Degradation of Chlorobenzoates and Chlorophenols by Methanogenic Consortia

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 28 april 1999
des namiddags te vier uur in de Aula

Aan mijn ouders

Cover: 3 dancing leaves, Frank Maarsen

ISBN 90 5808 017 X

The research described in this thesis was financially supported by a grant of the Innovatively-Oriented Program (IOP) Committee on Environmental Biotechnology (IOP-m 91212) established by the Dutch Ministry of Economics, and a grant from Paques BV Environmental Technology, Balk, The Netherlands.

STELLINGEN

1.
Vier uit slib afkomstige anaërobe, meso- en neutrofiele en onderling afhankelijke micro-organismen groeien slechts onder strikte voorwaarden samen.
Dit proefschrift

2.
Bij de bespreking van bacteriële aggregatie, bevoordeeld door begrazing door protozoën, hebben Bossier en Verstraete zich ten onrechte tot een theoretische beschouwing beperkt.
Bossier P. en Verstraete W. 1996. Triggers for microbial aggregation in activated sludge? Appl. Microbiol. Biotechnol. **45**, 1-6
Güde H. 1979. Grazing by protozoa as selection factor for activated sludge bacteria. Microb. Ecol. **5**, 225-237

3.
In 1995 was het achterhaald te stellen dat er slechts één obligaat anaëroob micro-organisme (namelijk *Desulfomonile tiedjei*) geïsoleerd was dat haloaromaten reductief dehalogeneerde.
Slater J.H., Bull A.T. en Hardman D.J. 1995. Microbial dehalogenation. Biodegradation **6**, 181-189
Madsen T. and Licht D. 1992. Isolation and characterization of an anaerobic chlorophenol-transforming bacterium. Appl. Environ. Microbiol. **58**, 2874-2878
Utkin I., Woese C. and Wiegel J. 1994. Isolation and characterization of *Desulfitobacterium dehalogenans* gen. nov., sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. Int. J. Syst. Bacteriol. **44**, 612-619

4.
De genetica is het recept, de fysiologie het gerecht; beiden zijn nodig en vullen elkaar aan.

5.
Bij de 3-chloorbenzoaat-omzetting door *Alcaligenes* sp. stam L6 is zuurstofafhankelijkheid op zich geen reden om een reductief dechloreringsmechanisme onwaarschijnlijk te noemen.
Krooneman J., Wieringa E.B., Moore E.R.B., Gerritse J., Prins R.A. en Gottschal J.C. 1996. Isolation of *Alcaligenes* sp. strain L6 at low oxygen concentrations and degradation of 3-chlorobenzoate via a pathway not involving (chloro)catechols. Appl. Environ. Microbiol. **62**, 2427-2434
Groenewegen P.E.J., Driessen A.J.M., Konings W.N. en De Bont J.A.M. 1990. Energy-dependent uptake of 4-chlorobenzoate in the coryneform bacterium NTB-1. J. Bacteriol. **172**, 419-423

6.

Het is niet onmogelijk, maar zeker nog niet eenvoudig om specifieke actieve biomassa in biofilms te bepalen.

Bauer-Kreisel P., Eiesenbeis M. en Scholz-Muramatsu H. 1996. Quantification of *Dehalospirillum multivorans* in mixed-culture biofilms with an enzyme-linked immunosorbent assay. Appl. Environ. Microbiol. **62**, 3050-3052

Davey H.M. en Kell D.B. 1996. Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. Microbiol. Rev. **60**, 641-696

7.

De constatering dat 2-broom-ethaansulfonaat niet alleen methanogenen, maar ook Eubacteria remt geeft aan dat het begrip "specifieke remmer" ook in dit geval niet in absolute zin moet worden begrepen.

Löffler F.E., Ritalahti K.M. en Tiedje J.M. 1997. Dechlorination of chloroethenes is inhibited by 2-bromoethanesulfonate in the absence of methanogens. Appl. Environ. Microbiol. **63**, 4982-4985

Stellingen behorend bij het proefschrift "Degradation of chlorobenzoates and chlorophenols by methanogenic consortia" van Karin A. Ennik-Maarsen, Wageningen, 28 april 1999.

DANKWOORD

Dit proefschrift is niet zonder de hulp van anderen tot stand gekomen. Een aantal mensen wil ik hiervoor bedanken en enkele daarvan met name noemen.

Ik wil graag mijn co-promotor Fons Stams bedanken voor de waardevolle voortgangsgesprekken en de snelle correcties van de manuscripten. Fons Stams en Gosse Schraa stonden aan het begin van het onderzoeksproject. Willem de Vos, mijn promotor, bedankt voor je adviezen en hulp in een later stadium van het onderzoek. Annemarie Louwerse, jou bedank ik voor het vele werk en de prettige samenwerking. Wim Roelofsen was er altijd bij problemen met de analytische apparatuur en later de computers. Een aantal mensen heeft me met technieken en met het reilen en zeilen op het lab wegwijs gemaakt, daarvoor bedank ik vooral Francis Koene-Cottaar, Caroline Plugge en Alette Langenhoff. In de eindfase van het onderzoek heb ik praktische of theoretische hulp gehad van Maarten de Gee, André van Lammeren, Imke Leenen en Hans Scholten. Nees Slotboom droeg zorg voor de financiële en materiële zaken. Mijn kamergenoten, onder wie Ine van Kuijk, Stefanie Oude Elferink, Xiuzhu (mevrouw) Dong en Sonja Parshina, zorgden voor een goed werkklimaat. Mijn studenten Lars, Sandra en Peter, bedank ik voor hun bijdrage. Verder bedank ik de (oud-)medewerkers en (oud-)studenten van het Laboratorium voor Microbiologie voor de prettige werksfeer en hun ondersteuning.

Karin

CONTENTS

Chapter 1

INTRODUCTION

1. General introduction

Chlorinated aromatic hydrocarbons have contaminated the environment through their use in industry as pesticides, solvents, dielectric and hydraulic fluids, heat transfer agents, and wood preservatives. They also originate from numerous industrial processes as byproducts in the manufacturing of other chlorinated compounds and in pulp bleaching effluents (Mohn & Tiedje, 1992; Dolfing & Beurskens, 1995; Field *et al.*, 1995; Puhakka & Melin, 1996). For instance, chlorine bleaching effluents from paper factories contain chlorocatechols, -guaiacols, -phenols, and other chlorinated aromatic and aliphatic compounds (Lepistö & Rintala, 1994). As a consequence, halogenated compounds often enter wastewater treatment plants (Verstraete *et al.*, 1996). In general, pollution with chlorinated compounds is problematic, because the majority of these compounds is toxic, little biodegradable, or both. The presence of chlorinated compounds in soils, sediments, surface or subsurface waters, and the atmosphere often but not always indicates pollution by anthropogenic industrial activity. Chlorinated aromatic compounds are also produced in natural processes such as volcanic eruptions. Furthermore, various chlorinated compounds are excreted by diverse living organisms such as fungi, insects, worms and blue-green algae. The function of such excreted chlorinated aromatic and aliphatic compounds for living organisms is usually to discourage a predator or competitor, because the chlorinated compound makes the prey distasteful, toxic, or both (Gribble, 1994; De Jong *et al.*, 1994).

Chlorinated aromatic compounds have been shown to be susceptible to chemical transformation. They can be degraded by photochemical oxidation and reduction, by oxidation with for instance hydroxyl radicals and ozone in the absence of light, and by hydroxylation with hydroxyl radicals resulting from ultrasound sonication (Wong & Crosby, 1981; Ollis *et al.*, 1991; Sedlak & Andren 1991; Boncz *et al.*, 1997; Uraki *et al.*, 1997).

This thesis decribes the biodegradation and toxicity of chlorinated aromatic compounds under methanogenic conditions. Most research under these anaerobic conditions has focused on the microbial degradation of relatively simple compounds like mono- or dichlorinated benzoates and phenols (Mohn & Tiedje, 1992). Therefore, this and the following chapters will mainly deal with (mono- and di-) chlorinated benzoates and phenols.

2. Biological degradation of aromatic compounds

Since chlorinated aromatic compounds consist of an aromatic nucleus with one or more chlorine atoms attached to the aromatic ring, the microbial metabolism of aromatic compounds in general will be described first. Benzene is the simplest aromatic hydrocarbon. The six aromatic electrons are delocalized and this makes an aromatic compound much more stable (less reactive) than an aliphatic compound. A substituent on the ring affects the reaction rate and reaction mode, by activating or deactivating the aromatic ring and directing the attack to a certain position on the ring. The initial step of the transformation of aromatic compounds is different for prokaryotes (e.g. bacteria) and eukaryotes (e.g. fungi, mammals) (Rochkind-Dubinsky *et al.*, 1987). Furthermore, in bacteria great differences exist for degradation of aromatics under aerobic and anaerobic conditions (Heider & Fuchs, 1997).

Under aerobic conditions, oxygen does not only serve as a terminal electron acceptor, but is also a reactant in the initial attack of the benzene ring. The first transformation step in eukaryotes is catalyzed by a monooxygenase, which incorporates a single oxygen atom onto the benzene ring (Armstrong, 1987; Middelhoven, 1993; Parkinson, 1996). Bacteria oxidize the aromatic compound with a mono- or dioxygenase (Schlegel, 1993). In a dioxygenase reaction both oxygen atoms from an oxygen molecule are incorporated into the ring at adjacent positions. Subsequently, two hydrogen atoms are removed by a dehydrogenase. The product from benzene dihydroxylation is catechol (1,2-dihydroxybenzene) (Figure 1a). Substituted benzenes can directly be dihydroxylated to substituted catechols. Alternatively, they are first oxidized and the product is subsequently dihydroxylated to (substituted) catechol. Phenol is oxidized by a monooxygenase and the product is catechol (Figure 1b) (Schlegel, 1993). The pathways for oxidation of toluene can start with a mono- or a dioxygenase reaction (summarized by Mikesell *et al.*, 1993). Central intermediates are catechol, protocatechuate (3,4-dihydroxybenzoate) and gentisate (2,5-dihydroxybenzoate) (Fuchs *et al.*, 1994). The opening of the ring can proceed if the molecule contains two hydroxyl groups *ortho*- or *para*-positioned. For *ortho*-positioned hydroxyl groups, there are two possible locations for ring cleavage: between the two hydroxyl groups or next to one hydroxyl group, called *ortho*- and *meta*-cleavage, respectively (Rochkind-Dubinsky *et al.*, 1987).

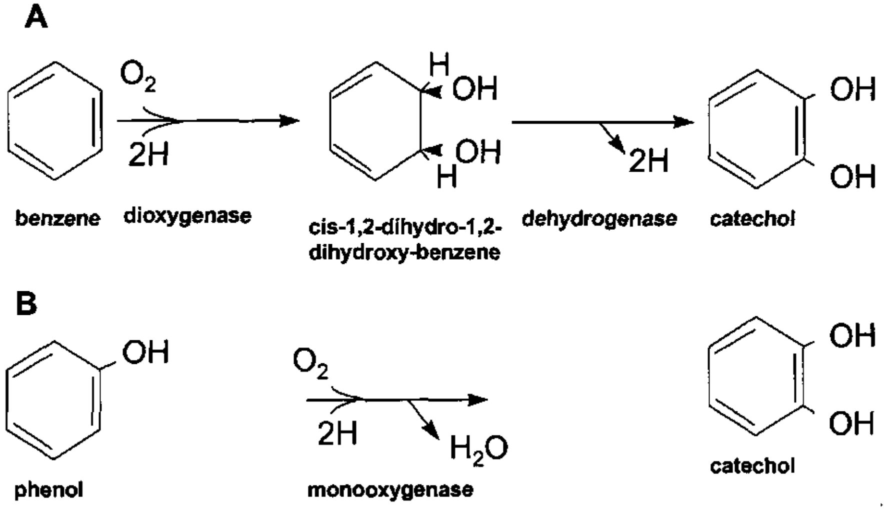

Figure 1. Initial hydroxylation reaction of benzene by a dioxygenase (A) and of phenol by a monooxygenase (B) (from Schlegel, 1993).

Microbial degradation of aromatic compounds under anaerobic conditions is different from aerobic aromatic degradation, because oxygen can not serve as a reactant. In the absence of molecular oxygen, the aromatic degradation requires carbon dioxide, water, coenzyme A and ATP or addition of reducing equivalents. A general sequence of events has been described by Fuchs *et al.* (1994). Firstly, an activation of chemically little reactive compounds takes place, typically by carboxylation, hydroxylation, or coenzyme A thioester formation. This is followed by reactions like reductive dehydroxylations or transhydroxylations to form one of the central intermediates. Known central intermediates are benzoyl-CoA, resorcinol (1,3-dihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene). The central intermediate is attacked by reductases, followed by hydrolytical cleavage of the ring (Fuchs *et al.*, 1994). Some aromatic compounds degraded via the benzoyl-CoA pathway are presented in Figure 2, illustrating the various activation reactions. Phenol, catechol and aniline are carboxylated, vanillate is demethoxylated, and benzyl alcohol is oxidized. Activation of 4-aminobenzoic acid occurs by CoA thioester formation. One of the possible pathways of anaerobic toluene degradation starts with a hydroxylation to benzyl alcohol (not shown in Figure 2) (Heider & Fuchs, 1997). Finally, reductive dechlorination of chlorophenols and chlorobenzoates is shown in Figure 2 (see section 2.1.1).

2.1. Biological degradation of chlorinated aromatic compounds

2.1.1 Initial degradation mechanisms

Previous reviews have focused on the transformation or biodegradation of chlorinated aromatic hydrocarbons by eukaryotes, including mammals (Armstrong, 1987; Parkinson, 1996) and fungi (Häggblom, 1992). Therefore, this section only decribes the biodegradation mechanisms occurring in bacteria.

The degradation of chlorinated aromatic compounds by bacteria can proceed in four ways (Häggblom, 1992; Commandeur & Parsons, 1994):
(1) dehalogenation after ring cleavage, (2) oxidative dehalogenation, (3) hydrolytic dehalogenation and (4) reductive dehalogenation (Figure 3).

Dehalogenation after ring cleavage (1) is a fortuitous reaction catalyzed by enzymes involved in the degradation of non-chlorinated compounds. The initial degradation steps before ring cleavage are analogous to the initial steps described for aromatic compounds. In case a chlorinated aromatic compound is oxidized, chlorocatechols are formed from e.g. chlorobenzoates, chlorobenzenes and chloroanilines in a reaction catalyzed by dioxygenases. Mono- and dichlorophenols with one non-substituted *ortho*-position are oxidized by a dioxygenase or a monooxygenase, also resulting in the formation of chlorocatechols.

Oxidative dehalogenation (2) is a fortuitous reaction as well. The chlorine atom is removed from the aromatic compound during catechol formation by a dehalogenating dioxygenase (Figure 3a). This has been described for degradation of lower chlorinated phenols and for chlorobenzoates by e.g. *Pseudomonas* strains (Häggblom, 1992).

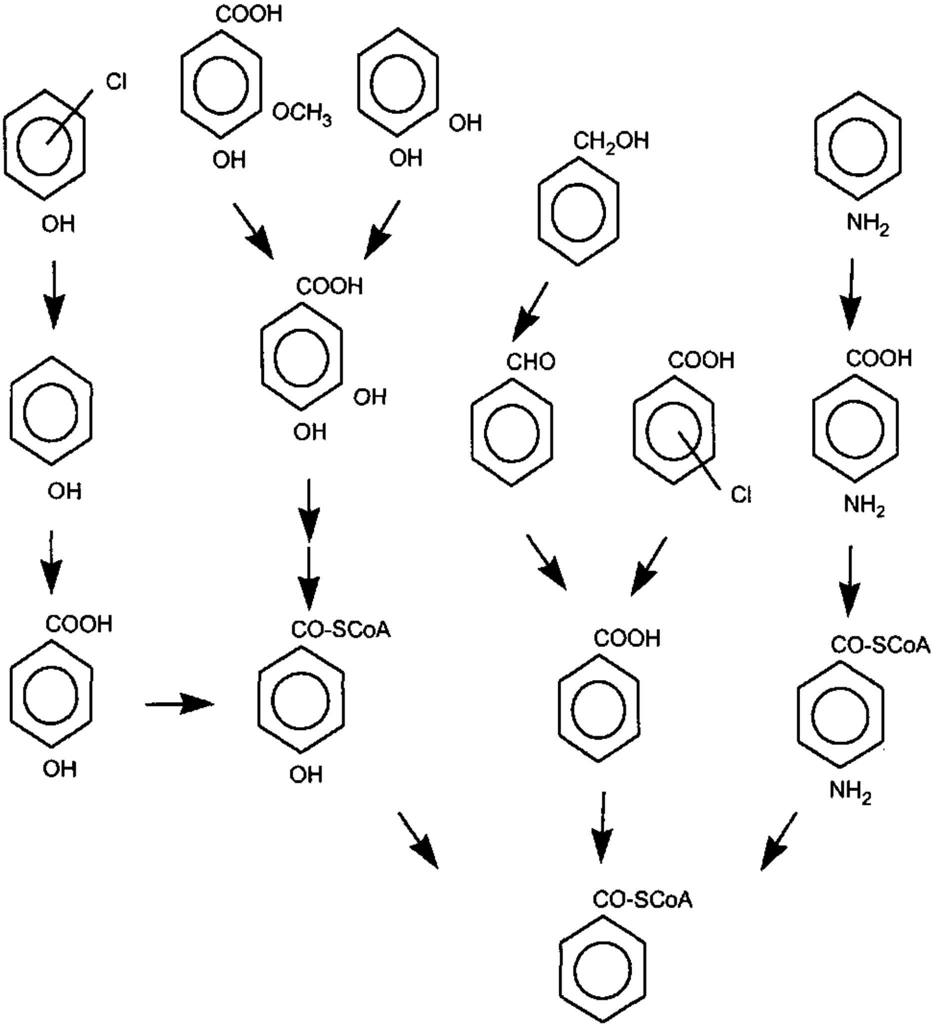

Figure 2. Formation of benzoyl-CoA in the anaerobic degradation of some aromatic compounds (from Heider & Fuchs, 1997). From left to right: chlorophenol, vanillate, catechol, benzyl alcohol, chlorobenzoate, and aniline.

Hydrolytic dehalogenation (3) is the replacement of the halogen atom by a hydroxyl group, derived from water (Figure 3b). A few examples of hydrolytic dehalogenation of chlorophenols and chlorobenzoates have been described. For instance, various bacteria convert 4-chlorobenzoate into 4-hydroxybenzoate (Häggblom, 1992).

Reductive dehalogenation (4) of aryl chlorides occurs by hydrogenolysis (Mohn & Tiedje, 1992). In this reaction two protons and two electrons are involved and the chlorine is not replaced by a hydroxyl group but by a hydrogen atom (Figure 3c). The protons were shown to be derived from water for the reductive dehalogenation of 3-chlorobenzoate by *Desulfomonile tiedjei* (Griffith *et al.*, 1992). Degradation by reductive dehalogenation has been shown for chlorobenzenes, -anilines, -catechols, -resorcinols, -phenols, -benzoates, -toluenes, and -phenoxyacetates, and polychlorinated biphenyls (PCBs) (Mohn & Tiedje, 1992, Ramanand *et al.*, 1993).

Combinations of the aryl dehalogenation types also occur. Higher chlorinated phenols, like pentachlorophenol (PCP), are first hydroxylated to a chlorinated *para*-hydroquinone. *Rhodococcus chlorophenolicus* strain PCP-1, later reclassified as *Mycobacterium chlorophenolicum* strain PCP-1 (Briglia *et al.*, 1994), probably uses a monooxygenase for this transformation. This step is followed by reductive and hydrolytic dechlorination steps, all before ring cleavage occurs. In the coryneform bacterium strain NTB-1 and *Corynebacterium sepedonicum* strain KZ-4, 2,4-dichlorobenzoate (2,4-DCB) is first reductively dechlorinated to 4-chlorobenzoate (4-CB), which is then hydrolytically dechlorinated (Van den Tweel *et al.*, 1987; Groenewegen *et al.*, 1990; Romanov & Hausinger, 1996). From the herbicides 2,4-dichlorophenoxyacetate (2,4-D) and 2,4,5-trichlorophenoxyacetate (2,4,5-T), first the ether bond is cleaved rendering chlorophenols, which subsequently, as stated above, can be degraded via several pathways (Häggblom, 1992; Commandeur & Parsons, 1994).

In many cases, the specificity for a halogen atom is such that from a specific position on the ring, a chlorine, bromine, and iodine atom can be removed at similar rates, while the (small) fluorine atom is more difficult to remove (e.g., Suflita *et al.*, 1982; Van den Tweel *et al.*, 1987; Van der Woude, 1996; Monserrate & Häggblom, 1997). During oxidative dehalogenation, the halogen atom which is fortuitously removed can be either chlorine, bromine, iodine, or fluorine (Häggblom, 1992; Commandeur & Parsons, 1994). Since fluorine analogs are usually not immediately defluorinated under anaerobic conditions, they are often used as a tool to examine degradation pathways of aromatic compounds (e.g., Aftring & Taylor, 1981; Sharak Genthner *et al.*, 1990; Londry *et al.*, 1997; Boersma *et al.*, 1998; Häggblom, 1998). However, a sulfate-reducing biculture has been described, in which a *Desulfovibrio*-like microorganism is possibly responsible for defluorination, suggesting that defluorination occurs during an initial step in the degradation of 2- and 4-fluorobenzoate (Drzyzga *et al.*, 1994).

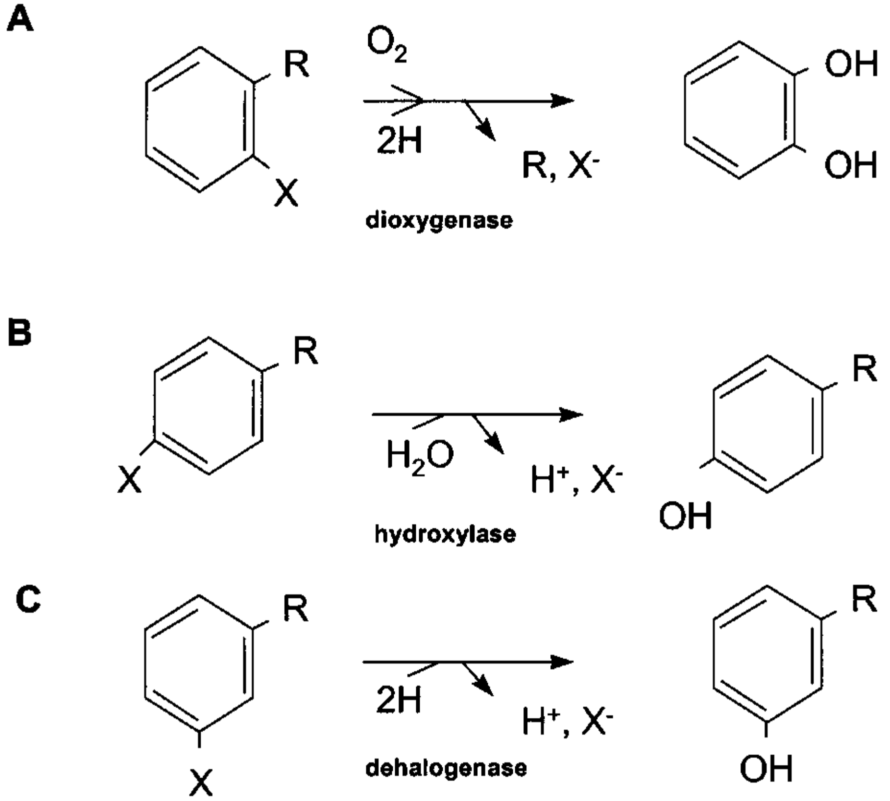

Figure 3. Oxidative (A), hydrolytic (B), and reductive (C) dehalogenation of haloaromatic compounds. R = e.g. COOH, OH, NH_2; X = F, Cl, Br, I (from Commandeur & Parsons, 1994).

2.1.2 Redox conditions

Under oxic conditions, all three initial dehalogenation mechanisms (oxidative, hydrolytic, and reductive dehalogenation) have been found, while under methanogenic conditions, only reductive dehalogenation takes place (Häggblom, 1992; Mohn & Tiedje, 1992; Commandeur & Parsons, 1994). Degradation of chloroaromatics under methanogenic conditions will be discussed in detail in section 2.2. Few investigations have concentrated on dehalogenation under other redox conditions. These will be discussed here. Some of these studies deal with the removal of other halogens than chlorine. Dehalogenation mechanisms have been clarified for several monohalobenzoates and -phenols (Table 1 and sections 2.1.2.1. to 2.1.2.4).

2.1.2.1 Sulfate-reducing conditions

In the presence of sulfate, reductive dehalogenation has been observed in soil, wastewater treatment reactors and aquatic sediments (e.g., Bosma *et al.*, 1988; Kohring *et al.*, 1989b; Lepistö & Rintala, 1994; Hale Booth *et al.*, 1997). In some cases the reductive dehalogenation or other degradation steps were coupled to sulfate reduction (e.g., King, 1988; Yonezawa *et al.*, 1994). In contrast, other authors observed that the rate or extent of reductive dehalogenation was negatively affected by sulfate (e.g., Kuhn *et al.*, 1990; Madsen & Aamand, 1991; Pecher *et al.*, 1995; Chang *et al.*, 1996, 1998). A coupling of sulfate reduction and chloroaromatic degradation was described in several other studies, but in these cases the dehalogenation type was unclear (Sharak Genthner *et al.*, 1989a; Häggblom & Young, 1990; Häggblom *et al.*, 1993b, 1995; Kennes *et al.*, 1996). The O-demethylation of 4,5,6-trichloroguaiacol (1-methoxy-2,3-dihydroxy-4,5,6-trichlorobenzene) to 3,4,5-trichlorocatechol was followed by reductive dechlorination steps (Allard *et al.*, 1992). Masunaga et al. (1996) speculated that there would be other reactions additional to reductive dechlorination in the degradation of chlorophenols, because more substrate disappeared than was reductively dechlorinated. Sulfite, as well as thiosulfate and sulfate, could serve as an electron acceptor during the degradation of 4-halophenols by an enrichment from estuarine sediment (Häggblom & Young, 1995). In this enrichment, the dechlorination mechanism for 4-CP was found to be reductive dehalogenation while sulfate reduction also took place (Häggblom, 1998).

Various sulfate-reducing bacteria have been isolated that can utilize haloaromatics (see also Table 2). Reductive dechlorination by *D. tiedjei* was under some conditions inhibited by sulfate (DeWeerd *et al.*, 1991). An explanation for the difference in sensitivity to sulfate of resting cells (weak inhibition) and growing cells (strong inhibition) was given by Townsend and Suflita (1997). Sulfate can repress the induction of the dehalogenase gene expression but not the activity of the dehalogenase enzyme. Two other strains have been characterized less extensively. Strain DSL-1 is a marine isolate which debrominates 2,4,6-tribromophenol to 4-bromophenol (Steward *et al.*, 1995). Another sulfate-reducer, isolated from swine manure, grows on phenol or 4-chlorophenol as sole carbon and energy source (Boopathy, 1995). In the presence of sulfite, *M. chlorophenolicum* strain PCP-1 hydrolytically dechlorinates PCP and 2,3,5-trichlorophenol, while *D. dehalogenans* reductively dechlorinates 3-Cl-4-OHPA (Uotila *et al.*, 1992; Mackiewicz & Wiegel, 1998).

In summary, chlorobenzoates, -benzenes, -phenols, bromophenols, 2,3,4,5-tetrachloroaniline, 4,5,6-trichloroguaiacol and 3,4,5-trichlorocatechol were degraded under sulfate-reducing conditions in the above mentioned investigations. The dehalogenating mechanisms found so far in the presence of sulfate or sulfite are reductive and hydrolytic dehalogenations.

2.1.2.2 Nitrate-reducing conditions

Chlorophenols were reductively dechlorinated in soil slurries with active denitrification activity (Sanford *et al.*, 1997). However, the dechlorination was not coupled to denitrification. Coupling of degradation of chlorobenzoates and 2-chlorophenol (2-CP) to denitrification was shown to occur in freshwater and estuarine sediments, but in these cases the dehalogenation mechanism was unclear (Sharak Genthner *et al.*, 1989a; Häggblom *et al.*, 1993b, 1996; Kazumi *et al.*, 1995a). The degradation of 4-CP in a denitrifying fluidized-bed reactor was established, although the degradation mechanism was unclear (Melin *et al.*, 1993).

Several bacteria in pure culture are able to perform dehalogenation while nitrate is reduced. The coryneform bacterium strain NTB-1 hydrolytically dechlorinates 4-CB under denitrifying conditions. The energy for carrier-mediated 4-CB uptake is supplied by denitrification (Groenewegen *et al.*, 1990). The denitrifying toluene-degrading bacterium strain T1 was genetically engineered, acquiring the nitrate-dependent ability to dechlorinate 4-CB hydrolytically (Coschigano *et al.*, 1994). *Desulfitobacterium dehalogenans* reduces nitrate to ammonium while it reductively dechlorinates 3-chloro-4-hydroxyphenylacetate (3-Cl-4-OHPA) (Mackiewicz & Wiegel, 1998). Four denitrifying *Pseudomonas* strains utilize 2- or 4-fluorobenzoate, from which the fluorine atom is most probably lost fortuitously, while nitrogen evolves (Taylor *et al.*, 1979; Schennen *et al.*, 1985). In short, during the degradation of chlorophenols and halobenzoates under nitrate-reducing conditions, the halogen atom is removed by hydrolytic dehalogenation, by reductive dehalogenation, or fortuitously.

2.1.2.3 Iron-reducing conditions

Investigations on aryl dehalogenation under iron(III)-reducing conditions first aimed to find a coupling between the formation of iron(II) and the mineralization of chloroaromatics in aquatic sediments (Kazumi *et al.*, 1995a, 1995b). With evidence for this coupling, the possibilities for reductive dechlorination and hydrolytic dechlorination as the initial degradation step were discussed (Dolfing, 1996; Kazumi *et al.*, 1996). Neither mechanism could theoretically be ruled out. With 2-bromophenol a reductive debromination mechanism and a stoichiometric conversion of 2-bromophenol and Fe(III) could be established (Monserrate & Häggblom, 1997). Reductive dechlorination of 3-CB in the presence of Fe(III) has also been found in freshwater sediments (Myers *et al.*, 1994). The degradation of chloroaromatic compounds in the presence of manganese(IV) has been investigated with 3-CB and 2,4-D. 3-CB was reductively dechlorinated. The transformation mechanisms of 2,4-D were not clarified, nor was it examined whether Mn(IV) reduction and reductive dechlorination were coupled (Myers *et al.*, 1994).

2.1.2.4 Photobiological conversion

Under anoxic conditions in the presence of light, phototrophic bacteria such as *Rhodopseudomonas palustris* can utilize (chloro-)aromatic compounds. *R. palustris* strain WS17 metabolizes monohalobenzoates as a cosubstrate, when grown on benzoate (Kamal & Wyndham, 1990; Van der Woude *et al.*, 1994). For *R. palustris* strain DCP3 benzoate is not required for the degradation of 3-CB and 3-bromobenzoate (Van der Woude *et al.*, 1994). In strain DCP3, high coenzyme A ligase activity and the presence of benzoate in cell extracts indicate that dechlorination of 3-CB, activated in the form of 3-chlorobenzoyl-CoA, takes place before the aromatic ring is reductively attacked (Van der Woude, 1996). The *R. palustris* strains BAH2 and PL1 carboxylate 2-CP to 3-Cl-4-OHPA, which is subsequently reductively dechlorinated (to 4-hydroxyphenylacetate) and further degraded (Noh *et al.*, 1995).

Table 1. Overview of publications in which the dehalogenating mechanism of monochlorobenzoates and -phenols under anaerobic conditions has been established. The underlined publication numbers refer to hydrolytic dehalogenation; in all other cases, reductive dehalogenation took place. CB = chlorobenzoate; CP = chlorophenol.

	Condition				
Chloroaromatic compound	Methanogenic	Sulfate-reducing	Denitrifying	Phototrophic	Iron(III)-reducing
2-CB	1, 2	-	-	-	-
3-CB	1, 2, 3, 4, 5, 6	1, 7, 8, 9	-	-	10
4-CB	-	-	11, 12	-	-
2-CP	1, 2, 13, 14, 15, 16, 17, 18, 19, 20, 21	1, 22, 23, 24, 25	26	27	-
3-CP	1, 2, 4, 16, 21, 28	23, 25	-	-	-
4-CP	4, 14, 21, 29, 30	23, 25, 31	-	-	-

1 Sharak Genthner *et al.*, 1989a; 2 Sharak Genthner *et al.*, 1989b; 3 Suflita *et al.*, 1982; 4 Gibson & Suflita, 1986; 5 van der Woude *et al.*, 1996; 6 Shelton & Tiedje, 1994; 7 Townsend *et al.*, 1997; 8 DeWeerd *et al.*, 1991; 9 Townsend & Suflita, 1997; 10 Myers *et al.*, 1994; 11 Groenewegen *et al.*, 1990; 12 Coschigano *et al.*, 1994; 13 Häggblom *et al.*, 1993a; 14 Kennes *et al.*, 1996; 15 Häggblom *et al.*, 1993b; 16 Hale *et al.*, 1990; 17 Basu *et al.*, 1996; 18 Dietrich & Winter, 1990; 19 Zhang & Wiegel, 1992; 20 Boyd *et al.*, 1983; 21 Christiansen, 1995; 22 Häggblom & Young, 1990; 23 Susarla *et al.*, 1997; 24 Monserrate & Häggblom, 1997; 25 Liu *et al.*, 1996; 26 Sanford *et al.*, 1997; 27 Noh *et al.*, 1995; 28 Juteau *et al.*, 1995; 29 Zhang & Wiegel, 1990; 30 Armenante *et al.*, 1995; 31 Häggblom, 1998.

2.2 Biodegradation of chlorinated aromatic compounds under methanogenic conditions

2.2.1 Cometabolic conversions and halorespiration

Reductive dehalogenation can occur as a co-metabolic reaction, resulting from the lack of specificity of enzymes and cofactors, but since the reductive dehalogenation reaction has a large negative Gibbs free energy change, the halogenated aromatic compound is a potential terminal electron acceptor (Dolfing & Harrison, 1992). Aspecific dehalogenation reactions have been reported for hexa- and pentachlorobenzene by the cofactors vitamin B_{12} and hematin, hexachlorobenzene by the methanogenic cofactor F_{430}, and penta-, tetra-, and trichlorophenols by vitamin B_{12} (Gantzer & Wackett, 1991; Smith & Woods, 1994). A specific reductive dechlorination reaction from which energy is conserved in an anaerobic respiration was first described for *D. tiedjei* (Mohn & Tiedje, 1992). Some reductively dechlorinating bacteria use only hydrogen or formate as electron donors for halorespiration, but for instance, *Desulfitobacterium* strains are also able to use organic compounds (lactate, pyruvate, butyrate, and yeast extract) (Holliger & Schumacher, 1994; Sanford *et al.*, 1996). The organization of the respiratory chain with chloroaromatic and chloroaliphatic compounds as electron acceptors is still unclear (Mohn & Tiedje, 1992; Holliger & Schumacher, 1994). The dehalogenating enzyme of *D. tiedjei* has been purified and may be an integral membrane protein (Ni *et al.*, 1995; Townsend & Suflita, 1996). Like the dehalogenase of *D. tiedjei*, the dehalogenating enzyme of *Desulfitobacterium chlororespirans* is a membrane bound reductase. Both use methyl viologen as an artificial electron donor (Löffler *et al.*, 1996). A number of bacteria is able to conserve energy from dechlorination, which suggests that the use of the chlorinated aromatic compound as a terminal electron acceptor leads to proton translocation (Table 2). Tetrachloroethene dehalogenases have been studied in more detail. The dehalogenating enzymes of *Dehalospirillum multivorans* and *Dehalobacter restrictus* both contain a corrinoid. The electron transfer chain of *D. restrictus* contains a hydrogenase at the periplasmic side of the membrane and a dehalogenase at the cytoplasmic side, while the dehalogenase of *D. multivorans* is recovered in the cytoplasmic soluble fraction (Wohlfarth & Diekert, 1997).

Dechlorination pathways have been examined in a number of investigations (for a discussion see Dolfing & Beurskens, 1995). Dechlorination is not necessarily the first degradation step, as was found for example with 2,4-dichlorophenoxyacetate and 3-chloro-4-hydroxybenzoate in samples from diverse anaerobic environments (Gibson & Suflita, 1986; Zhang & Wiegel, 1992). Dechlorination was either preceded or followed by acetate cleavage of 2,4-dichlorophenoxyacetate (Gibson & Suflita, 1986). Dehydroxylation preceded dechlorination of 3-chloro-4-hydroxybenzoate or vice versa (Zhang & Wiegel, 1992). However, under methanogenic conditions, dechlorination takes place before ring cleavage (see section 1.1). This implies

Table 2. Some morphological and physiological properties of bacteria which generate energy from reductive dechlorination under methanogenic conditions: *Desulfomonile tiedjei* (DeWeerd *et al.*, 1990; DeWeerd and Suflita, 1990; reviewed by Mohn & Tiedje, 1992), strain 2CP-1 (Cole *et al.*, 1994), *Desulfitobacterium dehalogenans* strain JW/IU-DC1 (Utkin *et al.*, 1994, 1995), *Desulfitobacterium* sp. strain PCE1 (Gerritse *et al.*, 1996), *D. hafniense* strain DCB-2 (Madsen & Licht, 1992; Christiansen & Ahring, 1996a), and *D. chlororespirans* strain C023 (Sanford *et al.*, 1996). CBs = chlorobenzoates; 3-Cl-4-OHPA = 3-chloro-4-hydroxyphenylacetate, PCE = tetrachloroethene, CPs = chlorophenols.

Strain or species	*D. tiedjei*	2CP-1	*D. dehalogenans*	*Desulfitobacterium* PCE1	*D. hafniense*	*D. chlororespirans*
Morphology	straight rod	thin rod	curved rod	curved rod	curved rod	curved rod
Gram + or -	-	-	+	+	-	-
Spores	-	-	-	-	+	+
Motility	-	-	+	+	+	+
Temperature range; optimum (°C)	30-38; 37	ND	13-45; 38	19-42; 34-37	22-45; 35-37	15-37; 37
pH range; optimum	6.5-7.8; 6.8-7.0	ND	6-9; 7.5	6.1-8.7; 7.2	ND	6.8-7.5; ND
Fermentation of pyruvate	+	-	+	+	+	+
Electron acceptors	SO_4^{2-}, SO_3^{2-} $S_2O_3^{2-}$	O_2, fumarate	SO_3^{2-}, $S_2O_3^{2-}$, S^0, NO_3^-, fumarate	SO_3^{2-}, $S_2O_3^{2-}$, fumarate	SO_3^{2-}, $S_2O_3^{2-}$, NO_3^-, Fe^{3+}	SO_3^{2-}, $S_2O_3^{2-}$, S^0
Chlorinated electron acceptors						
meta-CBs	+[a]	ND	-[a]	-	ND	-[a]
3-Cl-4-OHPA	ND	ND	+	+	+	+
PCE	+	ND	+	+	ND	-
Position of CPs	*meta*	*ortho*	*ortho*	*ortho*	*ortho, meta*	*ortho*

[a] dechlorination of 3-chloro-4-hydroxybenzoate

that the degradation of chlorinated aromatic compounds proceeds via the same central intermediates as the degradation of non-chlorinated aromatics. For many compounds the central intermediate is benzoate (or more precisely, benzoyl-CoA) (Figure 2). The further degradation of benzoate is a conversion to hydrogen, acetate, and carbon dioxide (Table 3). The conditions under which benzoate oxidation takes place and the role of methanogens and dechlorinating bacteria will be discussed below.

2.2.2 Syntrophic degradation of chloroaromatic compounds

Benzoate oxidation under standard conditions is an endergonic process: the Gibbs free energy change $\Delta G^{0\prime}$ is positive. The $\Delta G^{0\prime}$ value represents the situation in which the temperature is 298 K, the solute concentrations are 1 mol/litre, the pH is 7 and the partial pressures of the gases are 1 atm. When hydrogen is efficiently removed by hydrogen-consuming methanogenic archaea, the $\Delta G^{0\prime}$ value becomes negative and the benzoate-oxidizer can convert benzoate. This interaction in which the hydrogen-producer (acetogen) depends on the methanogen for degradation of a substrate, and the methanogen depends on the hydrogen-producer for hydrogen supply, is called syntrophism. Beside benzoate conversion, degradation of other aromatic compounds like phenol and hydroxybenzoate also occurs syntrophically. In methanogenic environments, syntrophic interactions are common in the degradation of fatty acids, amino acids, other organic acids, and alcohols (Schink, 1992; Stams 1994).

In addition to hydrogen, formate and acetate may influence a syntrophic conversion. Formate and hydrogen can both be produced by acetogens and consumed by methanogens, and some methanogens are able to interconvert formate and hydrogen. In a few examples, acetate is essential for degradation, e.g. the anaerobic degradation of acetone (Platen & Schink, 1987). Theoretical considerations and experimental evidence for the role of hydrogen, formate, and acetate have been reviewed (Stams, 1994; Schink, 1997).

The maximal hydrogen concentration for exergonic substrate conversion (10-32 Pa for different acetogens) is a little higher than the hydrogen threshold for methanogenesis (2.5-16 Pa for mesophilic methanogens) (Stams, 1994). The benzoate-oxidizing bacteria strain BZ-2, *Syntrophus buswellii* and *S. gentianae* convert benzoate until a hydrogen concentration of 2000, 25 and 100-1000 Pa is reached, respectively (Dolfing & Tiedje, 1991; Wallrabenstein & Schink, 1994; Schöcke & Schink, 1997). The concentration gradient between the hydrogen producer and the hydrogen consumer is rather gradual, allowing only low substrate conversion rates, but it becomes steeper if the distance between the two microorganisms is reduced. Consequently, the hydrogen flux, and thereby the syntrophic substrate conversion rate, increases (Schink & Thauer, 1988).

For a chlorinated aromatic compound such as 3-CB, the microbial interaction is more complex. When the electron donor for dechlorination is hydrogen (or formate), two groups of hydrogen consumers compete for hydrogen (or formate). An example is a triculture of *D. tiedjei*, the syntrophic benzoate-oxidizing strain BZ-2 and *Methanospirillum* strain PM-1. In this triculture reducing equivalents are shared between the dechlorinating bacteria and the methanogens apparently without competition (Dolfing & Tiedje, 1991). In the degradation of benzoate, three molecules of hydrogen are formed. One of these hydrogen molecules is required for reductive dechlorination of 3-CB, leaving two hydrogen molecules for methane formation. In the conversion of phenol or hydroxybenzoate, only two hydrogen molecules are released. During the breakdown of highly chlorinated compounds even more hydrogen molecules are invested in reductive dechlorination than are released in later stages of the mineralization. Therefore, when a highly chlorinated compound is the sole substrate, hydrogen supply is limiting. Dechlorinating bacteria should have an advantage in the competition for hydrogen with methanogens, because the $\Delta G^{0\prime}$ of the substrate conversion is inversely related to the steady-state concentration of hydrogen in a culture (Cord-Ruwisch *et al.*, 1988). The $\Delta G^{0\prime}$ is -33.9 kJ/mol H_2 for methanogenesis and -130 to -171 kJ/mol H_2 for reductive dechlorination of chloroaromatics (Stams, 1994; Dolfing & Harrison, 1992). The theoretical hydrogen threshold of dechlorinating bacteria is very low (Figure 4). The measured values for *D. tiedjei*, strain 2CP-1 and *D. chlororespirans* are in the range of 0.01-0.1 Pa (DeWeerd *et al.*, 1991; Sanford *et al.*, 1996), and thus lower than reported for hydrogenotrophic methanogens (1-10 Pa) (Stams, 1994).

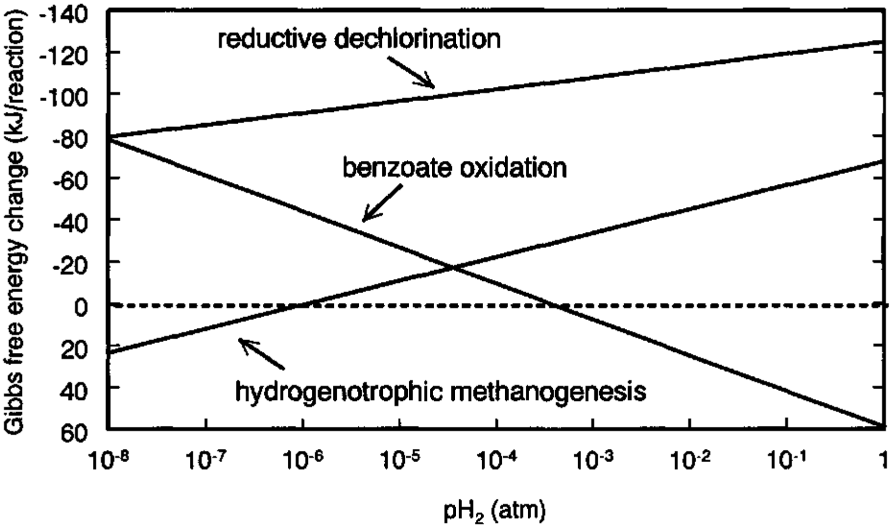

Figure 4. The $\Delta G'$ values per reaction for reductive dechlorination, syntrophic benzoate oxidation and hydrogenotrophic methanogenesis under standard conditions, but with variation in the hydrogen partial pressure (see also Table 3).

2.2.3 Effects of environmental conditions on the conversion of chloroaromatic compounds

The affinity constant and maximal substrate conversion rate are not the only factors affecting chloroaromatic biodegradation rates. In addition, the bioavailability should be high enough and the degradation of chloroaromatic compounds at high concentrations can be limited by their toxicity (Mohn & Tiedje, 1992; Holliger & Schumacher, 1994). Toxicity of compounds towards microorganisms can be expressed in several ways. A parameter to express toxicity of chlorinated aromatics to methanogens and methanogenic granular sludge is the concentration that causes 50% inhibition of the methane production rate (IC_{50} or 50% IC). This value was 0.03 mM PCP for some methanogens and methanogenic granular sludge, while for 2-CP, 3-CB and chlorobenzene it was 3 mM or higher (Patel *et al.*, 1991; Sierra-Alvarez & Lettinga, 1991). Most chlorinated aromatic compounds have a high solvent/water partition coefficient (Sierra-Alvarez & Lettinga, 1991; Kennedy *et al.*, 1992). One can make use of this property during enrichment of specific dechlorinating microorganisms. In this approach, the concentration in the water phase is kept low to prevent toxic effects, while the substrate supply is sufficient to provide a selective advantage for dechlorinating microorganisms. This method was applied to enrich for 1,2,3-trichlorobenzene-degrading microorganisms and resulted in high numbers of dechlorinating microorganisms, about 10^8/ml (Holliger *et al.*, 1992). Chlorophenols and chlorobenzoates are better water soluble than chlorobenzenes, and are therefore usually sufficiently bioavailable. In soil, the adsorption of chlorophenols to particles depends on the soil type. Additionally, the organic matter in soil reduces the bioavailability of the chlorophenols. Likewise, bioavailability can be a limiting factor for the biodegradation in sediments (Puhakka & Melin, 1996).

The pH can influence the degradation of chlorinated aromatic compounds in several ways (Dolfing & Beurskens, 1995). Chlorobenzoates are dissociated at neutral pH: the pK_a values are 2-4 (Dolfing & Beurskens, 1995). However, for weak acids such as chlorophenols, the pH is more important, because the dissociation constants (pK_a values) of chlorophenols are in the range of 5 to 9 (Kishino & Kobayashi, 1994). The undissociated acid acts as an uncoupler (Sikkema *et al.*, 1995), but is also the form in which the chlorophenol is taken up when passive transport takes place. Furthermore, the pH optima of dechlorinating microorganisms are different. The dechlorinating bacteria listed in Table 2 prefer a pH near neutrality, while a 2,4,6-trichlorophenol-dechlorinating enrichment from an anaerobic reactor only dechlorinated between pH 8 and 9 (Armenante *et al.*, 1993; Togna *et al.*, 1995).

Chlorobenzoate- and chlorophenol-degrading microorganisms differ in their temperature optima (see also Table 2). Samples from freshwater sediments had temperature optima for 2,4-dichlorophenol degradation between 30 and 43°C (Kohring *et al.*, 1989a; Zhang & Wiegel, 1990). Freshwater sediment microcosms, digested manure and granular sludge could reductively dechlorinate PCP or other chlorinated phenols at 50 to 55°C (Larsen *et al.*, 1991; Mohn & Kennedy, 1992; Lepistö & Rintala, 1994). Thermophilic (75°C) reductive dechlorination of 3-CB to benzoate by cocultures has been reported as well (Maloney *et al.*, 1997). Preliminary results indicated that at 75°C reductive dechlorination by pure cultures of archaeal freshwater strains occurred and that 2,4-dichlorophenol was converted into phenyl acetic acid (Tuttle *et al.*, 1996).

Additional electron donors and nutrients are often supplied to stimulate the degradation of chlorinated aromatic compounds (Mohn & Tiedje, 1992). Some effects that can result from electron donor addition are an enhanced dechlorination rate, a shorter lag phase, a greater extent of dechlorination, and a change in the dechlorination pathway (e.g., Gibson & Suflita, 1990; Kuhn *et al.*, 1990; Hendriksen *et al.*, 1991, 1992; Hendriksen & Ahring, 1992; Holliger *et al.*, 1992; Perkins *et al.*, 1994; Basu *et al.*, 1996). The supply of essential nutrients by for example yeast extract can also result in a higher dechlorination rate (Holliger *et al.*, 1992).

3. Construction of consortia from pure cultures and aggregation by anaerobic microorganisms

In methanogenic environments, biodegradation is usually accomplished by the action of more than one microorganism, and methanogens are responsible for the final mineralization step. A number of different microorganisms, which together biodegrade an organic compound, is often referred to as a consortium. Combining pure cultures in such a consortium can be useful for several purposes such as physiological and ecological studies and practical applications in bioremediation. Since some substrates are obligatory syntrophically degraded, the cocultivation of the involved microorganisms is even essential. However, additional microorganisms, like aceticlastic methanogens or dechlorinating bacteria, may influence the substrate conversion kinetics in obligate syntrophic degradation processes (Ahring & Westermann, 1987, 1988; Dolfing & Tiedje, 1991; Dong *et al.*, 1994a, b; Schöcke & Schink, 1997). In addition, immobilized microorganisms have been studied (Jones *et al.*, 1984; Dwyer *et al.*, 1986; Wu *et al.*, 1996) and applied in biodegradation and bioremediation (Ahring *et al.*, 1992; Cassidy *et al.*, 1996; Christiansen & Ahring, 1996b; Schmidt & Ahring, 1997; Hörber *et al.*, 1998). When the syntrophic partners are immobilized, the interbacterial distances are shorter than when they are suspended in the liquid, and consequently, the substrate conversion rate will be higher in the former situation.

3.1 Construction of methanogenic consortia from pure cultures

A thermophilic butyrate-degrading bacterium has been described that converted butyrate into acetate and hydrogen provided that a hydrogenotroph, *Methanobacterium thermoautotrophicum*, was present

(Ahring & Westermann, 1987). In a triculture with a thermophilic acetate-utilizing methanogen, butyrate was completely mineralized into methane and carbon dioxide. In the triculture, the butyrate degradation rate was higher than in the biculture. Elevated hydrogen and acetate concentrations inhibited butyrate degradation, but inhibition by acetate could be reversed after addition of the aceticlast to the biculture of the butyrate degrader and *M. thermoautotrophicum* (Ahring & Westermann, 1988). Under mesophilic conditions, the syntrophic butyrate oxidizer *Syntrophospora bryantii* and the hydrogenotroph *Methanospirillum hungatei* converted butyrate faster in the presence than in the absence of the aceticlast *Methanosaeta (Methanothrix) soehngenii* (Dong *et al.*, 1994a). Similar observations have been described for the mesophilic propionate-oxidizing bacterium *Syntrophobacter fumaroxidans* (Harmsen *et al.*, 1998). The propionate oxidation rate was higher in the presence than in the absence of *M. soehngenii*, but this methanogen could not replace *M. hungatei* (Dong *et al.*, 1994b). Calculations showed that low acetate concentrations alone could not pull propionate oxidation. *Syntrophus gentianae* degrades benzoate in syntrophy with *M. hungatei*. Acetate and methane are formed by benzoate oxidation. There exists a residual benzoate concentration, but the residual concentration can be lowered when *Methanosaeta concilii* is added (Schöcke & Schink, 1997). For the methanogenic degradation of isobutyrate, three successive conversions are performed in a triculture: isomerization to butyrate by strain WoG13, butyrate oxidation by *Syntrophomonas wolfei* and methane formation by *M. hungatei* (Matthies & Schink, 1992). Strain WoG13 grows by the fermentation of glutarate to butyrate, isobutyrate, carbon dioxide and small amounts of acetate. It also isomerizes butyrate and isobutyrate until approximately equal concentrations are reached. Growth on butyrate or isobutyrate by strain WoG13 is not possible. In the triculture, isobutyrate is completely converted via butyrate to acetate and methane. Hence, the addition of two extra species of microorganisms shifts the equilibrium of the isomerization reaction towards 100% butyrate (Matthies & Schink, 1992). *Desulfomonile tiedjei* DCB-1 grew by the conversion of 3-CB to benzoate and chloride with hydrogen or formate as an electron donor with doubling times of 45 to 46 days (Mohn & Tiedje, 1990). In a triculture with the syntrophic benzoate-degrading bacterial strain BZ-2 and the hydrogenotrophic methanogen *Methanospirillum* strain PM-1, the doubling time was 3 days (Dolfing & Tiedje, 1986). When benzoate was the substrate, benzoate oxidation was accelerated in the presence of 3-CB, because the hydrogen partial pressure decreased when dechlorination occurred (Dolfing & Tiedje, 1991). The proximity of the consortial members was important, as was demonstrated by the decreased dechlorination rate when *D. tiedjei* was separated from the strains BZ-2 and PM-1 by a membrane, permeable for (chloro-)benzoate and hydrogen (Tiedje & Stevens, 1988).

In the above mentioned examples, syntrophic conversions are made possible by two syntrophic partners, but an extra microorganism does affect the kinetics of this syntrophic conversion.

3.2 Aggregation by anaerobic microorganisms

In anaerobic (industrial) wastewater treatment reactors, methane is the end product of the biodegradation process. Such reactors are strictly anaerobic environments in which the biomass grows as aggregates. This implies that the interbacterial distances are small and selection is dependent on both adhesion properties and growth kinetics. Biofilms develop on a carrier material and/or self-aggregation takes place via flocculant to granular sludge. Granular sludge develops in particular in Upflow Anaerobic Sludge Blanket (UASB) reactors (for a review, see Lettinga, 1995). Immobilization of cells and enzymes by gel entrapment, in which cell adhesion is not required, has been most often used in the biotechnological production of certain useful compounds, like amino acids, organic acids, antibiotics, steroids, and enzymes (Cassidy *et al.*, 1996). Anaerobic degradation by gel-entrapped microorganisms may result in similar kinetics for freely suspended and immobilized microorganisms (Jones *et al.*, 1984), or may lead to significant differences between the two conditions (Dwyer *et al.*, 1986). Studies concerning aggregation in sludge granules comprise granular sludge formation by self-aggregation of defined cultures (Wu *et al.*, 1996) and incorporation of specific microorganisms in existing sludge (Ahring *et al.*, 1992).

Four different microorganisms, which together convert sucrose into methane and carbon dioxide, were immobilized in agar pellets (Jones *et al.*, 1984). *Escherichia coli* fermented sucrose to lactate, ethanol, acetate and hydrogen. *Desulfovibrio vulgaris* converted lactate and ethanol into acetate and reducing equivalents, which were partly used for sulfate reduction and released as hydrogen. *Methanosarcina barkeri* utilized acetate and

Methanobacterium formicicum utilized hydrogen and carbon dioxide. The total methane production and the methane production rate were higher when *Acetobacterium woodii* was present as well. *A. woodii* contributed to the pool of the methanogenic precursors acetate and hydrogen, since it could convert glucose or fructose as well as lactate or ethanol to acetate and hydrogen. The immobilization of this consortium in agar did not influence the total amount of methane produced nor the methane production rate (Jones *et al.*, 1984).

A phenol-degrading methanogenic enrichment consisted of a phenol-oxidizing bacterium, a hydrogen-utilizing methanogen and an acetate-utilizing methanogen (Dwyer *et al.*, 1986). These three microorganisms syntrophically degraded phenol into methane and carbon dioxide. The culture was immobilized in agar strands, containing a high biomass density (5 g protein/l gel). Phenol degradation kinetics of the immobilized consortium differed from those of non-immobilized cells, which was demonstrated by a decrease of the lag phase, of the apparent K_m, and of the methane production rate. Furthermore, the immobilization matrix protected the cells from toxic effects of phenol. Both the change in the kinetics and the protection from toxicity indicated a mass transfer limitation of phenol (Dwyer *et al.*, 1986).

Wu *et al.* (1996) were able to construct methanogenic granular sludge in a UASB reactor from five microbial species. These microorganisms degraded a mixture of acetate, propionate, and butyrate, three important intermediates in anaerobic degradation processes. The syntrophically butyrate-oxidizing bacterium strain BH aggregated with the hydrogenotrophic methanogen *M. formicicum*. The syntrophic propionate-oxidizing strain PT only aggregated when both *M. formicicum* and the aceticlast *Methanosaeta* sp. were present. This study revealed that these two methanogens were essential for granulation. The addition of the clump-forming *Methanosarcina* sp. altered the granule structure by making it more heterogeneous. The butyrate-oxidizing bacterium strain BH enhanced granulation.

Granular sludge from a UASB reactor may be applied for the degradation of specific compounds to which it has not been exposed before. Adaptation of the sludge may take a long time period, and therefore, an alternative strategy was developed by Ahring *et al.* (1992). Small (200-ml) UASB reactors were inoculated with granular sludge and *D. tiedjei*. The influent containing 3-CB was first recirculated and subsequently the flow was gradually increased. Finally, the hydraulic retention time was reduced to a value below the generation time of *D. tiedjei*, assuring that the cells were retained in the granules and did not grow in the liquid. The activity of the granules was measured and the presence of *D. tiedjei* cells inside the granules was analyzed immunologically (Ahring *et al.*, 1992). A similar approach was used with the PCP-dechlorinating bacterium *Desulfitobacterium hafniense* DCB-2, the acetate-utilizing methanogens *Methanosarcina mazeii* and *Methanosaeta concilii*, and the tetrachloroethene-dechlorinating bacterium *Dehalospirillum multivorans* (Christiansen & Ahring, 1996a, b; Schmidt & Ahring, 1997; Hörber *et al.*, 1998). Moreover, a consortium was incorporated into granular sludge. This consortium consisted of the 3-CB-dechlorinating bacterium *D. tiedjei*, the syntrophic benzoate oxidizer *Syntrophus buswellii* and the hydrogenotrophic methanogen *M. hungatei* (Ahring *et al.*, 1992). The final dechlorination rate of the sludge granules was higher when the consortium was immobilized than when only *D. tiedjei* was inoculated. These studies showed that immobilization of an extra species into granular sludge is possible, resulting in an additional degradation ability.

4. Outline of this thesis

The aim of this thesis was to investigate the biodegradation kinetics of chloroaromatic compounds by consortia under methanogenic conditions. Since such substrates can inhibit methanogenic degradation, toxicity was studied as well. Chlorinated phenols and benzoates were chosen in this study, because the biodegradation of these compounds has been examined most extensively. To gain insight in the fate of chlorinated aromatics and their effects on anaerobic treatment of industrial wastewater, sludge granules from UASB reactors were used in toxicity and enrichment studies (Chapters 2 and 3). In Chapter 2 the toxic effects of monochlorophenols on the performance of methanogenic granular sludge under different experimental conditions are described. The ability of anaerobic sludges to degrade monochlorophenols and the enrichment of 2-chlorophenol degraders are described in Chapter 3. A defined 3-chlorobenzoate-degrading consortium is characterized in Chapters 4, 5 and 6. Four microbial species were required for the complete mineralization of 3-CB to methane, carbon dioxide and chloride, namely *Desulfomonile tiedjei, Syntrophus buswellii, Methanospirillum hungatei* and *Methanosaeta concilii* (Table 3) (Ferry *et al.*, 1974; Mountfort & Bryant, 1982; Mountfort *et al.*, 1984; Patel, 1984; Shelton & Tiedje, 1984; DeWeerd *et al.*, 1990; Patel

& Sprott, 1990). Chapter 4 reports how balanced growth was obtained and describes the effects of the composition of the medium on 3-CB conversion by the consortium. Adhesion of cells to carrier materials and the growth stimulating effects of carrier materials on the members of the consortium are described in Chapter 5. Chapter 6 describes the immobilization of the consortium in a κ-carrageenan gel matrix and growth and substrate conversion of the immobilized consortium. The results presented in this thesis are summarized and discussed in Chapter 7.

Table 3. Complete methanogenic conversion of 3-chlorobenzoate by a consortium composed of four microbial species at two different pH_2 values (Stams, 1994).

Species	Conversion	ΔG' kJ/reaction	
		1 atm H_2	10^{-5} atm H_2
Desulfomonile tiedjei	$\text{3-CB}^- + H_2 \rightarrow$ benzoate$^-$ $+ H^+ + Cl^-$	-125	-96.4
Syntrophus buswellii	benzoate$^-$ $+ 7\ H_2O \rightarrow$ 3 acetate$^-$ $+ 3\ H_2 + HCO_3^- + 3\ H^+$	+58.9	-26.8
Methanospirillum hungatei	$2\ H_2 + 0.5\ HCO_3^- + 0.5\ H^+ \rightarrow 0.5\ CH_4 + 1.5\ H_2O$	-67.8	-10.7
Methanosaeta concilii	3 acetate$^-$ $+ 3\ H_2O \rightarrow 3\ CH_4 + 3\ HCO_3^-$	-93	-93
Consortium	$\text{3-CB}^- + 8.5\ H_2O \rightarrow 3.5\ CH_4 + Cl^- + 3.5\ H^+ + 3.5HCO_3^-$	-226.9	-226.9

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1. INTRODUCTION

Chapter 2

INFLUENCE OF MONOCHLOROPHENOLS ON METHANOGENIC ACTIVITY IN GRANULAR SLUDGE

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Water Research **32**, 2977-2982, 1998

ABSTRACT

The effects of monochlorophenols (MCPs) on volatile fatty acid degradation by methanogenic granular sludge from two full scale Upflow Anaerobic Sludge Blanket (UASB) reactors treating potato processing wastewater, were tested. Sludges 1 and 2 were derived from the first reactor, and sludge 3 from the second reactor. Methane production and utilization of acetate, propionate and butyrate were measured. Methane production by sludge 3 was more severely inhibited by MCPs (50% inhibition around 0.6 mM or less) than were sludges 1 and 2 (50% inhibition above 1 mM). Aceticlastic methanogens, propionate-degrading consortia and butyrate-degrading consortia of the two sludges had a different sensitivity. In sludge 3, the acetate utilization was most sensitive, while for the other sludges, butyrate utilization was inhibited most strongly. In an experiment with stored sludge, the inhibition was reversible, but depended on the exposure time. In addition, stored sludge was less active and more sensitive to MCPs compared to fresh sludge. An activation period after cold storage lowered the sensitivity of the sludge to 2-CP. These findings may be important when stored sludge is used to start up a reactor to treat wastewater that contains toxic compounds.

2. TOXICITY TESTS

INTRODUCTION

Halogenated phenolic compounds are used for wood preservation and as pesticides. They are formed during pulp bleaching and can also arise from natural sources (King, 1986; De Jong *et al.*, 1994; Rintala & Puhakka, 1994; Field *et al.*, 1995). Degradation of chlorophenols (CPs) under anaerobic conditions is well established, but because of their toxicity, CPs can severely impede anaerobic treatment of wastewater from pulp- and paper-industry (Sierra-Alvarez *et al.*, 1994).

In general, toxicity of a series of analogous phenolic compounds appears to be correlated with their hydrophobicity, which can be expressed as the 1-octanol/water partition coefficient (Sierra-Alvarez & Lettinga, 1991a). Monochlorophenols and monochloroanilines affect both the affinity constant and the maximum substrate conversion rate. Therefore, the toxic effect is most appropriately described as mixed inhibition (Davies-Venn *et al.*, 1992; Kim *et al.*, 1994). Most likely, phenolic compounds affect the functioning of the cytoplasmic membrane. The effect of aromatic compounds on membrane processes is reviewed by Sikkema *et al.* (1995).

There is some variation in the literature concerning the toxicity of halogenated aromatics to different bacterial groups. Colleran *et al.* (1992) reported that some important subpopulations in methanogenic sludges, i.e. aceticlastic methanogens, butyrate oxidizers and ethanol oxidizers, are similarly sensitive to halogenated aromatics, while hydrogenotrophs are less sensitive (Golden *et al.*, 1994; Kim *et al.*, 1996). In other studies, propionate degraders were found to be most sensitive (Johnson & Young, 1983; Wu *et al.*, 1993), whereas Kim *et al.* (1994) described that ethanol degraders are not as sensitive as aceticlastic methanogens. For a range of different types of aerobic bacteria and Clostridia, Ruckdeschel *et al.* (1987) found no significant differences between species. Furthermore, Blum and Speece (1991) reported that inhibition of aceticlastic methanogens and of aerobic heterotrophs occurs in the same order of magnitude of concentrations for many toxic organic compounds. One explanation for the variation in literature data may be that the specific growth rates or the physiological state under the experimental conditions determine the sensitivity. The physiological state of cells may affect the tolerance of an inhibitory compound, as well as the accumulation of halogenated compounds in the cell. Indications for this have been reported by several authors. Fast-growing

Pseudomonas aeruginosa cells were more inhibited by 3-CP and 4-CP than slower growing cells (Gilbert & Brown, 1978). Metabolically active cells of the alga *Chlorella* sp. accumulated more lindane than did dead cells (Hansen, 1979). High substrate levels may increase the tolerance of toxic compounds. Glucose addition decreases sensitivity to 2,4-dibromophenol in anaerobic marine sediments (King, 1986). In addition, in some cases higher phenol concentrations decrease inhibition of activated sludge by CPs (Beltrame *et al.*, 1988). Furthermore, starvation results in a higher sensitivity of anaerobic granules towards pulp wastewater (Kudo *et al.*, 1991).

The aim of our research was to study the toxicity of monochlorophenols (MCPs) on methanogenesis from acetate, propionate and butyrate by unadapted granular sludge. In addition, we studied effects of experimental conditions on the sensitivity of the sludge.

MATERIALS AND METHODS

Biomass. Methanogenic granular sludge was obtained from two Upflow Anaerobic Sludge Blanket (UASB) reactors both treating potato processing wastewater (Aviko, Steenderen, The Netherlands). During the weekends wastewater was not supplied. Instead, effluent water was recycled. Sludge was collected at different times (Table 1). Samples of reactor B consisted of smaller granules than samples from reactor A. Fresh samples of the sludges had a sludge volume index of 16 ml/g total solids. This sludge was not acclimated to MCPs. In the degradability test with sludge 3, the sludge was blended with two other types of methanogenic granular sludge, in a ratio of 1:1:1. One of these sludges was derived from a pilot plant UASB reactor, treating adsorbable organic halogens (AOX) (PAQUES, Balk, The Netherlands), the other was obtained from a full scale UASB reactor treating sugar refinery wastewater (CSM, Breda, The Netherlands).

Anaerobic toxicity assay. A medium was used as described by Holliger *et al.* (1993), with the modification that fermented yeast extract was not added. The granular sludge was preincubated at 37°C with a volatile fatty acids (VFA) mixture (acetate, propionate and butyrate; 20 mM each). When sludge 2 had been stored for 3 months, it was preincubated for 3 days. In the other experiments, sludge was preincubated for 1 day. Then, the sludge was centrifuged at 3100 x *g* for 5 minutes and rinsed with water. Sludge (2 ml, ±0.1 g of volatile suspended solids (VSS) per bottle) was transferred to 117-ml serum bottles containing 20 ml of medium. The bottles were sealed with rubber or viton stoppers and aluminum crimp seal caps. The gas phase was replaced by 1.8 bar N_2/CO_2 (80:20, v:v). All bottles were incubated with the VFA mixture at 37°C. No substrate was added in the endogenous activity controls. MCPs were added from aqueous stock solutions (20, 50 or 200 mM; pH 8-9). Each concentration was tested at least in duplicate. The gas phase was sampled 1 to 2 times per day. The endogenous methanogenic activity was subtracted in the calculation of methane production. The specific methanogenic activity was calculated from the slope of the methane production versus time curve in a period of 3 days, but when stored sludge was examined a period of 6 days was used. Activity was expressed as percentage of control activity (% ACT); the percentage of inhibition (% INH) is complementary: % INH = 100-% ACT. Acetate consumption was estimated from disappearance of acetate, taking into account the acetate formed from propionate (factor 1) and butyrate (factor 2).

The effect of exposure period to an MCP was tested with sludge 2, stored for 3 months. After 7 hours, 4 days or 7 days of incubation with substrate and MCP, sludge was centrifuged and washed twice with fresh medium at 4100 x *g* for 10 min. More than 95% of MCP was removed. Then, extra substrate was added without MCPs.

Degradability test. The same medium was used as in the anaerobic toxicity assay. Sludge 1, which had been stored for 2 months, was disintegrated under air; sludge 1, stored for 4 months, was disintegrated under a N_2/CO_2 atmosphere; and the sludge 3 blend was used as intact granules. Sludge (1-2 ml) was directly transferred to 117-ml serum bottles, containing 20 ml medium. The granular sludge was incubated at 37°C with a VFA mixture (acetate, propionate and butyrate; 5 mM each). After 1 day, one of the monochlorophenols was added in a concentration of 1 mM (sludges 1) or 0.1 mM (sludge 3 blend). Degradation of phenol (10 mM) was tested with only sludge 1, which had been stored for 2 months. Phenol was added from aqueous stock solutions (40 or 400 mM), pH 8-9.

Table 1. Characteristics of the sludges and experiments performed with the sludges.

Sludge number	Date of sampling	Reactor	Average influent-COD	Storage temperature	Storage time	Experiment type
1	November 1992	A	8	4°C	3 weeks	T
					2 months	D
					3 months	T
					4 months	D
					6 months	T
2	March 1994	A	10	10°C	-	T
					3 months	T[a]
3	October 1994	B	9	10°C	2 days	T
					3 months	D

COD: Chemical oxygen demand in g/l
T: Anaerobic toxicity assay
D: Anaerobic degradability test
[a] MCPs were added at the start of the experiment or were added later

Analyses. Methane and VFA were analyzed with a Packard-Becker 417 or Chrompack CP9001 or CP9000 gas chromatograph as described previously (Stams *et al.*, 1993). VSS was analyzed according to Dutch Standard Methods (NEN 3235 4.1). MCPs were measured with an LKB high-performance liquid chromatograph equipped with a ChromSep column (100x3.0 mm) ChromSpher Pesticides and an LKB 2158 Uvicord SD UV-detector. The mobile phase was an acetonitrile-0.01 M H_3PO_4 (20:80) mixture at a flow rate of 1 ml/min. The column was kept at room temperature. Samples (20μl) were injected by a Spectra-Physics autosampler AS1000.

RESULTS AND DISCUSSION

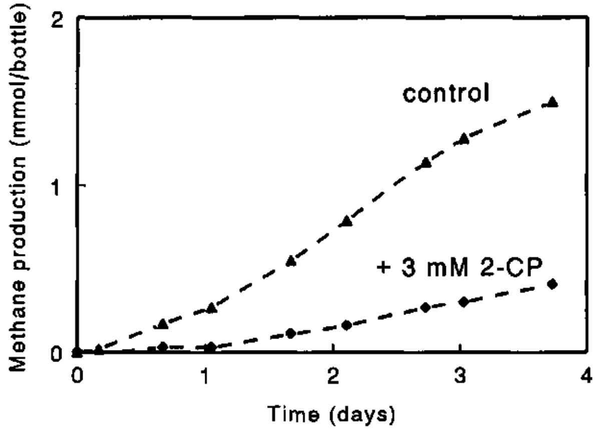

Figure 1. Methane production by fresh sludge (sludge 2) on a mixture of volatile fatty acids, with or without 2-chlorophenol.

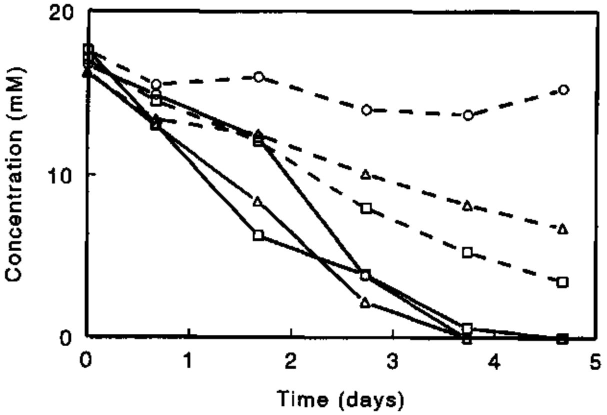

Figure 2. Utilization of volatile fatty acids by fresh sludge (sludge 2), in the presence and absence of 3 mM 2-chlorophenol. □: acetate; △: propionate; O: butyrate. —: control; - -: 3 mM 2-CP.

2. TOXICITY TESTS

Degradability of MCPs by the sludge. Phenol (10 mM) was transformed completely within 14 days of incubation, but the concentrations of the three MCPs stayed constant for 100 days in all sludge incubations. Therefore, it was concluded that, within the time course of the experiments, this sludge was not able to degrade MCPs.

Toxicity tests with fresh sludge. Methane production by granular sludge was measured in time (Figure 1). Tests were performed on a time scale of days instead of hours, because sometimes the degradation of propionate or butyrate was not maximal yet during the first two days. This is illustrated in Figure 2.

A toxicity test was performed with the three MCPs at different concentrations. The methanogenic activity of the sludge (referred to as sludge 3) was inhibited more by 3-CP and 4-CP than by 2-CP (Table 2). This order can be related to solvent/water partition coefficients (Kishino and Kobayashi, 1994). The concentration that caused 50% inhibition was around 0.6 mM for 2-CP and probably around 0.2 mM for 3-CP and 4-CP. For acetate-utilizing methanogenic granular sludges, 50% inhibition by MCPs was reported to occur at 3.2 mM 2-CP (Sierra-Alvarez & Lettinga, 1991a) and at 1.6 mM 2-CP or 4-CP (Golden *et al.*, 1994). Acetate-degrading methanogenic enrichment cultures were inhibited for 50% at 0.8 to 4.7 mM MCP (Blum & Speece, 1991; Wang *et al.*, 1991; Davies-Venn *et al.*, 1992). Apparently, sludge 3 had a very low tolerance to MCPs. Aceticlastic methanogenesis was inhibited stronger than syntrophic butyrate and propionate oxidation (Table 3).

Earlier observations in an experiment with only 2-CP with sludge 2 showed that butyrate oxidation was inhibited most strongly (Table 3). Inhibition of methanogenesis by this sludge was comparable to the inhibition observed in other methanogenic granular sludges (Sierra-Alvarez & Lettinga, 1991a; Golden *et al.*, 1994) and in methanogenic enrichment cultures (Blum & Speece, 1991; Wang *et al.*, 1991; Davies-Venn *et al.*, 1992; Kim *et al.*, 1994).

Although the two sludges were adapted to similar reactor conditions, they performed differently in the inhibition studies. This concerned the overall sensitivity as well as the relative sensitivity of several trophic groups.

Table 2. Inhibition of methanogenesis in different experiments.

Sludge	Storage time	Compound	CH_4-production mmol CH_4 g^{-1} VSS day^{-1}	% INH
1	3 weeks	control	6.5 (0.9)[a]	-
		1 mM 4-CP	5.2 (0.4)	20
1	3 months[b]	control	3.0 (0.0)	-
		1 mM 2-CP	1.4 (0.3)	54
		3 mM 2-CP	0	100
		1 mM 3-CP	0.3 (0.2)	91
		3 mM 3-CP	0	100
		3 mM 4-CP	0	100
2	-	control	6.5 (0.6)	-
		3 mM 2-CP	1.1 (0.7)	83
		5 mM 2-CP	0	100
2	3 months	control	4.2 (0.1)	-
		1 mM 2-CP	1.9 (0.3)	54
		3 mM 2-CP	0	100
3	2 days	control	6.4 (0.2)	-
		0.5 mM 2-CP	3.5 (0.1)	46
		1 mM 2-CP	2.0 (0.1)	68
		2 mM 2-CP	1.0 (0.0)	85
		3 mM 2-CP	0.05 (0.02)	99
		5 mM 2-CP	0	100
		0.5 mM 3-CP	1.7 (0.3)	73
		1 mM 3-CP	0.6 (0.2)	90
		2 mM 3-CP	0	100
		0.5 mM 4-CP	1.6 (0.1)	75
		1 mM 4-CP	0.62 (0.09)	90
		2 mM 4-CP	0	100

[a] The values in parentheses are standard deviations

[b] With sludge 1, after 3 or 6 months storage, similar results were found

Table 3. Average VFA utilization rates of 2 fresh sludges.

Sludge	Activity mmol l^{-1} g^{-1} VSS day^{-1}	Acetate	Propionate	Butyrate
2	Controls	243 (4)[a]	68 (2)	82 (3)
	3 mM 2-CP	61 (16)	29 (11)	9 (5)
3	Controls	350 (29)	81 (7)	91 (8)
	1 mM 2-CP	99 (5)	40 (0)	43 (2)
	1 mM 3-CP	32 (3)	25 (2)	19 (1)
	1 mM 4-CP	27 (2)	27 (1)	23 (1)

[a] The values in parentheses are standard deviations

The overall sensitivity, expressed as the percentage of inhibition of methanogenesis, has to be related to the characteristics of the granules. The specific methanogenic activity, the sludge volume index, and the ash content were almost equal for both sludges, but the amount of sludge per incubation was higher with sludge 3, the granules were smaller, they were collected at another time of the year and they had been stored for a short period of time (see Materials and methods). The smaller size of the granules may explain the higher sensitivity of sludge 3 in comparison with the other sludges. Smaller granules have relatively high surface areas, which may increase exposure of the bacteria to the MCPs.

Changes in the relative sensitivities of the tested trophic groups may point at a population shift or an activity change of the trophic groups. In the case of a population shift, the aceticlastic population and, to a lesser extent, the propionate-oxidizing population, may have shifted to a more sensitive population. All tested populations are interdependent, because acetate accumulation can inhibit syntrophic degradation steps and syntrophic acetogenesis determines the maximum acetate utilization rate. This implies that a slight variation in species composition can have a pronounced effect. In the case of an activity change, the physiological state of the bacteria may have influenced the tolerance of MCPs, comparable with observations of Gilbert and Brown (1978), King (1986) and Beltrame *et al.* (1988). Disappearance of acetate from the medium was usually more rapid than disappearance of propionate and much more rapid than disappearance of butyrate, except for sludge 3. On the other hand, when calculated activities of VFA degradation are compared, the differences are not so obvious. Activities on each volatile fatty acid seemed to be somewhat higher with sludge 3 than with sludge 2. Particularly activity changes can be expected when the influent of the reactors varies. However, the influent was rather constant. So it seems more likely that in both reactors a slightly different population had developed. In any case, it is not possible to determine which of the tested trophic groups should be considered as the most sensitive group towards MCPs.

Sensitivity of fresh and stored granular sludge. The activity of fresh and stored sludge (sludge 2), collected at the same time from the UASB reactor, was compared in the absence and presence of 2-CP. Stored sludge had a lower control methanogenic activity and exhibited a higher sensitivity to 2-CP, expressed as percentage inhibition (Table 2). The test with stored sludge was also performed in a slightly different way (data not shown). In that case, 2-CP was added after 3 days of incubation, while usually 2-CP was added immediately. For 2, 3 or 4 mM 2-CP % INH were respectively 40%, 65% and 96%. Addition of 1 mM 2-CP after 2 or more days of incubation with VFA, did not affect methane production significantly. Thus, the stored sludge became less sensitive to 2-CP than fresh sludge, exposed immediately to 2-CP.

A decrease of methanogenic activity with a VFA mixture after cold storage of granular sludge has been documented before (Shin *et al.*, 1993; Wu *et al.*, 1995). The lower tolerance to toxicants seems to be correlated to the lower activity, but it is not a consequence of the lower activity, since this possibility was excluded in the toxicity tests with fresh sludge. Both the decrease in activity and the increase in sensitivity may be caused by disintegration of the sludge structure. The spatial orientation which is needed for syntrophic degradation might have been disrupted and the surface exposed to the toxicant might have increased as well. An activation period increased the tolerance of stored sludge significantly. In such a period bacteria may generate sufficient metabolic energy to prevent toxic effects. Other studies showed that non-limiting amounts of an energy source can decrease the toxicity of compounds (Gilbert & Brown, 1978; King, 1986; Kudo *et al.*, 1991; Mohanty *et al.*, 1993).

Table 4. Second feeding experiment with stored sludge (sludge 2), fed with butyrate and exposed to 2-CP.

2-CP concentration	Duration of first feeding	% ACT after first feeding	% ACT after second feeding
3 mM	7 hours	n.c.[a]	90 (15)[b]
3 mM	4 days	0	2 (0)
1 mM	7 days	17 (2)	89 (2)
3 mM	7 days	2 (1)	5 (1)
5 mM	7 days	0	0

[a] n.c.: not calculated
[b] The values in parentheses are standard deviations

Effect of exposure time on inhibition. Toxicity may be reversible, but it also may become more severe when the toxicant has been removed and fresh substrate is added (Sierra-Alvarez & Lettinga, 1991b). Therefore, effects of exposure time were studied. Because in earlier experiments (Table 3) mainly butyrate oxidation was influenced by MCPs, these tests were only done with butyrate as substrate and 2-CP as toxicant (Table 4). In these incubations, there was hardly any acetate accumulation (data not shown). So methane production is directly related to butyrate oxidation. A second feeding was given: when (1) methane production had not started yet (7 hours), (2) the sludge was actively converting butyrate (4 days) or (3) butyrate had been converted completely (7 days). Methane production rates increased with time due to growth. Therefore, the methane production after the second butyrate feeding was expressed as percentage of the control activity after the second feeding. Restoration of activity depended on the concentration of 2-CP. Exposure to 1 mM 2-CP for 7 days hardly led to prolonged inhibitory effects. For 3 mM 2-CP, only bottles with a very short exposure time restored activity within the time that the controls were active. For many toxic compounds, recovery of activity occurs when either the exposure time is short enough or the concentration is low enough (Parkin *et al.*, 1983; Yang & Speece, 1985). Data from this study with granular sludge exposed to 2-CP are accordingly.

CONCLUSIONS

1. In accordance with other authors, we found that the isomer and concentration of the chlorophenol as well as the exposure time determine the toxicity of a chlorophenol. 3-CP and 4-CP were more toxic to the sludges than 2-CP was.
2. In addition to a lowered activity, as was described by others, storage of sludge also resulted in a higher sensitivity to 2-CP. However, a reactivation period of 2 to 3 days, preceding the exposure to 2-CP, resulted in a lower sensitivity to 2-CP.
3. When two reactors received the same wastewater, the sludges that developed had similar activities on the available substrates like volatile fatty acids. However, they did not exhibit similar sensitivity to chlorophenols to which they had never been exposed before. Therefore, toxicity data are limited in practical application.

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Chapter 3

REDUCTIVE DECHLORINATION OF 2-CHLOROPHENOL BY ENRICHMENT CULTURES

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ABSTRACT

Samples from anaerobic environments were studied for their ability to transform monochlorophenols under methanogenic conditions. Sludges from laboratory scale reactors adapted to either pentachlorophenol (PCP), benzoate or terephthalate, a peat slurry, and a mixture of polluted sediments were able to transform 2-chlorophenol (2-CP) under methanogenic conditions. In the sludges, reductive dechlorination of 2-CP to phenol occurred. In the peat slurry and in the sediment mixture, 2-CP was transformed to a product which is likely to be 3-chlorobenzoate (3-CB). The PCP-adapted sludge was enriched further by repeated transfer in media containing 2-CP. This resulted in a culture that reductively dechlorinated 2-CP to phenol and carboxylated and dehydroxylated 2-CP to 3-CB. Similarly, a culture, enriched from benzoate-adapted sludge, was obtained that dechlorinated 2-CP and dichlorophenols specifically at the *ortho*-position.

3. CHLOROPHENOL DEGRADATION

INTRODUCTION

Chlorophenolic compounds have been spread in the environment from several industrial sources, namely wood preservation, pulp bleaching effluents, and pesticide use, and by natural production (Field *et al.*, 1995). Chlorophenols are toxic but biologically degradable under aerobic and anaerobic conditions (Häggblom, 1992). In general, the anaerobic degradation of chlorophenols starts with a reductive dechlorination step (Mohn & Tiedje, 1992). Degradation has been reported for all three monochlorophenols (MCPs) in anaerobic sludges, sediments, and soils (Mohn & Tiedje, 1992). Cultures enriched for MCP-dechlorinating ability under methanogenic conditions have been described for 2-CP (Genthner *et al.*, 1989; Dietrich & Winter, 1990; Basu *et al.*, 1996; Themel *et al.*, 1996) and 3-CP (Genthner *et al.*, 1989). A culture which is able to dechlorinate 4-CP could only be maintained in the presence of sediment (Zhang & Wiegel, 1990). It appeared that sludges adapted to MCPs were able to dechlorinate higher chlorinated phenols as well (Boyd & Shelton, 1984; Mikesell & Boyd, 1986; Krumme & Boyd, 1988; Kong *et al.*, 1994).

Several pure cultures have been described which reductively dechlorinate *ortho*-chlorinated phenols under methanogenic conditions (Cole *et al.*, 1994; Utkin *et al.*, 1994; Bouchard *et al.*, 1996; Christiansen & Ahring, 1996; Gerritse *et al.*, 1996; Sanford *et al.*, 1996). One of the isolates, strain 2CP-1, belongs to the Cystobacteriaceae. Strain 2CP-1 utilizes a limited number of electron acceptors, namely 2-CP, 2,6-dichlorophenol (2,6-DCP), oxygen and fumarate, and it also dechlorinates 2,5-DCP (Cole *et al.*, 1994). The other species known up to now all belong to the genus *Desulfitobacterium*. *Desulfitobacterium* species are more versatile in electron acceptor utilization (Sanford *et al.*, 1996; Bouchard *et al.*, 1996). *D. dehalogenans* and *Desulfitobacterium* strain PCE1 are able to dechlorinate 2-CP (Utkin *et al.*, 1995; Gerritse *et al.*, 1996).

In this study, samples from methanogenic environments adapted to aromatic compounds, were investigated for their potential to transform MCPs. Furthermore, the metabolic capabilities of enrichments on 2-CP were characterized. One enrichment transformed 2-CP to phenol and 3-chlorobenzoate. The other enrichment has a specifically *ortho*-dechlorinating activity.

MATERIALS AND METHODS

Biological material. Pentachlorophenol(PCP)-degrading methanogenic granular sludge (sludge P) was obtained from a laboratory scale Upflow Anaerobic Sludge Blanket (UASB) reactor completely dechlorinating 6 mg/l PCP (PAQUES, Balk, The Netherlands). Benzoate-degrading methanogenic granular sludge (sludge B) was obtained from a 1-L scale UASB reactor, loaded with 0.17 mol benzoate l^{-1} day^{-1}. Terephthalate-degrading methanogenic flocculent sludge (sludge T) was obtained from a 4 l hybrid reactor. A mixture of polluted samples, derived from aerobic and anaerobic sludges and sediments which had been stored for 1 year at 4°C, was used. A peat slurry was used, which had been prepared in the following way. The first 5 cm of drained fertilized clay-containing peat, with 0.2 kg C kg^{-1}, was suspended with 3 parts of anaerobic distilled water under a nitrogen atmosphere, and incubated at 15°C. The biological materials were stored at 4°C.

Degradability test. Sludges B and P were homogenized with a mortar and pestle. The sludges, the sediment mixture and the peat slurry were transferred to 117-ml serum bottles containing 20 ml of medium. This medium was the same as described by Holliger *et al.* (1993), with the modification that fermented yeast extract was not added. A 5% (v/v) inoculum was used, but a 10% inoculum was applied for sludge P. The bottles were sealed with viton stoppers and aluminum crimp caps. The gas phase was replaced by 1.8 bar N_2/CO_2 (80:20, v:v). All bottles were incubated at 37°C with a volatile fatty acids (VFA) mixture (acetate, propionate and butyrate; 5 mM each). After a few days, 2-CP, 3-CP or 4-CP (Sigma Aldrich, Zwijndrecht, The Netherlands) was added in a concentration of 1 mM for sludge P and 0.1 mM for all other incubations. The choice for a preincubation period without CPs was based on the observation that reactivation after cold storage could reduce the toxic effect of CPs. For more details about this reactivation, the reader is referred to Chapter 2.

Experiments with culture P. Culture P, obtained from the incubation with sludge P, was transferred (10%) to fresh medium containing 1 or 3 mM 2-CP and a VFA mixture (acetate, propionate and butyrate; 5 mM each). Other electron donors were tested as well (concentrations in mM in parentheses): formate (5), glucose (5), pyruvate (20), acetate (5), propionate (5), butyrate (5), and 1.8 bar

H_2/CO_2 (80:20, v:v). In addition, the effect of 2-bromoethanesulfonic acid (BrES, 6 to 10 mM) and the effect of pasteurization (80°C for 1 h) on dechlorination were tested. From the second until the fifth transfer 0.2 g/l yeast extract and after that 5 g/l yeast extract or 0.5% (v/v) fermented yeast extract was added (Holliger *et al.*, 1993). Several medium supplements were tried instead of yeast extract: 1,4-naphthoquinone (0.2 mg/l), autoclaved granular sludge (10% v/v, Aviko, Steenderen, The Netherlands), rumen fluid (5% v/v). The following electron acceptors were added (concentrations in mM in parentheses): SO_4^{2-} (2 or 20), NO_3^- (3 or 30), 2,6-dichlorophenol (2,6-DCP, 0.1), 2,4,6-trichlorophenol (0.1), 3-CP (0.1), 4-CP (0.1). These electron acceptors were tested in the presence of an H_2/CO_2 atmosphere, fermented yeast extract and BrES. Both 3-CP and 4-CP were tested in the presence of 2-CP (1 mM). Attempts to isolate the dechlorinating bacteria were done by incubation in agar roll tubes.

Experiments with culture B. Culture B, obtained from sludge B, was transferred (maximally 1%) to fresh medium containing 0.1 or 0.5 mM 2-CP and 1 to 5 mM pyruvate. The following electron donors were tested: pyruvate, lactate, ethanol, formate, acetate, propionate, butyrate, benzoate, glucose, in a concentration of 5 mM, a VFA mixture (acetate, propionate and butyrate; 5 mM each), 1 mM phenol, and 1.8 bar H_2/CO_2 (80:20, v:v). The electron acceptor utilization was tested with pyruvate (20 mM) as electron donor for the following electron acceptors (concentrations in mM in parentheses): 2-CP (0.1) plus 3-CP (0.1), 2-CP (0.1) plus 4-CP (0.1), 2,3-DCP (0.1), 2,4-DCP (0.1), 2,5-DCP (0.1), 2,6-DCP (0.1), NO_3^- (30), fumarate (10), SO_4^{2-} (20), $S_2O_3^{2-}$ (20), SO_3^{2-} (20), 3-chloro-4-hydroxyphenylacetate (3-Cl-4-OHPA, 20). Utilization of 3-chloro-4-hydroxybenzoate (3-Cl-4-OHB, 1 mM) was tested with 5 mM pyruvate. The effect of BrES (10 mM), MoO_4^{2-} (0.5 or 10 mM) and the effect of pasteurization (80°C for 1 h) on dechlorination were tested. In a few incubations, yeast extract (0.5 g/l), fermented yeast extract (1% v/v), or trypticase (0.5 g/l) was added. To a dilution series of the ninth transfer 2-CP (1 mM) and glucose or pyruvate (2.5 mM) were added.

Analyses. Methane, hydrogen, and VFA were analyzed with a Packard-Becker 417 or Chrompack CP9001 or CP9000 gas chromatograph as described previously (Stams *et al.*, 1993). Glucose and organic acids were analyzed on an LKB high-performance liquid chromatograph as described previously (Stams *et* *al.*, 1993). Chlorophenolic compounds were measured as described in Chapter 2 or on a TSP high-performance liquid chromatograph, with a SpectraSystem P2000 pump, an AS3000 autosampler and a UV1000 UV-detector. When the TSP HPLC was used, the mobile phase was an acetonitrile-0.01 M H_3PO_4 mixture with a volume ratio of 15:85. Sulfide was determined colorimetrically (Trüper & Schlegel, 1964). N_2O was measured with a gas chromatograph as described previously (Scholten & Stams, 1995). SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, NO_3^-, and NO_2^- were analyzed with a high-performance liquid chromatograph (Scholten & Stams, 1995). NO_3^- was also determined colorimetrically in 0.05 M perchloric acid at a wavelength of 210 nm. In some incubations, a product was formed that coeluted with 3-chlorobenzoate on HPLC. Further identification of this product was done with gas chromatography-mass spectrometry (GC-MS). Supernatant of a 1.2-ml sample was mixed with 2 ml methanol and 0.2 ml H_2SO_4 for methylation. This mixture was incubated at 30°C for 30 minutes. Then, 1 ml chloroform and 2 ml water were added to each tube for extraction. The chloroform extracts were analyzed by GC-MS as described previously for hexachlorocyclohexane (Middeldorp *et al.*, 1996).

RESULTS

Transformation of MCPs by samples from anaerobic environments. The ability to transform MCPs was investigated in samples from different anaerobic environments. Methanogenic granular sludges which had not been adapted to aromatic compounds were not able to utilize MCPs, and neither was a granular sludge adapted to adsorbable organic halogens (AOX) (Chapter 2). Here, samples from environments containing aromatic compounds were investigated. In summary, in all samples, 2-CP was transformed (Figure 1A-E). 3-CP disappearance was measured only in the sediment mixture (Figure 1F). In none of the samples 4-CP transformation was established within 120 days. Products from MCP transformation were measured (Figure 1). With 3-CP, methane was formed from a volatile fatty acids (VFA) mixture, but the 3-CP transformation was not reproducible and could not be enriched for. From 2-CP, sludges P and B produced phenol, while sediment and peat incubations formed a product coeluting on HPLC with 3-chlorobenzoate. Since the 2-CP transformation rate was highest in sludges P and B, these sludges were used for further enrichments on 2-CP. These enrichments will be discussed below.

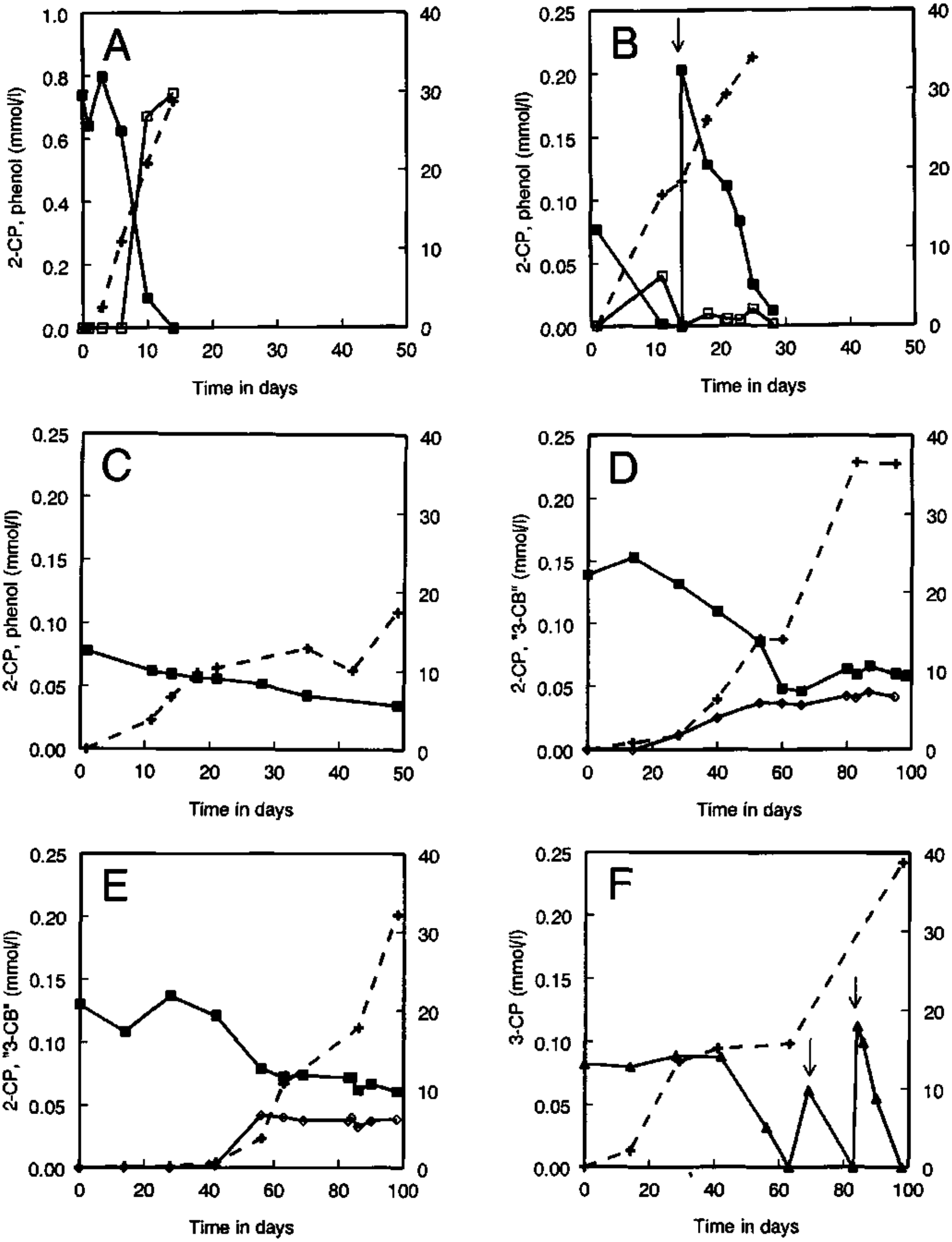

Figure 1. Transformation of 2-CP (■) or 3-CP (▲) and formation of phenol(□), 3-chlorobenzoate (◆) and methane (+) in samples from sludges P (A), B (B) and T (C), from a peat slurry (D) and from a polluted sediment mixture (E, F). Right y-axis: methane (mmol/l). In figure 1B and 1F, CP was repeatedly added.

Enrichment from pentachlorophenol-adapted sludge. Culture P degraded 2-CP in the presence of several electron donors (Table 1). The 2-CP degradation rate was highest with glucose. The dechlorination rate in the original sludge P was about 0.1 mmol l^{-1} day^{-1} (Figure 1). The enrichment degraded 2-CP at a lower rate (Table 1). Another difference with the original sludge was that the enrichment did not accumulate phenol. Culture P could degrade 2-CP at a high concentration, up to 5 mM. The dechlorinating activity was *ortho*-specific (Table 2).

Table 1. Transformation of 2-CP [μmol l^{-1} day^{-1}] by culture P in the presence of different electron donors. The numbers in parentheses are standard deviations. After the second transfer, an initial rapid dechlorination (phase 1) was followed by a slower dechlorination, when hydrogen, formate or no electron donor was added (phase 2).

	Dechlorination rate		
Electron donor	Second transfer	Fourth transfer	Eighth transfer
-	25 (8)[a], 7 (2)[b]		
Hydrogen	42 (13)[a], 13 (1)[b]		29 (1)
Formate	39 (6)[a], 6 (1)[b]		
VFA	30 (10)		
Acetate			32 (2)
Propionate			34 (3)
Butyrate			31 (3)
Glucose	55 (7)		
Pyruvate		16 (1)	

[a], phase 1, day 4 to 15, and [b], phase 2, day 15 to 77.

A complex substrate, e.g. yeast extract, improved the 2-CP degradation rate. Autoclaved granular sludge, rumen fluid, and fermented yeast extract could replace yeast extract in this respect. Naphthoquinone, an essential vitamin for dechlorination by *Desulfomonile tiedjei* (DeWeerd *et al.*, 1990), completely inhibited the degradation of 2-CP. BrES, an inhibitor of methanogenesis, hardly affected the 2-CP degradation rate, but it was necessary for the further enrichment of the 2-CP-degrading bacteria. The degradation of 2-CP stopped after pasteurization. Some incubations were done with nitrate or sulfate as electron acceptor. After the reduction of all nitrate or sulfate, the cultures were transferred back to fresh medium containing 2-CP. It appeared that after one transfer without 2-CP the ability to degrade 2-CP was lost.

Table 2. Electron acceptor utilization by culture P.

Electron acceptor	Utilization	Product
2-CP plus 3-CP	2-CP	phenol, 3-CB
2-CP plus 4-CP	2-CP	phenol, 3-CB
2,6-DCP	yes	phenol
2,4,6-DCP	no	-

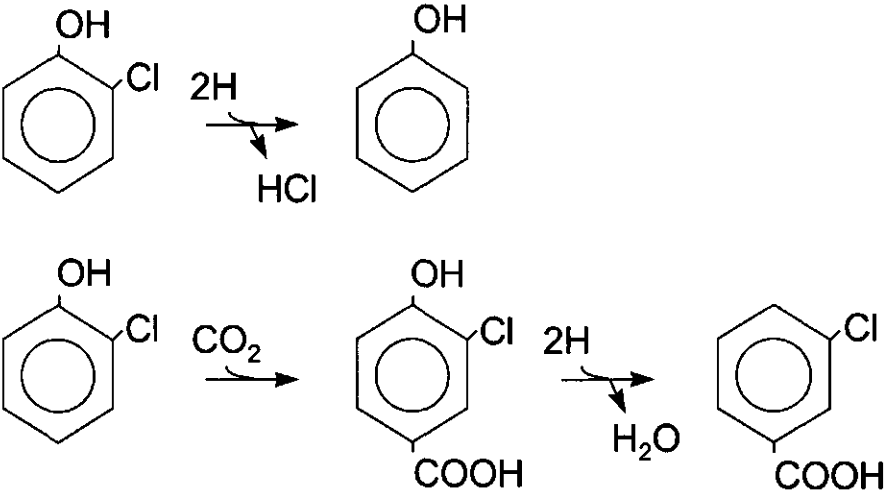

Figure 2. Observed transformation mechanisms of 2-chlorophenol to phenol or 3-chlorobenzoate

In addition to the ability to dechlorinate 2-CP to phenol, the culture was able to convert 2-CP to 3-chlorobenzoate. In the incubations of the tenth transfer, virtually all 2-CP was transformed to a product that coeluted with 3-chlorobenzoate on HPLC. A methylated sample from this incubation was analyzed on a gas chromatograph equipped with a mass spectrometer. This analysis confirmed the presence of a monochlorobenzoate. The 3-chlorobenzoate was most probably the product of a carboxylation and dehydroxylation of 2-CP (Figure 2). Unfortunately, attempts to isolate a 2-CP degrader failed and the 2-CP degrading activity was finally lost upon repeated transfer.

Enrichment from benzoate-adapted sludge. Benzoate-adapted sludge formed methane from 2-CP and a VFA mixture. After one transfer, phenol and benzoate accumulated transiently to high concentrations. The dechlorination rates were 11, 19, and 31 μmol l^{-1} day^{-1}, with the electron donors VFA, pyruvate, and hydrogen, respectively. From the second transfer only phenol was detected as a product (Figure 3). Analysis of samples from culture B established that no 3-chlorobenzoate was produced. In general, the dechlorination rate did not exceed 30 μmol l^{-1} day^{-1}.

The maximal 2-CP concentration added to culture B was 1.4 mM. Several electron donors were tested (Table 3). First, hydrogen, formate, VFA and ethanol seemed the most favourable electron donors, but later the dechlorination rate appeared to be higher in the presence of glucose, pyruvate and acetate. Therefore, the transfers were continued with pyruvate as an electron donor. In addition to 2-CP, 2,4-dichlorophenol (2,4-DCP) and 2,6-DCP were dechlorinated at the *ortho*-position (Table 4).

Since the determined dechlorination rates were often much lower in later than in the initial transfers, attempts were done to improve the conversion. Incubations supplied with yeast extract dechlorinated 1.4 times faster than incubations without yeast extract. At 30°C the dechlorination rate was lower than at 37°C, which was the usual incubation temperature. Furthermore, culture B was incubated with alternative electron acceptors (Table 4). Fumarate, nitrate, sulfate, sulfite and thiosulfate were reduced by culture B. In case of nitrate, nitrite accumulated to a high concentration. The cultures were transferred with these electron acceptors two times. After two transfers in the presence of fumarate, nitrate, or sulfate, and in the absence of 2-CP, the dechlorinating ability was lost, while after two transfers in the presence of thiosulfate and sulfite, the dechlorinating ability returned. The culture which had been transferred with thiosulfate instead of 2-CP, afterwards dechlorinated 2-CP after a long lag-phase. After alternating transfers in the presence of sulfite or 2-CP, the dechlorinating activity was lost.

Dechlorination continued in the presence of BrES and molybdate. This is in accordance with the findings that sulfate was not utilized by the dechlorinating bacteria (Table 4) and that no methane was produced. After pasteurization no dechlorination occurred.

In the dilution series, only up to 10^{-3} diluted cultures dechlorinated 2-CP with glucose. The 10^{-5} dilution did not exhibit dechlorinating activity and neither did it show growth on glucose. In the series with pyruvate, the results were similar. Since culture B contained several morphologically different microorganisms, no further attempts were made to isolate the dechlorinating bacteria.

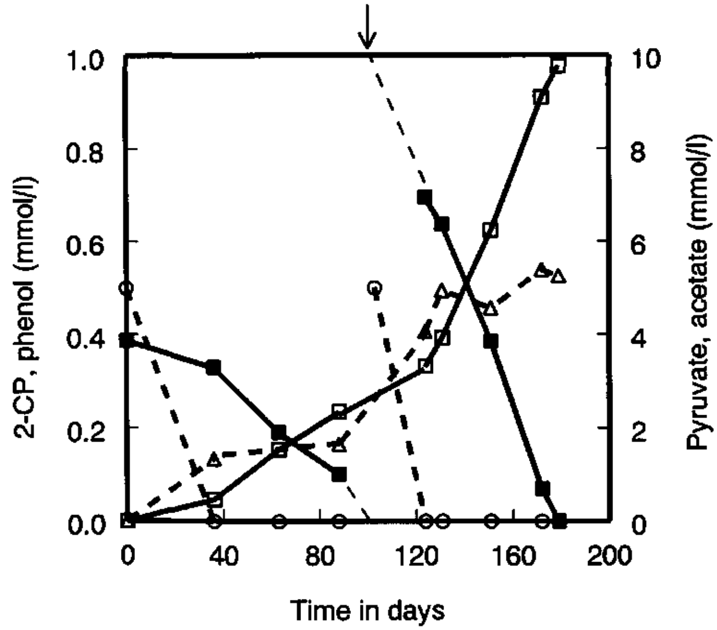

Figure 3. Reductive dechlorination of 2-CP (■), utilization of pyruvate (O), and formation of phenol (□) and acetate (x) by culture B. CP was repeatedly added.

Table 3. Dechlorination of 2-CP by culture B in the presence of different electron donors. BrES was added to the incubations with formate and hydrogen.

	Dechlorination		Electron donor utilization	
Electron donor	Third transfer[a]	Sixth transfer	Third transfer[b]	Sixth transfer[c]
Phenol	-		no	
Benzoate	-	1.0	n.m.[d]	no
Glucose	±	4.6	yes	yes
Pyruvate	+	2.3	yes	yes
Lactate	+	1.8	yes	yes
Formate, acetate, yeast extract	+ +[e]		yes	
Formate		1.1		yes
Hydrogen, acetate, yeast extract	+ +[e]		yes	
Hydrogen, yeast extract	+ +[e]	0.3	yes	n.c.[f]
Hydrogen, acetate	-		yes	
Ethanol	+ +	1.4	yes	yes
Acetate	±	2.2	n.c.	n.c.
Propionate		0.8		n.c.
Butyrate		1.4		no
VFA	+ +		n.c.	

[a], third transfer, product formation after 3 weeks. -, no product, ±, less than 0.015 mM, +, 0.015 to 0.05 mM, + +, 0.05 to 0.1 mM.

[b], after 3 weeks.

[c], after 8 weeks.

[d], n.m., not measured.

[e], the measured product is benzoate and not phenol.

[f], n.c., not clear.

Table 4. Electron acceptor utilization by culture B. After two transfers with sulfuroxy anions, fumarate or nitrate (1% inoculum), the cultures were transferred to medium containing 2-CP.

Electron acceptor	Utilization	Products	Dechlorination after 2 transfers with electron acceptor
2-CP plus 3-CP	2-CP	phenol	
2-CP plus 4-CP	2-CP	phenol	
2,4-DCP	yes	4-CP	
2,6-DCP	yes	2-CP, phenol	
2,3-DCP	no		
2,5-DCP	no		
3-Cl-4-OHPA	no		
3-Cl-4-OHB	no		
NO_3^-	yes	NO_2^-; no N_2O [a]	-
Fumarate	yes	succinate	-
SO_4^{2-}	yes	[b]	-
$S_2O_3^{2-}$	yes	[b]	+
SO_3^{2-}	yes	$S_2O_3^{2-}$; SO_4^{2-} [b]	+

[a], not tested for N_2-production.
[b], not tested for S^{2-}-production.

DISCUSSION

The ability to dechlorinate monochlorophenols is present in sludge which was adapted to other aromatic compounds, namely PCP, benzoate or terephthalate, and in polluted sediment. The dechlorinating ability was not observed in unadapted sludge, nor in sludge adapted to adsorbable organic halogens (AOX) (Chapter 2). Possibly, the incubation period of about 120 days was not long enough for detectable degradation of all the MCPs, because it may take several months before detectable degradation starts (Mohn & Tiedje, 1992; Häggblom *et al.*, 1993; Kong *et al.*, 1994; Basu *et al.*, 1996).

Except for 3-CP degradation in a sediment mixture, in this study the dechlorinating ability is restricted to dechlorination of the *ortho*-positioned chlorine atom. This is in accordance with the observations of a dominance of *ortho*-dechlorinating microorganisms in acclimated sludges and a natural adaptation to *ortho*-chlorinated phenols (Field *et al.*, 1995). The preference for *ortho*-dechlorination is explained by the carbon-chlorine bond charge, which is most negative at the *ortho*-position (Cozza & Woods, 1992). The opposite preference, namely a specificity for *meta*- and *para*-dechlorination, has been observed for chemical CP dechlorination by vitamin B_{12} (Smith & Woods, 1994). These authors proposed that the reaction mechanism was a nucleophilic aromatic substitution, which could explain the preference for *meta*- and *para*-dechlorination, because nucleophilic substitution occurs at the position on the ring with the largest positive charge. Alternatively, Dolfing and Beurskens (1995) proposed that the redox potential of the redox couples resulted in this regiospecificity, because a higher redox potential of the redox couple implies a larger net energy gain.

In this study, the transformation of 2-CP to 3-CB was observed in some of the incubations, namely in peat slurry, a sediment mixture, and in culture P. Bisaillon *et al.* (1993) described the transformation of 2-CP to 3-CB as being a cometabolic conversion of microorganisms that carboxylate and dehydroxylate phenol, in a culture enriched on phenol. *Ortho*-substituted phenols were transformed to *meta*-substituted benzoates and this transformation was induced by phenol (Bisaillon *et al.*, 1993). In culture P, carboxylation-dehydroxylation became eventually the most important transformation of 2-CP. However, if 2,6-DCP was the substrate, phenol but no 3-CB was produced. Biodegradation of chlorinated phenolic compounds has been observed more frequently to follow multiple pathways under methanogenic conditions. Although unacclimated anaerobic digester sludge dechlorinated PCP only at the *ortho*-position, PCP dechlorination by acclimated sludge starts at the *ortho*, *meta*, or *para* position (Nicholson *et al.*, 1992). 2,4,5-Trichlorophenoxyacetate is first converted to 2,4,5-trichlorophenol and probably acetate by a reduction of the aryl ether bond in anaerobic digester sludge, while in a pond sediment and methanogenic aquifer material it is first transformed to a dichlorophenoxyacetate (Gibson & Suflita, 1986, 1993). In a non-adapted pond sediment, 3-chloro-4-hydroxybenzoate is either first decarboxylated and then dechlorinated or vice versa (Zhang & Wiegel, 1992). Both 2-CP and 4-hydroxybenzoate are formed. In the presence of acetate, decarboxylation of 3-Cl-4-OHB to 2-CP was the predominant first degradation step, and 2-CP and benzoate accumulated transiently. As has been shown by Zhang and Wiegel (1992), in bioremediation an undesired transformation may be favoured if the conditions are not chosen correctly. In the samples described in this chapter, the 3-CB which was formed from 2-CP accumulated. However, 3-CB is expected to be mineralized in methanogenic environments, since at least one microorganism, *Desulfomonile tiedjei*, reductively dechlorinates 3-CB under methanogenic conditions (DeWeerd *et al.*, 1990). In Chapters 4, 5, and 6, the mineralization of 3-CB by a methanogenic consortium, containing *Desulfomonile tiedjei*, will be discussed.

The rates of 2-CP transformation in cultures P and B were low compared to the enrichment of Dietrich and Winter (1990) and strain 2CP-1 (Cole *et al.*, 1994), because the enrichment of Dietrich and Winter (1990) had a high dechlorination rate (about 1 mmol l^{-1} day^{-1}) and tolerated a high concentration of 2-CP (10 mM), and strain 2CP-1 (10 μg protein ml^{-1}) converted 2 mmol g $protein^{-1}$ h^{-1}, which is approximately 0.5 mmol l^{-1} day^{-1} (Cole *et al.*, 1994). However, culture B has a higher 2-CP tolerance than strain 2CP-1; complete inhibition of dechlorination by 2CP-1 occurs at about 1 mM 2-CP (unpublished results). The dechlorination rate of culture B is comparable to that of another enrichment (Basu *et al.*, 1996).

The dechlorinating microorganisms in culture P were no methanogens and did not form spores. The dechlorinating bacteria did not utilize sulfate and nitrate, indicating that the dechlorinators were no sulfate or nitrate reducers.

The dechlorinating microorganisms in culture B are no methanogens and do not form spores. They do not utilize sulfate, nitrate nor fumarate. There are indications that they are able to utilize sulfite and thiosulfate, but cannot compete with other microorganisms for these electron acceptors. First thiosulfate and later sulfate were formed from sulfite. Thiosulfate may have been formed by a chemical reaction of sulfite with sulfide. The formation of sulfate from sulfite or thiosulfate has been described for *Desulfovibrio sulfodismutans, D. vulgaris, Desulfobacter curvatus* and *D. tiedjei* (Bak & Pfennig, 1987; Mohn & Tiedje, 1990). These microorganisms disproportionate sulfite or thiosulfate to sulfate and sulfide. 3-Cl-4-OHPA and 3-Cl-4-OHB are structural analogs of 2-CP, which are not dechlorinated by culture B, although other *ortho* and *meta* dechlorinators utilize one or both of these chlorophenolic compounds. 3-Cl-4-OHB is dechlorinated in a freshwater sediment (Zhang & Wiegel, 1992) and by *Desulfomonile tiedjei* and *Desulfitobacterium* *chlororespirans* (DeWeerd & Suflita, 1990; Sanford *et al.*, 1996). 3-Cl-4-OHPA is utilized as an electron acceptor by most *Desulfitobacterium* species described up to now (Utkin *et al.*, 1994; Christiansen & Ahring, 1996; Gerritse *et al.*, 1996; Sanford *et al.*, 1996). Since 3-Cl-4-OHPA is hardly toxic, it allows high growth rates by these dechlorinating microorganisms.

In conclusion, our experiments confirm other observations that 2-CP degradation is more common than 3-CP and 4-CP degradation. Furthermore, in addition to reductive dechlorination of 2-CP, carboxylation-dehydroxylation of 2-CP to 3-CB could be an important transformation of 2-CP in methanogenic environments. Moreover, a culture could be enriched from benzoate-degrading granular sludge, of which the dechlorination activity was directed to the *ortho*-position of chlorophenols.

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3. CHLOROPHENOL DEGRADATION

Chapter 4

CONSTRUCTION AND GROWTH OF A 3-CHLOROBENZOATE-MINERALIZING METHANOGENIC CONSORTIUM CONSISTING OF FOUR MICROBIAL SPECIES

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ABSTRACT

To study the complete degradation of 3-chlorobenzoate (3-CB) under methanogenic conditions, a consortium was composed of the following four microorganisms. *Desulfomonile tiedjei* reductively dechlorinated 3-CB to benzoate, *Syntrophus buswellii* oxidized benzoate syntrophically to acetate and hydrogen, *Methanospirillum hungatei* formed methane from hydrogen and carbon dioxide, and *Methanosaeta concilii* converted acetate to methane and carbon dioxide. We investigated under which conditions dechlorination and mineralization can take place. For the balanced growth of the four microorganisms, the growth medium had to be optimized and initially supplied with an electron donor (e.g. pyruvate). Chlorobenzoates but not chlorophenols were dechlorinated by the consortium, although these were known to be utilized by *D. tiedjei* in pure culture. Furthermore, the toxicity of 3-CB decreased its mineralization rate, because the applied concentrations of 3-CB (up to 5 mM) were inhibiting *S. buswellii* and the methanogens, especially *M. concilii*. Omission of the bicarbonate buffer had a positive effect on methane production and a negative effect on dechlorination. Based on cell counts it could be calculated that a stable coculture contained approximately 18% *D. tiedjei*, 70% *S. buswellii*, 10% *M. hungatei*, and 3% *M. concilii*.

INTRODUCTION

Chlorinated benzoates are present in the environment as a result of their use as herbicides (Dolfing & Beurskens, 1995), and as products from aerobic degradation of polychlorinated biphenyls (Flanagan & May, 1993). Microbial degradation of chlorobenzoates takes place under aerobic as well as under anaerobic conditions. In the latter case, reductive dechlorination is often the first step in the breakdown of chlorobenzoates (Commandeur & Parsons, 1990). The microorganisms may benefit from the dechlorination of the chlorinated compound if it serves as a terminal electron acceptor (Mohn & Tiedje, 1992).

Desulfomonile tiedjei was the first isolated bacterium able to dechlorinate chlorobenzoates reductively under methanogenic conditions (DeWeerd *et al.*, 1990). This bacterium was derived from a consortium degrading 3-chlorobenzoate (3-CB), in which three organisms were required for the breakdown of this chlorobenzoate (Shelton & Tiedje, 1984; Dolfing & Tiedje, 1986). These three organisms, *D. tiedjei*, *Methanospirillum* PM-1, and strain BZ-2, a syntrophic benzoate oxidizer, were extensively studied as a triculture (reviewed by Mohn and Tiedje, 1992). This coculture, consisting of one hydrogen producer and two hydrogen consumers, converted 3-CB into methane, acetate, and chloride. A similar conversion was realized by a triculture consisting of *D. tiedjei*, *Syntrophus buswellii* and *Methanospirillum hungatei* that were immobilized in methanogenic granular sludge of an Upflow Anaerobic Sludge Blanket reactor (Ahring *et al.*, 1992).

In this study, a consortium of four organisms was constructed to accomplish complete mineralization of 3-CB to carbon dioxide, methane, and chloride. In order to optimize this complex metabolic conversion, we examined under which conditions 3-CB degradation and balanced growth of the four organisms can take place. The composition of the medium was adapted and the effect of other substrates and the toxicity of 3-CB were studied.

MATERIALS AND METHODS

Organisms. *Desulfomonile tiedjei* strain DCB-1 (DSM 6799), *Syntrophus buswellii* (DSM 2612M), *Methanospirillum hungatei* strain SK (DSM 3595) and *Methanosaeta concilii* strain GP6 (DSM 3671) were obtained from the Deutsche Sammlung von Mikroorganismen (DSM, Braunschweig, Germany).

Media, culture conditions and chlorinated compounds. The chlorobenzoates and chlorophenols were obtained from Sigma Aldrich, Zwijndrecht, The Netherlands. An anaerobic bicarbonate-buffered mineral medium was used as described by Stams *et al.* (1993), with the following exceptions: the alkaline trace element solution did not contain Na_2MoO_4, the vitamins were as described by DeWeerd *et al.* (1990), and the acid trace element solution was as described below. The final concentrations of the vitamins in the medium were as follows (in µg/l): pyridoxine, riboflavin, calcium pantothenate, *p*-aminobenzoate, cyanocobalamine, biotin, folic acid, lipoic acid, haemin, 45 each; nicotinamide 450; 1,4-naphthoquinone 180. The acid trace element solution contained (with final concentrations in the medium in µM): $FeCl_2 \cdot 4H_2O$ 4.75, $MnCl_2 \cdot 4H_2O$ 0.31, $CoCl_2 \cdot 6H_2O$ 0.27, $ZnCl_2$ 0.50, $CuCl_2$ 0.10, H_3BO_3 1.0, $Na_2MoO_4 \cdot 2H_2O$ 0.085, $NiCl_2 \cdot 6H_2O$ 0.054, HCl 50. In later studies, the acid trace element solution was replaced by the trace element solution used by Holliger *et al.* (1993) and thiamine was added (90 µg/l medium). *M. concilii*, *D. tiedjei* and *M. hungatei* were routinely grown with 20 mM acetate, 20 mM pyruvate with or without 5 mM 3-CB, and H_2/CO_2 (80:20, v/v) as substrates, respectively. *M. hungatei* cultures also contained acetate (1 mM) or a combination of casein tryptic peptone (0.5 g/l), yeast extract (0.5 g/l) and acetate (4 mM). *S. buswellii* was grown as a coculture with *M. hungatei*, using 20 mM benzoate, or a mixture of 10 mM benzoate and 10 mM crotonate as substrates. The four organisms were also subcultured as a coculture. The medium was inoculated with equal volumes of the four cultures (resulting in 1 to 10% inocula), or equal amounts of the cultures were used based on the ratio of optical densities. The medium contained 1 to 5 mM 3-CB. As additional substrates, 1 to 20 mM pyruvate, 1 mM benzoate, or 2.5% H_2 (v/v) in the gas phase were provided. When 3-CB was depleted, it was replenished. Routinely, microorganisms were grown in 120, 300, 600, or 1200-ml bottles, containing a multiple of 50 ml medium and a gas phase of 1.8 bar N_2/CO_2 or H_2/CO_2 (80:20, v:v).

Presence of electron donors. The cocultures were grown in the presence of 5 mM 3-CB. For the culture derived from pure cultures and a biculture, the following cultures were used (5% inoculum): *D. tiedjei*, grown on pyruvate, 3-CB and yeast extract, *M. hungatei*, grown on H_2/CO_2 (80:20, v:v), casein tryptic peptone, yeast extract and acetate, *M. concilii*, grown on acetate, and a biculture of *S. buswellii* and *M. hungatei*, grown on benzoate and crotonate. For the consortium derived from a transferred coculture, a 10% inoculum was used from a culture grown on 5 mM 3-CB and 5 mM pyruvate.

Utilization of electron acceptors. The consortium, grown on 10 mM 3-CB and 5 mM pyruvate, was transferred (10% inoculum) to fresh media, in bottles with rubber or viton stoppers. The medium contained 5 mM 3-CB and 5 mM pyruvate. Theoretically, 23.75 mmol methane per litre medium could be produced from the amounts of 3-CB and pyruvate which were added to the cultures. When approximately this amount of methane had been produced, the following substrates were added to duplicate bottles (final concentrations in mM): 3-CB (1), 3,5-dichlorobenzoate (3,5-DCB) (0.1), 2,5-DCB (0.1), 2,3-dichlorophenol (2,3-DCP) (0.01), or 2,5-DCP (0.01). To all bottles 1 mM benzoate was added. Controls contained 3-CB (1 mM) without benzoate. In other bottles, a mixture was used, consisting of the following substrates with concentrations in mM: 3-CB (0.1), 2,5-DCB (0.1), 2,5-DCP (0.1) and benzoate (1).

Toxicity of 3-CB. Cultures of *M. concilii* and bicultures of *S. buswellii* and *M. hungatei* were transferred (10%) to a medium with different concentrations of 3-CB, in duplicate. The bottles were supplied with 20 mM acetate and 0.1 mM $FeCl_3$ for *M. concilii* and 10 mM benzoate for the biculture of *S. buswellii* and *M. hungatei*. Duplicate cultures with a 5% inoculum of *M. hungatei* were incubated with H_2/CO_2 (80:20, v:v), casein tryptic peptone (0.5 g/l), yeast extract (0.5 g/l), acetate (2 mM) and different concentrations of 3-CB. Methane production was used to estimate the specific growth rate.

Effect of the presence of bicarbonate buffer. Cocultures of the four organisms, grown on 35 mM 3-CB and 5 mM pyruvate, were transferred (10%) in triplicate to medium from which sodium bicarbonate was omitted, and was or was not replaced by 50 mM NaCl to maintain the ionic strength. As substrates, 5 mM 3-CB and 2.5 mM pyruvate were added. Bottles with bicarbonate buffer were used as controls.

Cell counts. Suspended cells were counted with a Bürker-Türk counting chamber using a microscope (Wild, Heerbrugg, Switzerland) at a magnification of 400 times.

Protein determination. Cell pellets from 2 ml culture were resuspended in 1 ml 1 M NaOH and boiled for 15 minutes. The samples were treated further according to the protein determination method of Lowry *et al.* (1951).

Analytical methods. Methane, hydrogen and acetate were measured with a Packard-Becker 417 or Chrompack CP9001 or CP9000 gas chromatograph as described by Stams *et al.* (1993). Methane was also measured with a Packard-Becker 417 gas chromatograph with a flame ionization detector, instead of a thermal conductivity detector. A molecular sieve 5A column was used (110 cm X 2.1 mm, Chrompack, Middelburg, The Netherlands) at 70°C. Nitrogen was the carrier gas, with a flow of 20 ml/min. Acetate, pyruvate and lactate were analyzed on an LKB high-performance liquid chromatograph as described previously (Stams *et al.*, 1993). Benzoate and chlorobenzoates were measured on an LKB high-performance liquid chromatograph as described by Tros *et al.* (1996), or on a TSP high-performance liquid chromatograph, with a SpectraSystem P2000 pump, an AS3000 autosampler and a UV1000 UV-detector. The mobile phase was an acetonitrile-5 mM H_2SO_4 mixture with a volume ratio of 35:65. Chlorophenols were measured as described in Chapter 2.

RESULTS AND DISCUSSION

Development of a 3-CB-degrading tetraculture consortium. To study the complete mineralization of 3-CB under methanogenic conditions, a consortium was constructed, which consisted of the following four microorganisms. *Desulfomonile tiedjei* reductively dechlorinated 3-CB to benzoate and chloride, *Syntrophus buswellii* oxidized benzoate syntrophically to acetate, hydrogen, and carbon dioxide, *Methanospirillum hungatei* formed methane from hydrogen and carbon dioxide, and *Methanosaeta concilii* converted acetate to methane and carbon dioxide. A medium was developed that supported growth of all members of this consortium by replacing the acid trace element solution and adding thiamine. Inoculation of this medium with the four microorganisms of this consortium resulted in the complete degradation of 3-CB within 40 days of incubation (Figure 1). Methane was produced as the final product of this incubation, but intermediary products accumulated transiently, starting with the apparent stoichiometric conversion of 3-CB into benzoate and the formation of large amounts of acetate. However, after the second addition of 3-CB, benzoate and acetate hardly accumulated. This showed that the microorganisms were adapted to the coculture conditions (Figure 1).

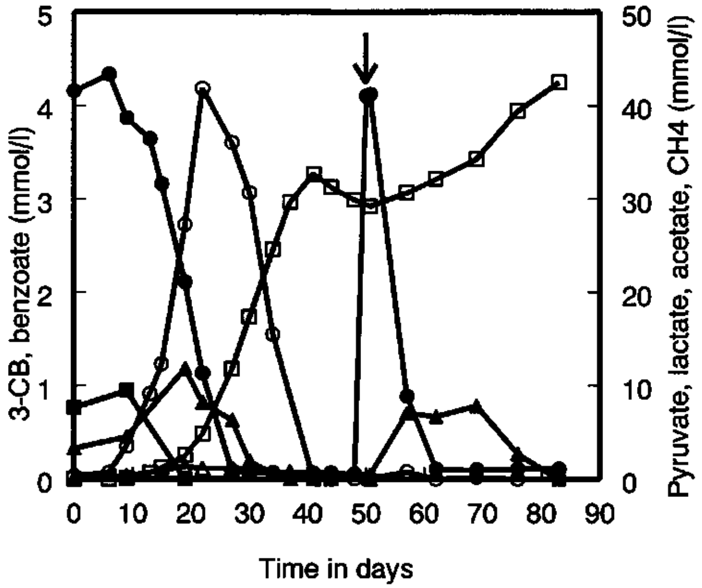

Figure 1. Substrate consumption and product formation by the consortium growing on 5 mM 3-CB and 20 mM pyruvate, and refed after 50 days with 5 mM 3-CB (arrow). Concentrations are in mmol/l medium. ●:3-CB; O:benzoate; ■:pyruvate; △:lactate; ▲:acetate; □:methane.

Presence of electron donors. Initially, the consortium was composed of pure cultures and a biculture, and subsequently, it was transferred as a coculture. A triculture consortium has been shown to be very stable after transfer (Dolfing & Tiedje, 1986). After transfer of the consortium of four microorganisms, no activity was measured if 3-CB was the only substrate, although an initially composed consortium could grow on 3-CB (Figure 2).This could be explained by the dependence of *D. tiedjei* on *S. buswellii* for the electron donor hydrogen, while *S. buswellii* could only release hydrogen if benzoate had been produced by *D. tiedjei*. Addition of benzoate, hydrogen or pyruvate resulted in an actively 3-CB-degrading consortium. Pyruvate was chosen, because it was successful as an electron donor for 3-CB dechlorination by *D. tiedjei* in pure culture (Shelton & Tiedje, 1984). Since in the presence of hydrogen or benzoate product inhibition can take place of *S. buswellii* and *D. tiedjei*, respectively, pyruvate was routinely used as an electron donor after every transfer. As a consequence, the flow of the intermediary products changed: (1) initially, interspecies hydrogen transfer occurred mainly between *S. buswellii* and *M. hungatei* and (2) extra acetate, derived from pyruvate oxidation, was available for *M. concilii*.

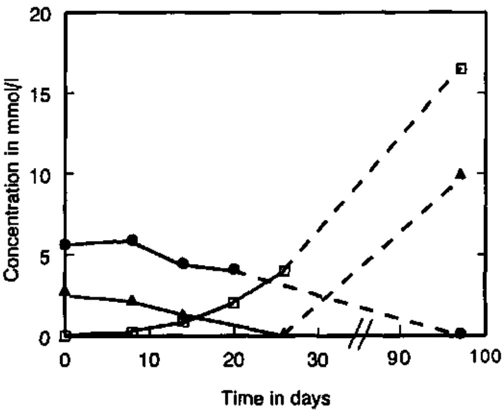

Figure 2. Substrate consumption and product formation by the consortium growing on 5 mM 3-CB. The consortium was composed of pure cultures of *D. tiedjei*, *M.hungatei*, and *M. concilii*, and a biculture of *S. buswellii* and *M. hungatei*. ●:3-CB; ▲:acetate; □:methane.

Utilization of electron acceptors. Dechlorination of 3-CB, 3,5-dichlorobenzoate (3,5-DCB) and 2,5-DCB is strongly accelerated in the presence of a benzoate oxidizer (Dolfing & Tiedje, 1991). *Meta*-substituted dichlorophenols (10 μM) are dechlorinated by *D. tiedjei* cell suspensions (Mohn & Kennedy, 1992). We tested dichlorobenzoates and dichlorophenols as substrates for the consortium, with benzoate as an electron donor. In all cases except when 2,5-DCP was the substrate, the consortium produced methane from benzoate or chlorobenzoates (Table 1). The chlorobenzoate dechlorination rates were lower than for the triculture consortium (Dolfing & Tiedje, 1991). The chlorophenols were not dechlorinated within 20 days. It was expected that chlorophenols would be dechlorinated within this period, because Mohn & Kennedy (1992) reported dechlorination of 3 to 5 μmol l^{-1} day^{-1} by a culture with cell numbers that were about 10 times higher than the cell numbers of the consortium we tested.

Table 1. Dechlorination rates and methane production rates in [mmol l^{-1} day^{-1}], by a coculture grown on 5 mM 3-CB and 5 mM pyruvate. After the growth on 5 mM 3-CB and 5 mM pyruvate, the bottles contained 60.4 (± 5.0) mg/l protein.

Substrates	dichlorobenzoate dechlorination	dichlorophenol dechlorination	3-chlorobenzoate dechlorination	methane production
3-CB without benzoate			0.5	1.2
3-CB			0.4	5.0
3,5-DCB	0.08			2.1
2,5-DCB	0.05			1.0
2,5-DCP		0		0
2,3-DCP		0		1.6
2,5-DCB, 2,5-DCP, 3-CB	0.1	0	0.1	1.4

Toxicity of 3-CB. Dolfing and Tiedje (1986) reported that for the triculture growing on 3-CB 3.2 mM 3-CB was optimal, while growth was decreased at 4.8 mM 3-CB. Since we usually added 5 mM 3-CB, we collected more data on the toxicity of 3-CB to the separate microorganisms. Toxicity tests were performed with growing cultures of *M. concilii*, *M. hungatei* and of *S. buswellii* in coculture with *M. hungatei* (Table 2). *M. concilii* was inhibited by all tested 3-CB concentrations. *M. hungatei* and *S. buswellii* were partially inhibited at concentrations of 3 to 5 mM 3-CB. Both *M. concilii* and *M. hungatei* were more sensitive to 3-CB in these experiments than was expected from data of Patel *et al.* (1991).

M. concilii appeared to be most sensitive to 3-CB. Since it also has the lowest specific growth rate, this microorganism limited the mineralization of 3-CB.

Table 2. Toxic effect of 3-CB on growing cultures of *M. concilii*, *M. hungatei* and a biculture of *S. buswellii* and *M. hungatei*.

3-CB added	Growth rate (day^{-1})			Methane production (μmol l^{-1} day^{-1})
(mM)	*M. concilii*	*M. hungatei*	*S. buswellii/ M. hungatei*	*S. buswellii/ M. hungatei*
0	0.064 (0.004)[a]	0.36 (0.05)	0.14 (0.1)	144 (1)
1	0.048 (0.005)	0.41 (0.04)	0.13 (0.01)	141 (11)
3	0.015 (0.010)	0.29 (0.004)	n.t.[b]	n.t.
5	0.024 (0.006)	0.23 (0.14)	0.10 (0.06)	92 (8)

[a] The values in parentheses are standard deviations. [b]: n.t., not tested.

Effect of the presence of bicarbonate buffer. The importance of the bicarbonate buffer was examined to get information about the effect of prolonged or continuous 3-CB degradation, during which the pH might change or during which the medium could not adequately be buffered (Figure 3). Bicarbonate was either omitted from the medium or replaced by the same concentration of sodium chloride. In the absence of buffer, the pH drop was indeed larger. The pH stayed in the range for growth of *D. tiedjei* on pyruvate (6.5-7.8) (DeWeerd *et al.*, 1990), but the lower pH reduced the dechlorination rate.

In the absence of buffer, not replaced by sodium chloride, the methane production rate was higher and acetate accumulated to a lower level or not at all. This indicates that a higher growth rate of *M. concilii* was due to the reduction of the sodium ion concentration. Sodium ions, in concentrations ranging from 60 to 110 mM, do not inhibit growth of *Methanosaeta soehngenii* (Huser *et al.*, 1982). However, effects of sodium ion concentrations below 60 mM on the growth rate of *M. concilii* in pure culture have not been described. Therefore, it may not be excluded that the increase in the growth rate of *M. concilii* may be an indirect effect occurring only in the consortium and not in a pure culture.

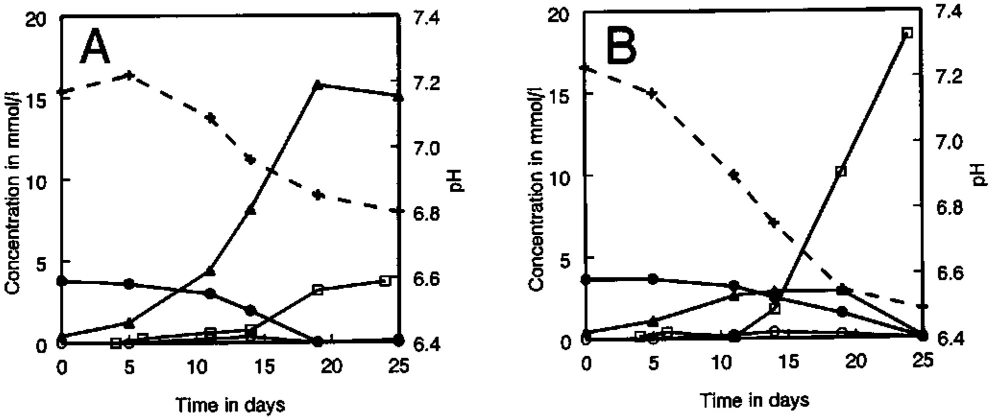

Figure 3. Substrate consumption, product formation and pH change in the consortium growing on 5 mM 3-CB and 2.5 mM pyruvate in presence (A) and absence (B) of a bicarbonate buffer. ● :3-CB; □:methane; x: pH value.

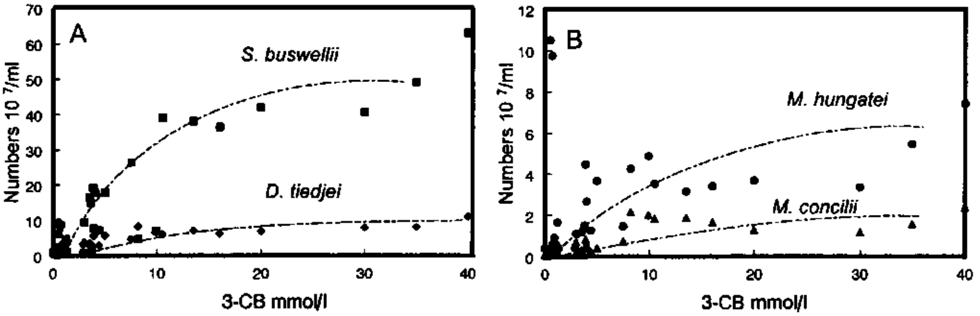

Figure 4. Average cell and filament numbers [10^7 ml^{-1}] from 60 cell counts of *D. tiedjei*, *S. buswellii* (A), *M. hungatei*, and *M. concilii* (B), in relation to the utilized concentration of 3-CB.

Cell counts. From the above described experiments and from experiments with the consortium not explicitly described in this chapter, data were collected about the cell numbers present in the batches (Figure 4). The ratio of the four microorganisms in the consortium could fluctuate, because *M. concilii* grew slower than the other microorganisms involved in the consortium and *S. buswellii* cell numbers sometimes seemed to decrease (possibly due to cell lysis). From the average values of the cell and filament numbers the following ratio of the four organisms could be established: 18% *D. tiedjei*, 70% *S. buswellii*, 10% *M. hungatei* and 3% *M. concilii*. The percentages do not represent the percentages of the biomass, because the cell weights differ greatly. The protein contents per cell were approximately 0.3 pg for *D. tiedjei*, 0.1 pg for *S. buswellii*, and 0.5 pg for *M. hungatei*. *M. concilii* usually tended to form large filaments. The ratio for the triculture consortium has been reported to be: 31% *D. tiedjei*, 70% BZ-1, and 4% *Methanospirillum* PM-1 (Dolfing & Tiedje, 1987). The relatively high numbers of *M. hungatei* that we found can be explained by the addition of pyruvate as an electron donor for *D. tiedjei*, which resulted in a larger amount of hydrogen available for methanogenesis.

CONCLUSIONS

A consortium consisting of four microorganisms mineralized 3-CB under methanogenic conditions. For a growing and 3-CB-mineralizing consortium, a specific growth medium as well as the initial presence of an electron donor (e.g. pyruvate) were required. The presence of pyruvate affected the ratio of cell numbers of the four microorganisms involved in the consortium. Dichlorobenzoates were degraded by the consortium, as has been described by other authors, but dichlorophenols were not. In accordance with other authors, toxic effects of 3-CB occurred in the mM-range, and 3-CB especially reduced the growth rate of *M. concilii*. The absence of bicarbonate buffer reduced the dechlorination rate and stimulated the methane production.

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Chapter 5

EFFECTS OF CARRIER MATERIALS ON THE DEGRADATION OF 3-CHLOROBENZOATE BY A DEFINED METHANOGENIC CONSORTIUM

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ABSTRACT

The degradation of 3-chlorobenzoate (3-CB) was studied under methanogenic conditions, using a consortium of the following four organisms: *Desulfomonile tiedjei*, a 3-CB-dechlorinating bacterium, *Syntrophus buswellii*, a syntrophic benzoate-oxidizer, *Methanospirillum hungatei*, a hydrogenotrophic methanogen, and *Methanosaeta concilii*, an aceticlastic methanogen. Complete mineralization of 3-CB took place in suspended batch cultures, in the presence of an added electron donor, e.g. pyruvate. Because of the possibility of interspecies hydrogen transfer between *S. buswellii* and both *D. tiedjei* and *M. hungatei*, the distance between these microorganisms was expected to affect 3-CB degradation. Therefore, we investigated the effects of the carrier materials glass, teflon, polystyrene, activated carbon, vermiculite and hydroxyapatite on the growth kinetics and 3-CB degradation by the consortium and pure cultures. The presence of polystyrene stimulated the dechlorination process, while the presence of vermiculite and granular sludge stimulated consumption of pyruvate and acetate. The growth rates of pure cultures of *M. hungatei* and bicultures of *S. buswellii* and *M. hungatei* were hardly influenced by the presence of carrier materials. However, the growth of *M. concilii* was stimulated by vermiculite and polystyrene, while the growth of *D. tiedjei* was stimulated by polystyrene, vermiculite and hydroxyapatite. Since there was little surface attachment of the cells, the effects of the carrier materials were probably related to adsorption and desorption of nutrients and/or toxic compounds rather than a consequence of a reduced interbacterial distance. Since the lag phase for dechlorination and the transient acetate accumulation limited 3-CB mineralization, the increased activity of *D. tiedjei* and *M. concilii* appeared to be important for the overall degradation process.

INTRODUCTION

In methanogenic environments, the oxidation of some reduced organic compounds, e.g. fatty acids and aromatic compounds, is endergonic under standard conditions. Therefore, these conversions can only take place when the products, particularly hydrogen, are efficiently utilized by methanogens (Schink & Thauer, 1988). The hydrogen-producing bacteria and hydrogen-consuming methanogens cooperate in a syntrophic relationship. The maximal hydrogen partial pressure for the oxidation reactions is in the same order of magnitude as the hydrogen threshold value for hydrogenotrophic methanogenesis. Therefore, the substrate conversion rates are relatively low. However, hydrogen diffusion and hence the hydrogen flux is accelerated when the distance between the syntrophic partners is small (Schink & Thauer, 1988; Stams, 1994; Schink, 1997). It has been confirmed experimentally that some acetogenic bacteria and hydrogenotrophic methanogens are juxtapositioned in aggregates. For instance, in granular sludge, mixed microcolonies of propionate-oxidizing bacteria and hydrogenotrophic methanogens were detected (Grotenhuis *et al.*, 1991; Harmsen *et al.*, 1996).

A special case of syntrophic degradation is the conversion of 3-chlorobenzoate (3-CB). A three-tiered 3-CB-degrading mixed culture with *D. tiedjei*, *Methanospirillum* PM-1 and strain BZ-2 was studied (Dolfing & Tiedje, 1986). *D. tiedjei* reductively dechlorinates 3-CB with hydrogen as electron donor and produces benzoate. Strain BZ-2 converts benzoate into hydrogen, carbon dioxide and acetate. This reaction can only occur at a low pH_2, which is realized by hydrogen consumption by *D. tiedjei* and *Methanospirillum* PM-1. This coculture, with one hydrogen producer and two hydrogen consumers, formed methane, acetate, and chloride from 3-CB in expected stoichiometries. Studies on the dechlorination by *D. tiedjei* as well as the interactions between the three members of the consortium have been reviewed by Mohn and Tiedje (1992).

In order to study aggregation by 3-CB-converting microorganisms, *D. tiedjei* was incorporated in methanogenic granular sludge (Ahring *et al.*, 1992). In this way, the sludge gained the ability to dechlorinate 3-CB. In another immobilization study, a combination of *D. tiedjei*, the syntrophic benzoate oxidizer *Syntrophus buswellii* and *M. hungatei* were incorporated in sludge from a UASB reactor. Other methanogens, namely *Methanosaeta concilii* and *Methanosarcina mazei*, could also be incorporated (Schmidt & Ahring, 1997).

In this study, a consortium of four organisms was used to achieve complete mineralization of 3-CB to carbon dioxide, methane, and chloride. For this purpose, we added the aceticlastic methanogen *M. concilii* to a similar consortium as used previously by Dolfing and Tiedje (1986) and Ahring *et al.* (1992). To increase the 3-CB degradation rate, a variety of carrier materials were added that could stimulate adherence and hence result in reduced interbacterial distances. The effect of carrier materials on growth kinetics was examined and the attachment of the cultures to carrier materials was studied.

MATERIALS AND METHODS

Organisms. *D. tiedjei* strain DCB-1 (DSM 6799), *S. buswellii* (DSM 2612M), *M. hungatei* strain SK (DSM 3595) and *M. concilii* strain GP6 (DSM 3671) were obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). *Pseudomonas fluorescens* p62 was obtained from our culture collection.

Media and culture conditions. *P. fluorescens* was cultivated in nutrient broth (BBL, Cockeysville, USA) (8 g/l) in shaken erlenmeyer flasks at 30°C. The other microorganisms were cultivated in an anaerobic bicarbonate-buffered mineral medium as described by Stams *et al.* (1993), but with the acid trace element solution replaced by the trace element solution used by Holliger *et al.* (1993) and vitamins as described by DeWeerd *et al.* (1990). The alkaline trace element solution did not contain Na_2MoO_4. The concentrations of the vitamins were as follows (in µg/l medium): pyridoxine, riboflavin, calcium pantothenate, *p*-aminobenzoate, cyanocobalamine, biotin, folic acid, lipoic acid, haemin, 45 each; nicotinamide 450; 1,4-naphthoquinone 180; thiamine 90. *M. concilii, D. tiedjei* and *M. hungatei* were routinely grown with 20 mM acetate, 20 mM pyruvate and H_2/CO_2 (80:20, v/v) as substrates, respectively. Bottles were inoculated with 10% of pregrown cultures. The medium used for *M. hungatei* contained additionally acetate (1 mM), or casein tryptic peptone (0.5 g/l), yeast extract (0.5 g/l) and acetate (4 mM). *S. buswellii* was transferred (10%) as a coculture with *M. hungatei*, with 20 mM benzoate. The four organisms were also subcultured as one coculture (experiments 1 and 2). The medium contained 5 mM 3-chlorobenzoate (3-CB) and 5 mM pyruvate. When 3-CB was depleted, extra 3-CB (without pyruvate) was added.

Materials and chemicals. 3-CB was used with a purity of 99% (Sigma Aldrich, Zwijndrecht, The Netherlands). Glass microscope coverslips (Rofa-Mavi, Beverwijk, The Netherlands) and fluor ethene propene-teflon film (Fluorplast, Raamsdonksveer, The Netherlands) with a surface area of 1.5 cm^2 were used in adhesion tests. In the growth experiments, the following materials were used: glass beads (0.3 mm diameter, Tamson, Zoetermeer, The Netherlands), teflon beads (a batch with various diameters), polystyrene beads (0.125 mm-0.25 mm, Akzo, Amsterdam, The Netherlands), vermiculite (powder or grained 0.25-0.5 mm, Sigma-Aldrich), activated carbon (±1.5 mm extra pure, Merck, Darmstadt, Germany), and hydroxyapatite (Merck). Granular sludge was derived from a full scale UASB reactor treating potato processing wastewater (Aviko, Steenderen, The Netherlands). Part of the sludge was γ-irradiated at a dose of 25 kGray and then washed with sterile medium. This sludge was autoclaved in the incubation bottles (20 minutes, 120°C). The rest of the sludge was autoclaved three times. First, it was autoclaved for 1.5 hours (120°C). After this, the sludge was incubated for one week at 37°C, washed with medium and autoclaved again (0.5 hours, 120°C). The sludge was distributed among the incubation bottles (10% (v/v) in the medium) and autoclaved for 20 minutes (120°C).

Growth experiments. Several growth experiments were performed under the culture conditions described above. A 10% inoculum was used with or without 20 g/l carrier material unless stated otherwise. Only changes in the culture conditions for the consortium (experiment 1 and 2), *D. tiedjei* (experiment 3 and 4), *M. concilii* (experiment 5 and 6), *S. buswellii* plus *M. hungatei* (experiment 7 and 8), and *M. hungatei* (no experiment number) are described here. In experiment 1 and 2, a 1% inoculum of the four-membered consortium was used with or without 20 g/l carrier material or 10% (v/v) granular sludge. In experiment 2, also a preincubation of media with vermiculite was tested. For this, bottles with medium and vermiculite were incubated 7 days without buffer and without sulfide. The bottles were centrifuged and decanted and in this way most of the vermiculite was removed. Afterwards, the gas phase was changed to N_2/CO_2, and buffer, sulfide, and inoculum were added. In experiment 4, the inoculum size was 5 x 10^5/ml instead of 10% (approximately 1 x 10^7/ml), and the substrates were 5 mM pyruvate and 1 mM 3-CB. In experiment 6, the complete medium was prepared a few days before incubation.

In experiment 1, few samples from the liquid phase were taken, so that numbers of cells and amounts of materials would be minimally influenced. The gas phase was sampled frequently to determine when 3-CB would be completely mineralized. From 5 mM 3-CB and 5 mM pyruvate, maximally 23.75 mmol/l medium methane could be produced. When the methane concentration approached 23.75 mmol/l medium, suspended cells and filaments were counted. Then, the materials were washed (0,9% NaCl, for 1 day) to see if attached cells would desorb from the materials (Wiesel *et al.*, 1993). A sample of the supernatant was counted. The bottles with vermiculite or polystyrene were washed again for one day. Polystyrene and vermiculite were allowed to precipitate. The precipitates were transferred to four bottles with fresh medium in a flow chamber. In the bottles, 20 mM pyruvate, benzoate, or acetate, or H_2/CO_2 (80:20, v/v) was added.

Formation of products was used to estimate the specific growth rate. In experiment 2, the lag phase for dechlorination was estimated from methane production, because methane concentrations could be measured more accurately than 3-CB concentrations. It was assumed that dechlorination and benzoate oxidation had the same lag phase. Chloride release was used to calculate the dechlorination rates. In the presence of glass, the dechlorination rates were estimated from the 3-CB concentrations. During benzoate oxidation and methane production by the biculture *S. buswellii/M. hungatei* it was difficult to distinguish whether or not exponential growth occurred. Because of this, the activity was estimated by assuming a constant methane production rate.

Suspended cells or filaments were counted with a Bürker-Türk counting chamber using a microscope (Wild, Heerbrugg, Switzerland) at a magnification of 400 times.

Analytical methods. Methane and acetate were measured with a Packard-Becker 417 or Chrompack CP9001 or CP9000 gas chromatograph as described by Stams *et al.* (1993). Methane was also measured with a Packard-Becker 417 gas chromatograph with a flame ionization detector, instead of a thermal conductivity detector. A molecular sieve 5A column was used (110 cm X 2.1 mm, Chrompack, Middelburg, The Netherlands) at 70°C. Nitrogen was the carrier gas, with a flow of 20 ml/min. Acetate and pyruvate were analyzed on an LKB high performance liquid chromatograph as described previously (Stams *et al.*, 1993). Benzoate and 3-CB were measured on an LKB high performance liquid chromatograph as described by Tros *et al.* (1996). The mobile phase was an acetonitrile-5 mM H_2SO_4 mixture with a volume ratio of 35:65. Chloride was analyzed with a Micro-chlor-o-counter as described previously (Holliger *et al.*, 1992).

RESULTS

A tetraculture consortium constructed from pure cultures is able to grow on 3-CB if an electron donor, for instance pyruvate, is initially present. This consortium is stable upon transfer (Chapter 4). To get insight into the physiological effects of the addition of carrier materials on the 3-CB-degrading consortium several experiments were done with the consortium and individual members of this consortium. The substrate conversion kinetics, as affected by the presence of carrier materials, as well as adhesion data were collected.

Attachment of the consortium to carrier materials during growth. The attachment of the complete consortium was studied in growing cultures. It was expected that attached organisms would benefit from the presence of carrier materials, because interspecies distances would be lowered and fluxes of especially hydrogen would be increased.

Addition of activated carbon and hydroxyapatite resulted in a strong inhibition of 3-CB conversion. In all other incubations, 3-CB was completely mineralized. Counts of suspended cells and filaments were performed for controls, and incubations with glass, teflon, vermiculite or polystyrene (Table 1). Since the numbers of cells in suspension were not significantly lowered in the presence of the tested carrier materials in comparison with control bottles, adhesion could not explain the enhancement of 3-CB mineralization in the presence of vermiculite or polystyrene.

After the suspended cells had been counted, the materials were washed. Attached cells desorbing from the materials were counted. High numbers were released from polystyrene and especially from vermiculite to the medium (Table 1). This indicated that the organisms had been attached loosely to these materials or had been coprecipitated with the materials. After another day of washing, polystyrene and vermiculite were allowed to precipitate. The precipitates were transferred to bottles with different substrates. In case of vermiculite, methane formation was found with pyruvate, acetate or hydrogen but no methane was found in the presence of benzoate. In case of polystyrene, methanogenic activity could be measured with pyruvate, but with benzoate, hydrogen, or acetate no activity was observed. Since syntrophic benzoate oxidation did not take place, *S. buswellii* was probably not present in sufficient numbers in these bottles. This may be explained by the small size of *S. buswellii* cells. The possible loss of *S. buswellii* cells and the release of many cells during the washing steps of vermiculite indicated that rather coprecipitation (mainly of larger cells and filaments) but not attachment had occurred. A similar effect of polystyrene was expected, because both vermiculite and polystyrene consist of small light particles.

Table 1. Average cell counts in 10^7/ml from experiment 1.

Species	*D. tiedjei*	*S. buswellii*	*M. hungatei*	*M. concilii*
Inoculum cell counts	0.06	0.4	0.05	0.01
Final cell counts of controls	1	14	6	0.3
Final cell counts with carrier materials	0.9	16	4	0.2
Washed vermiculite	0.2	7	2	0.04
Washed polystyrene	0.7	2	2	0.2

Adhesion tests with pure cultures. The initial attachment of microbial cells was tested in short term (4 h) adhesion tests. We used *Pseudomonas fluorescens* as a reference bacterium and found it to adhere well to both glass and teflon surfaces as was previously described (Rijnaarts *et al.*, 1993). *D. tiedjei* and *S. buswellii* adhered weakly to moderately and *M. hungatei* adhered even less (Table 2). *M. concilii* seemed to adhere weakly, although it is known to be hydrophobic and to form aggregates. In this experimental setup the adhesion properties of *M. concilii* were underestimated, because adhesion of filamentous organisms is limited. In this short term test without agitation, contact of the cells with the surface is determined by diffusion and filamentous organisms diffuse very slow (Rijnaarts *et al.*, 1993).

Table 2. Mean numbers of adherence (range in parentheses). The standard deviations were 50 to 100%.

	Initial density	Numbers adhered (10^6/cm^2)	
	(10^8/ml)	glass	teflon
D. tiedjei	5	0.2 (0.06-0.40)	0.3 (0.05-0.85)
S. buswellii	5	0.4 (0.19-0.70)	0.5 (0.26-0.94)
M. hungatei	4	0.05 (0.02-0.12)	0.12 (0.03-0.27)
M. concilii	1	0.009 (0.003-0.024)	0.01 (0.001-0.017)
P. fluorescens	5	0.5 (0.3-1.1)	1.5 (0.5-2.5)

Effects of materials on the conversion of 3-CB by the consortium. Attachment seemed not to be an explanation for the stimulatory effects of some of the materials on growth kinetics of the consortium (experiment 1 in Table 3). In the presence of vermiculite and polystyrene, the lag phase for methane production was approximately 16 days, while in the controls it was 23 days. The addition of teflon had no effect, while in the presence of glass, the results were not reproducible. By detailed measurement of the conversion steps of 3-CB, more information would be gained about the type of effect the carrier materials had. Since all organisms of this consortium could be incorporated in granular sludge (Ahring *et al.*, 1992; Schmidt & Ahring, 1997), and in experiment 1, addition of vermiculite and polystyrene reduced the time required for complete mineralization of 3-CB while the effect of glass was unclear, glass, vermiculite, polystyrene and killed granular sludge were included in this experiment (experiment 2). Since addition of vermiculite could have had an effect by coprecipitation as well as by inorganic nutrient release or uptake (England *et al.*, 1993), two types of incubations were done. In the first case, as in experiment 1, vermiculite was present in the bottles during the incubation of the consortium. Alternatively, vermiculite was added to and later removed from the bottles before addition of the consortium. The latter case was referred to as vermiculite preincubation. After the bottles were preincubated with addition of vermiculite, only a small part of the vermiculite was left during the incubation of the consortium. Coprecipitation could only play a minor role in the bottles, preincubated with vermiculite, but cation exchange could still be important.

Table 3. Effects of carrier materials on growth rates (μ, [day^{-1}]) of pure and mixed cultures. C, controls; G, glass; T, teflon; P, polystyrene; V, vermiculite; VP, vermiculite preincubation; H, hydroxyapatite; GS, granular sludge. When the standard deviation was larger than 25% or when the specific growth rate could not be calculated accurately, a range is given.

Experiment	1	2	2	2	3	4	5	6
Culture	consortium	consortium	consortium	consortium	*D. tiedjei*	*D. tiedjei*	*M. concilii*	*M. concilii*
Substrate	acetate	pyruvate	benzoate	acetate	pyruvate	pyruvate	acetate	acetate
C	0.09	0.23-0.53	0.23	0.09	0.083	0.15-0.16	0.13	0.10-0.20
G	0.11	0.31	0.08	0.08	0.091			0.18
T	0.14				0.091		0.12	
P	0.18	0.24-0.32	0.24	0.09	0.095	0.17-0.23	0.15	
V	0.21	0.5-0.6	0.21	0.12	0.102		0.20	
VP		0.49	0.24	0.16				
H	0				0.102			0.08
GS		0.28	0.23	0.13-0.19				

Substrate conversions were used to determine growth rates (Table 3) and to estimate lag phases of the different populations. Dechlorination of 3-CB seemed neither exponential nor linear in time. For calculations it was assumed to be linear (Table 4). In most cases, 3-CB dechlorination started later than pyruvate consumption. Therefore, pyruvate as well as hydrogen may have served as electron donor for dechlorination.

In experiment 2, intermediary products accumulated transiently (Figure 1). The controls first formed acetate from pyruvate, which took 20-25 days. At that time benzoate accumulation started, although methane production started within 1 to 2 weeks. In the controls, benzoate accumulated to approximately 0.4 mM. After 40 days, all 3-CB was dechlorinated. In the first 40 days, no acetate degradation occurred. In the controls, complete mineralization of 3-CB took 90 days. The average lag phase for growth of *D. tiedjei* on pyruvate was 5 to 10 days, but there was no lag phase for growth on pyruvate in the presence of granular sludge.

Table 4. Effects of carrier materials on substrate utilization [mmol l^{-1} day^{-1}] of pure and mixed cultures. C, controls; G, glass; T, teflon; P, polystyrene; V, vermiculite; VP, vermiculite preincubation; GS, granular sludge. When the standard deviation was larger than 25% or when the substrate conversion rate could not be calculated accurately, a range is given.

Experiment	2	4	7	8
Culture	consortium	*D. tiedjei*	*S. buswellii/* *M. hungatei*	*S. buswellii/* *M. hungatei*
Substrate	3-CB	3-CB	benzoate	benzoate
C	0.35	0.029		0.84
G	0.21		0.65	
T			0.66	
P	0.45	0.058	0.63	
V	0.17		0.67	1.01
VP	0.33			
GS	0.11-0.37			

In the presence of glass beads, the lag phase for dechlorination was more than 48 days and in addition, the lag phase for growth of *M. concilii* was prolonged, compared to the controls without carrier materials. The dechlorination rate, and as a result the benzoate oxidation rate, was lower in the presence of glass beads than in the absence of carrier materials. After 110 days, 3-CB was completely converted into methane, carbon dioxide and chloride.

Several positive effects were found with polystyrene, vermiculite and granular sludge. The addition of polystyrene mainly shortened the lag phase for dechlorination (Figure 1). Because of this shorter lag phase for dechlorination, the mineralization of 3-CB was completed in a shorter period, namely 50 to 110 days, with an average of 80 days. The addition of vermiculite enhanced particularly the growth rates of *D. tiedjei* (on pyruvate) and *M. concilii*. For the growth rate of *M. concilii*, this effect was even stronger in bottles which had been preincubated with vermiculite. Acetate accumulated to high concentrations in the controls, with glass, and with polystyrene, but not with vermiculite (Figure 1). Hence, the complete mineralization of 3-CB took 50 days for the bottles incubated with vermiculite and 40 days for bottles preincubated with vermiculite. In the presence of vermiculite, the lag phase for benzoate formation was less than 20 days. In the presence of granular sludge, benzoate started to accumulate immediately (max. 0.08 mM), so the lag phase for dechlorination was negligible. The addition of sludge also provided 0.3 to 1 mM extra acetate, which is a small amount compared to the acetate transiently produced from pyruvate and 3-CB. Methane production started immediately (Figure 1). The mineralization of 5 mM 3-CB was completed in 40 to 70 days.

Effects of materials on kinetics of pure cultures and a biculture. The effects of the presence of carrier materials on the growth rates or substrate utilization rates in the consortium may be characteristic for the coculture or for the individual members. Therefore, the substrate kinetics of growing cultures of *M. hungatei*, *D. tiedjei*, *M. concilii* and a biculture of *S. buswellii* and *M. hungatei* in the presence and absence of carrier materials were analyzed.

None of the carrier materials enhanced the growth rate of *M. hungatei* (results not shown). The growth of *D. tiedjei* was somewhat stimulated by vermiculite, but not by polystyrene (experiment 1 in Table 3; Figure 2).

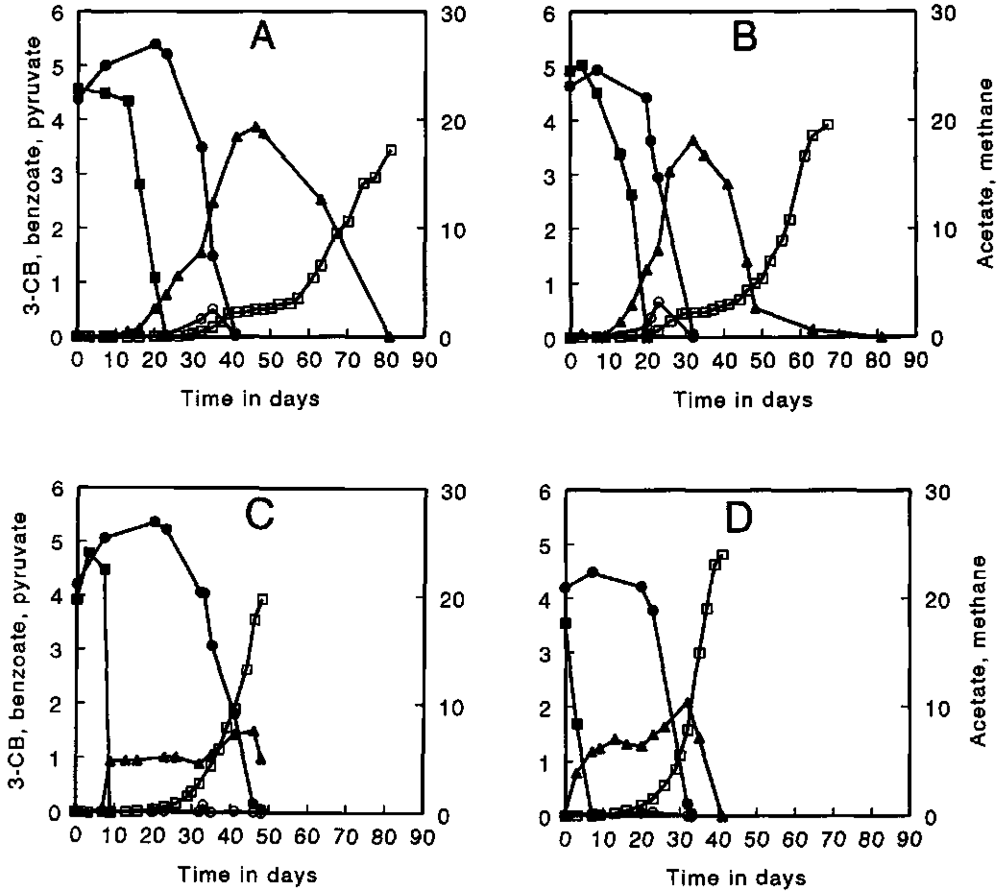

Figure 1. Substrate consumption and product formation by the consortium (experiment 2 in Table 3 and 4) in the absence of carrier materials and in the presence of polystyrene, vermiculite and granular sludge. Concentrations are in mmol/l medium. ■:pyruvate;●:3-CB; O:benzoate;▲:acetate; □:methane. A:control; B:polystyrene; C:vermiculite; D:sludge.

Only under the growth conditions of experiment 4, polystyrene enhanced the dechlorination rate, but not the growth rate on pyruvate (Table 3 and 4), which explains the shortened lag phase of the consortium in experiment 2. The growth rate of *M. concilii* was higher in the presence of vermiculite and also slightly higher in the presence of polystyrene (experiment 5 in Table 3). For the biculture of *S. buswellii* and *M. hungatei*, the methane production rates were compared (experiments 7 and 8 in Table 4). In experiment 8, vermiculite addition led to a higher methane production rate, compared to the controls. However, in experiment 7, the presence of vermiculite did not result in a higher methane production rate than in the other bottles. In the presence of hydroxyapatite the growth rate of *D. tiedjei* increased in comparison with the incubations in the absence of carrier materials (experiment 3 in Table 3), the growth rate of *M. concilii* decreased (experiment 6 in Table 3), and the activity of the biculture was completely inhibited. This explained the slow dechlorination and methane production by the consortium in the presence of hydroxyapatite (experiment 1).

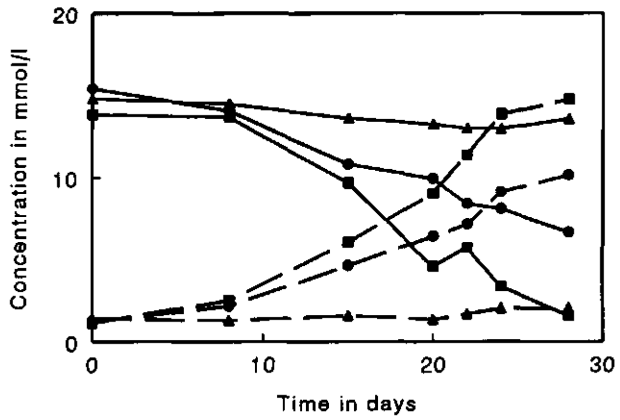

Figure 2. Pyruvate consumption (solid lines) and acetate production (dashed lines) by *D. tiedjei* in pure culture (experiment 3 in Table 3) in the absence of carrier materials and in the presence of vermiculite and activated carbon. ●:control; ■:vermiculite; ▲:activated carbon.

DISCUSSION

A 3-CB-degrading consortium was studied in the presence of carrier materials with the goal to improve the 3-CB mineralization rate. The attachment of the consortium members to the carrier materials played no significant role. However, some carrier materials affected the growth rates or the substrate conversion rates of the consortium or its individual members positively.

The presence of polystyrene, vermiculite and granular sludge influenced 3-CB mineralization by the consortium positively. This largely reflects the observed effects with pure cultures of *D. tiedjei* and *M. concilii*. In pure cultures the most pronounced positive effects were found on the growth rate of *M. concilii*, with the addition of vermiculite and polystyrene. The growth rates or methane production rates of the other microorganisms were less affected or not increased in the presence of carrier materials in comparison with the cultures grown in the absence of carrier materials. However, the higher growth rate of *M. concilii* had a large impact on the time required for complete mineralization of 3-CB by the consortium. This is illustrated by the effect of polystyrene compared to the effect of vermiculite and of sludge. In the presence of polystyrene, due to an increased dechlorination rate or a reduction of the lag phase, the complete mineralization of 3-CB was performed in a slightly shorter period than in the control bottles. In the presence of vermiculite and sludge the growth rate of *M. concilii* was higher than in the absence of these carrier materials. With vermiculite, mineralization was achieved in approximately 40 to 50 days, which was much less time than was needed with polystyrene. In two bottles with sludge the time needed for complete mineralization was even shorter than with vermiculite. One explanation for the beneficial effect of carrier materials may be that aceticlastic methanogenesis occurred so slowly, compared to dechlorination and benzoate oxidation, that it would always be the rate-limiting degradation step. This would imply that improvement of the process could only occur via stimulation of *M. concilii*. Alternatively, the large impact of acetate degradation might be explained by the prevention of acetate accumulation. Acetate accumulation inhibits benzoate oxidation by syntrophic benzoate-oxidizing bacteria (Dolfing & Tiedje, 1988; Warikoo *et al.*, 1996). In some bottles, a concentration of 20 mM acetate was reached. This is in the range of the K_i for benzoate oxidation by strain BZ-2 and strain SB, which are 40 mM and 10 mM acetate, respectively (Dolfing & Tiedje, 1988; Warikoo *et al.*, 1996). Furthermore, the Gibbs free energy change of benzoate oxidation depends on the acetate concentration. This implies that a decrease of the acetate concentration should either result in a higher hydrogen partial pressure as was reported for syntrophic benzoate oxidation by *Syntrophus gentianae* (Schöcke & Schink, 1997), or a higher substrate oxidation rate, which has been observed for instance for syntrophic propionate oxidation (Dong *et al.*, 1994). However, in experiment 2 the growth rate on benzoate was not affected by the presence of vermiculite or sludge. Therefore, it seems more likely that acetate conversion is the rate-limiting step for complete mineralization of 3-CB. This implies that the time required for the complete mineralization of 3-CB is decreased in the presence of vermiculite or sludge in comparison with the control cultures, because the growth of the aceticlastic methanogens is increased.

The dechlorination rates of the control bottles in experiment 2 are comparable to dechlorination rates of a similar consortium previously reported by Dolfing and Tiedje (1986, 1991a, 1991b). After growth on 3.2 mM 3-CB, the consortium contained 22-26 mg/l protein, and dechlorinated 17-27 μM 3-CB/h (Dolfing & Tiedje, 1991a; Dolfing & Tiedje, 1991b). The doubling time with benzoate as a substrate in experiment 2 was similar to the doubling time of the three-membered consortium (Dolfing & Tiedje, 1986).

The consortium was expected to aggregate or to attach to carrier materials, for the following reasons. *Methanosaeta, D. tiedjei* and strain BZ-2 form aggregates in syntrophic cocultures (Wu *et al.*, 1996; Tiedje & Stevens, 1988). *M. hungatei* is motile and thus able to move towards its syntrophic partner. *M. hungatei* also adheres to some hydrophilic polymers (Verrier *et al.*, 1988). In the growing consortium and in the adhesion tests we found little attachment. Possibly, the applied growth conditions were not optimal for attachment, or the strains had lost their ability to attach.

The presence of polystyrene affected the dechlorination in the consortium, by reducing the lag phase. In a pure culture of *D. tiedjei*, the presence of polystyrene mainly affected the dechlorination rate, and it had less effect on the growth rate on pyruvate. So, coprecipitation of *D. tiedjei* and *M. concilii* with polystyrene did not give rise to a higher growth rate of these microorganisms than in cultures incubated without carrier materials. The presence of polystyrene could have influenced dechlorination and methanogenesis in several other ways, not correlated to coprecipitation. Adsorption of 3-CB to the polystyrene beads could have reduced the toxicity of 3-CB (Dolfing & Tiedje, 1986), but there was no measurable adsorption. Small molecules, like styrene, 1-phenyl ethanol, or phenyl acetate may have diffused from the polystyrene beads. A low concentration of these toxic monomers, leaking from polystyrene, might have enhanced activity aspecifically. These molecules might also have induced 3-CB dechlorination. Because polystyrene seemed to affect especially dechlorination, induction of dechlorination seems most likely. However, the mentioned compounds have not been reported to act as inducers of dechlorination by *D. tiedjei* (Mohn & Tiedje, 1992).

Vermiculite was found to coprecipitate with cells and the presence of vermiculite could also have had an influence on the growth rate of *D. tiedjei* and *M. concilii* because of its great cation exchange capacity. In experiment 2, the growth rate of *M. concilii* was even higher in the bottles which were preincubated with vermiculite than in the bottles which were incubated with vermiculite. Since the preincubation was followed by centrifugation during which the medium was exposed to oxygen, an effect of vermiculite on the redox potential is less likely. Buffering of the pH and coprecipitation of cells probably played a minor role, because after the preincubation with vermiculite, only the smallest particles were left. It seems more likely that vermiculite adsorbed toxic metals, which were partially irreversibly removed after the preincubation. Furthermore, the iron concentration in solution might have been raised in the presence of vermiculite as well as after the preincubation of vermiculite. An effect on the iron concentration has been reported by Murray and Van den Berg (1981). In that study, a methanogenic enrichment on acetate was incubated with various carrier materials. The acetate conversion rate in the presence of carrier materials and in the presence of clay suspension supernatant was higher than in the absence of carrier materials. The high acetate conversion rate was due to an increased soluble iron concentration.

In the presence of granular sludge no lag phase was observed for pyruvate consumption, dechlorination and methane production. We did not find higher dechlorination rates than in the control bottles. The effect of the presence of granular sludge on dechlorination has been studied for an enrichment on polychlorinated biphenyls (Middeldorp, 1997). The lag phase for dechlorination was greatly reduced in the presence of granular sludge, while the dechlorination rate was much less affected. The effect on the lag phase was not due to redox potential changes, because the redox potential with sludge hardly differed from the controls. It has been suggested that the positive effect of the sludge was due to the supply of essential growth factors (Middeldorp, 1997).

Effects of the carrier materials on syntrophic benzoate oxidation by *S. buswellii* and *M. hungatei* could not be demonstrated in the biculture and the consortium. This is in accordance with the lack of attachment of the syntrophic partners. Although the organisms in the consortium seemed to have lost their ability to attach to surfaces, carrier materials still had a strong effect on the lag phases of degradation steps, and/or the growth of the populations which are involved in this consortium. By the reduction of the lag phase for dechlorination and the height and duration of the transient acetate accumulation, complete mineralization of 3-CB could be achieved in a shorter time period in the presence than in the absence of carrier materials.

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Chapter 6

A 3-CHLOROBENZOATE-DEGRADING METHANOGENIC CONSORTIUM IMMOBILIZED IN CARRAGEENAN GEL BEADS

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ABSTRACT

A tetraculture consortium was able to degrade 3-chlorobenzoate (3-CB) under methanogenic conditions. This consortium consisted of *Desulfomonile tiedjei*, a 3-CB-dechlorinating bacterium, *Syntrophus buswellii*, a syntrophic benzoate-oxidizer, *Methanospirillum hungatei*, a hydrogenotrophic methanogen, and *Methanosaeta concilii*, an aceticlastic methanogen. The tetraculture was immobilized in κ-carrageenan gel beads with K^+ as counterion. After the immobilization step, the protein content as well as the 3-CB conversion rate increased in time, indicating that growth occurred. Moreover, microscopic examination revealed the formation of microcolonies inside the gel beads. The immobilized cells had a lower specific 3-CB conversion rate than suspended cells, but the activity of the immobilized cells was maintained for a longer time period. Effects of varying cell ratios on the conversion of different substrates were examined. Addition of extra *D. tiedjei* or *M. hungatei* cells resulted in an increase of the 3-CB dechlorination rate, and addition of extra *D. tiedjei* cells led to an increased 3,5-dichlorobenzoate dechlorination rate. Extra *S. buswellii* cells led to an increase of the conversion rate of benzoate, but not of 3-CB unless crotonate was present as an additional electron acceptor. Since the cell ratios affected the interbacterial distances and thereby the hydrogen diffusion rate from *S. buswellii* to the hydrogen consuming *D. tiedjei* and *M. hungatei*, a calculation of the minimal distances was made. The changes in the minimal interbacterial distances could explain the measured changes in the substrate conversion rates.

INTRODUCTION

For the anaerobic degradation of organic compounds under methanogenic conditions the action of several microorganisms is required. Fermentation products and some aromatic compounds like benzoate are converted into acetate plus hydrogen in obligatory syntrophic conversions performed by acetogens. Substrate conversion by the acetogen can only proceed if hydrogen is maintained at a very low concentration by a hydrogen-consumer, usually a methanogen. This implies that the substrate conversion rate is determined by the hydrogen flux. The hydrogen flux itself, as described by Fick's diffusion equation, is affected by the cell densities of hydrogen producers and hydrogen consumers in two ways. Firstly, the surface area from which hydrogen diffuses is determined by the cell density of the hydrogen producer, and secondly, the distance between the hydrogen producer and the hydrogen consumer is determined by the cell densities of the hydrogen producer and the hydrogen consumer (Schink & Thauer, 1988; Stams, 1994; Schink, 1997).

A locally raised cell density can result from the immobilization of cells. Immobilization of cells is achieved by self-aggregation, attachment to a carrier material, or entrapment in a gel matrix. The physiological consequences of immobilization, the immobilization techniques, and the practical applications of immobilized cells have been studied extensively (see the review by Cassidy *et al.*, 1996). In many studies, the substrate utilization rates and product formation rates are higher for immobilized cells than for suspended cells, but in a few other studies the growth rates of immobilized cells were lower. The specific methanogenic activity is maintained somewhat longer for alginate imbedded than for suspended *Methanosarcina barkeri* cells converting methanol (Scherer, 1981). In some cases, the sensitivity of immobilized cells to toxic compounds is lower than that of suspended cells, as has been shown for a phenol-degrading methanogenic enrichment culture (Dwyer *et al.*, 1986). Cells imbedded in agar had a higher tolerance to phenol than non-immobilized cells. Additionally, the apparent K_m for phenol was lower and the lag period for phenol degradation was shorter for immobilized cells. However, the phenol mineralization rate decreased upon cell immobilization (Dwyer *et al.*, 1986).

A 3-chlorobenzoate (3-CB)-degrading consortium has been studied in a triculture, consisting of freely suspended cells of *Desulfomonile tiedjei*, *Methanospirillum* PM-1 and the benzoate-oxidizing bacterium strain BZ-2

(Dolfing & Tiedje, 1986; reviewed by Mohn and Tiedje, 1992). Cells of *D. tiedjei*, *Syntrophus buswellii* and *Methanospirillum hungatei* were immobilized by self-aggregation in methanogenic granular sludge in an Upflow Anaerobic Sludge Blanket reactor (Ahring *et al.*, 1992). In this consortium, *D. tiedjei* reductively dechlorinated 3-CB to benzoate and chloride, *S. buswellii* oxidized benzoate syntrophically to acetate and hydrogen, and *M. hungatei* formed methane from hydrogen. A consortium consisting of *D. tiedjei*, *S. buswellii*, *M. hungatei* and the aceticlast *Methanosaeta concilii* was able to mineralize 3-CB completely to methane, carbon dioxide and chloride (Chapter 4). Attachment of the consortium consisting of four microorganisms to carrier materials is described in Chapter 5. Here we describe a study on the gel entrapment of the consortium. The physiological impact of gel entrapment on 3-CB degradation and growth of the consortium was investigated by studying the 3-CB degradation rate, the growth yield and the colony formation in a carrageenan gel. Furthermore, the effects of additional cells on the conversion rates of several substrates were examined.

MATERIALS AND METHODS

Organisms. *D. tiedjei* strain DCB-1 (DSM 6799), *S. buswellii* (DSM 2612M), *M. hungatei* strain SK (DSM 3595) and *Methanosaeta concilii* strain GP6 (DSM 3671) were obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany).

Chemicals. Chlorobenzoates were derived from Sigma Aldrich, Zwijndrecht, The Netherlands. For the immobilization of the cells, κ-carrageenan (Genugel X0828, A/S Kobenhavns Pektinfabrik, Lille Skensved, Denmark) was dissolved (4% or 5% w/v) in medium without buffer, Na_2S, vitamins and $CaCl_2$. Subsequently, it was autoclaved and stored at 50°C until use.

Media and culture conditions. All microorganisms were cultivated in a medium as described in Chapter 5. In the immobilization experiments as well as in the suspended culture experiments that served as controls, $NaHCO_3$ was replaced by $KHCO_3$ (final concentration 50 mM), and 30 or 50 ml medium was used in 120-ml bottles.

The four microorganisms were subcultured as a coculture. The medium contained 1 to 5 mM 3-chlorobenzoate (3-CB) and 2.5 or 5 mM pyruvate. When 3-CB was depleted, extra 3-CB (without pyruvate) was added. In experiments 3 and 4, additional amounts of pure cultures were used. The pure cultures were cultivated with 20 to 40 mM pyruvate plus 1 mM 3-CB (*D. tiedjei*), 40 mM crotonate (*S. buswellii*), H_2/CO_2 (80:20, v:v) plus 1 mM acetate (*M. hungatei*), and 80 to 200 mM acetate plus 1 mM cysteine (*M. concilii*).

Table 1. Immobilization procedures. Specifications for the different experiments.

Experiment	Dripping method	Decane layer	KCl (M)	Temperature	Distribution method	Aseptic	Anaerobic
1A	pump + vessel	+	0.75	10°C	F[a]	+	-
1B	syringe	-	0.1	RT[b]	R[c]	-	+
1C	syringe	+	0.1	RT	R	-	+
2	pump + vessel or syringe	-	0.1	10°C	F	+	-
3	syringe	-	0.1	RT	F	+	-
4	syringe	-	0.1	RT	A[d]	-	+

[a]: Distribution of the beads into bottles with medium was done in a flow chamber
[b]: RT, room temperature
[c]: KCl solution was replaced by medium
[d]: Distribution of the beads into bottles with medium was done in an anaerobic chamber

Immobilization procedures. A carrageenan solution was gently mixed with an equal volume of a bacterial suspension at 37°C, yielding a 2.5% or 2% carrageenan solution (w/v). Different methods were applied to add this mixture dropwise in a solution containing KCl (Table 1). One method was to use a 10 or 20 ml syringe with a needle (diameter 1 mm) (Kierstan & Coughlan, 1985). The other method was to pump the mixture through tubings into a conic vessel (content 50 ml) with a needle (diameter 1 to 2 mm) (Hulst *et al.*, 1985). With the syringe the average diameter of the beads was about 4 mm and with the vessel about 5 mm. In the KCl solution, the beads were allowed to harden during 1 h. Subsequently, the beads were distributed among bottles containing medium. The head space was flushed and one or several substrates were added.

In experiment 1A, 10 g beads (approximately 10 ml) was added to 50 ml medium. Acetate, benzoate, glucose, 3-CB plus pyruvate (all 5 mM) and H_2/CO_2 (80:20, v:v) were added as substrates. Controls contained a suspended culture (10% inoculum) and 3-CB plus pyruvate, 5 mM each. Other controls contained sterile medium in the beads instead of a culture (empty beads) and acetate, benzoate, glucose, or 3-CB plus pyruvate (5 mM). In experiment 1B, 10 ml beads per bottle were used to which 50 ml medium was added. As substrates 5 mM 3-CB plus pyruvate were given. In experiment 1D, 50 ml medium was added to 4 ml beads. The controls contained a suspended culture (4% inoculum). Both the immobilized and suspended cultures were fed 5 mM 3-CB, 0.5 mM benzoate and 0.5 mM pyruvate. In experiment 1, all incubations were done at least in duplicate, except those containing empty beads and the glucose incubations, which served as negative controls.

In experiment 2, 10 g beads were added to a bottle with 50 ml medium. As substrates, 5 mM 3-CB and 1 mM pyruvate were added, or the same substrates with additionally 1 mM benzoate were used. All incubations were done in triplicate. Controls (in duplicate) contained 5 ml of a coculture, with the same substrate combinations as the bottles with beads. The inoculum contained about 5 mg/l protein. The sum of the cell numbers were 1.7×10^8 cells/g beads and 3.3×10^7 cells/ml suspension.

In experiment 3, cultures were diluted in medium without $CaCl_2$, Na_2S, $NaHCO_3$ and vitamins, before they were added to the carrageenan solution, and 5 g beads were added to 50 ml medium. The following substrates were provided: 5 mM 3-CB plus 1 mM pyruvate (duplicate), 5 mM 3-CB plus 1 mM benzoate (duplicate), 5 mM 3-CB (one bottle), and 5 mM benzoate (one bottle). Controls were supplied with the same substrates. The consortium which was used as inoculum contained 7×10^7 *D. tiedjei*, 3×10^8 *S. buswellii*, 5×10^7 *M. hungatei* and 1×10^7 *M. concilii* per ml. The inoculum size in the beads was: 0.1%, 1%, 50% *D. tiedjei*, 0.1%, 1%, 1% *S. buswellii*, 5%, 5%, 1% *M. hungatei* and 5%, 5%, 50% *M. concilii* in series 1, 2 and 3, respectively. In order to obtain higher numbers of two microorganisms, extra cells were supplied from pure cultures with higher cell numbers, resulting in high inoculum sizes without exceeding the available volume, i.e. the volume equal to that of the carrageenan solution. Since the beads occupied 10% of the volume of the medium, the inoculum size calculated for the total volume of the medium was 10 times lower. Controls received the same or a 10 times greater inoculum size in the medium. In the latter case, the average distances between the cells would equal those between the imbedded cells.

In experiment 4, 0.5 to 2.5 l cultures were harvested by centrifugation at 48000 x *g* for *M. hungatei* and 27500 x *g* for all other cultures. The cultures were washed in medium without $CaCl_2$ and vitamins and resuspended in approximately 2 to 3 ml. A consortium suspension was mixed with 5 ml carrageenan solution and supplemented with a similar solution without carrageenan or with a pure culture suspension, until a volume of 10 ml was reached. To 30 ml medium 3 g beads were added (in triplicate). The beads were incubated on a rotary shaker with 5 mM 3-CB, for 1½ days or overnight.

Experiments. In experiment 1, substrate consumption and product formation was measured. Experiment 1A without cells was a control to examine if the concentration of the substrates would be influenced by the presence of carrageenan. In experiment 2, after utilization of the substrates, 3-CB was replenished twice. After the experiment the protein content was determined. The yield was estimated, taking into account the minimal and maximal amounts of 3-CB and protein removed by sampling. In experiment 3, cell numbers were counted in the control incubations. The beads from immobilized consortium incubations were examined microscopically after utilization of the substrates. An interference contrast microscope (Nikon Optiphot) equipped with a CCD camera was used. In experiment 4 the addition of extra *D. tiedjei, S. buswellii* or *M. hungatei* was tested. On successive days the medium and the gas phase were replaced. Substrate was added, and samples were taken during 6 hours. The expected value (E(x)) of the distance (x) from *S. buswellii* to the closest *D. tiedjei* and/or *M. hungatei* cell was calculated with the assumptions that there was no influence of neighbouring *S. buswellii* cells, that cells were distributed homogeneously, and that cells did not occupy a volume themselves, with the equation $E(x) = 0.55396\ C^{-1/3}$. Cell numbers (C) of *D. tiedjei* and/or *M. hungatei* are in cell numbers per μm^3.

Protein determinations. Cell pellets from 2 ml culture were resuspended in 1 ml 1 M NaOH and boiled for 15 min. The samples were treated further according to the protein determination method of Lowry *et al.* (1951). From cultures which would be immobilized, cell pellets were resuspended in 1 ml 0.5 M NaOH. These suspensions were boiled for 30 min. The protein content was determined according to the method of Bradford (1976). The preparation of immobilized cells for protein determination was performed according to the slightly modified method described by Smith *et al.* (1991). At the end of experiment 2 and 4, a known amount of beads was frozen at -70°C and then freeze dried for at least 40 h. Freeze-dried carrageenan beads were incubated in distilled water at 60°C for at least 30 min and thereby dissolved. Then, an aliquot of the suspension was mixed with an equal volume of 1 M NaOH and boiled for 30 min. The protein content was determined according to the method of Bradford (1976). In these experiments, the standard was bovine serum albumin in a solution with the same concentration of carrageenan as the samples.

Cell counts. Suspended cells were counted with a Bürker-Türk counting chamber using a microscope (Wild, Heerbrugg, Switzerland) at a magnification of 400 times.

Analytical methods. Methane, hydrogen and acetate were measured with a Packard-Becker 417 or Chrompack CP9001 gas chromatograph as described by Stams *et al.* (1993). Methane was also measured with a Packard-Becker 417 gas chromatograph with a flame ionization detector, instead of a thermal conductivity detector. A molecular sieve 5A column was used (110 cm X 2.1 mm, Chrompack, Middelburg, The Netherlands) at 70°C. Nitrogen was the carrier gas, with a flow of 20 ml/min. Acetate, pyruvate and lactate were analyzed on an LKB high-performance liquid chromatograph as described previously (Stams *et al.*, 1993). Benzoate and chlorobenzoates were measured on an LKB high-performance liquid chromatograph as described by Tros *et al.* (1996), or on a TSP high-performance liquid chromatograph, with a SpectraSystem P2000 pump, an AS3000 autosampler and a UV1000 UV-detector. The mobile phase was an acetonitrile-5 mM H_2SO_4 mixture with a volume ratio of 35:65. Chlorophenols were measured as described previously (Chapter 2).

RESULTS

Immobilization procedures. Carrageenan is a carbohydrate polymer produced by seaweeds. It is known to meet physiological constraints when it is used as a matrix for imbedding bacteria. In order to obtain spherical strong gel beads, several authors varied the conditions for the actual immobilization step. These variations include cooling below 10°C, use of 0.1 or 0.75 M KCl to collect the beads, and a passage through a decane layer to obtain perfect spheres (Tosa *et al.*, 1979; Wijffels *et al.*, 1991). In the experiments described here we varied the temperature, the KCl concentration, the presence or absence of a decane layer, as well as the dripping method and the distribution method (experiments 1 and 2 in Table 1). The choice of the immobilization method was found to be crucial for the activity of the consortium in the beads (experiments 1 and 2, results not shown). The presence of a decane layer above the hardening solution severely inhibited microbial growth. Additionally, it seemed that 0.75 M KCl led to inhibition of substrate conversion. However, even after being pumped through tubings and a vessel, experiencing a temperature drop from 37°C to 10°C, and being briefly exposed to oxygen, the consortium was still capable of converting 3-CB at a high rate.

Effect of immobilization on substrate conversion and growth. The effect of immobilization on the activity, the specific activity and the maintenance of the activity were studied in growing cultures which received three consecutive additions of 3-CB. After the first substrate addition, the dechlorination rate was lower with immobilized cells than with cells that were suspended (Table 2). Furthermore, the dechlorination rate was lower when benzoate was supplied as a substrate additional to 3-CB and pyruvate than when only the latter two substrates were added. Dechlorination and methane production started immediately in immobilized cultures to which benzoate had been added as well as in the suspended cultures with and without benzoate, which served as controls. After the third addition of 3-CB, no benzoate accumulation occurred, which implied that the benzoate oxidation rates were equal to the dechlorination rates. When 3-CB had been added for the third time, all immobilized cultures immediately started to dechlorinate and to form methane, while only one of the suspended cultures converted 3-CB during the first four days. In summary, the dechlorination rates and methane production rates rather decreased than increased upon immobilization, but the activity of the immobilized cultures was maintained for a longer time period.

Table 2. 3-CB conversion rates, final protein contents and estimated yields ($Y_{est.}$) of the consortium immobilized and supplied three times with 5 mM 3-CB. In the first feeding also pyruvate or pyruvate plus benzoate were present (0.5 mM).

	First feeding 3-CB (mmol/l day)	Third feeding 3-CB (mmol/l day)	Protein (mg/l)	$Y_{est.}$ (g protein/mol 3-CB)
Immobilized cells (3-CB, pyruvate)[a]	0.28 (0.03)[b]	0.68 (0.14)	77 (16)	4.6-8.0
Controls	0.38 (0.06)	0.72 90.02)	57 (2)	2.8-4.8
Immobilized cells (3-CB, pyruvate, benzoate)	0.26 (0.02)	0.85 (0.12)	114 (13)	7.1-11.6
Controls[c]	0.31 (0.01)	0.48	53	2.8-4.8

[a]: because two of the six incubations had low activity, they were supplied with 3-CB only once or twice. Therefore, average values of the 3-CB conversion rate after the third feeding, the protein content, and the yield are based on four replicates

[b]: standard deviation in parentheses

[c]: one of the two incubations was not active after the third feeding

Growth of the consortium in a carrageenan matrix. To study the pattern of colony formation, the consortium was entrapped in carrageenan at low cell densities. Additional cells of one of the hydrogen consumers were imbedded to provide a low hydrogen partial pressure for benzoate oxidation by *S. buswellii*. Because the maximum specific growth rate of *M. concilii* is low compared to that of the other three consortium members, *M. concilii* was also added in higher numbers, relative to the ratio for a stable consortium (Chapter 4).

Methane production was only observed in immobilized consortia containing elevated cell numbers of *D. tiedjei* and *M. concilii*. The two active immobilized consortia had been either fed with 3-CB and pyruvate or with 3-CB and benzoate. Colonies of *D. tiedjei*, *S. buswellii*, and *M. hungatei* were visible (Figure 2). The shapes of the colonies of *S. buswellii* were globular, while those of *D. tiedjei* were moon-shaped, and the colonies of *M. hungatei* were rope-like. Furthermore, single *D. tiedjei* cells and filaments of *M. concilii* were visible. It was examined whether cells imbedded in the proximity of a syntrophic partner would grow, and cells far from a syntrophic partner would not. However, no spatial orientation of colonies of *S. buswellii* and *D. tiedjei* or *M. hungatei* was observed.

Colony formation was compared with the changes in the cell numbers of the suspended cultures, serving as controls. In all control cultures, cell numbers of *S. buswellii* and *M. hungatei* increased. In the controls with a 10 times higher inoculum size than in the immobilized cultures, cell numbers of *D. tiedjei* and *M. concilii* stayed constant, while in the controls with the same inoculum size as the immobilized consortium, *D.tiedjei* numbers clearly increased and *M. concilii* numbers hardly increased. The presence of colonies of *D. tiedjei, S. buswellii,* and *M. hungatei,* and the absence of colonies of *M. concilii* in the beads was in accordance with the inoculum size in the beads and with the increase in cell numbers in the controls with the same inoculum size as the immobilized consortium.

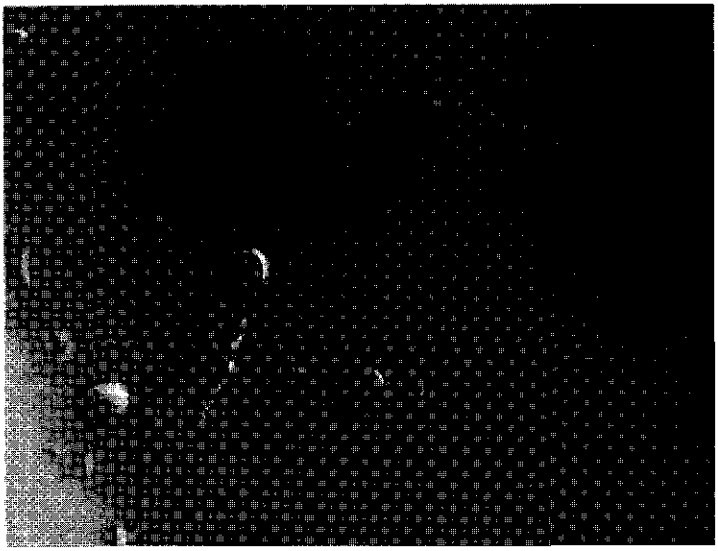

Figure 1. Microcolonies of *S. buswellii* (large, globular), *M. hungatei* (large, rope-like), and *D. tiedjei* (small, moon-shaped) grown in a κ-carrageenan gel bead with 5 mM 3-CB and 1 mM pyruvate.

Table 3. Substrate conversion rates [µmol/h] of the consortium, immobilized in high biomass densities. Substrates were provided in a concentration of 1 mM unless stated otherwise. The values in parentheses are standard deviations. The protein contents per bottle were, in mg, with extra *D. tiedjei* 20, controls 7.6, with extra *S. buswellii* 24, controls 12, with extra *M. hungatei* 5.3, controls 4.5. Values are marked if the substrate conversion rate was significantly altered in the presence of additional cells (*, increase, #, decrease, $\alpha = 0.10$).

	extra *D. tiedjei*		extra *S. buswellii*		extra *M. hungatei*	
Substrate	-	+	-	+	-	+
3-CB	9.0 (0.5)	10.2 (0.4)*	8.9 (0.1)	8.2 (0.5)#	5.9 (0.2)	6.5 (0.2)*
3-CB (5 mM)	13.3 (0.1)	16.8 (0.3)*	12.8 (0.1)	11.1 (0.7)#	6.2 (0.5)	7.7 (1.1)*
3-CB (with benzoate)			9.5 (0.3)	9.2 (0.3)		
3-CB (with crotonate)			5.9 (1.0)	7.5 (0.8)*		
benzoate	14.8 (0.7)	16.3 (0.9)*			7.5 (1.3)	7.9 (1.8)
benzoate (5 mM)	17.4 (1.5)	19.5 (0.7)*	14.1 (0.9)	35.7 (0.6)*		
benzoate (with 3-CB)			18.2 (0.5)	21.9 (3.7)		

Activity tests with varying cell ratios. The effect of the cell numbers of syntrophic partners on the substrate degradation rate was tested in beads in which the consortium was immobilized in high cell numbers per volume. Additionally, extra cells were added from a pure culture of *D. tiedjei*, *S. buswellii*, or *M. hungatei*. Since high cell numbers were used, it was assumed that cell growth was negligible and that thus the cell ratios and interbacterial distances stayed constant during the experiments.

A high activity was achieved within one or two days. In contrast to incubations of growing cultures, these cultures did not require a substrate additional to 3-CB. The intermediary products benzoate and acetate, which accumulated in other experiments, were only found in very low concentrations.

In general, higher substrate conversion rates were found with benzoate than with 3-CB as the substrate (Table 3). In the presence of benzoate, 3-CB dechlorination rates were higher than in the absence of benzoate, while in the presence of crotonate, the 3-CB dechlorination rates were lower. After the addition of a dichlorobenzoate, the conversion of 3-CB or benzoate on the subsequent day(s) was inhibited by the remaining amount of dichlorobenzoate (Table 4).

Table 4. Substrate conversion rates [μmol/h] of the consortium, immobilized in high biomass densities. Substrates were provided in a concentration of 1 mM. The values in parentheses are standard deviations. The protein contents per bottle are denoted above Table 3. Values are marked if the substrate conversion rate was significantly altered in the presence of additional cells (*, increase, $\alpha = 0.10$).

	extra *D. tiedjei*		extra *S. buswellii*		extra *M. hungatei*	
Substrate	-	+	-	+	-	+
3,5-DCB	6.2 (0.4)	9.1 (0.2)*	5.3 (2.1)	5.8 (0.4)	1.4 (0.2)	1.7 (0.5)
3,5-DCB (with acetate)	6.3 (0.3)	7.4 (0.1)*				
2,5-DCB					0.28 (0.06)	0.41 (0.09)*
3-CB					1.4 (0.1)	1.7 (0.5)
after 2,5-DCB					1.9 (0.3)	2.1 (0.6)
benzoate after 3,5-DCB					3.5 (0.9)	5.5 (1.0)*

The addition of extra cells of one species resulted in an increase (factor 1.1 to 2.5) of the substrate conversion in many cases (Tables 3 and 4). The calculated change of the inverse of the minimal distance (d^{-1}) from *S. buswellii* to the nearest syntrophic partner cell correlated with higher 3-CB dechlorination rates in the presence of additional *M. hungatei* cells (factor 1.1 to 1.2) and the lower 3-CB dechlorination rates in the presence of additional *S. buswellii* cells (factor 0.9) (Table 5). In the latter case, lower cell numbers of *D. tiedjei*, *M. hungatei* and *M. concilii* were present in the incubations with additional *S. buswellii* cells than in the control incubations. With additional *D. tiedjei* cells, the d^{-1} from *S. buswellii* to *D. tiedjei* increased with a factor of 3.1 and the d^{-1} from *S. buswellii* to *D. tiedjei* or *M. hungatei* increased with a factor of 2.6, while the 3-CB and 3,5-DCB dechlorination rates only increased with a factor of approximately 1.2. However, when 3,5-DCB was the substrate, the 3-CB dechlorination rate in the presence of additional *D. tiedjei* cells increased with a factor of 2.0 with (not shown) and 1.6 without acetate as a cosubstrate (Figure 2). The largest effects on the substrate conversion rates were found with additional *S. buswellii* cells, when benzoate was the substrate. In these incubations, the benzoate conversion rate increased with a factor of 2.5. The numbers and therefore the surface area of the *S. buswellii* cells were 7.9 times larger when extra *S. buswellii* cells were added than in the control incubations. Together with the decrease in the d^{-1} from *S. buswellii* to a syntrophic partner (factor 0.9), this would allow a maximal increase of the benzoate oxidation rate with a factor of 7.2, if there were no diffusion limitations or limitations in the hydrogen consumption rate by *M. hungatei*. Unexpectedly, extra *D. tiedjei* resulted in a higher benzoate conversion rate in the absence of 3-CB.

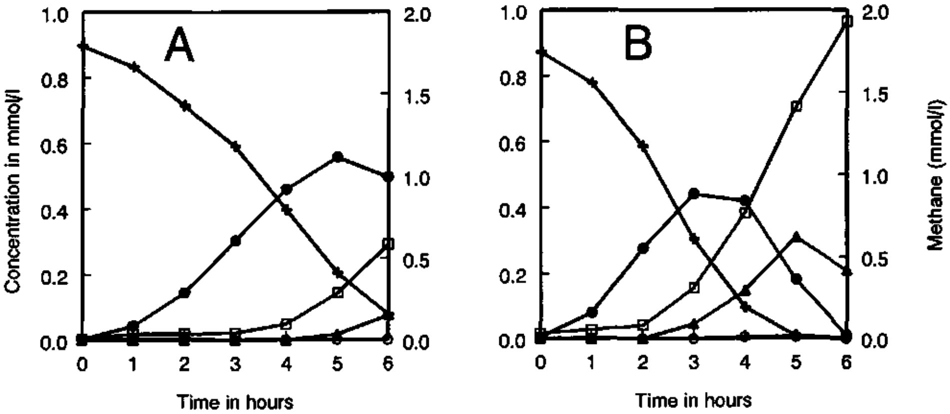

Figure 2. Conversion of 3,5-dichlorobenzoate by the concentrated consortium immobilized in κ-carrageenan gel beads in the absence (A) and presence (B) of additional *D. tiedjei* cells. ×:3,5-dichlorobenzoate; ●:3-CB; O:benzoate; ▲ :acetate; □:methane.

Table 5. The calculated expected value of the distance in µm from *S. buswellii* to the closest *D. tiedjei* and/or *M. hungatei* cell in the experiments in which the consortium was immobilized in high biomass densities, with or without extra cells of one species.

	Extra *D. tiedjei*		Extra *S. buswellii*		Extra *M. hungatei*	
	-	+	-	+	-	+
From *S. buswellii* to *D. tiedjei*	4.0	1.3	4.4	4.8	4.0	4.0
From *S. buswellii* to *M. hungatei*	4.6	4.6	5.4	5.9	4.4	3.0
From *S. buswellii* to either hydrogen consumer	3.4	1.3	3.8	4.1	3.3	2.7

DISCUSSION

A methanogenic tetraculture, degrading 3-CB, was successfully immobilized in κ-carrageenan gel beads. Depending on the purpose of the experiment, the immobilization method was varied. Although a decane layer above the hardening solution was not toxic to the bacteria in other studies (Buitelaar *et al.*, 1989; Wijffels *et al.*, 1991), the presence of a decane layer severely inhibited bacterial activity in our experiments. Therefore, the beads that were used in the experiments described here were not completely spherical. Growth of the microbial cells in the beads was confirmed with protein determinations, an increase of the 3-CB-degrading activity in time (Table 2), and colony formation (Figure 1). The appearance of the three-dimensional colonies of *M. hungatei* differed from the round, lobed surface colonies, grown on agar (Ferry *et al.*, 1974).

Especially when high initial cell numbers were used, the 3-CB degradation rate was very high, up to 0.56 mmol l^{-1} h^{-1} in the presence of extra *D. tiedjei* cells. For practical applications, in for instance wastewater treatment, carrageenan seems not to be suitable, because natural gel materials, particularly carrageenan, are dependent of specific counterions and have a low stability when exposed to mechanical forces. For such purposes, synthetic gels could be used, because they do not have these disadvantages (Leenen *et al.*, 1996).

Immobilized in high cell numbers per volume, the consortium converted benzoate at a higher rate than 3-CB, which is opposite to the findings with the triculture consortium described by Dolfing and Tiedje (1991). Since in our experiments relatively high numbers of *M. hungatei* were present in balanced cultures (Chapter 4), competition occurred for hydrogen between *D. tiedjei* and *M. hungatei*. With extra cells of *M. hungatei* or *D. tiedjei*, probably the hydrogen partial pressure decreased, resulting in a higher benzoate oxidation rate, a higher hydrogen production rate and therefore a higher 3-CB dechlorination rate. When benzoate was the substrate, there was sufficient benzoate supply and sufficient hydrogen removal capacity by *M. hungatei* to allow a higher benzoate oxidation rate by *S. buswellii* than when 3-CB was the substrate. This is in accordance with the observations that with additional *S. buswellii* cells, but not with additional *M. hungatei* cells, a higher benzoate conversion rate was observed in comparison with the control incubations.

As was expected, in the experiments in which high cell numbers were immobilized, the 3-CB dechlorination rate was affected by the addition of the cosubstrates benzoate and crotonate. While addition of benzoate resulted in a larger amount of hydrogen available for dechlorination, and thus a higher dechlorination rate, a lower dechlorination rate was observed after addition of crotonate which may have served as an extra electron sink. Approximately one third of the hydrogen was used for 3-CB dechlorination, one third for hydrogenotrophic methanogenesis and one third for crotonate reduction to butyrate (not shown). The exact ratio was dependent on the relative cell numbers, because with additional *S. buswellii* cells and fewer *D. tiedjei*, *M. hungatei* and *M. concilii* cells, less methane and more butyrate accumulated in comparison with in the control incubations.

The specific substrate conversion rates for 3-CB and benzoate from incubations with high biomass densities were higher than those reported for a triculture consortium (Dolfing & Tiedje, 1991). In the control incubations, specific 3-CB dechlorination rates were in the range of 0.8 to 1.8 mmol g^{-1} total protein h^{-1} and specific benzoate oxidation rates were between 1.2 and 2.3 mmol g^{-1} total protein h^{-1}. The specific activities for 3-CB were somewhat higher than Dolfing and Tiedje (1991) reported, namely 1.0 mmol g^{-1} total protein h^{-1}, while for benzoate the activities of the tetraculture were two to four times as high as the 0.6 mmol g^{-1} total protein h^{-1} reported by Dolfing and Tiedje (1991). The higher specific activities of cultures containing high cell numbers, also compared to growing tetracultures (experiment 2), are probably due to the reduction of the interbacterial distance, although effects of the immobilization method and protein determination method may not be excluded. In experiment 2, the cells were briefly exposed to oxygen during the immobilization, and cells that died as a result of the oxygen exposure did contribute to the protein content.

In this study, mass transfer limitations have not been considered in the interpretation of the specific activity changes when extra cells of one species were added. In general, effects of biomass imbedded in a polymer matrix have not been quantified adequately. However, most of the substrates and intermediary products (3-CB, benzoate, acetate, crotonate and butyrate) have similar diffusion coefficients. This excludes the possibility that in the presence of extra *D. tiedjei* cells, the 3-CB dechlorination rate was limited by diffusion through the gel, since the substrate conversion rate was higher when benzoate was the substrate than when 3-CB was the substrate. It is possible that in the presence of extra *S. buswellii* cells, the conversion rate of benzoate was limited by diffusion through the gel.

The work presented here shows that the effects of individual members of syntrophic consortia can be studied in immobilized cell systems, as the interbacterial distances can be fixed, enabling the quantification of hydrogen diffusional effects.

Acknowledgements

We would like to thank André van Lammeren and Maarten de Gee for their assistance to the microscopical examinations and mathematical calculations.

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6. IMMOBILIZATION IN GEL BEADS

Chapter 7

SUMMARY AND CONCLUDING REMARKS

Pollution of the environment with chlorinated organic compounds mainly results from (agro)industrial activity. In many studies, biodegradation is examined under anaerobic conditions, because highly chlorinated compounds are more easily degradable under anaerobic than under aerobic conditions (Field *et al.*, 1995). Problems arise because these compounds can inhibit methane formation and the microorganisms which are able to degrade these compounds may be absent in the methanogenic environment, like for instance wastewater treatment plants. This thesis concentrates on chlorinated aromatic compounds and aims to examine their biodegradation kinetics by methanogenic consortia. The biochemical degradation mechanisms of aromatic compounds under anaerobic conditions differ greatly from the aerobic conversions, because under aerobic conditions, oxygen is a reactant, while under anaerobic conditions, other activation steps are involved. The degradation of chlorinated aromatic compounds requires the elimination of chlorine atoms. Under methanogenic conditions, the initial degradation step is a reductive dechlorination. This conversion may yield biological useful energy, if the chlorinated compound is utilized as a terminal electron acceptor. The product of the reductive dechlorination step is an aromatic compound. For some of these compounds, for instance benzoate and phenol, the oxidation to acetate, carbon dioxide and hydrogen is endergonic under standard conditions. Therefore, this conversion can only occur when hydrogen-consuming organisms are present, which in turn depend on hydrogen-producers for substrate supply. This relationship is called syntrophism. Methanogenic archaea can act as syntrophic partners. Hydrogen can also be an electron donor for some dechlorinating microorganisms. Consequently, for the complete degradation of chlorinated aromatic compounds under methanogenic conditions, a consortium of different types of microorganisms is needed. Furthermore, the distance for hydrogen diffusion between syntrophic partners determines the conversion rate of the aromatic compounds. The interbacterial distances are small when the microorganisms exist as aggregated biomass in and/or on carrier materials, or as flocs or granules without the need for carrier materials. In upflow anaerobic sludge blanket

(UASB) reactors, the microorganisms are auto-immobilized as granular sludge. These aspects are dealt with in Chapter 1.

Chapter 2 describes the toxicity of monochlorophenols (MCPs) to granular sludge from a UASB reactor. Methane production from a mixture of acetate, propionate and butyrate was measured to test the influence of MCPs on the activity of the sludge. Since other substrates in addition to acetate were used, the sensitivity of syntrophs was also determined. The concentration, the isomeric form and the exposure time of the MCP determined the inhibition of methane formation. Different sensitivities were found for sludges, developed in reactors which had received similar influents, which may be due to differences in the population composition or the granule size. After cold storage of the sludge, the methane production rate decreased, and the sensitivity towards 2-chlorophenol (2-CP) increased. However, an activation period after cold storage could reduce the toxic effect. Inhibition of methane production occurred by MCP concentrations that were in the mM-range. A similar sensitivity to 2-CP was found for a 3-chlorobenzoate(3-CB)-degrading consortium (see Chapters 4, 5 and 6). Monochlorobenzoates (MCBs) were hardly toxic to the methanogenic granular sludge.

The *biodegradation* of MCPs was studied in samples from different anaerobic environments, most of which were granular sludges from UASB reactors (Chapter 3). 2-CP was transformed by granular sludge, a sediment mixture and a peat slurry. The first intermediary products which accumulated were phenol and most probably 3-CB. This indicated that 2-CP was reductively dechlorinated to phenol as well as carboxylated and dehydroxylated to 3-CB. Two enrichment cultures were obtained from the granular sludge. One converted 2-CP to phenol and later to 3-CB. Unfortunately, this culture lost its 2-CP-degrading activity. The other dechlorinated 2-CP and dichlorophenols specifically at the *ortho*-position, and did not form 3-CB.

Chapters 4, 5 and 6 describe the dechlorination and subsequent mineralization of the model compound 3-CB by a defined consortium, consisting of four microorganisms. *Desulfomonile tiedjei* reductively dechlorinated 3-CB to benzoate, *Syntrophus buswellii* oxidized benzoate syntrophically to acetate and hydrogen, *Methanospirillum hungatei* formed methane from hydrogen and carbon dioxide, and *Methanosaeta concilii* converted acetate to methane and carbon dioxide. The growth conditions were varied to optimize the dechlorination as well as the mineralization of 3-CB (Chapter 4). In addition, the effects of carrier materials (Chapter 5) and of the immobilization of the consortium in a gel (Chapter 6) are described. In Chapter 4, the composition of the 3-CB-degrading consortium is described. The growth medium was optimized to obtain balanced growth of the consortium. *D. tiedjei* utilized hydrogen for the dechlorination of 3-CB to benzoate, while hydrogen was only produced when *S. buswellii* oxidized benzoate. Therefore, to initiate the 3-CB conversion, an additional electron donor, e.g. pyruvate, was required. This contrasts with observations of Dolfing and Tiedje (1986), who studied a consortium consisting of *D. tiedjei*, the benzoate-oxidizing strain BZ-2 and *Methanospirillum* strain PM-1, and observed no dependence of an additional electron donor. Furthermore, the substrate spectrum, the toxicity of 3-CB towards individual consortium members and the necessity for buffering capacity were studied. Based on cell counts it was found that a stable consortium consisted of about 18% *D. tiedjei*, 70% *S. buswellii*, 10% *M. hungatei*, and 3% *M. concilii*.

Chapter 5 describes the effects of carrier materials on the 3-CB-degrading consortium. The consortium was incubated with 3-CB and pyruvate in the presence of a range of carrier materials. In the presence of polystyrene, the length of the lag phase for dechlorination was decreased. In the presence of vermiculite and granular sludge the consumption rates of pyruvate by *D. tiedjei* and acetate by *M. concilii* were higher than in the their absence. As a result, these three carrier materials decreased the time period for complete mineralization of 3-CB. Attachment of the consortium cells to the carrier materials was not an explanation for the stimulatory effects, because in pure cultures, similar effects were observed. Furthermore, cells adhered only weakly to glass and teflon sheets, and the majority of the cells in the consortium grew in suspension. The conditions for aggregation or adherence may not have been optimal, because Ahring *et al.* (1992) described the incorporation of *D. tiedjei*, *S. buswellii* and *M. hungatei* in granular sludge in a UASB reactor. On the other hand, Wu *et al.* (1996) described aggregation of syntrophic fatty acid-oxidizing bacteria with a few species of methanogens, but not with *M. hungatei*. Nevertheless, an improvement of the pyruvate and acetate consumption rates or the shortening of the lag phase for dechlorination could reduce the time for complete degradation of 3-CB up to 50%.

Immobilization of the consortium in κ-carrageenan gel beads is described in Chapter 6. It was demonstrated that 3-CB degradation and growth occurred in the gel beads. Subsequently, cells grown in suspension were concentrated and immobilized in different ratios. An increased dechlorination rate was achieved with extra *D. tiedjei* cells (for 3-CB and 3,5-dichlorobenzoate) and with extra *M. hungatei* cells (for 3-CB). Addition of extra *S. buswellii* cells only resulted in a higher specific activity of *D. tiedjei* cells. In that case, immobilized cell numbers of *D. tiedjei*, *M. hungatei*, and *M. concilii* were lower than without extra *S. buswellii*. The specific activity was around 1 x 10^{-15} mol 3-CB *D. tiedjei* $cell^{-1}$ h^{-1} in most of these experiments. In pure cultures with pyruvate, the dechlorinating activity of *D. tiedjei* is 0.1 pmol 3-CB $cell^{-1}$ day^{-1}, i.e. 4 x 10^{-15} mol 3-CB $cell^{-1}$ h^{-1} (Tiedje *et al.*, 1987). The interbacterial distances were determined by the cell densities. Therefore, a variation of the cell ratios had to affect the rate of the hydrogen diffusion between *S. buswellii* and the two hydrogen consumers, *D. tiedjei* and *M. hungatei*. The minimal interbacterial distances were calculated and compared with the substrate conversion rates. Indeed, the changes in the calculated distances could, at least partially, explain the changes in the measured substrate conversion rates. Unfortunately, better growth of cells in the proximity of syntrophic partner cells could neither be established with carrier materials (Chapter 5) nor in gel beads (Chapter 6). The performance of the immobilized consortium was not tested in continuous flow reactors (which would be more like wastewater treatment plants), but in batch cultures a high activity was achieved (up to 0.56 mM/h) and only low concentrations of intermediary products accumulated transiently.

In conclusion, the toxicity tests depict the sensitivity of granular sludge to MCPs and MCBs. Stored sludge which in practice is used again would perform better when the exposure to toxic compounds is preceded by a reactivation period. Furthermore, as has been frequently observed, 2-CP was most easily biodegraded, while the other two MCPs were not or hardly degraded. Methanogenic degradation of 2-CP has been observed to proceed via phenol. Here we found another pathway via 3-CB. In addition, it was possible to grow a completely mineralizing consortium on 3-CB. However, an external electron donor was important, and, under starting conditions, indispensable. The consortium did not aggregate nor adhere to surfaces, but it could convert 3-CB at a high rate when immobilized in a gel matrix.

7. SUMMARY

Dechlorination could be accelerated by an increase of the cell numbers of the dechlorinators or the hydrogenotrophic methanogens and not of the syntrophic benzoate oxidizers. In the presence of carrier materials, stimulation of the growth of the dechlorinating bacteria or the aceticlastic methanogens decreased the time period required for mineralization.

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7. SUMMARY

Chapter 8

SAMENVATTING

Milieuvervuiling met gechloreerde verbindingen is vooral een gevolg van (agro)industriële activiteit. Veel onderzoek naar de omzettingen van gechloreerde verbindingen door micro-organismen is gericht op de afbraak onder anaërobe (zuurstofloze) omstandigheden, omdat hoog-gechloreerde verbindingen (verbindingen die veel chlooratomen bevatten) onder anaërobe omstandigheden beter afbreekbaar zijn dan onder aërobe omstandigheden. Er ontstaan problemen omdat deze verbindingen methaanvorming (biogasvorming) kunnen remmen en de micro-organismen die deze verbindingen kunnen afbreken niet in alle gevallen aanwezig zijn. Deze micro-organismen zijn bijvoorbeeld vaak afwezig in afvalwaterzuiveringsinstallaties. In dit proefschrift is het onderzoek gericht geweest op een groep gechloreerde aromaten (benzoaten en fenolen) en heeft als doel gehad de afbraak van die verbindingen door methaanvormende consortia van micro-organismen te bestuderen. De biochemische mechanismen voor de afbraak van aromaten onder anaërobe omstandigheden verschillen sterk van die onder aërobe omstandigheden, omdat onder aërobe omstandigheden zuurstof een reactant in de afbraak is en er onder zuurstofloze condities (dus) andere reactiemechanismen nodig zijn om de aromatische ring af te breken. Bij de afbraak van gechloreerde aromaten komt er een extra stap bij, waarbij het chlooratoom verwijderd wordt. Onder omstandigheden waarbij methaan wordt gevormd (methanogene condities) begint de afbraak van chloorverbindingen met een reductieve dechlorering. Het chlooratoom wordt vervangen door een waterstofatoom. Deze omzetting kan biologisch bruikbare energie opleveren als de chloorverbinding als terminale electronenacceptor van een ademhalingsketen wordt gebruikt. Na de reductieve dechlorering blijft er een aromaat over (bijvoorbeeld benzoaat of fenol), die vervolgens geoxideerd wordt tot acetaat, waterstof en kooldioxide. Deze oxidatie van benzoaat en fenol levert geen energie onder standaardomstandigheden, maar kost juist energie. De reactie kan daardoor alleen verlopen als waterstof wordt weggenomen door een waterstofconsumerend partner-organisme, dat zelf voor de aanvoer van waterstof weer afhankelijk is van de waterstofproducent. Er is hier sprake van een syntrofe omzetting. De micro-organismen die als syntrofe partner van een benzoaat- of fenol-oxiderende bacterie kunnen optreden, zijn bijvoorbeeld methanogene archaea. Verder kunnen ook sommige dechlorerende bacteriën waterstof als electronendonor gebruiken. Bij de volledige afbraak van een gechloreerde verbinding tot kooldioxide, methaan en chloride is dus een consortium van verschillende micro-organismen nodig. Tenslotte bepaalt de afstand tussen de syntrofe partners, in verband met de afstand voor de waterstofdiffusie, de omzettingssnelheid van de aromatische verbinding. De afstanden tussen de bacteriën zijn kleiner als de micro-organismen niet vrij zweven in de vloeistof, maar aggregaten vormen op en/of in dragers of, zonder dragers, als vlokken of korrels. In anaërobe opstroomreactoren, de zogenaamde UASB-reactoren (een afkorting van Upflow Anaerobic Sludge Blanket reactor), aggregeren de micro-organismen spontaan tot een compacte biomassa, het korrelslib. Deze literatuurgegevens worden in Hoofdstuk 1 besproken.

Hoofdstuk 2 beschrijft de toxiciteit van monochloorfenolen (MCPs) voor korrelslib uit een UASB-reactor. Als maat voor de invloed van MCPs op de activiteit van het korrelslib werd methaanvorming uit een mengsel van acetaat, propionaat en butyraat gebruikt. Ook kon door het gebruik van meer substraten dan alleen acetaat ook de gevoeligheid van syntrofen voor MCPs worden bepaald. De remming van methaanvorming was afhankelijk van de concentratie, de MCP-isomeer en de duur van de blootstelling. Slibmonsters uit reactoren die vergelijkbaar afvalwater ontvingen, verschilden in hun gevoeligheid voor MCPs. Deze waarnemingen zijn mogelijk veroorzaakt door verschillen in de soortsamenstelling van de bacteriën of een ongelijke korrelgrootte. Het slib werd koud bewaard en opnieuw getest. De methaanvormingssnelheid was lager en de gevoeligheid voor 2-CP hoger. Echter, door een reactiveringsperiode na koude opslag verminderde het toxische effect. Remming van methaanvorming door MCPs vond plaats in het mM-gebied.

De afbraak van MCPs werd bestudeerd in monsters uit verschillende methanogene milieus, hoofdzakelijk korrelslib uit verschillende UASB-reactoren (Hoofdstuk 3). 2-CP werd omgezet door korrelslib, sedimentmateriaal en veen. De eerste intermediaire stoffen die ophoopten waren fenol en waarschijnlijk 3-chloorbenzoaat (3-CB). Dit wees erop dat 2-CP zowel reductief gedechloreerd werd tot fenol als gecarboxyleerd en gedehydroxyleerd tot 3-CB. Er werden twee ophopingen uit het slib verkregen. Een daarvan zette 2-CP eerst om in fenol en later in 3-CB. Deze cultuur verloor na verloop van tijd zijn 2-CP-afbrekende activiteit. De andere dechloreerde 2-CP en dichloorfenolen specifiek op de *ortho*-plaats en vormde geen 3-CB.

De Hoofdstukken 4, 5 en 6 beschrijven de dechlorering en de daaropvolgende mineralisatie (tot kooldioxide, methaan en chloride) van de modelverbinding 3-CB door een gedefinieerd consortium van vier soorten micro-organismen. *Desulfomonile tiedjei* dechloreerde 3-CB reductief tot benzoaat, *Syntrophus buswellii* oxideerde benzoaat syntroof tot acetaat en waterstof, *Methanospirillum hungatei* vormde methaan uit waterstof en kooldioxide en *Methanosaeta concilii* zette acetaat om in methaan en kooldioxide. Om de dechlorering en de uiteindelijke mineralisatie van 3-CB te optimaliseren werden de groeicondities gevarieerd (Hoofdstuk 4). Daarnaast werden de effecten van dragermaterialen (Hoofdstuk 5) en van de inbedding van het consortium in een gel (Hoofdstuk 6) bestudeerd. In Hoofdstuk 4 wordt de constructie van het 3-CB-afbrekende consortium beschreven. Voor een evenwichtige groei van de vier micro-organismen van het consortium werd het medium geoptimaliseerd. *D. tiedjei* gebruikte waterstof als electronendonor voor de dechlorering van 3-CB, terwijl de productie van waterstof alleen plaatsvond als *S. buswellii* benzoaat oxideerde. Voor het opstarten van de 3-CB-omzetting moest daarom een extra electronendonor worden toegevoegd, bijvoorbeeld pyruvaat. Verder werden het substraatspectrum, de toxiciteit van 3-CB voor de individuele soorten uit het consortium en het effect van een buffer bestudeerd. Een evenwichtig consortium bestond uit ongeveer 18% *D. tiedjei*, 70% *S. buswellii*, 10% *M. hungatei* en 3% *M. concilii* als percentage van het aantal cellen.

In Hoofdstuk 5 worden de effecten van dragermaterialen op het 3-CB-afbrekende consortium beschreven. Het consortium zette 3-CB en pyruvaat om in aanwezigheid van een aantal dragermaterialen. In aanwezigheid van polstyreenbollen was de aanloopfase voor de dechlorering begon verkort. In aanwezigheid van gemalen vermiculiet of dood korrelslib zette *D. tiedjei* pyruvaat sneller om en vormde *M. concilii* sneller methaan uit acetaat. Als gevolg hiervan duurde de volledige mineralisatie van 3-CB in aanwezigheid van polystyreen, vermiculiet of slib korter. De stimulerende werking van deze materialen kon niet verklaard worden door hechting van de cellen aan de dragermaterialen (en dus een verkorte afstand tussen de syntrofe partners), want in reinculturen traden vergelijkbare effecten op. Bovendien hechtten de cellen slecht aan glasplaatjes en teflonplakjes. Verder groeiden er evenveel cellen vrij zwevend in de vloeistof met dragermateriaal als zonder. Mogelijk waren de omstandigheden voor aggregatie of hechting aan dragers niet optimaal. Door de versnelde omzetting van pyruvaat en acetaat of de verkorting van de aanloopfase voor dechlorering kon niettemin de benodigde tijd voor volledige mineralisatie van 3-CB tot 50% worden teruggebracht.

In Hoofdstuk 6 werd onderzocht hoe het 3-CB-afbrekend consortium kon worden ingebed in gelbolletjes, bestaande uit κ-carrageen (een agar-achtig polysaccharide). 3-CB-afbraak en celgroei in de gelbollen werden aangetoond. Vervolgens werden voorgegroeide cellen geconcentreerd en in verschillende verhoudingen in de gel ingebed. Een hogere dechloreringssnelheid werd bereikt met extra *D. tiedjei*-cellen (met 3-CB en 3,5-dichloorbenzoaat) en met extra *M. hungatei*-cellen (met 3-CB). De toevoeging van extra *S. buswellii*-cellen leidde alleen tot een hogere specifieke activiteit van *D. tiedjei*-cellen. In dat geval waren de aantallen ingebedde cellen van *D. tiedjei, M. hungatei* en *M. concilii* lager dan in de bollen zonder extra *S. buswellii*-cellen. Met de celdichtheden veranderen ook de afstanden tussen de cellen. Dit betekent dat met een verandering in de verhoudingen tussen de aantallen van de verschillende soorten ook de waterstofdiffusiesnelheid van *S. buswellii* naar *D. tiedjei* en *M. hungatei* veranderde. De minimale interbacteriële afstanden werden berekend en vergeleken met de gemeten substraatomzettingssnelheden. Inderdaad konden de veranderingen in de berekende afstanden de veranderingen in de substraatom-zettingssnelheden ten minste deels verklaren. Helaas kon niet worden aangetoond dat de nabijheid van syntrofe partnercellen leidde tot een verhoogde groeisnelheid, noch met dragermaterialen (Hoofdstuk 5), noch in gelbollen (Hoofdstuk 6). Het gedrag van het ingebedde consortium is niet in continue reactoren getest, die een beter modelsysteem zouden zijn voor afvalwaterzuiveringsinstallaties. Niettemin werden in batchculturen hoge omzettingssnelheden behaald (tot 0,56 mM per uur) en tussenproducten hoopten zich tussentijds in slechts lage concentraties op.

De belangrijkste conclusies kunnen als volgt worden samengevat. De uitgevoerde toxiciteitstesten geven een beeld van de gevoeligheid van korrelslib voor MCPs. Koud bewaard slib was na een reactivatie voorafgaande aan de blootstelling aan toxische stoffen minder gevoelig. Hiervan zou gebruik gemaakt kunnen worden bij het (her-)opstarten van een UASB-reactor. Verder werd, zoals in verschillende andere studies ook is gevonden, 2-CP het makkelijkst afgebroken, terwijl 3-CP en 4-CP niet of nauwelijks afgebroken werden. Naast de bekende methanogene afbraakroute voor 2-CP via fenol werd tevens een route via 3-CB gevonden. Op 3-CB kon een consortium groeien, dat 3-CB volledig mineraliseerde. Echter, de aanwezigheid van een electronendonor was van belang en in het begin noodzakelijk. Het consortium aggregeerde niet en hechtte niet aan dragermaterialen, maar ingebed in gelbollen kon het 3-CB met een hoge snelheid omzetten. Door de toevoeging van extra cellen van de dechlorerende bacteriën of van de waterstofverbruikende methaanbacteriën, maar niet door toevoeging van de benzoaat-omzettende bacteriën, kon de dechloreringssnelheid worden verhoogd. In aanwezigheid van dragermaterialen werden dechlorerende bacteriën en acetaat-afbrekende methaanbacteriën gestimuleerd, hetgeen leidde tot een verkorting van de benodigde tijd voor mineralisatie.

8. SAMENVATTING

CURRICULUM VITAE

Karin Alexandra Maarsen werd op 2 juli 1968 te Amsterdam geboren. Ze behaalde het Atheneum-diploma aan de Scholengemeenschap Snellius te Amstelveen in 1986, en begon toen met de studie Biologie aan de Vrije Universiteit te Amsterdam. Tijdens de doctoraalfase specialiseerde ze zich in de moleculaire en cellulaire biologie, met het accent op microbiologie. Een microbieel fysiologische stage werd gelopen aan de Vrije Universiteit en daarbij werden symbiotische stikstofbindende bacteriën bestudeerd. Tijdens de theoretisch biologische stage aan de Vrije Universiteit maakte ze kennis met modellering van een voedselketen van zich delende organismen. Daarna volgde een moleculair biologische stage aan het Nederlands Kankerinstituut te Amsterdam met als onderwerp signaaltransductie. Haar scriptie handelde over protozoën in actief slib. Na het afstuderen in augustus 1992 is ze van september 1992 tot december 1996 als assistent in opleiding en toegevoegd onderzoeker aangesteld geweest aan het Laboratorium voor Microbiologie aan de Landbouwuniversiteit Wageningen. Het onderzoek betrof de afbraak van gechloreerde aromatische verbindingen door microbiële cocultures onder methanogene omstandigheden. De resultaten zijn in dit proefschrift samengevat.

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