palustris* has been elucidated and involves an initial ligation with coenzyme A and subsequent reductive dechlorination of 3-chlorobenzoyl-CoA to benzoyl-CoA, which is further degraded to CO_2 via acetyl-CoA (Egland et al., 2001).

1.3.4. Anaerobic halorespiration of chlorinated benzoates

Certain anaerobic microorganisms utilize chlorobenzoates as an electron acceptor. The oxidation of simple substrates such as hydrogen and organic acids is linked to reductive dechlorination of the chlorine group. In fact, 3-CBc is the first compound for which halorespiration was documented. The growth and ATP production of the bacterium, *Desulfomonile tiedjei* DCB-1, was shown to linked to the reductive dechlorination of 3-CBc to benzoate (Dolfing and Tiedje, 1987; Mohn and Tiedje, 1990; Louie and Mohn, 1999). Aside from 3-CBc, *Desulfomonile tiedjei* DCB-1 is able to halorespire a variety of other chlorobenzoates such as 3,5-DCBc, 2,5-DCBc, 3,4-DCBc, 3-chloro-4-hydroxybenzoate and 3-chloro-4-methyl-benzoate (Shelton and Tiedje, 1984; Deweerd et al., 1990; Mohn and Tiedje, 1990; 1992; Dolfing and Tiedje, 1991). Another strain, *Desulfomonile limimaris* strain DCB-M-T, was isolated from a marine environment and was also shown to link its growth with the reductive dehalogenation of 3-CBc (Sun et al., 2001).

The evidence for halorespiration of chlorinated benzoates by *Desulfomonile tiedjei* DCB-1 is based on several lines of evidence (Louie and Mohn, 1999). The first is that reductive dehalogenation was inhibited by a respiratory quinone inhibitor (Louie and Mohn, 1999), which suggests that a respiratory quinone is part of electron transport chain linked with reductive dehalogenation. Secondly, reductive dehalogenation activity depends on 1,4-naphthoquinone (Deweerd et al., 1990), a possible precursor for a respiratory quinone. Thirdly, the positions of the hydrogenase (when grown on pyruvate) or the formate dehydrogenase (when grown on formate) as well as that of the dehalogenase are consistent with the build up of a proton motive force required for the chemiosmotic production of ATP.

1.4. Microbial kinetics of chlorinated benzoate biodegradation

A summary of the microbial kinetic data available in the literature for chlorinated benzoates is presented in Table 1.5 (annex). A large number of aerobic bacterial strains have been identified that grow on monochlorobenzoates as a carbon and energy source with relatively high rates corresponding to doubling times ranging from 9.9 to 1.6 hours. Fast growth rates are also observed for some dichlorobenzoates, corresponding to doubling times ranging from 30.8 to 1.9 hours. By comparison, growth rates of anaerobic halorespiring bacteria are less rapid compared to aerobic bacteria utilizing chlorobenzoates for carbon and energy. The doubling times of halorespiring bacteria growing on monochlorobenzoates (mostly 3-CBc) as electron acceptors ranged from 231 to 8.4 h. Both aerobes and anaerobes displayed a high affinity for chlorinated benzoates as evidenced by relatively low half velocity constants for growth (k_s) ranging from 0.34 to 31.3 mg l^{-1} for aerobes and 1.1 to 58.3 mg l^{-1} for anaerobes. The specific activities of aerobic bacteria metabolizing mono- and dichlorinated benzoates ranged from several hundred up to 21,600 mg CBc g^{-1} dwt cells d^{-1}. The anaerobic halorespiring bacteria had specific activities that ranged from several hundred up to 3780 mg CBc g^{-1} dwt cells d^{-1}. The biggest difference between aerobes and anaerobes was the cell yield. The aerobes had cell yields ranging from 0.14 to 0.54 g dwt cells g^{-1} CBc consumed; whereas the anaerobes had cell yields ranging from 0.002 to 0.087 g dwt cells g^{-1} CBc converted.

2 Chlorophenols

2.1. Introduction

Chlorophenols include pentachlorophenol (PCP), tetrachlorophenol (TeCP), trichlorophenol (TCP), dichlorophenol (DCP), and chlorophenol (CP). Chlorophenols have been introduced into the environment through their use as biocides. 4-CP was used as an antiseptic for home, hospital and farm use. 2,4-DCP and 2,4,5-TCP were chemical intermediates, especially in the production of the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (WHO, 1989). The annual industrial production of chlorophenols was estimated at 0.2×10^6 metric tonnes in 1989 (WHO, 1989). PCP along with TCP and TeCP isomers were used historically as fungicides in wood preservative formulations (McAllister et al., 1996; Puhakka et al., 2000). PCP was also utilized as an herbicide in rice paddies in Japan (Kuwatsuka and Igarashi, 1975; Watanabe, 1977). Chlorophenols are observed as by-products if chlorine bleaching in the pulp and paper industry (Annachhatre and Gheewala, 1996). Several review articles are available on the biodegradation of chlorinated phenols. Annachhatre and Gheewala (1996) as well as Steiert and Crawford (1985) have authored short reviews on the biodegradation of chlorinated phenols. The pathways and genetic basis of chlorophenol biodegradation were summarized by Solyanikova and Golovleva (2004). Haggblom (1992) provided a review on lower chlorinated phenol biodegradation as part of larger review on the biodegradation of halogenated aromatic pesticides. McAllister *et al*. (1996) has written an extensive review on the biodegradation of PCP. Orser and Lange (1994) reviewed the genetic basis for aerobic PCP degradation.

2.2. Biodegradation of chlorophenols

2.2.1. Degradation of chlorophenols in the environment

The biodegradability of chlorinated phenolic compounds in the natural environment has been considered in a large number of studies. Indigenous microorganisms in forest soil were shown to degrade 2-CP (Lallai and Mura, 2004). Many chlorinated phenols incubated under aerobic conditions with a Canadian clay loam grassland soils were observed to be biodegraded (2-, 3-, and 4-CP; 2,4-, 2,6- and 3,4-DCP; 2,4,6-, and 2,4,5-TCP; and PCP) (Baker and Mayfield, 1980). However, 3,4,5-TCP and 2,3,4,5-TeCP were not degraded. Compounds lacking *meta*-substituted chlorines were completely degraded within a few days or weeks. Twenty-six soil samples from a screening of 170 samples were found to readily degrade 2,4,6-TCP, indicating a wide distribution of chlorinated phenol degraders in the environment (Kiyohara et al., 1989). Four chlorinated phenolic compounds (2-CP, 2,4,6-TCP, 2,4-DCP and PCP) were degraded by two subsurface soil samples collected form two different states in the U.S.A. (Smith and Novak, 1987). High concentrations of 2,4,6-TCP up to 5000 mg kg^{-1} were readily degraded by pristine forest soil, previously not exposed to chlorophenols (Sanchez et al., 2004). Additionally, the soil microbial community structure was not altered by doses of 2,4,6-TCP as high as 500 mg kg^{-1}. PCP supplied at 200 mg kg^{-1} was degraded by indigenous microorganisms in pristine grassland soils at an average rate of 1.2 mg kg^{-1} d^{-1} (Mahmood et al., 2005). Molecular fingerprinting of the soil microbial population was utilized to demonstrate the enrichment of PCP-degrading strains of bacteria. [^{13}C]PCP spiked into the soil could be recovered in the ribosomal RNA of the enriched microorganisms, demonstrating their ability to utilize PCP as a carbon source (Mahmood et al., 2005). PCP degradation was observed in Japanese soils where the compound has been used extensively as an herbicide (Kuwatsuka and Igarashi, 1975; Watanabe, 1977, 1978). Even soils from plots not exposed to the herbicide were shown to degraded PCP and contain 10^2 PCP-decomposing colonies/g soil (Watanabe, 1977, 1978).

Ten wetland soil samples from 5 states in the U.S.A. were examined for their ability to degrade PCP under aerobic conditions (D'Angelo and Reddy, 2000). Five of the samples caused PCP biodegradation with first-order rate constants of 0.139 to 0.338 d^{-1}. Approximately 75 to 100% of the PCP was eliminated in 30 days. Initially, pentachloroanisole was observed as a minor product in the soil incubations, indicating that some methylation had occurred.

Aquifer sediments from a PCP-contaminated site were shown to readily degraded PCP, 40 mg PCP l^{-1} was removed in 90 days; whereas no removal occurred in poisoned controls (Kao et al., 2004). Aerobic degradation of chlorinated phenols is also observed in surface water samples. Radiolabeled 3,4-DCP, 2,4,5-TCP and PCP were shown to be mineralized to CO_2 in two types of lake water samples (Larsson and Lemkemeier, 1989). Surface water from an estuarine river was shown to degrade 4-CP with a first-order rate constant of 0.384 d^{-1} (Hwang et al., 1986). Field-scale river channel mesocosms built on the Mississippi River were used to study the biodegradation of PCP (Pignatello et al., 1983). River channels exposed for several weeks to PCP developed microbial populations responsible for the biological mineralization of PCP to CO_2. Most of the PCP-degrading activity was due to microorganisms attached to rock or aquatic plant surfaces.

Abundant evidence for the anaerobic degradation of chlorinated phenols in the natural environment is also reported. Gibson and Suflita (1986) observed biodegradation of 4-CP, 2,4-DCP, 2,5-DCP, 3,4-DCP and 2,4,5-TCP in pond sediments. Additionally, 4-CP, 2,4-DCP and 2,5-DCP were biodegraded in methanogenic aquifer sediments. Lower chlorinated phenols and phenols were detected as intermediates of degradation. The biotransformation of 2-CP was observed in Lake Michigan sediments (Becker et al., 1999). All isomers of monochlorinated phenol were degraded by river sediments of the upper Hudson River in New York State under both methanogenic and sulfate reducing conditions (Haggblom et al., 1993). Phenol, benzoate and 3-chlorobenzoate were observed as intermediates of the biotransformation. Several investigators have observed the biodegradation of various DCP isomers in freshwater sediments. Susarla *et al.* (1997a) measured the kinetics of anaerobic degradation of all 6 DCP isomers in lake sediments from Japan and observed first-order rate constants ranging from 0.018 d^{-1} for 3,4-DCP to 0.091 d^{-1} for 2,5-DCP. Monochlorophenol and phenol were observed as intermediates in the degradation. Three isomers of DCP, 2,3-DCP, 2,4-DCP and 2,6-DCP were anaerobically degraded with first-order rates constants of 0.017 to 0.033 d^{-1} and 0.087 to 0.139 d^{-1}, respectively; for two different pond sediments collected in Southeastern U.S. that were not previously exposed to the chemicals (Hale et al., 1990). Lake sediment samples from the Southeastern U.S. were also shown to anaerobically degrade 2,4-DCP, with 4-CP occurring as a major intermediate in the process (Kohring et al., 1989; Zhang and Wiegel, 1990).

Anaerobic PCP degradation was observed in five of ten wetland soil samples incubated under methanogenic conditions (D'Angelo and Reddy, 2000). The first-order rate constants of PCP degradation ranged from 0.082 to 0.38 d^{-1}. TeCP, TCP and DCP were observed as intermediates in the anaerobic degradation of PCP. Tetrachloro-1,4-hydroquinone was shown to be dehydroxylated and dechlorinated by a mixed culture derived from Baltimore harbor sediments to yield 2,3,5-TCP (Milliken et al., 2004).

Several studies evaluated the anaerobic degradation of chlorinated phenols in marine and estuarine sediments. Anoxic estuarine sediments from Taiwan were found to degrade 2-CP, 3-CP, 4-CP. 2,5-DCP, 3,4-DCP, 3,5- DCP and PCP (Liu et al., 1996). PCP degradation was shown to be linked to sulfate reduction. Estuarine sediments from the lower Hudson River in New York were able to degrade 2-CP, 3-CP and 4-CP under sulfate reducing conditions (Haggblom et al., 1993). Estuarine sediments from the Chesapeake Bay biotransformed 2,4-DCP to 4-CP when incubated under methanogenic conditions (Warner et al., 2002). Similarly, 2,4-DCP and 3,4-DCP were dechlorinated by marine sediments to 4-CP and 3-CP, respectively (Boothe et al., 1997). The monochlorinated phenol intermediates did not persist since they were also degraded in the marine sediments. Marine sediments collected off the coast of Sweden were observed to dechlorinate PCP, yielding 2,3,4,5-TeCP, 3,4,5-TCP and 3,5-DCP as products (Abrahamsson and Klick, 1991).

Municipal anaerobic digester sludge not previously exposed chlorinated phenols also has been shown to degrade chlorophenols. All monochlorinated isomers were degraded by anaerobically-digested primary sewage sludge (Boyd and Shelton, 1984). Additionally, dichlorophenols (except 3,4-DCP and 3,5-DCP) were degraded via monochlorophenol intermediates. Municipal anaerobic digester sludge was shown to dechlorinate 2-CP to phenol and selective inhibitors demonstrated that the population responsible for the dechlorination reaction was associated with syntrophic acetogens (Basu et al., 2005). The ability of municipal anaerobic digester sludge to degrade 12 chlorinated phenolic compounds was tested and 10 of the compounds were shown to be degraded (Wang et al., 1998). The digester sludge culture did not degrade PCP or 2,3,4,6-TeCP. In another study, all nineteen possible isomers of chlorinated phenols were degraded by methanogenic sludge with first-order rate constants ranging from 0.00046 to 0.161 d^{-1} (Takeuchi et al., 2000). Dechlorination of *ortho*-chlorines occurred at the fastest rate; whereas dechlorination of *para*-chlorines occurred at the slowest rate. First-order rate constants decreased with decreasing number of chlorine substituents.

2.2.2. Degradation of chlorophenols in engineered systems

Numerous studies are available on the biological treatment of chlorinated phenols in effluents, groundwater, soil and compost. Biotreatment schemes are based on aerobic, anaerobic and combined anaerobic-aerobic schemes.

Table 2.1 (annex) summarizes the results on the aerobic biodegradation of PCP and other higher chlorinated phenols in bioreactors. The highest rates of PCP biodegradation were observed in a reactor with immobilized biofilms developed on soil retained in geotextiles. The reactor was able to convert 22.8 g PCP $l^{-1}_{reactor}$ d^{-1} (Karamanev and Samson, 1998). To obtain these high conversion rates, high effluent concentrations PCP concentrations of 10 to 20 mg l^{-1} were required to overcome biofilm diffusion limitations and thus only partial removal of PCP was achieved. Likewise, the high rates were not sustainable for long due to biomass clogging of geotextile. Volumetric rates in all the other aerobic bioreactor studies were one to two orders of magnitude lower; however, the reactor operation in most cases was sustainable for long periods of time. Acclimated biomass in activated sludge reactors removed PCP at volumetric rates of 40 to 100 mg PCP $l^{-1}_{reactor}$ d^{-1} (Edgehill and Finn, 1983a; Moos et al., 1983). ^{14}C-Labelled PCP was used to confirm that PCP was being mineralized to CO_2 and incorporated into cell biomass (Moos et al., 1983). Several studies conducted in fluidized bed reactors evaluated the treatment of lower concentrations of chlorophenols, characteristic of contaminated groundwater (Valo et al., 1990; Jarvinen et al., 1994; Puhakka et al., 1995b; Melin et al., 1997; Melin et al., 1998a; Melin et al., 1998b). At room temperature, mixtures of PCP, 2,3,4,6-TeCP and 2,4,6-TCP were mineralized by 99.9% at loading rates of 1 g chlorophenols $l^{-1}_{reactor}$ d^{-1} when supplied as the sole source of carbon and energy. At groundwater temperatures of 7°C, similar levels of elimination were achieved at loading rates of 0.74 g chlorophenols $l^{-1}_{reactor}$ d^{-1} and 80% removal was achieved at 2.1 g chlorophenols $l^{-1}_{reactor}$ d^{-1} (Jarvinen et al., 1994; Puhakka et al., 1995b). The recovery of inorganic chloride was in agreement with the complete mineralization of the chlorophenols (Jarvinen et al., 1994). Aerobic fluidized bed reactors were also effective in treating PCP as the sole carbon and energy source. PCP (2.5 mg l^{-1}) was removed with greater than 99% efficiency at loading rates of 0.12 g PCP $l^{-1}_{reactor}$ d^{-1}. One study considered the biodegradation of PCP in groundwater at low aqueous concentrations of less than 1 mg l^{-1} (Schmidt et al., 1999). PCP was eliminated to a concentration below 0.002 mg l^{-1} in batch reactors.

In addition to aerobic reactors based on bacterial biomass, several studies have employed white-rot fungi to promote chlorophenol biodegradation. Bioreactors composed of wood chips seeded with the white-rot fungus, *Phanerochaete chrysosporium*, eliminated 4-CP and 2,4-DCP (Yum and Peirce, 1998b, a). A bioreactor with the white-rot fungus, *Trametes versicolor*, immobilized on polyurethane foam, effectively removed PCP (Pallerla and Chambers, 1998).

Table 2.2 (annex) summarizes the results on the anaerobic biodegradation of PCP in bioreactors. Several research groups have examined the biodegradation of PCP in upward-flow anaerobic sludge blanket (UASB) reactors supplied with electron donating

substrates to promote reductive dechlorination (Hendriksen et al., 1992; Wu et al., 1993; Tartakovsky et al., 2001a; Ye et al., 2004; Lanthier et al., 2005; Shen et al., 2006). In these reactors, the sludge contains microorganisms enriched over extended periods of time to PCP dechlorination. High efficiencies of PCP removal are observed at volumetric loadings loading ranging from 2.2 to 220 mg PCP $l^{-1}_{reactor}\,d^{-1}$ Similar results were also obtained in UASB reactors bioaugmented with PCP-dechlorinating microorganisms *Desulfitobacterium frappieri* PCP-1 (Tartakovsky et al., 1999) or strain DCB-2 (Christiansen and Ahring, 1996b) so that extended periods of microbial enrichment could be avoided. Specific PCP-degrading activities of biomass recovered from the UASB reactors ranged from 1.7 to 14.6 mg PCP g^{-1} VSS d^{-1} (Hendriksen et al., 1992; Wu et al., 1993; Tartakovsky et al., 2001a). In addition to UASB reactors, anaerobic fluidized bed reactors and fixed-film reactors effectively removed PCP at volumetric loadings of 5 to 313 mg PCP $l^{-1}_{reactor}\,d^{-1}$. Molecular ecology techniques were used to describe the bacterial population in the fixed-film reactor that was operated with PCP for 225 days (Lanthier et al., 2005). A known chlorophenol halorespiring species, *Desulfitobacterium hafniense*, accounted for 19% of the microorganisms in the biofilms. Evidence of PCP mineralization was obtained by conversion of [^{14}C]PCP to radiolabeled CO_2 and CH_4 with biomass granules recovered from a UASB reactor (Wu et al., 1993) or by high recovery of the chlorine in PCP as inorganic chloride in the reactor effluents (Hendriksen et al., 1992; Mohn et al., 1999; Tartakovsky et al., 1999). In some cases, lower chlorinated phenols were observed as products of PCP degradation (Magar et al., 1999; Tartakovsky et al., 1999; 2001a). PCP removal at volumetric loads of up to 0.5 g PCP g PCP $l^{-1}_{reactor}\,d^{-1}$ were observed in a fluidized bed reactor with a granular activated carbon (GAC) support matrix. However, the contribution due to biodegradation alone versus GAC adsorption is not clear since a GAC replacement rate of 0.25 to 0.5 g $l^{-1}_{reactor}\,d^{-1}$ was utilized (Khodadoust et al., 1997).

A laboratory-scale municipal sludge digester was set up to study PCP degradation fed initially at a concentration 8 mg PCP l^{-1} (Chen et al., 2006). After 11 days, *ortho*-dechlorination of PCP was observed as evidenced by the formation of 2,3,4,5-TeCP and 2,4,5-TCP. After 13 days, evidence for *para*-dechlorination was observed based on the detection of 3,5-DCP. The first evidence of *meta*-dechlorination occurred on day 36 with the detection of 3-CP (Chen et al., 2006).

Anaerobic bioreactors have also been utilized to degrade lower chlorinated phenols. Three studies evaluated UASB reactors for the degradation of 2,4-DCP with readily biodegradable cosubstrates (Ning et al., 1997; Kennedy et al., 2001; Sponza and Ulukoy, 2005). Volumetric loading of up to 144 mg $l^{-1}_{reactor}\,d^{-1}$ with 78.7% 24-DCP have been reported when molasses was utilized as the electron donor (Sponza and Ulukoy, 2005). The specific 2,4-DCP-degrading activity of the granules obtained from the UASB reactors was 4.1 mg DCP g^{-1} VSS d^{-1} (Ning et al., 1997). The anaerobic degradation of five isomers of TCP was observed in laboratory-scale UASB reactors treating each compound individually (Droste et al., 1998). A hybrid expanded granular sludge bed (EGSB) – anaerobic filter was evaluated for anaerobic 2,4,6-TCP degradation at 15°C with ethanol and volatile fatty acids as electron donor (Collins et al., 2005). Treatment was tested up to volumetric loads of 25 mg $l^{-1}_{reactor}\,d^{-1}$ with nearly 100% removal efficiency when the load was gradually increased. Specific TCP-degrading activity of the biofilm was 5.3 and 8.5 mg TCP g^{-1} VSS d^{-1} at 15 and 37°C, respectively (Collins et al., 2005). Chlorinated catechols, chlorinated vanillins and chlorinated guaiacols in bleaching effluent were eliminated in a UASB by greater than 80% when supplemented with cosubstrate (Parker et al., 1993). A mixture of chlorophenols, chloroguaiacols and chloroveratroles were also treated in a UASB reactor supplemented with acetate, methanol and sucrose as electron donor (Woods et al., 1989). The removal efficiency ranged from 64.9 to 96.5% for different chlorophenolic compounds.

The degradation of 3-CP or a mixture of 2-CP, 3-CP and 4-CP as sole sources of carbon and energy was evaluated in hybrid UASB anaerobic filter reactors (Krumme and Boyd, 1988). The reactors could accommodate a volumetric loading of 20 g CP per m^3 reactor per day with greater than 90% removal of chlorophenols. Conversion of at least 40% of the ^{14}C-labelled 4-CP fed to the bioreactor to methane and CO_2 was demonstrated. Most anaerobic bioreactor studies have evaluated chlorophenol degradation under methanogenic conditions. However, one study researched the treatment of 4-CP in a fluidized bed reactor under denitrifying conditions (Melin et al., 1993). From 1.9 to 2.5 mg of 4-CP were degraded per mg of NO_3^--N reduced which was slightly above the theoretical

value of 1.8. The mass balance based on inorganic chloride released in the reactor provided further evidence of 4-CP degradation in the bioreactor.

Several researchers have evaluated the biodegradation of chlorinated phenols in a system combining anaerobic-aerobic bioreactors. A sequence of a UASB reactor and an aerobic suspended growth reactor eliminated 86% of 2,4-DCP in the presence of glucose; whereas, each individual reactor accomplished either 52 or 78% removal, respectively (Atuanya et al., 2000). A similar sequence of UASB and an aerobic continuously stirred tank reactor (CSTR) resulted in 87% removal of 2,4-DCP supplied at 120 mg l^{-1} (Sponza and Ulukoy, 2005). A sequence of an anaerobic filter combined with an aerobic bioreactor was utilized to treat 2,4,6-TCP supplied at 13 mg l^{-1} (Armenante et al., 1999). In the anaerobic reactor, a mixture of organic acids (acetate, formate and succinate) supplied electrons to dechlorinate 2,4,6-TCP to 4-CP. Subsequently in the aerobic reactor 4-CP was degraded.

Many biological treatment methods are utilized to clean up soils contaminated with chlorinated phenols. One approach towards aerobic bioremediation is the addition of nutrients and organic amendments to enhance the indigenous microbial population. Contaminated soil (1365 mg kg^{-1} PCP) from a wood treatment facility was treated in a biopile with inorganic or organic nutrient treatments (Miller et al., 2004). The best results were obtained with municipal solid waste compost treatment, which reduced the levels of PCP by 82% after one year. Compost was mixed with chlorophenol-contaminated soil from a sawmill in a pilot-scale soil bioremediation experiment (Laine and Jorgensen, 1997). Over 90% of the chlorophenols were removed during the composting period of 8 weeks with highly contaminated soils of 850 mg PCP kg^{-1} soil. A parallel bench-scale soil composting system spiked with ^{14}C-PCP demonstrated that 60% of the PCP was mineralized in 4 weeks (Laine and Jorgensen, 1997). In a similar study, contaminated soil from a sawmill site was mixed with farm animal manure and composted (Jaspers et al., 2002). Disappearance of PCP (250 mg/kg dry weight) was rapid and virtually complete within 6 days. Evidence for anaerobic reductive dechlorination during composting was observed based on the accumulation of lower chlorinated phenols, which were subsequently eliminated. ^{14}C-PCP was added to the compost and was shown to be mineralized by 85% within 5 days. Even without organic additives, the indigenous microflora mineralized 82% of 30 mg ^{14}C-PCP per kg added to soil in 7 months (Miethling and Karlson, 1996). The maximum rates of degradation reached 0.5 mg PCP kg^{-1} soil d^{-1}. PCP degradation in forest soil was enhanced from 55% to 90% by addition of aerobic activated sludge in the soil (Lallai and Mura, 2004).

Another approach towards bioremediation is the addition of chlorophenol-degrading microorganisms to soil. The addition of 10^6 PCP-utilizing *Arthrobacter* cells per g of dry soil reduced the half-life for PCP removal in soils from 2 weeks to less than 1day (Edgehill and Finn, 1983b). Addition of *Sphingomonas chlorophenolica* RA2 (10^8 cells g^{-1} of dry soil) greatly accelerated the mineralization of 30 mg ^{14}C-PCP per kg added to soil (Miethling and Karlson, 1996). The PCP was mineralized by 80% within one month; whereas non-inoculated controls required 7 months. Additionally, *Sphingomonas chlorophenolica* RA2 enabled mineralization of soils spiked with 100 mg [^{14}C]PCP kg^{-1}; whereas only negligible mineralization occurred in soils lacking inoculation. The success of bioremediation depends on the survival of the PCP-degrading strains in the soil. Recombinant strain *Sphingomonas chlorophenolica* was constructed by inserting a green fluorescent protein gene as a reported in site of a PCP-degrading gene (Oh et al., 2004). The recombinant strain survived at leats 6 days in non-sterile soil and 28 days in sterile soil.

In some studies, bioremediation of PCP contaminated soils is stimulated by addition of mixed cultures enriched for PCP degradation. A PCP-degrading enrichment culture degraded 99% of PCP in a sandy loam soil contaminated with 450 mg PCP kg^{-1} in 130 days. PCP-remediated soil enriched with PCP-degrading microorganisms (10^2 cells g^{-1} of dry soil) rapidly mineralized 6 mg ^{14}C-PCP per kg soil by 56% in 4 weeks (Laine and Jorgensen, 1996). In other studies, various white-rot fungal strains have been utilized as inoculum to stimulate PCP degradation. The white-rot fungus, *Phanerochaete sordida*, was utilized in a field-scale test to treat a wood preservation contaminated soil containing 1058 mg PCP kg^{-1} soil (Lamar et al., 1994). The inoculated plots decreased the PCP concentration by 64% in 20 weeks; whereas uninoculated control plots decreased the PCP concentration by 18 to 26%. In a similar field study, *Phanerochaete sordida*, *Phanerochaete chrysisporium* and *Trametes hirsuta* removed 89, 72 and 55% of PCP from a soil

contaminated with 672 mg PCP kg^{-1} soil (Lamar et al., 1993). Degradation of PCP was evaluated with the white-rot fungus, *Lentinula edodes*, in sterile and non-sterile soils (Okeke et al., 1997). In a 10 week long experiment, 99 and 42% elimination of PCP was observed with *Lentinula edodes* inoculated into sterile and non-sterile soil. During white-rot fungal PCP bioremediation in soil, a large fraction of the PCP is converted to non-extractable bound-residue and a small fraction (8-13%) is converted to the methylation product, pentachloroanisole (Lamar and Dietrich, 1990; Tuomela et al., 1999). In one experiment, partial mineralization of ^{14}C-PCP to CO_2 (29%) was observed (Tuomela et al., 1999). New white-rot fungal isolates discovered in New Zealand were tested for their ability to mineralize radiolabeled PCP spiked at a rate of 200 mg kg^{-1} into highly contaminated PCP soil (Walter et al., 2004). Up to 41% of the ^{14}C label was converted to $^{14}CO_2$ whereas in non-inoculated controls only 6% was mineralized by indigenous soil microflora.

Besides aerobic methods, anaerobic strategies are utilized for the bioremediation of soils contaminated with chlorinated phenols. Anaerobic sewage sludge applied to soil at a rate of 5 g dry weight/kg was used to enhance the degradation of PCP under anaerobic conditions (Mikesell and Boyd, 1988). PCP present at 10 to 30 mg kg^{-1} was degraded to less than 0.5 mg kg^{-1} in within a month. The PCP was converted to mono-, di- and trichlorophenols, which accounted for 80% of the PCP eliminated. In another study, a halorespiring bacterium, *Desulfitobacterium frappieri* strain PCP-1, was added to PCP contaminated soil slurries to promote reductive dechlorination (Beaudet et al., 1998). The PCP removal was greater than 90% in soils contaminated with up to 200 mg PCP per kg. The main product of the biotransformation was 3-CP. In the sequel study, *Desulfitobacterium frappieri* strain PCP-1 was inoculated into rotating biological contactor reactors (RBC) treating soil slurries of PCP contaminated soil (Lanthier et al., 2000). PCP was completely removed in less than 9 days in soils contaminated with 189 mg PCP kg^{-1} of soil. In non-inoculated reactors, the indigenous microorganisms of some soils were also able to degrade PCP.

2.3. Microbiology and biochemistry of chlorophenol biodegradation

Chlorophenols are subject to both aerobic and anaerobic metabolism. Under aerobic conditions, both lower and higher chlorinated phenols can serve as sole electron and carbon sources supporting growth and in some cases chlorophenols are aerobically cometabolized. Under anaerobic conditions, chlorinated phenols are subject to reductive dechlorination when suitable electron-donating substrates are available. Halorespiring bacteria are known which can use chlorophenols as electron acceptors to support growth. Lower chlorinated phenols are also used as a carbon and energy source by anaerobic microbial consortia.

2.3.1. Aerobic bacterial cometabolism of chlorophenols

Several examples of aerobic cometabolism of chlorinated phenols are reported. A *Nocardia* strain and three *Pseudomonas* strains isolated from soil with benzene as a sole carbon source were observed to cometabolize chlorophenols (Haider et al., 1974) when precultivated on benzene. ^{14}C-Labelled 2-CP and 4-CP were extensively mineralized to $^{14}CO_2$ by 60 to 85% within 7 days. Under the same conditions, two of the *Pseudomonas* partially mineralized DCP and TCP supplied as isomer mixtures. Toluene-grown cells of *Pseudomonas putida* were also shown to effectively oxidized 2-CP, 3-CP, 4-CP, 2,4-DCP, 2,3-DCP, 2,5-DCP, 3,4-DCP and 2,4,5-TCP, generating in most cases chlorocatechols as intermediates (Spain and Gibson, 1988). Methylstyrene served as primary substrate in the oxidation of 2-CP and 4-CP by *Pseudomonas putida* (Bestetti et al., 1992). *O*-Cresol served as a primary substrate for the oxidation of 2,4-DCP by *Alcaligenes eutrophus* JMP222 to 3,5-dichlorocatechol (Koh et al., 1997). Benzoate-induced cells of *Rhodococcus erythropolis* M1 cometabolized 2-CP, 4-CP and 2,4-DCP. In several studies the analogue substrate, phenol, has been utilized by various bacterial strains as the primary substrate to support the cometabolism of 2-CP (Cobos-Vasconcelos et al., 2006; Loh and Wu, 2006), 4-CP (Krug et al., 1985; Li et al., 1991; Saez and Rittmann, 1991; Kim and Hao, 1999; Kim et al., 2002; Cobos-Vasconcelos et al., 2006; Loh and Wu, 2006) or 2,4-DCP (Beltrame et al., 1982; Cobos-Vasconcelos et al., 2006). There are also examples of chlorophenols serving as primary substrates allowing for the cometabolism of other chlorophenols. *Pseudomonas* strain B13 cometabolized 3-CP and 2-CP with 4-CP as growth substrate (Knackmuss and Hellwig, 1978). 3-CP served as a primary substrate supporting 3,5-DCP cometabolism (Liu et al., 1991) and PCP served as a primary substrate supporting 3,4,5-TCP and 2,3,5,6-TeCP cometabolism (Liu et al., 1991). 2,4,6-Trichlorophenol served as a growth substrate of either *Azobacter* sp., *Streptomyces rochei* or *Pseudomonas pickettii* to support the cooxidation of a large number of other chlorophenols (Li et al., 1991; Golovleva et al., 1992; Kiyohara et al., 1992). Dichlorophenoxy acetic acid or trichlorophenoxyacetic acid-grown cells of *Arthrobacter* or *Pseudomonas cepacia* also supported the degradation of numerous chlorophenols (Bollag et al., 1968b; Kilbane et al., 1982; Karns et al., 1983). Finally, ammonia in a nitrifying bioreactor served as a primary substrate supporting the cooxidation of 2,4,6-TCP (Nevalainen et al., 1993). PCP-grown cells of the PCP-degrading bacterium, *Sphingomonas chlorophenolica*, have the ability to mineralize 2,3,6-TCP, 2,4,6-TCP and 2,3,4,6-TeCP as evidenced by chloride release (Yang et al., 2005).

The primary substrates considered above are all expected to induce either dioxygenases or monooxygenases facilitating the co-oxidation of the cometabolized chlorophenols. However, primary substrates such as sugar, which do not require oxygenases for metabolism, have also been implicated in the cometabolism of 2-CP (Haider et al., 1974), 4-CP (Haider et al., 1974; Wang and Loh, 1999), 2,4-DCP (Beltrame et al., 1982) and PCP (Banerji and Bajpai, 1994; Yang et al., 2005). Sugars as primary substrate may support cometabolism by generating NADH required by oxygenases (Wang and Loh, 1999).

2.3.2. Aerobic bacterial growth on chlorophenols as a sole source of carbon and energy

A large variety of bacteria are known which can utilize chlorophenols as a carbon and energy source under aerobic conditions. The earliest reports of bacterial utilization of chlorophenols include those of Chu and Kirsch (1972) and Tyler and Finn (1974). Chu and Kirsch (1972)) described a bacterial strain (KC 3) capable of mineralizing [^{14}C]PCP to ^{14}CO2 when supplied as a sole carbon source. PCP mineralization was shown to be linked to cell growth. Tyler and Finn (1974) described *Pseudomonas* sp. strain NCIB9340 that grew on 2,4-DCP with a maximum rate of 2.88 d^{-1}. Since then a wide variety of bacterial strains have been shown to utilize chlorophenols, examples of which are shown in Table 2.3 The table lists taxonomic names of the isolates as reported in the original manuscripts. Recent evidence suggests that many of the aerobic chlorophenol-degrading strains belong to the genera, *Mycobacterium* and *Sphingomonas*. *Rhodococcus chlorophenolicum* PCP-1 and *Rhodoccocus* sp. strain CG-1 and CP-2 should be classified as *Mycobacterium chlorophenolicum* (Haggblom and Valo, 1995). *Arthrobacter* sp. strain ATCC33790, *Flavobacterium* sp. strain ATCC39723 and *Pseudomonas* sp RA2 and SR3 should be classified as *Sphingomonas chlorophenolica* (Nohynek et al., 1995; Ederer et al., 1997). *Alcaligenes eutrophus* JMP134(pJP4) has been reclassified as *Ralstonia eutropha* JMP134(pJP4) (Coenye et al., 1999), and *Comamonas testosteroni* as *Herbaspirillum chlorophenolicum* (Im et al., 2004).

There is extensive evidence that chlorophenols are mineralized by bacteria that utilize the compounds as a carbon and energy source. The evidence is based on the stoichiometric release of inorganic chloride (Tyler and Finn, 1974; Edgehill and Finn, 1982; Saber and Crawford, 1985; Li et al., 1991; Radehaus and Schmidt, 1992; Resnick and Chapman, 1994; Mannisto et al., 1999; Hollender et al., 2000; Yang et al., 2005), the concomitant production of biomass linked to chlorophenol utilization (Tyler and Finn, 1974; Edgehill and Finn, 1982; Radehaus and Schmidt, 1992; Hu et al., 1994; Resnick and Chapman, 1994; Rutgers et al., 1997) or the conversion of ^{14}C-labeled chlorophenols to $^{14}CO_2$ (Chu and Kirsch, 1972; Saber and Crawford, 1985; Apajalahti and Salkinoja Salonen, 1986; Haggblom et al., 1988b).
Two main strategies are used to degrade chlorophenols by aerobic bacteria utilizing these compounds as a carbon and energy source (Solyanikova and Golovleva, 2004). Lower chlorinated phenols (1 to 2 chlorine substituents) are initially attacked by monooxygenases yielding chlorocatechols as the first intermediates (chlorocatechol pathway), which are subject to ring cleavage prior to dechlorination. On the other hand, polychlorinated phenols (3 to 5 chlorines) are converted to chlorohydroquinones as the initial intermediates (hydroquinone pathway). Subsequent reactions progressively remove chlorines from the ring prior to ring cleavage.

The chlorocatechol pathway will be exemplified for the chlorophenol, 2,4-DCP, as shown in Figure 2.1. 2,4-DCP attack is initiated by a monooxygenases forming 3,5-dichlorocatechol (Bollag et al., 1968b; Finkel'shtein et al., 2000). The chlorocatechol is *ortho* cleaved yielding 2,4-dichloromuconic acid (Bollag et al., 1968a; Tiedje et al., 1969). The first dechlorination takes when a lactonizing enzyme converts the dichloromuconic acid to 2-chloro-4-carboxymethylene but-2-enolide (Bollag et al., 1968a; Tiedje et al., 1969; Sharpee et al., 1973).

Table 2.3. Aerobic bacterial strains capable of growing on chlorinated phenols as a sole source of carbon and energy.

Bacterial Strain	Congener	Reference
Alcaligenes sp. strain A7-2	2-CP	(Schwien & Schmidt, 1982; Menke & Rehm, 1992)
Alcaligenes xylosoxidans strain JH1	2-CP	(Hollender et al., 2000)
Pseudomonas pickettii strain LD1	2-CP	(Fava et al., 1995; Kafkewitz et al., 1996)
Rhodococcus opacus strain 1G	2-CP	(Finkel'shtein et al., 2000)
Streptomyces rochei strain 303	2-CP	(Golovleva et al., 1992)
Pseudomonas pickettii strain LD1	3-CP	(Fava et al., 1995)
Rhodococcus opacus strain 1G	3-CP	(Finkel'shtein et al., 2000)
Alcaligenes xylosoxidans strain JH1	3-CP	(Hollender et al., 2000)
Pseudomonas sp. Strain B-13	4-CP	(Knackmuss & Hellwig, 1978)
Pseudomonas pickettii strain LD1	4-CP	(Fava et al., 1995; Kafkewitz et al., 1996)
Rhodococcus opacus strain 1G	4-CP	(Finkel'shtein et al., 2000)
Alcaligenes sp. strain A7-2	4-CP	(Schwien & Schmidt, 1982; Menke & Rehm, 1992)
Alcaligenes xylosoxidans strain JH1	4-CP	(Hollender et al., 2000)
Arthrobacter ureafaciens strain CPR706	4-CP	(Bae et al., 1997b)
Arthrobacter chlorophenolicus strain A6	4-CP	(Westerberg et al., 2000; Nordin et al., 2005)
Comamonas testosteroni strain CPW301	4-CP	(Bae et al., 1997a)
Pseudomonas sp strain DP-4	24-DCP	(Tarao & Seto, 2000)
Rhodococcus opacus strain 1G	24-DCP	(Finkel'shtein et al., 2000)
Rhodococcus erythropolis	24-DCP	(Goswami et al., 2002)
Pseudomonas sp strain NCIB9340	24-DCP	(Tyler and Finn, 1974)
Streptomyces rochei strain 303	24-DCP	(Golovleva et al., 1992)
Streptomyces rochei strain 303	26-DCP	(Golovleva et al., 1992)
Sphingomonas sp. strain P5	26-DCP	(Rutgers et al., 1997)
Arthrobacter strain NC	246-TCP	(Stanlake & Finn, 1982)
Pseudomonas saccharophila	246-TCP	(Puhakka et al., 1995a)
Pseudomonas pickettii	246-TCP	(Kiyohara et al., 1992)
Rhodopseudomonas sp. strain K13	246-TCP	(Mannisto et al., 1999)
Nocardioides sp. strain K44	246-TCP	(Mannisto et al., 1999; Mannisto et al., 2001)
Unidentified strain K112	246-TCP	(Mannisto et al., 1999)
Sphingomonas strains K74 and MT1	246-TCP	(Mannisto et al., 2001)
Alcaligenes eutrophus JMP134(pJP4)	246-TCP	(Valenzuela et al., 1997)
Ralstonia eutropha JMP134(pJP4)	246-TCP	(Matus et al., 2003; Xun & Webster, 2004)
Azotobacter sp strain Gp1	246-TCP	(Li et al., 1991)
Streptomyces rochei 303	246-TCP	(Golovleva et al., 1992)
Novosphingobium lentum strain MT1	246-TCP	(Tiirola et al., 2005)

Continued on next page

Table 2.3 (*Continued*). Aerobic bacterial strains capable of growing on chlorinated phenols as a sole source of carbon and energy.

Bacterial Strain	Congener	Reference
Rhodococcus chlorophenolicus	2345-TeCP	(Apajalahti & Salkinoja Salonen, 1986; 1987b)
Rhodococcus chlorophenolicus	234-TCP	(Apajalahti & Salkinoja Salonen, 1986)
Mycobacterium sp. strain CG-2	235-TCP	(Haggblom et al., 1988b)
Rhodococcus chlorophenolicus	235-TCP	(Apajalahti & Salkinoja Salonen, 1986; 1987b)
Rhodococcus sp. CP-2 and CG1	235-TCP	(Haggblom et al., 1988b)
Sphingomonas sp. strain P5	236-TCP	(Rutgers et al., 1997)
Pseudomonas saccharophila	2346-TeCP	(Puhakka et al., 1995a)
Rhodopseudomonas sp. K13	2346-TeCP	(Mannisto et al., 1999)
Nocardioides sp. strain K44	2346-TeCP	(Mannisto et al., 1999; 2001)
Unidentified strain K112	2346-TeCP	(Mannisto et al., 1999)
Sphingomonas K74 and MT1	2346-TeCP	(Mannisto et al., 2001)
Sphingomonas sp. strain P5	2346-TeCP	(Rutgers et al., 1997)
Rhodococcus chlorophenolicus	2346-TeCP	(Apajalahti & Salkinoja Salonen, 1986; 1987b)
Novosphingobium lentum MT1	2346-TeCP	(Tiirola et al., 2005)
Mycobacterium sp. strain CG-2	2356-TeCP	(Haggblom et al., 1988b)
Rh. chlorophenolicus PCP-1	2356-TeCP	(Apajalahti & Salkinoja Salonen, 1986; 1987b)
Rhodococcus sp. CP-2 and CG1	2356-TeCP	(Haggblom et al., 1988b)
Pseudomonas spp. UG25 and UG30	PCP	(Leung et al., 1997)
Pseudomonas sp. RA2	PCP	(Radehaus & Schmidt, 1992)
Pseudomonas sp. strain SR3	PCP	(Resnick &Chapman, 1994)
Pseudomonas sp. strain IST103	PCP	(Thakur et al., 2001; 2002)
Pseudomonas mendocina NSYSU	PCP	(Kao et al., 2005)
Mycobacterium sp. strain CG-2	PCP	(Haggblom et al., 1988b)
Mycobacterium chlorophenolicum PCP-1	PCP	(Wittmann et al., 1998)
Sphingomonas sp. strain P5	PCP	(Rutgers et al., 1997)
Sphingomonas chlorophenolica RA2	PCP	(Nohynek et al., 1995; Ederer et al., 1997; Wittmann et al., 1998)
Novosphingobium lentum MT1	PCP	(Tiirola et al., 2005)
Sphingomonas chlorophenolica	PCP	(Yang et al., 2006)
Rhodococcus chlorophenolicus PCP-1	PCP	(Apajalahti et al., 1986; Apajalahti & Salkinoja Salonen, 1986; 1987b)
Rhodococcus sp. CP-2 and CG1	PCP	(Haggblom et al., 1988b)
Strain KC-3	PCP	(Chu and Kirsch, 1972)
Flavobacterium sp ATCC39723	PCP	(Hu et al., 1994; Orser & Lange, 1994)
Flavobacterium sp.	PCP	(Gonzalez & Hu, 1991)
Flavobacterium strains	PCP	(Saber & Crawford, 1985; Steiert & Crawford, 1986)
Arthrobacter strain NC	PCP	(Stanlake & Finn, 1982)

The butenolide is converted to 2-chloromaleylacetic acid which is further metabolized to succinate (Tiedje et al., 1969). The 2,4-dichlorophenol monooxygenases of *Ralstonia eutropha* JMP134 (formerly *Alcaligenes eutrophus*) and from an unidentified bacterial strain S1 were purified and characterized as a heterodimer and a homotetramer, respectively (Farhana and New, 1997; Makdessi and Lechner, 1997). Like other chlorophenol hydroxylases described in the literature (Solyanikova and Golovleva, 2004), 2,4-dichlorophenol hydroxylases lack heme groups and contain FAD as the prosthetic group.

In most strains studied, the conversion of chlorocatechols generally proceeds via *ortho*-cleavage. The alternative pathway via *meta*-cleavage is less common because the 2,3-dioxygenase is inactivated by 3-chlorocatechols (3-CC) and the cleavage product from 4-CC, 5-chloro-2-oxymuconic semialdehyde, is toxic to bacteria (Solyanikova and Golovleva, 2004). Nevertheless, a few bacterial strains are able to degrade lower chlorinated phenols *meta*-cleavage (Bae et al., 1997a; Koh et al., 1997). A 2,3-dioxygenase has been isolated from *Pseudomonas putida* GJ31 which can catalyzes the *meta*-cleavage of 3-CC without being inactivated and resulting in the release of the chloro-group as chloride (Kaschabek et al., 1998).

The other main pathway of chlorophenol degradation is via chlorohydroquinone intermediates. The chlorohydroquinone pathway will be exemplified with PCP, as shown in Figure 2.2. The pathway begins with a hydroxylation in the *para* position resulting in the formation of *p*-tetrachlorohydroquinone (TeCHQ) (Steiert and Crawford, 1986; Apajalahti and Salkinojasalonen, 1987b; Fetzner, 1998; Solyanikova and Golovleva, 2004). In *Sphingomonas chlorophenolica* ATCC 39723 (formerly *Flavobacterium* sp), the hydroxylation is carried out by PCP 4-monooxygenase (Pcp B) which is a soluble flavoprotein requiring NADPH (Xun et al., 1992a; Orser and Lange, 1994; Tiirola et al., 2002). Pcp B momooxygenase requires oxygen from O_2 (Xun et al., 1992a). Similar monooxygenases are found in strains of other PCP-degrading aerobic bacteria (Orser and Lange, 1994; Leung et al., 1997; Thakur et al., 2002). TeCHQ is sequentially dechlorinated in two steps to 2,6-dichloro-1,4-hydroquinone (2,6-DCHQ) by a reductive dehalogenase, which is a glutathione S-transferase (GST), known as Pcp C (Xun et al., 1992b; Orser et al., 1993). 2,6-DCHQ is oxidized by 2,6-DCHQ 1,2-dioxygenase (Pcp A) which requires O_2 and results in the formation 2-chloromaleylacetate as well as the liberation of one of the chlorogroups as chloride (Ohtsubo et al., 1999; Xu et al., 1999; Xun et al., 1999). Recently, a hydroxyquinone hydratase was discovered in *Sphingobium chlorophenolicum* which converts hydroxyl-1,4-quinone to 1,2,4,5-tetrahydroxybenzene, an intermediate that is subsequently auto-oxidized to 2,5-dihydroxyquinone in the presence of O_2 (Bohuslavek et al., 2005). The hydratase may provide an alternative pathway of metabolizing the aromatic ring.

In *Mycobacterium chlorophenolicum* PCP1 and *Mycobacterium fortuitum* CG-2 (formerly *Rhodoccocus* strains), PCP hydroxylation to TeCHQ is carried out by a membrane bound cytochrome P-450 type enzyme (Uotila et al., 1991; 1992). The hydroxylation reaction can use oxygen from H_2O based as evidenced fromm experiments with ^{18}O labelled H_2O (Apajalahti and Salkinoja Salonen, 1987b). TeCHQ is subsequently converted to dichloro-1,2,4-trihydroxybenzene by a sequence of hydrolytic and reductive dechlorinations without any accumulation of a trichlorinated intermediate (Apajalahti and Salkinoja Salonen, 1987a). The dichloro-1,2,4-trihydroxybenzene metabolite is then subject to two reductive dehalogenation steps leading to formation of the nonchlorinated metabolite 1,2,4-trihydroxybenzene (Apajalahti and Salkinoja Salonen, 1987a).

Several unique pathways of chlorophenol degradation by aerobic bacteria have come to light. 4-CP degradation by *Arthrobacter ureafaciens* strain CPR706 and *Arthrobacter chlorophenolicus* strain A6, is initiated by a dechlorination yielding 1,4-hydroquinone as the intermediate (Bae et al., 1997b; Nordin et al., 2005). The proposed pathway involves the conversion of 4-CP to hydroquinone to hydroxyquinol to maleylacetate (Nordin et al., 2005). A ring cleaving hydroxyquinol dioxygenase is a key enzyme in the pathway. When a gene coding for the dioxygenase is disrupted, *Arthrobacter chlorophenolicus* has negligible growth on 4-CP (Nordin et al., 2005). The monooxygenase from *Ralstonia eutropha* strain JMP134(pJP4) catalyzes successive dechlorination reactions of 2,4,6-TCP to 2,6-dichlorohydroquinone and then to 6-chlorohydroxyquinol prior to ring cleavage by a hydroxyquinol dioxygenase (Matus et al., 2003). A unique property of the monooxygenase is that the second dechlorination reaction does not require O_2, instead the dechlorination of

2,6-dichlorohydroquinone is catalyzed by a hydrolytic activity associated with the monooxygenase (Xun and Webster, 2004).

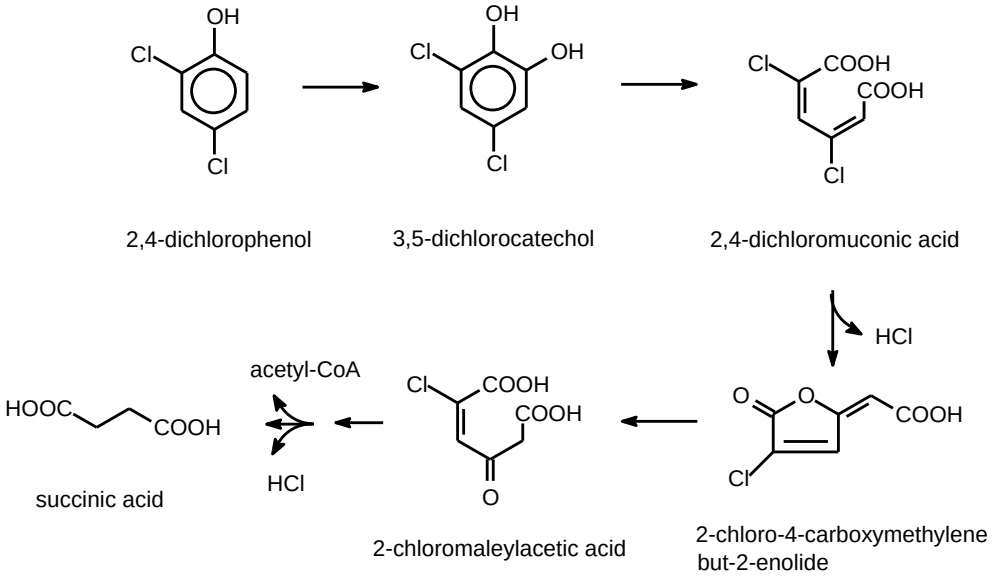

Figure 2.1. *Proposed pathway of the aerobic degradation of 2,4-dichlorophenol by bacteria (Bollag et al., 1968a; 1968b; Tiedje et al., 1969; Sharpee et al., 1973; Farhana and New, 1997; Ennik-Maarsen, 1999; Finkel'shtein et al., 2000).*

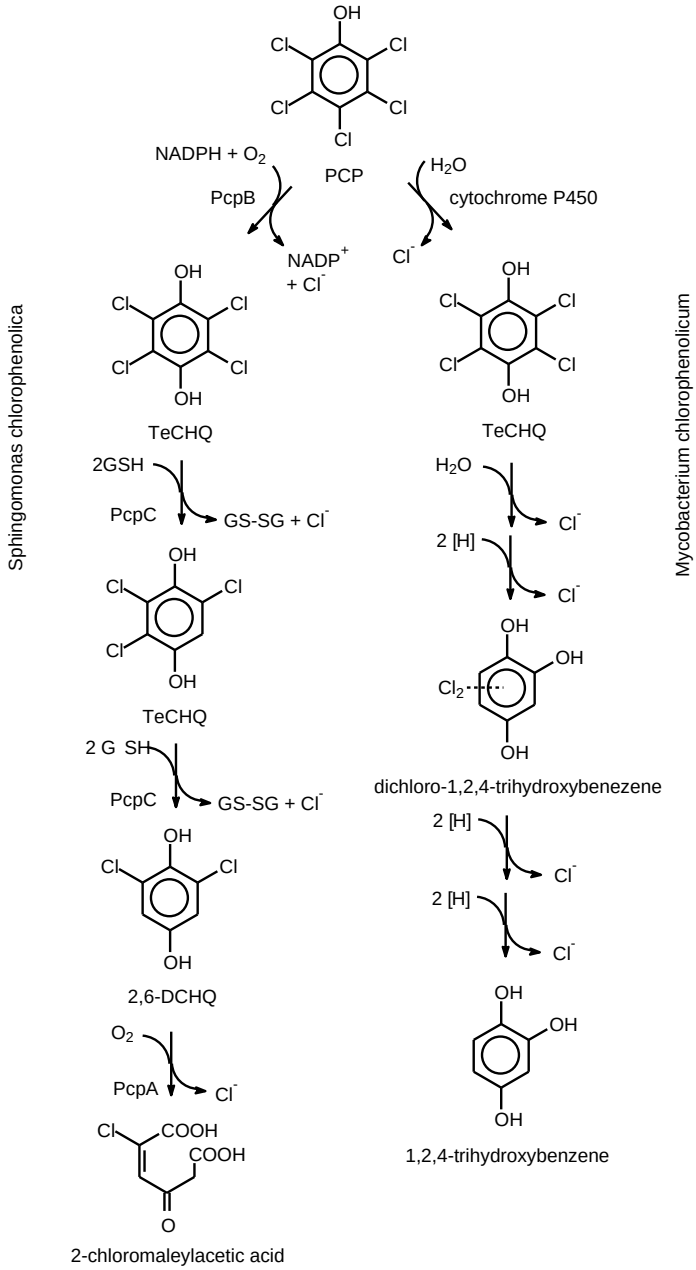

Figure 2.2. *Proposed pathway of the aerobic degradation of pentachlorophenol by bacteria. Pathway on left is for Sphingomonas (Steiert and Crawford, 1986) and pathway on right is for Mycobacterium (Uotila et al., 1991; 1992).*

Several aerobic bacteria implicated in the rapid degradation of higher chlorinated phenols are known to convert chlorophenols to methylated biotransformation products. *Mycobacterium* strains were shown to O-methylate a variety of chlorophenols (Haggblom et al., 1988b). The preferred substrates of the methylation were hydroxyl groups doubly flanked by chlorine groups. Chlorinated hydroquinones were readily O-methylated (Haggblom et al., 1988a), accounting for the occurrence of methylated chlorohydroquinone intermediates during PCP biodegradation (Suzuki, 1983). Direct methylation of PCP resulted in the formation of pentachloroanisole in soil (D'Angelo and Reddy, 2000).

2.3.3. Aerobic fungal degradation of chlorophenols

Many fungi and yeasts are capable of cometabolizing chlorophenols. A wide variety of fungi from different taxanomic groups (Ascomycetes, Basidiomycetes, Zygomycetes, Deuteromycetes) were shown to have the capacity to remove PCP in an extensive screening program (Seiglemurandi et al., 1991; 1992; Benoitguyod et al., 1994). Phenol-degrading strains of the fungus, *Penicillium* and a phenol-degrading yeast, *Candida maltosa* oxidized monochlorinated phenols (Polnisch et al., 1992; Hofrichter et al., 1993; 1994; Marr et al., 1996). 4-Chlorophenol was converted to 4-chlorocatechol, 3-chloropehnol was converted to chlorohydroquinone, 4-chlorocatechol and 5-chlorocatechol as well as chloropyrogallol. The chlorocatechol intermediates were oxidized further, 4-chlorocatechol was oxidized and dehalogenated to 4-carboxymethylenebut-2-ene-4-olide; whereas, 3-chlorocatechol was oxidized to 2-chloromuconic acid (Polnisch et al., 1992; Hofrichter et al., 1994). *Candida albicans* PDY-07, a strain isolated from activated sludge, was able to grow on 4-CP as a sole source of carbon and energy (Wen et al., 2006). The soil fungus, *Trichoderma harzianum*, converted PCP to pentachloroanisole (Rigot and Matsumura, 2002). A soil fungus, *Mortierella* sp. was shown to biotransform 24-DCP via two pathways (Nakagawa et al., 2006). One pathway involved hydroxylation and methylation yielding 2,4-dichloroguaiacol; whereas the other pathway resulted in the replacement of the 4-chloro-group with a hydroxyl group and subsequent dechlorination of the 2-chlorogroup to yield hydroquinone. *Aspergillus awamori* strain NRRL 3112 degraded 2,4-DCP supplied at high concentrations up to 3 g l^{-1} (Stoilova et al., 2006).

Wood-degrading fungi are well established as excellent degraders of chlorophenols. These fungi are divided into two categories: brown-rot fungi, which degrade wood polysaccharides but lack lignin-degrading enzymes, and white-rot fungi, which posses both lignin- and polysaccharide-degrading enzymes and thus are able to completely degrade wood. The brown-rot fungi, *Gloeophyllum striatum* and *Gloeophyllum trabeum*, degraded 2,4-dichlorophenol and pentachlorophenol up to 54% and 27% respectively, based on ^{14}C-labeled substrate converted to $^{14}CO_2$ (Fahr et al., 1999). A Fenton-type reaction mechanism was suggested based on the formation of 4-chlorocatechol and 3,5-dichlorocatechol from the incubation of 2,4-DCP with *Gloeophyllum striatum*, since similar metabolites were produced with Fenton's reagent and the reaction could be inhibited by a hydroxyl radical scavenger, mannitol (Schlosser et al., 2000).

The literature is particularly rich in studies on chlorophenol degradation by white-rot fungi due to the involvement of lignin-degrading enzymes in the process. A comprehensive review of chlorophenol degradation by white-rot fungi was recently provided by Field (2003). In whole cultures, a high degree of chlorophenol compound mineralization is reported. The extent of [^{14}C]pentachlorophenol (PCP) mineralization to $^{14}CO_2$ has been reported up to 70% (Mileski et al., 1988; Lin et al., 1990) and the mineralization of PCP-organochlorine to chloride has been reported up to 62% (Alleman et al., 1995; Aiken and Logan, 1996). Similarly, mineralization of [^{14}C]2,4,6-TCP (Reddy et al., 1998), [^{14}C]2,4,5-TCP (Joshi and Gold, 1993) and [^{14}C]2,4-DCP (Valli and Gold, 1991) to $^{14}CO_2$ by 58, 61 and 50%, respectively, was observed. Recently, newly isolated white-rot fungal strains from New Zealand where also shown to mineralize PCP (Walter et al., 2004). These included a *Trametes versicolor* strain that mineralized radiolabeled PCP by 17% in 30 days.

Chlorophenol degradation in white-rot fungi is initiated by extracellular ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (phenol oxidase). LiP and MnP were shown to oxidize PCP to tetrachloro-1,4-benzoquinone (TeCBQ) (Hammel and Tardone, 1988; Reddy and Gold, 2000) or 2,4,6-TCP to 2,6-dichloro-1,4-benzoquinone (TCBQ) (Hammel and Tardone, 1988; Reddy et al., 1998), releasing the chlorines in the *para* position as chloride. The chlorinated benzoquinones are subject to reduction by quinone reductase activity of fungal cells forming chlorinated hydroquinones such as TeCHQ in the case of PCP degradation (Reddy and Gold, 2000). The degradation of chlorinated hydroquinones in white-rot fungi proceeds via two general pathways. In one pathway, cycles of hydroquinone oxidation by extracellular ligninolytic enzymes and subsequent quinone reduction by cells result in the hydroxylation and dechlorination of the chlorinated phenols leading to the formation of tri- and tetrahydroxybenzenes (Valli and Gold, 1991; Joshi and Gold, 1993; Reddy et al., 1998). In the other pathway, TeCHQ is progressively reductively dechlorinated by the combined activity of glutathione-S-transferase and glutathione conjugate reductase (Reddy and Gold,

2000, 2001) in a fashion similar to the bacteria, *e.g. Sphingomonas*. Reductive dechlorination eventually leads to formation of the non-chlorinated 1,4-hydroquinone (Reddy and Gold, 2000), which is subsequently oxidized to yield 1,2,4-trihydroxybenzene (Reddy et al., 1998).

White-rot fungi are also effective in O-methylating chlorinated phenols. Pentachloroanisole formation from PCP metabolism is well established (Lamar and Dietrich, 1990; Okeke et al., 1997; Tuomela et al., 1999; Chung et al., 2001). Also the conversion of to 3,4- and 2,4-dichlorophenols to their respective anisoles has been reported (Coulter et al., 1993; Deschler et al., 1998). During degradation of chlorinated phenols, chlorinated hydroquinone intermediates are occasionally subject to methylation, leading to the formation of chlorinated 1,4-dimethoxybenzenes or chlorinated 4-methoxyphenols (Valli and Gold, 1991; Reddy et al., 1998; Reddy and Gold, 2000).

Finally it should be noted that ligninolytic enzymes are also effective in catalyzing the oxidative coupling of chlorophenols (Sjoblad and Bollag, 1977; Dec and Bollag, 1994; Ruttimann Johnson and Lamar, 1996; Ullah et al., 2000). The reactions are particularly prevalent if the extracellular oxidative enzymes are functioning in the absence of fungal cells. The polymerization reactions result in the incorporation of chlorophenols into humic like polymers (Hatcher et al., 1993).

2.3.4. Anaerobic biodegradation of chlorinated phenols

Chlorinated phenols are readily metabolized by bacteria under anaerobic conditions. Anaerobic conditions favor the reductive dechlorination, which results from the displacement of chloro-groups by hydrogen atoms. Reductive dechlorination generally requires the input of electron donating substrates. For most of the studies it is impossible to discern whether the reductive dechlorination is cometabolic or beneficial to microorganisms. In a few cases, reductive dechlorination is known to be linked to growth due to the use of the chlorinated phenols as electron acceptors (halorespiration). Likewise, there are a few examples in which chlorinated phenols are clearly the carbon and energy source (the electron donor) to microorganisms which will be discussed first.

2.3.5. Anaerobic metabolism of chlorophenols serving as carbon and energy source

Several examples are known in which chlorinated phenols are completely degraded when supplied as sole carbon and energy sources to microbial enrichments cultures. Anaerobic bioreactors have been operated under methanogenic conditions with monochlorinated phenols as the only substrate for extended periods of time ranging from 190 to 400 days. In these experiments, mineralization of 3-CP and 4-CP to CO_2 and CH_4 was demonstrated by mass balances or by the use of ^{14}C-labeled substrates (Krumme and Boyd, 1988). Sulfate-reducing consortia enriched from estuarine sediment were maintained on either 2-CP, 3-CP, or 4-CP as the only source of carbon and energy for over 5 years (Haggblom and Young, 1995). Detailed studies on the stoichiometry of the 4-CP-degrading enrichment culture established that sulfate reduction concomitant with 4-CP degradation accounted for 81% of the theoretical expected from the complete oxidation of the chlorophenol, clearly suggesting 4-CP oxidation was linked to sulfate reduction. Sulfate or other sulfoxy ions (sulfite or thiosulfate) was required as an electron acceptor for 4-CP degradation by the enrichment. Phenol was observed as a temporal intermediate during 4-CP degradation (Haggblom, 1998) and phenol is readily metabolized by the enrichment (Haggblom and Young, 1995). Sulfate-reducing enrichment cultures capable of degrading 2-CP or 4-CP which were derived from Hudson River sediments were also shown to couple the mineralization of the chlorophenols to sulfate reduction (Haggblom et al., 1993). Iron-reducing 2-CP, 3-CP or 4-CP enrichment cultures were developed from Hudson River sediments (Kazumi et al., 1995). Fe^{2+} recovered from Fe^{3+} reduction could be completely accounted for by the chlorophenol oxidation, providing strong evidence that the chlorophenol degradation was linked to iron reduction. Only recently has chlorophenol degradation linked to denitrification been demonstrated with an enrichment culture utilizing 2-CP derived from activated sludge (Bae et al., 2002). The consumption of nitrate was

equivalent to the expected amount based on the stoichiometry of 2-CP denitrification. In the absence of nitrate, 2-CP was not degraded.

Enrichment cultures from freshwater sediments degrading 2-CP or 3-CP under methanogenic conditions converted the chlorophenols to methane and CO_2 with temporal accumulation of phenol and benzoate as intermediates (Genthner et al., 1989b). Similar methanogenic enrichment cultures developed from municipal digester sludge mineralized radiolabeled [^{14}C]4-CP, [^{14}C]2-CP and [^{14}C]2,4-DCP by greater than 90% to $^{14}CH_4$ and $^{14}CO_2$ (Boyd and Shelton, 1984). The results taken as a whole suggest that chlorophenols utilized as a carbon and energy source are first reductively dechlorinated to phenol. Subsequent phenol mineralization provides the energy and carbon to support growth as well as electrons to support the initial dechlorination. The presence of benzoate as an intermediate is in keeping with the accepted route of anaerobic phenol degradation in which phenol is carboxylated in the *para* position and subsequently dehydroxylated to yield benzoate (Londry and Fedorak, 1992; 1993; Bisaillon et al., 1993; Letourneau et al., 1995). In fact, 2-chlorophenol is sometimes carboxylated and dehydroxylated prior to reductive dechlorination, resulting in the formation of 3-chlorobenzoate as an intermediate (Bisaillon et al., 1993; Becker et al., 1999; Ennik-Maarsen, 1999). Pathways of 2-CP and 4-CP biodegradation are shown in Figure 2.3.

2.3.6. Anaerobic metabolism of chlorophenols by mixed cultures and enrichment cultures

There are many studies carried out with enrichment cultures demonstrating the reductive dechlorination of chlorophenols. In such studies, it is often difficult to determine if the reductive dechlorination is cometabolic or serving as an electron acceptor to support growth of halorespiring bacteria. However, a recent study revealed that biofilms from an anaerobic bioreactor enriched with PCP-dechlorinating organisms contained 19% halorespiring microorganisms (Lanthier et al., 2005). Intermediates from the reductive dechlorination of PCP in enrichment cultures with added electron donor are summarized in Table 2.4 (annex). The most common reductive dechlorination pattern encountered with PCP is one in which there are initial *ortho*-dechlorinations, followed by a *para*-dechlorination, followed by *meta*-dechlorinations leading to phenol. This dechlorination pattern would result in a PCP degradation pathway illustrated in Figure 2.4. There are also exceptions to this pattern, for example *para*-dechlorination of PCP occurred prior to *ortho*-dechlorination which was followed by *meta-dechlorination* to phenol in a culture previously enriched on 3,4-DCP (Bryant et al., 1991). PCP dechlorination initiated by *meta*-dechlorination was observed in a fluidized bed reactor, leading to the accumulation of 2,4,6-TCP which was subsequently *ortho*- and *para*-dechlorinated to phenol (Mohn et al., 1999). Based on the use of [^{14}C]PCP, it has been established that PCP is ultimately converted to CH_4 and CO_2 (Mikesell and Boyd, 1986; Wu et al., 1993), most likely due to utilization of the dechlorinated phenol.

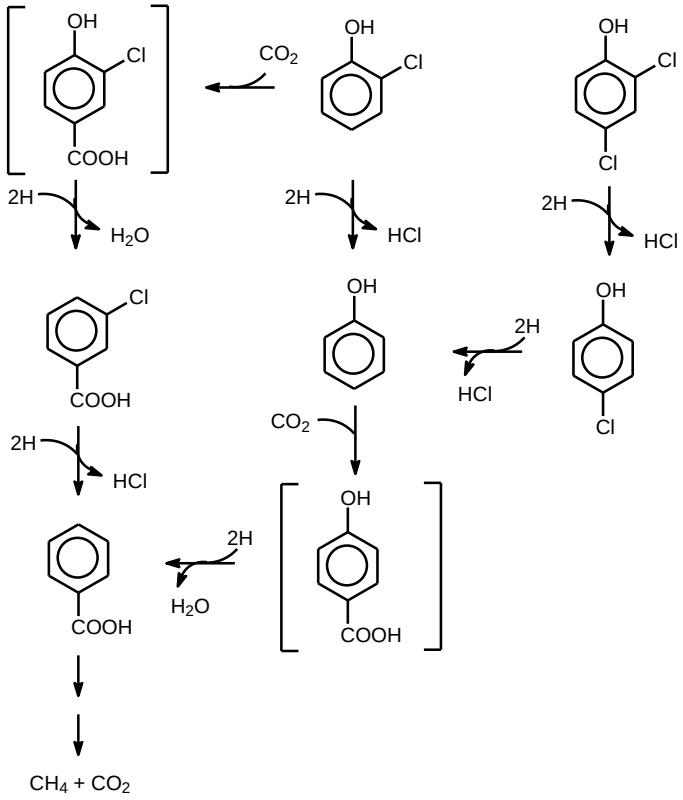

Figure 2.3. *Pathways of anaerobic 2-CP, 4-CP and 2,4-DCP biodegradation (Becker et al., 1999; Bisaillon et al., 1993; Boyd and Shelton, 1984; Chang et al., 1996; Genthner et al., 1989; Larsen et al., 1991; Madsen and Aamand, 1991: Orser et al.. 1993: Puhakka et al.. 2000: Zhang and Wiegel. 1990: Xun*

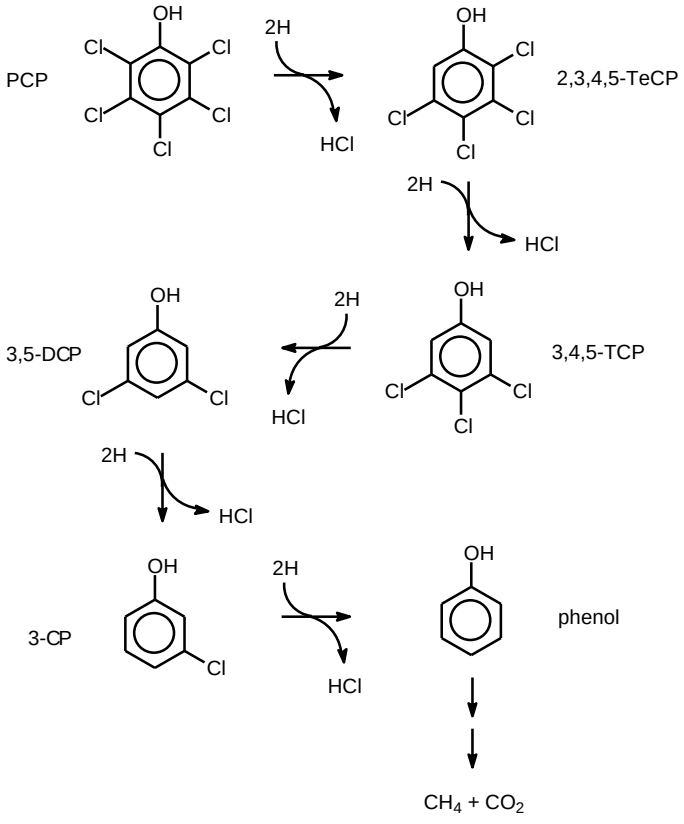

Figure2.4. *The most commonly encountered reductive dechlorination pathway during anaerobic PCP biodegradation: ortho-dechlorination, followed by para-dechlorination, followed by meta-dechlorination (Mikesell and Boyd, 1986; Londry and Fedorak, 1993).*

The reductive dechlorination of 2,4,6-TCP was evaluated in an enrichment culture developed from sewage sludge (Madsen and Aamand, 1992). 2,4,6-TCP was also preferably transformed by *ortho*-dechlorinations to 4-CP. A similar conversion of 2,4,6-TCP to 4-CP was observed in an anaerobic filter (Armenante et al., 1999). Another enrichment culture from sewage sludge was able to reductively dechlorinate 2-CP and 2,6-DCP to phenol and HCl in medium containing yeast extract and peptone (Dietrich and Winter, 1990). The same enrichment *ortho*-dechlorinated 2,4-DCP to 4-CP (Dietrich and Winter, 1990). Pond sediments previously exposed to 2,4-DCP also reductively dechlorinated 2,4-DCP to 4-CP (Hale et al., 1990). A sediment-free 2,4-DCP-degrading enrichment cultures obtained from lake sediments degraded 2,4-DCP via 4-CP, phenol and benzoate (Zhang and Wiegel, 1990) as shown in Figure 2.3.

2.3.7. Halorespiration of chlorophenols

The use of chlorophenols by anaerobic microorganisms as an electron acceptor to support microbial growth during the oxidation of simple electron donating substrates is well established. Halorespiration in a mixed culture responsible for *ortho*-dechlorination of PCP to 3,4,5-TCP was demonstrated with kinetic evidence (Stuart and Woods, 1998). During the incubation the pseudo first-order rate of PCP dechlorination increased 6-fold. The large increase in the rate constants suggests growth of a bacterial population capable of using PCP as a terminal electron acceptor. Several bacteria capable of haloprespiration with chlorophenol have been isolated as summarized in Table 2.5 (annex). Growth linked to the use of chlorophenols as electron acceptor has been demonstrated for nearly all of these isolates (Cole et al., 1994; Utkin et al., 1994; Sanford et al., 1996; Sun et al., 2000; Breitenstein et al., 2001; He and Sanford, 2002). The majority of the chlorophenol halorespiring bacterial isolates belong to the genus *Desulfitobacterium* (Villemur et al., 2006). Most of these strains exclusively cause the reductive *ortho* dehalogenation of chlorophenols. Only *Desulfitobacterium* strains belonging to the species *D. hafniense* (formerly *frappieri*) are capable of multiple patterns of reductive dehalogenation, catalyzing *ortho*, *meta* and *para* dechlorinations (Bouchard et al., 1996; Tartakovsky et al., 1999; Breitenstein et al., 2001; Villemur et al., 2006). Aside from *Desulfitobacterium*, strains belonging to the genera *Desulfovibrio* (Sun et al., 2000) and *Anaeromyxobacter* (He and Sanford, 2002; Sanford et al., 2002) are also known to halorespire chlorophenols by catalyzing *ortho*-dechlorinations. *Desulfomonile tiedjei* DCB-1 *meta*-dechlorinated polychlorophenols; however, this reaction was only feasible with cells previously cultivated on 3-CBc as electron acceptor (Mohn and Kennedy, 1992). Common electron donors supporting the halorespiration of chlorophenols include 3- to 4-carbon organic acids, ethanol, formate or hydrogen as shown in Table 2.5 (annex).

Several reductive dehalogenases have been purified from *Desulfitobacterium* strains capable of halorespiring chlorophenols (Loffler et al., 1996; Christiansen et al., 1998; van de Pas et al., 1999; 2001; Boyer et al., 2003; Villemur et al., 2006). Most of the dehalogenases purified so far have activity towards *ortho*-dechlorination of chlorophenols. Like all reductive dehalogenases from halorespiring bacteria the *ortho*-chlorophenol reductive dehalogenases (*o*-CRD) are corrinoid containing Fe/S-proteins (Christiansen et al., 1998; van de Pas et al., 1999; Boyer et al., 2003). However, based on N-terminal sequence data the *o*-CRDs were shown to be distinct enzymes from reductive dehalogenases used for trichloroethene and teterachloroethene by *Desulfitobacterium* (van de Pas et al., 2001). All of the *o*-CRDs isolated so far have only been shown to dechlorinate chlorophenols with reduced methyl viologen as artificial electron donor. The *o*-CRD of *Desulfitobacterium hafniense* PCP-1 had a very low *para*-dechlorinating activity with 345-TCP (Boyer et al., 2003). The *o*-CRD of *Desulfitobacterium dehalogenans* has a MW of 48 kDa and utilized 3C4HPA, 23-DCP, 24-DCP, 26D-CP and PCP as substrates (van de Pas et al., 1999). The V_{max} was 28 U mg^{-1} protein and the K_m was 20 μM for 3-chlorophenylacetic acid (3C4HPA). The corresponding genes of the protein were identified: *cprA*, encoding the dehalogenase; and *cprB*, encoding an integral membrane protein, responsible for anchoring the dehalogenase in the membrane (van de Pas et al., 1999). An *o*-CRD of 37 KDa from *Desulfitobacterium hafniense* PCP-1 had activities with PCP, 2345-TeCP, 246-TCP, 235-TCP, 234-TCP, 236-TCP and 23-DCP when incubated (Boyer et al., 2003). The highest activity of 0.2 U mg^{-1} protein was obtained with PCP. The K_m for 246-TCP and PCP was 18.3 and 26.8 μM, respectively. A membrane-bound *o*-CRD from

Desulfitobacterium hafniense of 47 KDa catalyzed the dechlorination of 3C4HPA at a rate of 6.2 U mg^{-1} protein (Christiansen et al., 1998). Lastly a membrane-bound *o*-CRD from *Desulfitobacterium chlororespirans* Co23, containing 64 and 37 kDa subunits, catalyzed the dechlorination of 3C4HBc at a rate of 18.0 U mg^{-1} protein (Loffler et al., 1996).

Based on distinct patterns of induction, it is suggested the *ortho*- and *meta/para*-chlorophenol reductive dehalogenases are distinct (Bouchard et al., 1996). Recently, the second chlorophenol reductase was discovered in *Desulfitobacterium hafniense* PCP-1 with specific activity towards *meta*-chlorines such as the chlorophenolic compound, 3,5-DCP (Thibodeau et al., 2004). The gene for the *meta*-chlorophenol reductase (*m*-CRD) has been designated *cprA5* (Gauthier et al., 2006). *Desulfitobacterium* strains capable of chlorophenol dechlorination were screened for the presence of *cprA* and *cprA5* genes. All strains contained *cprA*; whereas, only strains in the species *D. hafniense* also had *cprA5* in accordance with the unique ability of this species to catalyze *meta*-dechlorinations (Gauthier et al., 2006).

2.4. Kinetic data on chlorophenol degradation

Table 2.6 (annex) summarizes the kinetic data available on chlorophenol biodegradation. High growth rates were observed for aerobic bacteria specialized in the utilization of chlorophenols as a carbon and energy source. Growth rates of approximately 2 d^{-1} or more were commonly observed for 24-DCP, 246-TCP and PCP. Most of the growth rates of anaerobic halorespiring bacteria were recorded with monochlorophenols and were of comparable magnitude, ranging from 0.19 to 4.75 d^{-1}. Only one study recorded the growth of a halorespiring bacterium on PCP which was 0.41 d^{-1} (Stuart and Woods, 1998). Cell yields during aerobic growth on chlorinated decreased with increasing chlorine number. This was partly due to of the molecular weight of chlorine (which is not incorporated into biomass) and partly because of lower carbon conversion efficiencies. Aerobic cell yields with DCPs and TCPs were between 0.132 and 0.421 g dwt biomass g^{-1} chlorophenol metabolized; whereas, the values ranged from 0.054 to 0.190 g dwt biomass g^{-1} chlorophenol metabolized for PCP. Cell yields observed during halorespiration are relatively low and range from 0.016 to 0.052 g dwt biomass g^{-1} chlorophenol biotransformed. Remarkably, high specific activities are noted for halorespiration of chlorophenols. Rates ranging from 213 to 178,872 mg chlorophenol biotransformed g^{-1} dwt biomass d^{-1} have been found with most of the rates exceeding 10,000. The combination of relatively high growth rates and low cell yields accounts for the high specific activities that far exceed those of aerobic chlorophenol-degrading bacteria. The specific activities of aerobic bacteria utilizing chlorophenols as growth substrates mostly ranged between 29 to 851, excluding two outliers for PCP of 7,111 and 12,280 mg PCP biotransformed g^{-1} dwt biomass d^{-1}. Reductive dechlorination of chlorophenols in anaerobic reactor biofilms was observed between 0.37 to 12.9 mg chlorophenol biotransformed g^{-1} dwt biomass d^{-1}. Half-velocity coefficients are generally low during chlorophenolic compound biodegradation indicating a high affinity. Most microorganisms had K_s or K_m values between 0.01 and 11.7 mg l^{-1}, the only exception was a value of 112 mg l^{-1} for cometabolism by an aerobic bacterium. Microorganisms, which were previously enriched in bioreactors treating low concentrations of chlorinated phenols in groundwater, displayed remarkable affinities as judged from half-velocity coefficients as low as 0.014 to 0.016 mg l^{-1} under both aerobic (Melin et al., 1997) and anaerobic conditions (Magar et al., 1999).

3 Polychlorinated dibenzo-*p*-dioxins

3.1. Introduction

Chlorinated dioxins refer to two families of tricyclic, planar, aromatic compounds. One of these families is the polychlorinated dibenzo-*p*-dioxins (PCDD) with 75 possible congeners and the other is the polychlorinated dibenzofurans (PCDF) with 135 different congeners. Dioxins were introduced into the biosphere on a large scale as by-products from the manufacture of chlorinated phenols, which started to gain importance in the late 1930's as pesticides (Hutzinger et al., 1985). Dioxins have also been released into the environment by incineration of wastes (McKay, 2002; Tuppurainen et al., 2003). Aside from the anthropogenic input, dioxins are present naturally in the environment as evidenced by low levels detected in archived samples of soils and plant tissue from periods prior to the industrial revolution (Alcock and Jones, 1996; Green et al., 2004). The natural formation of octochloro- and heptachloro-dioxin congeners has been demonstrated during composting (Krauss et al., 1994) and during sewage treatment (Klimm et al., 1998). Oxidative enzymes such as peroxidases can catalyze the coupling of chlorophenols into dioxins (Oberg and Rappe, 1992; Wittsiepe et al., 2000), which could account for the natural formation of chlorinated dioxins. Another natural source of chlorinated dioxins is forest fires, 130 pounds of PCDDs are estimated to be produced by Canadian forest fires annually (Gribble, 1994).

PCDD and PCDF are stable hydrophobic contaminants which persist in the environment (Hutzinger et al., 1985; Alcock and Jones, 1996). Congeners with lateral chlorine atoms, such as in 2,3,7,8-tetrachlorodibenzo-*p*-dioxins (2378-TeCDD) are highly toxic to mammals (Landers and Bunce, 1991; Pohjanvirta and Tuomisto, 1994) and other organisms (Boening, 1998). Dioxins have a high tendency to become adsorbed onto soil and sediments as well as bioaccumulate in organisms (Matsumura and Benezet, 1973; Hutzinger et al., 1985). It has long been recognized that dioxins are subject to photodegradation (Crosby and Wong, 1977; Hutzinger et al., 1985; McPeters and Overcash, 1993). Loss due to biodegradation, on the other hand, has only recently been widely accepted as an important environmental fate of dioxins, due studies initiated in the mid-1980's that demonstrated the microbial conversion of PCDD and PCDF by isolated microorganisms. Previously, there has only been one comprehensive review article on the biodegradation of chlorinated dioxins, which was published by Wittich in 1998 (Wittich, 1998).

The nomenclature to be used for mono-, di-, tr-, tetra-, penta-, hexa-, hepta- and octochloro- dibenzo-*p*-dioxins/dibenzofurans in this chapter will be CDD/F, DCDD/F, TCDD/F, TeCDD/F, QCDD/F, HCDD/F, HpCDD/F and OCDD/F, respectively.

3.2. Biodegradation of Chlorinated Dioxins

3.2.1. Degradation of chlorinated dioxins in the environment

Evidence for the biodegradation of chlorinated dioxins in the environment is available in a few studies conducted with, either, soil, surface water or sediments. Dated sediment cores from aquatic depositional environments have the potential to provide chronologies of pollutant input as well as supply information on possible fates such as biodegradation (Alcock and Jones, 1996). A study with dated sediment cores from Lake Ketelmeer (The Netherlands), a sedimentation area of the River Rhine, confirmed significant disappearance of 4 higher chlorinated congeners of dioxins when compared with archived sediment samples (Beurskens, 1995). The average half life calculated for these 4 congeners was 12 years. The results indicated slow biodegradation of these 4 congeners; however, 13 other

dioxin congeners evaluated were persistent. A study of dated estuarine sediment cores collected in Queensland, Australia, indicated that sediment age (since deposition) was correlated with increasing proportions of lower chlorinated PCDDs that corresponded to decreasing proportions of OCDD (Gaus et al., 2002). The lower chlorinated congeners, which accumulated in older sediments, had characteristic substitution patterns expected from the anaerobic microbial dechlorination of OCDD. Lastly, another study of dated sediment cores from the Baltic sea indicated minimum half-lives of chlorinated dioxins and chlorinated dibenzofurans that ranged from 30 (for OCDF and HpCDF) to 170 years (for HpCDD) (Kjeller and Rappe, 1995).

Evidence for biodegradation of PCDD and PCDF in anaerobic sediments has been obtained in numerous microcosm studies where sediments collected from rivers, estuaries and bays have been spiked with specific congeners of chlorinated dioxin together with an electron donating substrate (Adriaens et al., 1995; Adriaens and Grbic-Galic, 1994; Ballerstedt et al., 1997; Beurskens et al., 1995; Bunge et al., 2001; Fu et al., 1999). In these studies, the spiked dioxins are converted to lower chlorinated dioxins, representing products of biologically mediated reductive dechlorination. The time scale for the bioconversions in the microcosms ranges from months to a year; whereas in the field many years are implicated. The difference may be due to the many-fold higher dioxin concentrations used in the microcosms, typically added as a fresh spike and together with an adequate supply of electron donating substrates. In one microcosms study, anaerobic dechlorination was also demonstrated for aged 2378TeCDD pollution in the sediments (Barkovskii and Adriaens, 1996).

Reports on the biodegradation of chlorinated dioxins in soil are conflicting. On the one hand, there are studies which indicate that chlorinated dioxins are persistent. One such study considered chlorinated dioxins that were introduced into soil via land application of sewage sludge (Wilson et al., 1997). Chlorinated dioxin concentrations did not significantly change after 260 days of monitoring. On the other hand, the evidence obtained in other experiments suggests that dioxins are degraded in soil. The concentration of 2378TeCDD was monitored over 10 years in soil contaminated with *agent orange* (chlorophenoxy acetates). The chlorophenoxy acetates and the 2378TeCDD were shown to significantly decrease in concentration (Young, 2006). Biodegradation was also observed in soil microcosms spiked with 1 to 100 ppm of 2378TeCDD (Kearney et al., 1972). From 37 to 44% of added 2378TeCDD was eliminated in one year. In a similar soil microcosm, [^{14}C]27DCDD was converted in 10 weeks to $^{14}CO_2$ (5%) and an unidentified polar metabolite isolated on a thin-layer chromatography plate (Kearney et al., 1972).

The biotransformation of 2378TeCDD was evaluated in outdoor pond water (Matsumura et al., 1983). The apparent half-life of 2378TCDD was approximately 1 year, based on the measured recoveries of 2378TCDD of 49.7 and 29.4% after 12 and 25 months, respectively.

3.2.2. Biodegradation of chlorinated dioxins in engineered systems

There are limited research results available on the biodegradation of dioxins in engineered systems. A number of studies have been conducted, evaluating concentrations of chlorinated dioxins during municipal waste treatment (McLachlan et al., 1996; Rogers, 1996; Stevens et al., 2003; Oleszek-Kudlak et al., 2005). Chlorinated dioxins have low volatility and are highly hydrophobic so they tend to adsorb to sludge solids. Municipal digested sludge contains from 10 to 40 ppb of chlorinated dioxins on a dry weight basis (Rogers, 1996; Stevens et al., 2001). The predominant isomers detected are the higher chlorinated isomers, especially hepta- and octochloro-congeners. Most studies are in agreement that chlorinated dioxins are not significantly degraded during anaerobic sludge digestion (Disse et al., 1995; Stevens et al., 2003; Oleszek-Kudlak et al., 2005). Microcosms studies provide differing results. In one study, the incubation of 1234TeCDD with anaerobic digester sludge for 13 months resulted in no evidence for its degradation based on monitoring for the formation of dechlorinated daughter products (Ballerstedt et al., 1997). However, another microcosm study evaluating the disappearance of 2378TeCDD in anaerobic sludge found that the compound was removed by 86% in 90 days, while there was no significant removal in poisoned controls (Kao et al., 2001). Significant losses of

many chlorinated dioxin congeners were observed during the aerobic digestion of sludge (Disse et al., 1995).

There is limited experience with the bioremediation of dioxins. Most of the studies have considered the impact of adding dioxin-degrading bacterial strains to soils artificially contaminated with defined dioxin congeners. The results, summarized in Table 3.1, indicate that bacterial strains added to the soils metabolize from 32 to 100% of mono- to trichloro-DD/DF congeners supplied at concentrations ranging from 1 to 10 ppm within a week. In some cases, the addition of dioxin-degrading bacterial strains to real contaminated soil was evaluated, resulting in 8.3% to 10% after 7 days of incubation (Habe et al., 2001b; 2002b). The dioxin-degrading strain *Sphingomonas wittichii* RW1 was used to bioremediate PCDD in real contaminated sample of incinerator fly ash (Nam et al., 2005). After 15 days, 75.5% of the toxic PCDDs were removed. Only 20.2% removal was observed in a control receiving heat-killed inoculum, providing strong evidence for biodegradation.

Table 3.1. Bioremediation of soils artificially contaminated with chlorinated dioxins and bioaugmented with bacterial strains.

Bacterial Strain	**Dioxin**	**Conc.** (ppb)	**Incubation Time** (d)	**Removal** (%)	**Reference**
Pseudomonas resinovorans CA10	23-DCDD	1,000	14	100.0	(Widada et al., 2002)
Terrabacter sp. DBF63	28-DCDF 2-CDF 2-CDD 28-DCDF 23-DCDF	1,000 1,000 1,000 1,000 1,000	7 5 5 5 5	90.0 89.0 65.0 78.0 32.0	(Habe et al., 2002b)
Sphingomonas sp. KA1	2-CDD 23-DCDD	1,000 1,000	7 7	96.0 70.0	(Habe et al., 2002a)
Pseudomonas sp. CA10	2-CDD 23-DCDD 123-TCDD	1,000 1,000 1,000	5 5 5	97.0 89.0 52.0	(Habe et al., 2001b)
Pseudomonas sp. CA10	2-CDD	10,000	7	98.5	(Halden et al., 1999)

Another approach towards bioremediation has been the addition of large amounts of organic matter to soil. Autoclaved compost was added as an organic nutrient to a heavily polluted soil, which resulted in the decrease of PCDD and PCDF concentrations by 22% after 3 months (Nam et al., 2005). The pattern in the time course of the congener removal was interpreted to reflect anaerobic reductive dechlorination. Two studies evaluated anaerobic bioremediation of soil and sediments. In one study, a soil artificially contaminated with 2378-TeCDD (96 ppb) was treated with anaerobic sludge supplied as an inoculum and sludge cake supplied as an electron donor (Kao et al., 2001). After a 90-d incubation period, 86% of the 2378-TeCDD added was removed, while controls poisoned with a mixture of $HgCl_2$ and NaN_3 caused only 2% removal. A zero-order rate constant of 1 ppb d^{-1} was reported. In the other study, addition of organic acids was used as strategy to remediate chlorinated dioxins in polluted river sediments (Yoshida et al., 2005). Up to 32% removal of PCDD/PCDF from the sediments was achieved after 210 days of incubation. A poisoned control (paraformaldehyde) did not cause any removal, confirming biodegradation as the main removal mechanism. The best results were obtained in anaerobic microcosms exposed at the surface to air because lower chlorinated congeners formed from reductive dechlorination were subsequently oxidized by aerobic bacteria. The authors demonstrated the formation of catechol and salicylic acid which was indicative of occurrence of aerobic bacteria.

3.3. Microbiology and biochemistry of chlorinated dioxin biodegradation

Chlorinated dioxins are subject to both aerobic and anaerobic metabolism. Under aerobic conditions, lower chlorinated dioxins are susceptible to partial degradation during cometabolic metabolism. In only a few cases have chlorinated dioxins been reported to serve as growth substrates, and these cases are restricted to monochlorinated congeners. Under anaerobic conditions, chlorinated dioxins are subject to reductive dechlorination when suitable electron-donating substrates are available. Recently, several strains of halorespiring bacteria have been reported to utilize polychlorinated dioxins as electron acceptors to support microbial growth.

3.3.1. Aerobic bacterial cometabolism of chlorinated dioxins

Table 3.2 (annex) summarizes literature data on the aerobic degradation of chlorinated dioxins by aerobic bacterial strains. A quick survey of the Table reveals that most of the evidence for aerobic biodegradation of chlorinated dibenzo-*p*-dioxins and chlorinated dibenzofurans has been obtained with monochloro- or dichloro-congeners, which collectively account for 84% of the reported cases of aerobic bacterial chlorinated dioxin degradation. Most of the remaining reports concern trichloro- and tetrachloro-congeners. These findings are in keeping with a general trend that aerobic the biodegradability of chlorinated dioxins increases with a decreasing number of chlorine groups (Du et al., 2001; Keim et al., 1999; Parsons and Storms, 1989; Wilkes et al., 1996; Schreiner et al., 1997). As general rule, PCDD/F with 5 chlorine groups or more are not prone to aerobic degradation. One important exception is the recent report that *Sphingomonas wittichii* strain RW1 is able to slowly biotransform 123478-HCDD (Nam et al., 2006).

Aerobic bacterial biodegradation of chlorinated dioxins occurs via cometabolism in the overwhelming majority of literature reports (91%). The most widely used primary substrate to support chlorinated dioxin cometabolism is the non-halogenated analogue, dibenzofuran (DF) (Table 3.2 - annex -). In addition to DF, non-halogenated dibenzo-*p*-dioxin (DD) (Hong et al., 2002), biphenyl (BP) (Parsons et al., 1998), carbazole (CAR) (Habe et al., 2001a), *o*-dichlorobenzene (*o*-DCB) (Du et al., 2001), and benzoic (Bc) or 3-methoxybenzoic acid (3MBc) (Parsons and Storms, 1989) represent common examples of other primary substrates utilized. These primary substrates most likely induce dioxygenases that may be involved in the degradation of the chlorinated dioxins. The most commonly used primary substrates are biphenylic compounds (DF, DD, CAR, BP) which most likely induce angular dioxygenases implicated in the degradation of DF and DD (Wittich, 1998; Nojiri and Omori, 2002) as well as chlorinated DFs and DDs (Habe et al., 2001a).

The most common biodegradation pathways for the aerobic degradation of chlorinated dioxins are illustrated in Figures 3.1 and 3.2 for 2-CDD and 2-CDF, respectively. The best chlorinated dioxin-degrading bacterial strains initiate the attack of the biphenylic dioxin compounds with angular dioxygenases (Habe et al., 2001a; Nojiri and Omori, 2002). Angular dioxygenases attack a ring adjacent to the ether oxygen bridging the two rings (position 1,10a in dibenzo-*p*-dioxin and position 4,4a in dibenzofuran). Three angular dioxygensases have been described and cloned. These are carbazole 1,9a dioxygenase (CARDO) from *Pseudomonas* sp. strain CA10 (Habe et al., 2001a), dibenzofuran 4,4a dioxygenase (DFDO) from *Terrabacter* sp. strain DBF63 (Habe et al., 2001a) and a dibenzo-*p*-dioxygenase 1,10a from (Wittich et al., 1992; Armengaud et al., 1998). CARDO and DFDO cloned into and expressed by *Eschericia coli* were able to catalyze the biotransformation of 2-CDF, 28-DCDF, 2-CDD, 23-DCDD, 27- DCDD and 123-TCDD (with the exception of 27DCDD which was transformed by DFDO) (Habe et al., 2001a).

The angular dioxygenases catalyze the formation of diols which are spontaneously form chlorinated 2,2',3-trihydroxydiphenyl ethers (THDE) and chlorinated 2,2',3-trihydroxybiphenyl (THB) in the case of PCDD and PCDF, respectively. THDE and THB are subsequently oxidized by dioxygenases causing ring opening by *meta* cleavage of the dihydroxylated ring. The ring opened products is metabolized further yielding chlorinated catechols or chlorinated salicylates from PCDD or PCDF, respectively (Figures 3.1 and

3.2). In addition to chlorinated salicylates, metabolism of PCDF sometimes yields chlorinated 2-methyl-4H-chroman-4-ones, especially when the PCDF has chlorogroups on both rings (Keim et al., 1999; Fukuda et al., 2002) as indicated in Table 3.2 (annex).

Aside from angular dioxygenases, dioxygenase activity has also been found in some bacterial strains, accounting for dioxygenation in the lateral positions of chlorinated dioxins. Examples include dihydrodiols recovered from 2-CDD metabolism by *Beijerinckia* sp. strain B8/36 (Klecka and Gibson, 1980) and dihydroxy-2-CDD metabolites from the incubation of *Alcaligenes* sp. strain JB1 (Parsons and Storms, 1989). Bacterial cytochrome P450 from *Bacillus megaterium* has the ability to cause monoxygenation of 23-DCDD and 237-TCDD yielding hydroxylated metabolites of these dioxins (Sulistyaningdyah et al., 2004).

3.3.2. Aerobic bacterial growth utilizing chlorinated dioxins as a sole carbon and energy source

There are surprisingly few well documented examples of chlorinated dioxins serving as a sole source of carbon and energy for pure bacterial strains. *Pseudomonas veronii* PH-03 has been shown to utilize 1-CDD and 2-CDD, growing on the aliphatic acids generated from ring cleavage but accumulating 3-chlorocatechol (CC) and 4-CC, respectively; as dead products from the chlorinated ring (Hong et al., 2004). Similarly, *Sphingomonas* sp. strain RW1 can grow on 4-CDF (Arfmann et al., 1997). In this case, the 5-carbon aliphatic acid, 2-hydroxypenta- 2,4-dienoate released from ring cleavage is the substrate that provides carbon and energy; whereas, 3-chlorosalicylic acid (CSA) accumulates as the dead-end product. Complete mineralization of chlorinated dioxins has only been achieved in co-cultures, which combine a CDF-degrader with CSA-degrader. A coculture of *Sphingomonas* sp. strain RW16 together with *Pseudomonas* sp RW10 completely degraded 2-CDF and 3-CDF (Wittich et al., 1999). *Pseudomonas* sp RW10 was responsible for mineralizing 5-CSA and 4-CSA that accumulated from 2-CDF and 3-CDF, respectively. About 60% of the chlorine in 3-CDF was recovered as inorganic chloride, indicating an extensive mineralization of 3-CDF by the coculture. A coculture constructed from *Sphingomonas* sp. strain RW1 and *Burkholderia* sp. strain JWS was used to completely degrade 4-CDF (Arfmann et al., 1997). *Burkholderia* sp. strain JWS was able to utilize 3-CSA accumulating from 4-CDF degradation for growth. This coculture released 86% of the chlorine added with 4-CDF.

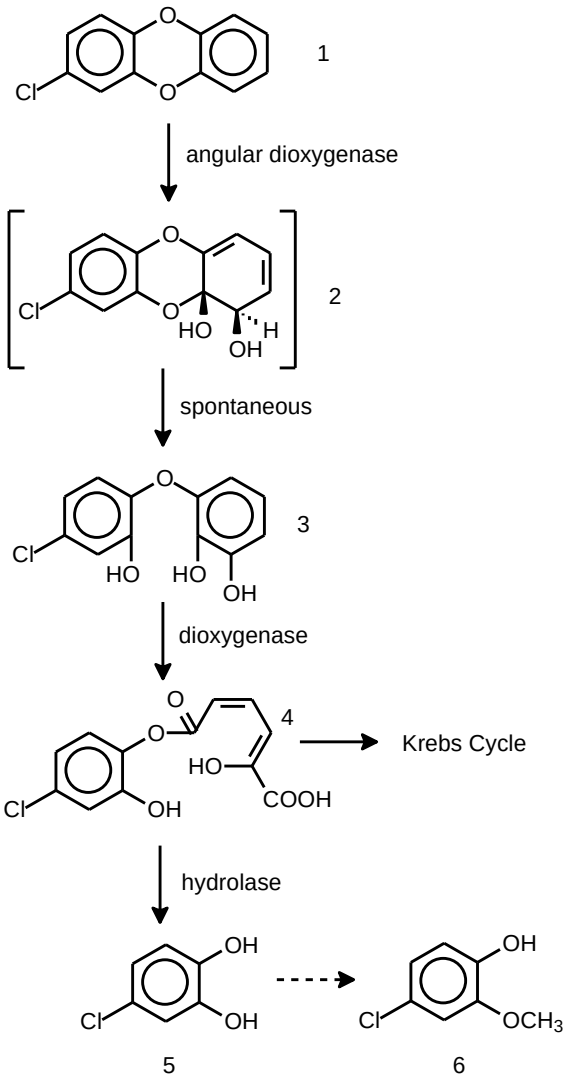

Figure 3.1. *Pathway of 2-CDD biodegradation by aerobic bacteria. Compound definitions: 2-chlorodibenzo-p-dioxin (1); 8-chloro-cis-1,10a-dihydroxy-1-hydro-dibenzo-p-dioxin (2); 4'-chloro-2,2',3-trihydroxydiphenyl ether (3); 2-hydroxy-6-oxo-6-(4-chloro-2-hydroxyphenoxy)-2,4-hexadienoic acid (4); 4-chlorocatechol (5); 4-chloroguiacol (6).*

<u>References</u>: (Wittich, 1998; Habe et al., 2001a; Kimura and Urushigawa, 2001; Nojiri and Omori, 2002; Hong et al., 2004)

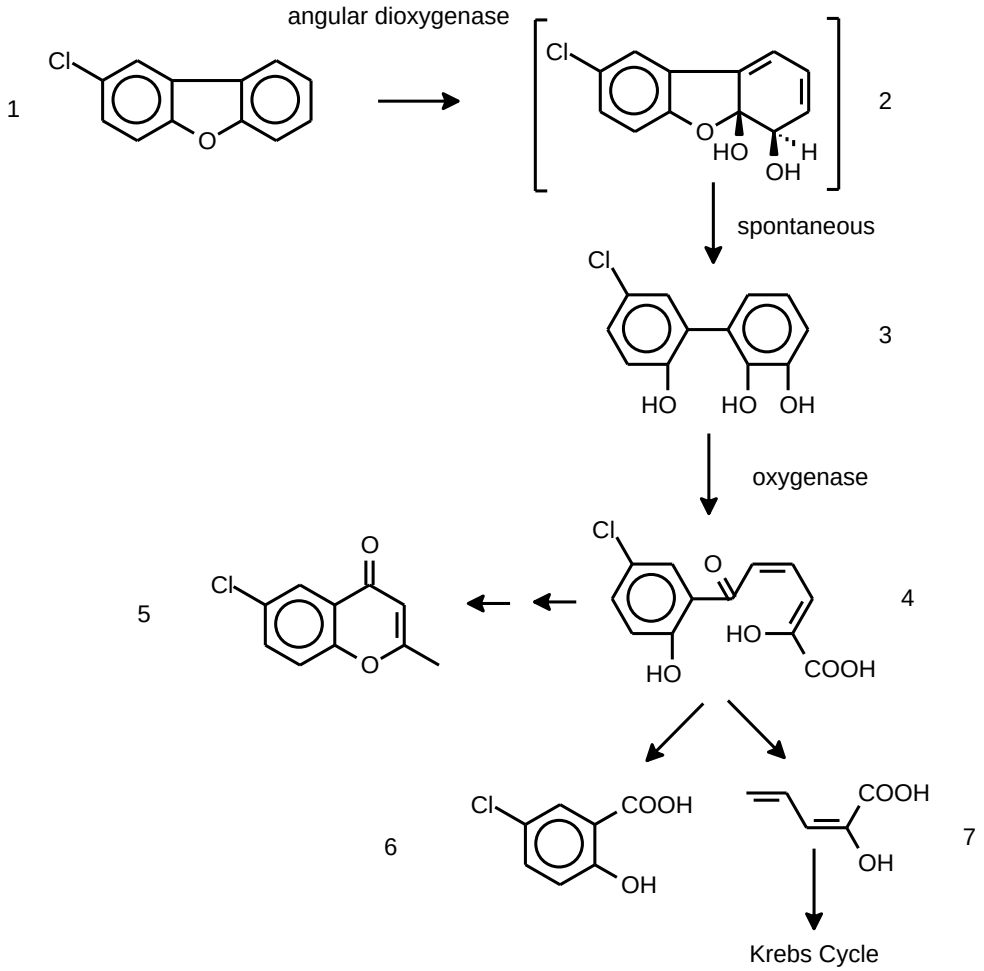

Figure 3.2. *Pathway of 2-CDF biodegradation by aerobic bacteria. Compound definitions: 2-chlorodibenzofuran (1); 8-chloro-cis-4,4a-dihydroxy-4-hydro-dibenzofuran (2); 5'-chloro-2,2',3-trihydroxybiphenyl (3); 2-hydroxy-6-oxo-6-(5-chloro-2-hydroxyphenyl)-2,4-hexadienoic acid (4); 6-chloro-2-methyl-4H-chroman-4-one (5); 5-chlorosalicylic acid (6); 2-hydroxypenta- 2,4-dienoate (7). References: (Harms et al., 1991; Wilkes et al., 1996; Arfmann et al., 1997; Wittich, 1998; Wittich et al., 1999; Habe et al., 2001a; Nojiri and Omori, 2002).*

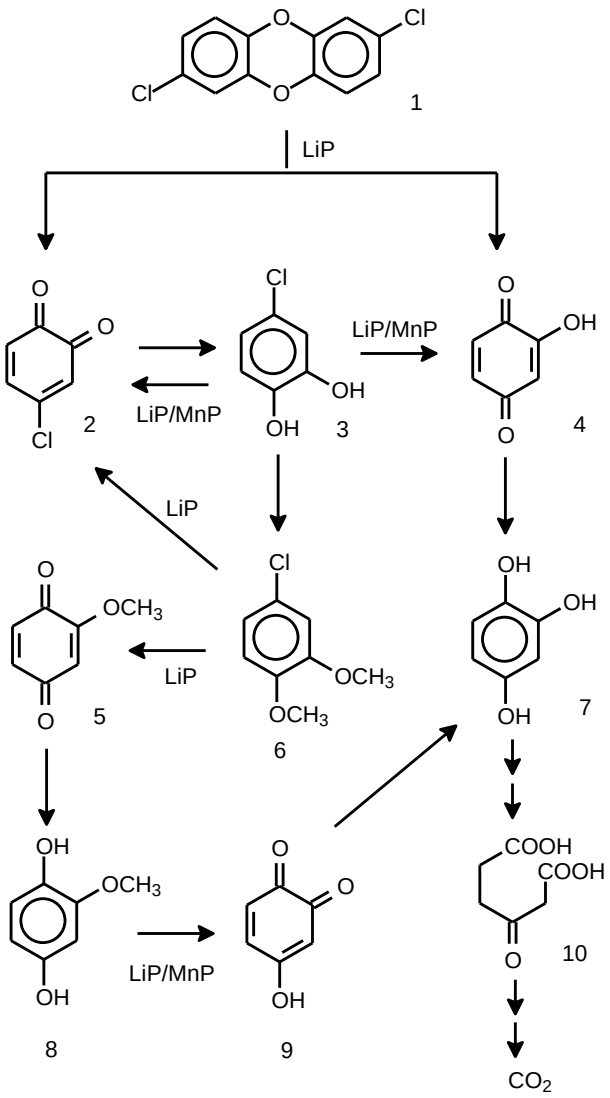

***Figure 3.3.**. Proposed pathway of 2,7-dichlorodibenzo-p-dioxin (27-DCDD) degradation by the white rot fungus, Phanerochaete chrysosporium (Valli et al., 1992). Legend: 27-DCDD (1); 4-chloro-1,2-benzoquinone (2), 4-chlorocatechol (3); 2-hydroxy-1,4-benzoquinone (4), 2-methoxy-1,4-benzoquinone (5); 4-chloroveratrole (6); 1,2,4-trihydroxybenzene (7); 2-methoxyhydroquinone (8); 4-hydoxy-1,2-benzoquinone (9); β-ketoadipic acid (10); lignin peroxidase (LiP); manganese peroxidase (MnP).*

3.3.3. Aerobic fungal cometabolism of chlorinated dioxins

The only evidence for the degradation of chlorinated dioxins by fungi is limited to wood-, or litter-degrading white rot fungi. The white-rot fungi constitute the most important group of organisms responsible for the degradation of nature's most complex polymer, lignin. Lignin is formed from the random polymerization of phenyl propanoid units. White-rot fungi use extracellular oxidative enzymes to initiate the attack of lignin. These oxidative enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP), are also capable of oxidizing a variety of xenobiotic pollutants (Field et al., 1993), including chlorinated dioxins (Hammel et al., 1986; Valli et al., 1992). In the first study evaluating the potential of white-rot fungi to degrade chlorinated dioxins, *Phanerochaete chrysosporium* was shown to mineralize [^{14}C]2378-TeCDD by 2.2% to $^{14}CO_2$ in 30 days (Bumpus et al., 1985a). The mechanism of DD and 2-CDD oxidation by LiP from *Phanerochaete chrysosporium* involves successive one-electron oxidations, with the cation radical of DD being the initial intermediate of the process (Hammel et al., 1986). In an elaborate study evaluating the oxidation of 27-DCDD by whole cultures and LiP of *Phanerochaete chrysosporium*, a pathway of degradation was elucidated (Valli et al., 1992). 27-DCDD supplied at 6.9 mg l^{-1} was degraded by 50% in 27 days by *Phanerochaete chrysosporium* only under conditions of low-nutrient nitrogen required for the induction of ligninolytic enzymes. Purified LiP catalyzed the conversion of 27-DCDD to 4-chloro-1,2-benzoquinone (2CBQ), 2-hydroxy-1,4-benzoquinone (2HBQ) and chloride. The molar yield of chloride accounted for 19% of the 27-DCDD removed. In whole

cultures, the formation of 2CBQ and 1,2,4-trihydroxybenzene (124THB) was demonstrated after short incubation of 48 h with 27-DCDD. These metabolites were added exogenously to the cultures *in vivo* and LiP *in vitro* to elaborate the pathway. The overall pathway shown in Figure 3.3 involves cycles of oxidation by LiP and/or MnP forming quinones, followed by reduction to hydroquinones or catechols and subsequent methylations to methoxybenzenes. 124THB is subject to ring cleavage, which could lead to mineralization to CO_2.

The ability of white-rot fungi to degrade chlorinated dioxins is not limited to lower chlorinated congeners. *Phanerochaete sordida* and *Phanerochaete chrysosporium* were able to remove 34% and 48% of a higher chlorinated dioxins supplied as a mixture of penta-, hexa-, hepta-, and octo- CD/CF (collectively 0.0005 mg l^{-1}) after 7 and 14 days of incubation, respectively (Takada et al., 1996). A biological removal mechanism is implicated since the removal is reported in comparison to heat-killed controls and the removal was stimulated by addition of glucose. Additional evidence for a biotransformation reaction was the recovery of 4,5-dichlorocatechol (DCC) and tetrachlorocatechol (TeCC) as metabolites of 2378-TeCDD and OCDD (0.005 mg l^{-1}) incubations (10 days) with *Phanerochaete sordida*, respectively (Takada et al., 1996).

Aside from *Phanerochaete*, several other genera of white-rot fungi have been described with outstanding abilities to degrade dioxins. Three strains of fungi, *Phlebia lindtneri*, *Phlebia* sp. MG-60, and an unidentified strain MZ-227, were shown to mineralize [^{14}C]27-DCDD (7.0 mg l^{-1}) to $^{14}CO_2$ by 5.0, 5.2 and 6.5%, respectively, after 30 days (Mori and Kondo, 2002a). In screening studies that followed, other *Phlebia* strains (BMC3014, BMC9152, and BMC9160) were identified as good 27-DCDD-degraders (Kamei et al., 2005). These strains degraded from 32.4-51.1, 18.4-27.8, 11.9-21.1, 14.2-21.5% of 27-DCDD (6.9 mg l^{-1}), 278-TCDD (7.9 mg l^{-1}), 1289-TeCDD (8.7 mg l^{-1}) and 1267-TeCDD (8.7 mg l^{-1}), respectively, after 14 days. *Phlebia brevispora* eliminated 1368-TeCDD by 28% in 28 days and produced 7-methoxy-1368-TeCDD as a metabolite along with traces of hydroxyl-TeCDD, dimethoxy-TeCDD, dimethoxy-TCDD as well as 3,5-DCC (Kamei et al., 2005). *Phlebia lindtneri* was shown to remove 27-DCDD (6.9 mg l^{-1}) by 55% in 20 days (Mori and Kondo, 2002b). The same strain mineralized [^{14}C]28-DCDF and [^{14}C]27-DCDD (6.9 mg l^{-1}) to $^{14}CO_2$ by 6 and 17%, respectively, in 5 days (Mori and Kondo, 2002b). Additionally, 3-hydroxy-28-DCDF and 3-hydroxy-27-DCDD were identified as metabolites (Mori and Kondo, 2002b). *Panellus stipticus* was identified as another outstanding chlorinated dioxin degrader from another screening study (Sato et al., 2002). *Panellus stypticus* completely eliminated 27-DCDD (2.8 mg l^{-1}) in 40 days. 4CC was identified as a metabolite of the degradation.

3.3.4. Anaerobic reductive dechlorination of chlorinated dioxins

Chlorinated dioxins undergo reductive dechlorination in anaerobic environments. The first evidence was obtained by spiking anaerobic sediment microcosms with highly chlorinated congeners of dioxins, 1234678-HpCDD, 1234678-HpCDF, 123478-HCDD, and 12468-PeCDF (Adriaens and Grbicgalic, 1994). These higher chlorinated congeners were shown to be eliminated faster in undisturbed sediments compared to heat-killed sediments, establishing a biological mechanism of removal. The removal rates of PCDD/F in live microcosms were from 19 to 56% higher than in autoclaved controls. Long-term incubation of the congeners up to years revealed the accumulation of lower chlorinated biotransformation products, accounting for up to 30% of the PCDD spiked (Adriaens et al., 1995). In live microcosms, the early studies demonstrated that 1234678-HpCDD and 123478-HCDD were dechlorinated to TeCDD congeners. Likewise, 12468-PCDF was converted to to TeCDF congeners. Dechlorination of the PCDD/F was also observed in the heat-killed control microcosms, however, the extent of dechlorination was less and the dechlorination was limited to removal of only one to two chlorines (Adriaens et al., 1995; Barkovskii and Adriaens, 1998). Further studies revealed that model humic compounds and vitamin B_{12} served as redox mediating compounds catalyzing the reduction of the abiotic reduction of the highly chlorinated (octo- to penta-) PCDD/F with either sulfide (present in reduced anaerobic medium) or added Ti(III)citrate (Adriaens et al., 1996; Barkovskii and Adriaens, 1998). In abiotic reactions, the humic model compounds catalyzed the slow removal of one or two chlorine atoms; whereas OCDD was converted in low yields (<10%)

to TeCDD by vitamin B_{12}. The results clearly indicate that chemical reduction mechanisms are implicated in some of the initial dechlorination reactions of highly chlorinated.

More extensive dechlorination of the higher chlorinated dioxins requires the activity of microorganisms in the sediment. In live anaerobic sediment microcosms incubated with 5.3 mg OCDD per liter for 7 months, tri-, di- and mono- chlorinated CDD congeners were identified, albeit in low molar yields (0.4%) (Barkovskii and Adriaens, 1996, 1998). A number of additional studies have evaluated the bioconversion of penta-, tetra-, tri- and di-chlorinated dioxins added to anaerobic sediments, enrichment cultures and pure cultures of halorespiring bacteria. The results from these studies are summarized in Table 3.3 (annex). Several trends can be recognized. Firstly, that PeCDD and TeCDD has been consistently dechlorinated by two or more chlorine groups to mono-tri- CDD in numerous studies. This confirms the potential of microorganisms to extensively dechlorinate PCDDs. Secondly, previous enrichment of anaerobic cultures derived from sediments with a halogenated compound serving as an electron acceptor (bromophenols, chlorinated benzenes, *etc*) is associated with a greater extent of test compound removal and a higher degree of dechlorination. This observation would suggest that the alternative halogenated compounds contribute to the growth of halorespiring bacteria that can utilize PCDD/F as an electron acceptor. Finally, two studies have shown that pure cultures of known halorespiring bacteria from the genus, *Dehaloccocoides*, can effectively dechlorinate various PCDD congeners (Bunge et al., 2003; Fennell et al., 2004). *Dehaloccocoides* sp. strain CBDB1 known for its ability to dechlorinate chlorinated benzenes caused extensive dechlorination of 1234-TeCDD as well as intermediates to 2-CDD (Bunge et al., 2003). *Dehaloccocoides* sp. strain CBDB1 can be sustained for several transfers with 1234-TeCDD as the sole electron acceptor, suggesting that this strain uses 1234-TeCDD as the primary electron acceptor for growth. On the other hand, *Dehalococcoides ethenogenes* strain 195, known for its ability to dehalogenate perchloroethene (PCE), only catalyzed the efficient removal of one chlorine atom from 1234-TeCDD to form 124-TCDD as the main product (Fennell et al., 2004). The ability of *Dehalococcoides ethenogenes* strain 195 to utilize 1234-TeCDD as the primary electron acceptor for growth was not demonstrated since PCE was added during the assay to support the growth of the strain.

Several pathways of PCDD dechlorination have been observed. The most commonly studied model compound for evaluating reductive dechlorination has been 1234-TeCDD (Table 3.3 –annex-). A summary of the pathways observed is provided in Figure 3.4. nitial dechlorination is either observed in a lateral position such as with *Dehalococcoides ethenogenes* strain 195 (Fennell et al., 2004), forming 124-TCDD (labeled “195”), or in the *peri* position such as with *Dehaloccocoides* sp. strain CBDB1 (Bunge et al., 2003) (labeled “CBDB1”), forming 123-TCDD. Whereas 124T-CDD is not significantly further degraded by strain 195, organisms present in enrichment cultures and sediment microcosms appear to be capable of continuing the dechlorination of 124-TCDD via *peri* dechlorinations to 13-DCDD and 2-CDD (Vargas et al., 2001; Fennell et al., 2004). Strain CBDB1 can continue dechlorinating its first major intermediate, 123-TCB, via a second *peri* dechlorination to 23-DCDD and a subsequent lateral dechlorination to 2-CDD (Bunge et al., 2003).

Several sediment microcosms and enrichment cultures display both types of activities (195 and CBDB1) (Beurskens et al., 1995; Bunge et al., 2001). One pattern involves organisms solely capable of catalyzing lateral dechlorinations; whereas, the other involves organisms that can catalyze both *peri*- and lateral dechlorinations. Distinct dechlorination patterns were also observed during the reductive dechlorination of OCDD in a microbial consortium derived from anaerobic sediments (Barkovskii and Adriaens, 1996). Non-spore forming cells were responsible for the *peri*-dechlorination of higher PCDD, resulting in the formation of the toxic 2378-TeCDD intermediate. Spore-forming cells (cells that survive a pasteurization treatment), on the other hand, performed lateral dechlorinations leading to non-2378-TeCDD intermediates; however, the spore-formers were unable to dechlorinate beyond TCDD congeners. The results suggest that there is a diversity of microorganisms that catalyze reductive dechlorinations of chlorinated dioxins.

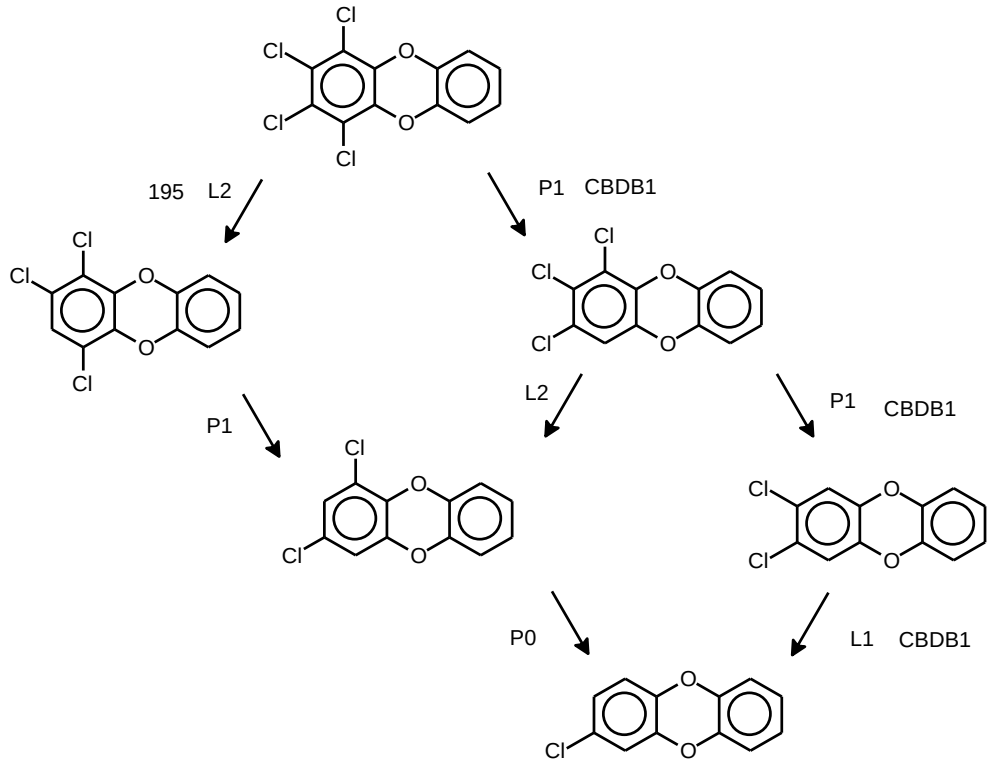

Figure 3.4*. Anaerobic biotransformation pattern of 1234-TeCDD in anaerobic sediments, enrichment cultures and pure cultures of Dehalococcoides spp. (Ahn et al., 2005; Beurskens et al., 1995; Ballerstedt et al., 1997; Bunge et al., 2001; 2003; Fennell et al., 2004; Vargas et al., 2001). Legend: lateral dechlorination surrounded by 2 vicinal chlorines (L2); lateral dechlorination adjacent with 1 vicinal chlorine (L1); peri- dechlorination adjacent with 1 vicinal chlorine (P1); peri-dechlorination with no vicinal chlorine (P0); predominant dechlorination reactions carried out by Dehalococcoides sp.CBDB1 (Bunge et al., 2003) (CDDB1); predominant dechlorination reactions carried out by D. ethenogenes 195 (Fennell*

3.4. Kinetics of chlorinated dioxin biodegradation

The only reliable data on the microbial kinetics of chlorinated dioxin degradation are those obtained with lower chlorinated congeners under aerobic conditions (Table 3.4 -annex-). Most aerobic bacterial strains have been tested under cometabolic conditions. Specific activities of substrate consumption range from an incredibly rapid rate of 42,749 mg g^{-1} dwt cells d^{-1} for 3-CDF metabolism by dibenzofuran grown cells of *Pseudomonas* sp. HH69 (Harms et al., 1991) to moderate rates ranging from several hundered to several thousand mg g^{-1} dwt cells d^{-1} for other monochlorinated dioxin congeners. As the chlorine number increases for either the chlorinated dibenzo-*p*-dioxin or chlorinated dibenzofuran series, the specific substrate consumption activities as well as the specific O_2-uptake activities decrease by orders of magnitude (Table 3.4 -annex-). The results indicate that the oxygenolytic attack of dioxins by dioxygenases is greatly impeded by the electron withdrawing properties of multiple chlorine groups.

Microbial kinetic data is lacking for the anaerobic dechlorination of higher chlorinated congeners. One study reported zero-order rate constants in mixed cultures for the bioconversion of 1234-TeCDD of 6.0×10^{-7} and 1.1×10^{-6} mg g^{-1} dwt d^{-1} in anaerobic

sediments and digester sludge, respectively (Kao et al., 2001). The remaining kinetic data are limited to half-lives in sediments or culture fluids. Half-lives in anaerobic river sediment microcosms are reported to be 4.1, 2.0, 2.1 and 1.0 years for HeCDD, HCDD, HeCDF and PeCDF, respectively; and in anaerobic aquifer sediments are reported to be 2.9, 2.9, 2.5 and 3.5 years for HeCDD, HCDD, HeCDF and PeCDF, respectively (Adriaens and Grbicgalic, 1994). An anaerobic enrichment culture derived from lake sediments degraded 1234-TeCDD with a half-life of 15.5 days (Beurskens, 1995).

4 Chlorinated Benzenes

4.1. Introduction

Chlorobenzenes include chlorobenzene (CB), dichlorobenzene (DCB), trichlorobenzene (TCB), tetrachlorobenzene (TeCB), pentachlorobenzene (QCB) and hexachlorobenzene (HCB). Chlorobenzenes are important industrial intermediates and solvents. Their widespread use has resulted in broad distribution of these compounds in the environment. Only a few review articles are available that are specific for the biodegradation of chlorobenzenes. Wang and Jones (1994) reviewed the fate of chlorobenzenes in soils, including a section summarizing previous research on the biodegradation of various chlorobenzene compounds by bacterial strains and soil. Van Agteren *et al*. (1998) wrote a chapter on the aerobic and anaerobic biodegradability of chlorobenzenes in their *Handbook on Biodegradation and Biological treatment of Hazardous Organic Compounds*. Adrian and Görisch (2002) published a comprehensive review on the anaerobic biotransformation of chlorobenzenes.

4.2. Biodegradation of Chlorobenzenes

4.2.1. Degradation of chlorobenzenes in the environment

Numerous studies have evaluated the biodegradability of chlorobenzenes in either aerobic or anaerobic environments. Conflicting results have been attained with respect to the importance of biodegradation as a fate of chlorobenzenes added to aerobic soil. Two studies indicate that the major mechanism of chlorobenzene loss is due to volatilization with biodegradation playing only a minor role. One of the studies arrived at this conclusion while testing 1,3-DCB, 1,2,3-TCB, 1,2,3,5-TeCB and QCB (Wang and Jones, 1994b). The other study drew the same conclusion for monochlorinated CB (Brahushi et al., 2002). However, other studies provide careful aerobic biodegradation measurements of chlorinated benzenes in contaminated soil incubated in enclosed vessels or microcosms (Marinucci and Bartha, 1979; Schroll et al., 2004). Based on chloride release and increase in optical density (due to cell growth), it was determined that CB, 1,3-DCB, 1,4-DCB, 1,2,4-TCB, 1,2,3,.4-TeCB and 1,2,4,5-TeCB were biodegradable when incubated in soil slurries (Feidieker et al., 1994). In moist pristine soil, 1,2,3-TCB and 1,2,4-TCB were biologically mineralized at a rate of 0.012 and 0.052 nmol CO_2 g^{-1} soil dry weight d^{-1}, respectively (Marinucci and Bartha, 1979). Radiolabeled 1,2,4-TCB was incubated aerobically in a microcosm with either agricultural soil or soil from an industrial site (Schroll et al., 2004). Very little mineralization of radiolabeled 1,2,4-TCB was observed with the agricultural soil; however, the industrially impacted soil was responsible for 62% mineralization of [^{14}C]TCB to $^{14}CO_2$ in 23 days. Nishino *et al*. (1994; 1999) were able to readily isolate indigenous CB degraders from chronically contaminated soils. The number of CB-degrading bacteria per gram soil-slurry increased with the CB concentration of a given site, the highest counts of 5×10^6 CB-degraders g^{-1} were observed at the most contaminated site with 28 mg CB l^{-1} (Nishino et al., 1994). The natural microbial consortium in a soil-groundwater microcosm prepared from the most heavily contaminated site mineralized CB by 54% in 7 days (Nishino et al., 1992). In a similar study, 8 different aerobic isolates capable of growth on CB as a sole source of carbon and energy were readily obtained from a contaminated aquifer (van der Meer et al., 1998). Groundwater from another contaminated aquifer readily mineralized CB and 1,4-DCB based on O_2 uptake data (Dermietzel and Vieth, 2002). The corresponding half-lives at 14°C for the degradation of CB ranged from 3 to 29 days. Experiments with [^{14}C]1,4-DCB revealed that two-thirds of the compound was mineralized

in the groundwater to $^{14}CO_2$ (Dermietzel and Vieth, 2002). Evidence for the biodegradation of 1,2-DCB, 1,3-DCB, 1,4-DCB and 1,2,4-DCB in the contaminated groundwater was also obtained by measurements of inorganic chloride release (Dermietzel and Vieth, 2002). Finally, biodegradation of CB and 1,2,4-TCB was also reported in freshwater and estuarine surface waters (Bartholomew and Pfaender, 1983).

Evidence for the anaerobic biotransformation of chlorinated benzenes has also been found in the natural environment. The best example comes from the comparison of historically archived sediments from 1972 with recently sampled and dated sediment core data from a large inland lake in the Netherlands, Ketelmeer (Beurskens et al., 1993a). The Ketelmeer is the sedimentation basin of the inflowing Rhine River. HCB in the early 1970 layer of the recently sampled sediments was 80% lower than the HCB in archived sediments. Well-known anaerobic biotransformation products of HCB, 1,3,5-TCB and 1,3-DCB, were 2.1 and 5.7-fold higher in the 1970 layer compared to the archived sample, respectively. A maximum half-life of HCB in the sediments was estimated to be 7 years. To confirm that anaerobic dechlorination was the dominant process for HCB removal, laboratory microcosms of the sediment samples were shown to catalyze the biological dechlorination of HCB to 1,3,5-TCB and 1,3-DCB (Beurskens et al., 1993a). Microcosms prepared from historically contaminated estuarine sediment were also shown to anaerobically dechlorinate HCB with a half-life of approximately 1 year (Prytula and Pavlostathis, 1996). Anaerobic microcosms prepared from a freshwater lake in Japan, dechlorinated HCB with a half-life of 63 days and 1,2,4-TCB, 1,2,3-TCB, 1,4-DCB and 1,3-DCB were observed as important biotransformation products (Susarla et al., 1997b). Chlorinated benzenes were tested in a sulfate-reducing river sediment from Japan (Masunaga et al., 1996). The half-life of HCB was only 27 days and, again, 1,2,4-TCB, 1,2,3-TCB, 1,4-DCB and 1,3-DCB were observed as the main biotransformation products. The half-lives of QCB, TeCB and TCB were in the same order of magnitude as HCB; however, those of 1,4-DCB and 1,3-DCB were distinctly higher, corresponding to 385 and 433 d, respectively (Masunaga et al., 1996).

Monochlorinated CB is generally regarded as persistent in anaerobic environments. However, CB was shown to be degraded *in situ* at the fringe of an anoxic aquifer (with a long history of CB contamination) based on evidence from isotopic fractionation data and incorporation of ^{13}C-labeled CB into long chain fatty acids of bacteria (Kaschl et al., 2005; Kastner et al., 2006).

4.2.2. Biodegradation of chlorobenzenes in engineered systems

There are multiple examples in which engineered treatment systems have been utilized to degrade various congeners of chlorinated benzenes under aerobic, anaerobic as well as sequential anaerobic-aerobic conditions. Several research groups have explored the use of aerobic bioreactors to treat groundwater contaminated with lower chlorinated benzenes (Alfreider et al., 2002; Feidieker et al., 1995; Klecka et al., 1996; Lapertot et al., 2006; Nishino et al., 1994). At one chlorinated solvents site, two 135-liter fixed-film pilot scale bioreactors were operated to degrade CB present in the groundwater. One reactor was inoculated with a CB-degrading strain *Pseudomonas* sp. strain JS150; while the other reactor was allowed to become colonized with the indigenous CB-degrading microorganisms from the site (Nishino et al., 1994). The reactors treated groundwater containing approximately 1 mg l^{-1} CB and the removal efficiencies ranged from 87 to 95%. The introduced strain could not be recovered after 3 weeks, indicating that the indigenous microorganisms were responsible for CB degradation. A similar reactor was established in the laboratory and confirmed the pilot-study results with hydraulic residence times as low as 30 minutes. A field-scale fluidized bed reactor (V= 0.8 m^3) supplied with granular activated carbon (GAC) as biofilm support was evaluated for the removal of CB in groundwater (Klecka et al., 1996). The bioreactor was inoculated with aerobic activated sludge. The reactor removed CB from an average ground water concentration of 145 mg l^{-1} to levels of 1 mg l^{-1} in the effluent. The average load of CB removed was 4.8 g CB l^{-1}_{reactor} d^{-1}. The elimination of dissolved oxygen accounted for 45% of the theoretical oxygen demand of CB, suggesting that at least 45% of the CB removed was due to biodegradation. The degradation of a mixture of CB and 1,2-DCB was evaluated in an aerobic bioreactor operated with sequenced pulses and continuously (Lapertot et al., 2006). The maximum conversion capacity of the aerobic bioreactor was 5.6 and 11.3 k chlorobenzene mixture l^{-1}_{reactor} d^{-1}., for the sequenced pulse and continuous operation modes, respectively. An *in situ*

bioreactor filled with aquifer sediments was used to aerobically degrade CB in anoxic groundwater by supplying oxygen via hydrogen peroxide (Vogt et al., 2004a). The reactor effectively removed 17.7 mg l^{-1} of CB with a supply of 29.2 mg l^{-1} of H_2O_2; however, with time higher H_2O_2 concentrations were required due to a shift in the bacterial population. Finally, the aerobic cooxidation of CB-contaminated groundwater utilizing methane as the primary substrate was explored in a soil column (7.9 L) packed with 13.4 aquifer sediments and colonized with a natural mixed culture of methanotrophic bacteria (Jechorek et al., 2003). The column, which operated with a hydraulic retention time of 1.5 days, was highly effective in the removal of CB, reducing the influent concentration of 25-30 CB mg l^{-1} to 0.04 CB mg l^{-1} or less.

Degradation of mixtures of chlorinated benzenes has also studied in slow sand filter columns under aerobic conditions (Zacharias et al., 1995; Bosma et al., 1996). In one study, contaminated water was filtered through 40 kg of sand placed in 32-liter columns with a hydraulic retention time of 3.2 hours. The sum of all chlorobenzenes in the influent and effluent was 3.81 and 0.014 mg l^{-1} respectively; corresponding to a removal efficiency of 99.6%. As evidence of biodegradation, the inorganic chloride concentration was shown to increase in the column. In another study, the removal of chlorobenzenes during infiltration of water into sand dunes as part of a drinking water treatment scheme was evaluated with laboratory-scale columns (V= 0.6 or 5.7 l) filled with sand (Bosma et al., 1996). Under aerobic conditions, CB, 1,2-DCB, 1,3-DCB, 1,4-DCB and 1,2,4-TCB were removed by more than 99, 90, 30, 90 and 40%, respectively.

Several studies considered the removal of CB in waste gases in biotrickle reactors (Oh and Bartha, 1994; Mpanias and Baltzis, 1998; Seignez et al., 2004; Mathur et al., 2006). In the first study, a laboratory biotrickle column of 1.57 l was packed with perlite and used to treat CB and 1,2-DCB vapors supplied at concentrations of 1.2 and 0.7 g m^{-3} air, respectively (Oh and Bartha, 1994). The volumetric removal rates of the compounds in the trickling biofilter were 0.122 and 0.053 g l^{-1}_{reactor} d^{-1} for CB and 1,2-DCB, respectively. Inorganic chloride accumulated in the liquid phase of the reactor and corresponded to 72% of the removal of CB and 1,2-DCB, indicating a high level of mineralization. The second study evaluated the removal of CB vapors in a 14.5-liter laboratory trickle filter packed with ceramic saddles with liquid recirculation in counter flow to the gas (Mpanias and Baltzis, 1998). Maximum volumetric removal rates of up to 1.5 g l^{-1}_{reactor} d^{-1}. were obtained. In the third study, a 40-liter biotrickle reactor with cylindrical PVC as packing (Seignez et al., 2004) was used treat mixtures of CB and 1,2-DCB. At steady state after 3 months of operation, the reactor achieved maximum volumetric loading rates of 5.2 g l^{-1}_{reactor} d^{-1}. with removal efficiencies in the range of 95-99%. In the fourth study, CB was treated in a 2-liter biotrickle filter with coal packing and inoculated with a mixed culture from activated sludge (Mathur et al., 2006). The average CB elimination capacity of the column was 1.9 g l^{-1}_{reactor} d^{-1}.

Also anaerobic bioreactor and bioremediation systems have been utilized to treat higher chlorinated benzenes. A large number of chlorinated benzene congeners were susceptible to biotransformation under anaerobic conditions in sediment or sand columns (Bosma et al., 1988; 1996; Van Der Meer et al., 1992). Laboratory columns packed with Rhine River sediments or sand were capable of transforming most congeners of chlorinated benzenes under methanogenic conditions by 90 to >99% with the exception of CB (Bosma et al., 1988; 1996). CB was the main biotransformation product of DCBs, whereas 1,3- and/or 1,4-DCB were the main biotransformation products of TCBs, TeCBs, QCB and HCB. Similar experiments conducted under sulfate-reducing conditions revealed that 1,2,3-TCB, 1,2,4,-TCB, 1,2,3,4-TeCB, QCB and HCB were eliminated by more than 90% (Van Der Meer et al., 1992). None of the congeners were subject to biotransformation under denitrifying conditions (Bosma et al., 1996). The reductive dechlorination of HCB was observed to readily occur in anaerobic sewage sludge (Fathepure et al., 1988; Dionisi et al., 2006). HCB (50 mg l^{-1}) was completely converted within 3 weeks primarily to 1,3,5-TCB and to a lesser extent to 1,2,4-TCB and DCBs (Fathepure et al., 1988). 1,3,5-TCB (21.5 mg kg^{-1} dwt) present in municipal anaerobic digester sludge was removed by 53% when incubated anaerobically for 175 days with yeast extract as electron donor (Dionisi et al., 2006).

Several studies evaluated the anaerobic bioremediation of chlorinated benzenes in soil (Ramanand et al., 1993; Rosenbrock et al., 1997; Brahushi et al., 2004). In the first study,

bioremediation of HCB was evaluated by providing anaerobic conditions to the different soils (Rosenbrock et al., 1997). In some soils, the endogenous organic matter provided electron donor to support HCB dechlorination; whereas in other soils with low organic matter content, organic substrate addition was required. In both cases, dechlorination of radiolabeled [^{36}Cl]HCB (spiked at 30 mg kg^{-1} soil) to $^{36}Cl^-$ was demonstrated accounting for about 40% dechlorination in 140 days. In the second study, a soil slurry contaminated with a mixture of HCB (0.029 mM), QCB (0.074 mM) and 1,2,4-TCB (1.14 mM) was converted almost stoichiometrically to CB (1.01 mM) after 140 days of incubation under methanogenic conditions with H_2 as electron donor (Ramanand et al., 1993). In the third study, HCB in agricultural soil was bioremediated by flooding the soil in laboratory microcosms (Brahushi et al., 2004). After 20 weeks of incubation only 1% of applied [^{14}C]HCB radiolabeled could be recovered in the extractable fraction. Products of reductive dechlorination, most notably 1,3,5-TCB, accounted for approximately 75% of the HCB removed from the soil.

The sequential anaerobic-aerobic treatment of HCB was evaluated in one study (Fathepure and Vogel, 1991). A two-stage biological treatment scheme was tested for the biodegradation of HCB (0.075 mg l^{-1}) utilizing laboratory-scale anaerobic and aerobic biofilm reactors (each 0.25 l) operated in series, having a hydraulic retention times of 37.5 and 2.24 h, respectively (Fathepure and Vogel, 1991). During the anaerobic stage, acetate was found to be the best electron-donating substrate, supporting 98.7% removal of HCB, which was recovered mostly as 1,2,3-TCB (60%) and 1,2-DCB (10%). Experiments with [^{14}C]HCB revealed that HCB was mineralized by 23% to $^{14}CO_2$ during the sequential anaerobic-aerobic treatment and the total metabolism to both $^{14}CO_2$ and [^{14}C] in non-volatile intermediates was 94%.

4.3. Microbiology and biochemistry of chlorobenzene biodegradation

Chlorobenzenes are subject to both aerobic and anaerobic metabolism. Under aerobic conditions, lower chlorinated benzene congeners can serve as a growth supporting substrates and in some cases become cometabolized. Under anaerobic conditions, higher chlorinated benzene congeners are subject to reductive dechlorination provided electron-donating substrates are available. Recent evidence also points to the use of higher chlorinated benzenes as electron acceptors supporting halorespiration.

4.3.1. Aerobic bacterial cometabolism of chlorobenzenes

Several examples of aerobic cometabolism of chlorinated benzenes are reported. CB-grown cells of the bacterium *Pseudomonas* sp. strain JS150 were able to oxidize 1,2-DCB and 1,3-DB which were otherwise not growth substrates (Haigler et al., 1992). The cometabolism of 1,2-DCB and 1,2,4-TCB by *Pseudomonas aeruginosa* strain RHO1 was reported while the strain utilized growth substrates CB and 1,4-DB (Brunsbach and Reineke, 1994). The cooxidation of 1,2,3-TCB by the methane oxidizing bacterium, *Methylosinus trichosporium* OB3b, was studied (Sullivan and Chase, 1996). Cells grown with methane under low copper conditions to stimulate soluble methane monooxygenase metabolized 1,2,3-TCB to 2,3,4- and 3,4,5- trichlorophenol when provided with formate as cosubstrate. Another methanotrophic strain, *Methylocystis* sp. GB 14, was found to cooxidize CB with methane-grown cells cultivated in copper-free medium (Jechorek et al., 2003). Under variable culture conditions, 80% of CB was eliminated by *Methylocystis* sp. GB 14, and chlorophenols were recovered as products accounting for 53% recovery of CB metabolized. Cooxidation of CB by propane-grown cells of the bacterium, *Mycobacterium vaccae* strain JOB-5, has also been reported (Burback and Perry, 1993). The main product from the conversion was 4-chlorophenol.

Finally, there are several reports describing the metabolism of chlorinated benzene by *Pseudomonas putida* strains. Glutamate-grown cells of *Pseudomonas putida* MST were shown to cometabolize CB to 3-chlorocatechol (Bestetti et al., 1992). Benzene-grown cells of *Pseudomonas putida* cometabolized 1,2-DCB to 2,3-, 3,4- and 2.6-dichlorophenols (Ballschmiter and Scholz, 1981). A benzene-grown mixed culture from soil cometabolized

1,3,5-TCB to 2,4,6-trichlorophenol (Ballschmiter and Scholz, 1981). In a similar study, almost all congeners of chlorinated benzenes ranging from mono- to tetrachlorobenzenes were hydroxylated to chlorophenols by a benzene-grown mixed culture from soil (Ballschmiter et al., 1977). Chlorocatechols were additionally recovered from experiments with the dichlorobenzenes (Ballschmiter and Scholz, 1980). In the experiments with benzene as the primary substrate, the results suggest the involvement of a monooxygenase in the cometabolism of chlorobenzene. The initial formation of an epoxide is postulated. The epoxide is subsequently rapidly converted to a chlorophenol. An additional reaction with monooxygenase results in the conversion of the chlorophenol intermediates to chlorocatechols (Ballschmiter and Scholz, 1980). The monooxygenase from *Pseudomonas putida* has been isolated and it is responsible for the hydroxylation of chlorinated benzenes (Jones et al., 2001). Site-directed mutagenesis has been used to improve the activity and broaden the substrate specificity of the monooxygenase to include QCB and HCB (Jones et al., 2001; Chen et al., 2002b). The pentachlorophenol (PCP)-degrading bacterium, *Sphingobium chlorophenolicum* ATCC 39723, was genetically engineered to include genes for a mutant form of the monooxygenase cytochrome P450 that can oxidize HCB to PCP (Yan et al., 2006a). Glutamate-grown cells of the genetically engineered strain were able to metabolize HCB and PCP was shown to be an intermediate.

4.3.2. Aerobic bacterial growth on chlorinated benzenes as sole carbon and energy source

The evidence that several chlorinated benzenes can be utilized as a sole source of carbon and energy is overwhelming. The first report of bacterial growth on a chlorinated benzene was that of an unidentified strain, WR1306, utilizing CB (Reineke and Knackmuss, 1984). Since then, a wide variety of bacterial strains have been shown to utilize, CB, 1,2-DCB, 1,3-DCB, 1-4-DCB, 1,2,4-TCB, 1,2,4,5-TeCB and 1,2,3,4-TeCB as a growth substrates as outlined in Table 4.1. In most of these studies, sound evidence for the mineralization of the chlorinated benzene has been provided such as stoichiometric release of chloride (Reineke and Knackmuss, 1984; Debont et al., 1986; Schraa et al., 1986; Spain and Nishino, 1987; Haigler et al., 1988; Sander et al., 1991; Spiess et al., 1995; Sommer and Gorisch, 1997; Potrawfke et al., 1998a) or mineralization of ^{14}C-labeled chlorinated benzenes as $^{14}CO_2$ (Marinucci and Bartha, 1979; Haigler et al., 1988; Nishino et al., 1992). The degradative attack of chlorinated benzenes by these strains is initiated with dioxygenases to produce chlorinated dihydrodiol intermediates that are subsequently rearomatized by dihydrodiol dehydroganeses, yielding chlorocatechols as intermediates (Spain and Nishino, 1987; Haigler et al., 1988; Sander et al., 1991; Spiess et al., 1995; Beil et al., 1997; Mars et al., 1997; Beil et al., 1998; Potrawfke et al., 1998a; van der Meer et al., 1998). A chlorobenzene dioxygenase from *Burkholderia* sp. strain PS12 was cloned into *Escherichia coli*, which could express an active form of the enzyme (Beil et al., 1997). The heterologous recombinat chlorobenzene dioxygenase converted 1,2,4,5-TeCB to an unstable tetrachlorodihydrodiol intermediate, which spontaneously rearomatizes with concomitant chloride elimination to the corresponding 3,4,6- trichlorocatechol. In most other cases, the initial dioxygenation results in the formation of stable dihydrodiol intermediates with the same number of chlorine groups as the original substrate (Debont et al., 1986; Haigler et al., 1988; Sander et al., 1991; Spiess et al., 1995; Chartrain et al., 2000). The heterologous recombinant chlorobenzene dioxygenase for example converted 1,2,4-TCB to the corresponding stable trichlorodihydrodiol (Beil et al., 1997). Theses *cis* dihydrodiol intermediates are oxidized to the corresponding chlorocatechols by dihydrodiol dehydrogenases (Spain and Nishino, 1987; Haigler et al., 1988; Sander et al., 1991; Spiess et al., 1995; Spiess and Gorisch, 1996; Sommer and Gorisch, 1997). The chlorocatechols are subsequently oxidized by either one of two types of chlorocatechol dioxygenases, causing either *ortho*-cleavage (catechol 1,2-dioxygenase) to chloromucconic acids (Schraa et al., 1986; Spain and Nishino, 1987; Haigler et al., 1988; Spiess et al., 1995; Sander et al., 1991; Sommer and Gorisch, 1997; Potrawfke et al., 1998a) or *meta* cleavage (catechol 2,3-dioxygenase) to 2-hydroxy-6-chlorocarbonyl mucconic acid (acylchloride) (Klecka and Gibson, 1981; Bartels et al., 1984; Pettigrew et al., 1991; Mars et al., 1997). Chloromuconic acids are metabolized further to intermediates of the Kreb's cycle as shown in Figure 4.1. The formation of a reactive acylchloride by *meta*-cleavage usually results in inactivation of the catechol dioxygenase and eventually cell death (Klecka and Gibson, 1981; Bartels et al., 1984). However, *Pseudomonas putida* GJ31 was found to have a *meta*-cleavage enzyme resistant to suicide and converted chlorocatechol to 2-hydroxy-

cis,*cis*-muconic acid, which was mineralized further (Mars et al., 1997) as shown in Figure 4.1.

4.3.3. Degradation of chlorobenzenes by fungi

The white-rot fungus, *Phanerochaete chrysosporium*, partially mineralizes radiolabeled mono- and dichlorobenzenes to $^{14}CO_2$ by 12-28% (Yadav et al., 1995a). The metabolism of chlorobenzene by *Phanerochaete chrysosporium* was very limited in low-nitrogen medium and greatly improved in high-nitrogen, indicating that ligninolytic enzymes were most likely not involved. Hexachlorobenzene was shown to be partially eliminated from soil by a *Lentinus* isolate (Matheus et al., 2000). Two white-rot fungi, *Phanerochaete chrysosporium* and *Pleurotus pulmonarius*, were used to inoculate a contaminated soil that contained a mixture of chlorinated pollutants, including 17.4 and 10.3 mg kg^{-1} of 1,2,3,4-TeCB and 1,2,4,5-TeCB, respectively (D'Annibale et al., 2005). After 30 days of incubation, the TeCB congeners were completely eliminated in soils inoculated with the white-rot fungi; whereas they were only eliminated by 50% with the indigenous soil population.

4.3.4. Anaerobic cometabolism of chlorobenzenes

Reductive dechlorination of chlorinated benzenes under anaerobic conditions is a well established biotransformation process occurring either as a fortuitous cometabolic reaction or energy yielding halorespiration (Adrian and Gorisch, 2002). The slow reductive biotransformation of 1,2,4-TCB to 1,2-DCB and CB in the presence of H_2 by *Staphylococcus epidermidis* isolated from the gastrointestinal tract of rats constitutes one of the first reported examples of chlorinated benzene cometabolism (Tsuchiya and Yamaha, 1984). Ruptured cells of *Staphylococcus epidermidis* were also capable of converting TCB when supplied with NADH as electron donor. Other examples include the conversion HCB to several TCB (1,3,5- and 1,2,4-) and DCB (1,2-, 1,3- and 1,4-) isomers in anaerobic sewage sludge (Fathepure et al., 1988; Yuan et al., 1999). The main product recovered from the conversion was 1,3,5-TCB, accounting for almost 90% of HCB added (Fathepure et al., 1988).

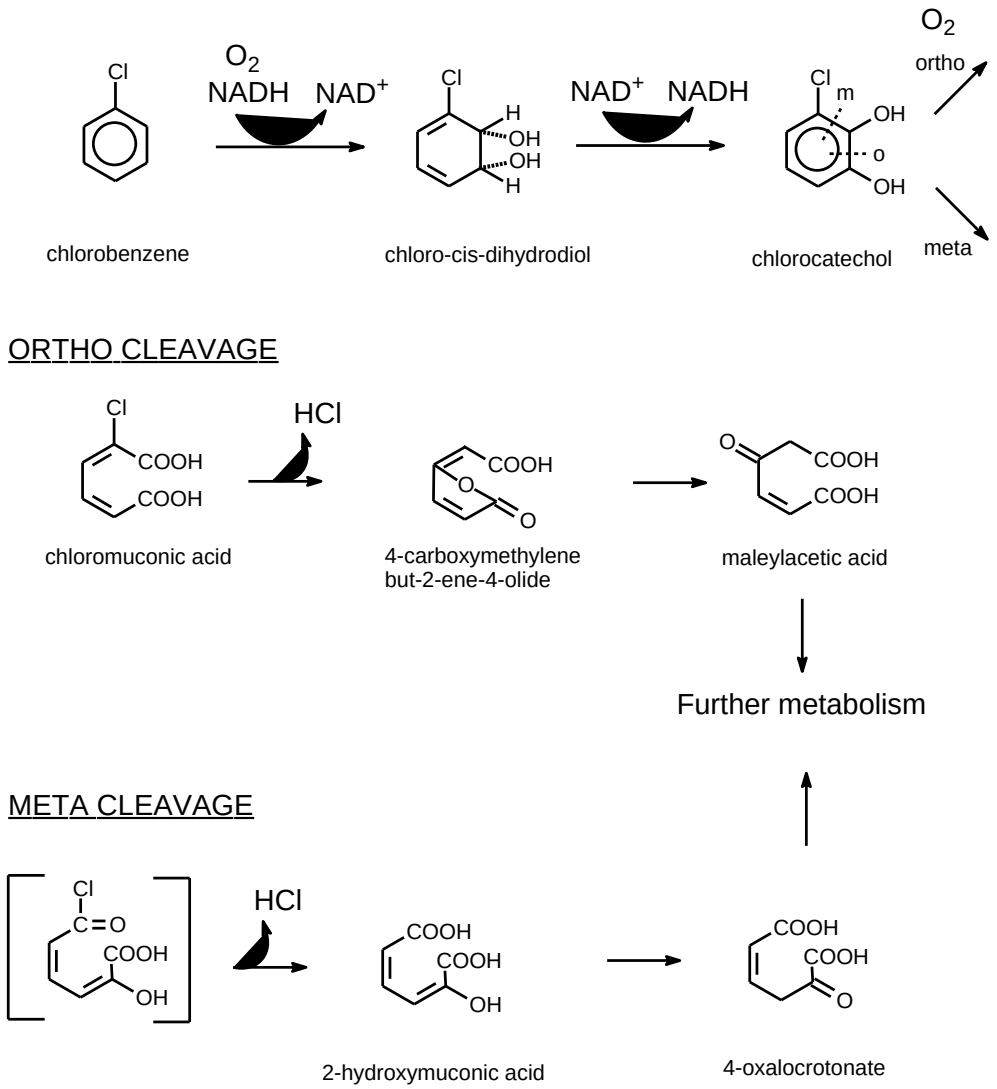

Figure 4.1. *Pathways of aerobic degradation of chlorobenzenes by strains utilizing the chlorinated compound as a growth substrate (Reineke and Knackmuss, 1984; Debont et al., 1986; Schraa et al., 1986; Spain and Nishino, 1987; Haigler et al., 1988; Sander et al., 1991; Spiess et al., 1995; Mars et al.,*

Table 4.1. Aerobic bacterial strains capable of growing on chlorinated benzenes as a sole source of carbon and energy.

Bacterial Strain	Congener	Reference
Burkholderia sp. strain PS12	CB	(Sander et al., 1991)
Burkholderia sp. strain PS14	CB	(Sander et al., 1991)
Escherichia hermanii	CB	(Kiernicka et al., 1999)
Hydrid strain WR1313	CB	(Oltmanns et al., 1988)
Pseudomonas aeruginosa RHO1	CB	(Brunsbach & Reineke, 1994)
Pseudomonas putida GJ31	CB	(Oldenhuis et al., 1989; Mars et al., 1997)
Pseudomonas sp. strain JS100	CB	(Haigler et al., 1988)
Pseudomonas sp. strain JS150	CB	(Haigler et al., 1992)
Pseudomonas sp. strain JS6	CB	(Pettigrew et al., 1991)
Ralstonia sp. strain JS705	CB	(van der Meer et al., 1998)
Rhodococcus phenolicus	CB	(Rehfuss & Urban, 2005)
Rhodococcus sp.	CB	(Vogt et al., 2004a)
Pseudomonas sp.	CB	(Vogt et al., 2004a)
Xanthobacter sp.	CB	(Vogt et al., 2004a)
Paenibacillus sp.	CB	(Vogt et al., 2004a)
Kocuria sp.	CB	(Vogt et al., 2004a)
Stenotrophomonas sp.	CB	(Vogt et al., 2004a)
Unidentified strain 1469	CB	(Nishino et al., 1992)
Unidentified strain 1474	CB	(Nishino et al., 1994)
Unidentified strain WR1306	CB	(Reineke & Knackmuss, 1984)
Planococcus sp strain ZD22	CB	(Li et al., 2006)
Acidovorax facilis 13517	CB	(Vogt et al., 2004a; Vogt et al., 2004b)
Cellulomonas turbata B529	CB	(Vogt et al., 2004b)
Pseudomonas veronii 13547	CB	(Vogt et al., 2004b)
Pseudomonas veronii B549	CB	(Vogt et al., 2004b)
Paenibacillus polymyxa B550	CB	(Vogt et al., 2004b)
Burkholderia sp. strain PS12	1,2-DCB	(Sander et al., 1991)
Burkholderia sp. strain PS14	1,2-DCB	(Sander et al., 1991; Rapp & Timmis, 1999)
Pseudomonas sp. strain GJ60	1,2-DCB	(Oldenhuis et al., 1989)
Pseudomonas sp. strain P51	1,3-DCB	(Van Der Meer et al., 1987)

Continued on next page

Table 4.1. ***(Continued).*** Aerobic bacterial strains capable of growing on chlorinated benzenes as a sole source of carbon and energy.

Bacterial Strain	Congener	Reference
Alcaligenes sp. strain OBB65	1,3-DCB	(Debont et al., 1986)
Burkholderia sp. strain PS12	1,3-DCB	(Sander et al., 1991)
Burkholderia sp. strain PS14	1,3-DCB	(Sander et al., 1991; Rapp & Timmis, 1999)
Pseudomonas sp. strain JS100	1,2-DCB	(Haigler et al., 1988)
Pseudomonas sp. strain P5	1,2-DCB	(Van Der Meer et al., 1987)
Acidovorax avenae	1,2-DCB	(Monferran et al., 2005)
Alcaligenes sp. strain R3	1,4-DCB	(Oltmanns et al., 1988)
Burkholderia sp. strain PS12	1,4-DCB	(Sander et al., 1991)
Burkholderia sp. strain PS14	1,4-DCB	(Sander et al., 1991; Rapp & Timmis, 1999)
Hydrid strain WR1323	1,4-DCB	(Oltmanns et al., 1988)
Pseudomonas aeruginosa RHO1	1,4-DCB	(Oltmanns et al., 1988; Brunsbach &Reineke, 1994)
Pseudomonas sp. B1	1,4-DCB	(Oltmanns et al., 1988)
Pseudomonas sp. strain JS150	1,4-DCB	(Haigler et al., 1992)
Pseudomonas sp. strain JS6	1,4-DCB	(Spain and Nishino, 1987)
Pseudomonas sp. strain P51	1,4-DCB	(Van Der Meer et al., 1987)
Sphingomonas (*Alcaligenes*) A175	1,4-DCB	(Schraa et al., 1986)
Unidentified strain 1474	1,4-DCB	(Nishino et al., 1994)
Xanthobacter flavus 14p1	1,4-DCB	(Sommer and Gorisch, 1997)
Rhodococcus phenolicus	1,4-DCB	(Rehfuss and Urban, 2005)
Burkholderia sp. strain PS12	1,2,4-TCB	(Sander et al., 1991)
Burkholderia sp. strain PS14	1,2,4-TCB	(Sander et al., 1991; Rapp & Timmis, 1999; Rapp, 2001)
Pseudomonas sp. strain P51	1,2,4-TCB	(Van Der Meer et al., 1987)
Pseudomonas chlororaphis RW71	1,2,3,4-TeCB	(Potrawfke et al., 1998a)
Burkholderia (*Pseudomonas*) PS12	1,2,4,5-TeCB	(Beil et al., 1997; Beil et al., 1998)
Burkholderia sp. strain PS14	1,2,4,5-TeCB	(Sander et al., 1991; Rapp & Timmis, 1999)

4.3.5. Anaerobic metabolism of chlorobenzenes by enrichment cultures

Several researchers have developed anaerobic enrichment cultures that dehalogenate the chlorinated benzenes at considerably faster rates than the original inocula. Examples of anaerobic enrichment cultures and the isomers dechlorinated are shown in Table 4.2. The fact that organisms have become enriched by dechlorinating chlorinated benzenes suggests some kind of benefit is derived from the process (Holliger et al., 1992; Beurskens et al., 1994; Adrian et al., 2000a; Chen et al., 2002a). The evidence is supported by demonstrating that population growth is linked the dechlorination of the chlorinated benzenes (Adrian et al., 2000a). In most studies the dechlorination pattern follows the thermodynamically most favorable reaction (Beurskens et al., 1994), as shown in Figure 4.2. This entails a preferential dechlorination of doubly flanked over single flanked chlorines. Thus, the most frequently observed dechlorination pattern proceeds via 1,2,3,5-TeCB to 1,3,5-TCB which is dechlorinated further to 1,3-DCB. One study has observed the exception to the trend, in which single flanked chlorines are preferentially dechlorinated, resulting in a pathway to CB proceeding via 1,2,3,4-TeCB, 1,2,3-TCB, 1,2-DCB (Ramanand et al., 1993).

In recent years, considerable progress has been made in identifying halorespiring organisms responsible for growth-linked dechlorination of chlorinated benzenes. A phylogenetic survey using 16S RNA genes and applied to an anaerobic TCB-transforming microbial community obtained from a fluidized bed reactor revealed the presence of the halorespiring bacterium, *Dehalobacter* sp (von Wintzingerode et al., 1999). The occurrence of halorespiring bacteria in the TCB-dechlorinating community was further confirmed by hybridization with molecular probes based on conserved regions of reductive dehalogenase genes (*PceA* and *CprA*) from known halorespiring bacteria (von Wintzingerode et al., 2001). These findings support the hypothesis that reductive dechlorination of TCB occurs via a respiratory pathway.

A pure culture capable of dechlorinating TeCB and TCB was isolated and characterized (Adrian et al., 2000b; Jayachandran et al., 2003). *Dehaloccocoides* strain CBDB1, a bacterium that is closely related to known PCE halorespiring bacterial strains, links its growth to the oxidation of hydrogen at the expense of respiring the chlorinated benzenes. The chlorinated benzene substrate range and products of *Dehaloccocoides* strain CBDB1 are shown in Table 4.3. The initial preference of dechlorination is doubly flanked chlorines, followed by single flanked chlorines. Chlorines that are not flanked at all are not dechlorinated by *Dehaloccocoides* strain CBDB1. Cell-free extracts prepared from this strain displayed dehalogenase activity towards many congeners of chlorinated benzenes, utilizing methyl viologen as an artificial electron donor (Holscher et al., 2003). Rates ranged from 0.3 to 355 nkat/mg protein for 1,2,4-TCB to 1,2,3,4-TeCB, respectively. The activity was associated with the membrane fraction of the cells and specific inhibitors indicated the involvement of corrinoid cofactors.

A pure culture of *Dehalococcoides ethenogens* strain 195, known for its ability to halorespire perchloroethene (PCE) to ethene, was shown to dechlorinate various highly chlorinated benzene congeners when PCE was also present as an electron acceptor to support growth (Fennell et al., 2004). The daughter products from the reductive dechlorination with either HCB or QCB were 1,2,3,5-TeCB, 1,2,4-TCB and 1,3,5-TCB. The daughter products from the dechlorination of 1,2,4,5-TeCB were 1,2,4-TCB, 1,4-DCB and 1,3-DCB. The daughter products from the reductive dechlorination of 1,2,3,4-TeCB were 1,2,4-TCB, 1,3,5-TCB and 1,3-DCB. By comparison, the congener, 1,2,3,5-TeCB, was dechlorinated very slowly by *Dehalococcoides ethenogens* strain 195.

Table 4.2. Anaerobic enrichment cultures reductively dehalogenating chlorinated benzene compounds.

Original Inoculum	Congener(s)	Electron Donor	Product(s)	Reference
River sediment	123-TCB	Lactate or H_2	13-DCB	(Holliger et al., 1992)
Mixture of sediments	124-TCB	Lactate, glucose, ethanol, methanol, propionate, acetate, H_2	14-DCB, CB	(Middeldorp et al., 1997)
Bioreactor	Mix 123-TCB and 124-TCB	Pyruvate or formate	DCBs	(Adrian et al., 1998)
Bioreactor	Mix 123-TCB and 124-TCB	Pyruvate or H_2	DCBs	(Adrian et al., 2000a)
PCB-enrichment culture (with DF-1)	QCB or HCB	Formate	1235-TeCB, 1,3,5-TCB	(Wu et al., 2002a)
Lake sediment	HCB	Lactate	QCB, 1235-TeCB, 1245-TeCB, 124-TCB, 135-TCB	(Beurskens et al., 1994)
River sediments	HCB	Yeast extract	1,3,5-TCB	(Chen et al., 2002a)
123-TCB enrichment from river sediments	HCB	Yeast extract	QCB, 1235-TeCB, 125-TCB, 124-TCB, 13DCB	(Chang et al., 1998) (Chang et al., 1997)
HCB enrichment from sediment	HCB	Surfactant (Tween 61)	135-TCB, 14-DCB, 13-DCB	(Yeh & Pavlostathis, 2005)

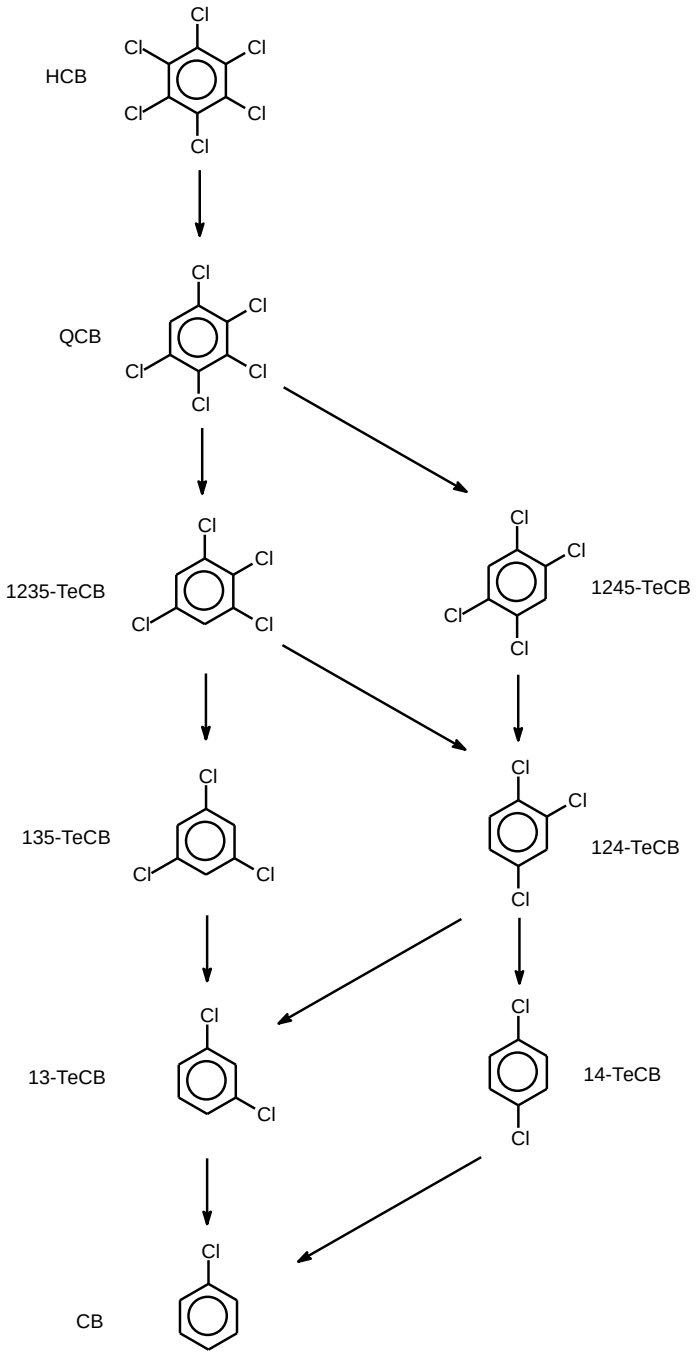

Figure 4.2. *Most common pathways of anaerobic reductive dechlorination of hexachlorobenzene by microbial enrichment cultures and environmental samples (Fathepure et al., 1988; Holliger et al., 1992; Beurskens et al., 1994; Middeldorp et al., 1997; Adrian et al., 1998; Chang et al., 1998; Adrian and Gorisch, 2002; Chen et al., 2002a; Wu et al., 2002).*

Table 4.3. Range of chlorinated benzene congeners utilized by the halorespiring bacterium, *Dehaloccocoides* strain CBDB1, as terminal electron-acceptor and products from the dechlorination (Adrian et al., 2000b; Jayachandran et al., 2003; Griebler et al., 2004). Hydrogen is utilized as the electron donor.

Chlorinated benzene congeners utilized	Transient intermediates	Final reduced products
123-TCB		13-DCB
124-TCB		13-DCB and 14-DCB
1234-TeCB	124-TCB	13-DCB and 14-DCB
1245-TeCB	124-TCB	13-DCB and 14-DCB
1235-TeCB		135-TCB
QCB	1245-TeCB, 1235-TeCB and 124-TCB	135-TCB, 13-DCB and 14-DCB
HCB	QCB, 1245-TeCB, 1235-TeCB and 124-TCB	135-TCB, 13-DCB and 14-DCB

*In the case of QCB, a highly enriched culture instead of a pure culture was utilized.

A highly enriched culture cultivated by growth-linked dehalogenation of the PCB congener, 2,3,4,5-tetrachlorobiphenyl, was shown to also dechlorinate HCB (Wu et al., 2002b). The culture dechlorinated HCB to 1,3,5-TCB, via QCB and 1,2,3,5-TeCB. Using molecular ecology tools, the uncultured bacterium DF-1 was shown to be the responsible dehalogenator of HCB. Bacterium DF-1 had a closer relationship to known PCE- and TCE-dechlorinating strains of *Dehalococcoides* than to *Dehaloccocoides* strain CBDB1 (only 89% base pair homology).

4.4. Microbial kinetics of chlorobenzene biodegradation

Microbial kinetic data for chlorinated benzene degradation are presented in Table 4.4 (annex). Aerobic growth rates on chlorinated benzenes as a sole source of carbon and energy is moderate to high ranging from 1.11 to 13.2 d^{-1}. These values correspond to doubling times of 1.3 to 15 hours. The literature is in good agreement concerning the specific activity of aerobic bacteria growing on chlorinated benzenes, with the exception of one value, the range is from 520 to 2459 mg g^{-1} dwt d^{-1}. Likewise, there is good agreement on the cell yield from the aerobic growth on chlorinated benzenes with all but one value in the range of 0.33 to 0.43 g dwt g^{-1} chlorinated benzene consumed. Only one author, Naziruddin et al., 1995 has provided information on the half velocity constant, K_s, which indicates a value of a few mg l^{-1} for an aerobic mixed culture.

Less information is available on the biotransformation kinetics under anaerobic conditions. However, specific activities of chlorinated benzene biotransformation are highly dependent on the degree to which an anaerobic culture has been purified and enriched. The specific activities of chlorinated benzene dehalogenation in unadapted aquatic sediments ranged from 10^{-5} to 10^{-4} mg chlorinated benzene transformed g^{-1} dwt sediments d^{-1}. Rates in enrichment cultures or anaerobic reactor biofilms ranged from 10^{-2} to 10^{2} mg chlorinated benzene transformed g^{-1} dwt biomass d^{-1}. Rates in the halorespiring bacterium, *Dehalococcoides* sp. CBDB1, were 10^{4} to 10^{5} mg chlorinated benzene transformed g^{-1} dwt biomass d^{-1} for chlorinated benzenes used as the growth electron acceptor. The specific activities were even higher, up to 10^{6} mg chlorinated benzene transformed g^{-1} dwt biomass d^{-1}, for chlorinated benzenes when cometabolized with another electron acceptor (Jayachandran et al., 2003). Therefore, halorespiration of chlorobenzenes is at least one order of magnitude faster than the oxidation of chlorobenzene by pure cultures of aerobic bacteria. The cell yields of halorespiration averaged 0.057 g dwt biomass g^{-1} chlorinated benzene transformed.

5 Polychlorinated Biphenyls

5.1. Introduction

The term polychlorinated biphenyls (PCBs) refers to a large group of chlorinated biphenyls with 209 possible congeners. In practice, PCB contamination results from commercial preparations composed of complex mixtures containing 60-90 congeners with commercial names such as Aroclor, Fenclor, Kanechlor and Phenclor, among others. Studies documenting PCB degradation frequently refer to Aroclor which was produced in the United States. Commercial preparations of Aroclor are specified with a 4-digit code. The first two numbers in the code refer to the parent structure (12 indicating biphenyl) and the second two digits refers to the weight percentage of chlorine. For example, Aroclor 1242, 1248, 1254 and 1260 refer to PCB mixtures with an average weight percentage of chlorine of 42, 48, 54 and 60%, respectively. Some studies have utilized single congeners, which will be abbreviated as CBp, DCBp, TCBp, TeCBp, PeCBp, HCBp, HeCBp, OCBp and NCBp for mono-, di-, tri, tetra-, penta-, hexa, hepta, octa- and nona- chlorobiphenyl, respectively.

The main source of PCB into the environment is from their manufacture and use from 1929 to 1978 as transformer fluids, hydraulic fluids and other industrial products (Bedard, 2003). Several hundred million kilograms were released into the environment. Due to their hydrophobic properties, PCBs tend to become adsorbed by natural organic matter in soil, sediments and sludges. The majority of the PCBs released into aquatic environment have, therefore, partitioned into aquatic sediments (Wiegel and Wu, 2000; Bedard, 2003).

Many review articles on PCB biodegradation are available. Borja *et al.* (2005) (Borja et al., 2005) provide a recent overview of both aerobic and anaerobic pathways of PCB biodegradation. Bedard (2003) offers a summary of PCB degradation in aquatic sediments including both anaerobic and aerobic processes. Wiegel and Wu (2000) give a detailed review of anaerobic dechlorination of PCB. Abramowiz (1990) reviewed the PCB congeners degraded by aerobic strains of bacteria. Furukawa (2000) and Seeger et al. (1997) and, more recently, Pieper (2005) provide an overview of the pathways, biochemistry and genetics of the aerobic PCB degradation. Abraham et al. (2002) and Ohtsubo et al. (2004) reviewed some recent advances in PCB biodegradation with an outlook towards applications in bioremediation; both reviews emphasize advances on aerobic degradation.

5.2. Biodegradation of polychlorinated biphenyls

5.2.1. Biodegradation of PCBs in the environment

There are multiple lines of evidence for anaerobic biotransformation of PCB in aquatic sediments. Bedard (2003) reviewed evidence for the anaerobic natural attenuation of PCB at 16 sites located in Canada, Japan, The Netherlands and United States. PCB concentrations in dated sediment layers were compared with archived sediments and were shown to be significantly decreased (Beurskens et al., 1993b). Sediment samples from various historically polluted sites display congener patterns with less chlorine substitutions than the original contaminating commercial PCB mixture (Brown et al., 1987; Sokol et al., 1994; Bedard and May, 1996; Vanier et al., 1996; Li et al., 2005; Magar et al., 2005b; Pakdeesusuk et al., 2005; Bzdusek et al., 2006a; 2006b). The most compelling evidence for the *in situ* anaerobic biotransformation of PCBs was obtained from sediment cores samples in Lake Hartwell (South Carolina, USA) in which the concentration *para*- and *meta*-chlorines was shown to decrease with sediment layer age; whereas resistant *ortho*-

chlorines were shown to be stable with core depth (Magar et al., 2005a). Collectively these results provide strong evidence for the occurrence of reductive dechlorination in aquatic sediments. Additionally, anaerobic microcosms studies indicate the ability of sampled sediments to reductively dechlorinate spiked PCB (Mavoungou et al., 1991; Fish and Principe, 1994; Williams, 1994; Sokol et al., 1998; Wu et al., 1998; Chang et al., 2001; Bedard et al., 2005; Pakdeesusuk et al., 2005; Yan et al., 2006b, a; Zanaroli et al., 2006) as well as aged PCB naturally present in the sample (Zanaroli et al., 2006). The lack of biotransformation in heat-killed controls suggests that the dechlorination is catalyzed by biological processes (Fish and Principe, 1994; Rysavy et al., 2005). Evidence for aerobic degradation of PCB has also been observed in the field based on the detection of characteristic metabolites in sediment cores, *e.g.* chlorinated benzoates and 2,3-dihydro-2,3-dihydroxy-2'-chlorobiphenyl and 2,3-dihydroxy- 2'-chlorobiphenyl (Flanagan and May, 1993b).

5.2.2. Biodegradation of PCB in engineered systems

Anaerobic and aerobic methods have been utilized to promote the biodegradation of PCB in engineered systems. The main approach towards the anaerobic bioremediation of PCB has been to supply electron-donating substrates to facilitate reductive dechlorination. Hudson River sediments spiked with Aroclor 1242 were treated with or without electron donating substrates (Nies and Vogel, 1990). Addition of glucose, acetone and methanol promoted a decrease in the higher chlorinated congeners and an increase in lower chlorinated congeners concomitant with the release of 21, 22 and 13% release of chloride after 22 weeks of incubation. No dechlorination occurred if electron-donating substrate was excluded. In a similar study, a mixture of volatile fatty acids (VFA) was tested as an electron donating substrate to stimulate the dechlorination of PCB in Hudson River sediments spiked with Aroclor 1242 (Alder et al., 1993). VFA addition stimulated the initial rates of dechlorination enabling 65% removal of *para*- and *meta*-chlorines in 2 months while 11 months were required to reach the same final extent of dechlorination without added VFA. H_2 produced from zero-valent iron (Fe^0) was shown to significantly stimulate the reductive dechlorination of spiked HeCBp by harbor sediments (Rysavy et al., 2005). The chlorine:biphenyl ratio decreased by 11% in 250 days. The reductive dechlorination of Aroclor 1248-spiked sediments was promoted in an anaerobic batch reactor with recycle of the liquid phase, utilizing landfill leachate as the electron donor (Pagano et al., 1995). The chlorine to biphenyl ratio was decreased up to 23% in 7 weeks. Biosurfactant additions enhanced reductive dechlorination of anaerobic sediments spiked with Aroclor 1248 (Cho et al., 2004). The enhancement was attributed to an increased bioavailability of the PCB. Anaerobic dechlorination of Aroclor 1242 has also been investigated in a continuously-fed anaerobic upward-flow anaerobic sludge blanket (UASB) reactor (Tartakovsky et al., 2000). Arochlor 1242 was successfully eliminated up to volumetric loading rates of 5.6 g PCB m^{-3} reactor d^{-1}. The removal was due in part to sludge adsorption but recovery of inorganic chloride in the reactor effluent suggested a large percentage of biological dechlorination. The maximal dechlorination rate was 0.6 mg PCB g^{-1} VSS d^{-1}. Granular sludge from a UASB had higher activity towards the dechlorination of 2,3,4-TCBp to 2,4-DCBp with ethanol and formate as electron donors compared to other electron donors such as pyruvate (Nollet et al., 2005)

The main strategy towards promoting the aerobic degradation of PCB has been through the addition of oxygen, cosubstrates, surfactants, inducers and in some cases bioaugmentation of PCB degrading bacteria. Biphenyl, which is an important primary substrate supporting PCB cometabolism, has successfully been utilized to stimulate aerobic degradation of PCB contaminated soil and sediments (Brunner et al., 1985; Harkness et al., 1993). Brunner *et al.* (Brunner et al., 1985) studied the impact of adding biphenyl and a PCB-degrading *Acinetobacter* strain to soil on the mineralization [^{14}C]Aroclor 1242. Biphenyl supplementation permitted up to 27% conversion of the radiolabel label to ^{14}CO2 in 62 days, while treatments lacking biphenyl were only mineralized by <1%. Biphenyl also enhanced mineralization of the non-bioaugmented treatments, enabling up to 20% mineralization by the natural microflora in the soil. A field study evaluated the aerobic degradation of preexisting PCB contamination in Hudson River sediment previously subjected to anaerobic dechlorination (Harkness et al., 1993). A combined treatment, containing O_2, biphenyl and inorganic nutrients, enhanced PCB biodegradation by 37 to

55% in 73days. Bioaugmentation with a PCB degrading strain *Alcaligenes eutrophus* H850 did not help, indicating the natural microflora was sufficient to catalyze the aerobic degradation of the PCB. In a similar study, Anid *et al*. (1993) utilized H_2O_2 to promote the aerobic degradation of PCB previously dechlorinated by anaerobic conditions. Carvone, a terpenoid compound known to induce aerobic PCB degradation, was explored as an additive to enhance bioremediation of Aroclor 1242 in soil (Gilbert and Crowley, 1998). Repeated application of the carvone-induced bacterial strain *Arthrobacter* sp. B1B enabled 27% degradation of PCB, while addition of carvone alone enabled 10% degradation of PCB. Surfactants were tested in combination with bioaugmentation for the aerobic bioremediation of an Aroclor 1242-polluted soil (Lajoie et al., 1993; Singer et al., 2000). The surfactant sorbitan trioleate was added with or without repeated application of the bacterial strains *Arthrobacter* sp. B1B and *Ralstonia eutrophus* H850, which were induced by carvone and salicylic acid, respectively. PCB removals ranged from 55-59% with the combined bioaugmentation surfactant treatment or 30-36% with surfactant treatment only that relied on the natural microflora. Cyclodextrins, which are alternative compounds to aid in the solubilization of apolar compounds, have also been found to stimulate the aerobic degradation of PCB in soil (Fava et al., 1998). Composting of PCB-contaminated soil from a former paper mill mixed with yard trimmings was evaluated at field scale (Michel et al., 2001). Up to a 40% loss of PCBs were observed with high levels of yard trimmings. Congener specific PCB analysis indicated that PCB congeners of only 1 to 3 chlorines per biphenyl were preferentially degraded.

PCBs have also been successfully degraded in an aerobic bioreactor packed bed reactor (Fava et al., 1996a). A three-membered bacterial co-culture was immobilized on beads or foam cubes and biphenyl was supplied to the reactor by vapor phase to support the cometabolism of PCB. Model DCBp congeners and Aroclor 1221 were extensively dechlorinated in the bioreactor as evidenced by release of inorganic chloride and removal of PCB. A coculture of *Acinetobacter* spp. was utilized to degrade mono-, di- and tetrachlorinated biphenyl congeners in a fixed-bed reactor with polyurethane foam boards as support for bacterial biofilms (Adriaens and Focht, 1990). Benzoate was supplied as the primary substrate together with biphenyl vapors as an inducer of PCB cometabolism by *Acinetobacter* strain P6. Chlorobenzoates generated from 4,4'-DCBp, 3,4-DCBp and 3,3',4,4'-TeCBp were further metabolized by *Acinetobacter* strain 4-CB1. The PCBs were converted highly to chlorobenzoates, ring fission products, inorganic chloride as well as partial conversion to CO_2 (6.5-11% for DCBp's).
Tucker *et al*. (1975) studied the degradation of various Aroclor preparations in a laboratory aerobic activated sludge plant. The biodegradability was inversely correlated with the degree of chlorination as shown in Figure 5.1. For example, Aroclor 1221 was degraded by 80%; whereas, 1254 was only degraded by 20%. Borja et al. (2006) demonstrated 95% removal of a mixture PCB in an aerobic fluidized-bed biofilm reactor operated with one day retention time in sequenced batches with biphenyl as a carbon source.

The sequential anaerobic aerobic treatment of Aroclor 1242 was evaluated with river sediments (Rodrigues et al., 2006). First, the sediments were incubated for 1 year under anaerobic conditions to generate lower chlorinated congeners. Subsequently, the sediment containing the lower chlorinated congeners was treated aerobically after inoculating with two genetically engineered aerobic bacteria, *Burkholderia xenovorans* strain LB400 and strain RHA1(fcb), which are capable of growing on 2-CBp and 4-chlorobenzoate, respectively. The bacterial strains were able to significantly reduce the lower chlorinated congeners remaining after the anaerobic phase by 57% in 30 days. Degradation of Aroclor 1242 was also studied in a granular biofilm reactor with limited aeration providing both anaerobic and aerobic conditions simultaneously (Tartakovsky et al., 2001b). The PCB removal efficiency was high with only 16 to 19% PCB recovered from the effluent and biomass. The specific rate of PCB removal was 1.43 mg PCB g^{-1} VSS d^{-1}.

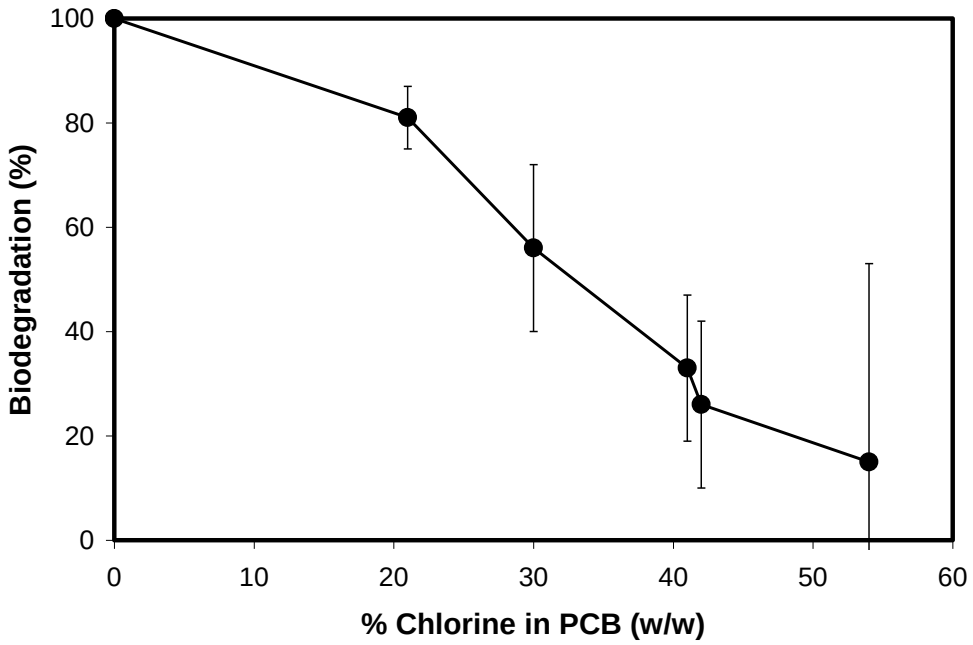

Figure 5.1. *Biodegradation of biphenyl and commercial mixtures of PCB after 48 h in a laboratory-scale semi-continuous aerobic activated sludge reactor as a function of the chlorine content of the PCB mixture. The liquid volume of the reactor was 1.5 l, the PCB mixtures were added at a rate of 1 mg each 48 hours. Reference: Tucker*

5.3. Microbiology and biochemistry of PCB biodegradation

PCBs are subject to both aerobic and anaerobic metabolism. Under aerobic conditions, lower chlorinated PCB congeners can be cometabolized as well as serve as a growth supporting substrates. Under anaerobic conditions, higher PCB congeners are subject to reductive dechlorination provided that electron-donating substrates are available.

5.3.1. Aerobic bacterial cometabolism of PCBs

A large number of aerobic bacteria are capable of cometabolizing PCB with biphenyl as the primary substrate. The biphenyl metabolic enzymes encoded by the *bph* gene cluster are responsible for the attack of PCB (Abramowicz, 1990; Furukawa, 2000). PCB-degrading bacteria are found among the gram negative genera *Pseudomonas*, *Alcaligenes*, *Achromobacter*, *Burkholderia*, *Comamonas*, *Sphingomonas*, *Ralstonia* and *Acinetobacter* and among the Gram-positive genera *Rhodococcus*, *Corynebacterium* and *Bacillus* (Furukawa, 2000; Pieper, 2005). In an extensive screening program, bacteria cometabolizing lower chlorinated congeners of PCB were isolated from almost all contaminated soil samples tested, indicating a ubiquitous presence of aerobic PCB-degrading bacteria (Bedard et al., 1986). Nearly all of these isolates were capable of degrading PCB congeners with only two chloro groups (*eg*. 2,3-, 2,4'or 4,4'-DCBp) but as the chlorine number increased, progressively less and less strains were able to metabolize the PCB congeners (Bedard et al., 1986; Abramowicz, 1990). The trend reflects the general observation that aerobic PCB biodegradability decreases with increased chlorine (Furukawa, 2000). PCB congeners with double *ortho* substitutions are also poorly degraded (Furukawa, 2000; Bedard, 2003), presumably due to steric hindrance of the responsible dioxygenase, biphenyl 2,3-dioxygenase. Of all the bacterial cultures isolated to date, strains *Burkholderia xenovorans* LB400 (formerly *Pseudomonas* sp.; *Burkholderia cepacia* (Goris et al., 2004)) and *Ralstonia eutropha* H850 (formerly *Alcaligenes eutrophus*), display the broadest substrate specificity (Abramowicz, 1990; Erickson and Mondello, 1993; Gibson et al., 1993). These bacteria have a biphenyl 3,4-dioxygenase

enabling them to attack some of the double *ortho*-substituted congeners (Bedard et al., 1986; 1987; Bopp, 1986).

PCB metabolism by the biphenyl degradation pathway is illustrated in Figure 5.2. During cometabolism, the PCB is oxidized by biphenyl-2,3-dioxygenase leading to the formation of the corresponding *cis*-dihydriol. The *cis*-dihydriol is subsequently oxidized by a dehydrogenase to a 2,3-dihydroxy intermediate, which in turn is oxidized by an oxygenase to the *meta* cleavage product, 2-hydroxy-6-oxo-6-(chloro)phenylhexa-2,4-dienoic acid (HOPDA). If the second ring of the PCB contains chlorine, the aliphatic portion branch of HOPDA may contain a chlorine substitution. Recently, a glutathione *S*-transferase (BphK) was shown to catalyze the replacement of the aliphatic chlorines substituted on a double bonded C with a hydrogen atom (Fortin et al., 2006). The aliphatic portion of HOPDA is cleaved from the remaining aromatic ring to yield chlorobenzoic acid and an aliphatic acid, 2-hydroxy-penta-2,4-dienoic acid (Furukawa, 2000; Bedard, 2003). The aliphatic acid is metabolized via acetyl-CoA through the tricarboxylic acid cycle ultimately leading to CO_2. The chlorobenzoic acids are typically not metabolized by the PCB-degrader; however, chlorobenzoic acids are degraded in coculture with bacterial strains specialized in the metabolism of chlorobenzoic acids (Sylvestre et al., 1985; Adriaens et al., 1989; Havel and Reineke, 1991; Fava et al., 1994; Potrawfke et al., 1998b; Rodrigues et al., 2001). In co-cultures, high levels of PCB mineralization can be achieved as evidenced by inorganic chloride formation (Adriaens et al., 1989; Havel and Reineke, 1991; Fava, 1996b; Potrawfke et al., 1998b; Rodrigues et al., 2001). In a similar fashion, genetic engineering has been utilized to merge chlorobenzoic acid degrading and PCB degrading genes in a single organism to accomplish extensive degradation of PCB (Brenner et al., 1994; McCullar et al., 1994; Stratford et al., 1996; Potrawfke et al., 1998b; Hrywna et al., 1999; Pieper, 2005; Rodrigues et al., 2006).

In recent years, exceptional bacterial strains have been discovered. A psychrotrophic (low temperature) bacterium, *Hydrogenophaga taeniospiralis* IA3-A, was isolated that cometabolizes DCBp congeners and lower chlorinated PCB congeners in Aroclor 1221 at 5°C (Lambo and Patel, 2006). A bacteria isolated from soil, *Paenibacillus* sp KBC101, was shown to degraded highly chlorinated congeners; including NCBp congeners, which were degraded by 40% in 3 days by resting cells grown on biphenyl (Sakai et al., 2005). A marine bacterium lacking biphenyl dioxygenase genes, *Pseudomonas* CH07, was also isolated that degraded higher PCB congeners including HeCBp (De et al., 2006).

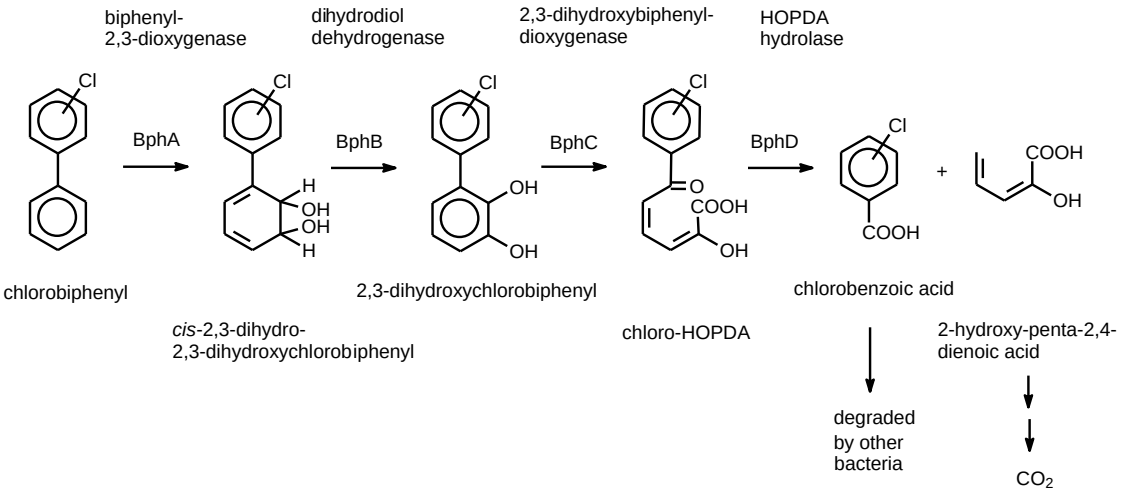

Figure 5.2. *Pathway of aerobic PCB degradation by biphenyl-oxidizing bacteria. References: (Furukawa, 2000; Bedard, 2003; Pieper, 2005).*

5.3.2. Aerobic bacterial growth on PCB as sole carbon and energy source

Some bacteria have the ability to grow by utilizing PCB congeners of one or two chlorines as sole sources of carbon and energy. Examples of growth on 4CBp are common (Furukawa et al., 1978; Masse et al., 1984; Shields et al., 1985; Furukawa and Miyazaki, 1986; Barton and Crawford, 1988; Ahmad et al., 1990); whereas examples on 2CBp and 3CBp are less frequent (Bedard et al., 1987; Parsons et al., 1988; Hickey et al., 1992). *Burkholderia cepacia* P166 (formerly *Pseudomonas cepacia*) utilizes 4CBp, 2CBp and 3CBp as growth substrates (Arensdorf and Focht, 1994). However, growth on 2CBp and 3CBp is restricted due to accumulation of toxic intermediates. Unrestricted growth on 4CBp is attributable to the ability of the strains to readily mineralize the 4-chlorobenzoate intermediate without formation of any toxic intermediates. The initial growth rate on 4CBp was 0.132 h^{-1}, about half of that achieved with biphenyl as growth substrate. In 2 days, 1 mM 4CBp was converted to 0.53 mM 4-chlorobenzoic acid and 0.34 mM inorganic chloride. Resting cells of *Burkholderia cepacia* P166 rapidly converted 4CBp stoichiometrically to chloride with transient accumulation of 4-chlorobenzoate. A pathway in which 4-chlorobenzoate is converted to 4-chlorocatechol and subsequently to its *meta* cleaved intermediate, 5-chloro-2-hydroxymuconic semialdehyde, was elucidated in a subsequent publication (Arensdorf and Focht, 1995). The *meta*-cleaved product was further mineralized via intermediates 5-hydroxymuconate, 5-chloro-2-oxopent-4-enoate, 5-chloro-4-hydroxy-2-oxopentanoate, and chloroacetate. The inactivation of biomass during the metabolism of 2CBp and 3CBp has been rationalized by the formation of 2- and 3-chlorocatechols via 2- and 3-chlorobenzoates (Arensdorf and Focht, 1994), which are also *meta* cleaved yielding acyl halides that are reactive with biological macromolecules (Bartels et al., 1984). An unidentified bacterial isolate, strain SK-3 was able to grow on a dichlorinated biphenyl with a chlorine group on each ring, 2,4'-DCBp (Kim and Picardal, 2001). While utilizing 2,4'-DCBp, strain SK-3 had a growth rate 0.016 h^{-1} and the production of inorganic chloride implied release of chlorine from both rings.

Metabolism of chlorobenzoates is not a requirement for growth on monochlorinated PCBs. The five-carbon fragment released from the non-chlorinated ring, 2-oxopent-4-dienoate, is an easily biodegradable carbon source that can support growth (Furukawa, 2000; Sakai et al., 2003). The majority of strains can grow by use of one PCB ring and accumulate chlorobenzoates from the other ring (Masse et al., 1984; Barton and Crawford, 1988; Bevinakatti and Ninnekar, 1993; Kobayashi et al., 1996; Hrywna et al., 1999; Kim and Picardal, 2001). An unidentified bacterial strain, SK-4, grew on 2,2'-dichlorobiphenyl and 2,4'- dichlorobiphenyl producing stoichiometric amounts of 2- and 4-chlorobenzoate at a growth rate of 0.019 and 0.041 h^{-1}, respectively (Kim and Picardal, 2001). The discovery of genes responsible for the metabolism of 2-oxopent-4-dienoate to Kreb's cycle intermediates provides a rationale for explaining growth of strains on PCB even though they accumulate chlorobenzoates as dead-end metabolites (Hofer et al., 1994; Sakai et al., 2003).

5.3.3. Aerobic fungal cometabolism of PCBs

PCBs are metabolized by various white-rot fungi. As a general trend, the extent of PCB mineralization by white-rot fungi decreases with increasing chlorine number. Radiolabeled tri, di- and mono-chlorinated biphenyls are mineralized to $^{14}CO_2$, with values ranging from 11 to 16% (Thomas et al., 1992; Dietrich et al., 1995; Beaudette et al., 1998). Hexa- and tetrachlorobiphenyls were less extensively mineralized for 0.4 to 1.4% indicating that the evidence for their mineralization is not very conclusive, since radiolabeled impurities could have also accounted for the mineralization (Bumpus et al., 1985b; Thomas et al., 1992; Dietrich et al., 1995). In one study, where very low concentrations of 2,4,2',4'-terachlorobiphenyl were utilized, 9.6% mineralization could be demonstrated (Thomas et al., 1992). Similar trends were also observed with technical PCB mixtures. For example, *Phanerochaete chrysosporium* decreased PCB concentrations of Aroclor 1242, 1254, and 1260 (42, 54 and 60% chlorinated) by 60.9, 30.5, and 17.6%, respectively (Yadav et al., 1995b). Congeners of lower chlorine number were degraded more extensively compared to those of higher chlorine number during the remediation of the technical mix Delor 103 by

Pleurotus ostreatus (Kubatova et al., 2001). Little information is available on the types of metabolites formed during the degradation of PCBs by white-rot fungi. Two metabolites, 4-chlorobenzoic acid and 4-chlorobenzyl alcohol, have been observed during the degradation of 4,4'-dichlorobiphenyl by *P*. *chrysosporium* (Dietrich et al., 1995). The white-rot fungus, *Phlebia brevispora*, degraded TeCBp, PeCBp and HCBp congeners to methoxylated intermediates as well as *para*-dechlorinated methoxylated intermediates (Kamei et al., 2006).

In addition to white-rot fungi, there is one report of the degradation of a PCB technical mixture (Chlophen A) by the filamentous fungus, *Aspergillus niger* (Dmochewitz and Ballschmiter, 1988). Only the three Chlophen mixtures (A30, A50 and A60 of 42, 54 and 60% chlorine content, respectively) were tested as substrate, but only the mixture with the lowest total chlorine content was biodegradable. In a similar study, *Aspergillus flavus* could only metabolize Aroclor 1221 but not technical mixtures with greater chlorine content (Murado et al., 1976). The PCB congener 4,4'-DCBp was observed to be hydroxylated by the yeast *Saccharomyces cerevisiae* (Layton et al., 2002).

5.3.4. Anaerobic metabolism of PCBs

Anaerobic reductive dechlorination of PCB was first observed by Brown et al. (1987). The authors compared congener patterns of PCB mixtures in anaerobic sediments from six spill sites and compared the patterns to technical mixtures of PCB implicated in the contamination of the sediment. The comparison of the congener patterns led the authors to conclude that that the higher PCB congeners were subject to reductive dechlorination, resulting in an accumulation of lower chlorinated congeners. Afterwards, Quensen et al. (1988) confirmed the role of microorganisms in the anaerobic biotransformation of PCB by incubating sediment from the Hudson River with PCBs in laboratory microcosms. After 16 weeks, 53% of the total chlorine in Aroclor 1242 applied at 700 ppm was removed. The proportion of mono- and dichlorobiphenyls increased from 9 to 88 percent. Heat-killed controls did not convert the PCB. The research was continued, investigating the ability of anaerobic sediment microcosms to dechlorinate Aroclor 1242, 1248, 1254 and 1260 (Quensen et al., 1990). The results demonstrated that the PCB congeners were mostly attacked in the *para* and *meta* positions, resulting in the accumulation of lower congeners with *ortho* chlorines. The loss of *para*- and *meta*-chlorines is advantageous from a toxicological point of view since congeners with these chlorine groups are mainly responsible for toxicity (Kimbrough and Goyer, 1985; Safe, 1989; Quensen et al., 1998). The maximum rate of PCB dechlorination observed in the laboratory microcosm study was 0.3 μg Cl g^{-1} sediment $week^{-1}$ (Quensen et al., 1990). Since these initial studies, a large number of research groups world-wide have succeeded in demonstrating the anaerobic dechlorination of PCB in anaerobic sediment cultures from different environmental sources and under a wide variety of conditions (Nies and Vogel, 1990; Ye et al., 1992; Alder et al., 1993; Boyle et al., 1993; Rhee et al., 1993; Ofjord et al., 1994; Williams, 1994; Pagano et al., 1995; Berkaw et al., 1996). An overview of these studies has been provided by Bedard (2003) and Wiegel and Wu (2000).

The involvement of different microorganisms in the reductive dechlorination of PCB was first suggested by enrichment cultures with different behavior derived from the same sediment source. For example, cultures derived from Hudson River sediments after pasteurization were able to catalyze mainly *meta*-dechlorination, while the original untreated sediment culture maintained the capacity to dechlorinate both *meta*- and *para*-chlorine groups (Ye et al., 1992). The result clearly demonstrates the involvement of more than one population of dechlorinating bacteria. Similarly, organisms from Woods Pond sediment showed distinct patterns of dechlorination depending on temperature of cultivation (Wiegel and Wu, 2000). Eight dechlorination processes have been identified based on careful examination of congener loss and product accumulation patterns in different sediment samples (Wiegel and Wu, 2000; Bedard, 2003). The patterns are shown in Table 5.1. The documented patterns only include *meta*- and *para*-dechlorination, reflecting the relatively infrequent observation of *ortho*-dechlorination in many of the initial sediment studies (Brown et al., 1987; Quensen et al., 1988; 1990; Nies and Vogel, 1990; Alder et al., 1993; Ofjord et al., 1994) or later studies with enriched cultures developed from the same sediments (Bedard et al., 2005). Poor *ortho*-dechlorination is also observed in the field

(Bedard et al., 2005). However, some studies with either enrichment cultures or sediment samples spiked with a defined congener have revealed many examples where *ortho*-dechlorination has taken place (Van Dort and Bedard, 1991; Williams, 1994; Berkaw et al., 1996; Hartkamp et al., 1996; Natarajan et al., 1996; Wu et al., 1997; Cutter et al., 1998; Quensen et al., 1998; Kuipers et al., 1999; Wiegel and Wu, 2000). While mono- and dichlorobiphenyl congeners are generally the products of PCB dechlorination, there are examples of PCB dechlorination to biphenyl. Examples include the conversion of 4,4'-DCBp (Mavoungou et al., 1991), 3,4,5-TCBp (Williams, 1994), 3,4, 3',4'-TeCBp (Rhee et al., 1993) or 2,3,4,5,6-PCBp (Natarajan et al., 1996) to biphenyl. Pathways of reductive PCB dechlorination are shown in Figure 5.3, illustrating *meta*-, *para*- and *ortho*-dechlorination.

The observation that rates of PCB dechlorination can increase upon repeated exposure to PCBs indicates that the organisms responsible for the reaction are benefiting from the reaction and growing. This realization has led to the concept of priming the growth of reductive PCB dechlorinating microorganisms with simple PCB congeners (Bedard et al., 1996; Klasson et al., 1996; Van Dort et al., 1997) or alternative halogenated substrates such as halogenated benzoates or chlorophenols (Deweerd and Bedard, 1999; Cho et al., 2002a) or bromobiphenyls (Bedard et al., 1998; Wu et al., 1999; Chang et al., 2001).

Table 5.1. Patterns of reductive dechlorination of PCBs (Wiegel and Wu, 2000; Bedard, 2003).

Pattern	Targeted Chlorine	Reactive Chloro Groups[c]
P	Flanked *para*	3<u>4</u>, 23<u>4</u>, 2<u>4</u>5, 23<u>4</u>5, 23<u>4</u>56
H	Flanked *para*[a]	3<u>4</u>, 2<u>3</u>4, 2<u>4</u>5, 23<u>4</u>5
H'	Flanked *para*[a,b]	2<u>3</u>, 3<u>4</u>, 2<u>3</u>4, 2<u>4</u>5, 23<u>4</u>5
N	Flanked *meta*	2<u>3</u>4, 236, 2<u>4</u>5, 2<u>3</u>4<u>5</u>, 2<u>3</u>46, 2<u>3</u>4<u>5</u>6
M	Flanked and unflanked *meta*	<u>3</u>, 2<u>3</u>, 2<u>5</u>, <u>3</u>4, 2<u>3</u>4, 2<u>3</u>6
Q	Flanked and unflanked *para*[a,b]	<u>4</u>, 2<u>3</u>, 2<u>4</u>, 3<u>4</u>, 2<u>3</u>4, 2<u>4</u>5, 2<u>4</u>6
LP	Flanked and unflanked *para*	2<u>4</u>, 2<u>4</u>5, 2<u>4</u>6
T	Double flanked *meta*	2<u>3</u>45

[a] Double flanked *meta* of 234 is also targeted.
[b] Flanked *meta* of 23 is also targeted.
[c] Underlined group is dechlorinated.

Priming is based on the hypothesis that high concentrations of an appropriate substrate which is readily susceptible to microbial dehalogenation will selectively promote the growth of microorganisms capable of utilizing analogous PCB compounds as electron acceptors (Bedard, 2003). Through the estimation of PCB-dechlorinator population size with the most probable number method, it was shown that addition of 2,6-dibromobiphenyl increased the population of PCB-dechlorinators (Wu et al., 1999). The number of microorganisms capable of dehalogenating PCBs in Aroclor 1260 increased approximately 1000-fold after priming for 48 d with 2,6-dibromobiphenyl (1050 μmol l^{-1} of slurry) in the presence of 10 mM malate. The addition of 2,6-dibromobiphenyl primed exclusively *meta*-dechlorination of the PCBs (Process N), resulting in large decreases (75-88%) of the hexa- through nona-chlorobiphenyls in only 5-8 months. Simple congeners such as 2,3,6-TCBp have been utilized to rapidly enrich cultures of PCB dechlorinators from river sediments (Boyle et al., 1993; Bedard et al., 2006), which could dechlorinate 100-fold faster compared to the original sediment cultures. A highly enriched culture developed by repeated supplementation of 2,3,6-TCBp had a specific dechlorinating activity of 1.13 pmol of 2,3,6-TCBp converted to 2,6-DCBp day^{-1} bacterial $cell^{-1}$ (Boyle et al., 1993).

The present knowledge of organisms involved in anaerobic PCB conversion has been obtained from either molecular fingerprinting techniques applied to PCB-dechlorinating enrichment cultures or from the use of pure cultures of halorespiring bacteria. The denaturing gradient gel electrophoresis (DGGE) of 16S rDNA technique was utilized to identify microorganisms in an enrichment culture catalyzing the *ortho*-dechlorination of

2,3,5,6-TeCBp with acetate as electron donor (Cutter et al., 2001). One of the predominant bands in the DGGE gel, designated bacterium o-17, was highly dependent on the presence of the PCB and was proposed as the responsible *ortho*-dechlorinating organism.

Bacterium O-17 had a sequence with has high sequence similarity with the green non-sulphur bacteria which includes *Dehalococcoides ethenogenes* (a known halorespiring bacterium). Bacterium O-17 has the ability to dechlorinate 8 PCB congeners, including single-flanked *ortho*-PCB chlorines; however, double-flanked chlorines of PCBs were preferentially dechlorinated (May et al., 2006).

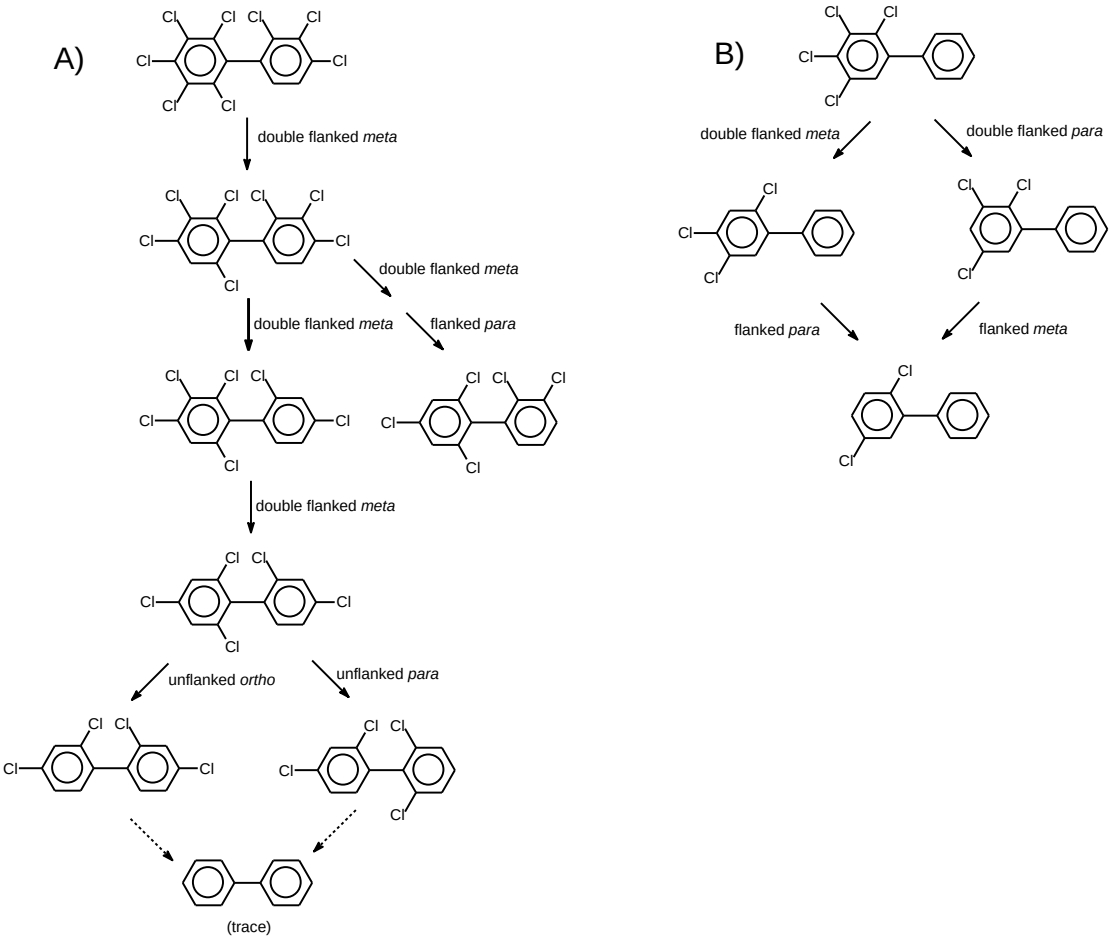

Figure 5.3. *Examples of anaerobic pathways of reductive dechlorination observed in sediment microcosms and anaerobic enrichment cultures. Dotted arrows indicate slow reactions. References are indicated in text.*

The dechlorination of three of the PCBs (23456-PeCBp, 2346-TeCBp, and 2356-TeCBp) could be sustained for three or more sequential transfers, suggesting that bacterium o-17 was utilizing these congeners as a terminal electron acceptor. However, other PCB congeners (234-TCBp and 235-TCBp) were only subject to cometabolic reductive dechlorination as long as more highly chlorinated parent compounds were present in the culture (May et al., 2006). DGGE was also utilized to identify the responsible organisms of a highly enriched culture catalyzing the *para*-dechlorination of flanked chlorines from 2,3,4,5-TeCBp (Wu et al., 2002a). The band associated with the presence of 2,3,4,5-TeCBp was sequenced and also found to have a high sequence similarity (89%) to Bacterium o-17 and thus also to *Dehalococcoides*. Additionally, a *meta*- and *para*-dechlorinating enrichment culture derived from lake sediments and adapted to the dechlorination of Aroclor 1242 was analyzed for the presence of 16S rDNA unique to the

genus *Clostridium* (Hou and Dutta, 2000). The analysis revealed the presence nine *Clostridium* strains in the sediment but did not provide proof that these bacteria were responsible for dechlorination. The occurrence of *Clostridium*, which are spore-formers, would be consistent with the survival of PCB dechlorinating activity after pasteurization (Ye et al., 1992). An analysis of 16S rRNA genes in an highly-enriched acetate-oxidizing *ortho*-dechlorinating enrichment culture adapted to 2,3,5,6-TeCBp revealed the presence of sequences closely related to *Dehalococcoides* as well as several sequences corresponding to undescribed species of the δ-subgroup of the class *Proteobacteria* the low-G+C gram-positive subgroup and the subgroup the *Thermotogales* (Pulliam Holoman et al., 1998). Sequences corresponding to methanogens and a *Clostridium* could be eliminated from the enrichment culture without great loss in dechlorinating activity, suggesting that these organisms were not implicated in the dechlorination.

Several studies evaluated the structure of microbial populations in PCB-dechlorinating enrichment cultures using clone libraries. Utilizing universal bacterial primers as well as primers targeting *Dehalococcoides*, 16S rRNA gene sequences closely related to *Dehalococcoides* were shown to be present in anaerobic cultures enriched from estuarine, marine and river sediments with 2,3,4,5-TeCBp (Yan et al., 2006b). 16S-rRNA gene sequences closely related of another halorespiring bacterial group, *Dehalobacter*, were found in addition to those of *Dehalococcoides* in enrichment cultures providing the most extensive dechlorination of 2,3,4,5-TeCBp (Yan et al., 2006b). A sediment free enrichment culture primarily responsible for *meta*-dechlorination of Aroclor 1260 (process N dechlorination) was shown to contain *Dehalococcoides* based on restriction fragment length of polymorphism (RFLP) analysis of 16S rRNA gene clones (Bedard et al., 2006). A 16S rRNA gene library was made with a specific primer set that was custom made for bacterium o-17 and closely related bacteria (Watts et al., 2005). Using this primer set it was determined that bacteria of this group were present in microcosms enriched from harbor sediments with HCBp. Closely related 16S rRNA gene sequences were also detected in sediments from a river tributary with confirmed PCB-dechlorinating activity. Phylogenetic comparison of these detected 16S rRNA gene sequences revealed a relatively diverse group of organisms within the dehalogenating *Chloroflexi*, some of which are phylogentically close but distinct from the genus *Dehaloccocoides* (Fagervold et al., 2005; Watts et al., 2005).

One study has shown that a pure culture of halorespiring bacterium from the genus *Dehalococcoides* can catalyze reductive dechlorination of PCBs. *Dehalococcoides ethenogens* sp. strain 195 cometabolically converted 2,3,4,5,6-PeCBp to TeCBp and 2,4,6-TCBp while utilizing perchloroethene (PCE) as its primary electron acceptor (Fennell et al., 2004).

Presently, no information is available on the dehalogenases responsible for PCB dechlorination. There are, however, two reports which indicate that vitamin B_{12}, a commonly occurring coenzyme of anaerobes, is capable of catalyzing the dechlorination of certain PCB congeners. With reduced dithiothreitrol as reductant, vitamin B_{12} catalyzed the *para*- and *meta*-dechlorination of 2,3,4,5,6-PCBp (Assafanid et al., 1992). Vitamin B_{12} reduced with titanium(III) citrate catalyzed the reduction of sediment-sorbed 2,3,4,5,6-PCBp (Woods et al., 1999). In 42 days, 40% of the chlorine was removed at a temperature of 30ºC. Experiments conducted in aqueous solutions revealed a preference for dechlorination of flanked chlorines.

5.4. Microbial kinetics of PCB biodegradation

Compared to the large body of information available on the biodegradation of PCBs in the literature, there is a surprising deficiency in information on microbial kinetic parameters. The kinetic information available is summarized in Table 5.2 (annex). Growth rates of wild type aerobic bacteria growing on mono-chlorinated biphenyls ranged from 1.04 to 3.17 d^{-1}, which were clearly higher than the rates obtained for wild type aerobic strains growing on DCBp's, ranging from 0.38 to 1.11 d^{-1}. The two genetically engineered strains capable of utilizing chlorobenzoate intermediates had among the fastest growth rates reported, 2.38 to 4.16 d^{-1}, corresponding to doubling times of only 4 to 7 hours (Hrywna et al., 1999).

Pseudo first-order rate constants (1^{st} order rate constants normalized to 1 g dwt cells l^{-1}) for the cometabolic aerobic degradation of PCBs ranged from 17 to 637 l g^{-1} dwt d^{-1}. Bacteria responsible for the aerobic cometabolism of PCB congeners a high affinity for PCB as demonstrated by low half velocity constants observed (mg l^{-1} range).

There is no kinetic data available on the growth rates of halorespiring bacteria utilizing PCB as a terminal electron acceptor. One study does report the composite growth rate of a mixed anaerobic culture reductively dehalogenating Aroclor 1248 which corresponds to a doubling time of 83 hours (Rhee et al., 2001). Specific activities of higher PCB dechlorination in anaerobic sediment microcosms are very low, ranging from 6.0×10^{-6} to 2.7×10^{-2} mg PCB reductively dechlorinated g^{-1} dwt sediment d^{-1}. The specific activities in anaerobic or mixed anaerobic/aerobic biofilms enriched for PCB dechlorination are orders of magnitude higher, 0.6 to 1.4 mg PCB reductively dechlorinated g^{-1} dwt biofilm d^{-1}. Even though considerable progress has been made with the isolation of sediment free enrichment cultures responsible for the reductive dehalogenation of PCB, basic kinetic information such as specific activities are not yet available. The kinetics of PCB reductive dechlorination in the field have been estimated from sediment core data. Beurskens *et al*. (1993b) estimated a half-life of several higher PCB congeners of approximately 9 years for lake sediments from the Ketelmeer (deposition basin of the Rhine river, located in the Netherlands). Magar *et al.* (2005) estimated dechlorination rates in Lake Hartwell (South Carlina, USA) of 0.039 to 0.232 mol Cl^- mol^{-1} PCB y^{-1}, which corresponds to an average of 16 years for the biotransformation of a PCB molecule (assuming a *para*- and a *meta*-dechlorination will occur per PCB). The rates in the field are 1 to 2 orders of magnitude lower than laboratory microcosms (Magar et al., 2005a), most likely due to lower concentrations and more limited bioavailability of aged pollution in the field. Nonetheless, the field evidence does confirm that reductive dechlorination occurs at measurable rates even at the low concentrations prevailing in aquatic sediments.

6 Conclusions

The biodegradability of various chlorinated aromatics (chlorophenols, chlorobenzoates, polychlorinated biphenyls, chlorobenzenes and chlorinated dioxins) was reviewed in this manuscript. Compelling evidence was provided for the biodegradation of compounds in each of categories under a variety of physiological and redox conditions. Many microorganisms gain energy and benefit from the biodegradation by utilizing the chlorinated aromatic compound as an electron donor or as an electron acceptor. If utilized as an electron donor and carbon source, the chlorinated aromatic compounds are oxidized to CO_2 and chloride. If utilized as an electron acceptor, the chlorinated aromatic compounds become reductively dehalogenated to lower chlorinated congeners in a process known as haloresipiration. Biodegradation can also result from the cometabolism in which case the chlorinated aromatics are transformed while microorganisms are utilizing other substrates to gain energy.

Common strategies responsible for the enzymatic attack of chlorinated aromatics have been identified. Under aerobic conditions microorganisms utilize three main mechanisms of initiating the degradation. The most common approach is through the use of oxygenases (either monooxygenases or dioxygenases) which insert oxygen from O_2 into the compound prior to ring cleavage. Some aerobic organisms utilize a unique hydrolytic dehalogenases that replace a Cl-group with a hydroxyl group exemplified by the degradation of 4-chlorobenzoate. The third most common approach is a dehalogenation catalyzed by a glutathione S-transferase (replacement Cl-group with the sulfhydryl group of glutathione) exemplified by the dehalogenation of polychlorinated hydroquinones, which are intermediates in the aerobic pathway of PCP degradation. The predominant mechanism of initiating degradation of chloroaromatics under anaerobic conditions is via reductive hydrogenolysis in which a Cl-group is replaced by a hydrogen atom. The dehalogenation of chlorinated benzenes, PCBs and chlorinated dioxins are catalyzed by halorespiring bacteria in the genus *Dehalococcoides* (or closely related genera); whereas the dehalogenation of chlorophenols and chlorinated benzoates are catalyzed predominantly by halorespiring bacteria in the genus *Desulfitobacterium* and *Desulfomonile*, respectively. The biochemical strategies dictate certain patterns of biodegradability.

This review has elucidated general patterns of biodegradability. Chlorinated aromatic compounds with oxygen containing functional groups behaved distinctly from aromatics lacking these groups. The degree of chlorination dramatically affects the biodegradability potential under different redox conditions of aromatic compounds lacking free oxygen groups. On the other hand, aromatics with oxygen containing functional groups are potentially fully biodegraded under either anaerobic or aerobic conditions regardless of the degree of chlorination.

Compounds without freely available oxygen containing functional groups (chlorinated benzenes, PCBs and chlorinated dioxins) have a shared biodegradability pattern. Highly chlorinated congeners are not degraded under aerobic conditions; whereas, they are more prone to reductive dechlorination under anaerobic conditions. Conversely, lower chlorinated congeners are readily degraded under aerobic conditions but are less susceptible to reductive dechlorination by anaerobes. Chlorinated aromatic hydrocarbons with just one chlorine substituent are essentially recalcitrant under anaerobic conditions. Chlorine is a highly oxidized substituent thus it is logical that that as the chlorine number goes up or down there will be a decreasing ability for enzymes to oxidize or reduce the aromatic hydrocarbons, respectively. The complete biodegradation of highly chlorinated congeners therefore requires successive anaerobic-aerobic conditions. An initial anaerobic biotransformation converts higher chlorinated congeners to lower chlorinated congeners, which can subsequently be mineralized to CO_2 and chloride by microorganisms under aerobic conditions.

Compounds with freely available oxygen containing functional groups (chlorophenols and chlorobenzoates) behave differently. Both lower and highly chlorinated congeners such as PCP can be completely degraded to CO_2 and chloride by aerobic bacteria. Aerobic bacterial strains utilizing higher chlorophenols grow at similar rates compared to strains specialized in utilizing lower chlorinated phenols. Likewise, both lower and chlorinated congeners of chlorinated benzoates and chlorophenol can be dehalogenated by anaerobic microorganisms. Even mono-chlorinated congeners such as 3-chlorobenzoate and 2-chlorophenol can be fully degraded in methanogenic consortia to CH_4, CO_2 and Cl^- as a sole source of carbon and energy. The oxygen containing functional groups provide an enzymatic "handlebar" to initiate biodegradation on aromatic skeletons that otherwise would be inert.

Chlorobenzoates are readily biodegraded in the environment to CO_2 and Cl^-. A large list of taxonomically diverse bacterial strains is known to utilize 3-chlorobenzoate and 4-chlorobenzoate as a sole source of carbon and energy under aerobic conditions attesting to a high level of biodiversity. Due to the high biodiversity, multiple pathways are responsible for the mineralization of chlorobenzoates, ranging from pathways initiated by dioxygenases to a pathways initiated by a hydrolytic dehalogenase. In addition to monochlorinated benzoates, several bacterial strains have been isolated that can grow on dichloro-, and trichloro- isomers of chlorobenzoates. The highest aerobic growth rates and specific activities observed for chlorobenzoate was 10.4 d^{-1} and 21.6 g g^{-1} dwt d^{-1}, respectively. Volumetric rates of chlorobenzoate degradation in aerobic bioreactors ranged up to 10.9 g $l^{-1}_{reactor}$ d^{-1}. Chlorobenzoates are also biodegraded under anaerobic conditions. Halorespiring bacteria (*e.g. Desulfomonile tiedjei*) can catalyze the reductive dechlorination of 3-chlorobenzoate and once dechlorinated, other microorganisms in anaerobic environments can mineralize the benzoic acid skeleton to CO_2 and CH_4. The growth rates and specific activities of halorespiring bacteria range up to 1.98 d^{-1} and 3.8 g g^{-1} dwt d^{-1}, respectively. Volumetric rates of chlorobenzoate degradation in anaerobic bioreactors ranged up to 0.5 g $l^{-1}_{reactor}$ d^{-1}. Various dichloro- and trichlorobenzoates are also known to be dechlorinated in anaerobic sediments.

Numerous bacteria are known which can utilize chlorophenols as a carbon and energy source under aerobic conditions. The most well studied strains utilizing PCP belong to the genera *Mycobacterium* and *Sphingomonas*. There is extensive evidence that chlorophenols are mineralized by these bacteria to $chloride^-$ and CO_2. Two main strategies are used by aerobic bacteria for the degradation of chlorophenols. Lower chlorinated phenols for the most part are initially attacked by monooxygenases yielding chlorocatechols as the first intermediates. On the other hand, polychlorinated phenols are converted to chlorohydroquinones as the initial intermediates. The growth rates and specific activities of aerobic PCP-utilizing bacteria range up to 3.7 d^{-1} and 12.3 g g^{-1} dwt d^{-1}, respectively. Volumetric rates of degradation with polychlorinated phenol mixtures in aerobic bioreactors (with long-term operation) ranged up to 1.7 g $l^{-1}_{reactor}$ d^{-1}. Under anaerobic conditions, halorespiring bacteria are responsible for reductive dechlorination of lower and higher chlorinated phenols. Many strains of halorespiring bacteria have the capacity to eliminate *ortho*-chlorines; however only bacteria from the species *Desulfitobacterium hafniense* (formerly *frappieri*) can eliminate *para*- and *meta*-chlorines in addition to *ortho*-chlorines. The growth rates of these halorespiring bacteria range up to 1.63 d^{-1} on 2-chlorophenol. Once dechlorinated, the phenolic carbon skeletons are subsequently completely converted to CH_4 and CO_2 by other anaerobic microorganisms in the environment. Volumetric rates of degradation with PCP in anaerobic bioreactors ranged up to 0.3 g $l^{-1}_{reactor}$ d^{-1}.

Chlorobenzenes with four or less chlorine groups are susceptible to oxidation by aerobic bacteria, including bacteria (*Burkholderia*, *Pseudomonas etc.*) that grow on such compounds as the sole source of carbon and energy with growth rates ranging between 1.1 to 13.2 d^{-1} and specific activities ranging up to 9.7 g g^{-1} dwt d^{-1}. Sound evidence for the mineralization of chlorobenzenes has been provided based on stoichiometric release of chloride or mineralization of ^{14}C-labeled chlorobenzenes to $^{14}CO_2$. The degradative attack of chlorobenzenes by these strains is initiated with dioxygenases eventually yielding chlorocatechols as intermediates in a pathway leading to CO_2 and Cl^-. In engineered bioreactor systems, the maximum volumetric rate of chlorobenzene degradation reported was 11.3 g $l^{-1}_{reactor}$ d^{-1}. Higher chlorobenzenes are readily reductively dechlorinated to lower chlorinated benzenes in anaerobic environments. Halorespiring bacteria from the genus *Dehalococcoides* are implicated in this conversion. In laboratory studies,

hexachlorobenzene (HCBc) was converted with a specific activity as high as 41.4 g g^{-1} dwt d^{-1} by *Dehalococcoides* strain CBDB1. Lower chlorinated benzenes are less readily converted, and mono-chlorinated benzene is recalcitrant to biotransformation under anaerobic conditions. The evidence for the dehalogenation of highly chlorinated benzenes in anaerobic sediment cores is compelling. Geochronological evaluation of the cores indicates half-lives of HCBc of 7 year; whereas the half-lives in measured in sediment microcosms range from 1 month to 1 year.

Polychlorinated biphenyls (PCB) refer to a large group of chlorinated biphenyls with 209 possible congeners. Higher chlorinated biphenyls are susceptible to reductive dehalogenation resulting in the formation of lower chlorinated biphenyls in anaerobic environments. There is strong evidence that certain bacteria from the genus *Dehalococcoides* as well as *Chloroflexi* bacteria closely related to *Dehalococcoides* can grow by linking the oxidation of hydrogen gas to the reductive dechlorination of PCB. Lower chlorinated biphenyls are susceptible to oxidation by aerobic bacteria. In the field *meta*- and *para*-chlorines are more susceptible to reductive dechlorination than *ortho*-chlorines. Geochronological studies conducted in lake sediments indicate half-lives of highly chlorinated PCB congeners ranging from 9 to 16 years. The lower chlorinated biphenyls can be cometabolized aerobically by many bacteria with biphenyl as the primary substrate. However, some bacteria have the ability to grow by utilizing PCB congeners containing only one or two chlorines as sole sources of carbon and energy. The classic examples is the growth of *Burkholderia cepacia* on 4-chlorobiphenyl. *B*. *cepacia* utilizes 4-chlorobiphenyl with a growth rate of 3.2 d^{-1}. Chlorobenzoates tend to accumulating as a product of the reaction and are typically not metabolized by an aerobic PCB-degrading bacterial strain. However, in mixed cultures, chlorobenzoates are further metabolized to CO_2 and Cl^- by other bacteria. Likewise bacteria have been genetically engineered by merging chlorobenzoate-degrading and PCB-degrading genes in a single organism to accomplish extensive degradation of PCB.

Chlorinated dioxins may not be important in mass like the other chlorinated aromatic pollutants; however, because of their toxicity they have been the subject of great public and scientific scrutiny. The biodegradation of chlorinated dioxins is well established. Lower chlorinated dioxins can be degraded by aerobic bacteria from the genera of *Sphingomonas*, *Pseudomonas* and *Burkholderia*. Most studies have evaluated the cometabolism monochlorinated dioxins with unsubstituted dioxin as the primary substrate. The degradation is usually initiated by unique dioxygenases that attack the angular position of the molecule creating unstable hemiacetals that decompose spontaneously to trihydroxylated intermediates. Chlorinated dioxins can also be attacked cometabolically under aerobic conditions with white rot fungi that utilize extracellular lignin degrading peroxidases. Recently bacteria that can grow on monochlorinated dibenzo-*p*-doixins as a sole source of carbon and energy have also been characterized (*Pseudomonas veronii*). Higher chlorinated dioxins are known to be reductively dechlorinated in anaerobic sediments. Similar to PCB and chlorinated benzenes, halorespiring bacterium from the genus *Dehalococcoides* are implicated in the dechlorination reactions. Anaerobic sediments have been shown to convert tetrachloro- to octachlorodipenzo-*p*-dioxins to lower chlorinated dioxins including monochlorinated congeners.

Taken as a whole, the evidence in the literature suggests that chlorinated aromatic compounds are subject to biodegradation in the environment as part of the natural chlorine cycle. It is no longer valid to assume that chlorinated aromatics compounds will persist indefinitely in the environment. Risk assessment models should take into account that biodegradation is most likely to be one of the major components in the natural attenuation processes affecting chlorinated aromatics.

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