Stellingen

behorend bij het proefschrift "Transformation of chlorinated compounds by methanogenic granular sludge" van Miriam H.A. van Eekert

Wageningen, 21 juni 1999

1. De bewering van Bachmann (1988) dat anaërobie vermeden zou moeten worden bij de sanering van met HCH vervuilde gronden is onjuist, aangezien zelfs de (meest persistente) beta-isomeer van HCH gemakkelijk onder anaërobe omstandigheden blijkt te worden afgebroken.
   Bachmann, A., P. Walet, P. Wijnen, W. de Bruin, J. L. M. Huntjens, W. Roelofsen, and A. J. B. Zehnder. 1988. Appl. Environ. Microbiol. 54:143-149.
   Jagnow, G., K. Haider, and P. C. Ellwardt. 1977. Arch. Microbiol. 115:285-292.
   Middeldorp, P. J. M., M. Jaspers, A. J. B. Zehnder, and G. Schraa. 1996. Environ. Sci. Technol. 30:2345-2349.
   Dit proefschrift

2. De belangrijke mate waarin gechloreerde verbindingen in aanwezigheid van een methanogeen korrelslib worden omgezet via abiotische (chemische) reacties benadrukt het grote belang van dit soort omzettingen in het anaërobe milieu.
   Dit proefschrift

3. Het feit dat in methanogeen korrelslib facultatief anaërobe bacteriën voorkomen die in aanwezigheid van zuurstof en/of nitraat produkten kunnen omzetten die onder methanogene omstandigheden zijn gevormd, wijst op een zeer grote microbiële diversiteit van de bacteriële consortia welke aanwezig zijn in de anaërobe waterzuiverings-installatie waaruit betreffend korrelslib afkomstig is.
   Dit proefschrift

4. Het leggen van een verband tussen tussen Gibbs vrije energieën van reacties en het optreden van bepaalde dechloreringsroutes is niet altijd gerechtvaardigd, omdat de correlatie vaak gebaseerd is op verschillen van enkele procenten tussen de omzettingsroutes.
   J.E. Beurskens, C.G.M. Dekker, H. van den Heuvel, M. Swart, J. de Wolf en J. Dolfing, 1994. Environ. Sci. Technol. 28: 701-706.

5. Het is onjuist om bij de bepaling van kinetische afbraakconstanten voor de dechlorering van tetrachlooretheen uit te gaan van afbraak via 1,1-dichlooretheen als de mogelijkheid van dechlorering via 1,2-dichlooretheen niet kan worden uitgesloten.
   R.S. Skeen, J. Gao and B.S. Hooker, 1995. Biotechnol. Bioeng. 48: 659-666.

6. De bewering van Rosenbrock et al. (1997) dat in hun experimenten geen correlatie tussen dechloreringssnelheden en inherente anorganische elektronacceptoren wordt gevonden, wordt niet onderbouwd door de experimentele resultaten: de afname in de concentraties van de elektronacceptoren zijn niet in de tijd gemeten en voor één van de onderzochte gronden werd juist wel een verband tussen methaanvorming en dechlorering gevonden.
   P. Rosenbrock, R. Martens, F. Buscot, J.C. Munch, 1997. Appl. Microbiol. Biotechnol. 48: 115-120.

7. Moleculair ecologische technieken worden bij microbiologisch onderzoek nog te veel als doel en te weinig als middel gebruikt.

8. Een stukje van een bloemkool is op zichzelf een bloemkooltje.
   (vrij uit: 't Giet zoals 't giet (van Daniel Lohues, Skik 1998))

9. Gezien de ceremoniële aard van de promotieplechtigheid zouden alle gepromoveerde leden van een promotiecommissie gerechtigd dienen te zijn een toga te dragen, en niet uitsluitend de hoogleraren.

10. De pretentie die spreekt uit de eerste zinnen van hoofdstuk 2 ("Ziezo, de opzet is gelukt. We zijn onder elkaar. De onreine meelezers zijn hals over kop gevlucht voor al die spookachtige letters.") van "de Procedure" van Harry Mulisch, waarmee de schrijver suggereert slechts geïnteresseerd te zijn in een lezerspubliek met een groot doorzettingsvermogen, is in tegenspraak met het hoge "boeket-reeks"-gehalte van het plot van "De ontdekking van de hemel" van dezelfde schrijver.

11. Een lekkend riool kan goed zijn voor het milieu.

12. Een goed functionerende UASB reactor is uitermate rustgevend.

13. Voor een bredere specialisatie van AIO's zou het goed zijn wanneer ze bij twee - qua discipline - verschillende leerstoelgroepen zouden werken, hoewel na vier jaar de kans bestaat op verwarring met betrekking tot de verkregen specialisatie.

14. Tijdens de NAVO bombardementen op Kosovo en Servië is gebleken dat met hoogwaardige technologie gestuurde precisiebombardementen toch mensenwerk zijn en derhalve dramatische gevolgen kunnen hebben.

TRANSFORMATION

OF

CHLORINATED COMPOUNDS

BY

METHANOGENIC GRANULAR SLUDGE

Miriam van Eekert

Promotor: **dr. ir. G. Lettinga**
hoogleraar in de anaerobe zuiveringstechnologie en hergebruik

Copromotoren: **dr. ir. G. Schraa**
unversitair docent bij de leerstoelgroep Microbiologie

dr. J.A. Field
onderzoeker bij het departement Agro-, Milieu- en Systeemtechnologie

Transformation of chlorinated compounds by methanogenic granular sludge

M.H.A. van Eekert

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 maandag 21 juni 1999
des namiddags te half twee in de Aula.

Ontwerp van omslag: Jan-Hein Arens
John Stohr
Foto: Hardy Temmink

The research described in this thesis was financially supported by a grant (project 92014) from the Innovative Research Program on Environmental Biotechnology (IOP-Milieubiotechnologie) of the Ministries of Economic Afffairs and Housing, Physical Planning and Environment

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Eekert, M.H.A. van

Transformation of chlorinated compounds by methanogenic granular sludge / M.H.A. van Eekert [S.I. : s.n.]
Thesis Wageningen Agricultural University, Wageningen, the Netherlands - With ref. - With summary
ISBN: 90-5808-068-4
Subject headings: transformation/chlorinated compounds/anaerobic/granular sludge/UASB

Aan mijn ouders

Abstract

Eekert, M.H.A. van, 1999. **Transformation of chlorinated compounds by methanogenic granular sludge.** Doctoral Thesis, Wageningen Agricultural University, Wageningen, The Netherlands 150 pages.

Chlorinated compounds are an important group of contaminants often found in sediments, groundwater, soils, wastewaters, and off-gasses. Many of these pollutants are found on the EPA list of Priority Pollutants indicating their potential hazard for the environment. Initial degradation can occur via dechlorination reactions which are carried out by specific halorespiring bacteria or by acetogenic and/or methanogenic bacteria which dechlorinate via aspecific reactions. The dechlorination rates of halorespiring bacteria are generally high, due to the high degree of substrate specificity. However, these bacteria are limited in the number of chlorinated compounds they can degrade. This may be a disadvantage when they are put in to use for the remediation of environments with a mixture of chlorinated compounds. In those cases, a better strategy is the application of acetogenic and methanogenic bacteria which have a broad substrate specificity.

This research has evaluated the aspecific dechlorinating ability of unadapted acetogenic and methanogenic bacteria in methanogenic granular sludge. The long-term goal of the research is the evaluation of the applicability of unadapted methanogenic consortia in the bioremediation of wastestreams contaminated with mixtures of pollutants.

Chlorinated alkanes and cycloalkanes were completely or substantially degraded to lower- or non-chlorinated compounds by methanogenic (granular) sludge without prior adaptation. Chlorinated methanes were degraded mainly via oxidative and substitutive reactions to non-halogenated compounds like CO_2, while chloroethanes were dechlorinated via dichloroelimination and reductive hydrogenolysis to lower chlorinated ethenes and ethanes. The cycloalkanes, α- and β-HCH, were degraded via different reactions to a mixture of benzene and chlorobenzene. The degradation of chlorinated alkenes (tetrachloroethene and lower chlorinated ethenes) and aromatic compounds (hexachlorobenzene, and pentachlorophenol) by unadapted consortia takes place at low rates. This dechlorination of chloroethenes and pentachlorophenol is much slower compared to (cyclo)alkanes. Sometimes a long lag phase is required (lower chlorinated benzenes). PCBs were not degraded. All the degradation products, lower chlorinated alkanes and alkenes, and (chloro)benzene in case of HCH, are biodegradable under aerobic conditions.

Contaminated soils often contain many bacteria, which are able to perform remediation provided that the environmental conditions are favorable. In that case, the addition of granular sludge does not seem to be necessary for the degradation the chlorinated compounds. However, the presence of a methanogenic bacterial consortium can lead to e.g., a decrease of the redox-potential, thus favoring the environmental conditions for other, more specialized dechlorinating bacteria. Another application of granular sludge may be the use of the facultative anaerobic or microaerophilic bacteria in the sludge. These bacteria can degrade the persistent dechlorination products under higher redox-conditions. Altogether, the broad spectrum applicability of the unadapted methanogenic granular sludge may make it a useful first "tool" in the (in situ) bioremediation of contaminated environments.

Voorwoord

Nooit gedacht, maar nu is het dan eindelijk klaar! Het onderzoek beschreven in dit boekje, uitgevoerd op de vakgroepen Microbiologie en Milieutechnologie, werd gekenmerkt door (te) véél van alles: véél batches, véél head-spacemetingen, maar ook twee (of meer) bureaus, twee postvakjes, meerdere koffiepauzes per dag, twee keer zo veel taart (alhoewel dat in de praktijk behoorlijk kan tegenvallen door het synchroon lopen van koffiepauzes) en natuurlijk twee keer zo veel collega's....

Als eerste wil ik het triumviraat van wijze mannen bedanken dat mij in de afgelopen jaren heeft begeleid. Jim Field, Gosse Schraa en Fons Stams hebben een grote invloed gehad op de inhoud van dit boekje.
Jim, bedankt voor alles! De onnavolgbare wijze waarop je elke keer weer klaar stond voor de wetenschappelijke en de persoonlijke (er is inderdaad nog zo veel meer.....) begeleiding zal ik niet snel vergeten.
Gosse (meneer Schraa), je zette regelmatig de puntjes op de I, hebt heel veel dingen voor me geregeld en ik heb goede herinneringen aan de vele aangename gesprekken over de microbiologische aspecten van het onderzoek en hele andere zaken.
Fons, ik heb de samenwerking gedurende de afgelopen jaren erg gewaardeerd. Dank je wel voor het snelle doorlezen van de manuscripten!
Naast dit illustere drietal, wil ik Gatze Lettinga bedanken omdat hij als promotor wilde optreden, na mij reeds tijdens mijn studentenjaren enthousiast te hebben gemaakt voor de anaërobe zuivering.

De studenten (in volgorde van opkomst): Astrid van Rhee, Nienke Riezenbos, Niels van Ras, Thomas Schröder, Antoinette de Groot en Geert Mentink hebben vol geestdrift mee-geholpen met het uitvoeren van de vele batch-testen. Hoewel het soms saai werk was (dat wist ik echt wel!) hebben jullie een enorme bijdrage geleverd aan de totstandkoming van dit boekje. Also, many thanks to Maria Carmen Veiga, who did some valuble experiments on the inhibition of the dechlorination of carbon tetrachloride.

Voor de ondersteuning van de labactiviteiten ben ik dank verschuldigd aan Wim Roelofsen, Ilse Gerrits-Bennehey, Johannes van der Laan, Sjoerd Hobma, Jo Jacobs-Ackermans, Caroline Plugge en Johan de Wolf. Daarnaast hebben met name Nees Slotboom, Ans Broersma, Frits Lap, Jannie Wennekes, Liesbeth Kesaulya-Monster en Heleen Vos hulp van administratieve en andere aard verleend. De dames uit de Micro- en Biotechnion-kantine zorgden voor een lekkere kop koffie en een gezellig praatje. De medewerkers van de werkplaats in het Biotechnion stonden altijd klaar om de reactoren, maar vooral de grote hoeveelheid oververhitte pompen weer te repareren...

Bij de xeno-groep en later de micfys-groep op Microbiologie heb ik voornamelijk de ochtenduren doorgebracht. Het 's ochtends vroeg met z'n allen koffie drinken was een aangename start van de dag, voordat ik de GC-ruimte of het lab indook. Iedereen van de micfys-groep èn de rest op Microbiologie bedankt voor de gezelligheid en goede sfeer!

Het grote aantal kamergenoten (Martin, Bea, Sandra, Marijke, Wendela, Johan, Caroline, Judith) dat ik bij Microbiologie heb "versleten" doet echter vermoeden dat het met mijn ochtendhumeur lastig in één kamer vertoeven is. Alsnog mijn excuses, maar ik heb wel veel lol gehad!
Francis, je hebt het het langst met mij op een kamer uitgehouden! Bedankt voor de hulp en de gezelligheid, zowel binnen als buiten het lab, en het functioneren als mijn referentie-kader. Hans Scholten en Hermie Harmsen hebben onder het nuttigen van wat biertjes gezorgd voor de broodnodige relativering van het bestaan tijdens mijn vroege AIO-jaren.

Bij Milieutechnologie heb ik het genoegen gehad met Albert Janssen, en daarna met Luc Bonten en Adrie Veeken de kamer te delen, waarbij ik vooral A3 wil bedanken voor het op tijd aangeven wanneer hij iets niet snapte, waardoor ik zelf ook weer moest gaan nadenken! Robbert Kleerebezem en Frank van der Zee zijn gedurende de afgelopen zes jaar erg belangrijk geweest, zowel in de wetenschap als daarbuiten. Ik ga in ieder geval niet meer onbezonnen een 30°-kamer in....
Het was een leuke tijd op MT en ik wil iedereen bedanken voor de support en gezelligheid, met name de "dames van MT": Anita, Erna, Anneke, en Ilse en natuurlijk Marjo voor het op peil brengen van mijn fietsconditie. Verder André, Jules, Salih en de rest van "het Bordes", Vinnie, de (inmiddels ook niet meer zo) "jonge AIOs" voor het laten opbloeien van mijn moedergevoelens en Brian Donlon, Elias Razo, Shyung Hwu, Jim Collins, en Lourdinha Florencio, die het leven op en buiten de vakgroep door de jaren heen op allerlei verschillende gebieden hebben veraangenaamd.
En natuurlijk mag de "vrijdagavond Loburg club" niet onvermeld blijven, het was en is mij een waar genoegen het glas met jullie te heffen!

Verder dank aan al mijn vrienden die zich bij tijd en wijle toch hebben moeten afgevraagd waarom het allemaal zo lang moest duren, maar gelukkig niet altijd vroegen hoe het er mee stond... Jan-Hein Arens heeft in rap tempo de omslag ontworpen en John Stohr heeft daar zeker ook een aandeel in gehad. Heren, dank voor het snelle werk! Waar Carnaval al niet toe kan leiden....

Mijn ouders hebben mij door de jaren heen altijd gesteund in wat ik wilde doen ook al lag wat ik wilde soms wat verder van hun bed. Guus, je hebt me geleerd wat doorzetten is! Mijn vader maakt dit niet meer mee, maar ik denk dat hij toch wel een beetje trots op me zou zijn geweest. Hein, je zette mij taalkundig regelmatig op het rechte spoor, hetgeen erg nodig was, dank!

Tenslotte, Hardy.... We hebben de laatste tijd lekker samen gewerkt, jij in B'kom en ik op het Biot. Deze support (ik voelde me zo schuldig als ik niet óók wat ging doen), heeft de afronding van het proefschrift zeker niet tegengewerkt. Bovendien is de Nederlandse samenvatting door jou vakkundig geredigeerd. Maar eerst en vooral, heb je het leven gedurende het laatste deel van mijn promotietijd een stuk mooier gemaakt!

Het is af! Allemaal bedankt (zéker degenen die ik vergeten ben)!

Contents

1

General introduction

Introduction

Chlorinated compounds are an important group of contaminants found in sediments, wastewaters, groundwater, soils and off-gasses. Many chlorinated compounds are present on the EPA list of priority pollutants indicating their potential hazard for the environment [107]. They were (and are) used as degreasing agents and solvents in the (metal)industry, as cooling fluid in refrigerators (chlorofluorocarbons), as pesticides, fungicides or insecticides, or as dielectric fluid or flame retardant (Table 1.1). Some chlorinated chemicals are produced as by-products during the manufacturing of other chemicals (e.g. α-, β-, and δ-hexachlorocyclo-hexane (α-, β-, δ-HCH) during the production of γ-HCH (lindane)).

Although these compounds are generally considered to be xenobiotic compounds, their origin is not solely confined to human activities. Chemical, physical and biological processes in soil and atmosphere lead to the formation of halogenated compounds. Around 2000 chlorinated compounds are released into the environment by plants, marine organisms, bacteria, mammals and via other biogenic routes [71]. The natural production of e.g., methyl chloride by algae and fungi as well as during volcanic eruptions, exceeds the anthropogenic production by two to three orders of magnitude [45,71]. The capacity to produce chlorinated aliphatic and aromatic compounds is also widely present among fungi [92].

Although "natural" processes have been active for long periods of time, still a large part of the chlorinated priority pollutants is of anthropogenic origin. The annual production of chlorinated compounds varies among the different groups of compounds (Table 1.1). The amount which ultimately ends up in the environment is dependent upon its use. The use of chlorinated pesticides implies that the total amount produced is also released in the environment; whereas compounds like VC and 12DCA, which serve as intermediates in the production of PVC and other chlorinated compounds, will end up in the atmosphere in minor amounts only.

The remediation of wastestreams and sites which are contaminated with chlorinated compounds has been widely researched in the past few decades, and the possibility of the application of microorganisms to "assist" in the clean-up of pollution under both aerobic and anaerobic conditions has been recognized. Many compounds, like PCE and β-HCH, which were previously considered to be resistant to degradation have been found to be microbiologically degradable.

This thesis deals with the dechlorination of several chlorinated compounds (Fig. 1.1) by acetogenic and methanogenic bacteria in microbial consortia, which have not been priorily exposed to chlorinated compounds. These bacteria are known to transform chlorinated

compounds gratuitously and they have no direct benefit form the dechlorination process. Because of the aspecific nature of these reactions, acetogenic and methanogenic bacteria may be suitable for the dechlorination of mixtures of chlorinated compounds.

Granular sludge from upflow anaerobic sludge blanket (UASB) reactors was chosen as the source of acetogenic and methanogenic bacteria. UASB reactors are successfully used for the treatment of different types of industrial wastewaters [109]. Also, granular sludge is able to remove chlorinated compounds from wastewater [36,37,72,78]. However, little is known about the dechlorinating capacity of unadapted methanogenic and acetogenic bacteria in granular sludge. The aim of the research described in this thesis was to get more insight in the degradation of chlorinated compounds by unadapted anaerobic bacteria and the role of abiotic conversions in these degradation processes.

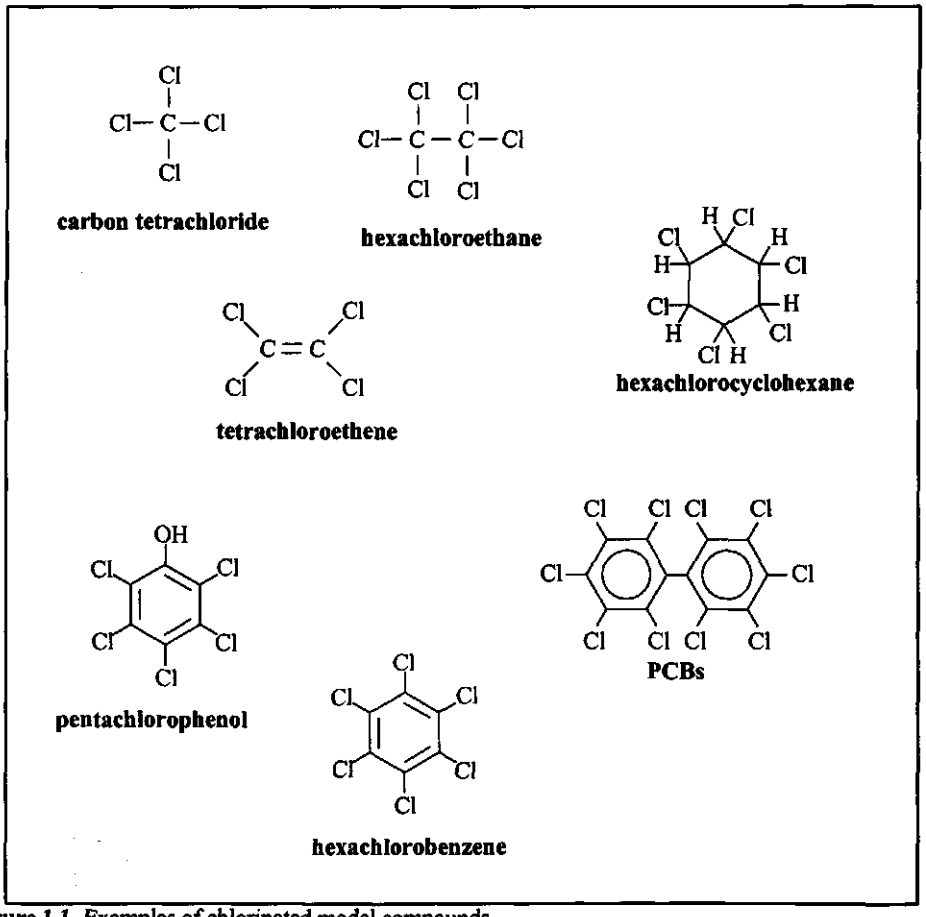


Figure 1.1 Examples of chlorinated model compounds

Table 1.1 General properties of selected chlorinated compounds. Data taken from [154] unless otherwise stated

Compound	Chemical formula	Acronym	Production worldwide (10^6 kg (year)$^{-1}$)	%[a]	Examples of application	Natural sources
Chloromethanes						
methylchloride	CH_3Cl	MC	400[b]		methylation reactions[c]	marine algae, kelp, fungi, cedar, volcanoes, forest fires, cultivated mushrooms[d]
dichloromethane	CH_2Cl_2	DCM	600 (1994)	100	solvent, degreasing	forest fires, barley[d]
chloroform	$CHCl_3$	CF	250 (1978-1980)	5-10	intermediate, solvent	marine algae, fungi[e], cedar, lemon, orange, moss, barley, drill wells, volcanoes[d]
carbon tetrachloride	CCl_4	CT	143 (1991)	5-10	intermediate, solvent, degreasing	volcanoes, oceans, marine algae, drill wells[d]
Chloroethanes						
chloroethane	C_2H_5Cl	CA	0.4[b]		ethylation reactions[c]	
1,1-dichloroethane	$C_2H_4Cl_2$	11DCA	<10 (1996)	1-10	feedstock 111TCA[c]	-
1,2-dichloroethane	$C_2H_4Cl_2$	12DCA	33,600 (1995)	1-4	intermediate VC[c]	-
1,1,1-trichloroethane	$C_2H_3Cl_3$	111TCA	900 (1990)	83	dry cleaning, solvent[c]	oceans[d]
1,1,2-trichloroethane	$C_2H_3Cl_3$	112TCA	200 (1996)	10	intermediate, solvent	-
1,1,1,2-tetrachloroethane	$C_2H_2Cl_4$	1112TeCA	0[f]			
1,1,2,2-tetrachloroethane	$C_2H_2Cl_4$	1122TeCA	200 (1996)	?	intermediate, solvent	-
pentachloroethane	C_2HCl_5	PCA	NA[g]			
hexachloroethane	C_2Cl_6	HCA	NA[g]			
Chloroethenes						
vinylchloride	C_2H_3Cl	VC	7.7-16,7 (1985)	0-5	intermediate PVC	-
1,1-dichloroethene	$C_2H_2Cl_2$	11DCE	200 (1981)	1-5	intermediate 111TCA[c]	-
1,2-dichloroethene	$C_2H_2Cl_2$	c/tDCE	<0.5 (1986)		intermediate, solvent	-
trichloroethene	C_2HCl_3	TCE	600 (1978-1980)	90-100	solvent, degreasing	oceans, volcanoes[d]
tetrachloroethene	C_2Cl_4	TeCE	750 (1982)	100	solvent, degreasing	oceans, volcanoes[d]

Table 1.1 continued

Compound	Chemical formula	Acronym	Production worldwide (10^6 kg (year)$^{-1}$)	%[a]	Examples of application	Non-antropogenic sources
Cycloalkanes						
α-hexachlorocyclohexane	$C_6H_6Cl_6$	α-HCH	100 (1983)	100		-
β-hexachlorocyclohexane	$C_6H_6Cl_6$	β-HCH	15 (1983)	100		-
γ-hexachlorocyclohexane	$C_6H_6Cl_6$	γ-HCH	38 (1986)	100	insecticide	-
δ-hexachlorocyclohexane[h]	$C_6H_6Cl_6$	δ-HCH	9			-
Chlorobenzenes						
chlorobenzene	C_6H_5Cl	CB	480 (1985)	?	intermediate dyes and others	-
1,2-dichlorobenzene	$C_6H_4Cl_2$	12DCB	80	>25	intermediate dyes, pesticides[c]	-
1,3-dichlorobenzene	$C_6H_4Cl_2$	13DCB	30 (1994)	0-2	intermediate, solvent	-
1,4-dichlorobenzene	$C_6H_4Cl_2$	14DCB	80	75	"toilet-stones", intermediate in dyes	-
1,2,4-trichlorobenzene	$C_6H_3Cl_3$	124TCB	30	1-2	termite poison, solvent	-
hexachlorobenzene	C_6Cl_6	HCB	5 (1993)	0.1-1	PCP production, seed coating	-
Chlorophenols						
2-chlorophenol	C_6H_4ClOH	2CP	10 (1996)	2	desinfectant	-
3-chlorophenol	C_6H_4ClOH	3CP	3-10 (1996)	1	production of dyes	-
4-chlorophenol	C_6H_4ClOH	4CP	20-30 (1996)	10	intermediate	
2,4-dichlorophenol	$C_6H_3Cl_2OH$	24DCP	70 (1985)	10	woodpreservative	fungi
2,4,5-trichlorophenol	$C_6H_2Cl_3OH$	245TCP	<5 (1996)	<10	intermediate, fungicide	-
2,4,6-trichlorophenol	$C_6H_2Cl_3OH$	246TCP	<10 (1996)	?	intermediate, insecticide	forest fires, humines
pentachlorophenol	C_6Cl_5OH	PCP	35 (1985)	?	woodpreservative, fungicide	-
Polychlorinated biphenyls		PCB	2000[k]		flame-retardant, dielectric fluid	volcanoes[d]

[a] Percentage of total production ending up in the environment
[b] Data taken from Pearson [145]
[c] Data taken from Fetzner [61]
[d] Data taken from Gribble [71]
[e] Data taken from Hoekstra et al. [80]
[f] Not commercially produced [135]
[g] No data available
[h] Data taken from Slooff and Matthijssen [167]
[k] Total production worldwide until 1988 [45]

Anaerobic degradation of chlorinated compounds

The degradation mechanism of halogenated compounds depends on the nature of the redox conditions, but also on the presence and activity of the (co)enzymes required for transformation [183]. The degradation of halogenated compounds has been observed under different redox conditions, but higher halogenated compounds are especially susceptible to dehalogenation via reductive pathways [183]. Consequently, higher halogenated compounds are more easily degraded under anaerobic than aerobic conditions. The nature of the halogen atom also determines the rate of reduction. For the metal catalyzed reduction of arylhalides the reactivity decreases in the order I>Br>Cl>F [188]. This is probably due to the decreasing atomic radius combined with the electronegativity. Since the bond enthalpy increases in that order, it indicates that the C-I bond is "broken" more easily than the C-F bond [114]. This is also reflected in the free energy of formation (G_f°) of the ions (formed during the dehalogenation process) which under the same conditions is lower for a I^--ion (aq) than for a F^--ion [76]. Since the research described in this thesis focuses on the transformation of higher chlorinated compounds, this introduction will mainly deal with dechlorination reactions, which occur under anaerobic conditions. Degradation of chlorinated compounds under aerobic conditions has been reviewed by others, e.g. [27,90,151].

Reduction, dehydrochlorination and substitution reactions are the dechlorination mechanisms most commonly observed under anaerobic conditions (Fig. 1.2). The reduction reactions require the input of electrons and mainly occur via biologically mediated reactions. Four types of reduction reactions are distinguished: reductive hydrogenolysis, dichloroelimination, hydrolytic reduction and coupling. Reductive hydrogenolysis requires the input of two electrons, and a chlorine atom is replaced by a hydrogen atom (Fig. 1.2). In dichloroelimination reactions, two chlorine atoms may be removed from two adjacent C-atoms with the subsequent formation of a double bound between the carbon atoms. The hydrolytic reduction of CT is believed to take place with bulk electron donors which are weaker than Ti(III)citrate, like, e.g., dithiotreitol (DTT) [112]. CO and acetate could be formed via this pathway from CT and 111TCA, respectively [64,85,184]. Reductive coupling is a process which is only of minor importance (e.g., the formation of ethane from DCM by cofactor F_{430} with Ti(III)citrate as electron donor [100]).

Substitution and dehydrochlorination reactions can take place without the presence of a catalyst, provided that environmental conditions are suitable. Besides water, nucleophiles like hydroxyl and sulfhydryl groups (SH-) can also function as nucleophiles in the substitution reactions (Fig. 1.2). Dehydrochlorination reactions do not proceed rapidly in water under

physiological conditions. The rate with which these substitution and dehydrochlorination reactions take place is also dependent on the number of chlorine atoms in the molecule [183]. Abiotic conversions of chlorinated compounds will be discussed in more detail in one of the following sections.

Chlorinated compounds may serve as electron acceptors in degradation processes. The redox potentials of the half reactions are of the same order of magnitude as the reduction of oxygen and nitrate (Fig 1.3).

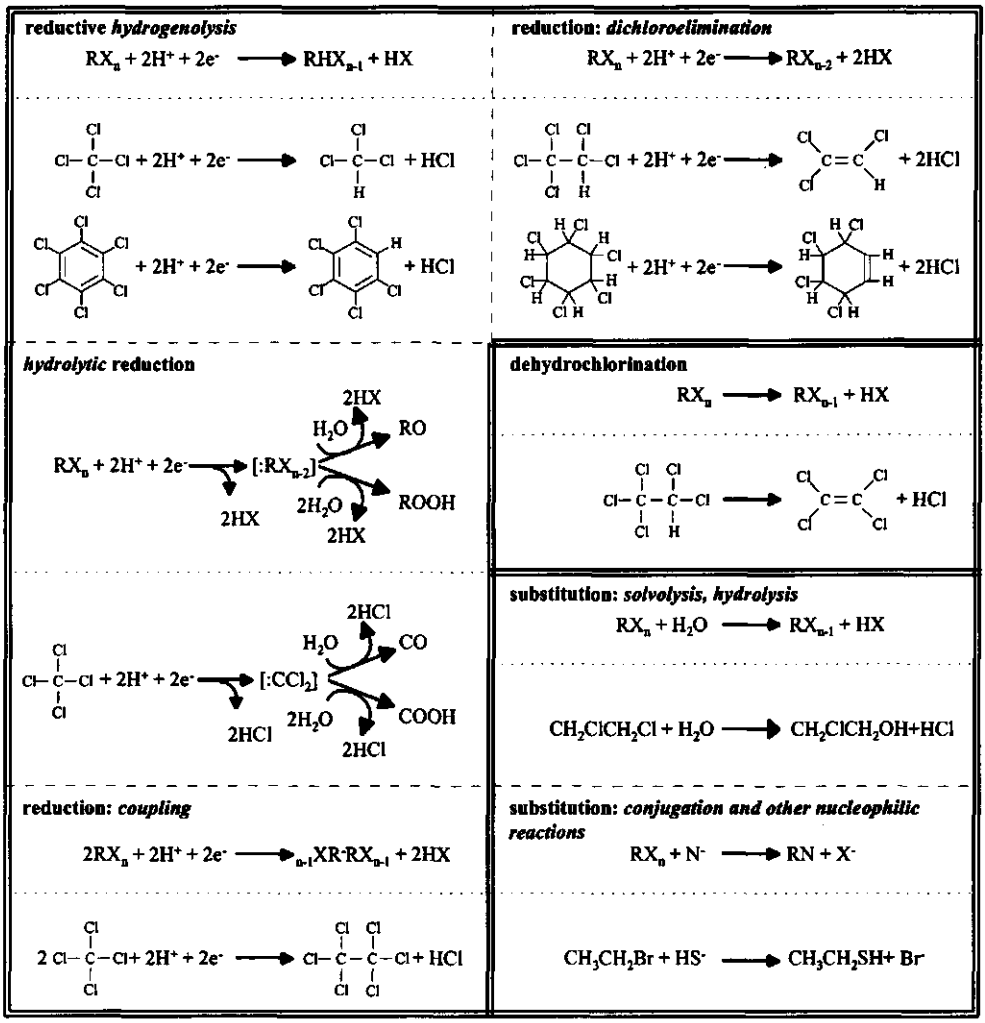


Figure 1.2 Dechlorination types commonly observed in anaerobic environments (N⁻=nucleophile) (Information is partly obtained from others [85,183]).

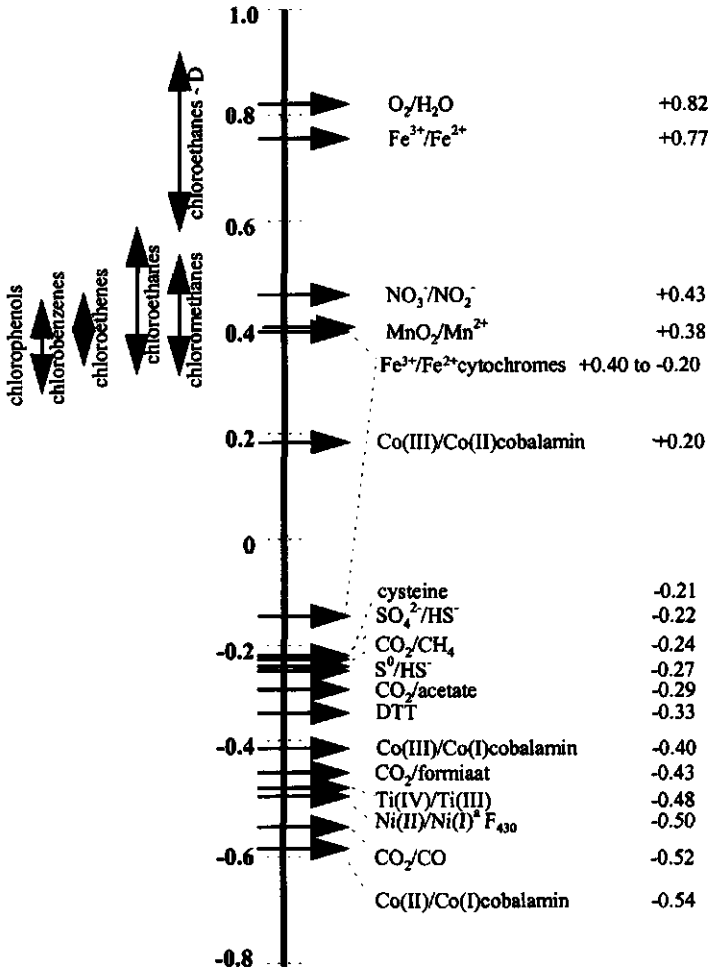


Figure 1.3 Reduction potentials (in V) under standard conditions (E'_0) of redox couples involved in reductive hydrogenolysis of different groups of chlorinated compounds, the dichloroelimination (D) of chlorinated ethanes, and other relevant redox processes. Data are taken from [2,30,46,84,100,103,169,177,192]. The redox couples of the chlorinated compounds are calculated using data from [14,45,47,76].
[a] Value taken from Krone *et al.* [100]. Holliger *et al.* [81] give a value of -0.65 or -0.62 V for native and diepimeric F_{430}, respectively. However, these values were measured at pH 10.2.

Especially dichloroelimination reactions can compete with oxygen as an electron acceptor. Reductive hydrogenolysis of chloromethanes, -ethanes, -ethenes and chlorinated aromatic compounds takes place at half redox potentials between 0.23 and 0.6 V corresponding to a Gibbs free energy of -43.4 to -115.8 kJ/mole.

Several bacteria have been isolated which are able to use energy gained by reductive dechlorination, for growth. This process is called halorespiration. Compounds like TeCE, but also chlorinated aromatic compounds are able to sustain the growth of bacteria. Because the dechlorination is carried out by enzyme systems which are usually limited to the transformation of one or two different compounds, this process is generally referred to as specific dechlorination. *Desulfomonile tiedjei* [43] was one of the first known specifically dechlorinating bacteria, and was isolated from a 3-chlorobenzoate (3-CBa) degrading consortium. This bacterium is able to dechlorinate 3-CBa, PCP, lower chlorinated phenols, and TeCE [57,180]. Several other TeCE dechlorinating bacteria have been isolated so far. Usually, they are able to remove only one or two chlorine atoms. *Dehalobacter restrictus*, *Dehalospirillum multivorans*, and strain TT4B are able to reductively dechlorinate TeCE and/or TCE to cDCE [82,104,162]. *Desulfitobacterium* strain PCE-1 [68] dechlorinates TeCE as well but only to TCE and minor amounts of DCE. An exception is *Dehalococcoides ethenogenes*, which transforms TeCE to VC and ethene [124]. The dechlorination rates of these specifically PCE dechlorinating bacteria may be as high as 20-450 μmol (mg protein)$^{-1}$ day^{-1}. *Desulfitobacterium* strain PCE-1, as well as other *Desulfitobacterium* sp., like *D. dehalogenans*, *D. chlororespirans*, *D. frappieri*, *D. hafniense*, and strain 2CP-1, were also able to dechlorinate 3-chloro-4-hydroxyphenylacetate, PCP and other chlorinated phenols [19,33,38,68,158,182]. H_2 seems to be the preferred electron donor in the reductive dechlorination process. The TeCE dechlorinating strain TT4B is the only bacterium that couples TeCE dechlorination to the oxidation of acetate.

Abiotic versus enzymatic dechlorination

The dechlorination of chlorinated compounds in mixed cultures as a whole can be considered as a combination of abiotic and biotic conversions. Abiotic conversions are reactions in which bulk chemicals in the medium or in the mixed culture serve as a reactant in a dechlorination reaction or when a component of the medium or in the sludge acts as a mediator in dechlorination reactions. An example of the first reactions is the reduction of CT to CF in the presence of sulfide as the bulk electron donor (e.g., [40]). The reactions which involve a medium or sludge component as the mediator could be considered as biological reactions. However, in the research described in this thesis these reactions, e.g., the dechlorination of CT by vitamin B_{12} (e.g., [102]), are considered abiotic, although the cofactors (and other mediators) are biologically generated. The enzymatic (biological)

conversions can also be divided in two groups, the cometabolic and the specific dechlorination reactions. In this thesis, the focus will be on the abiotic and the cometabolic pathways used by methanogenic and acetogenic bacteria for dechlorination. Bacteria which are able to use chlorinated compounds as electron acceptor (halorespiring bacteria) will not be discussed in detail, since these conversions are not expected to take place with unadapted granular sludge. Usually, when the source material has not been previously exposed to the xenobiotic compound, long adaptation periods are required for the enrichment of the appropriate halorespiring bacteria.

Abiotic conversion - bulk chemical dechlorination

When chlorinated compounds are degraded via abiotic chemical hydrolysis and/or dehydrochlorination (Fig. 1.2) reactions in the absence of a catalyst, reaction rates usually are low. In general, lower chlorinated aliphatic compounds are hydrolyzed with half-lives varying from several months to several years, while higher chlorinated compounds are transformed less easily with half-lives up to several hundreds to thousands of years. Dehydrochlorination reactions generally occur faster (half-lives of several days for the higher chlorinated compounds). Lower chlorinated aliphatics preferably are hydrolyzed instead of dehydrochlorinated [183].

Chlorinated alkanes are more easily degraded via bulk chemical substitution reactions than chlorinated ethenes and chlorinated aromatic compounds. With sulfide [40], iron sulfide [24], or Fe^{2+} [40,123] as nucleophile, higher chlorinated ethanes and methanes like HCA and CT are known to be dechlorinated (also in natural waters [131]). These reactions are accelerated by the presence of electron mediators such as natural organic matter with structures like humic acids, anthrahydroquinone disulfonate, (mercapto)juglone, or bipyridine [24,40,146,147] and also by the presence of metal porphyrins (see "abiotic conversions - the dechlorinating ability of cofactors"). The half-life of HCA and CT at 50°C was 7 to 140 days with Fe^{2+} or sulfide; whereas the addition of humic acids decreased the half-lifes by approximately one order of magnitude [40]. The products formed from chloroethanes and methanes are usually lower chlorinated ethenes and methanes, respectively. 1122TeCA was also transformed (to TCE) both in the absence and presence of a reducing agent like sulfide or Ti(III)citrate, while transformation products of 112TCA and 12DCA could not be detected [28]. In other experiments however, 12DCA was dechlorinated abiotically via substitution reactions in the presence of sulfide (half-life 6.1 years) [6]. While electron donors like sulfide

[175] or compounds like pyrite [99] or other bulk chemical electron donors like Fe(II)sulfate could sustain the dechlorination of CT, CF and 111TCA were not dechlorinated [48,96]. PCE and TCE, however, were reported to be reductively dechlorinated in sulfide containing medium [94].

Hydrolysis and dehydrochlorination of chlorinated ethanes, CT [67,91,155], and HCH [137] has also been found (Table 1.2). The half-life of these reactions was dramatically decreased by using more extreme conditions like higher pH or higher temperatures.

Zero-valent metals like Fe(0), Zn(0), Mg(0), Sn(0) could also serve as an indirect electron donor in the dechlorination of chlorinated methanes [17,115,123,161], chloroethanes [60] and/or chloroethenes [142,156,164]. The reaction mechanism is not completely clarified. The reactions could occur as a direct oxidative corrosion of the zero valent metal by the chlorinated compound or by a combined oxidation of the zero valent metal by water and a subsequent reduction of the chlorinated compound [123]. However, metals in granular sludge are not likely to be present in their zero-valent state and this topic will not be discussed in more detail.

Besides the purely chemical reactions mentioned above, chlorinated compounds like CT and lower chlorinated methanes, and chloroethenes can also be dechlorinated by alternative methods like sonolytic transformation e.g., [87], photocatalytic reduction or oxidation [25,31,32,74,113], or catalytic dehydrochlorination with a noble metal catalyst and hydrogen gas e.g., [163]. These processes do commonly not occur in granular sludge during the biotransformation of chlorinated compounds under anaerobic conditions and will therefore not be discussed any further.

Table 1.2 Hydrolysis and dehydrochlorination half-lives (in years, at pH 7 and 20 to 25 °C) for several chlorinated compounds.

Compounds	**Half-life**	**Ref.**
CT	40.5	[91]
CF	1850	[91]
HCA	$1.8 \cdot 10^9$	[91]
PCA, TeCA	0.01 - 50	[91]
TCA, DCA	1.7 - 139.2	[6,67,91,186]
chlorinated ethenes	10^6 to 10^{10}	[91]
α- and γ-HCH	20 to 50[a]	[137]

[a]These rates were measured at pH 8 and 5°C

Abiotic conversions - the dechlorinating ability of cofactors

Many enzymes commonly found in anaerobic bacteria contain transition metal cofactors in their active center (Fig. 1.4). These cofactors may consist of cobalt (vitamin B_{12}), nickel (cofactor F_{430}) or iron (hematin or cytochromes) in a porphyrin ring. These transition metals contain a partially filled *d*-orbital which allows them to exist in different oxidation states [187]. Research has shown that corrinoids, like vitamin B_{12}, and other porphyrin rings are able to mediate the *in vitro* dechlorination of chlorinated methanes [100,102,112] and chloroethanes [160]. Also, HCHs [121,122], chlorinated ethenes [66,69,70], chlorobenzenes [3,66], chlorophenols [168] and PCBs [3] can be dechlorinated in reactions catalyzed by vitamin B_{12} and other cofactors. In these reactions, the electrons needed for dechlorination are provided by the transition metal in the cofactor, but the presence of a bulk electron donor like Ti(III) or dithiotreitol (DTT) or sulfide ensures that the cofactors are recycled (Fig. 1.5).

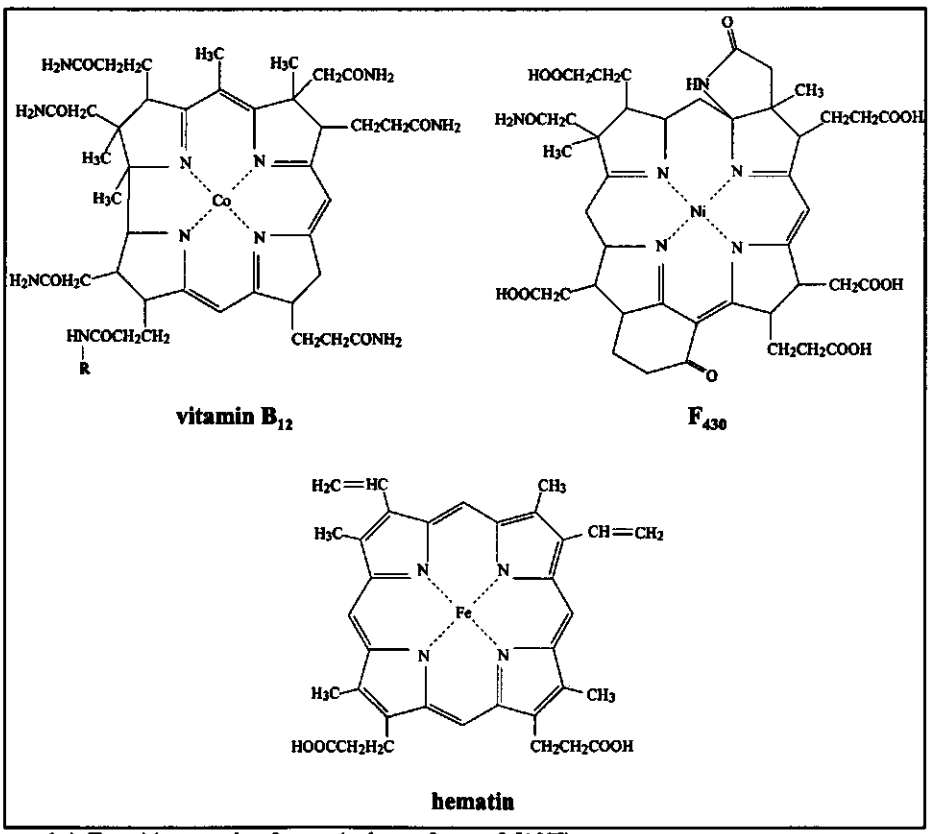


Figure 1.4 Transition metal cofactors (redrawn from ref. [187])

From the data in figure 1.3, it is clear that vitamin B_{12} with the cobalt ion in its Co(I) state and other cofactors like cofactor F_{430} ($E^{0'}$ = -0.4 to -0.6 V) can easily serve as electron donors for dechlorination. For cytochromes ($E^{0'}$ = -0.2 to +0.4 V) and Co(II)-cobalamin ($E^{0'}$ = +0.2 V) however, this may be more difficult. Also, not all the bulk reductants can reduce the oxidized vitamin B_{12} and cofactor F_{430}. Strong reductants like Ti(III) ($E^{0'}$ = -0.48 mV) are able to reduce these mediators completely; whereas, only partial reduction will occur in the presence of DTT or sulfide [112].

Many different reaction conditions have been tested (Table 1.3). In general, it can be said that the use of a stronger bulk electron donor (Ti(III) vs. DTT (Fig. 1.3)) leads to the formation of more reduced products, because the cobalt atom is reduced to its Co(I) state instead of Co(II). This is shown for CT reduced by vitamin B_{12}. With Ti(III)citrate as the bulk electron donor, CT is mostly reduced via reductive hydrogenolysis to CH_4; whereas, DTT leads to the formation of DCM and relatively more CO (the latter via hydrolytic reduction). With cystein, mostly CF but also DCM and CS_2 are formed [112].

The overall dechlorination rates of chlorinated methanes, but also of other chlorinated compounds, decrease with the number of chlorine atoms in the molecule [102]. Also, the nature of the transition metal seems to influence the products formed and the reduction rates. For chlorinated ethanes, vitamin B_{12} produces more reduced products compared to hematin [160]. With chloroethenes, the rates decrease in the order vitamin B_{12} > cofactor F_{430} > hematin [66]. Both vitamin B_{12} and hematin can reductively dechlorinate chlorinated benzenes, whereas, cofactor F_{430} can not [66]. Other metals are also able to mediate these kind of reactions, e.g., γ-HCH was found to be dechlorinated by porphyrin rings containing magnesium (chlorophyll), molybdenum, or vanadium [121].

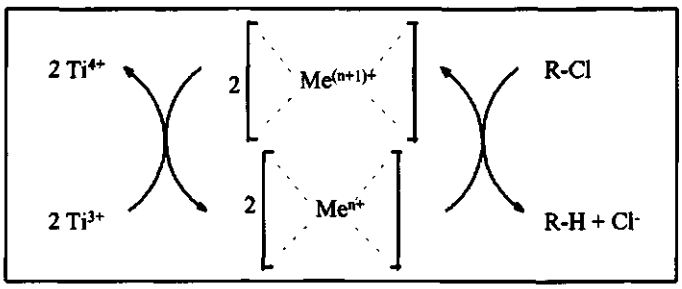


Figure 1.5 Schematic dechlorination of a chlorinated compound mediated by a transition metal cofactor and with Ti(III) as an example of an bulk electron donor.

Table 1.3 Transformation of chlorinated compounds catalyzed by vitamin B_{12}, cofactor F_{430}, or hematin with Ti(III)citrate (Ti), dithiotreitol (DTT), cystein (C) or sulfide (S) as the bulk reductant (red).

	Transformation products observed[a]	Red.	Ref.
Vitamin B_{12}/corrinoid/cobalamin			
Chloromethanes	RH to CH_4, formation of CO, CO_2, and NSR[b]	Ti	[2,29,30,96,102,
	RH of CT to DCM, CO, and NSR	DTT	103,112,122]
	RH of DCM to CH_4	DTT	
	RH of CT to CF, formation of CO, CS_2 (tr), NSR	C	
Chloroethanes	D/RH/H of HCA, PCA, TeCAs to lower chloroethanes/ethenes;	Ti	[84,160]
	RH of 111TCA; RH/D of 12DCA to CA and ethene	Ti	
γ-HCH	Formation of CB	DTT	[121,122]
Chloroethenes	RH of TeCE to ethene, and ethane.	Ti/DTT	[22,66,69,70,108]
	11DCE dechlorination via acetylene		
Hexachloro-1,3-butadiene	RH to pentachloro-1,3-butadiene and reduction to trichloro-1-buten-3-yn and 1-butyn-3-yn	Ti	[18]
Chlorobenzenes	RH of HCB to QCB and TeCB, QCB to TeCB	Ti/DTT	[3,66]
Chlorophenols	RH of PCP, TeCPs, TCPs; removal of one chlorine	Ti	[66,168]
(23456Cl)-PCB	RH, removal of one chlorine	DTT	[3]
Cofactor F_{430}			
Chloromethanes	RH of CT to CH_4, formation of traces of ethane;	Ti	[66,100,103]
	Coupling of DCM to traces of ethane	Ti	
Chloroethanes	RH/D of 12DCA to CA and ethene	Ti	[84]
Chloroethenes	RH to ethene	Ti/DTT	[66]
Hematin/iron porphyrins			
Chloromethanes	RH of CT to CF, CO, and unknown products	Ti	[26,66,96]
	RH to CF (and further?); No transformation of DCM	C/S	
Chloroethanes	D/RH/H of HCA, PCA, TeCAs to lower chloroethanes/ethenes	Ti	[96,147,160,190]
	D of HCA to TeCE (prereduced)		
	D of 1122TeCA to 11DCE;	S	
	RH of 111TCA	Ti/C/S	
	No transformation of 112TCA and 11DCA	S	
γ-HCH	Formation of CB	DTT	[121]
Chloroethenes	RH to VC	Ti/DTT	[66]
Chlorobenzenes	RH of HCB to QCB	Ti	[66]

[a]RH=reductive hydrogenolysis, D=dichloroelimination, H=dehydrochlorination; [b]NSR=non strippable residue

Many of these porphyrins are present in anaerobic microorganisms as part of enzymes constitutively present (e.g., methyltetrahydrofolate B_{12} methyltransferase and other carboxyl and methyl group transferring enzymes, methyl-S-coenzyme M reductase, cytochromes, and ribonucleotide reductase [187]). These enzymes play a role in the transformation of chlorinated compounds by the different groups of anaerobic bacteria. The role of corrinoids and cofactor F_{430} in the dechlorination of 12DCA by acetogenic and methanogenic bacteria was already established [51,84]. The role of transition metal containing cofactors in specific enzyme systems has recently been reviewed by others [53,61,192].

Enzymatic dechlorination - pure cultures of cometabolically dechlorinating bacteria

The reductive dechlorination can occur via cometabolic pathways. Cometabolism is generally referred to as "the metabolism of a non-growth substrate in which no apparent benefit is accrued by the metabolizing organism", and is considered to be a function of broad enzyme specificity, imprecise induction specificity, and other processes [189]. Therefore, cometabolic reactions occur gratuitously, and involve (parts of) enzymes, that normally are mediators of common (constitutively present) catabolic pathways in the bacterium (Fig 1.6). A cometabolic reaction does not yield energy which can be used for growth. It occurs because the substrate (in this research chlorinated) fits into an enzyme which is already present. Another substrate as carbon and energy source is required to support growth of the cometabolically dechlorinating bacteria. Cometabolic dechlorination does not require the production of specialized enzymes. This is in contrast with the specialized halorespiring bacteria, which were mentioned earlier.

Acetogenic and methanogenic bacteria contain high levels of cofactors like vitamin B_{12} and F_{430}. These are involved in major anaerobic pathways, such as the acetyl-CoA pathway and the formation of methane [44,62,88,105,125]. The cofactor containing enzyme systems are believed to play a role in the cometabolic dechlorination process as was shown for the degradation of 12DCA in pure cultures of methanogenic bacteria [84]. However, other unknown processes may also play a role. Both for *Acetobacterium woodii* and a *Methanosarcina* sp., an unidentified catalyzing factor was found which is involved in the dechlorination of CT [138,171].

Many chlorinated compounds are degraded via cometabolic pathways by acetogenic and methanogenic bacteria (Table 1.4) [12,51,52,56,64,84,89,130,176].

Table 1.4 Summary of literature data concerning cometabolic dechlorination of several chlorinated compounds grown with different primary substrates (PS).

Compound	Microorganism	PS	Products	Rate[a]	Ref
$CFCl_3$	Methanosarcina barkeri	MeoH, H_2	$CHFCl_2$, F^-, CO	2.4	[101]
CF_2Cl_2	Clostridium pasteurianum	glucose		$8{\cdot}10^{-5}$	[118]
CT	Pseudomonas putida	camphor,	CF,...		[111]
	P. sp. strain KC	various	CF,...		[26]
	Shewanella putrefaciens	lactate	CF,....	0.0014	[148]
	Desulfobacterium autotrophicum	lactate	CF, DCM, NI	15.6	[50,51]
	Acetobacterium woodii	fructose	CF, DCM, MC, CO,CO_2, NI[b]	5.44-30	[51,52,171]
	Clostridium sp. TCAIIB	various	CF, DCM, NI	0.28	[64,65]
	Methanobacterium thermoautotrophicum	H_2	CO_2, CF, DCM, NI	0.78	[50,51]
	Methanosarcina thermophila	Fe(0)	CF,...		[100,138]
	M. barkeri	CO	DCM	0.48	
CF	Desulfobacterium autotrophicum	lactate	DCM		[50]
	Acetobacterium woodii	fructose	DCM, MC	1.21	[52]
	M. thermoautotrophicum	H_2	DCM		[50]
	Methanosarcina sp.	methanol	DCM,....	0.48	[100,130]
	M. barkeri	CO	DCM		
DCM	Acetobacterium woodii	fructose	MC	<0.09	[52]
	Methanosarcina barkeri	CO	MC	0.12	[100]
MC	Acetobacterium woodii	fructose	ND[c]	0.35	[52]
Cl_3CNO_2	Pseudomonas putida	camphor	....CH_3NO_2		[26]
HCA	Pseudomonas putida G786	camphor	TeCE	1.90	[117]
PCA	Pseudomonas putida G786	camphor	TCE	1.28	[117]
1112TeCA	Pseudomonas putida G786	camphor	11DCE	0.2	[117]
111TCA	Desulfobacterium autotrophicum	lactate	11DCA		[50]
	Acetobacterium woodii	fructose	11DCA	3.37	[52]
	Clostridium sp. TCAIIB	various	DCA,.....	0.28	[64,65]
	Methanobacterium thermoautotrophicum	H_2	11DCA		[50]
12DCA	Methanobacterium thermoautotrophicum	H_2	ethene		[12,50,83]
	Methanococcus deltae	H_2	ethene		[12]
	M. thermolithoautotrophicus	H_2			
	Methanosarcina barkeri	methanol	CA, ethene		[83]
	M. mazei	methanol	CA, ethene		
	Methanothrix soehngenii	acetate	CA, ethene		[83]
CA	Methanosarcina barkeri	methanol	ethane		[83]

Table 1.4 continued on next page

Tabel 1.4 continued

Compound	Microorganism	PS	Products	Rate[a]	Ref
TeCE	Desulfomonile tiedjei	pyruvate	TCE	$2.3 \cdot 10^{-3}$	[56]
	Acetobacterium woodii	fructose	TCE	<0.09	[52,176]
	Sporomusa ovata	methanol	TCE	0.24	
	Methanobacterium thermoautotrophicum	H_2	TCE		[50]
	Methanosarcina sp.	methanol	TCE	$8.4 \cdot 10^{-4}$	[55,56]
	M. mazei	methanol		$4.8 \cdot 10^{-4}$	
12DCE	Methanobacterium thermoautotrophicum	H_2	acetylene		[12]
	Methanococcus deltae	H_2	acetylene		[12]
	M. thermolithoautotrophicus	H_2			
α-HCH	Citrobacter freundii, Clostridium sp.	various			[89]
β-HCH	Citrobacter freundii, Clostridium sp.	various			[89]
γ-HCH	Clostridium sp.	various	γ-TeCCH, CB	0.032[d]	[79,89]
	Citrobacter freundii, Bacillus sp., others	various			[89]
δ-HCH	Citrobacter freundii, Clostridium sp.	various			[89]
PCP	Desulfomonile tiedjei	formate	TCP	1.3	[133]

[a] rate in mmol g^{-1} protein day^{-1} unless stated otherwise
[b] NI=not identified
[c] ND=not detected
[d] mmol g^{-1} dry weigth day^{-1}

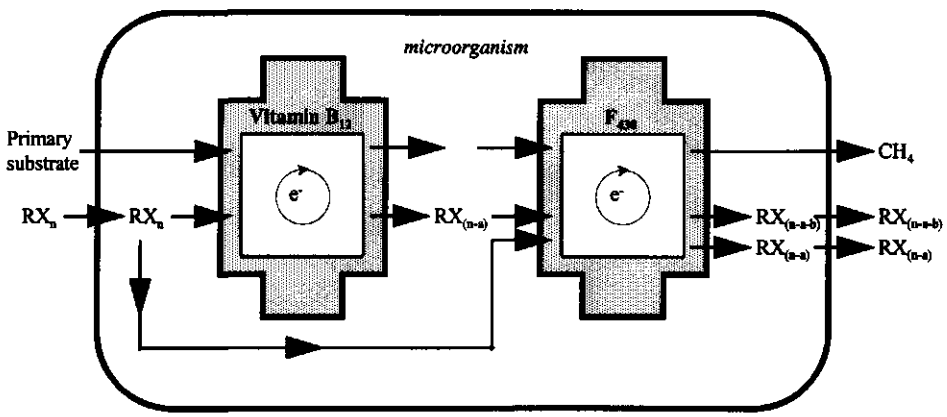


Figure 1.6 Schematic diagram of the role of biologically generated cofactors like vitamin B_{12} and cofactor F_{430} in the reductive cometabolic transformation of chlorinated compounds (RX_n) by methanogenic bacteria.

Also, cytochrome containing (facultative) anaerobes like *Pseudomonas putida, Shewanella putrefaciens* or *Desulfobacterium autotrophicum* are able to dechlorinate via cometabolic pathways. The products formed are diverse. Chlorinated methanes are usually completely dechlorinated (Table 1.4). In the case of higher chlorinated ethanes and ethenes, and PCP no more than two chlorine atoms are removed. γ-HCH, and other hexachlorocyclohexane isomers are also known to be converted. The end products of γ-HCH dechlorination have been identified as chlorobenzene and benzene. So far, aromatic compounds, except for PCP, are not known to be degraded via cometabolic pathways. This, can be explained by the fact that the transformation of chlorinated aromatics in general is considered to require specific enzyme systems [179]. The aspecificity of cometabolic reactions is shown by the broad chlorinated substrate spectrum of e.g., *Methanosarcina barkeri* and *Acetobacterium woodii.*

Only a few chlorinated compounds are used as carbon or energy source or as electron acceptor. The homoacetogenic bacterium *Acetobacterium dehalogenans* (formerly known as strain MC) uses MC as energy source by fermenting it to acetate [126,181]. DCM is used as carbon and energy source by *Dehalobactericum formicoaceticum* [119,120], which transforms DCM to acetate and formate. DCM and MC are degraded at 1.3 and 2.2 mmol (g protein)$^{-1}$ day^{-1}, respectively [120,126]). These rates are significantly higher than most of the rates mentioned in Table 1.4. The aforementioned specific dechlorinating bacteria which use chlorinated compounds as electron acceptor have been isolated with only TeCE, TCE, chlorinated phenols or 3-chlorobenzoate as electron acceptor. It has been postulated that (γ-)HCH may act as an alternative electron acceptor of the Stickland reaction, thus associating reductive dechlorination to ATP synthesis [140].

Dechlorination by granular sludge and other mixed cultures

Toxicity of chlorinated compounds towards methanogenic consortia. Granular sludge is a microbial community consisting of different anaerobic bacteria which are grown in UASB systems. UASB reactors are applied in many different industries [109]. However, anaerobic wastewater treatment systems are considered to be very sensitive towards toxic compounds. Especially, methanogenesis, which is the rate limiting step in anaerobic wastewater treatment, was found to be relatively easily affected by the presence of chlorinated compounds. This, may be due to the relatively low growth yield, which leads to a greater impact of a toxic substance. Only the chlorinated methanes are known to have a direct inhibitory effect on the mechanism of methane formation. Surprisingly, toxic concentrations reported for the different

compounds are relatively wide (Table 1.5). This is probably caused by the differences in the exposure time, sludge condition, primary substrates and other factors [54]. In contrast with the chlorinated aromatic compounds, a relation between the structure of an aliphatic chlorinated compound and its toxicity has not (yet) been established. For aromatic compounds, the methanogenic toxicity increases with an increasing number of chlorine atoms in the molecule [54]. Also, the octanol water coefficient was found to be positively correlated to the methanogenic inhibition [166]. For chlorinated aliphatics only a limited relationship between the electric dipole moment and toxicity has been found [159]. The precise mechanism by which toxicity of chlorinated compounds functions has not yet been elucidated. It is only known that PCP is able to uncouple oxidative phosphorylation [144], and the toxicity of higher chlorinated methanes was suggested to be caused by their structural analogy with (metabolic precursors of) methane [159]. Although the IC_{50} concentrations of several of the aliphatic compound are relatively low, it was found that a methanogenic consortium is able to recover largely from the exposure to chlorinated compounds within 48 hours [159].

Dechlorinating capacity of granular sludge and mixed cultures. Microbial transformations of chlorinated methanes [172], chlorinated fluorocarbons [141,170], chloroethanes (e.g., [39,173,184,186]), chlorinated ethenes (e.g., [8,128,185]), and 1,2-dichloropropane [116] have been observed in water, soils and sediments. Aromatic compounds, like chlorinated benzenes (e.g., [14,139,157]), PCBs and Aroclor mixtures (e.g., [9-11,143,150,152]), chlorinated phenols (e.g., [75,93,174]), or organic pesticides (e.g., DDT, and mirex) [134] and compounds like chlorinated dioxins [1,5,7,15] are also degraded under anaerobic conditions. The products formed are dependent on the microorganisms present and the environmental conditions applied. Usually, the degradation has been found to be more complete in the presence of mixed microbial cultures than pure bacterial cultures which can be explained by a variety of reasons. Firstly, the different microorganisms present will combine their dechlorinating activities. Secondly, and probably more important is that most studies have been carried out with inocula that have been previously exposed to chlorinated compounds, and that they often consisted of long term experiments. This may have lead to more extensive dechlorination, because specific dechlorinating mixed cultures had time to develop. The microorganisms in mixed cultures can also benefit from each others presence through syntrophic interactions, leading to a more complete dechlorination.

Table 1.5 Ranges for the methanogenic toxicity (as IC_{50}: 50% inhibition concentration) for chlorinated compounds

Chlorinated compound	IC_{50} (μM)	Ref.
CT	14.2 - 50	[16,149,159,178]
CF	4.2 - 14.2	[16,149,159,165,178]
DCM	84.7 - 1177	[16,159,178]
MC	990	[16]
HCA	92.8 - 126.7	[16,159]
PCA	54.3 - 123.6	[16,159]
1112TeCA	10.1 - 20.9	[16,159]
1122TeCA	24.4 - 107.3	[16,159]
111TCA	3.9 - 15.0	[16,159]
112TCA	10.5 - 43.5	[16,159]
11DCA	62.6	[16]
12DCA	135 - 961	[12,16,159]
TeCE	60.2 - >602	[13,16,95,159]
TCE	98.6 - 1301	[16,95,159]
12DCE	<135.0 - >1081	[12,16]
cDCE	551.1	[159]
tDCE	439.6 - 494.9	[16,159]
11DCE	79.4	[16]
TeCB	93	[16]
TCB	132 - 4132	[16,166]
DCB	585 - 1769	[16,166]
CB	2400 - 3380	[16,166]
PCP	0.15 - 30	[16,144,166]
TeCP	0.56	[16]
TCP	9.1 - 590	[16,166]
DCP	85.9 - 920	[16,166]
CP	200 - 4280	[16,41,54,166,191]

This may also be the reason for the difficulties which are encountered during the enrichment of dechlorinating bacteria and the isolation of pure cultures of specifically dechlorinating bacteria. The degradation of chlorinated compounds by mixed cultures can be considered to be a combination of abiotic and biological conversions. Chlorinated ethanes, like 1122TeCA

and 111TCA, have been found to be degraded via a combination of abiotic and biotic reactions leading to the formation of lower chlorinated and completely dechlorinated compounds [28,184], whereas dechlorination by pure microbial cultures, (albeit in circumstances which are not comparable,) often only results in the removal of one or two chlorine atoms via reductive processes.

Basically, there are two ways of obtaining the suitable dechlorinating activity in granular sludge from UASB reactors. One is to enrich for a specific dechlorinating population during long-term reactor operation and another is to incorporate specific bacterial cultures into the sludge or to construct new granules to obtain the desired activity. Research on the latter method has been limited. The introduction of the PCP degrading bacterium DCB-2 into dead granular sludge led to the transformation of PCP to 345TCP even at hydraulic retention times which were much lower than the doubling time of the incorporated bacterium [34]. Pure cultures of chlorinated aryl degrading bacteria like *Desulfomonile tiedje* [35] and *Dechlorosporium hafniense* [37] were also successfully incorporated in granular sludge, thus introducing dechlorinating activity towards 3-chlorobenzoate and PCP, respectively. Inoculation of TeCE degrading granular sludge with *Dehalospirillum multivorans* led to a more extensive dechlorination to cDCE during the test period of around 80 days; whereas uninoculated sludge only formed TCE [86]. Similar results have been obtained for PCB dechlorination by constructed methanogenic granules [136]. Still, little is known about the stability of these consortia over longer periods of time.

In most cases, the desired microbial community has been be obtained by enrichment in the (anaerobic) reactor and the degradation of many different compounds was established. Bouwer and McCarty [20] took anaerobic sewage sludge from a laboratory scale reactor, fed it with chlorinated compounds and found immediate transformation of CT and 1122TeCA. Other compounds like CF, 12DCA, 111TCA and TeCE required a lag phase. In other studies, bacterial consortia were first exposed to a mixture of chlorinated aliphatic and /or aromatic compounds to obtain some dechlorinating activity (e.g., for 1122TeCA [28]). By taking sewage sludge from a municipal wastewater treatment plant which was run with 40% industrial wastewater, HCB [58], TeCE and CF [59], and PCP dechlorinating activities [129] were obtained. Sometimes dechlorinating activity is also present in sewage sludge which has only been exposed to municipal wastewater (e.g., for TeCE [63] or HCH [23]).

Most research on removal of chlorinated compounds in anaerobic reactors has dealt with long term treatment of mixed contaminant wastestreams or compounds like chloroguaiacols [193] in wastewater of the pulp and paper industry ([49,97,98,106,153,165]). Information on

the dechlorinating capacity of granular sludge to degrade specific compounds is limited. Sometimes granular sludge is used as part of a mixture of sludges, soils and sediments which is used as an inoculum for the enrichment of certain bacteria [21,127]. So far, the majority of the research on dechlorination of pure compounds by granular sludge has been focused on the transformation of TeCE [36,72] and chlorinated phenols [77,132,194]. The results obtained are somewhat diverse. TeCE was converted at concentrations up to 102 μM to DCE (with a mixture of other carbon sources [72]), while lower concentrations of 27 μM (with ethanol, [36]) and around 60 μM (with formate and acetate [86]) led to the formation of tDCE and TCE, respectively. For PCP dechlorination in UASB reactors it has been found that the addition of extra electron donor was beneficial for the dechlorination process [78]. Apparently, PCP toxicity can be easily overcome. This is probably due to its degradation and not because of tolerance. Within 200 days of reactor operation, PCP (at concentrations of 150 to 225 μM) was completely converted to methane and CO_2 with a mixture of methanol and volatile fatty acids as carbon source.

Research objectives and background of experimental set-up

The ability to degrade chlorinated compounds obviously is widespread among different groups of bacteria. Some chlorinated compounds can be degraded by specifically dechlorinating bacteria. The rates obtained by these bacteria are high (20-450 mmol (g protein)$^{-1}$ day^{-1}). However, these bacteria are usually limited to the dechlorination of one or two compounds. This may be disadvantageous when a wastestream is contaminated with a mixture of chlorinated compounds, each one requiring its own specific dechlorinating enzyme. Anaerobic acetogenic and methanogenic bacteria are able to transform chlorinated compounds via aspecific cometabolic pathways. The dechlorination rates are relatively low (Table 1.4), but, because the dechlorination is aspecific, these low rates may be compensated by the broad spectrum of chlorinated compounds which can be degraded and the high biomass density of certain mixed communities. This research has evaluated the aspecific dechlorinating ability of unadapted acetogenic and methanogenic bacteria using methanogenic granular sludge. Granular sludge from UASB reactors was believed to be a suitable source of bacteria, because it has a high density of acetogenic and methanogenic bacteria [110]. This high biomass concentration could lead to high rates of dechlorination. The research described in this thesis deals mainly with the capacity of the anaerobic bacteria in granular sludge to transform chlorinated compounds. Although much research has been carried out in the field of

anaerobic wastewater treatment, still little is known about the dechlorinating ability of unadapted granular sludge. The aim of the research was to get more insight in the possible role of acetogenic and methanogenic bacteria for treatment of contaminated wastestreams. Another objective of the research was the evaluation of the applicability of unadapted methanogenic consortia for the bioremediation of wastestreams contaminated with mixtures of pollutants.

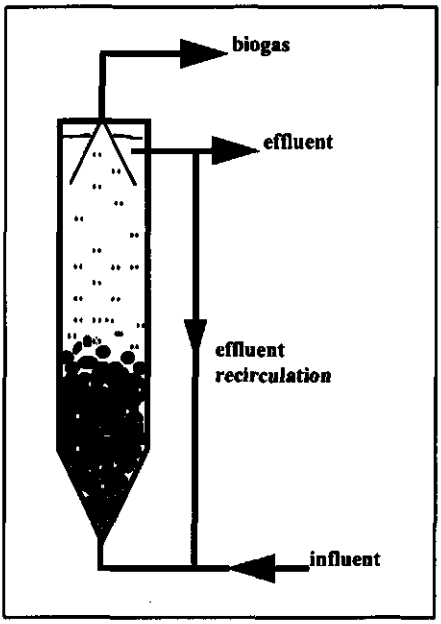


Figure 1.7 Schematic view of a lab scale UASB reactor

Three methanogenic consortia were grown in laboratory scale reactors (10 liters) with methanol, a mixture of volatile fatty acids (VFA: acetate, propionate, and butyrate) or sucrose (Fig. 1.7). These main substrates were chosen for several reasons. Firstly, they represent substrates commonly used by methanogenic consortia in UASB reactors applied for wastewater treatment. Secondly, methanol grown microorganisms are known to contain elevated amounts of vitamin B_{12}. Thirdly and foremostly, the substrates were chosen because a long term operation of UASB reactors with these substrates should result in three different microbial populations. Several stages can be distinguished during the anaerobic conversion of organic material (Fig. 1.8). Complex organic materials like polysaccharides, lipids and proteins are hydrolyzed in the first stage, leading to the formation of sugar mono- and oligomers, long chain fatty acids, amino acids, respectively, and H_2. These compounds are fermented to fatty acids, alcohols, H_2, and other fermentation products in the second stage. Acetogenic bacteria convert the compounds further to acetate, H_2, formate, CO_2. These compounds and methanol, which can be formed in the degradation of e.g., pectine, are subsequently degraded to methane in the final stage (methanogenesis) [73]. Sludge grown on sucrose will consist of more fermentative bacteria compared to VFA grown sludge, while methanogenic methanol grown sludge will have a completely different microbial population.

The sludges were not adapted to any halogenated compound prior to the experiments. By autoclaving the sludges and evaluating product formation during the transformation of chlorinated compounds, a distinction was made between the biological enzymatic activity of

living sludge and chemical reactions in autoclaved sludge. The abiotic reactions could be the result of either the bulk chemicals present in the sludge and the action of (biologically generated) cofactors, or the complex organic matter behaving as redox mediator. The cofactors responsible for the dechlorination (vitamin B_{12}, cofactor F_{430}, and hematin) consist of metal-containing porphyrin rings that are heat-stable [4,42]. By autoclaving the sludge, the (specific) enzyme activity in the sludge is inactivated; whereas, the cofactors and mediators remain stable. The presence of dechlorinating activity in the autoclaved sludge together with the instantaneous dechlorinating activity in living sludge, point towards the involvement of aspecific cometabolic processes in the transformation of chlorinated compounds.

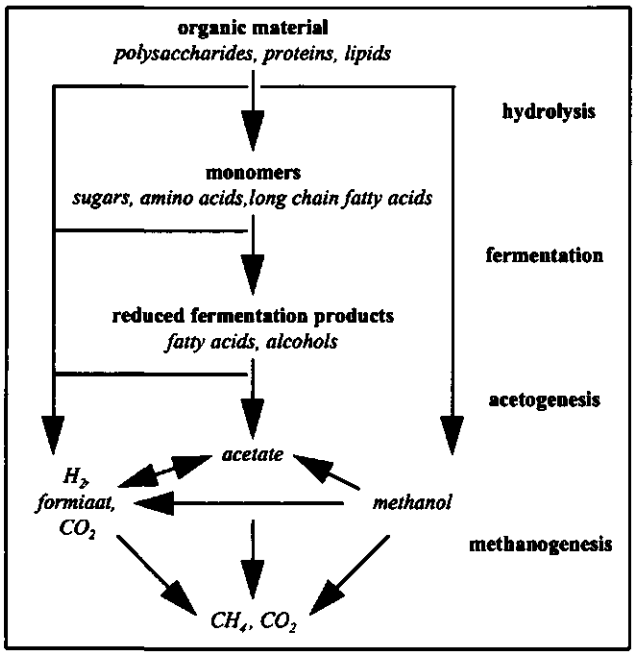


Figure 1.8 Simplified scheme of the transformation of organic material under anaerobic conditions.

Scope and structure of thesis

The first part of the thesis deals with the degradation of chlorinated alkanes. Chapter 2 describes the fate and degradation of carbon tetrachloride (CT) and lower chlorinated methanes by the three sludges. For this purpose, the granular sludges were incubated with CT and its lower chlorinated daughter products. The product formation by both living and

autoclaved sludge was determined and degradation rates were calculated. The influence of the substrate and biomass concentration were also tested with CT (Chapter 3). The dechlorination of other groups of chlorinated compounds were mainly tested with methanol-fed sludge. Chapters 4 and 5 describe the transformation of chlorinated ethanes and β-hexachlorocyclohexane, respectively. To investigate the dechlorinating activity of acetogenic and methanogenic bacteria in soil, α-and β-hexachlorocyclohexane contaminated soil was incubated and the products formed were evaluated (Chapter 5). Chapter 5 also describes experiments which were carried out to determine the effect of the addition of granular sludge to contaminated soil on the dechlorinating activity of the indigenous microbial population.

The transformation of chlorinated ethenes (Chapter 6) and chlorinated aromatic compounds like chlorobenzenes, PCP, and higher chlorinated PCBs (Chapter 7) was also investigated.

Finally, the results are discussed and summarized in chapter 8. The possible application of granular sludge for the remediation of contaminated wastestreams is also evaluated in this chapter. *Tenslotte worden in hoofdstuk 9 de resultaten van dit proefschrift in het nederlands samengevat.*

References

1. **Adriaens, P., Q. Z. Fu, and D. Grbicgalic.** 1995. Bioavailability and transformation of highly chlorinated dibenzo-p-dioxins and dibenzofurans in anaerobic soils and sediments. Environ. Sci. Technol. **29:**2252-2260.
2. **Assaf-Anid, N., K. F. Hayes, and T. M. Vogel.** 1994. Reductive dechlorination of carbon tetrachloride by cobalamin(II) in the presence of dithiothreitol - mechanistic study, effect of redox potential and pH. Environ. Sci. Technol. **28:**246-252.
3. **Assaf-Anid, N., L. Nies, and T. M. Vogel.** 1992. Reductive dechlorination of a polychlorinated biphenyl congener and hexachlorobenzene by vitamin B_{12}. Appl. Environ. Microbiol. **58:**1057-1060.
4. **Atkinson, B., and F. Mavituna.** 1991. Biochemical engineering and biotechnology handbook, 2nd ed. Stockton Press, New York.
5. **Ballerstedt, H., A. Kraus, and U. Lechner.** 1997. Reductive dechlorination of 1,2,3,4-tetrachlorodibenzo-p-dioxin and its products by anaerobic mixed cultures from Saale river sediment. Environ. Sci. Technol. **31:**1749-1753.
6. **Barbash, J. E., and M. Reinhard.** 1989. Abiotic dehalogenation of 1,2-dichloroethane and 1,2-dibromoethane in aqueous solution containing hydrogen sulfide. Environ. Sci. Technol. **23:**1349-1357.
7. **Barkovskii, A. L., and P. Adriaens.** 1996. Microbial dechlorination of historically present and freshly spiked chlorinated dioxins and diversity of dioxin-dechlorinating populations. Appl. Environ. Microbiol. **62:**4556-4562.

8. **Barrio-Lage, G., F. Z. Parsons, R. S. Nassar, and P. A. Lorenzo.** 1986. Sequential dehalogenation of chlorinated ethenes. Environ. Sci. Technol. **20:**96-99.
9. **Bedard, D. L.** 1990. Bacterial transformation of polychlorinated biphenyls. *In* Advan. Appl. Biotechnol. pp 369-88.
10. **Bedard, D. L., S. C. Bunnell, and L. A. Smullen.** 1996. Stimulation of microbial para-dechlorination of polychlorinated biphenyls that have persisted in Housatonic river sediments for decades. Environ. Sci. Technol. **30:**687-694.
11. **Bedard, D. L., R. E. Wagner, M. J. Brennan, M. L. Haberl, and J. J. F. Brown.** 1987. Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53:**1094-102.
12. **Belay, N., and L. Daniels.** 1987. Production of ethane, ethylene and acetylene from halogenated hydrocarbons by methanogenic bacteria. Appl. Environ. Microbiol. **53:**1604-1610.
13. **Berededsamuel, Y., J. N. Petersen, and R. S. Skeen.** 1996. Effect of perchloroethylene (PCE) on methane and acetate production by a methanogenic consortium. Appl. Biochem. Biotechnol. **57-8:**915-922.
14. **Beurskens, J. E. M., C. G. C. Dekker, H. van den Heuvel, M. Swart, and J. de Wolf.** 1994. Dechlorination of chlorinated benzenes by an anaerobic microbial consortium that selectively mediates the thermodynamic most favorable reactions. Environ. Sci. Technol. **28:**701-706.
15. **Beurskens, J. E. M., M. Toussaint, J. De Wolf, J. M. D. van der Steen, P. C. Slot, L. C. M. Commandeur, and J. R. Parsons.** 1995. Dehalogenation of chlorinated dioxins by an anaerobic microbial consortium from sediment. Environ. Toxicol. Chem. **14:**939-943.
16. **Blum, D. J. W., and R. E. Speece.** 1991. A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. Res. J. Wat. Poll. Contr. Fed. **63:**198-207.
17. **Boronina, T., K. J. Klabunde, and G. Sergeev.** 1995. Destruction of organohalides in water using metal particles: carbon tetrachloride/water reactions with magnesium, tin, and zinc. Environ. Sci. Technol. **29:**1511-1517.
18. **Bosma, T. N. P., F. H. M. Cottaar, M. A. Posthumus, C. J. Teunis, V. A. van Veldhuizen, G. Schraa, and A. J. B. Zehnder.** 1994. Comparison of reductive dechlorination of hexachloro-1,3-butadiene in Rhine sediment and model systems with hydroxycobalamin. Environ. Sci. Technol. **28:**1124-1128.
19. **Bouchard, B., R. Beaudet, R. Villemur, G. McSween, F. Lepine, and J.-G. Bisaillon.** 1996. Isolation and characterization of *Desulfitobacterium frappieri* sp. nov., an anaerobic bacterium which reductively dechlorinates pentachlorophenol to 3-chlorophenol. Int. J. Syst. Bacteriol. **46:**1010-1015.
20. **Bouwer, E. J., and P. L. McCarty.** 1983. Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl. Environ. Microbiol. **45:**1286-1294.
21. **Bruin, W. P. de, M. J. J. Kotterman, M. A. Posthumus, G. Schraa, and A. J. B. Zehnder.** 1992. Complete biological reductive transformation of tetrachloroethene to ethane. Appl. Environ. Microbiol. **58:**1996-2000.
22. **Burris, D. R., C. A. Delcomyn, M. H. Smith, and A. L. Roberts.** 1996. Reductive dechlorination of tetrachloroethylene and trichloroethylene catalyzed by vitamin b-12 in homogeneous and heterogeneous systems. Environ. Sci. Technol. **30:**3047-3052.
23. **Buser, H., and M. D. Muller.** 1995. Isomer and enantioselective degradation of hexachlorocyclohexane isomers in sewage sludge under anaerobic conditions. Environ. Sci. Technol. **29:**664-672.
24. **Butler, E. C., and K. F. Hayes.** 1998. Effects of solution composition and pH on the reductive dechlorination of hexachloroethane by iron sulfide. Environ. Sci. Technol. **32:**1276-1284.

25. **Calza, P., C. Minero, and E. Pelizzetti.** 1997. Photocatalytically assisted hydrolysis of chlorinated methanes under anaerobic conditions. Environ. Sci. Technol. **31:**2198-2203.
26. **Castro, C. E., R. S. Wade, and N. O. Belser.** 1985. Biodehalogenation: reactions of cytochrome P-450 with polyhalomethanes. Biochemistry. **24:**204-210.
27. **Chaudhry, G. R., and S. Chapalamadugu.** 1991. Biodegradation of halogenated organic compounds. Microbiol. Rev. **55:**59-79.
28. **Chen, C., J. A. Puhakka, and J. F. Ferguson.** 1996. Transformations of 1,1,2,2-tetrachloroethane under methanogenic conditions. Environ. Sci. Technol. **30:**542-547.
29. **Chiu, P., and M. Reinhard.** 1995. Metallocoenzyme-mediated reductive transformation of carbon tetrachloride in titanium(III) citrate aqueous solution. Environ. Sci. Technol. **29:**595-603.
30. **Chiu, P.-C., and M. Reinhard.** 1996. Transformation of carbon tetrachloride by reduced vitamin B_{12} in aqueous cysteine solution. Environ. Sci. Technol. **30:**1882-1889.
31. **Choi, W., and M. R. Hoffmann.** 1995. Photoreductive mechanism of CCl4 degradation on TiO2 particles and effects of electron donors. Environ. Sci. Technol. **29:**1646-1654.
32. **Choi, W. Y., and M. R. Hoffmann.** 1997. Novel photocatalytic mechanisms for $CHCl_3$, $CHBr_3$, and CCl_3CO_2- degradation and the fate of photogenerated trihalomethyl radicals on TiO_2. Environ. Sci. Technol. **31:**89-95.
33. **Christiansen, N., and B. K. Ahring.** 1996. *Desulfitobacterium hafniense* sp. nov., an anaerobic reductively dechlorinating bacterium. Int. J. Syst. Bacteriol. **46:**442-448.
34. **Christiansen, N., and B. K. Ahring.** 1996. Introduction of a *de novo* bioremediation activity into anaerobic granular sludge using the dechlorinating bacterium DCB-2. Antonie van Leeuwenhoek. **69:**61-66.
35. **Christiansen, N., and B. K. Ahring.** 1994. Introduction of bioremediation ability into granular sludge. Wat. Sci. Technol. **29:**189-193.
36. **Christiansen, N., S. R. Christensen, E. Arvin, and B. K. Ahring.** 1997. Transformation of tetrachloroethene in an upflow anaerobic sludge blanket reactor. Appl. Microbiol. Biotechnol. **47:**91-94.
37. **Christiansen, N., H. V. Hendriksen, K. T. Jarvinen, and B. K. Ahring.** 1995. Degradation of chlorinated aromatic compounds in UASB reactors. Wat. Sci. Technol. **31:**249-259.
38. **Cole, J. R., A. L. Cascarelli, W. W. Mohn, and J. M. Tiedje.** 1994. Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol. Appl. Environ. Microbiol. **60:**3536-3542.
39. **Crosby, D. G., and E. Leitis.** 1969. Reduction of hexachloroethane to tetrachloroethylene in groundwater. J. Contam. Hydrol. **1:**133-142.
40. **Curtis, G. P., and M. Reinhard.** 1994. Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthrahydroquinone disulfonate and humic acid. Environ. Sci. Technol. **28:**2393-2401.
41. **Davies-Venn, C., J. C. Young, and H. H. Tabak.** 1992. Impact of chlorophenols and chloroanilines on the kinetics of acetoclastic methanogens. Environ. Sci. Tech. **26:**1627-1635.
42. **Dawson, R. M. C., D. C. Elliot, W. H. Elliott, and K. M. Jones.** 1986. Data for biochemical research, 3rd ed. Clarendon Press, Oxford.
43. **DeWeerd, K. A., L. Mandelco, S. Tanner, C. R. Woese, and J. M. Sulfita.** 1990. *Desulfomonile tiedje* gen. nov. and sp. nov., a novel anaerobic, dehalogenating, sulfate-reducing bacterium. Arch. Microbiol. **154:**23-30.
44. **Diekert, G., U. Konheiser, K. Pichulla, and R. K. Thauer.** 1981. Nickel requirement and factor F_{430} content of methanogenic bacteria. J. Bacteriol. **148:**459-464.

45. **Dolfing, J., and J. E. M. Beurskens.** 1995. The microbial logic and environmental significance of reductive dehalogenation, p. 143-206. *In* J. G. Jones (ed.), Adv. Microb. Ecol., vol. 14. Plenum Press, NewYork.
46. **Dolfing, J., and B. K. Harrison.** 1992. Gibbs free energy of formation of halogenated aromatic compounds and their potential role as electron acceptors in anaerobic environments. Environ. Sci. Technol. **26:**2213-2218.
47. **Dolfing, J., and D. B. Janssen.** 1994. Estimates of Gibbs free energies of formation of chlorinated aliphatic compounds. Biodegradation. **5:**21-28.
48. **Doong, R.-A., and S.-C. Wu.** 1992. Reductive dechlorination of chlorinated hydrocarbons in aqueous solutions containing ferrous and sulfide ions. Chemosphere. **24:**1063-1075.
49. **Duff, S. J. B., K. J. Kennedy, and A. J. Brady.** 1995. Treatment of dilute phenol PCP wastewaters using the upflow anaerobic sludge blanket (UASB) reactor. Wat. Res. **29:**645-651.
50. **Egli, C., R. Scholtz, A. M. Cook, and T. Leisinger.** 1987. Anaerobic dechlorination of tetrachloromethane and 1,2-dichloroethane to degradable products by pure cultures of *Desulfobacterium* sp. and *Methanobacterium* sp. FEMS Microbiol. Lett. **43:**257-61.
51. **Egli, C., S. Stromeyer, A. M. Cook, and T. Leisinger.** 1990. Transformation of tetra- and trichloromethane to CO_2 by anaerobic bacteria is a non-enzymic process. FEMS Microbiol. Lett. **68:**207-212.
52. **Egli, C., T. Tschan, R. Scholtz, A. M. Cook, and T. Leisinger.** 1988. Transformation of tetrachloromethane to dichloromethane and carbon dioxide by *Acetobacterium woodii*. Appl. Environ. Microbiol. **54:**2819-24.
53. **El Fantroussi, S., H. Naveau, and S. N. Agathos.** 1998. Anaerobic dechlorinating bacteria. Biotechnol. Progr. **14:**167-188.
54. **Ennik-Maarsen, K. A., A. Louwerse, W. Roelofsen, and A. J. M. Stams.** 1998. Influence of monochlorophenols on methanogenic activity in granular sludge. Water Res. **32:**2977-2982.
55. **Fathepure, B. Z., and S. A. Boyd.** 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. FEMS Microbiol. Lett. **49:**149-156.
56. **Fathepure, B. Z., J. P. Nengu, and S. A. Boyd.** 1987. Anaerobic bacteria that dechlorinate perchloroethene. Appl. Environ. Microbiol. **53:**2671-2674.
57. **Fathepure, B. Z., and J. M. Tiedje.** 1994. Reductive dechlorination of tetrachloroethylene by a chlorobenzoate-enriched biofilm reactor. Environ. Sci. Technol. **28:**746-752.
58. **Fathepure, B. Z., J. M. Tiedje, and S. A. Boyd.** 1988. Reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in anaerobic sewage sludge. Appl. Environ. Microbiol. **54:**327-330.
59. **Fathepure, B. Z., and T. M. Vogel.** 1991. Complete degradation of polychlorinated hydrocarbons by a two-stage biofilm reactor. Appl. Environ. Microbiol. **57:**3418-3422.
60. **Fennelly, J. P., and A. L. Roberts.** 1998. Reaction of 1,1,1-trichloroethane with zero-valent metals and bimetallic reductants. Environ. Sci. Technol. **32:**1980-1988.
61. **Fetzner, S.** 1998. Bacterial dehalogenation. Appl. Microbiol. Biotechnol. **50:**633-657.
62. **Florencio, L.** The fate of methanol in anaerobic bioreactors. 1994. Ph.D. Agricultural University, Wageningen.
63. **Freedman, D. L., and J. M. Gossett.** 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl. Environ. Microbiol. **55:**2144-2151.
64. **Gälli, R., and P. L. McCarty.** 1989. Biotransformation of 1,1,1-trichloroethane, trichloromethane, and tetrachloromethane by a *Clostridium* sp. Appl. Environ. Microbiol. **55:**837-844.
65. **Gälli, R., and P. L. McCarty.** 1989. Kinetics of biotransformation of 1,1,1-trichloroethane by *Clostridium* sp. strain TCAIIB. Appl. Environ. Microbiol. **55:**845-851.

66. **Gantzer, C. J., and L. P. Wackett.** 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. Environ. Sci. Technol. **25**:715-722.
67. **Gerkens, R. R., and J. A. Franklin.** 1989. The rate of degradation of 1,1,1-trichloroethane in water by hydrolysis and dehydrochlorination. Chemosphere. **19**:1929-1937.
68. **Gerritse, J., V. Renard, T. M. Pedro Gomes, P. A. Lawson, M. D. Collins, and J. C. Gottschal.** 1996. *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or ortho-chlorinated phenols. Arch. Microbiol. **165**:132-140.
69. **Glod, G., W. Angst, C. Holliger, and R. P. Schwarzenbach.** 1997. Corrinoid-mediated reduction of tetrachloroethene, trichloroethene, and trichlorofluoroethene in homogeneous aqueous solution: reaction kinetics and reaction mechanisms. Environ. Sci. Technol. **31**:253-260.
70. **Glod, G., U. Brodmann, W. Angst, C. Holliger, and R. P. Schwarzenbach.** 1997. Cobalamin mediated reduction of cis and trans dichloroethene, 1,1 dichloroethene, and vinyl chloride in homogeneous aqueous solution: reaction kinetics and mechanistic considerations. Environ. Sci. Technol. **31**:3154-3160.
71. **Gribble, G. W.** 1994. The natural production of chlorinated compounds. Environ. Sci. Technol. **28**:310A-319A.
72. **Guiot, S. R., X. Kuang, C. Beaulieu, A. Corriveau, and J. Hawari.** Anaerobic and aerobic/anaerobic treatment for tetrachloroethylene (PCE). 1995. Proceedings of the Third International In Situ and On-Site Bioreclamation Symposium, 4 - Bioremediation of chlorinated solvents, San Diego. pp 297-305.
73. **Gujer, W., and A. J. B. Zehnder.** 1983. Conversion processes in anaerobic digestion. Wat. Sci. Technol. **15**:127-167.
74. **Haarstrick, A., O. M. Kut, and E. Heinzle.** 1996. TiO2-assisted degradation of environmentally relevant organic compounds in wastewater using a novel fluidized bed photoreactor. Environ. Sci. Technol. **30**:817-824.
75. **Haggblom, M. M., and L. Y. Young.** 1995. Anaerobic degradation of halogenated phenols by sulfate-reducing consortia. Appl. Environ. Microbiol. **61**:1546-1550.
76. **Hanselmann, K. W.** 1991. Microbial energetics applied to waste repositories. Experientia. **47**:645-687.
77. **Hendriksen, H. V., and B. K. Ahring.** 1992. Metabolism and kinetics of pentachlorophenol transformation in anaerobic granular sludge. Appl. Microbiol. Biotechnol. **37**:662-666.
78. **Hendriksen, H. V., S. Larsen, and B. K. Ahring.** 1992. Influence of a supplemental carbon source on anaerobic dechlorination of pentachlorophenol in granular sludge. Appl. Environ. Microbiol. **58**:365-370.
79. **Heritage, A. D., and I. C. MacRae.** 1979. Degradation of hexachlorocyclohexanes and structurally related substances by *Clostridium sphenoides*. Austr. J. Biol. Sci. **32**:493-500.
80. **Hoekstra, E. J., F. J. M. Verhagen, J. A. Field, E. W. B. De Leer, and U. A. T. Brinkman.** 1998. Natural production of chloroform by fungi. Phytochem. **49**:91-97.
81. **Holliger, C., A. J. Pierik, E. J. Reijerse, and W. R. Hagen.** 1993. A spectroelectrochemical study of factor F_{430} nickel (II/I) from methanogenic bacteria in aqueous solution. J. Am. Chem. Soc. **115**:5651 ev.
82. **Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder.** 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. **59**:2991-2997.
83. **Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder.** 1990. Reductive dechlorination of 1,2-dichloroethane and chloroethane by cell suspensions of methanogenic bacteria. Biodegradation. **1**:253-261.

84. **Holliger, C., G. Schraa, E. Stupperich, A. J. M. Stams, and A. J. Zehnder.** 1992. Evidence for the involvement of corrinoids and factor F_{430} in the reductive dechlorination of 1,2-dichloroethane by *Methanosarcina barkeri*. J. Bacteriol. **174**:4427-4434.
85. **Holliger, H. C.** Reductive dehalogenation by anaerobic bacteria. 1992. Ph.D. Thesis. Agricultural University, Wageningen.
86. **Hörber, C., N. Christiansen, E. Arvin, and B. K. Ahring.** 1998. Improved dechlorinating performance of upflow anaerobic sludge blanket reactors by incorporation of *Dehalospirillum multivorans* into granular sludge. Appl. Environ. Microbiol. **64**:1860-1863.
87. **Hua, I., and M. R. Hoffmann.** 1996. Kinetics and mechanism of the sonolytic degradation of CCl4: Intermediates and byproducts. Environ. Sci. Technol. **30**:864-871.
88. **Inoue, K., S. Kageyama, K. Miki, T. Morinaga, Y. Kamagata, K. Nakamura, and E. Mikami.** 1992. Vitamin B_{12} production by *Acetobacterium* sp. and its tetrachloromethane-resistant mutants. J. Ferm. Bioeng. **73**:76-78.
89. **Jagnow, G., K. Haider, and P. C. Ellwardt.** 1977. Anaerobic dechlorination and degradation of hexachlorocyclohexane isomers by anaerobic and facultative anaerobic bacteria. Arch. Microbiol. **115**:285-292.
90. **Janssen, D. B., and W. d. Koning.** 1995. Development and application of bacterial cultures for the removal of chlorinated aliphatics. Wat. Sci. Technol. **31**:237-247.
91. **Jeffers, P. M., L. M. Ward, L. M. Woytowitch, and N. L. Wolfe.** 1989. Homogeneous hydrolysis rate constants for selected chlorinated methanes, ethanes , ethenes, and propanes. Environ. Sci. Technol. **23**:965.
92. **Jong, E. de, and J. A. Field.** 1997. Sulfur Tuft and Turkey Tail: Biosynthesis and biodegradation of organohalogens by basidiomycetes. Annu. Rev. Microbiol. **51**:375-414.
93. **Juteau, P., R. Beaudet, G. Mcsween, F. Lepine, and J. G. Bisaillon.** 1995. Study of the reductive dechlorination of pentachlorophenol by a methanogenic consortium. Can. J. Microbiol. **41**:862-868.
94. **Kästner, M.** 1991. Reductive dechlorination of tri- and tetrachloroethylenes depends on transition from aerobic tot anaerobic conditions. Appl. Environ. Microbiol. **57**:2039-2046.
95. **Kennes, C., M. C. Veiga, and L. Bhatnagar.** 1998. Methanogenic and perchloroethylene-dechlorinating activity of anaerobic granular sludge. Appl. Microbiol. Biotechnol. **50**:484-488.
96. **Klecka, G. M., and S. J. Gonsior.** 1984. Reductive dechlorination of chlorinated methanes and ethanes by reduced iron (II) porphyrins. Chemosphere. **13**:391-402.
97. **Kortekaas, S., M. Soto, T. Vicent, J. A. Field, and G. Lettinga.** 1995. Contribution of extractives to methanogenic toxicity of hemp black liquor. J. Ferm. Bioeng. **80**:383-388.
98. **Kortekaas, S., R. R. Wijngaarde, J. W. Klomp, G. Lettinga, and J. A. Field.** 1998. Anaerobic treatment of hemp thermomechanical pulping wastewater. Water Res. **32**:3362-3370.
99. **Kriegman-King, M. R., and M. Reinhard.** 1994. Transformation of carbon tetrachloride by pyrite in aqueous solution. Environ. Sci. Technol. **28**:692-700.
100. **Krone, U. E., K. Laufer, R. K. Thauer, and H. P. C. Hogenkamp.** 1989. Coenzyme F_{430} as a possible catalyst for the reductive dehalogenation of chlorinated C1 hydrocarbons in methanogenic bacteria. Biochemistry. **28**:10061-10065.
101. **Krone, U. E., and R. K. Thauer.** 1992. Dehalogenation of trichlorofluoromethane (CFC 11) by *Methanosarcina barkeri*. FEMS Microbiol. Lett. **90**:201-204.
102. **Krone, U. E., R. K. Thauer, and H. P. C. Hogenkamp.** 1989. Reductive dehalogenation of chlorinated C1-hydrocarbons mediated by corrinoids. Biochemistry. **28**:4908-4914.
103. **Krone, U. E., R. K. Thauer, H. P. C. Hogenkamp, and K. Steinbach.** 1991. Reductive formation of carbon monoxide from CCl4 and freons 11, 12 and 13 catalyzed by corrinoids. Biochemistry. **30**:2713-2719.

104. **Krumholz, L. R., R. Sharp, and S. S. Fishbain.** 1996. A freshwater anaerobe coupling acetate oxidation to tetrachloroethylene dehalogenation. Appl. Environ. Microbiol. **62:**4108-4113.
105. **Krzycki, J., and J. G. Zeikus.** 1980. Quantification of corrinoids in methanogenic bacteria. Curr. Microbiol. **3:**243-245.
106. **Kudo, A., K. Kennedy, and E. Andras.** 1991. Anaerobic (UASB) treatment of pulp (CTMP) wastewater and the toxicity on granules. Wat. Sci. Tech. **23:**1919-1928.
107. **Leisinger, T.** 1983. Microorganisms and xenobiotic compounds. Experientia. **39:**1183-1191.
108. **Lesage, S., S. Brown, and K. Millar.** 1998. A different mechanism for the reductive dechlorination of chlorinated ethenes: Kinetic and spectroscopic evidence. Environ. Sci. Technol. **32:**2264-2272.
109. **Lettinga, G., and L. W. Hulshoff Pol.** 1991. UASB-processes design for various types of wastewaters. Wat. Sci. Technol. **24:**87-107.
110. **Lettinga, G., A. F. M. van Velsen, S. W. Hobma, W. de Zeeuw, and A. Klapwijk.** 1980. Use of upflow anaerobic (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioeng. **22:**677-734.
111. **Lewis, T. A., and R. L. Crawford.** 1993. Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas* sp. Strain KC. Appl. Environ. Microbiol. **59:**1635-1641.
112. **Lewis, T. A., M. J. Morra, and P. D. Brown.** 1996. Comparative product analysis of carbon tetrachloride dehalogenation catalyzed by cobalt corrins in the presence of thiol or titanium (III) reducing agents. Environ. Sci. Technol. **30:**292-300.
113. **Lichtin, N. N., and M. Avudaithai.** 1996. Tio2-photocatalyzed oxidative degradation of CH3CN, CH3OH, C2HCl3, and CH2Cl2 supplied as vapors and in aqueous solution under similar conditions. Environ. Sci. Technol. **30:**2014-2020.
114. **Lide, D. R., and H. P. R. Frederikse (ed.).** 1996. CRC Handbook of Chemistry and Physics, 77th ed. CRC Press, Boca Raton, Ann Arbor, London, Tokyo.
115. **Lipczynska-Kochany, E., S. Harms, R. Milburn, G. Sprah, and N. Nadarajah.** 1994. Degradation of carbon tetrachloride in the presence of iron and sulpher containing compounds. Chemosphere. **29:**1477-1489.
116. **Löffler, F. E., J. E. Champine, K. M. Ritalahti, S. J. Sprague, and J. M. Tiedje.** 1997. Complete reductive dechlorination of 1,2 dichloropropane by anaerobic bacteria. Appl. Environ. Microbiol. **63:**2870-2875.
117. **Logan, M. S. P., L. M. Newman, C. A. Schanke, and L. P. Wacket.** 1993. Cosubstrate effects in reductive dehalogenation by *Pseudomonas putida* G786 expressing cytochrome P-450 CAM. Biodegradation. **4:**39-50.
118. **Lovley, D. R., and J. C. Woodward.** 1992. Consumption of freons CFC-11 and CFC-12 by anaerobic sediments and soils. Environ. Sci. Technol. **26:**925-929.
119. **Magli, A., M. Messmer, and T. Leisinger.** 1998. Metabolism of dichloromethane by the strict anaerobe *Dehalobacterium formicoaceticum*. Appl. Environ. Microbiol. **64:**646-650.
120. **Mägli, A., M. Wendt, and T. Leisinger.** 1996. Isolation and characterization of *Dehalobacterium formicoaceticum* gen. nov. sp. nov., a strictly anaerobic bacterium utilizing dichloromethane as source of carbon and energy. Arch. Microbiol. **166:**101-108.
121. **Marks, T. S., J. D. Allpress, and A. Maule.** 1989. Dehalogenation of lindane by a variety of porphyrins and corrins. Appl. Environ. Microbiol. **55:**1258-1261.
122. **Marks, T. S., and A. Maule.** 1992. The use of immobilized porphyrins and corrins to dehalogenate organochlorine pollutants. Appl. Microbiol. Biotechnol. **38:**413-416.
123. **Matheson, L. J., and P. G. Tratnyek.** 1994. Reductive dehalogenation of chlorinated methanes by iron metal. Environ. Sci. Technol. **28:**2045-2053.

124. **Maymogatell, X., Y. Chien, J. M. Gossett, and S. H. Zinder.** 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Sci. **276:**1568-1571.
125. **Mazumder, T. K., N. Nishio, S. Fukuzaki, and S. Nagai.** 1987. Production of extracellular vitamin B-12 compounds from methanol by *Methanosarcina barkeri*. Appl. Microbiol. Biotechnol. **26:**511-516.
126. **Messmer, M., G. Wohlfarth, and G. Diekert.** 1993. Methyl chloride metabolism of the strictly anaerobic, methyl chloride-utilizing homoacetogen strain MC. Arch. Microbiol. **160:**383-387.
127. **Middeldorp, P. J. M., M. Jaspers, A. J. B. Zehnder, and G. Schraa.** 1996. Biotransformation of alpha-, beta-, gamma-, and delta-hexachlorocyclohexane under methanogenic conditions. Environ. Sci. Technol. **30:**2345-2349.
128. **Middeldorp, P. J. M., M. A. van Aalst, H. H. M. Rijnaarts, F. J. M. Stams, H. F. de Kreuk, G. Schraa, and T. N. P. Bosma.** 1998. Stimulation of reductive dechlorination for in situ bioremediation of a soil contaminated with chlorinated ethenes. Water Sci. Technol. **37:**105-110.
129. **Mikesell, M. D., and S. A. Boyd.** 1986. Complete reductive dechlorination and mineralization of pentachlorophenol by anaerobic microorganisms. Appl. Environ. Microbiol. **52:**861-865.
130. **Mikesell, M. D., and S. A. Boyd.** 1990. Dechlorination of chloroform by *Methanosarcina* strains. Appl. Environ. Microbiol. **56:**1198-1201.
131. **Miller, P. L., D. Vasudevan, P. M. Gschwend, and A. L. Roberts.** 1998. Transformation of hexachloroethane in a sulfidic natural water. Environ. Sci. Technol. **32:**1269-1275.
132. **Mohn, W. W., and K. J. Kennedy.** 1992. Limited degradation of chlorophenols by anaerobic sludge granules. Appl. Environ. Microbiol. **58:**2131-2136.
133. **Mohn, W. W., and K. J. Kennedy.** 1992. Reductive dehalogenation of chlorophenols by *Desulfomonile tiedjei* strain DCB-1. Appl. Environ. Microbiol. **58:**1367-1370.
134. **Mohn, W. W., and J. M. Tiedje.** 1992. Microbial reductive dehalogenation. Microbiol. Rev. **56:**482-507.
135. **Mross, K. G., and J. K. Konietzko.** 1991. Chlorinated ethanes. *In* O. Hutzinger (ed.), The handbook of environmental chemistry, vol. 3G. Springer - Verlag, Berlin Heidelberg.
136. **Natarajan, M. R., W. M. Wu, J. Nye, H. Wang, L. Bhatnagar, and M. K. Jain.** 1996. Dechlorination of polychlorinated biphenyl congeners by an anaerobic microbial consortium. Appl. Microbiol. Biotechnol. **46:**673-677.
137. **Ngabe, B., T. F. Bidleman, and R. L. Falconer.** 1993. Base hydrolysis of α- and γ-hexachlorocyclohexane. Environ. Sci. Technol. **27:**1930-1933.
138. **Novak, P. J., L. Daniels, and G. F. Parkin.** 1998. Rapid dechlorination of carbon tetrachloride and chloroform by extracellular agents in cultures of *Methanosarcina thermophila*. Environ. Sci. Technol. **32:**3132-3136.
139. **Nowak, J., N. H. Kirsch, W. Hegemann, and H. J. Stan.** 1996. Total reductive dechlorination of chlorobenzenes to benzene by a methanogenic mixed culture enriched from Saale river sediment. Appl. Microbiol. Biotechnol. **45:**700-709.
140. **Ohisa, N., N. Kurihara, and M. Nakajima.** 1982. ATP synthesis associated with the conversion of hexachlorocyclohexane related compounds. Arch. Microbiol. **131:**330-333.
141. **Oremland, R. S., D. J. Lonergan, C. W. Culbertson, and D. R. Lovley.** 1996. Microbial degradation of hydrochlorofluorocarbons (CHCl2F and CHCl2CF3) in soils and sediments. Appl. Environ. Microbiol. **62:**1818-1821.
142. **Orth, W. S., and R. W. Gillham.** 1996. Dechlorination of trichloroethene in aqueous solution using Fe^0. Environ. Sci. Technol. **30:**66-71.
143. **Pagano, J. J., R. J. Scrudato, R. N. Roberts, and J. C. Bemis.** 1995. Reductive dechlorination of PCB-contaminated sediments in an anaerobic bioreactor system. Environ. Sci. Technol. **29:**2584-2589.

144. **Patel, G. B., B. J. Agnew, and C. J. Dicaire.** 1991. Inhibition of pure cultures of methanogens by benzene ring compounds. Appl. Environ. Microbiol. **57:**2969-2974.
145. **Pearson, C. R.** 1982. C1 and C2 halocarbons, p. 69-88. *In* O. Hutzinger (ed.), The handbook of environmental chemistry, vol. 3B. Springer-Verlag, Berlin.
146. **Perlinger, J. A., W. Angst, and R. P. Schwarzenbach.** 1996. Kinetics of the reduction of hexachloroethane by juglone in solutions containing hydrogen. Environ. Sci. Technol. **30:**3408-3417.
147. **Perlinger, J. A., J. Buschmann, W. Angst, and R. P. Schwarzenbach.** 1998. Iron porphyrin and mercaptojuglone mediated reduction of polyhalogenated methanes and ethanes in homogeneous aqueous solution. Environ. Sci. Technol. **32:**2431-2437.
148. **Petrovskis, E. A., T. M. Vogel, and P. Adriaens.** 1994. Effects of electron acceptors and donors on transformation of tetrachloromethane by *Shewanella putreficans* MR-1. FEMS Microbiol. Lett. **121:**357-364.
149. **Prins, R. A., C. J. v. Nevel, and D. I. Demeyer.** 1972. Pure culture studies of inhibitors for methanogenic bacteria. Antonie van Leeuwenhoek. **38:**281-287.
150. **Quensen, I. J. F., S. A. Boyd, and J. M. Tiedje.** 1990. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. Appl. Environ. Microbiol. **56:**2360-2369.
151. **Reineke, W., and H.-J. Knackmuss.** 1988. Microbial degradation of haloaromatics. Ann. Rev. Microbiol. **42:**263-287.
152. **Rhee, G. Y., R. C. Sokol, C. M. Bethoney, and B. Bush.** 1993. A long-term study of anaerobic dechlorination of PCB congeners by sediment microorganisms - pathways and mass balance. Environ. Toxicol. Chem. **12:**1829-1834.
153. **Rintala, J. A., and J. A. Puhakka.** 1994. Anaerobic treatment in pulp- and paper-mill waste management - a review. Biores. Technol. **47:**1-18.
154. **Rippen.** 1994. Rippen Handbuch Umweltchemikalien. *In* G. Rippen (ed.). Ecomed, Landsberg/Lech.
155. **Roberts, A. L., and P. M. Gschwend.** 1991. Mechanism of pentachloroethane dehydrochlorination to tetrachloroethene. Environ. Sci. Technol. **25:**76-86.
156. **Roberts, A. L., L. A. Totten, W. A. Arnold, D. R. Burris, and T. J. Campbell.** 1996. Reductive elimination of chlorinated ethylenes by zero valent metals. Environ. Sci. Technol. **30:**2654-2659.
157. **Rosenbrock, P., R. Martens, F. Buscot, and J. C. Munch.** 1997. Initiation of [Cl36]hexachlorobenzene dechlorination in three different soils under artificially induced anaerobic conditions. Appl. Microbiol. Biotechnol. **48:**115-120.
158. **Sanford, R. A., J. R. Cole, F. E. Loffler, and J. N. Tiedje.** 1996. Characterization of *Desulfitobacterium chlororespirans* sp nov, which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. Appl. Environ. Microbiol. **62:**3800-3808.
159. **Sanz, J. L., N. Rodriguez, and R. Amils.** 1997. Effect of chlorinated aliphatic hydrocarbons on the acetoclastic methanogenic activity of granular sludge. Appl. Microbiol. Biotechnol. **47:**324-328.
160. **Schanke, C. A., and L. P. Wackett.** 1992. Environmental reductive elimination reactions of polychlorinated ethanes mimicked by transition-metal coenzymes. Environ. Sci. Technol. **26:**830-833.
161. **Scherer, M. M., J. C. Westall, M. Ziomekmoroz, and P. G. Tratnyek.** 1997. Kinetics of carbon tetrachloride reduction at an oxide free iron electrode. Environ. Sci. Technol. **31:**2385-2391.
162. **Scholz Muramatsu, H., A. Neumann, M. Messmer, E. Moore, and G. Diekert.** 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Arch. Microbiol. **163:**48-56.

163. **Schreier, C. G., and M. Reinhard.** 1995. Catalytic hydrodehalogenation of chlorinated ethylenes using palladium and hydrogen for the treatment of contaminated water. Chemosphere. **31:**3475-3487.
164. **Schreier, C. G., and M. Reinhard.** 1994. Transformation of chlorinated organic compounds by iron and manganese powders in buffered water and in landfill leachate. Chemosphere. **29:**1743-1753.
165. **Sierra-Alvarez, R., J. A. Field, S. Kortekaas, and G. Lettinga.** 1994. Overview of the anaerobic toxicity caused by organic forest industry wastewater pollutants. Wat. Sci. Technol. **29:**353-363.
166. **Sierra-Alvarez, R., and G. Lettinga.** 1991. The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. Appl. Microbiol. Biotechnol. **34:**544-550.
167. **Slooff, W., and A. J. C. M. Matthijsen.** 1988. Integrated criteria document hexachlorocyclohexanes. National Institute of Public Health and Environmental Protection. Report nr.: 758473011
168. **Smith, M. H., and S. L. Woods.** 1994. Regiospecificity of chlorophenol reductive dechlorination by vitamin B_{12}s. Appl. Environ. Microbiol. **60:**4111-4115.
169. **Sober, H. A. (ed.).** 1970. CRC Handbook of biochemistry, 2nd ed. The chemical rubber Co., Cleveland.
170. **Sonier, D. N., N. L. Duran, and G. B. Smith.** 1994. Dechlorination of trichlrofluoromethane (CFC-11) by sulfate-reducing bacteria from an aquifer contaminated with halogenated aliphatic compounds. Appl. Environ. Microbiol. **60:**4567-4572.
171. **Stromeyer, S. A., K. Stumpf, A. M. Cook, and T. Leisinger.** 1992. Anaerobic degradation of tetrachloromethane by *Acetobacterium woodii*: separation of dechlorinative activities in cell extracts and roles for vitamin B_{12} and other factors. Biodegradation. **3:**113-123.
172. **Stromeyer, S. A., W. Winkelbauer, H. Kohler, A. M. Cook, and T. Leisinger.** 1991. Dichlorormethane utilized by an anaerobic mixed culture: acetogenesis and methanogenesis. Biodegradation. **2:**129-137.
173. **Stucki, G., and M. Thuer.** 1995. Experiences of a large-scale application of 1,2-dichloroethane degrading microorganisms for groundwater treatment. Environ. Sci. Technol. **29:**2339-2345.
174. **Susarla, S., Y. Yonezawa, J. Nakanishi, and S. Masunaga.** 1997. Anaerobic transformation of kinetics and pathways of chlorophenols in fresh water lake sediment. Wat. Sci. Technol. **36:**99-105.
175. **Tanaka, K.** 1997. Abiotic degradation of tetrachloromethane in anaerobic culture media. J. Fermen. Bioeng. **83:**118-120.
176. **Terzenbach, D. P., and M. Blaut.** 1994. Transformation of tetrachloroethylene to trichloroethylene by homoacetogenic bacteria. FEMS Microbiol. Lett. **123:**213-218.
177. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41:**100-180.
178. **Thiel, P. G.** 1969. The effect of methane analogues on methanogenesis in anaerobic digestion. Water Res. **3:**215-223.
179. **Tiedje, J. M., S. A. Boyd, and B. Z. Fathepure.** 1987. Anaerobic degradation of chlorinated aromatic hydrocarbons. Dev. Ind. Microbiol. **27:**117-127.
180. **Townsend, G. T., and J. M. Suflita.** 1997. Influence of sulfur oxyanions on reductive dehalogenation activities in *Desulfomonile tiedjei*. Appl. Environ. Microbiol. **63:**3594-3599.
181. **Traunecker, J., A. Preuss, and G. Diekert.** 1991. Isolation and characterization of a methylchloride utilizing, strictly anaerobic bacterium. Arch. Microbiol. **156:**416-421.
182. **Utkin, I., C. Woese, and J. Wiegel.** 1994. Isolation and characterization of *Desulfitobacterium dehalogenans* gen. nov. sp. nov, an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. Int. J. Syst. Bacteriol. **22:**612-619.
183. **Vogel, T. M., C. S. Criddle, and P. L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21:**722-736.

184. **Vogel, T. M., and P. L. McCarty.** 1987. Abiotic and biotic transformations of 1,1,1-trichloroethane under methanogenic conditions. Environ. Sci. Tech. **21**:1208-1213.
185. **Vogel, T. M., and P. L. McCarty.** 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinylchloride, and carbondioxide under methanogenic conditions. Appl. Environ. Microbiol. **49**:1080-1083.
186. **Vogel, T. M., and P. L. McCarty.** 1987. Rate of abiotic formation of 1,1-dichloroethylene from 1,1,1-trichloroethane in groundwater. J. Contam. Hydrol. **1**:299-308.
187. **Wackett, J. L., W. H. Orme-Johnson, and C. T. Walsh.** 1989. Transition metal enzymes in bacterial metabolism. *In* T. J. Beveridge and R. J. Doyle (ed.), Metal ions and bacteria. John Wiley & Sons, New York.
188. **Wackett, J. L., and C. A. Schanke.** 1992. Mechanisms of reductive dehalogenation by transition metal cofactors found in anaerobic bacteria, p. 330-345. *In* H. Sigel and A. Sigel (ed.), Metal ions in biological systems, vol. vol 28. Marcel Dekker, Inc., New York.
189. **Wackett, L. P.** 1996. Co-metabolism: is the emperor wearing clothes. Curr. Opin. Biotechnol. 7:321-325.
190. **Wade, R. S., and C. E. Castro.** 1973. Oxidation of iron (II) porphyrins by alkyl halides. J. Amer. Chem. Soc. **95**:226-230.
191. **Wang, Y.-T., H. D. Gabbard, and P.-C. Pai.** 1991. Inhibition of acetate methanogenesis by phenols. J. Environ. Eng. **117**:487-500.
192. **Wohlfarth, G., and G. Diekert.** 1997. Anaerobic dehalogenases. Curr. Opin. Biotechnol. **8**:290-295.
193. **Woods, S. L., J. F. Ferguson, and M. M. Benjamin.** 1989. Characterization of chlorophenol and chloromethoxybenzene biodegradation during anaerobic treatment. Environ. Sci. Technol. **23**:62-68.
194. **Wu, W., L. Bhatnagar, and J. G. Zeikus.** 1993. Performance of anaerobic granules for degradation of pentachlorophenol. Appl. Environ. Microbiol. **59**:389-397.

2

Degradation and fate of carbon tetrachloride in unadapted methanogenic granular sludge

The potential of granular sludge from upflow anaerobic sludge blanket (UASB) reactors for bioremediation of chlorinated pollutants was evaluated by using carbon tetrachloride (CT) as a model compound. Granular sludges cultivated in UASB reactors on methanol, a volatile fatty acid mixture or sucrose readily degraded CT supplied at a concentration of 1,500 nmol/batch (approximately 10 μM) without any prior exposure to organohalogens. The maximum degradation rate was 1.9 μmol of CT g^{-1} volatile suspended solids day^{-1}. The main end products of CT degradation were CO_2 and Cl^-, and the yields of these end products were 44 and 68%, respectively, of the initial amounts of [^{14}C]CT and CT-Cl. Lower chlorinated methanes accumulated in minor amounts temporarily. Autoclaved (dead) sludges were capable of degrading CT at a two- to three-fold lower rates than living sludges degraded CT, indicating that abiotic processes (mediated by cofactors or other sludge components) played an important role in the degradation observed. Reduced components in the autoclaved sludge were vital for CT degradation. A major part (51%) of the CT was converted abiotically to CS_2. The amount of CO_2 produced (23%) was lower and the amount of Cl^- produced (86%) was slightly higher with autoclaved sludge than with living sludge. Both living and autoclaved sludges could degrade chloroform. However, only living sludge degraded dichloromethane and methylchloride. These results indicate that reductive dehalogenation, which was mediated better by living sludge than by autoclaved sludge, is only a minor pathway for CT degradation. The main pathway involves substitutive and oxidative dechlorination reactions that lead to the formation of CO_2. Granular sludge, therefore, has outstanding potential for the gratuitous dechlorination of CT to safe end products.

Published as: **M.H.A. van Eekert, T.J. Schröder, A.J.M. Stams, G. Schraa, and J.A. Field,** 1998. Appl. Environ. Microbiol., 64(7): 2350-2356.

Introduction

Chlorinated compounds are commonly found pollutants in the environment. Carbon tetrachloride (CT) is among the top 45 organic chemicals produced by the United States chemical industry, with 143,000 tons produced in 1991 [1]. CT is used as a solvent in, for example, the chemical cleaning and metal industries. Like many other halogenated hydrocarbons, CT is a suspected carcinogen and therefore is a public health concern.

Higher chlorinated compounds are degraded more easily under anaerobic conditions than under aerobic conditions [44]. The initial degradation of these compounds, often a dechlorination, can be carried out by specific halorespiring bacteria [10,40,43]. However, acetogenic and methanogenic bacteria are able to transform chlorinated compounds via aspecific reactions. It has been suggested that the dechlorination reactions are mediated by cofactors like vitamin B_{12} and other corrinoids and by cofactor F_{430}. These metallo-porphyrins, which contain cobalt, nickel, or iron, are parts of enzymes that catalyze common pathways present in anaerobic bacteria, like the acetyl coenzyme A pathway and methane formation. Acetogenic and methanogenic bacteria contain elevated levels of such cofactors [11,19,26,32]. The concentrations of cofactors in the bacteria are strongly dependent on the substrate used for growth. Some microorganisms, like *Methanosarcina barkeri* grown on methanol, are known to excrete 40 to 70% of the corrinoids produced into the culture medium [32]. On the other hand, acetogenic bacteria do not contain cofactor F_{430}, whereas the cofactor levels in methanogens can be as high as 800 nmol g^{-1} dry weight [11]. The dechlorination rates with the cofactors *in vitro* are lower than the rates of transformation via specific enzyme reactions.

A variety of dechlorination processes may be involved during the degradation of CT by unadapted sludge. Dechlorination can occur either chemically or by aspecific and specific biological reactions. Chemically, CT can be transformed in the presence of pyrite (FeS_2), iron, or sulfide as a bulk electron donor [9,22]. Aspecific biological reactions are carried out without a lag phase and are catalyzed by cofactors which are either free or bound to enzymes in the cell. The specific biological reactions usually require a long adaptation period. This time span is often necessary to enrich for the appropriate bacteria in the consortium. Two strictly anaerobic acetogenic bacteria, which use methylchloride (MC) or dichloromethane (DCM) to support growth, have been isolated [31,33]. Although the dehalogenation of CT by unadapted (pure) cultures is largely attributed to the action of vitamin B_{12} and other corrinoids present in the cells, other unknown dechlorinating mechanisms may also play a role in the dechlorination of halogenated compounds [41].

In this research we evaluated the aspecific dechlorinating ability of unadapted acetogenic and methanogenic bacteria by using methanogenic granular sludge from upflow anaerobic sludge blanket (UASB) reactors and CT as a model compound. The sludge used had a high biomass content [27], which was enriched with acetogenic and methanogenic bacteria. By autoclaving the sludges and evaluating product formation, we distinguished between biological processes and abiotic processes (mediated by cofactors or reactions with sludge components) that occurred during the transformation of CT.

Materials and methods

Chemicals. CT, chloroform (CF), and DCM (all pro analysis quality; E. Merck, Amsterdam, The Netherlands), as well as MC (purity, >99%; Hoekloos, Schiedam, The Netherlands), [^{14}C]CT (specific activity, 0.15 GBq mmol^{-1}; NEN Life Science Products, Boston, Mass.), and [^{13}C]CT (Isotec Inc., Miamisburg, Ohio), were used as received without further purification.

Granular sludge. The granular sludge was grown in three UASB reactors which originally had been inoculated with granular sludge from a full scale UASB reactor treating sugar beet refinery wastewater (CSM, Breda, The Netherlands). The reactors (volume, 10 liters) were fed with methanol, a mixture of volatile fatty acids (VFA) or sucrose, as well as a mineral medium containing (per liter) 1,040 mg of NH_4Cl, 170 mg of KH_2PO_4, 170 mg of $(NH_4)_2SO_4$, 150 mg of $MgCl_2 \cdot 6H_2O$, 270 mg of KCl, and 18 mg of yeast extract. Trace elements were added by using a stock solution whose composition has been described previously [45]. The hydraulic retention time in each of the reactors was 12 h, and the operating temperature was 30°C. The sludge content of each reactor was approximately 20 g of volatile suspended solids (VSS) liter^{-1}. The methanogenic activity of the sludges was approximately 0.40 g of chemical oxygen demand (COD) g^{-1} VSS day^{-1}. The loading rate of the sucrose-fed reactor was approximately 5 kg m^{-3} day^{-1} based on COD, which corresponded to 12 mM sucrose in the influent. Sodium bicarbonate (23 mM) was added to maintain pH stability. The VFA- and methanol-fed reactors were operated at a loading rate of 10 kg COD m^{-3} day^{-1}. The VFA (the acetate, propionate, and butyrate concentrations in the influent were 19, 14, and 13 mM, respectively) were neutralized with sodium hydroxide. Methanol was added at a concentration of 100 mM along with 30 mM $NaHCO_3$. The COD removal efficiencies were at least 85%. No VFA were present in the effluents of the three reactors. The reactors were run for at least 6 months prior to sludge sampling.

Batch experiments. The sludges were washed two times with demineralized water and one time with basal medium to remove residual soluble substrate before the sludges were used in the batch experiments. Approximately 2-g (wet weight portions) of granular sludge were transferred to 120-ml

serum flasks containing 20 ml of basal medium, as described previously [18]. When chlorine balances were determined, the medium was slightly modified by replacing the chloride salts of calcium and magnesium with $Ca(OH)_2$ and $MgHPO_4 \cdot 2H_2O$. The pH of the batches remained 7.2 to 7.3. The gas phase consisted of 80% N_2 and 20% CO_2. The flasks were sealed with Viton stoppers (Maag Technic AG, Dübendorf, Switzerland). The appropriate primary substrate (1.5 g COD l^{-1}) and chlorinated methane were added to each batch where required. CT, CF, and DCM were added by using solutions prepared in anaerobic water, and MC was added as a gas with a gas tight syringe (final concentration, approximately 1500 nmol/batch). The batches were incubated statically at 30°C in the dark. The possible loss of the chlorinated compounds due to leakage through the stoppers was checked by using separate batches of medium to which no sludge was added.

Abiotic involvement of the cofactors. The abiotic involvement of cofactors was tested by autoclaving the granular sludge, which inactivated all microbial activity. The granular sludge was autoclaved in basal medium for 90 minutes at 120°C three days prior to the start of the experiment and again for 30 minutes on the day that the experiment was started.

Corrinoid content of granular sludge. The corrinoid content of the granular sludge was determined by previously described methods which were adjusted to facilitate the granular sludge determination [18,20,42]. The absorption spectrum (λ, 200 to 800 nm) of a purified sample was determined with a Beckman spectrophotometer. Purity was calculated by using the ratio of absorbance at 361 nm (A_{361}) to A_{548} (the A_{361}/A_{548} ratios of calibration samples were 3.12 ± 0.12). The corrinoid concentration was quantified spectrophotometrically. A molar extinction coefficient of 7,316 M^{-1} cm^{-1} at 548 nm was determined with a calibration curve and was used in the calculations.

Experiments with [^{14}C]CT. The formation of CO_2 from CT was investigated by following the degradation of [^{14}C]CT. Experiments were carried out in 26-ml tubes containing 11 ml of medium and 2 ml of living or autoclaved crushed (to facilitate addition to the test tubes) sludge. The sludge was crushed by pressing a sludge suspension through sterile needles with decreasing diameters (the smallest needle was a Microlance needle 3 [25G5/8, 0.5 by 16mm]). Labeled CT (total amount, around 2.5×10^5 dpm/tube) together with unlabeled CT was added dissolved in water to obtain the desired concentration. In case of the experiments with living sludge, the chlorinated compound was added in small portions (150 nmol CT/tube). This low concentration was used to avoid the formation of CF at concentrations higher than the 50% inhibition concentration of CF for acetoclastic methanogens (1.7 mg l^{-1}) [37]. [^{14}C]CT was added again after the previously added [^{14}C]CT was completely transformed. A primary substrate was not used in these experiments to avoid high pressures in the tube and to obtain low background methane concentrations. Prior experiments had shown that there were no major differences in the CT degradation when a primary substrate was not added. For each measurement six tubes were sacrificed. To dissolve all of the $^{14}CO_2$ in the liquid

phase, 1 ml of a 5 M NaOH solution was added to three tubes. The remaining three tubes were amended with 1.5 ml of 1 M HCl to remove all of the CO_2 and bicarbonate from the liquid phase. To determine the amount of $^{14}CO_2$ formed, the six tubes were treated identically. A 2 ml sample was taken from each tube and centrifuged at 15,000 x *g* for 5 minutes. The supernatant was stripped with air (50 ml minute^{-1}) for 5 min. To 0.5 ml of the sample 4.5 ml of scintillation liquid was added (Ultima Gold, Packard Instrument BV, Groningen, The Netherlands) and the resulting mixture was counted for 3 minutes with a scintillation counter (model 1211 Rackbeta; LKB). The amount measured in the NaOH-treated tubes represented the total radioactivity (i.e., the activity of the nonvolatile compounds plus CO_2). The amount measured in the HCl-amended tubes represented the total activity minus the CO_2 activity. The amount of $^{14}CO_2$ produced was calculated from the difference between the NaOH- and HCl-amended incubation preparations and was compared with a calibration curve prepared with $NaH^{14}CO_3$ and sludge (recovery rate, 93 to 99% of the $H^{14}CO_3$). The pellet (sludge) of the centrifuged samples was washed in 1 ml of demineralized water, centrifuged again, and dissolved in 1 ml of 5 M NaOH. Samples (0.25 ml) of the dissolved pellet were counted in scintillation liquid. The activities in these samples represented the amount of carbon incorporated into the biomass and the amount of [^{14}C]CT adsorbed to the sludge. Chlorinated methane and CS_2 concentrations were monitored in tubes which were simultaneously incubated with [^{12}C]CT.

Experiments with [^{13}C]CT. The formation of acetate, formate, and methane from CT was measured by using [^{13}C]CT, that was added dissolved in water. The setup of the experiment was identical to that of the [^{14}C]CT experiments described above. For each measurement six tubes were sacrificed. From each of the first three tubes a sample was taken and centrifuged at 15,000 x *g* for 5 minutes. Part of the supernatant was acidified with formic acid and stored at -20°C until the [^{13}C]acetate analysis was conducted. Another part of the supernatant was used to determine the formate concentration after acidification with HCl. For the $^{13}CH_4$ measurement another three tubes were acidified to pH 2 with 1 M HCl and stored at 4°C until further analysis.

Analytical methods. Total masses of the chlorinated methanes, carbon disulfide, H_2, and methane were determined by headspace analysis. CT, CF, DCM, and carbon disulfide were analyzed by injecting 0.2 ml of headspace gas into a model 436 Chrompack gas chromatograph (GC) equipped with a flame ionization detector connected to a Sil 5CB column (25 m by 0.32 mm by 1.2 μm) and a splitter-injector (ratio 1:50). The operating temperatures of the injector, column, and detector were 250, 50, and 300°C, respectively. The carrier gas was N_2 with an inlet pressure of 50 kPa. The retention times were 5.3, 3.8, 2.5, and 2.7 minutes for CT, CF, DCM, and carbon disulfide, respectively. MC was analyzed by injecting 0.2 ml of headspace gas into a model 438A Chrompack GC equipped with a flame ionization detector connected to a Poraplot Q column (25 m by 0.32 mm by 10 μm) and a splitter-injector (ratio 1:40). Operating temperatures of the injector, column, and detector were 225, 140, and 250°C, respectively. The retention time of MC was 2.3 minutes. The

retention times and peak areas of all chlorinated methanes were determined with a Shimadzu model C-3A integrator. The lower detection limits of the chlorinated methanes were 30, 20, 38, and 30 nmol/batch for CT, CF, DCM, and MC, respectively. Hydrogen and methane were analyzed by injecting 0.4 ml of gas from the headspace into a model 417 Packard GC equipped with a thermal conductivity detector (100 mA) connected to a molecular sieve column (13X, 180 by 0.25 inch, 60 to 80 mesh). The temperatures of the column and detector were 100°C. Calibration curves were constructed by adding the required amount of the chlorinated methane, H_2, or methane to a serum bottle containing 20 ml of basal medium without sludge. Sludge was omitted to avoid degradation. The bottles were allowed to equilibrate overnight at 30°C.

Chloride and formate concentrations were determined by high-performance liquid chromatography as described previously [38]. The detection limits were 10 μM. Bromide and lactate were used as internal standards for chloride analysis and formate analysis, respectively. [^{13}C]acetate and $^{13}CH_4$ contents were determined by a GC-mass spectrometry analysis of liquid and gas phase samples as previously described [39]. For the acetate analysis *m/z* 62 acetate (100 μM) was added as an internal standard. The detection limit for [^{13}C]acetate was 25 μM (375 nmol/tube) with a maximum background level of 2 mM [^{12}C]acetate. The detection limit for $^{13}CH_4$ was 10 μM (130 nmol/tube) with a maximum background level of 300 μM $^{12}CH_4$. VFA and methanol concentrations were determined by GC as described previously [15]. The COD for methanol, VFA, and sucrose solutions were determined by standard methods [3]. The COD conversion factors (g g^{-1}) utilized were 1.07, 1.50, 1.07, 1.52, and 1.82 for sucrose, methanol, acetate, propionate, and butyrate, respectively. The VSS content of the sludge was determined by subtracting the ash content from the dry weight after the sludge was incubated overnight at 105°C. The ash content was determined after the dry sludge was heated at 600°C for 90 min.

Results

Degradation of CT by the unadapted sludges. CT was rapidly degraded by unadapted methanogenic consortia (Fig. 2.1). The degradation occurred without any lag phase, irrespective of whether the preparations were supplemented with primary substrate. The addition of primary substrate, however, was associated with a slight enhancement of CT removal. The maximum rates of CT elimination were 1.3, 1.2, and 1.9 μmol CT g^{-1} VSS day^{-1} for methanol-, VFA-, and sucrose-fed sludges, respectively. The autoclaved sludge was also able to cause significant removal of CT, but the rate was generally only one-third to one-half the rate observed with living sludge. No significant removal of CT occurred when the compound was incubated in sterile medium in the absence of sludge.

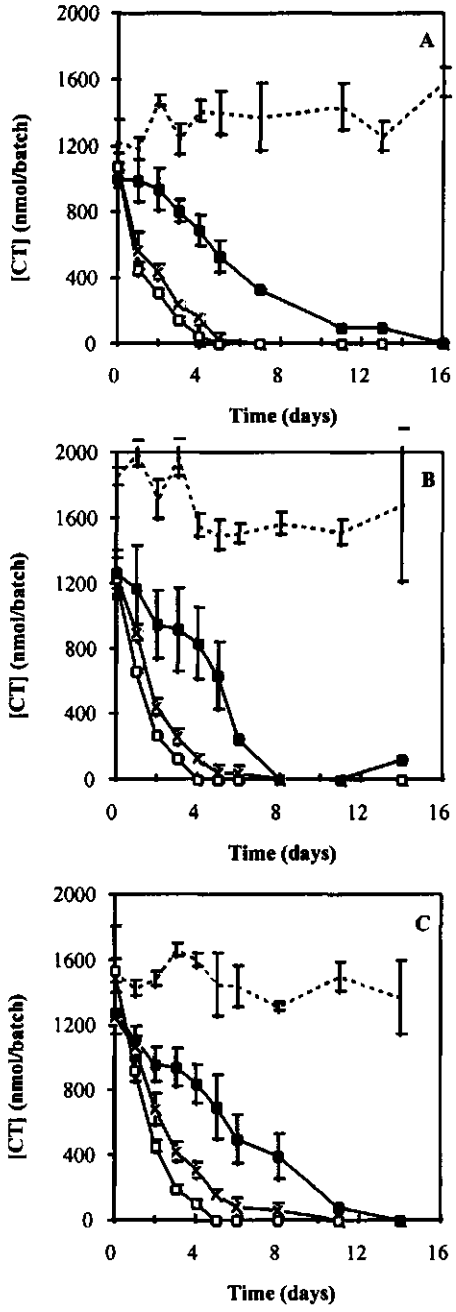


Figure 2.1 Disappearance of CT in the presence of unadapted living sludge with (□) or without (x) primary substrate, in the presence of autoclaved sludge (■), or in sterile medium in the absence of sludge (dashed line). The sludge was grown in a methanol-fed (A), VFA-fed (B), or sucrose-fed (C) UASB reactor. The VSS contents of the batches for living sludge without primary substrate, living sludge with primary substrate, and autoclaved sludge were 128, 122, and 136 mg VSS/batch, respectively, for methanol fed sludge, 263, 259, and 268 mg VSS/batch, respectively, for VFA-fed sludge, and 192, 197, and 228 mg VSS/batch, respectively, for sucrose-fed sludge. The error bars indicate the standard deviations based on triplicate incubations.

Inhibition and stimulation of CT elimination by autoclaved sludge. Autoclaved sludge from the VFA-fed reactor was incubated with different concentrations of H_2O_2 (1 to 5%) to determine whether it inhibited the CT-degrading capacity of the autoclaved sludge (Fig. 2.2A). The H_2O_2 treatments resulted in bleaching of the normally black sludge granules resulting in white granules. All concentrations of H_2O_2 tested were sufficient to eliminate all of the CT removal capacity of the autoclaved sludge. Addition of reducing equivalents in the form of Ti(III)citrate (300 μM) to autoclaved sludge led to an increase in the CT degradation rate in the first 6 h of incubation, showing the importance of electron availability for the conversion of CT by autoclaved sludge (Fig. 2.2B). To examine the involvement of vitamin B_{12} in the dechlorination of CT by autoclaved sludge, 1-iodopropane was tested at concentrations of 0 up to 100 mM (results not shown). 1-Iodopropane is a known inhibitor of reductive dehalogenation mediated by vitamin B_{12} because of its covalent binding to the cofactor [6]. No significant effect on CT-removal by autoclaved sludge cultivated in the VFA-fed reactor was found at 1-iodopropane concentrations up to 50 mM. Limited inhibition (60% of the CT removal rate) was observed at a 1-iodopropane concentration of 100 mM.

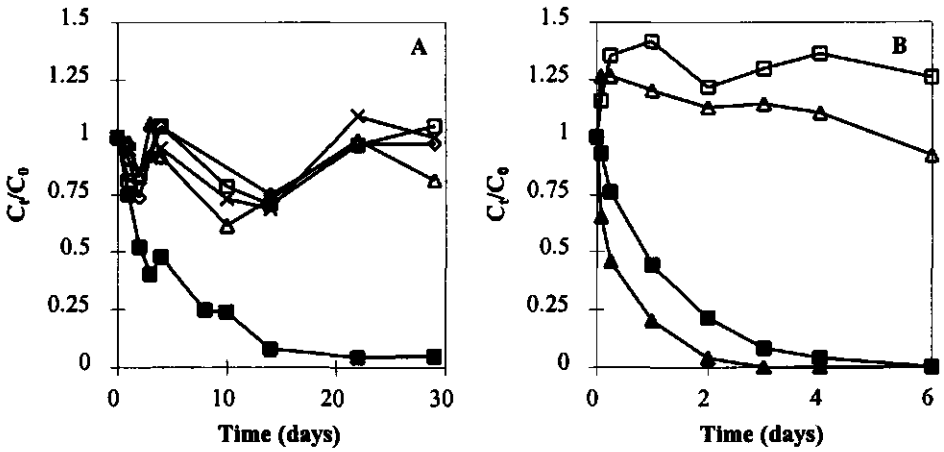


Figure 2.2 (A) CT elimination by autoclaved sludge (243 mg VSS/batch) from the VFA-fed reactor in the presence of H_2O_2, given as the concentration at time t divided by the concentration at time zero (C_t/C_0). Symbols: ■, no H_2O_2; □ , 1% (vol/vol) H_2O_2;▲ , 2% (vol/vol) H_2O_2; x, 3.5% (vol/vol) H_2O_2; ◆ , 5% (vol/vol) H_2O_2. The initial CT concentration was 3,900 nmol/batch.
(B) CT-elimination by autoclaved sludge (296 mg VSS/batch) from the methanol-fed reactor with (▲) and without (■) 300 μM Ti(III)citrate. Also shown are the concentrations in blanks with no sludge added with (△) and without (□) 300 μM Ti(III)citrate. The initial CT-concentration was 1,500 nmol/batch.

Identification of lower chlorinated methanes during CT degradation. Lower chlorinated methanes were detected as intermediates during incubation of CT with living and autoclaved sucrose-fed anaerobic granular sludges. Similar results were obtained for the VFA- and methanol-fed sludges. CF, DCM, and MC were identified as transient intermediates during incubation with living sludge (Fig. 2.3A). The recovery of the different intermediates was never more than 10% of the initial amount of CT. When the primary substrate was omitted, the lower chlorinated methanes were generally detectable for longer periods of time in the system. When CT was incubated with autoclaved sludge, CF and DCM could be detected as products, but no MC was formed (Fig. 2.3B). The intermediates were more stable in the presence of autoclaved sludge. The maximum molar yield of the intermediates was less than 8% of the CT initially present.

Degradability of lower chlorinated methanes by unadapted sludge. Degradation of CT, degradation of CF, degradation of DCM, and degradation of MC were examined individually with living and autoclaved sludges from the methanol-fed reactor (Fig. 2.4). Living sludge was able to degrade all of the halomethanes (Fig. 2.4A). CT, CF, and DCM were transformed to lower chlorinated methanes. During degradation of CT by living sludge, the molar yield of intermediates was similar to the molar yield obtained with the sucrose sludge (Fig. 2.3A). The maximum yields of DCM and MC during the degradation of CF were 24 and 6%, respectively. Only 14% of the DCM degraded was recovered as MC.

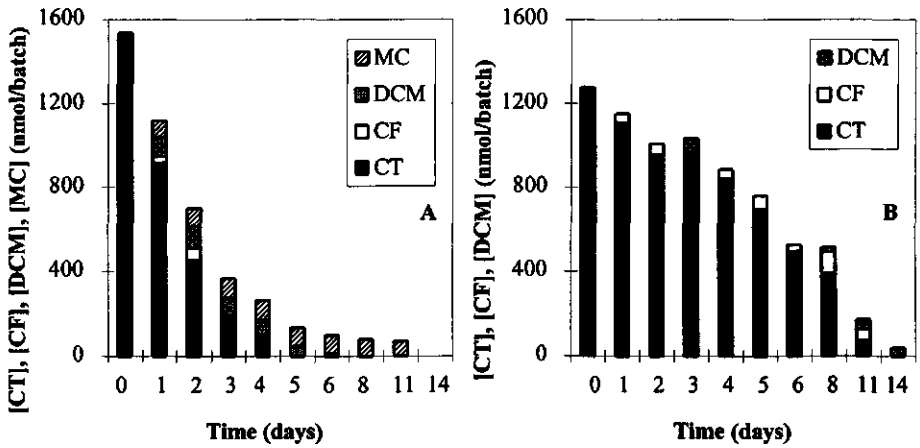


Figure 2.3 Degradation of CT and subsequent formation of intermediates by living (A) and autoclaved (B) granular sludges from the sucrose-fed reactor.

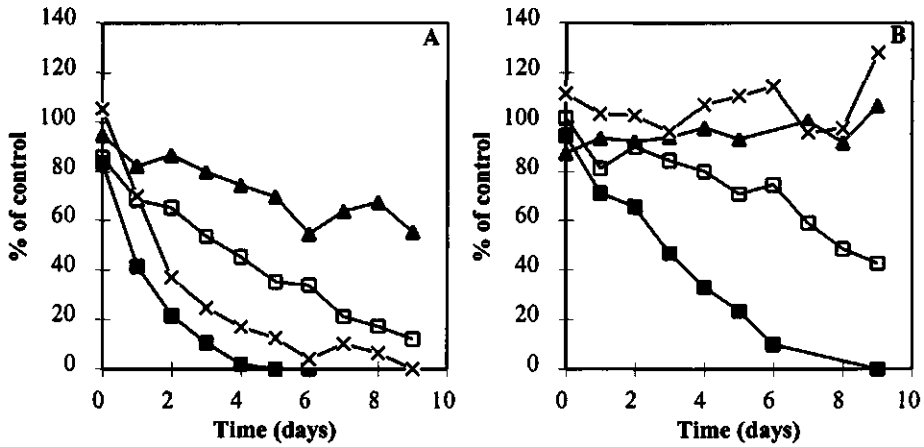


Figure 2.4 Degradation of CT (■) and the lower chlorinated methanes CF (□), DCM (▲), and MC (x) by living (A) and autoclaved (B) sludges from the methanol-fed reactor. The concentrations were normalized against the concentrations found in parallel incubated sterile blanks lacking sludge. The VSS contents of the batches for living and autoclaved sludges were 142 and 148 mg VSS/batch, respectively, in CT incubations, 136 and 134 mg VSS/batch, respectively, in CF incubations, 129 and 142 mg VSS/batch, respectively, in DCM incubations, and 134 and 144 mg VSS/batch, respectively in MC incubations.

The autoclaved sludge rapidly eliminated CT, whereas CF was only partially removed (52%) within 9 days. The maximum yield of DCM was 5% of the initial CF concentration. DCM and MC were not degraded by autoclaved sludge (Fig. 2.4B).

Chlorine balance. The chlorine balance after CT degradation was determined with both living and autoclaved sludges from the methanol-fed reactor (Table 2.1). The amounts of chlorine present in the products as chlorinated methanes or chloride (corrected for the background levels in the sludge) were measured after 6 days for living sludge and after 11 days for autoclaved sludge. After 6 days of incubation, 55 to 70% of the CT chlorine initially present in the incubations was recovered as chloride with living sludge. After 6 days, the incubation preparations were spiked once more with CT, and the chlorine was released as chloride with similar yields (results not shown). Compared to living sludge, the autoclaved sludge released more chloride from CT. Up to 86% of the initial amount of CT chlorine was recovered as chloride after 11 days (Table 2.1). These results indicate that there was almost complete dechlorination of CT to nonchlorinated end products. Consequently, adsorption did not play a major role in the mechanism of CT removal by living or autoclaved sludge.

Table 2.1. Chlorine balance for degradation of CT by living (methanol grown) sludge with (+PS) and without (-PS) primary substrate and by autoclaved sludge.

Prepn	Chlorine concn (nmol Cl/batch)						% Recovery	
	Zero time	Day 6 or 11					Cl[a]	Total[b]
	CT	CT	CF	DCM	MC	Cl^-[c]		
Living, -PS	5049 ± 360[d]	BDL[e]	BDL	351 ± 20	25 ± 35	2806 ± 265	55.6	63.0
Living, +PS	4925 ± 113	BDL	BDL	244 ± 17	91 ± 5	3359 ± 268	68.2	75.0
Autoclaved	5566 ± 131	BDL	BDL	112 ± 25	BDL	4771 ± 892	85.7	89.5

[a] Efficiency of chloride release compared to CT at zero time.
[b] Total amount of products (chlorinated methanes and chloride) compared to CT at zero time.
[c] Amount of chlorine released corrected for the sludge background chloride level.
[d] Mean ± standard deviation based on three determinations.
[e] BDL, below detection limit

Production of CO_2, CS_2, formate, acetate and CH_4 from CT. [^{14}C]CT was to a large extent (44%) degraded to $^{14}CO_2$ by living sludge (Table 2.2). Addition of 50 mM 2-bromo-ethane-sulfonic acid (BESA), a specific inhibitor of methanogenesis, resulted in the formation of more lower chlorinated methanes. Some (8-15%) of the [^{14}C]CT added was found to be associated with the sludge. In the presence of autoclaved sludge, 51% of the [^{14}C]CT could be recovered as CS_2, and 23% was transformed to $^{14}CO_2$. Approximately 20% of the [^{14}C]CT was adsorbed to the sludge. Methane, formate, and acetate could not be detected as products of [^{13}C]CT degradation by either living or autoclaved sludge, but the detection limits for these compounds were rather high. Altogether, a substantial amount of the CT added could be recovered as (labeled) products. Some possible products, like CO, could not be analyzed for, and it was not possible to measure the radioactivity in the gas phase. This resulted in an incomplete carbon balance.

Determination of the corrinoid content of granular sludges fed with different substrates. The vitamin B_{12} contents of the sludges grown in the three reactors were 0.97, 0.45, and 0.60 mg g^{-1} VSS for methanol-, VFA-, and sucrose-fed sludges, respectively.

Discussion

This research shows that methanogenic consortia grown in anaerobic wastewater treatment systems like an UASB reactor are able to degrade CT without any prior adaptation. CT is extensively dechlorinated, and CO_2 and Cl^- are the main products formed

Table 2.2 Products formed after 30 days of incubation of CT with unadapted living methanol-grown granular sludge in the presence or absence of 50 mM BESA and with autoclaved methanol-grown sludge and in medium with no sludge added.

	Amount of CT and products from [^{13}C]CT and [^{14}C]CT (% of initial CT)							
Prepn	CT	LCM[a]	CS_2	$^{14}CO_2$	$^{14}C_{biomass}$[b]	$[^{13}C]_{formate}$	$^{13}CH_4$	$[^{13}C]_{acetate}$
Medium	98.3[c]	0	0	0	-	-	-	-
Autoclaved	2.9	5.1	50.6	23.3	18.8	BDL[d]	BDL	BDL
Living	0	6.8	0	44.2	15.1	BDL	BDL	BDL
Living+BESA	0	28.0	0	34.9	8.8	BDL	BDL	BDL

[a] Lower chlorinated methanes (CF, DCM, and MC)
[b] CT carbon incorporated into biomass and adsorbed to the biomass
[c] Mean based on at least three determinations
[d] BDL, below detection limit

by unadapted living sludge. The presence of BESA, a specific inhibitor of methanogenesis, led to accumulation of lower chlorinated methanes and less conversion to CO_2, which showed the importance of methanogenesis in the dechlorination process. Autoclaved sludge was also able to degrade CT to an unusually high extent. The ability of the autoclaved sludge to degrade CT supports the hypothesis that cofactors, such as F_{430}, or cobalamines, such as vitamin B_{12}, are involved in the dechlorination of this compound. The main products of degradation of CT by autoclaved sludge were CS_2, CO_2, and Cl^-. The rates of CT dechlorination by granular sludge observed in this study (1 to 2 μmol g^{-1} VSS day^{-1}) are comparable the rates of dechlorination observed with adapted anaerobic sludge (>0.4 μmol g^{-1} VSS day^{-1}) [30], but lower than the rates of dechlorination of CT by pure cultures like *Acetobacterium woodii* (30 mmol g^{-1} protein day^{-1}), *Methanobacterium thermoautotrophicum* (0.8 mmol g^{-1} protein day^{-1}), and *Desulfobacterium autotrophicum* (15.4 mmol g^{-1} protein day^{-1}) [12,13].

Different mechanisms may have played a role in the degradation of CT by granular sludge. Biological catalysts, as well as abiotic mechanisms mediated by enzyme cofactors as catalysts and chemical mechanisms (without mediation by a catalyst), can be responsible for CT degradation. Biologically, CT degradation has been observed under redox conditions varying from nitrate reducing to methanogenic. Both pure and mixed microbial cultures have been found to degrade CT, and the products formed are usually lower chlorinated methanes and nonchlorinated products like CO_2. Sometimes CS_2 and VFA are also formed [4,5,13,16,28,36]. It has been suggested that there are two major pathways are that are used by these bacteria to degrade CT. First, there is a reductive route, in which

lower chlorinated methanes are formed, which is catalyzed by corrinoids and cofactor F_{430}. Second, CT is transformed via oxidative or substitutive pathways to CO_2 [12] (Fig. 2.5). Both pathways are heat stable and there seems to be a shift toward the oxidative or substitutive routes after the cultures are autoclaved (similar to our results), probably due to the loss of protein-mediated electron transfer [12]. The formation of CO_2 by living cells may be attributed to CO or formate produced by vitamin B_{12} which is further transformed by CO dehydrogenase [25] (Fig. 2.5). CO or formate is formed via reductive dechlorination of CT by a two electron transfer via dichlorocarbene, which is subsequently hydrolyzed [29]. The pathway for the formation of CO_2 by autoclaved cells has to our knowledge not been elucidated yet. It has been observed that the amount of methane formed via the reductive dechlorination of MC never exceeds 5% of the total amount of CT or CF added in methanogenic mixed cultures [5] or pure cultures [34].

Chemical dechlorination of CT (without a catalyst) has been observed in the presence of Fe^{2+} (250 μM, pH 7.2) or sulfide (250 μM HS^-, pH 7.8) at 50°C [9] and in the presence of pyrite [22]. Among the products formed were CF, CS_2, CO_2, and formate. The formation of CO_2 was ascribed to the hydrolysis of CS_2. However, we did not observe this reaction in our experimental setup when CS_2 was incubated in the presence of autoclaved sludge (data not shown). Nevertheless, organic molecules present in the (autoclaved) sludge could act as a mediator in the chemical conversion and thus increase reaction rates [9]. Since CT was not degraded in blanks which contained 1 mM sulfide and no sludge, the results clearly indicate that a potential chemical catalyst originated from the sludge. The amount of iron in granular sludge from a reactor run under conditions similar to those used for growing the sludge in our experiments was around 10 mg g of total suspended solids^{-1} [14]. This may result in a concentration as high as 1 mM in the medium.

Many *in vitro* studies have shown that metallocofactors like vitamin B_{12} and other cobalamines, as well as cofactor F_{430} [17,23] and iron porphyrins [21], are capable of catalyzing degradation of CT and other chlorinated alkanes when a suitable electron donor is present. These reactions are abiotic, but the cofactors which are usually present as parts of enzymes may also function as mediators in biological systems. Vitamin B_{12} dechlorinates CT via reductive, oxidative, and substitutive pathways, depending on the electron donor used [2,7,8,24,25,29]. By comparing the products formed during degradation of CT by living and autoclaved cells of *A. woodii* and by vitamin B_{12}, it was shown that vitamin B_{12} may be largely responsible for the dechlorination of CT by this bacterium [41].

The ^{13}C experiments showed that CT is not transformed to CH_4 as a major product by methanogenic consortia. Furthermore, neither formate nor acetate was a major product. This finding, together with the small amounts of lower chlorinated methane intermediates detected, indicates that reductive dehalogenation is only a minor pathway in the degradation of CT by unadapted granular sludge. CS_2 was detected in incubations with autoclaved sludge, indicating that chemical (abiotic) transformations may be involved in the removal of CT. Apparently, living sludge maintains a redox potential low enough to prevent CS_2 formation. Since the formation of CO_2 from CT takes place immediately at the beginning of incubation (data not shown), it seems likely that the CO_2 is formed by a direct substitution reaction from CT (Fig. 2.5). Whether the reaction takes place via CS_2 or CO remains uncertain. Clearly, net oxidative and substitutive pathways are predominant in CT degradation by methanogenic granular sludge. The difference in product formation shows that the pathways used by living and autoclaved sludges are different.

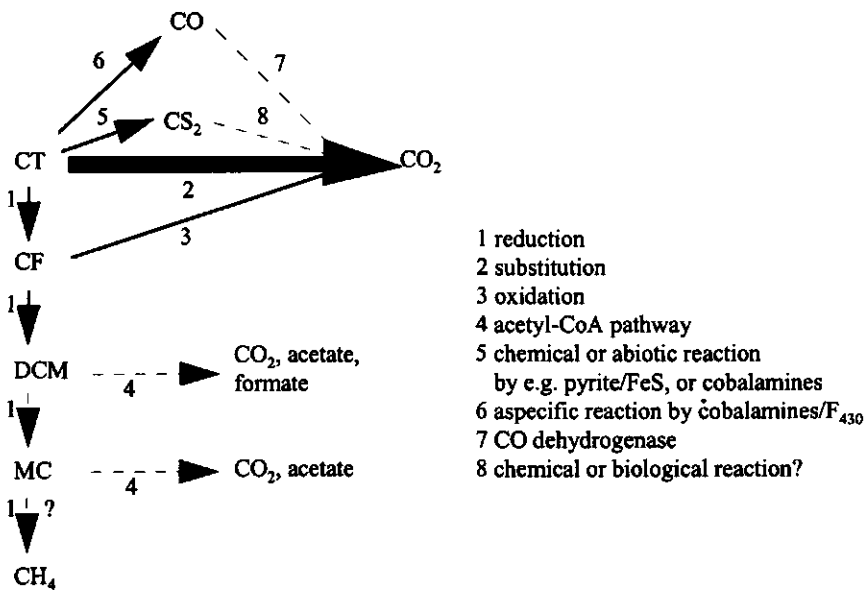


Figure 2.5 Possible pathways for CT degradation by unadapted granular sludge. The solid lines indicate transformations carried out by both living and autoclaved sludges. The dashed lines indicate transformations carried out only by living sludge. The numbers indicate the following processes which take place: 1, reduction; 2, substitution; 3, oxidation; 4, acetyl coenzyme A pathway; 5, chemical reaction with, for example pyrite (FeS_2) or cobalamines; 6, aspecific reactions with cobalamines or F_{430}; 7, CO dehydrogenase; 8, chemical or biological reaction (?).

There were no significant differences in dechlorination rate and product formation among the sludge grown on methanol, the sludge grown on VFA, and the sludge grown on sucrose. This was not expected because methylotrophic bacteria are known to have higher corrinoid contents than nonmethylotrophic bacteria [19,26,32]. We assumed that autoclaving the sludge solubilized the intracellular vitamin B_{12}. Our research showed that a 1.5- to 2-fold-higher corrinoid content in methanol-grown sludge did not lead to an increase in the CT degradation rate compared to sucrose- or VFA-fed sludge. Also, 1-iodopropane was found to be only a very weak inhibitor of dechlorination. We concluded that not the corrinoid content but the limited availability of electrons for dechlorination may have been the rate limiting factor in the degradation of CT. This could be an explanation for the faster degradation in biological (living) systems than in autoclaved sludge. The fact that degradation was slightly stimulated by adding primary substrate to living sludge also suggests that there was a shortage of reducing equivalents. Moreover, the addition of Ti(III)citrate enhanced the CT degradation by autoclaved sludge, and oxidation of all reducing equivalents with H_2O_2 led to complete inhibition of CT removal. However, the latter could also have been caused by a disruption of the cofactor structure [35]. The diffusion rate of the chlorinated compound into the sludge and cells may also have influenced the reaction rate. We did indeed observe enhanced CT degradation when we used crushed sludge incubated in a rotary shaking incubator (which decreased the mass transport limitation) instead of granular sludge (unpublished results).

Unadapted methanogenic granular sludge was shown to be a suitable source of dechlorinating activity. Although the degradation rates are low, dechlorination of CT is carried out without prior adaptation, and the degradation of CT is extensive and leads to nonhazardous products like CO_2. The presence of living sludge is essential to maintain sufficient reducing conditions. The dechlorination rate can potentially be increased by crushing the sludge or by incubating the preparation with shaking, thus decreasing the mass transport limitation.

Acknowledgments

We thank Wim Roelofsen for technical assistance and Serve Kengen and Hans Scholten for valuable discussions.

This research was supported by a grant (project 92014) from the Innovative Research Program on Environmental Biotechnology (IOP-Milieubiotechnologie) of the Ministries of Economic Affairs and Housing, Physical Planning, and Environment.

References

1. **Anonymous.** 1993. Facts and figures for the chemical industry. Chem. Eng. News:40-45.
2. **Assaf-Anid, N., K. F. Hayes, and T. M. Vogel.** 1994. Reductive dechlorination of carbon tetrachloride by cobalamin(II) in the presence of dithiothreitol - mechanistic study, effect of redox potential and pH. Environ. Sci. Technol. **28:**246-252.
3. **American Public Health Association.** 1985. Standard methods for examination of water and wastewater, vol. 16th ed. APHA, Washington DC.
4. **Becker, J. G., and D. L. Freedman.** 1994. Use of cyanocobalamin to enhance anaerobic biodegradation of chloroform. Environ. Sci. Technol. **28:**1942-1949.
5. **Bouwer, E. J., and P. L. McCarty.** 1983. Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl. Environ. Microbiol. **45:**1286-1294.
6. **Brot, N., and H. Weissbach.** 1965. Enzymatic synthesis of methionine. Chemical alkylation of the enzyme-bound cobamide. J. Biol. Chem. **240:**3064-3070.
7. **Chiu, P., and M. Reinhard.** 1995. Metallocoenzyme-mediated reductive transformation of carbon tetrachloride in titanium(III) citrate aqueous solution. Environ. Sci. Technol. **29:**595-603.
8. **Chiu, P.-C., and M. Reinhard.** 1996. Transformation of carbon tetrachloride by reduced vitamin B_{12} in aqueous cysteine solution. Environ. Sci. Technol. **30:**1882-1889.
9. **Curtis, G. P., and M. Reinhard.** 1994. Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthrahydroquinone disulfonate and humic acid. Environ. Sci. Technol. **28:**2393-2401.
10. **DeWeerd, K. A., L. Mandelco, S. Tanner, C. R. Woese, and J. M. Sulfita.** 1990. *Desulfomonile tiedje* gen. nov. and sp. nov., a novel anaerobic, dehalogenating, sulfate-reducing bacterium. Arch. Microbiol. **154:**23-30.
11. **Diekert, G., U. Konheiser, K. Pichulla, and R. K. Thauer.** 1981. Nickel requirement and factor F_{430} content of methanogenic bacteria. J. Bacteriol. **148:**459-464.
12. **Egli, C., S. Stromeyer, A. M. Cook, and T. Leisinger.** 1990. Transformation of tetra- and trichloromethane to CO_2 by anaerobic bacteria is a non-enzymic process. FEMS Microbiol. Lett. **68:**207-212.
13. **Egli, C., T. Tschan, R. Scholtz, A. M. Cook, and T. Leisinger.** 1988. Transformation of tetrachloromethane to dichloromethane and carbon dioxide by *Acetobacterium woodii*. Appl. Environ. Microbiol. **54:**2819-24.
14. **Florencio, L., P. Jenicek, J. A. Field, and G. Lettinga.** 1993. Effect of cobalt on the anaerobic degradation of methanol. J. Ferm. Bioeng. **75:**368-374.
15. **Florencio, L., A. Nozhevnikova, A. van Langerak, A. J. M. Stams, J. A. Field, and G. Lettinga.** 1993. Acidophilic degradation of methanol by a methanogenic enrichment culture. FEMS Microbiol. Lett. **109:**1-6.
16. **Hashsham, S. A., R. Scholze, and D. L. Freedman.** 1995. Cobalamin-enhanced anaerobic biotransformation of carbon tetrachloride. Environ. Sci. Technol. **29:**2856-2863.
17. **Holliger, C., G. Schraa, E. Stupperich, A. J. M. Stams, and A. J. Zehnder.** 1992. Evidence for the involvement of corrinoids and factor F_{430} in the reductive dechlorination of 1,2-dichloroethane by *Methanosarcina barkeri*. J. Bacteriol. **174:**4427-4434.
18. **Holliger, H. C.** Reductive dehalogenation by anaerobic bacteria. 1992. Ph.D. Thesis. Agricultural University, Wageningen.
19. **Inoue, K., S. Kageyama, K. Miki, T. Morinaga, Y. Kamagata, K. Nakamura, and E. Mikami.** 1992. Vitamin B_{12} production by *Acetobacterium* sp. and its tetrachloromethane-resistant mutants. J. Ferm. Bioeng. **73:**76-78.

20. **Kengen, S. W. M., P. J. H. Daas, E. F. G. Diuts, J. T. Keltjens, C. v. d. Drift, and G. D. Vogels.** 1992. Isolation of a 5-hydroxybenzimidazolyl cobamide-containing enzyme involved in the methyltetrahydromethanopterin: coenzyme M methyltransferase reaction in *Methanobacterium thermoautotrophicum*. Biochim. Biophys. Acta. **1118**:249-260.
21. **Klecka, G. M., and S. J. Gonsior.** 1984. Reductive dechlorination of chlorinated methanes and ethanes by reduced iron (II) porphyrins. Chemosphere. **13**:391-402.
22. **Kriegman-King, M. R., and M. Reinhard.** 1994. Transformation of carbon tetrachloride by pyrite in aqueous solution. Environ. Sci. Technol. **28**:692-700.
23. **Krone, U. E., K. Laufer, R. K. Thauer, and H. P. C. Hogenkamp.** 1989. Coenzyme F_{430} as a possible catalyst for the reductive dehalogenation of chlorinated C1 hydrocarbons in methanogenic bacteria. Biochemistry. **28**:10061-10065.
24. **Krone, U. E., R. K. Thauer, and H. P. C. Hogenkamp.** 1989. Reductive dehalogenation of chlorinated C1-hydrocarbons mediated by corrinoids. Biochemistry. **28**:4908-4914.
25. **Krone, U. E., R. K. Thauer, H. P. C. Hogenkamp, and K. Steinbach.** 1991. Reductive formation of carbon monoxide from CCl4 and freons 11, 12 and 13 catalyzed by corrinoids. Biochemistry. **30**:2713-2719.
26. **Krzycki, J., and J. G. Zeikus.** 1980. Quantification of corrinoids in methanogenic bacteria. Curr. Microbiol. **3**:243-245.
27. **Lettinga, G., A. F. M. van Velsen, S. W. Hobma, W. de Zeeuw, and A. Klapwijk.** 1980. Use of upflow anaerobic (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioeng. **22**:677-734.
28. **Lewis, T. A., and R. L. Crawford.** 1993. Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas* sp. Strain KC. Appl. Environ. Microbiol. **59**:1635-1641.
29. **Lewis, T. A., M. J. Morra, and P. D. Brown.** 1996. Comparative product analysis of carbon tetrachloride dehalogenation catalyzed by cobalt corrins in the presence of thiol or titanium (III) reducing agents. Environ. Sci. Technol. **30**:292-300.
30. **Long, J. L., H. D. Stensel, J. F. Ferguson, S. E. Strand, and J. E. Ongerth.** 1993. Anaerobic and aerobic treatment of chlorinated aliphatic compounds. J. Environ. Eng. - ASCE. **119**:300-320.
31. **Mägli, A., M. Wendt, and T. Leisinger.** 1996. Isolation and characterization of *Dehalobacterium formicoaceticum* gen. nov. sp. nov., a strictly anaerobic bacterium utilizing dichloromethane as source of carbon and energy. Arch. Microbiol. **166**:101-108.
32. **Mazumder, T. K., N. Nishio, S. Fukuzaki, and S. Nagai.** 1987. Production of extracellular vitamin B-12 compounds from methanol by *Methanosarcina barkeri*. Appl. Microbiol. Biotechnol. **26**:511-516.
33. **Messmer, M., G. Wohlfarth, and G. Diekert.** 1993. Methyl chloride metabolism of the strictly anaerobic, methyl chloride-utilizing homoacetogen strain MC. Arch. Microbiol. **160**:383-387.
34. **Mikesell, M. D., and S. A. Boyd.** 1990. Dechlorination of chloroform by *Methanosarcina* strains. Appl. Environ. Microbiol. **56**:1198-1201.
35. **Nazhat, N. B., B. T. Golding, G. R. A. Johnson, and P. Jones.** 1989. Destruction of vitamin B_{12} by reaction with ascorbate: the role of hydrogen peroxide and the oxidation state of cobalt. J. Inorg. Biochem. **36**:75-81.
36. **Picardal, F., R. G. Arnold, and B. B. Huey.** 1995. Effects of electron donor and acceptor conditions on reductive dehalogenation of tetrachloromethane by *Shewanella putrefaciens* 200. Appl. Environ. Microbiol. **61**:8-12.
37. **Sanz, J. L., N. Rodriguez, and R. Amils.** 1997. Effect of chlorinated aliphatic hydrocarbons on the acetoclastic methanogenic activity of granular sludge. Appl. Microbiol. Biotechnol. **47**:324-328.
38. **Scholten, J. C. M., and A. J. M. Stams.** 1995. The effect of sulfate and nitrate on methane formation in a freshwater sediment. Antonie van Leeuwenhoek. **68**:309-315.

39. **Scholten, J. C. M., J. Vogelaar, A. van Ittersum, K. Hordijk, W. Roelofsen, and A. J. M. Stams.** Submitted. Kinetics of 13C-acetate transformation in a freshwater sediment under different redox conditions. Appl. Environ. Microbiol.
40. **Scholz Muramatsu, H., A. Neumann, M. Messmer, E. Moore, and G. Diekert.** 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Arch. Microbiol. **163**:48-56.
41. **Stromeyer, S. A., K. Stumpf, A. M. Cook, and T. Leisinger.** 1992. Anaerobic degradation of tetrachloromethane by *Acetobacterium woodii*: separation of dechlorinative activities in cell extracts and roles for vitamin B_{12} and other factors. Biodegradation. **3**:113-123.
42. **Stupperich, E., I. Steiner, and M. Ruhleman.** 1986. Isolation and analysis of bacterial cobamides by high-performance liquid chromatography. Anal. Biochem. **155**:365-370.
43. **Utkin, I., D. D. Dalton, and J. Wiegel.** 1995. Specificity of reductive dehalogenation of substituted ortho-chlorophenols by *Desulfitobacterium dehalogenans* JW/IU-DC1 (vol 61, pg 346, 1995). Appl. Environ. Microbiol. **61**:1677.
44. **Vogel, T. M., C. S. Criddle, and P. L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21**:722-736.
45. **Zehnder, A. J. B., B. A. Huser, T. D. Brock, and K. Wuhrmann.** 1980. Characterization of an acetate-decarboxylating, non-hydrogen-oxidizing methane bacterium. Arch. Microbiol. **124**:1-11.

3

Transformation of carbon tetrachloride by methanogenic granular sludge: Effect of substrate and biomass concentrations

The effect of the carbon tetrachloride (CT) and the biomass concentration on the CT transformation rate was investigated with granular sludge from upflow anaerobic sludge blanket (UASB) reactors fed with methanol, volatile fatty acids or sucrose as the primary substrate. The sludges were able to degrade high concentrations of CT (0.25-0.4 mM), although acetogenesis and methanogenesis were severely inhibited. The conversion was faster at the high CT concentrations compared to low concentrations (10 μM). At the low CT concentration, the rates were increased by crushing the sludge or incubating under shaken conditions. Increasing the amount of biomass led to a higher specific pseudo first order rate constant normalized to the biomass present. The higher specific rate constants may have been caused by a decrease in the redox potential leading to more favorable conditions for dechlorination at higher biomass concentrations. CT dechlorination was also observed in autoclaved sludge and at temperatures beyond the biological optimum. These observations indicate that the dechlorinating activity of granular sludge is partially abiotic.

Introduction

CT, which is used as a solvent in the chemical cleaning and metal industry, is among the top 10 on the EPA list of priority pollutants. CT has been found to be degraded microbiologically under different redox conditions. Products, which are formed under methanogenic or acetogenic conditions, are usually lower chlorinated methanes and non chlorinated compounds like CO_2, volatile fatty acids (VFA) and to a lesser extent CS_2 [4,13,17]. Recently, granular sludge from upflow anaerobic sludge blanket (UASB) reactors was found to transform CT at low concentrations (10 μM in the waterphase) without prior exposure to halogenated compounds [11]. CO_2 and Cl^- were the main degradation products. Lower chlorinated methanes, like chloroform (CF) and dichloromethane (DCM) were detected only in minor amounts as transient intermediates. CT was also degraded by autoclaved (dead) sludge indicating that abiotic processes (mediated by enzyme metallo-cofactors or other sludge components) played an important role in the observed degradation. The same study showed that reduced components in the autoclaved sludge were involved in CT-degradation by autoclaved sludge. Previously, the direct dechlorination of CT by the cofactors vitamin B_{12} and cofactor F_{430} was demonstrated in defined systems containing Ti(III)citrate as the bulk reductant [20,21].

The dechlorination rates found in our research of 1 to 2 μmol g^{-1} VSS day^{-1} [11] were comparable to those found by other researchers who had utilized adapted sludge (> 0.4 μmol g^{-1} VSS day^{-1}) [23]. But these values were lower than the rates found with anaerobic consortia (21-35 μmol g^{-1} VSS day^{-1}) originating from an anaerobic digester [7]. Furthermore, much higher transformation rates were reported for CT transformation by pure cultures of anaerobes like *Acetobacterium woodii* (30 mmol g^{-1} protein day^{-1}), *Methanobacterium thermoautotrophicum* (0.8 mmol g^{-1} protein day^{-1}) or *Desulfobacterium autotrophicum* (15 mmol g^{-1} protein day^{-1}) [12,13].

In this research, we investigated the effect of increased CT or biomass concentration on the degradation of CT by anaerobic granular sludge from UASB reactors fed with methanol, VFA or sucrose as the primary substrate. For this purpose, granular sludge was incubated with high (0.25-0.4 mM) and low (10 μM) concentrations of CT. The effect of higher biomass concentrations on the degradation rate was also investigated. The influence of mass transport limitations on the transformation of CT was determined by incubating the sludge in a rotary shaker incubator as opposed to incubation under stationary conditions. The transformation of CT by autoclaved sludge was studied at different temperatures.

Materials and methods

Chemicals. CT, CF, DCM (all p.a. quality from E. Merck, Amsterdam, The Netherlands), and MC (purity >99%, Hoekloos, Schiedam, the Netherlands) were used as received without further purification.

Granular sludge. The granular sludge was grown in three UASB reactors with methanol, a VFA mixture (acetate, propionate, and butyrate) or sucrose as the main carbon source. Originally, they had been inoculated with granular sludge from a full scale UASB-reactor treating sugar-beet refinery wastewater (CSM, Breda, The Netherlands) and were operated as described earlier [11]. COD-removal efficiencies were higher than 85%. No VFA were detectable in the effluent of the three reactors. The reactors had been operated for at least 1 year prior to sludge sampling. The sludges were washed two times with demineralized water and one time with basal medium [18] to remove residual soluble substrate before use in the batch experiments. When appropriate, the sludge granules were crushed prior to the experiment by pressing the sludge suspension through sterile needles with decreasing diameter (smallest needle: Microlance 3, 25G5/8, 0.5x16 mm). It was confirmed that the methanogenic activity of the sludge was not affected by this treatment (data not shown). Other authors [1], also found no inhibitory effect of the crushing of the granular sludge on the maximum methanogenic activity.

Batch experiments. Granular sludge was transferred to 120 ml serum flasks containing 20 ml basal medium (unless stated otherwise), as described earlier by Holliger [18], but without the addition of the fermented yeast-extract. When chlorine balances had to be determined the medium (40 ml) was modified by replacing the chloride salts of calcium and magnesium with $Ca(OH)_2$ and $MgHPO_4 \cdot 2H_2O$. The pH in the batches remained constant at 7.2-7.3. The appropriate primary substrate was initially added at 2 g COD l^{-1} and new doses of primary substrate (1 to 2 g COD l^{-1}) were added every week. CT was added as a pure compound or as a solution prepared in anaerobic water. The batches were incubated statically at 30°C in the dark unless stated otherwise. The possible loss of the chlorinated compound due to leaking through the stoppers was checked in separate batches with sterile medium without any sludge addition. All experiments were carried out in triplicate unless stated otherwise.

Involvement of biologically generated cofactors in abiotic transformations. The involvement of abiotic transformations catalyzed by sludge components was tested by autoclaving the granular sludge, thus inactivating all microbial activity. The granular sludge was autoclaved in basal medium for 90 minutes at 120°C three days prior to the start of the experiment and again for 30 minutes on the day the experiment was started.

The effect of mass transport limitation and of the amount of biomass on the dechlorination rate. Autoclaved or living methanogenic sludge (0.25, 1.0, or 2.5 grams (wet weight) per bottle) from the reactor fed with VFA, was added to 40 ml basal medium in 120-ml serum bottles. CT was added dissolved in water (final concentration 1500-2000 nmol/bottle). The addition of primary substrate was omitted to avoid interference with the chloride measurement. Previous experiments indicated that the primary substrates were not required for the dechlorination of low concentrations of CT [11]. The batches were incubated at 30°C in a rotary shaking incubator (110 rpm) unless otherwise stated.

Influence of temperature on the degradation rate of CT by autoclaved sludge. Autoclaved sludge from the reactor fed with methanol was used to investigate the chemical nature of the transformation of CT at different temperatures. Sludge was added to the basal medium and CT was added as a stock solution dissolved in water (1500-2000 nmol/batch). After equilibration at 30°C, the amount of CT in the batches was measured to ensure that equal amounts of CT were present. The batches were allowed to equilibrate for 1.5-2 hours at the desired temperature (10, 30, 50, and 70°C). Afterwards the CT concentration was measured via head space analysis. The amount of CT in the headspace was correlated to the total amount of CT in the batches via calibration curves, as described earlier (Chapter 2). The temperature of the batches was kept constant during measurement. The stability of CT at the different temperatures was checked in batches containing sterile medium without sludge. The CT concentration was calculated with calibration curves made at 30°C, since the Henry constant of CT at 10, 50, and 70°C is unknown. The amount of CT measured via headspace analysis at time t at temperature T ($[CT]_{T,t}$) was corrected via the concentration of the blank ($[CT]_{blank,T,t}$) at time t and the concentration of the blank at time 0 at 30°C ($[CT]_{blank,30,0}$) according to equation (1):

$$[CT]_{normalized,T,t} = [CT]_{T,t} / ([CT]_{blank,T,t} / [CT]_{blank,30,0}) \quad (1)$$

The experiments were carried out with autoclaved crushed sludge incubated on a rotary incubator (130±10 rpm).

Analytical methods. Total masses of the chlorinated methanes, carbon disulfide, H_2, and methane were determined via headspace analysis using gas chromatographic methods described earlier [11]. Chloride concentrations were determined using HPLC as described previously [25]. Bromide was used as internal standard. VFA and methanol concentrations were determined by GC as described earlier [14]. The COD for methanol, VFA, and sucrose solutions was determined according to standard methods [3]. COD conversion factors (g g^{-1}) utilized were 1.07, 1.50, 1.07, 1.52, and 1.82 for sucrose, methanol, acetate, propionate, and butyrate, respectively. The VSS content of the sludge was determined by subtracting the ash-content from the dry weight after incubating the sludge

overnight at 105°C. The ash-content was determined after heating the dry sludge at 600°C for 90 minutes.

Pseudo first order rate constants (k) were calculated from the CT concentration ($[CT]_t$) measured at time (t) and fitted according to the equation (2):

$$[CT]_t/[CT]_0 = e^{(-kt)} \quad (2)$$

with $[CT]_0$ being the CT concentration at time zero. The error in the value of k was minimized with the least square method. To facilitate the comparison between the different experiments the pseudo first order rate constants were converted to second order rate constants via normalization to biomass by dividing the pseudo first order rate constant by the amount of biomass (g VSS) present in the batch.

Results

Degradation of the high concentration of CT by granular sludge. Granular sludges grown in reactors fed with methanol, a mixture of VFA or sucrose were able to degrade CT supplied at a high concentration (45-70 µmol/batch equivalent to 0.25-0.4 mM in the waterphase) without prior adaptation. The extent of CT removal was between 85 and 100% after 107 days. CF and DCM were formed as shown for the sludge from the reactor fed with sucrose (Fig. 3.1). The recovery of these lower chlorinated methanes was not largely affected by the source of the granular sludge. The accumulation of CF was enhanced by primary substrate addition during the incubation in the case of the sludge from the sucrose-fed reactor. This sludge transformed 17% of the CT to CF without primary substrate addition and 34% with the addition of sucrose. In contrast, CT conversion to CF by the sludges from the reactors fed with methanol or VFA was not influenced by the addition of primary substrate during the incubation. The recovery of CF was 16 and 12% for the methanol and VFA grown sludge, respectively. The DCM recovery in all cases was never higher than 4% of the CT initially present and MC could not be detected as a product. A chlorine balance was measured with sludge from the reactor fed with methanol. Approximately 109% and 63% of the CT-chlorine removed (and not accounted for by remaining CT, CF or DCM) was recovered as chloride after the incubations without and with primary substrate additions, respectively (data not shown). These results indicate that a major part of the CT-chlorine was mineralized by the unadapted sludge. Neither lower chlorinated methanes nor chloride were formed when CT was incubated in batches without sludge.

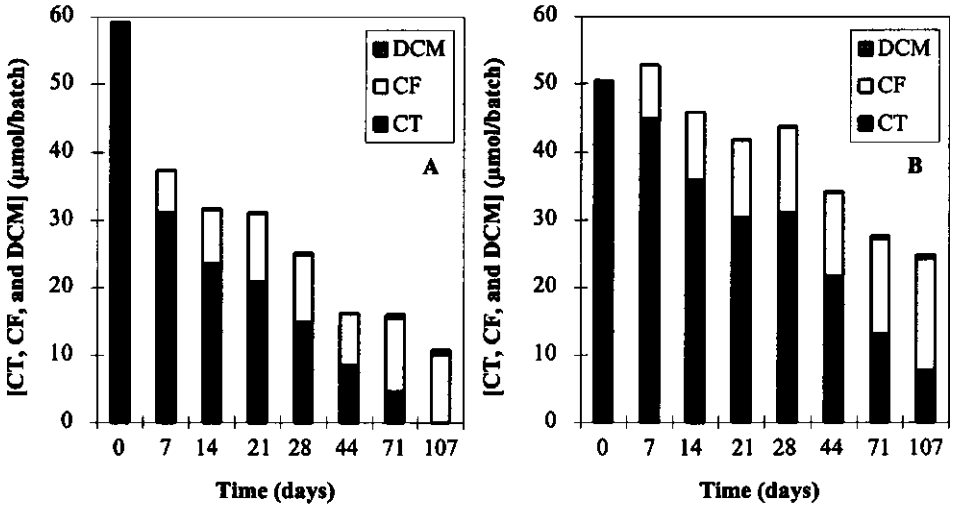


Figure 3.1 The degradation of high CT concentrations (0.25-0.4 mM) by granular sludge sampled from the reactor fed with sucrose. Panel A: Incubations without addition of primary substrate. Panel B: Incubations with weekly addition of primary substrate. (The results shown are mean values of triplicate incubations; the standard deviations of the triplicates were less than 20% of the mean value).

During the first 44 days of the incubation, primary substrate utilization and methane production were monitored. Methanogenesis was completely inhibited in the incubations with the three different sludges. A small amount of VFA (0.5 g COD l^{-1} of which 40% was acetate) was formed in incubations with the methanol fed sludge to which 8 g methanol-COD l^{-1} was added. Around 50% of the methanol was removed. However, products other than VFA could not be found and H_2 was not formed. Without the addition of any primary substrate similar amounts of VFA (0.6 g COD l^{-1} of which 24% of acetate) were formed, indicating that the VFA originated from substrate endogenously present in the sludge and not from the methanol substrate. The granular sludge fed with VFA did not convert the primary substrate added to the incubations, while the sucrose-fed sludge converted the added sucrose largely (75-90%) to VFA (21% acetate, 70% propionate). When the addition of primary substrate was omitted both the sludges from the VFA and sucrose-fed reactors also formed VFA, 1.3 and 1.1 g VFA-COD l^{-1}, respectively. Only the fermentative bacteria in the sucrose fed sludge survived the CT and CF toxicity and were thus able to generate reducing equivalents from the sucrose that was added. Therefore, the CF production was higher in the sucrose fed sludge to which primary substrate was added compared to the incubations without primary substrate.

Pseudo first order rate constants were calculated for the transformation of CT. An example of such a first order fit is depicted in Figure 3.2 for VFA grown sludge. The rate constants were normalized for the amount of biomass present, because the dechlorination rate was also dependent on the biomass concentration. Therefore, the degradation of CT is best described by second order rate kinetics. These second order rate constants were compared with rate constants obtained upon incubation with a low CT concentration (approximately 10 μM in the waterphase) (Table 3.1). The addition of primary substrate slightly increased the rate constants of CT at low concentrations, but at high CT concentrations the effect was less obvious as would be expected from the lack of primary substrate utilization in those incubations. The rate constants at higher concentrations are generally lower than those at lower concentrations. However, the absolute initial rates, which were calculated from the linear decrease in CT concentration within the first 14 days of incubation normalized to the biomass concentration, were approximately 1 order of magnitude greater at the higher CT concentrations (Table 3.1). The absolute rates however, decreased in time, which could be caused by inhibition by the chlorinated methanes or a deficiency in an unknown medium component which is essential for dechlorination.

An attempt was made to fit the data of both low and high CT concentration to the Michaelis-Menten kinetic model according to equations (3) and (4):

$$d[CT]/dt = -V * X \tag{3}$$

where

$$V = V_{max} * ([CT]/(K_m + [CT]) \tag{4}$$

(with [CT]: CT concentration at time t; K_m: substrate half saturation constant; V: the transformation rate at time t; V_{max}: the maximum transformation rate; and X: the biomass concentration).

Modeling of the results from all concentration ranges with Michaelis-Menten kinetics was not possible. For lower CT concentrations, K_m values of 10 to 230 nmol/batch were found corresponding to maximum transformation rates ($V_{initial}$) in the range of the values given in Table 3.1. However, the results did not correlate with values found for higher CT concentrations. The K_m calculated for higher CT concentrations was generally found to be in the order of 500 to 1000 times higher than those calculated for the low CT concentration.

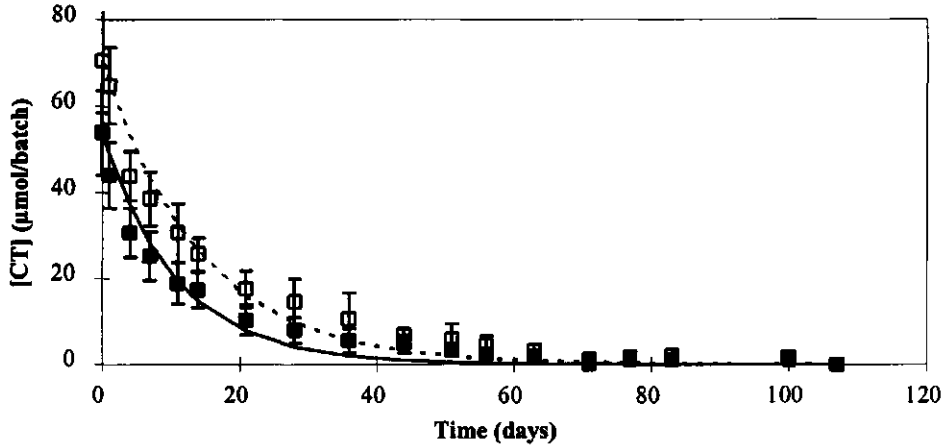


Figure 3.2 First order transformation curves for CT degradation by granular sludge sampled from the reactor fed with VFA with (□) and without (■) primary substrate. Error bars indicate standard deviation of triplicate incubations.

Table 3.1 Second order rate constants (k in day^{-1} g^{-1} VSS) and absolute initial rates ($v_{initial}$ in µmol g^{-1} VSS day^{-1}, mean of triplicate incubations ± standard deviation) determined at low (10 µM in the waterphase) and high (25-40 mM in the waterphase) CT concentrations. and with (PS) and without (No PS) primary substrate.

Sludge	Low concentration[a]				High concentration[b]			
	No PS		PS		No PS		PS	
	k	$v_{initial}$	k	$v_{initial}$	k	$v_{initial}$	k	$v_{initial}$
Methanol	4.1	1.3 ± 0.4	6.0	1.3 ± 0.1	0.5	13.9 ± 0.6	0.5	14.9 ± 0.5
VFA	1.9	1.2 ± 0.1	2.8	1.0 ± 0.0	0.3	13.0 ± 2.5	0.4	10.7 ± 2.1
Sucrose	1.8	1.7 ± 0.3	3.1	1.9 ± 0.2	0.4	12.6 ± 1.0	0.1	8.3 ± 2.0

[a] These rates were calculated from data taken from [11]
[b] These rates were determined during the first 14 days of incubation

Influence of the amount of biomass on the rate of CT degradation. The influence of the amount of biomass on the degradation rate of CT was determined with both living and autoclaved granular sludge from the reactor fed with VFA. Rate constants for the removal of CT were calculated during incubation under shaken and stationary conditions (Fig. 3.3). The normalized (second order) rate constant was 21 times higher with living sludge, upon incubation in a rotary shaking incubator than under stationary conditions. The rate of dechlorination by autoclaved sludge was increased five fold to 31 day^{-1} g^{-1} VSS if the sludge was crushed (Fig. 3.4). This indicates that mass transport limitations play an

important role in the CT degradation rate under stationary conditions. The biomass-normalized rate constant is equal for different amounts of autoclaved sludge incubated under shaken conditions. However, for living sludge the biomass-normalized rate constant increases with increasing amounts of biomass showing a beneficial effect of the presence of a larger amount of living sludge (Fig. 3.3). In all cases, the CT-chlorine transformed was recovered as chloride (data not shown), indicating that CT was dechlorinated and not adsorbed.

The influence of temperature on the dechlorination of CT by autoclaved sludge. Crushed autoclaved sludge was incubated at different temperatures in a rotary shaker incubator. Rate constants were determined and normalized for the amount of biomass in the batches (Fig. 3.4). Incubation under shaken conditions was shown to be beneficial for the CT transformation rate (Fig. 3.3). The reaction rate shows a doubling or tripling of the rate constant per 20 degrees increase in temperature. Chemical reaction rates in general are known to increase 2- to 3-fold with each 10 degrees increase in temperature. In the whole temperature range studied, the temperature effects on abiotic transformation are consistent with an activation energy of 46 to 71 kJ mol^{-1} $°C^{-1}$ (r^2=0.9567).

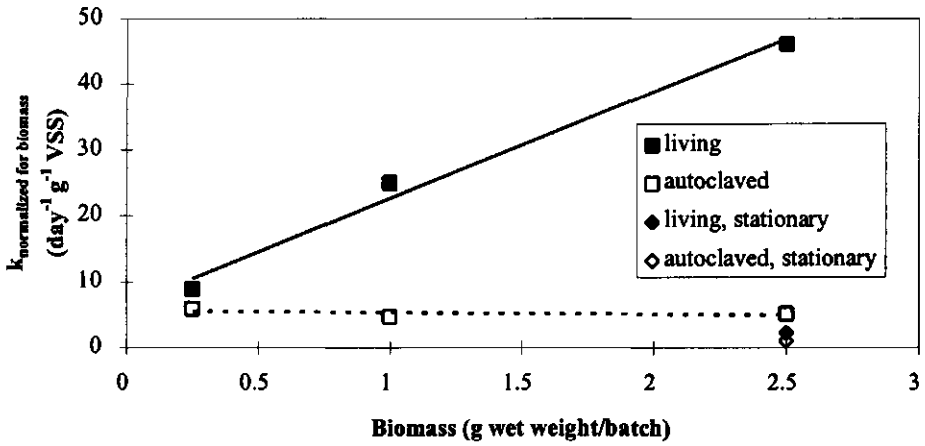


Figure 3.3 Second order rate constants determined for the degradation of CT (supplied at the concentration of approx. 10 μM) with different amounts of living and autoclaved biomass incubated under shaken or stationary conditions.

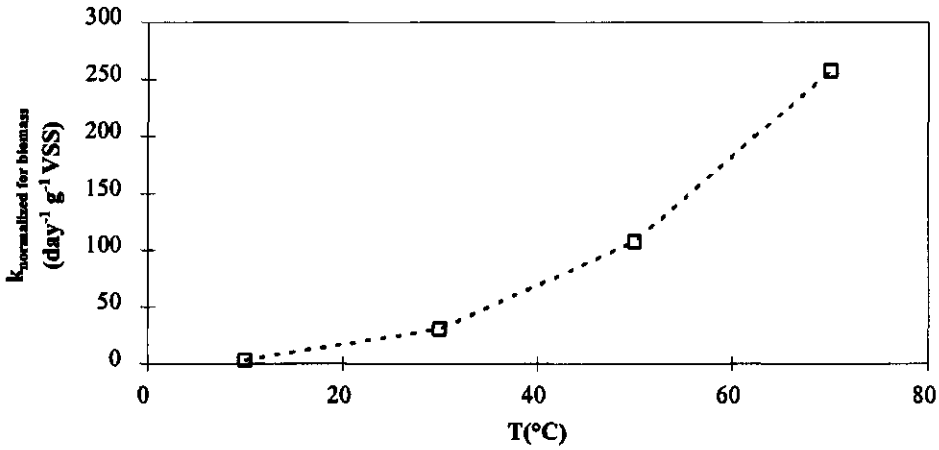


Figure 3.4 Second order rate constants for CT removal by autoclaved crushed sludge incubated under shaken conditions at different temperatures.

Discussion

Unadapted granular sludges were found to degrade CT applied at high concentrations (0.25-0.4 mM) without prior exposure. CF was the main product identified accounting for 10-35% of the carbon. The chlorine balance showed that the majority of the CT-Cl removed could be recovered as chloride. This supports the hypothesis that CT is partially converted to CO_2 as was shown for the degradation of C_{14}-labeled CT at a lower concentration (10 μM) [11].

Mass transfer limitations influence the transformation rate of CT by the granular methanogenic sludge. Compared to the conversion of CT at a low concentration, the initial absolute transformation rates were one order of magnitude higher at 0.25-0.4 mM (Table 3.1). This, together with the fact that incubation under shaken conditions (Fig. 3.3) increased the reaction rate, stresses that mass transfer affects the transformation of CT by methanogenic granular sludge. Similar results were found previously, but in those cases, the research dealt with more soluble substrates like volatile fatty acids [1,5,22]. Furthermore, the rate constants for the dechlorination of CT by autoclaved sludge were higher in the presence of crushed sludge compared to whole granules. Both the transfer of CT from the gas to the liquid phase and diffusion inside the anaerobic granule could be limiting factors for the conversion rates of CT.

An interesting observation from this study was that the rate constant was significantly higher at increased biomass concentrations. A plausible explanation could be that a lower redox potential is achieved with more biological activity which is favorable for the reduction of CT. Previously, variations of biomass at very low concentrations (0.56 and 1.7 mg VSS l^{-1}) were shown not to affect the normalized rate constants [7]. However, the biomass concentrations in this study (0.75 to 7.3 g VSS l^{-1}) were orders of magnitude higher. The importance of the biological activity on the normalized rate constants is stressed further by the lower values at high CT concentrations which severely inhibit acetogenic and methanogenic activities. Also the normalized rate constant was constant at all sludge concentrations with autoclaved sludge. Primary substrate additions which resulted in biological activity at low CT concentrations increased the rate constant but had no effect at high CT concentrations which were too inhibitory for substrate utilization. At low CT concentrations, the effect of supplementing the incubations with primary substrate may have increased the rate constant via the generation of more reducing equivalents by the metabolism of the primary substrate.

The high CT concentration appears to have inhibited both the CF degradation both via the oxidative (to CO_2) or the reductive pathway (to DCM). The percentage of CF accumulation was higher than at lower CT concentrations. Previously, we have shown that CF was further converted to DCM and MC at the low concentration [11]. Maybe, CT applied at a high concentration is degraded via a different pathway compared to the low concentration. Either the high CT concentration or the CF which accumulated was responsible for the severe inhibition of the acetogenesis and methanogenesis. CT and CF are known to inhibit the acetyl-CoA pathway via covalent binding to vitamin B_{12}, a coenzyme in the acetyl CoA pathway [16], thus preventing the generation of reducing equivalents.

At the high CT concentration applied (0.25-0.4 mM), the methanogenic activity of the three sludges was completely inhibited. The IC_{50} (50% inhibition concentration) of CT and CF towards acetoclastic methanogens is 60 and 14.2 μM (7.7 and 1.7 mg l^{-1}), respectively [24]. The observation that both methanogenesis and CT transformation processes are simultaneously inhibited does not necessarily imply that methanogens are involved in the actual CT transformation. However, their presence may be necessary to maintain certain environmental conditions such as the supply of metalloenzyme cofactors or a low redox potential as was suggested earlier for the transformation of β-hexachlorocyclohexane (β-HCH) [10]. The inhibition of methanogenesis could have resulted in relatively higher redox

potentials leading to lower rate constants. Interestingly, a correlation between redox potential and pseudo first order rate constants was found for the transformation of CT by cobalamin [2]. A correlation between the amount of CCl_4 transformed (C_t/C_0) and the redox potential was also found by other authors [7,8]. Dechlorination reaction rate constants were found to be higher at more negative redox potentials.

The finding that the dechlorination occurred in the presence of autoclaved sludge indicates that the reactions observed in the presence of living sludge were in part abiotic. Abiotic dechlorination was catalyzed by sludge components fueled by reducing equivalents, since dechlorinating activity was not observed in blanks containing medium and lacking autoclaved sludge. Therefore, a possible donor of reducing equivalents and/or mediator or reaction component should originate from the sludge. Vitamin B_{12} and other cofactors like cofactor F_{430}, which are present in anaerobic bacteria are known to catalyze the dechlorination of CT [15,20,21]. Whether the responsible sludge components are enzyme cofactors (vitamin B_{12} or F_{430}) or chemical compounds like e.g., iron or sulfide, which are also known to mediate CT dechlorination [6,9,19] remains to be investigated.

Acknowledgments

This research was supported by a grant (project 92014) from the Innovative Research Program on Environmental Biotechnology (IOP-Milieubiotechnologie) of the Ministries of Economic Affairs and Housing, Physical Planning, and Environment.

References

1. **Alphenaar, P. A., M. C. Perez, and G. Lettinga.** 1993. The influence of substrate transport limitation on porosity and methanogenic activity of anaerobic sludge granules. Appl. Microbiol. Biotechnol. **39**:276-280.
2. **Assaf-Anid, N., K. F. Hayes, and T. M. Vogel.** 1994. Reductive dechlorination of carbon tetrachloride by cobalamin(II) in the presence of dithiothreitol - mechanistic study, effect of redox potential and pH. Environ. Sci. Technol. **28**:246-252.
3. **American Public Health Association.** 1985. Standard methods for examination of water and wastewater, 16th ed. APHA, Washington DC.
4. **Bouwer, E. J., and P. L. McCarty.** 1983. Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl. Environ. Microbiol. **45**:1286-1294.
5. **Boyd, S. A., and D. R. Shelton.** 1984. Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. Appl. Environ. Microbiol. **47**:272-277.
6. **Curtis, G. P., and M. Reinhard.** 1994. Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthrahydroquinone disulfonate and humic acid. Environ. Sci. Technol. **28**:2393-2401.

7. **Doong, R.-A., T. F. Chen, and W. H. Chang.** 1996. Effects of electron donor and microbial concentration on the enhanced dechlorination of carbon tetrachloride by anaerobic consortia - short contribution. Appl. Microbiol. Biotechnol. **46:**183-186.
8. **Doong, R.-A., S. Wu, and T. Chen.** 1996. Anaerobic biotransformation of polychlorinated methane and ethene under various redox conditions. Chemosphere. **32:**377-390.
9. **Doong, R.-A., and S.-C. Wu.** 1992. Reductive dechlorination of chlorinated hydrocarbons in aqueous solutions containing ferrous and sulfide ions. Chemosphere. **24:**1063-1075.
10. **Eekert, M. H. A. van, N. J. P. van Ras, G. H. Mentink, A. J. M. Stams, J. A. Field, and G. Schraa.** 1998. Anaerobic transformation of β-hexachlorocyclohexane by methanogenic granular sludge and soil microflora. Environ. Sci. Technol. **32:**3299-3304.
11. **Eekert, M. H. A. van, T. J. Schröder, A. J. M. Stams, G. Schraa, and J. A. Field.** 1998. Degradation and fate of carbon tetrachloride in unadapted methanogenic granular sludge. Appl. Environ. Microbiol. **64:**2350-2356.
12. **Egli, C., S. Stromeyer, A. M. Cook, and T. Leisinger.** 1990. Transformation of tetra- and trichloromethane to CO_2 by anaerobic bacteria is a non-enzymic process. FEMS Microbiol. Lett. **68:**207-212.
13. **Egli, C., T. Tschan, R. Scholtz, A. M. Cook, and T. Leisinger.** 1988. Transformation of tetrachloromethane to dichloromethane and carbon dioxide by *Acetobacterium woodii*. Appl. Environ. Microbiol. **54:**2819-24.
14. **Florencio, L., A. Nozhevnikova, A. van Langerak, A. J. M. Stams, J. A. Field, and G. Lettinga.** 1993. Acidophilic degradation of methanol by a methanogenic enrichment culture. FEMS Microbiol. Lett. **109:**1-6.
15. **Gantzer, C. J., and L. P. Wackett.** 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. Environ. Sci. Technol. **25:**715-722.
16. **Ghambeer, R. K., H. G. Wood, M. Schulman, and L. Ljungdahl.** 1971. Total synthesis of acetate from CO_2. III. Inhibition by alkylhalides of the synthesis from CO_2, methyltetrahydrofolate, and methyl-B_{12} by *Clostridium thermoaceticum*. Arch. Biochem. Biophys. **143:**471-484.
17. **Hashsham, S. A., R. Scholze, and D. L. Freedman.** 1995. Cobalamin-enhanced anaerobic biotransformation of carbon tetrachloride. Environ. Sci. Technol. **29:**2856-2863.
18. **Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder.** 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. **59:**2991-2997.
19. **Kriegman-King, M. R., and M. Reinhard.** 1994. Transformation of carbon tetrachloride by pyrite in aqueous solution. Environ. Sci. Technol. **28:**692-700.
20. **Krone, U. E., K. Laufer, R. K. Thauer, and H. P. C. Hogenkamp.** 1989. Coenzyme F_{430} as a possible catalyst for the reductive dehalogenation of chlorinated C1 hydrocarbons in methanogenic bacteria. Biochemistry. **28:**10061-10065.
21. **Krone, U. E., R. K. Thauer, and H. P. C. Hogenkamp.** 1989. Reductive dehalogenation of chlorinated C1-hydrocarbons mediated by corrinoids. Biochemistry. **28:**4908-4914.
22. **Lier, J. B. van, J. L. S. Martin, and G. Lettinga.** 1996. Effect of temperature on the anaerobic thermophilic conversion of volatile fatty acids by dispersed and granular sludge. Water Res. **30:**199-207.
23. **Long, J. L., H. D. Stensel, J. F. Ferguson, S. E. Strand, and J. E. Ongerth.** 1993. Anaerobic and aerobic treatment of chlorinated aliphatic compounds. J. Environ. Eng. - ASCE. **119:**300-320.
24. **Sanz, J. L., N. Rodriguez, and R. Amils.** 1997. Effect of chlorinated aliphatic hydrocarbons on the acetoclastic methanogenic activity of granular sludge. Appl. Microbiol. Biotechnol. **47:**324-328.
25. **Scholten, J. C. M., and A. J. M. Stams.** 1995. The effect of sulfate and nitrate on methane formation in a freshwater sediment. Antonie van Leeuwenhoek. **68:**309-315.

4

Gratuitous dechlorination of chloroethanes by methanogenic granular sludge

The dechlorinating activity of a methanogenic granular sludge from a methanol-fed upflow anaerobic sludge blanket reactor was investigated with chlorinated ethanes. This unadapted methanogenic consortium degraded all chloroethanes tested. The product formation rates decreased with the number of chlorine substituents. The higher chlorinated ethanes were also converted, although at a lower rate, in the presence of autoclaved (dead) sludge, indicating the involvement of reduced heat-stable cofactors like vitamin B_{12} and F_{430}. Direct chemical dechlorination of hexa-, penta- and tetrachloroethanes was also observed in medium without sludge, although at a much lower rate. The results show the importance of cometabolic and abiotic (chemical) conversions for the transformation of chlorinated ethanes by the methanogenic consortium. The type of reactions and the products formed were correlated with the Gibbs free energy change ($\Delta G^{0'}$). Reductive hydrogenolysis and dichloroelimination were important dechlorinating mechanisms. Generally, these reactions have a higher $\Delta G^{0'}$ value than dehydrochlorination reactions, which occurred less frequently during the transformation of chloroethanes by the methanogenic granular sludge.

Published as: **M.H.A. van Eekert, A.J.M. Stams, J.A. Field, and G. Schraa,** 1999. Appl. Microbiol. Biotechnol, 51: 46-52.

Introduction

Chlorinated ethanes are often found as pollutants in groundwater. Their anaerobic degradation is a ubiquitous capacity among fermentative [15], sulfate-reducing [13], and pure as well as mixed methanogenic bacterial cultures [6,20,25]. Higher chlorinated ethanes are converted to non- or lower chlorinated ethanes and ethenes via three mechanisms. The first two mechanisms, reductive hydrogenolysis and dichloroelimination, require the input of two electrons resulting in the release of one or two chlorine atoms, respectively. Dichloroelimination also leads to the net formation of a double bond between vicinal carbon atoms. The third mechanism, dehydrochlorination, is a non-redox reaction by which an HCl-group is released and a double bond is formed between two neighboring carbon atoms [31]. Partial mineralization of 1,1,1-trichloroethane has been observed with adapted methanogenic consortia [5,32]. Despite the research that has been carried out so far, little is known about the dechlorinating capacity of unadapted methanogenic consortia.

Methanogenic consortia like granular sludges grown in upflow anaerobic sludge blanket (UASB) reactors have a high biomass content. The biomass consists mainly of acetogenic and methanogenic bacteria [23]. They contain high amounts of metalloenzyme cofactors like vitamin B_{12}, and cofactor F_{430} [22,28]. These cofactors are involved in dominant pathways of anaerobic bacteria, e.g., the acetyl CoA-pathway for CO_2 fixation and the last step of methane formation. The degradation of chlorinated compounds takes place via pathways catalyzed by enzymes containing these cofactors and is assumed to be cometabolic. The broad spectrum of chlorinated compounds that is degraded by the cofactors *in vitro* [16,20,27,30] suggests the applicability of granular sludge from UASB reactors as a suitable source of dechlorinating activity without prior adaptation. The relatively low rate of dechlorination may be compensated by the high amount of biomass.

In this study, we have investigated the dechlorination of chlorinated ethanes by an unadapted methanogenic consortium grown in a UASB reactor with methanol as a carbon source. Reaction pathways and rates were determined in living and dead (autoclaved) sludge. This enabled a distinction between abiotic conversions that involve biologically generated cofactors and reactions which involve more specific enzymes.

Materials and methods

Chemicals. Hexachloroethane (HCA), pentachloroethane (PCA), 1,1,1,2-tetrachloroethane (1112TeCA), 1,1,2,2-tetrachloroethane (1122TeCA), 1,1,1-trichloroethane (111TCA), 1,1,2-

trichloroethane (112TCA), 1,1-dichloroethane (11DCA), 1,2-dichloroethane (12DCA), tetrachloroethene (TeCE), trichloroethene (TCE), 1,1-dichloroethene (11DCE), *cis*-1,2-dichloroethene (cDCE), and *trans*-1,2-dichloroethene (tDCE) were purchased (p.a. quality) from E. Merck (Amsterdam, The Netherlands). Chloroethane (CA), ethane (A), vinylchloride (VC), and ethene (E) were obtained from Hoekloos, Schiedam, The Netherlands (purity >99%).

Granular sludge. The methanogenic granular sludge was grown in a UASB reactor fed with methanol as the sole carbon source. Originally, the reactor had been inoculated with granular sludge from a full scale UASB-reactor treating sugar-beet refinery wastewater (CSM, Breda, The Netherlands) and was operated as described earlier [12]. The COD (Chemical Oxygen Demand) removal efficiency was higher than 85%. No volatile fatty acids (VFA) were present in the effluent of the reactor. The reactor had been operated for at least 2 years prior to sludge sampling.

Batch experiments. Prior to use in the batch experiments, the sludge was washed two times with demineralized water and one time with basal medium to remove residual soluble substrates. Approximately 2 grams (wet weight) of granular sludge were transferred to 120-ml serum flasks containing 40-ml basal medium, as described earlier by Holliger et al. [19], but without the addition of the fermented yeast-extract. The sludge granules were first crushed for 10 seconds in a blender and then the sludge was pressed through sterile needles with decreasing diameter (smallest needle: Microlance 3, 25G5/8, 0.5x16). It was confirmed that the methanogenic activity of the sludge was not affected by this treatment.

The pH in the batches was 7.2-7.3. The gas phase consisted of N_2 (80%) and CO_2 (20%). The bottles were sealed with Viton stoppers (Maag Technic AG, Dübendorf, Switzerland). Methanol was added at 0.5 g COD l^{-1} per bottle. New doses of methanol were added whenever the previous dose was completely converted to methane. The total amount of methanol added was 2.8 g COD l^{-1} (58 mM) for HCA, PCA, TeCA and TCA amended batches and 3.4 g COD l^{-1} (71 mM) for the batches containing DCA. The chloroethanes (approximately 1500 nmol/batch) were added dissolved in acetone (total amount of acetone added 74 to 128 µl). The bottles were incubated statically at 30°C.

Abiotic involvement of the biologically generated cofactors. The granular sludge was autoclaved (inactivating all microbial activity) in basal medium for 90 minutes at 120°C three days prior to the start of the experiment and again for 30 minutes on the day the experiment was started.

Analytical methods. Total masses of chlorinated ethanes, chlorinated ethenes, and methane were determined via headspace analysis. HCA, PCA, and TeCAs and PCE, TCE and DCEs were analyzed by injecting 0.2 ml headspace into a 436 Chrompack gas chromatograph (GC) equipped with a flame ionization detector (FID) connected to a Sil 5CB column (25 m x 0.32 mm x 1.2 µm) and a splitter injector (ratio 1:50). Operating temperatures of the injector and detector were 250 and 300 °C,

respectively. The oven was operated with the following temperature program: 50°C (1 minute), and 10°C $minute^{-1}$ increase in temperature to 200°C. For the determination of TCA and DCA, and DCE concentrations the oven temperature was 60°C. Carrier gas was N_2 with an inlet pressure of 50 kPa. CA and VC were determined by injecting 0.2 ml headspace in a 438 A Chrompack GC equipped with a FID connected to a Poraplot Q column (25 m x 0.32 mm x 10 μm) and a splitter-injector (ratio 1:40). Operating temperatures of injector, column and detector were 225, 150 and 250 °C, respectively. The retention times and peak areas of all chlorinated ethanes and ethenes were determined with a Shimadzu C-3A integrator. Calibration curves were made by adding the desired amount of chlorinated ethane or chlorinated ethene to a serum bottle with 40 ml basal medium. The bottles were equilibrated overnight at 30°C.

Methanol concentrations were determined as described by Florencio et al. [14]. The COD for methanol was determined according to standard methods [2]. A COD conversion factor ($g\ g^{-1}$) of 1.5 was used. The volatile suspended solids (VSS) content of the sludge was determined by subtracting the ash-content from the dry weight after incubating the sludge overnight at 105°C. The ash content was determined after heating the dry sludge at 600°C for 90 minutes.

Results

Removal of chloroethanes. The chlorinated ethanes were rapidly dechlorinated in the presence of living methanogenic sludge (Fig. 4.1). HCA, PCA and 1112TeCA (the latter compound is not shown in Fig. 4.1) which were initially present at approximately 1500 nmol/batch were removed within 2 days. Dechlorination of 1122TeCA and both trichloroethanes also started immediately but at a lower rate. Methane formation was not inhibited by the chlorinated compounds at the concentrations applied (data not shown).

All chlorinated ethanes except 111TCA, 11DCA, and 12DCA were also substantially transformed in the presence of autoclaved sludge (Fig. 4.2). As was observed with the living sludge, HCA, PCA and 1112TeCA were dechlorinated faster by autoclaved sludge than the lower chlorinated ethanes.

HCA, PCA, both TeCAs, and 112TCA were also converted by direct chemical reactions in sterile medium without sludge (data not shown) but in most cases to a much lower extent: HCA (33%), 1112TeCA (38%), 1122TeCA (68%), and 112TCA (44%) (Table 4.1). Only PCA was completely transformed in the absence of sludge.

The maximum removal rates of the chloroethanes with and without sludge are given in Table 4.2. In medium without sludge, only higher chlorinated ethanes with chlorine atoms on both carbon atoms were transformed. The rates were lower than with sludge present. The removal rates were higher when more chlorine atoms were present on the molecule.

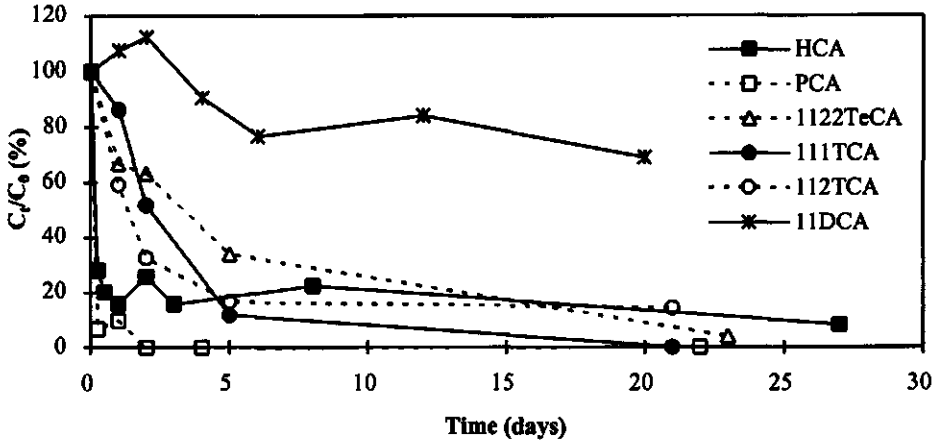


Figure 4.1 Transformation of chlorinated ethanes by living methanogenic methanol grown sludge (C_t/C_0 is the concentration in time divided by the concentration at the start of the experiment). The amount of sludge for each chlorinated ethane was 82.5 mg VSS/batch for HCA and PCA, and 79.5 mg VSS/batch for 1122TeCA, 111TCA, 112TCA, and 11DCA. Data points are mean values of duplicate or triplicate incubations.

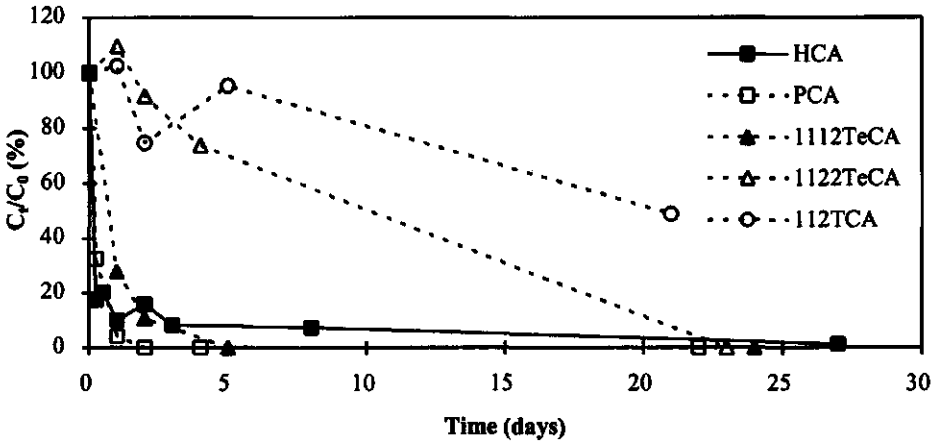


Figure 4.2 Transformation of chlorinated ethanes by autoclaved methanogenic methanol grown sludge (C_t/C_0 is the concentration in time divided by the concentration at the start of the experiment). The amount of sludge was 73.1 mg VSS/batch for each chlorinated ethane. Data points are mean values of duplicate or triplicate incubations.

Product formation. The chlorinated ethanes were typically converted to lower chlorinated ethanes and ethenes (Table 4.1). PCA and 1122TeCA were detected as transient intermediates during the first two days of incubation of living and autoclaved sludge with HCA and PCA, respectively. 112TCA was detected as an intermediate of 1122TeCA transformation with living sludge only.

Table 4.1 Transformation of chlorinated ethanes by living (L) and autoclaved (Au) sludge from a methanol fed UASB-reactor and in medium without any sludge added (M).[a]

	Sludge	Δ%[b]	Main Product[c]	Minor Products[d]
HCA	M	33.1	TeCE (18.4)	
	Au	98.7	TeCE (54.8)	TCE (6.2), c/tDCE (tr)
	L	91.8	TeCE (41.8)	TCE (10.0), DCE (tr)
PCA	M	99	TeCE (104)	TCE (tr)
	Au	100	TCE (24.6)	TeCE (9.3), DCE (6.4)
	L	100	TCE (34.9)	TeCE (6.3), DCE (8.0)
1112TeCA	M	38.2		11DCE (3.6)
	Au	100	11DCE (15.0)	
	L	100	11DCE (34.9)	VC (7.8), tDCE/E/A (tr)
1122TeCA	M	68.1	TCE (40.7)	c/tDCE (tr)
	Au	100	TCE (17.6), cDCE (68.4), tDCE (32.9)	VC (tr)
	L	96.1	cDCE (73.0), tDCE (31.5)	TCE (7.0), VC (11.3), E/A (tr)
111TCA	M	0		
	Au	44.9	no products	
	L	100	11DCA (36.3)	11DCE (12.2), CA (4.8), E/A (tr)
112TCA	M	44.3		
	Au	51.1	VC (18.4)	
	L	85.8	VC (39.5)	12DCA (10.9), E (3.8), 11DCE/A (tr)
11DCA	M	0		
	Au	10.1	no products	
	L	31.1	CA (14.5)	A (tr)
12DCA	M	0		
	Au	13.2	no products	
	L	37.0	CA (10.3)	E (9.7), A (tr)

[a] Data are mean values of duplicate or triplicate incubations. Standard deviations are in general within 20% of the mean value.
[b] The percentage of chlorinated ethane converted, measured after approximately 25 days.
[c] For each chlorinated ethane the amount of each product formed at approximately day 25 is given. Percentages are calculated from the amount of chlorinated ethane at the start of the experiment.
[d] tr = product formation <2%.

The presence of living sludge usually led to the formation of higher amounts of lower chlorinated compounds than with autoclaved sludge or without sludge. In general, the dechlorination products also had a lower number of chlorine atoms in incubations with living sludge compared to autoclaved sludge. Incubation with living sludge usually led to the formation of both chloroethanes and chloroethenes; whereas, the presence of autoclaved sludge resulted in transformation to chloroethenes only. For HCA and PCA the yields of lower chlorinated ethenes and/or ethanes were similar for living and autoclaved sludge. 111TCA, 11DCA, and 12DCA were mainly converted via reductive hydrogenolysis by living sludge only, leading to the formation of lower chlorinated ethanes like 11DCA and CA.

In sterile medium without sludge added, the dechlorination products usually had a higher number of chlorine substituents than with living or with autoclaved sludge present. Only chloroethenes were detected as products of the dechlorination that occurred in medium without sludge.

In many cases (e.g., HCA, PCA, and 111TCA in the presence of autoclaved sludge or 1112TeCA conversion by living sludge), the carbon balance could not be completely closed with the identified products. In this experiment however, only chloroethanes and chloroethenes as well as ethane and ethene were analyzed. Although not measured in this study, the microbiological formation of other products like acetate or other hydrolysis products and CO_2 has been reported [15]. It is likely that these conversions also took place in the presence of methanogenic sludge.

Table 4.2 Chloroethane removal rates[a] by autoclaved (Au) or living (L) sludge or in medium without sludge (M).

		HCA	PCA	TeCA		TCA		DCA	
				1112	1122	111	112	11	12
M	(nmol day^{-1})	60	590	10	110	0	70	0	0
Au	(nmol day^{-1})	1040	1290	1000	130	0	20	5	10
L	(nmol day^{-1})	980	1220	1390	330	280	280	20	30
Au	(µmol g^{-1} VSS day^{-1})	14.2	17.6	13.7	1.8	0	0.3	0.1	0.1
L	(µmol g^{-1} VSS day^{-1})	11.9	14.8	16.8	4.2	3.5	3.5	0.3	0.4

[a] Rates are determined at the maximum values within the first five days. For DCA the rates were determined during the first twenty days of incubation.

Discussion

In this study, chloroethanes were dechlorinated to lower chlorinated ethenes and ethanes in the presence of living methanogenic sludge. The lack of any acclimatization period suggests the involvement of cometabolic processes. All chlorinated ethanes, ranging from hexachlorinated down to dichlorinated ethanes were transformed, indicating a high dechlorination potential of the unadapted methanogenic sludge from UASB reactors. Earlier work has shown that unadapted methanogenic granular sludge was also able to degrade chlorinated methanes [12].

Most of the chloroethanes were also found to be transformed by autoclaved sludge. This points to the involvement of the biologically generated cofactors (like vitamin B_{12} and F_{430}) and/or other abiotic (chemical) processes in the degradation of these compounds. The transformation of chlorinated ethanes has been shown to occur *in vitro* in the presence of reduced iron complexes [21] and other transition metal complexes like vitamin B_{12} and cofactor F_{430} [20,30] or humic acid like substances [8]. Holliger et al. [20] provided evidence for the involvement of the biologically generated cofactors in the reductive dechlorination of DCA by methanogenic bacteria. The higher chlorinated ethanes were found to be even more susceptible to dechlorination mediated by vitamin B_{12} [30]. Combined with the results found in our experiments, this information points to the involvement of cometabolic processes mediated by the cofactors like vitamin B_{12} in the transformation of chloroethanes by methanogenic granular sludge. The fact that the higher chlorinated ethanes were also reduced in medium without sludge indicates that direct chemical reactions with medium components like sulfide also play a role in the conversion of some of these compounds. It has been shown that chlorinated alkanes like carbon tetrachloride can be dechlorinated in reactions mediated by vitamin B_{12} with sulfide as the bulk electron donor [24]. Also, a direct reaction of chloroethanes with (poly)sulfide(s) has been observed [3,8,29]. While in sterile medium, the chlorinated ethanes were converted chemically via dichloroelimination and dehydrochlorination reactions, the presence of sludge led to fewer dehydrochlorination and more reductive hydrogenolysis and dichloroelimination reactions resulting in lower chlorinated compounds. Higher chlorinated ethanes are dechlorinated via a combination of abiotic (cofactor mediated and direct chemical) and biological pathways by adapted consortia as was shown for 111TCA [32], 1122TeCA [6], and HCA [7]. 111TCA and both DCAs are probably converted via more specific enzyme systems, i.e., not by unbound cofactors, since these compounds were not transformed by autoclaved sludge. The presence of living sludge seems to be essential for more extensive dechlorination.

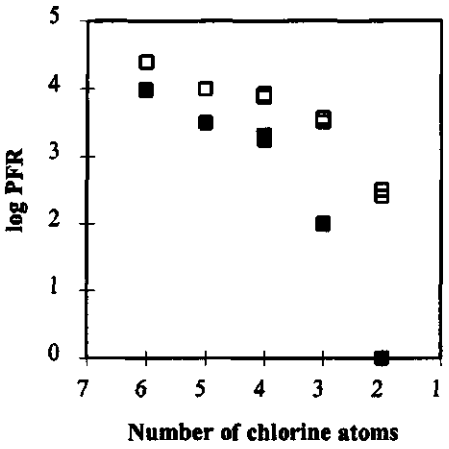


Figure 4.3 The logarithm of the product formation rate (PFR) (calculated in nmol g^{-1} VSS day^{-1}) with autoclaved (■) and living (□) sludge as a function of the number of chlorine atoms in the molecule.

The reaction rates found in our experiments increased with increasing chlorine number (Fig. 4.3). Similar observations have been found previously for chloromethanes and chloroethenes [31]. The rates are lower with autoclaved sludge compared to living sludge. The difference in rate between living and autoclaved sludge increases with a decreasing number of chlorine atoms in the molecule (Fig. 4.3). While the free energies of formation (G_f^0), a measure of the compounds "chemical stability", of chlorinated ethanes seem to be relatively independent of the number of chlorine atom substituents in the molecule (Table 4.3), the dechlorination of the higher chlorinated ethanes leads to more negative Gibbs free energy of reaction ($\Delta G^{0'}$) values compared to the lower chlorinated ethanes, irrespective of the mechanism. Based on thermodynamics, it has been hypothesized that the dechlorination reactions with more negative $\Delta G^{0'}$ values proceed before the others, because of the resemblance with the preferential use of inorganic electron acceptors by microorganisms [10]. This was indeed found for the reductive hydrogenolysis of hexachlorobenzene by two different enrichment cultures [4,10]. Similar results were found for the reductive dechlorination of PCBs [9]. In this case, we should expect mainly dichloroeliminations for higher chlorinated ethanes, since the $\Delta G^{0'}$ values of dichloroelimination reactions are usually 40-70 kJ $mole^{-1}$ more negative than those for reductive hydrogenolysis. This difference becomes smaller when the number of chlorine atoms in the molecule decreases suggesting a higher probability of reductive hydrogenolysis of dichloroethanes than of HCA. The same can be said for base catalyzed dehydrochlorination reactions. Both assumptions are consistent with the results obtained in our research. If a one step dechlorination to the observed products is assumed, there is a tendency towards more reductive hydrogenolysis compared to dichloroelimination reactions for tri- and dichlorinated ethanes. The higher reaction rates found for higher chlorinated ethanes than for lower chlorinated ethanes were not in agreement with the

differences in $\Delta G^{0'}$ values. However, $\Delta G^{0'}$ values are only related to equilibrium constants and are not a measure for reaction rates. Dehydrochlorination (a non-biological reaction) is only a minor pathway for these tri- and dichloroethanes by the methanogenic consortium.

The methanogenic consortium studied here was able to dechlorinate a variety of compounds comparable to adapted consortia and pure cultures described in literature. Therefore, granular sludge may be suitable for the remediation of waste streams that are contaminated with more than one chlorinated aliphatic compound. Most of the dechlorinated products formed are degradable under aerobic conditions, which makes the application of methanogenic sludge a suitable first step in the bioremediation of such waste streams. The results presented here also stress the importance of abiotic reaction mechanisms in the anaerobic transformation of chlorinated compounds.

Table 4.3 Free energy of formation of chlorinated ethanes in the gaseous state (G_f° (g) (kJ mole^{-1})), and $\Delta G^{0'}$ (kJ mole^{-1}) of dechlorination reactions[a] which could occur in the conversion of chlorinated ethanes.

Substrate	G_f° (g)[b]	$\Delta G^{0'}$[c]		
		Reductive hydrogenolysis	Dichloroelimination	Dehydrochlorination
HCA	-54.9	-189.7	-265.1	
PCA	-70.2	-180.6 and -191.6	-248.6	-155.2
1112TeCA	-80.3	-162.4 and -171.0	-232.4	-147.8
1122TeCA	-85.5	-159.8	-221.3 and -225.6	-136.6
111TCA	-76.1	-170.8		-149.8
112TCA	-77.4	-162.6 and -166.4	-205.0	-141.2 to -145.6
11DCA	-73.2	-139.2		-122.6
12DCA	-73.8	-135.0	-187.9	-118.4
CA	-43.0	-151.7		-132.7

[a] Reductive hydrogenolysis: $RCl + H_2 \rightarrow RH + H^+ + Cl^-$; Dichloroelimination: $RCl_2 + H_2 \rightarrow R + 2H^+ + 2Cl^-$; Dehydrochlorination: $RHCl + OH^- \rightarrow R + Cl^- + H_2O$ (assuming the release of a proton when OH^- originates from H_2O).

[b] Data for free energy of formation (G_f° (g)) are taken from Dolfing and Janssen [11].

[c] $\Delta G^{0'}$ values of all possible reactions are calculated according to $\Delta G^{0'} = \Sigma G_f^\circ$(products)-$\Sigma G_f^\circ$(substrates) using hydrogen as the electron donor at pH 7 and 298.15 K. All compounds are in the aqueous phase at 1 mole/kg activity except hydrogen which is in the gaseous state and H^+ which is at 10^{-7} mole kg^{-1} activity. Data for free energy of formation (G_f°) of Cl^-, H_2O, and OH^- (-131.2, -237.2, and -157.3 kJ/mole, respectively) are taken from Hanselmann [18]. Data for the Henry constants are taken from Dolfing and Janssen [11] (original data: Mackay and Shiu [26], Ashworth et al. [1] and Gossett [17]).

Acknowledgments

This research was supported by a grant (project 92014) from the Innovative Research Program on Environmental Biotechnology (IOP-Milieubiotechnologie) of the Ministries of Economic Affairs and Housing, Physical Planning, and Environment.

References

1. **Ashworth, R. A., G. B. Howe, M. E. Mullins, and T. N. Rogers.** 1988. Air-water partitioning coefficients of organics in dilute aqueous solutions. J. Hazard. Mater. **18:**25-36.
2. **American Public Health Association.** 1985. Standard methods for examination of water and wastewater, vol. 16th ed. APHA, Washington DC.
3. **Barbash, J. E., and M. Reinhard.** 1989. Abiotic dehalogenation of 1,2-dichloroethane and 1,2-dibromoethane in aqueous solution containing hydrogen sulfide. Environ. Sci. Technol. **23:**1349-1357.
4. **Beurskens, J. E. M., C. G. C. Dekker, H. van den Heuvel, M. Swart, and J. De Wolf.** 1994. Dechlorination of chlorinated benzenes by an anaerobic microbial consortium that selectively mediates the thermodynamic most favorable reactions. Environ. Sci. Technol. **28:**701-706.
5. **Bouwer, E. J., and P. L. McCarty.** 1983. Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl. Environ. Microbiol. **45:**1286-1294.
6. **Chen, C., J. A. Puhakka, and J. F. Ferguson.** 1996. Transformations of 1,1,2,2-tetrachloroethane under methanogenic conditions. Environ. Sci. Technol. **30:**542-547.
7. **Criddle, C. S., P. L. McCarty, M. C. Elliott, and J. F. Barker.** 1986. Reduction of hexachloroethane to tetrachloroethylene in groundwater. J. Contam. Hydrol. **1:**133-142.
8. **Curtis, G. P., and M. Reinhard.** 1994. Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthrahydroquinone disulfonate and humic acid. Environ. Sci. Technol. **28:**2393-2401.
9. **Dolfing, J., and J. E. M. Beurskens.** 1995. The microbial logic and environmental significance of reductive dehalogenation, p. 143-206. *In* J. G. Jones (ed.), Adv. Microb. Ecol., vol. 14. Plenum Press, NewYork.
10. **Dolfing, J., and B. K. Harrison.** 1993. Redox and reduction potentials as parameters to predict the degradation pathway of chlorinated benzenes in anaerobic environments. FEMS Microbiol. Ecol. **13:**23-30.
11. **Dolfing, J., and D. B. Janssen.** 1994. Estimates of Gibbs free energies of formation of chlorinated aliphatic compounds. Biodegradation. **5:**21-28.
12. **Eekert, M. H. A. van, T. J. Schröder, A. J. M. Stams, G. Schraa, and J. A. Field.** 1998. Degradation and fate of carbon tetrachloride in unadapted methanogenic granular sludge. Appl. Environ. Microbiol. **64:**2350-2356.
13. **Egli, C., R. Scholtz, A. M. Cook, and T. Leisinger.** 1987. Anaerobic dechlorination of tetrachloromethane and 1,2-dichloroethane to degradable products by pure cultures of *Desulfobacterium* sp. and *Methanobacterium* sp. FEMS Microbiol. Lett. **43:**257-61.
14. **Florencio, L., P. Jenicek, J. A. Field, and G. Lettinga.** 1993. Effect of cobalt on the anaerobic degradation of methanol. J. Ferm. Bioeng. **75:**368-374.
15. **Gälli, R., and P. L. McCarty.** 1989. Biotransformation of 1,1,1-trichloroethane, trichloromethane, and tetrachloromethane by a *Clostridium* sp. Appl. Environ. Microbiol. **55:**837-844.
16. **Gantzer, C. J., and L. P. Wackett.** 1991. Reductive dechlorination catalyzed by bacterial

transition-metal coenzymes. Environ. Sci. Technol. **25:**715-722.
17. **Gossett, J. M.** 1987. Measurements on Henry's law constants for C1 and C2 chlorinated hydrocarbons. Environ. Sci. Technol. **21:**202-208.
18. **Hanselmann, K. W.** 1991. Microbial energetics applied to waste repositories. Experientia. **47:**645-687.
19. **Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder.** 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. **59:**2991-2997.
20. **Holliger, C., G. Schraa, E. Stupperich, A. J. M. Stams, and A. J. Zehnder.** 1992. Evidence for the involvement of corrinoids and factor F_{430} in the reductive dechlorination of 1,2-dichloroethane by *Methanosarcina barkeri*. J. Bacteriol. **174:**4427-4434.
21. **Klecka, G. M., and S. J. Gonsior.** 1984. Reductive dechlorination of chlorinated methanes and ethanes by reduced iron (II) porphyrins. Chemosphere. **13:**391-402.
22. **Krzycki, J., and J. G. Zeikus.** 1980. Quantification of corrinoids in methanogenic bacteria. Curr. Microbiol. **3:**243-245.
23. **Lettinga, G., A. F. M. van Velsen, S. W. Hobma, W. de Zeeuw, and A. Klapwijk.** 1980. Use of upflow anaerobic (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioeng. **22:**677-734.
24. **Lewis, T. A., M. J. Morra, and P. D. Brown.** 1996. Comparative product analysis of carbon tetrachloride dehalogenation catalyzed by cobalt corrins in the presence of thiol or titanium (III) reducing agents. Environ. Sci. Technol. **30:**292-300.
25. **Long, J. L., H. D. Stensel, J. F. Ferguson, S. E. Strand, and J. E. Ongerth.** 1993. Anaerobic and aerobic treatment of chlorinated aliphatic compounds. J. Environ. Eng. - ASCE. **119:**300-320.
26. **Mackay, D., and W. Y. Shiu.** 1981. A critical review of Henry's law constants for chemicals of environmental interest. J. Phys. Chem. Ref. Data. **10:**1175-1199.
27. **Marks, T. S., J. D. Allpress, and A. Maule.** 1989. Dehalogenation of lindane by a variety of porphyrins and corrins. Appl. Environ. Microbiol. **55:**1258-1261.
28. **Mazumder, T. K., N. Nishio, S. Fukuzaki, and S. Nagai.** 1987. Production of extracellular vitamin B-12 compounds from methanol by *Methanosarcina barkeri*. Appl. Microbiol. Biotechnol. **26:**511-516.
29. **Miller, P. L., D. Vasudevan, P. M. Gschwend, and A. L. Roberts.** 1998. Transformation of hexachloroethane in a sulfidic natural water. Environ. Sci. Technol. **32:**1269-1275.
30. **Schanke, C. A., and L. P. Wackett.** 1992. Environmental reductive elimination reactions of polychlorinated ethanes mimicked by transition-metal coenzymes. Environ. Sci. Technol. **26:**830-833.
31. **Vogel, T. M., C. S. Criddle, and P. L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21:**722-736.
32. **Vogel, T. M., and P. L. McCarty.** 1987. Abiotic and biotic transformations of 1,1,1-trichloroethane under methanogenic conditions. Environ. Sci. Technol. **21:**1208-1213.

5

Anaerobic transformation of β-hexachlorocyclohexane by methanogenic granular sludge and soil microflora

β-hexachlorocyclohexane (β-HCH) was microbiologically transformed under anaerobic conditions by methanogenic granular sludges from upflow anaerobic sludge blanket (UASB) reactors fed with methanol, volatile fatty acids (VFA), or sucrose as substrates. These sludges, which had not priorily been exposed to β-HCH transformed β-HCH to benzene and chlorobenzene. Usually 2-3 times as much benzene as chlorobenzene was formed. The transformation rates ranged from 0.37 to 0.46 μmol β-HCH g^{-1} volatile suspended solids (VSS) day^{-1} at 30°C. β-HCH was not transformed by autoclaved (sterile) sludge, indicating the biotic nature of the reaction.

β- and also α-HCH present in contaminated soil were both found to be converted to chlorobenzene and benzene upon incubation of the soil under anaerobic conditions. Equal amounts of benzene and chlorobenzene were formed.

The results show that β-HCH transforming bacteria are present in different anaerobic environments. This finding may be of importance for the application of anaerobic bioremediation on sites contaminated with HCH isomers.

Published as: **M.H.A. van Eekert, N.J.P. van Ras, G.H. Mentink, H.H.M. Rijnaarts, A.J.M. Stams, J.A. Field, and G. Schraa,** 1998. Environ. Sci. Technol., 32:3299-3304.

Introduction

The use of lindane, the γ-isomer of hexachlorocyclohexane (HCH), as an insecticide in agriculture and forestry still leads to environmental problems in many countries [8,18,21]. Moreover, during the manufacturing process of γ-HCH by chlorination of benzene under UV light, a mixture of HCH isomers is produced of which the γ-isomer (usually only 10-15% of total HCH) is the only effective insecticide component. Initially, the technical mixture as a whole was applied. Currently however, in most countries only the use of γ–HCH is permitted. The ineffective α-, β-, δ-, and ε-isomer (55-70%, 5-14%, 2-10%, and 1-4% of the technical mixture, respectively [40]) are separated from γ-HCH and dumped at waste sites resulting in polluted soils and groundwaters.

Since the early 1970s, the microbiological transformation of HCH has been extensively studied both under aerobic (e.g., [2,3,10,35]) and anaerobic conditions (e.g., [15,20,26,31]). In most cases the β–isomer of HCH was found to be least susceptible to microbial dechlorination. This is probably due to the spatial (all equatorial) arrangement of the chlorine atoms as was postulated by Beurskens *et al.* [5]. In soils contaminated with HCH, rapid degradation of the α- and γ-isomer of HCH was found under aerobic conditions by the microbial population naturally present [3,10,13,32,35,42]. α-HCH in contaminated soil was found to be partially (20-40%) mineralized to CO_2 [3]. A *Pseudomonas* sp. that can use α-, γ- and δ-HCH as the sole carbon and energy source was isolated from a contaminated soil [34,38]. Limited aerobic conversion (5% mineralization) of β-HCH was observed, but only in the presence of acetate as a primary substrate [35].

In anaerobic (flooded) soils, α– and γ–HCH are almost always transformed [3,8,28], whereas the β-isomer is mostly found to be persistent under methanogenic conditions. In studies with both pure cultures (*Clostridium* spp., *Citrobacter freundii*, Bacillaceae, and Enterobacteriaceae) [16,17,20] and mixed cultures [6,29], γ-HCH was also found to be converted. It was postulated that only those bacteria capable of generating H_2 (from substrates like glucose and pyruvate) during fermentation, are able to transform γ-HCH anaerobically [20]. Maximum dechlorination rates, which were observed during the stationary growth phase, decreased in the following order $\gamma>\alpha>\beta\geq\delta$ [20]. Anaerobic mixed cultures like sewage sludge also dechlorinated γ-HCH and α-HCH, but the δ-isomer, and especially the β-isomer were converted very slowly. In this case, limited dechlorination (5-20%) was also observed with sterilized sludge, and this process was significantly faster than hydrolysis in water. The enhanced chemical transformation was attributed to unknown compounds in the sludge and was postulated to involve surface-catalyzed reactions [6]. Recently, an enrichment culture was

obtained from a mixture of polluted sediments, soils, sludges, and granular sludge from an upflow anaerobic sludge blanket (UASB) reactor. This culture is able to dechlorinate β-HCH anaerobically via δ-tetrachlorocyclohexene (δ-TeCCH) to chlorobenzene (CB) (67%) and benzene (19%). The enrichment culture transforms α-, γ-, and δ-HCH as well [31]. TeCCH isomers are often observed as the primary intermediates after an initial dichloroelimination reaction during anaerobic biotransformation [16,17,20].

In this study, we describe the anaerobic dechlorination of β-HCH by granular methanogenic sludge from UASB reactors fed with different primary substrates. The granular sludge from UASB reactors has a high biomass content [25] and consists mainly of acetogenic and methanogenic bacteria. These anaerobic bacteria contain large amounts of corrinoids and other cofactors like F_{430} (e.g., [7,23,30]). These factors are part of enzymes, which catalyze important pathways in anaerobic bacteria, like the acetyl-CoA pathway and the last step in methane formation. Corrinoids and other transition metal-containing porphyrins *in vitro* have been found to transform lindane to TeCCH, chlorobenzene and benzene [4,5,24,27]. Additionally, we have investigated the effect of the addition of granular methanogenic sludge on the conversion of α– and β-HCH in contaminated soil.

Materials and methods

Chemicals. α– and β–hexachlorocyclohexane (HCH, 97-99.6% purity, obtained from Promochem, Wesel, Germany), benzene (extra pure 99.5%), chlorobenzene (purity 99%), and chloroform (p.a. quality, all obtained from E. Merck, Amsterdam, The Netherlands) were used as received without further purification.

Granular sludge. The granular sludge was grown in three UASB reactors with methanol, a mixture of volatile fatty acids (VFA) (acetate, propionate, and butyrate), or sucrose as the main carbon source. Originally, they had been inoculated with granular sludge from a full-scale UASB reactor treating sugar beet refinery wastewater (CSM, Breda, The Netherlands) and were operated as described previously [11]. COD removal efficiencies were higher than 85%. No VFA were present in the effluent of the three reactors. The reactors had been operated for at least 2 years prior to sludge sampling. The sludges were washed two times with demineralized water and one time with basal medium to remove residual soluble substrate before use in the batch experiments. When appropriate, the sludge granules were crushed prior to the experiment by pressing the sludge suspension through sterile needles with decreasing diameter (smallest needle: Microlance 3, 25G5/8, 0.5x16 mm). It was confirmed that the methanogenic activity of the sludge was not affected by this treatment.

Transformation of β–HCH by granular sludge under methanogenic conditions in batch experiments. Approximately 2 grams of the granules or approximately 2 ml of the crushed sludge was transferred to 120-ml serum flasks containing 43 ml of basal medium, as described earlier by Holliger *et al.*, but without the addition of fermented yeast extract [19]. When chlorine balances had to be determined, the medium was modified by replacing the chloride salts of calcium and magnesium with $Ca(OH)_2$ and $MgHPO_4 \cdot 2H_2O$. The pH in the batches remained 7.2-7.3. The gas phase consisted of N_2 (80%) and CO_2 (20%). The batches were sealed with Viton stoppers (Maag Technic AG, Dübendorf, Switzerland). β–HCH was added dissolved in acetone to a final concentration of 20 μM in the liquid phase. The appropriate primary substrate (0.4-0.5 g COD l^{-1}) was added weekly by adding 0.2 ml of a stock solution (2 M methanol or 0.31 M sodium acetate·$2H_2O$, 0.23 M sodium propionate, and 0.21 M sodium butyrate, or 0.42 M sucrose buffered with 0.63 M $NaHCO_3$). The batches were incubated statically at 30°C in the dark unless otherwise stated. The possible loss of the chlorinated compounds due to leaking through the stoppers was checked in separate batches with medium (no sludge added).

Experiments with soil contaminated with α– and β–HCH under methanogenic conditions. The sandy soil used in the experiment originated from a HCH-contaminated site in Hengelo, Overijssel (The Netherlands), where it was stored in open air covered with foil. The soil was contaminated with (concentrations in mg/kg dry soil): α–HCH (37), β–HCH (35), γ-HCH (5), δ-HCH (2), and ε-HCH (2). Cu (20), Zn (50), Pb (4.7), and Hg (50) were also present as co-contaminants. The soil was sieved, and the fraction with a particle size below 2 mm was used. The experiments were carried out with 65 g of sieved soil transferred to 500-ml serum flasks containing 65 ml of basal medium, as described earlier by Holliger *et al.*, but without the addition of fermented yeast extract [19]. The pH in the batches was 7.5-7.8. The gas phase consisted of N_2 (80%) and CO_2 (20%). The batches were sealed with Viton stoppers (Maag Technic AG, Dübendorf, Switzerland). Lactate (0.5 g COD l^{-1} = 5 mM) was added to the batches weekly. The batches were incubated on a rotary shaker (130 rpm) at 30°C in the dark. The effect of the addition of sludge to the soil on the removal of the HCH isomers was tested with methanol (2.6 g dry weight/kg of soil) or VFA (5.4 g dry weight/kg soil) grown sludge from UASB reactors, as described previously [11]. In the latter experiments, 0.5 g COD l^{-1} methanol (equal to 10 mM) or VFA (a mixture of 1.9 mM acetate, 1.4 mM propionate, and 1.3 mM butyrate) was added weekly to the methanol or VFA sludge-amended batches, respectively.

Abiotic transformation of HCH by methanogenic sludge and in contaminated soil. Abiotic transformation was tested by autoclaving the granular sludge or soil, thus inactivating all microbial activity. The granular sludge or soil was autoclaved in basal medium for 1 hour at 120°C three times, namely, 4 and 2 days before the start of the experiment and again on the day the experiment was started.

Analytical methods. The HCH and TeCCH concentration in the batches with methanogenic sludge were determined in the liquid phase after extraction of 1 ml of sample with 1 ml of chloroform. For the

analysis of the HCH and TeCCH concentration in batches with soil, 4 ml of soil suspension was extracted with 4 ml of chloroform. The extraction mixtures were sonified for 15 minutes followed by a 24-h extraction in an end-over-end-shaker. The HCH and TeCCH concentrations were determined in the chloroform extracts with a GC/MS method previously described using external HCH standards [31]. Before analysis, the chloroform extract of the soil extraction-mixtures was membrane filtered (0.22 μm, Millex-FG13, Millipore, The Netherlands) to remove residual soil particles.

Total masses of benzene, chlorobenzene, H_2, and methane were determined via headspace analysis. Benzene and chlorobenzene were analyzed by injecting 0.2 ml of headspace gas into a 436 Chrompack gas chromatograph (GC) equipped with a flame ionization detector (FID) connected to a Sil 5CB column (25 m x 0.32 mm x 1.2 μm) and with split-injection (ratio 1:50). Operating temperatures of the injector and detector were 250 and 300 °C, respectively. The oven temperature conditions were 40°C (6 minutes), followed by a temperature increase of 5°C $minute^{-1}$ to 100°C. Carrier gas was N_2 with an inlet pressure of 50 kPa. The retention times were 6.2 and 14 minutes for benzene and chlorobenzene, respectively. The retention times and peak areas were determined with a Shimadzu C-3A integrator. Hydrogen and methane were analyzed by injecting 0.4 ml of gas from the headspace in a 417 Packard GC equipped with a thermal conductivity detector (TCD) (100 mA) connected to a molecular sieve column (13X, 180 x ¼inch, 60-80 mesh). The temperature of the column and detector were 100°C. Calibration curves were made by adding the required amount of the (chloro-) benzene, H_2, or methane to a 120-ml serum bottle with 43 ml of basal medium containing 2 grams of granular or crushed sludge. The bottles were allowed to equilibrate overnight at 30°C. The calibration curves for the batches containing soil were made accordingly in 500-ml bottles.

Chloride concentrations were determined using HPLC as described previously [37]. The detection limit was 10 μM. Bromide was used as internal standard. VFA and methanol concentrations were determined by GC as described earlier [14]. The COD for sucrose, methanol, and VFA solutions was determined according to standard methods [1]. COD conversion factors ($g\ g^{-1}$) utilized were 1.07 for sucrose, 1.50 for methanol, and 1.07, 1.52, 1.82, and 1.07 for acetic, propionic, butyric, and lactic acids, respectively. The volatile suspended solids (VSS) content of the sludge was determined by subtracting the ash content from the dry weight after incubating the sludge overnight at 105°C. The ash content was determined after heating the dry sludge at 600°C for 90 min.

Results and discussion

Transformation of β–HCH by unadapted granular sludge. Three methanogenic consortia grown on methanol, VFA, or sucrose as the primary substrate were able to transform β-HCH at a concentration of 20 μM without prior adaptation (Fig. 5.1). The initial β-HCH removal rates were very similar for the three sludges and ranged from 0.37 to 0.46 μmol β-HCH g^{-1} VSS day^{-1} (Table 5.1). Chlorobenzene and benzene were identified as the end products.

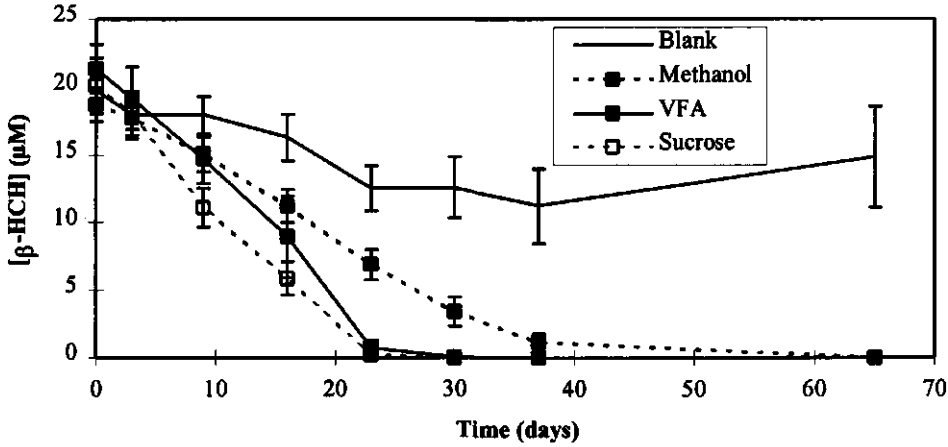


Figure 5.1 Biotransformation of β-HCH by unadapted methanogenic granular sludge grown on methanol, VFA, or sucrose. VSS contents of the incubations (mg VSS/batch) are 58.1, 107, and 86.0 for methanol, VFA and sucrose fed sludge, respectively. Data are mean values of triplicate incubations.

After 86 days, approximately 60-73% of the β-HCH removed was recovered as chlorobenzene and benzene. Furthermore, 68-115% of the β-HCH chlorine removed was recovered as chloride after incubation with the methanogenic sludges. Apparently, although 30-40% of the β-HCH initially present could not be accounted for as chlorobenzene and benzene, adsorption of β–HCH to the sludge was not an important mechanism by which β-HCH was removed, since a major part of the HCH chlorine was recovered as chloride. With the three sludges tested, more benzene than chlorobenzene was formed as is shown for methanol-grown sludge (Fig. 5.2). TeCCH was never observed as a transient intermediate. The methanogenic activity of the sludges was not inhibited by the presence of β-HCH (data not shown). Crushing the sludge did not affect methanogenic activity or β-HCH removal. β-HCH was not transformed by autoclaved sludge or in blanks without sludge. In the controls with autoclaved sludge or medium without sludge, formation of chlorobenzene or benzene and an increase of the chloride concentration were not observed.

Our previous research has shown that methanogenic sludges were also able to degrade chlorinated methanes and chloroethanes without having been priorily adapted to these compounds [11,12]. The sludges, which have corrinoid contents between 0.5 and 1.0 mg g^{-1} VSS [11], dechlorinated carbon tetrachloride mainly to CO_2, while chloroethanes were degraded to lower chlorinated ethanes and ethenes via dichloroelimination and reductive

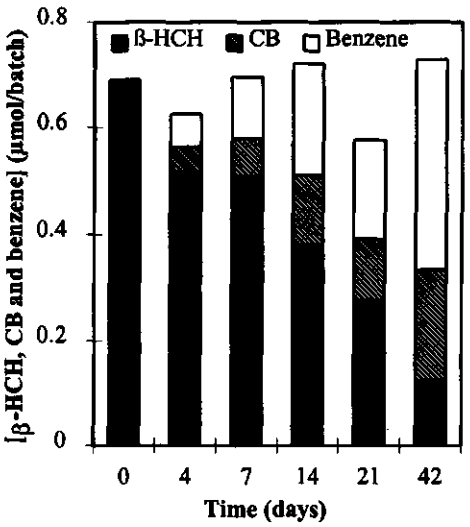


Figure 5.2 Biotransformation of β-HCH by crushed methanol-degrading methanogenic sludge (39.2 mg VSS/batch) and subsequent formation of chlorobenzene (CB) and benzene (primary substrate 100 mM methanol). Data are mean values of triplicate incubations (standard deviation was usually within 10% of mean value).

hydrogenolysis reactions. Higher chlorinated methanes and ethanes were also partially degraded at lower rates in the presence of autoclaved (sterile) sludge. These reactions were partly accounted for by the ability of heat-stable transition metal-containing cofactors to transform chlorinated methanes and ethanes [22,36]. Lindane (γ-HCH) has also been found to be dechlorinated *in vitro* via TeCCH to chlorobenzene by a variety of metal-containing porphyrins, including cobalt containing corrins, like vitamin B_{12} [27]. In contrast to these results, β-HCH was not transformed by our autoclaved sludge. Apparently, the cofactors present are not able to mediate the abiotic transformation of β-HCH. The relative inertness of β-HCH is considered to be due to the equatorial arrangement of the chlorine atoms in the molecule [5]. Possibly, stronger electron donors like titanium(III)citrate or dithiotreitol are required for β-HCH transformation by autoclaved sludge. We observed an enhancement of the transformation of carbon tetrachloride in the presence of autoclaved sludge after the addition of titanium(III)citrate [11].

Although other studies have reported dechlorination and transformation of β-HCH to occur under anaerobic conditions, this research shows the nearly complete transformation of β-HCH to a mixture of benzene and chlorobenzene by an unadapted methanogenic

Table 5.1. Biotransformation of β-HCH by methanogenic sludge grown on different primary substrates[a].

		Methanol	VFA	Sucrose
β–HCH removal rate	μmol β–HCH g^{-1} VSS day^{-1}	0.41	0.37	0.46
$(CB+Benzene)_{recovered}$	% of $(\beta\text{-HCH})_{removed}$	63.5 ± 7.0	72.9 ± 11.1	60.3 ± 5.2
CB:Benzene		1:1.41 ± 0.05	1:1.07 ± 0.03	1:1.39 ± 0.15
$(Cl^-)_{recovered}$	% of $(\beta\text{-HCH-chlorine})_{removed}$	88.2 ± 5.9	68.4 ± 8.2	114.6 ± 6.6

[a] Removal rates were determined over the first 23 (VFA and sucrose fed sludge) or 30 (methanol fed sludge) days and products were analyzed after 86 days.

consortium without the necessity of enriching for the β-HCH dechlorinating bacteria. Middeldorp *et al.* [31] were the first to show extensive biotransformation of β-HCH, and they postulated the following pathway. The transformation of β-HCH would involve two dichloroelimination reactions via δ-TeCCH to dichlorocyclohexadiene (DCCH), followed by either another dichloroelimination to benzene or a dehydrochlorination reaction to chlorobenzene. We did not observe the formation of TeCCH, but it is likely that the methanogenic sludges degrade β-HCH via the same pathway. The fact that TeCCH was not observed as an intermediate is consistent with observations made by Heritage *et al.* [16], who found degradation of δ–TeCCH while β-HCH was not degraded by washed cell suspensions of *Clostridium sphenoides*. Buser *et al.* [6] also did not observe the formation of intermediates with sewage sludge. Apparently, the first step in the transformation of HCH is the rate limiting step.

The ratio of chlorobenzene and benzene production (CB:B ratio) in the β-HCH transformation by the methanogenic sludge. The amount of benzene was 2.6-2.9 and 1.9-2.2 times higher than the amount of chlorobenzene formed for granular sludge and crushed sludge, respectively. The reason for the higher amount of benzene formed by granular sludge as compared to crushed sludge is not yet clear. We believe that benzene is formed microbiologically and that the chlorobenzene formation is a spontaneously occurring abiotic non-redox reaction [4]. We speculate that the amount of benzene formed is influenced by the syntrophic bacterial relationships inside the granules. Crushing of the sludge will result in disturbances of the interactions between the bacteria responsible for the benzene formation, and will lead to a larger formation of chlorobenzene.

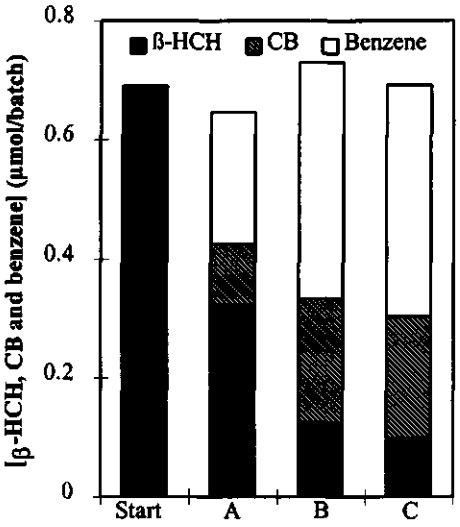


Figure 5.3 Biotransformation of β-HCH and subsequent formation of chlorobenzene (CB) and benzene by crushed methanogenic sludge (39.2 mg VSS/batch) under different primary substrate (methanol) addition regimes. Given are the amounts of β-HCH at the start of the experiment and the β-HCH, CB, and benzene concentrations after 42 days without primary substrate added (A), with 100 mM methanol added at once at the beginning of the experiment (B), and with a primary substrate addition of 10 mM methanol week^{-1} (C). Data are mean values of triplicate incubations (standard deviation was usually within 10% of mean value).

The effect of the limitation of available electrons on the β–HCH dechlorination and on the CB:B ratio was investigated with methanol-grown sludge. A regular (weekly) addition of primary substrate was expected to increase the relative amount of benzene formed. However, the addition of the primary substrate in small portions (10 mM methanol week^{-1}) did not result in a larger amount of benzene formed as compared to the benzene formed after the single addition of 100 mM methanol at the beginning of the experiment (Fig. 5.3). Although β-HCH was dechlorinated less extensively in the absence of primary substrate, the ratio of chlorobenzene and benzene formed did not change significantly. The limited dechlorination that did occur in the absence of exogenous primary substrate was probably supported by substrates indigenously present in the sludge.

An explanation for the difference in the chlorobenzene and benzene production ratio in the enrichment culture [31] and in our methanogenic sludge may be that different microorganisms are present. A further characterization of the bacteria responsible for the dechlorination of β-HCH is currently under investigation in our laboratory.

The effect of the redox condition on the transformation of β-HCH. For a long time, β-HCH has been reported to be persistent under anaerobic conditions [16,33] or only partially or slowly converted [6,20]. The study by Middeldorp *et al.* [31] and results presented here demonstrate that, given the proper conditions, biotransformation is possible. The complete biotransformation of β-HCH to benzene and chlorobenzene in our study may be caused by the

relatively high biomass concentration, the low redox potential due to methanogenic conditions, and/or the presence of sulfide in the incubations. The incubations with sludge were completely methanogenic, indicating that the redox potential should be below -250 mV [41]. In a study by Buser *et al.* [6], the use of a relatively large amount of biomass (250 grams of anaerobic sewage sludge per 300-ml bottles) only led to a very slow conversion of β-HCH (approx. 3 ng day^{-1}). The gas production by this mixed culture was low, which suggests that the redox conditions were not methanogenic. Siddaramappa *et al.* found that the transformation of β-HCH in soil required a redox potential lower than -40 mV [39]. Pure cultures of fermenting bacteria, like *Clostridium* and *Citrobacter* sp., degraded β-HCH at least partially. Within 6 days, 20-30% of the β-HCH initially present (around 35 μM) was dechlorinated, and 5-25% of the HCH chlorine was recovered as chloride. Further conversion of the compounds was not measured so it remains unclear whether the β-HCH ultimately would have been completely transformed by the pure cultures [20].

The presence of sulfide may also be important for a rapid dechlorination of β-HCH, because the redox potential in the incubations will be lowered by the addition of sulfide to the medium. The media used in most studies do not contain sulfide as a reductant. Middeldorp *et al.* [31] did use sulfide in their experiments with the enrichment culture, and they obtained high dechlorination rates (over 3 μM day^{-1}) and even when methanogenesis was completely inhibited, dechlorination of β-HCH continued. Nevertheless, there was always a lag-phase before dechlorination of β-HCH occurred, indicating that the initial environmental conditions in the incubations were not suitable to support immediate dechlorination. On the other hand, it is also possible that the initial number of dechlorinating microorganisms was low. In our case, the presence of a large amount of methanogenic biomass, which was able to lower the redox potential, may have been the reason that a lag phase was absent. The importance of methanogenesis is also supported by the fact that in the presence of 62% (v/v) oxygen in the headspace, which is sufficient to inhibit methanogenesis completely, β-HCH was not degraded (data not shown). Altogether, the presence of sulfide (leading to reduced conditions) may be essential for a rapid dechlorination of β-HCH, but the ability of a large amount of biomass to induce methanogenic conditions also seems to be important to ensure the instantaneous dechlorination of β-HCH. The influence of the presence of sulfide on β-HCH transformation requires further investigation.

Transformation of α- and β-HCH by endogenous microorganisms in contaminated soil. α- and β-HCH in contaminated soil were readily transformed when the soil was incubated under anaerobic conditions with sulfide and lactate containing medium (Fig. 5.4). The concentrations of γ-, δ- and ε-HCH were too low to measure. Chlorobenzene and benzene were formed in a ratio of 1:1. TeCCH was not detected as a transient intermediate. Lactate, added as electron donor, was converted to acetate and propionate; methane was not formed. Benzene and chlorobenzene were not found when lactate was excluded from the medium, underlining the importance of primary substrate addition for the conversion of HCH in soil. The HCH isomers were also not dechlorinated in autoclaved (sterile) soil. The addition of small amounts of granular sludge (2.5-5.5 g dry weight/kg of soil) from the UASB reactor fed with methanol or VFA did not enhance the α- and β–HCH removal rate.

The behavior of β–HCH in soils incubated under anaerobic conditions was previously reported to vary from persistence [3,9] to disappearance [26,39]. Our study confirms that transformation is feasible and identifies the products that are formed. The soil that we used in our experiments had been stored in an open air dump site, and therefore it seems unlikely that the redox conditions were methanogenic during storage. However, the presence of low concentrations of chlorobenzene and benzene in the soil indicates that perhaps some anaerobic HCH-dechlorinating bacteria were already present (in the soil). Upon anaerobic incubation of the soil in medium containing sulfide and lactate, transformation of α- and β-HCH started immediately. In contrast to these results, β-HCH in contaminated soil was not dechlorinated in anaerobic medium without sulfide present (data not shown). Small additions of methanogenic sludge (2.5-5.5 g dry weight/kg of soil), did not increase the initial β-HCH transformation rate in sulfide containing medium. However, the presence of large amounts of methanogenic sludge (approximately 100 g dry weight/kg of soil) in anaerobic medium without sulfide present did enhance the removal of HCH in contaminated soil (data not shown). It is possible that the large amount of added sludge biomass reduced the redox potential sufficiently to make this β-HCH conversion possible. These results show that β-HCH dechlorinating bacteria also can be found in soil that is polluted with HCH isomers. In addition to characterization studies of the responsible bacteria, also the optimal conditions needed for *in situ* HCH dechlorination and subsequent mineralization of the benzene and chlorobenzene formed in polluted soils are under investigation. The effect of the redox condition in the soil, the need for the addition of extra electron donor, and the effect of a limited availability of β-HCH are also topics of current research.

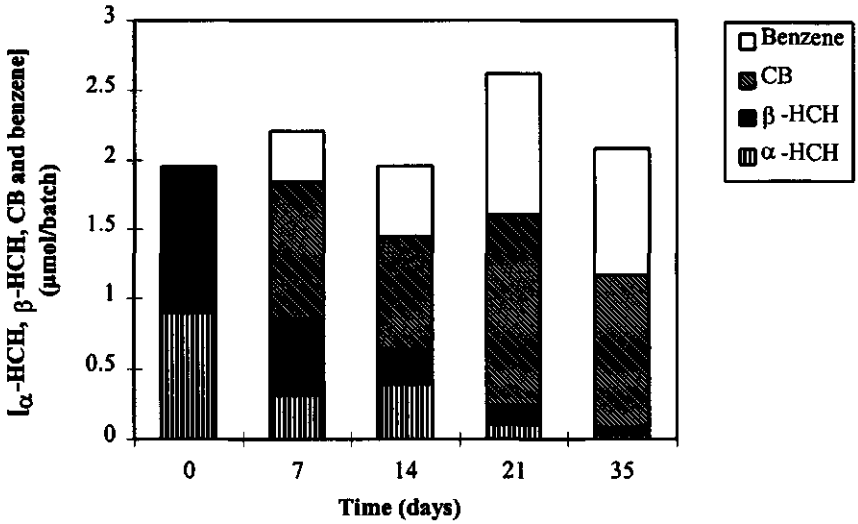


Figure 5.4 Biotransformation of α– and β-HCH in polluted soil under methanogenic conditions by the endogenous microbial population. Data are mean values of triplicate incubations (standard deviation was usually within 20% of mean value).

Acknowledgments

We thank Peter Middeldorp for valuable discussions. This research was supported by a grant (project 92014) from the Innovative Research Program on Environmental Biotechnology (IOP-Milieubiotechnologie) of the Ministries of Economic Affairs and Housing, Physical Planning, and Environment and by a grant from the Dutch Research Program for Biotechnological In-Situ Sanitation (CUR/NOBIS project 97-1-08).

References

1. **American Public Health Association.** 1985. Standard methods for examination of water and wastewater, vol. 16th ed. APHA, Washington DC.
2. **Bachmann, A., W. de Bruin, J. C. Jumelet, H. H. M. Rijnaarts, and A. J. B. Zehnder.** 1988. Aerobic biomineralization of alpha-hexachlorocyclohexane. Appl. Environ. Microbiol. **54**:548-554.
3. **Bachmann, A., P. Walet, P. Wijnen, W. de Bruin, J. L. M. Huntjens, W. Roelofsen, and A. J. B. Zehnder.** 1988. Biodegradation of alpha- and beta-hexachlorocyclohexane in a soil slurry under different redox conditions. Appl. Environ. Microbiol. **54**:143-149.
4. **Baker, M. T., R. M. Nelson, and R. A. van Dyke.** 1985. The formation of chlorobenzene and benzene by the reductive metabolism of lindane in rat liver microsomes. Arch. Biochem. Biophys. **236**:506-514.
5. **Beurskens, J. E. M., A. J. M. Stams, A. J. B. Zehnder, and A. Bachmann.** 1991. Relative biochemical reactivity of three hexachlorocyclohexane isomers. Ecotoxicol. Environ. Safety. **21**:128-136.

6. **Buser, H., and M. D. Muller.** 1995. Isomer and enantioselective degradation of hexechlorocyclohexane isomers in sewage sludge under anaerobic conditions. Environ. Sci. Technol. **29**:664-672.
7. **Diekert, G., U. Konheiser, K. Pichulla, and R. K. Thauer.** 1981. Nickel requirement and factor F_{430} content of methanogenic bacteria. J. Bacteriol. **148**:459-464.
8. **Doelman, P., L. Haanstra, E. de Ruiter, and J. Slange.** 1985. Rate of microbial degradation of high concentrations of α-hexachlorocyclohexane in soil under aerobic and anaerobic conditions. Chemosphere. **14**:565-570.
9. **Doelman, P., L. Haanstra, and A. Vos.** 1988. Microbial degradation by the autochthonous soil population of alpha and beta HCH under anaerobic field conditions in temperate regions. Chemosphere. **17**:481-487.
10. **Doelman, P., L. Haanstra, and A. Vos.** 1988. Microbial sanitation of soil alpha and beta HCH under glasshouse conditions. Chemosphere. **17**:489-492.
11. **Eekert, M. H. A. van, T. J. Schröder, A. J. M. Stams, G. Schraa, and J. A. Field.** 1998. Degradation and fate of carbon tetrachloride in unadapted methanogenic granular sludge. Appl. Environ. Microbiol. **64**:2350-2356.
12. **Eekert, M. H. A. van, A. J. M. Stams, J. A. Field, and G. Schraa.** 1999. Gratuitous dechlorination of chloroethanes by methanogenic granular sludge. Appl. Microbiol. Biotechnol. **51**:46-52.
13. **Engst, R., W. Fritsche, R. Knoll, M. Kujawa, R. M. Macholz, and G. Straube.** 1979. Interim results of studies of microbial isomerization of gamma-hexachlorocyclohexane. Bull. Environ. Contam. Toxicol. **22**:699-707.
14. **Florencio, L., A. Nozhevnikova, A. van Langerak, A. J. M. Stams, J. A. Field, and G. Lettinga.** 1993. Acidophilic degradation of methanol by a methanogenic enrichment culture. FEMS Microbiol. Lett. **109**:1-6.
15. **Haider, K., and G. Jagnow.** 1975. Abbau von ^{14}C-, ^{3}H- und ^{36}Cl-markierten γ-hexachlor-cyclohexan durch anaeroben bodenmikroorganismen. Arch. Microbiol. **104**:113-121.
16. **Heritage, A. D., and I. C. MacRae.** 1979. Degradation of hexachlorocyclohexanes and structurally related substances by *Clostridium sphenoides*. Austr. J. Biol. Sci. **32**:493-500.
17. **Heritage, A. D., and I. C. MacRae.** 1977. Identification of intermediates formed during the degradation of hexachlorocyclohexanes by *Clostridium sphenoides*. Appl. Environ. Microbiol. **33**:1295-1297.
18. **Hernandez, L. M., M. A. Fernandez, and M. J. Gonzalez.** 1991. Lindane pollution near an industrial source in northeast Spain. Bull. Environ. Contam. Toxicol. **46**:9-13.
19. **Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder.** 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. **59**:2991-2997.
20. **Jagnow, G., K. Haider, and P. C. Ellwardt.** 1977. Anaerobic dechlorination and degradation of hexachlorocyclohexane isomers by anaerobic and facultative anaerobic bacteria. Arch. Microbiol. **115**:285-292.
21. **Johri, A. K., M. Dua, D. Tuteja, R. Saxena, D. M. Saxena, and R. Lal.** 1996. Genetic manipulations of microorganisms for the degradation of hexachlorocyclohexane. FEMS Microbiol. Rev. **19**:69-84.
22. **Krone, U. E., R. K. Thauer, and H. P. C. Hogenkamp.** 1989. Reductive dehalogenation of chlorinated C1-hydrocarbons mediated by corrinoids. Biochemistry. **28**:4908-4914.
23. **Krzycki, J., and J. G. Zeikus.** 1980. Quantification of corrinoids in methanogenic bacteria. Curr. Microbiol. **3**:243-245.
24. **Kurihari, N., K. Tanaka, and M. Nakajima.** 1979. Anaerobic metabolism of lindane and related compounds by liver microsomes, p. 557-561. *In* H. Geussbuehler, G. T. Braks, and P. C. Kearny

(ed.), Advances in Pesticide Science, vol. Part III. Pergamon Press, Oxford, New York, Toronto, Sydney, Paris, Frankfurt.

25. **Lettinga, G., A. F. M. van Velsen, S. W. Hobma, W. de Zeeuw, and A. Klapwijk.** 1980. Use of upflow anaerobic (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioeng. **22:**677-734.
26. **MacRae, I. C., K. Ragu, and T. F. Castro.** 1967. Persistence and biodegradation of four common isomers of benzene hexachloride in submerged soils. J. Agric. Food Chem. **15:**911-914.
27. **Marks, T. S., J. D. Allpress, and A. Maule.** 1989. Dehalogenation of lindane by a variety of porphyrins and corrins. Appl. Environ. Microbiol. **55:**1258-1261.
28. **Mathur, S. P., and J. G. Saha.** 1975. Microbial degradation of lindane-C^{14} in a flooded sandy loam soil. Soil Science. **120:**301-307.
29. **Maule, A., S. Plyte, and A. V. Quirk.** 1987. Dehalogenation of organochlorine insecticides by mixed anaerobic microbial populations. Pest. Biochem. Physiol. **27:**229-236.
30. **Mazumder, T. K., N. Nishio, S. Fukuzaki, and S. Nagai.** 1987. Production of extracellular vitamin B-12 compounds from methanol by *Methanosarcina barkeri*. Appl. Microbiol. Biotechnol. **26:**511-516.
31. **Middeldorp, P. J. M., M. Jaspers, A. J. B. Zehnder, and G. Schraa.** 1996. Biotransformation of alpha-, beta-, gamma-, and delta-hexachlorocyclohexane under methanogenic conditions. Environ. Sci. Technol. **30:**2345-2349.
32. **Nagasawa, S., R. Kikuchi, Y. Nagata, M. Takagi, and M. Matsuo.** 1993. Aerobic mineralization of γ-HCH by Pseudomonas paucimobilis UT26. Chemosphere. **26:**1719-1728.
33. **Ohisa, N., and M. Yamaguchi.** 1979. *Clostridium* species and γ-BCH degradation in paddy soil. Soil Biol. Biochem. **11:**645-649.
34. **Sahu, S., K. K. Patnaik, and N. Sethunathan.** 1992. Dehydrochlorination of δ-isomer of hexachlorocyclohexane by a soil bacterium, *Pseudomonas* sp. Bull. Environ. Contam. Toxicol. **48:**265-268.
35. **Sahu, S. K., K. K. Patnaik, S. Bhuyan, B. Sreedharan, N. Kurihari, T. K. Adhya, and N. Sethunathan.** 1995. Mineralization of α-, γ-, and β-isomers of hexachlorocyclohexane by a soil bacterium under aerobic conditions. J. Agric. Food Chem. **43:**833-837.
36. **Schanke, C. A., and L. P. Wackett.** 1992. Environmental reductive elimination reactions of polychlorinated ethanes mimicked by transition-metal coenzymes. Environ. Sci. Technol. **26:**830-833.
37. **Scholten, J. C. M., and A. J. M. Stams.** 1995. The effect of sulfate and nitrate on methane formation in a freshwater sediment. Antonie van Leeuwenhoek. **68:**309-315.
38. **Senoo, K., and H. Wada.** 1990. γ-HCH-decomposing ability of several strains of *Pseudomonas paucimobilis*. Soil Sci. Plant Nutr. **36:**677-678.
39. **Siddaramappa, R., and N. Sethunathan.** 1975. Persistence of gamma-BHC and beta-BHC in indian rice soil under flooded conditions. Pestic. Sci. **6:**395-403.
40. **Slooff, W., and A. J. C. M. Matthijsen.** 1988. Integrated criteria document hexachlorocyclohexanes. National Institute of Public Health and Environmental Protection. Report nr.: 758473011
41. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41:**100-180.
42. **Tu, C. M.** 1976. Utilization and degradation of lindane by soil microorganisms. Arch. Microbiol. **108:**259-263.

6

Constitutive dechlorination of chlorinated ethenes by a methanol degrading methanogenic consortium

The ability of granular methanogenic sludge to dechlorinate chloroethenes was investigated with unadapted sludge from an upflow anaerobic sludge blanket (UASB) reactor fed with methanol. The sludge degraded chlorinated ethenes, but the degradation rates were low. The addition of primary substrate was necessary to sustain dechlorination. The dechlorinating activity seemed to be constitutively present in the anaerobic bacteria. Usually, one chlorine atom was removed via reductive hydrogenolysis. Only TCE was converted to substantial amounts of VC. 11DCE was observed to be an important intermediate in the dechlorination by unadapted granular sludge, although this compound was not commonly observed previously. Furthermore, the dechlorination of 11DCE was faster than the dechlorination of the other chloroethenes.

Introduction

Chlorinated ethenes are commonly used cleaning and degreasing agents in the metal and dry cleaning industry. They are often found as priority pollutants in ground and drinking water. It is believed that almost all of the tetra- and trichloroethene produced ultimately ends up in the environment [10].

Tetrachloroethene (TeCE) is only microbiologically degradable under anaerobic conditions; whereas, the other chlorinated ethenes are biodegradable under both anaerobic and aerobic conditions [1]. Reductive hydrogenolysis is the predominant pathway found under sulfidogenic and methanogenic conditions by mixed cultures [3,5,29,33,34,40]. Several pure cultures have been isolated that can use TeCE as the terminal electron acceptor and use the energy gained in the reductive dechlorination process for growth [23,31,35,36]. Pure cultures of acetogenic and methanogenic bacteria are able to dechlorinate chlorinated ethenes via cometabolic pathways (e.g., [12-14,38]).

Research concerning the transformation of chlorinated ethenes by granular sludge from upflow anaerobic sludge blanket (UASB) reactors is described in this chapter. Granular sludge has a high biomass content [28] which is highly enriched with acetogenic and methanogenic bacteria. These bacteria contain elevated amounts of cofactors like vitamin B_{12} and cofactor F_{430} [9,27,32], which are involved in the metabolic pathways commonly found in anaerobic bacteria. These cofactors are known to mediate the dechlorination of chlorinated ethenes *in vitro* [17,20,21]. TeCE is dechlorinated by adapted granular sludge from UASB reactors [5,7,22] with a variety of primary substrates in long term experiments. The main end products formed, described so far, were *cis*- and *trans*-1,2-dichloroethene. We evaluated the dechlorinating activity of unadapted granular sludge to degrade chloroethenes. By autoclaving the sludge we tried to differentiate between abiotic (chemical and aspecific (mediated by cofactors like vitamin B_{12})) dechlorination and dechlorination processes in the sludge catalyzed by specific enzyme systems.

Materials and methods

Chemicals. Tetrachloroethene (TeCE), trichloroethene (TCE), and 1,1-dichloroethene (11DCE) were obtained from E. Merck (Amsterdam, The Netherlands). *Cis*-1,2-dichloroethene (cDCE) was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands), and *trans*-1,2-dichloroethene (tDCE) from Janssen Chimica ('s Hertogenbosch, The Netherlands). Vinylchloride (VC), ethene, and ethane were obtained from Hoekloos, Schiedam, The Netherlands. All chemicals (pro analysis quality) were used as received without further purification.

Granular sludge. The granular sludge was cultivated in a UASB reactor with methanol as the main carbon source. Originally, the reactor had been inoculated with granular sludge from a full scale UASB-reactor treating sugar-beet refinery wastewater (CSM, Breda, The Netherlands) and were operated as described earlier [11]. The reactors had been operated for at least 1 year prior to sludge sampling. The sludges were washed two times with demineralized water and one time with basal medium [23] to remove residual soluble substrate before use in the batch experiments. The sludge granules were crushed prior to the experiment by pressing the sludge suspension through sterile needles with decreasing diameter (smallest needle: Microlance 3, 25G5/8, 0.5x16 mm). It was confirmed that the methanogenic activity of the sludge was not affected by this treatment.

Batch experiments. The transformation of chlorinated ethenes was tested with 2 grams (wet weight) of sludge (resulting in a VSS content of 1.3 g VSS l^{-1}) from the reactor fed with methanol in 120-ml batches containing 40-ml basal medium as described earlier by Holliger [23], but without the addition of the fermented yeast-extract. The pH in the batches was 7.2-7.3. Methanol was added weekly to a final concentration of 0.5 g COD l^{-1}. The chlorinated ethenes were added dissolved in 100 µl acetone or in the case of vinylchloride as a gas with a gas tight syringe (final concentration approximately 1500 nmol chlorinated ethene per batch). The added amount of acetone was not toxic for the methanogenic bacteria. The chlorinated ethenes did not inhibit the methane formation by living sludge at the concentration applied (1000 to 1500 nmol/batch). Approximately 70 to 75% of the methanol added was converted to methane in all cases (data not shown). In experiments where the effect of the nature and amount of electron donor added on the dechlorination of TeCE and TCE was studied (VSS content: 1.7 g VSS l^{-1}), special care was taken to prevent acetone from entering the system and the chlorinated compounds were added dissolved in water. The batches were incubated statically at 30°C in the dark unless stated otherwise. The possible loss of the chlorinated compound due to leaking through the stoppers or chemical reactions in the medium was checked in separate batches with sterile medium without any sludge addition. All experiments were carried out in triplicate unless stated otherwise.

Abiotic involvement of the cofactors. The abiotic involvement of cofactors was tested by autoclaving the granular sludge, thus inactivating all microbial activity. The granular sludge was autoclaved in basal medium for 90 minutes at 120°C three days prior to the start of the experiment and again for 30 minutes on the day the experiment was started.

Analytical methods. Total masses of chlorinated ethenes, hydrogen, and methane were determined via headspace analysis. TeCE, TCE and DCEs were analyzed by injecting 0.2 ml headspace into a 436 Chrompack gas chromatograph (GC) equipped with a flame ionization detector (FID) connected to a Sil 5CB column (25 m x 0.32 mm x 1.2 µm) with split injection (ratio 1:50). Operating temperatures of the injector, oven, and detector were 250, 60, and 300 °C, respectively. The carrier

gas was N_2 with an inlet pressure of 50 kPa. The retention times were 10.2, 5.0, 3.0, 2.6, and 2.3 minutes for TeCE, TCE, cDCE, tDCE, and 11DCE, respectively. Ethene, ethane, and VC were determined by injecting 0.2 ml headspace in a 438 A Chrompack GC equipped with a FID connected to a Poraplot Q column (25 m x 0.32 mm x 10 µm) and split injection (ratio 1:40). Operating temperatures of injector and detector were 225 and 250 °C, respectively. The oven was operated with the following temperature program: 50°C (2 minutes), and 15°C $minute^{-1}$ increase in temperature to 110°C. The retention time of ethene, ethane, and VC were 2.0, 2.4, and 8.0 minutes, respectively. The retention times and peak areas of all chlorinated ethenes were determined with a Shimadzu C-3A integrator. Calibration curves were made by adding the desired amount of chlorinated ethene to a serum bottle with 40 ml basal medium. The bottles were equilibrated overnight at 30°C. Hydrogen and methane were determined as described previously [11].

VFA and methanol concentrations were determined by GC as described earlier [16]. The COD for methanol, and VFA solutions was determined according to standard methods [2]. COD conversion factors ($g\ g^{-1}$) utilized were 1.50, 1.07, 1.52, and 1.82 for methanol, acetate, propionate, and butyrate, respectively. The VSS content of the sludge was determined by subtracting the ash-content from the dry weight after incubating the sludge overnight at 105°C. The ash-content was determined after heating the dry sludge at 600°C for 90 minutes.

Results

TeCE. TeCE was instantaneously dechlorinated by living sludge from the UASB reactor fed with methanol as the primary substrate (Fig 6.1). In the presence of autoclaved sludge TeCE was also removed from the system, but to a lesser extent. The TeCE concentration remained constant in time in the blanks without sludge. The formation of products was only observed in the presence of living sludge. Initially, only one chlorine atom was removed, and TCE was formed as the main product. However, as the incubation was continued, lower chlorinated ethenes (the DCE isomers, VC and ethene) started to accumulate, albeit in very small amounts (all below 30 nmol/batch). The total amount of chloroethenes formed (around 150 nmol/batch) could not completely balance the amount of TeCE removed (around 300 nmol/batch). Therefore, the remainder of the TeCE removed must have adsorbed to the sludge. This assumption is confirmed by the fact TeCE was also removed in the presence of autoclaved (dead) sludge (Fig. 6.1), but without the formation of lower chlorinated ethenes.

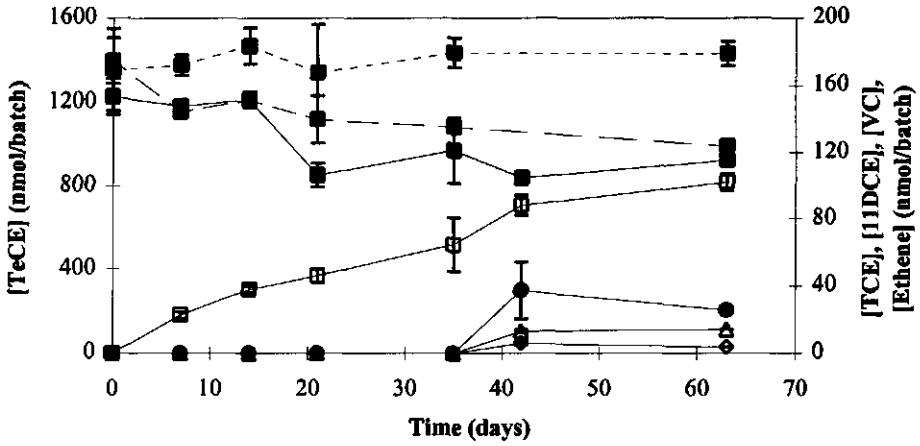


Figure 6.1 Dechlorination of TeCE by living (■, solid line) and autoclaved (■, — —) sludge and in blanks without sludge (■, - - -) (TCE: □, 11DCE: ◆, VC: ●, and ethene: ▲).

TCE. Living sludge dechlorinated TCE without prior acclimation, and substantial amounts of VC were formed (Fig 6.2). Minor amounts of DCE isomers were detected. cDCE and tDCE (not shown in Fig. 6.2) were formed in the last days of incubation (concentrations below 20 nmol/batch). The ethene formation started after day 35 and the concentration remained also below 20 nmol/batch. Not all of the TCE removed from the system could be accounted for as lower chlorinated ethenes. Therefore, a part of the TCE removed may have adsorbed to the sludge. This is confirmed by the fact that TCE was also, but to a lesser extent, removed in the presence of autoclaved sludge and in medium without sludge. In this case however, products could not be detected.

DCE and VC. 11DCE was the only DCE isomer which was converted to substantial amounts of VC (Fig. 6.3). The 11DCE removed could be completely accounted for by VC production (around 450 nmol/batch in 63 days). A small amount of ethene (10 to 20 nmol/batch) was formed and ethane was not detected (data not shown). 11DCE was not removed in the presence of autoclaved sludge or in blanks without sludge.

cDCE and tDCE were also transformed by living sludge but at a much lower rate (data not shown). The VC production after 63 days was around 60 nmol/batch for the cDCE and tDCE incubations, and ethene was formed in minor amounts only (10 to 20 nmol/batch). The formation of ethane was not observed. cDCE and tDCE were not

removed in the presence of autoclaved sludge or in blanks without sludge. VC was dechlorinated by living sludge to minor amounts of ethene (around 40 nmol/batch after 49 days; data not shown). VC was not removed in incubations with autoclaved sludge or in blanks without sludge.

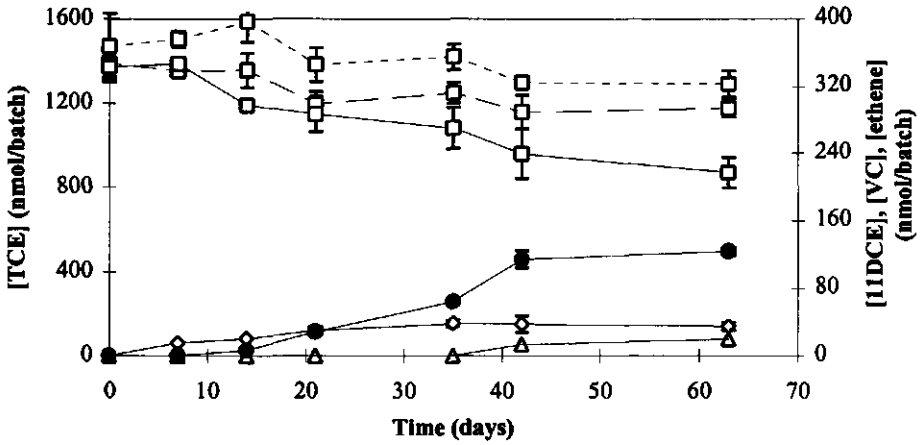


Figure 6.2 Dechlorination of TCE by living (□, solid line) and autoclaved (□, — —) sludge and in blanks without sludge (□, - - -) (11DCE: ◆, VC: ●, and ethene: ▲).

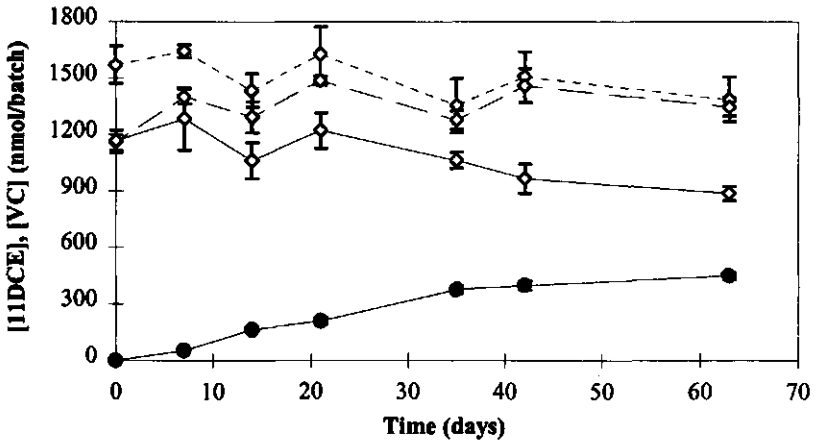


Figure 6.3 Dechlorination of 11DCE by living (◆, solid line) and autoclaved (◆, — —) sludge and in blanks without sludge (◆, - - -) (VC: ●).

Dechlorination rates.

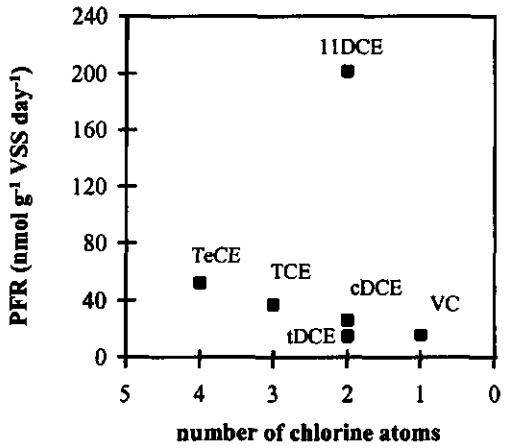


Figure 6.4 The product formation rate (PFR) (calculated in nmol g^{-1} VSS day^{-1}) with living sludge as a function of the number of chlorine atoms in the molecule.

The initial product formation rates (PFR) (for the first 14 to 21 days) for the incubation of TeCE and lower chlorinated ethenes in the presence of living sludge were calculated. The PFR increased with the amount of chlorine atoms in the molecule (Fig. 6.4). 11DCE is an exception; it was dechlorinated approximately 10 times faster than the other DCE isomers. The dechlorination was best described by first order transformation kinetics. The rate constants obtained were normalized for the biomass concentration. These calculated rate order constants were 0.048, 0.058, 0.017, 0.023, 0.129, and 0.013 day^{-1} g^{-1} VSS for TeCE, TCE, cDCE, tDCE, 11DCE, and VC respectively.

Primary substrate addition. To determine whether the primary substrate dosage regime had any influence on the dechlorination of chloroethenes, the living sludge was incubated with TCE under different regimes (Fig. 6.5). With living sludge, increasing the frequency of primary substrate dosage did enhance the removal of the chlorinated ethenes (comparison of slow and fast in Figure 6.5), but the VSS content of the batches receiving a daily dosage of primary substrate was higher than in the incubations receiving weekly dosages (1.3 and 1.7 g^{-1} VSS, respectively). Apparently, the transformation of the chlorinated ethenes is not limited by the amount of electron donor available. However, the supply of very low dosages of primary substrate (Fig 6.5, low) resulted in less lower chlorinated ethenes after 7 days compared to the fast addition of electron donor, indicating that addition of primary substrate is essential for dechlorination. When H_2 was supplied the dechlorination was not enhanced. This was not expected because hydrogen is expected to be the ultimate electron donor for reductive dechlorination. Mass transport limitation may have played a role, since the batches were incubated under static conditions. When methanol and hydrogen were supplied alternately, results were in between the results with

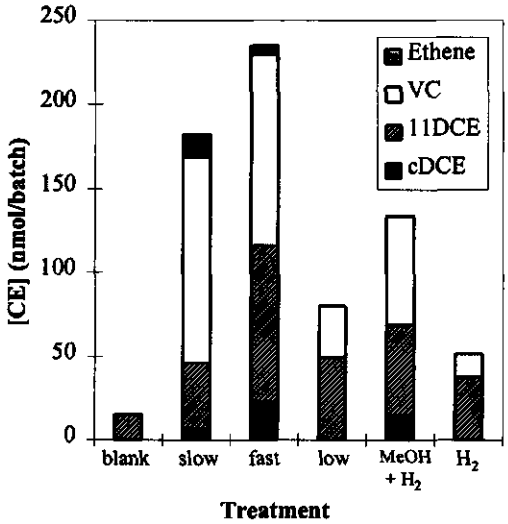


Figure 6.5 Influence of primary substrate addition on the dechlorination of TCE by sludge from the reactor fed with methanol. Concentrations of the lower chlorinated ethenes (CE) are represented after 7 weeks in medium without sludge added (blank), after 7 weeks of feeding 0.5 g COD l^{-1} $week^{-1}$ (slow) and after 7 days of feeding 0.5 g COD l^{-1} day^{-1} (fast) or 0.05 g COD l^{-1} day^{-1} (low), or 0.5 g COD l^{-1} day^{-1} MeOH and 10 ml H_2 alternately (MeOH + H_2), or 10 ml day^{-1} (0.2 g COD l^{-1}) H_2 (H_2).

only methanol or only hydrogen supplied. Surprisingly, in this experiment, small amounts of 11DCE were observed in blanks without sludge (Fig. 6.5, blank).

Discussion

This research shows that anaerobic granular methanogenic sludge is able to transform TeCE, TCE, DCE and to a lesser extent VC without having been priorily adapted to any of these compounds. The dechlorinating activity seems to be constitutively present in the sludge. The methanogenic consortium used in these experiments was able to remove at least one chlorine atom from the chlorinated substrates. The chlorinated ethenes were not dechlorinated in the presence of autoclaved sludge, indicating that the reactions were biologically catalyzed. Consequently abiotic dechlorination catalyzed by cofactors like vitamin B_{12} and cofactor F_{430} is very limited.

The results found in our research suggest that the methanogenic sludge from the UASB reactors dechlorinates TeCE via an unusual pathway. Apparently, TeCE is

transformed via 11DCE, instead of cDCE. cDCE and tDCE were degraded very slowly by the granular sludge used in our experiments and 11DCE is the only intermediate that could be transformed to relatively high amounts of VC. Otherwise, we would have observed the accumulation of cDCE and/or tDCE. cDCE is an intermediate or endproduct, that is most commonly found in dechlorination studies with TeCE [5,6,8,18,22,29,33]). Pure cultures of anaerobic bacteria which dechlorinate with specific enzyme systems usually dechlorinate TeCE to cDCE [23,26,31,35]. Only *Dehalococcoides ethenogenes* strain 195 is known to form 11DCE as a transient intermediate [30]. In a few studies, 11DCE was detected as a minor end product [7,8,19,24,33,34]. Most researchers that found dechlorination products beyond DCE, like VC and ethene, assume that cDCE is an intermediate. Nevertheless the possibility of rapid 11DCE dechlorination can not be ruled out, because it is very rarely tested.

Several patterns for TeCE dechlorination are known for anaerobic granular sludge varying from the formation of cDCE [22] to the formation of mainly tDCE [7]. The formation of 11DCE as the main intermediate or end product in the dechlorination of TeCE by sludge from UASB reactors was to our knowledge never observed. A methanol enriched anaerobic sediment consortium was found to transform TeCE via 11DCE as an important intermediate [37]. However, in that study like in the experiments described here, there was never a complete recovery of chloroethene products and cDCE and tDCE could have been undetectable due to relatively high detection limits. Similar to the findings here, 11DCE dechlorination rates by the methanol enriched consortium were around 10 times higher than TeCE and TCE dechlorination rates. 11DCE was also rapidly degraded by both (adapted) anaerobic sludge and anaerobic microcosms [4,29].

The formation of 11DCE from TCE may have been caused by an abiotic reaction mediated by sulfide present in the medium. In sterile medium containing 0.6 mM Na_2S, Kästner [25] observed the formation of 11DCE from TeCE or TCE via abiotic (chemical) reactions at a rate of around 5 to 10 nmol day^{-1}. No other DCE isomers were formed abiotically [25]. The sulfide concentration in our medium is around 1 mM, which could explain the abiotic dechlorination of TCE to 11DCE and further biotic dechlorination to VC observed. The abiotic dechlorination rate observed by Kästner [25] correlates nicely with the VC production rate found for 11DCE dechlorination (10-15 nmol day^{-1}, Fig. 6.3) in our experiments. This would also explain the low amounts of 11DCE accumulating in the TCE dechlorination experiments. However, rapid formation of 11DCE was not observed in the TeCE incubations. The TCE concentration in the first few weeks of the

experiment may have been too low to detect the chemical formation of 11DCE and subsequent dechlorination to VC. Also, TeCE or TCE were not significantly degraded in medium or in the presence of autoclaved sludge, which should have been the case if the observed reaction would have been strictly chemical. Apparently, the presence of enzyme systems in the living sludge is essential for the chemical dechlorination.

The rates with which the chlorinated ethenes are transformed by the unadapted methanogenic consortia described here are much lower than those observed in other studies with pure cultures of dechlorinating bacteria (*Dehalobacter restrictus*, *Dehalospirillum multivorans*, Strain MS-1), that vary from 24 to 450 μmol mg^{-1} protein day^{-1} [23,35,36]. Pure cultures of cometabolically degrading methanogens and acetogens show somewhat lower conversion rates but still higher than the rates found in this study. *Methanosarcina* sp, *Methanosarcina mazei*, *Sporomusa ovata* (all grown on methanol), and *Acetobacterium woodii* (on fructose) dechlorinated TeCE at rates up to 235 nmol mg^{-1} protein day^{-1} [12-14,38]. The rates found in our study (10 to 200 nmol g^{-1} VSS day^{-1}) are also much lower than those found in (UASB) reactor studies which were as high as 300 μmol g^{-1} VSS day^{-1} [6-8,15,22]. The reason for the relatively low rate measured in our experiments is not quite clear. In the experiments described here, the rates were measured in batch experiments under static incubation conditions. Such a rate determination tends to give lower values than rate measurements conducted directly in reactors. The low rates could have been caused by a variety of reasons. Firstly, our sludge was not adapted to chlorinated ethenes in contrast with the sludges used in most of the reactor studies. These sludges were usually exposed to the chloroethenes for a prolonged period of time. Secondly, mass transport limitations could have played a major role comparable to the effects described for carbon tetrachloride dechlorination (Chapter 3). Overall, the dechlorination rate decreases with the amount of chlorine atoms in the molecule (Fig. 6.3). These results correlate nicely with those found for chlorinated ethanes (Chapter 4) and results found by others [39].

The dechlorination of TeCE and lower chlorinated ethenes seems to be conducted by whole enzyme systems, since the compounds were not transformed in the presence of autoclaved sludge. Nevertheless, these (cometabolic) enzyme systems seem to be present constitutively, because dechlorination of the chloroethenes did not require a lagphase. Reductive hydrogenolysis appears to be the predominant dechlorination pathway found in the presence of granular methanogenic sludge. However, since lower chlorinated ethenes were the only products measured, and the balance could not always be recovered, the formation of other products from the chloroethenes can not be excluded. The results found

in this research indicate that unadapted granular sludge could be applied in the remediation of wastestreams contaminated with chlorinated ethenes. The lower dechlorination rates observed are partly compensated by the more extensive dechlorination by granular sludge as compared to pure cultures of cometabolic and specific dechlorinators. The dechlorination products formed are aerobically degradable.

Acknowledgments

This research was supported by a grant (project 92014) from the Innovative Research Program on Environmental Biotechnology (IOP-Milieubiotechnologie) of the Ministries of Economic Affairs and Housing, Physical Planning, and Environment.

References

1. **Agteren, M. H. van, S. Keuning, and D. B. Janssen.** 1998. Handbook on biodegradation and biological treatment of hazardous organic compounds, vol. 2. Kluwer Academic Publishers, Dordrecht.
2. **American Public Health Association.** 1985. Standard methods for examination of water and wastewater, 16th ed. APHA, Washington DC.
3. **Bagley, D. M., and J. M. Gossett.** 1990. Tetrachloroethene transformation to trichloroethene and cis-1,2-dichloroethene by sulfate-reducing enrichment cultures. Appl. Environ. Microbiol. **56:**2511-2516.
4. **Barrio-Lage, G., F. Z. Parsons, R. S. Nassar, and P. A. Lorenzo.** 1986. Sequential dehalogenation of chlorinated ethenes. Environ. Sci. Tech. **20:**96-99.
5. **Bruin, W. P. de, M. J. J. Kotterman, M. A. Posthumus, G. Schraa, and A. J. B. Zehnder.** 1992. Complete biological reductive transformation of tetrachloroethene to ethane. Appl. Environ. Microbiol. **58:**1996-2000.
6. **Carter, S. R., and W. J. Jewell.** 1993. Biotransformation of tetrachloroethylene by anaerobic attached-films at low temperatures. Water Res. **27:**607-615.
7. **Christiansen, N., S. R. Christensen, E. Arvin, and B. K. Ahring.** 1997. Transformation of tetrachloroethene in an upflow anaerobic sludge blanket reactor. Appl. Microbiol. Biotechnol. **47:**91-94.
8. **Chu, K. H., and W. J. Jewell.** 1994. Treatment of tetrachloroethylene with anaerobic attached film process. J. Environ. Eng. - ASCE. **120:**58-71.
9. **Diekert, G., U. Konheiser, K. Pichulla, and R. K. Thauer.** 1981. Nickel requirement and factor F_{430} content of methanogenic bacteria. J. Bacteriol. **148:**459-464.
10. **Dolfing, J., and J. E. M. Beurskens.** 1995. The microbial logic and environmental significance of reductive dehalogenation, p. 143-206. *In* J. G. Jones (ed.), Adv. Microb. Ecol., vol. 14. Plenum Press, NewYork.
11. **Eekert, M. H. A. van, T. J. Schröder, A. J. M. Stams, G. Schraa, and J. A. Field.** 1998. Degradation and fate of carbon tetrachloride in unadapted methanogenic granular sludge. Appl. Environ. Microbiol. **64:**2350-2356.
12. **Egli, C., T. Tschan, R. Scholtz, A. M. Cook, and T. Leisinger.** 1988. Transformation of tetrachloromethane to dichloromethane and carbon dioxide by *Acetobacterium woodii*. Appl.

Environ. Microbiol. **54**:2819-24.
13. **Fathepure, B. Z., and S. A. Boyd.** 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. FEMS Microbiol. Lett. **49**:149-156.
14. **Fathepure, B. Z., J. P. Nengu, and S. A. Boyd.** 1987. Anaerobic bacteria that dechlorinate perchloroethene. Appl. Environ. Microbiol. **53**:2671-2674.
15. **Fathepure, B. Z., and J. M. Tiedje.** 1994. Reductive dechlorination of tetrachloroethylene by a chlorobenzoate-enriched biofilm reactor. Environ. Sci. Technol. **28**:746-752.
16. **Florencio, L., A. Nozhevnikova, A. van Langerak, A. J. M. Stams, J. A. Field, and G. Lettinga.** 1993. Acidophilic degradation of methanol by a methanogenic enrichment culture. FEMS Microbiol. Lett. **109**:1-6.
17. **Gantzer, C. J., and L. P. Wackett.** 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. Environ. Sci. Technol. **25**:715-722.
18. **Garant, H., and L. R. Lynd.** 1996. Perchloroethylene utilization by methanogenic fed-batch cultures - acclimation and degradation. Appl. Biochem. Biotechnol. **57-8**:895-904.
19. **Gerritse, J., G. Kloetstra, A. Borger, G. Dalstra, A. Alphenaar, and J. C. Gottschal.** 1997. Complete degradation of tetrachloroethene in coupled anoxic and oxic chemostats. Appl. Microbiol. Biotechnol. **48**:553-562.
20. **Glod, G., W. Angst, C. Holliger, and R. P. Schwarzenbach.** 1997. Corrinoid-mediated reduction of tetrachloroethene, trichloroethene, and trichlorofluoroethene in homogeneous aqueous solution: reaction kinetics and reaction mechanisms. Environ. Sci. Technol. **31**:253-260.
21. **Glod, G., U. Brodmann, W. Angst, C. Holliger, and R. P. Schwarzenbach.** 1997. Cobalamin mediated reduction of cis and trans dichloroethene, 1,1 dichloroethene, and vinyl chloride in homogeneous aqueous solution: reaction kinetics and mechanistic considerations. Environ. Sci. Technol. **31**:3154-3160.
22. **Guiot, S. R., X. Kuang, C. Beaulieu, A. Corriveau, and J. Hawari.** Anaerobic and aerobic/anaerobic treatment for tetrachloroethylene (PCE). 1995. Proceedings of the Third International In Situ and On-Site Bioreclamation Symposium, 4 - Bioremediation of chlorinated solvents, San Diego. 297-305.
23. **Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder.** 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. **59**:2991-2997.
24. **Jablonski, P. E., and J. G. Ferry.** 1992. Reductive dechlorination of trichloroethylene by the CO-reduced CO dehydrogenase enzyme complex from *Methanosarcina thermophila*. FEMS Microbiol. Lett. **96**:55-60.
25. **Kästner, M.** 1991. Reductive dechlorination of tri- and tetrachloroethylenes depends on transition from aerobic tot anaerobic conditions. Appl. Environ. Microbiol. **57**:2039-2046.
26. **Krumholz, L. R., R. Sharp, and S. S. Fishbain.** 1996. A freshwater anaerobe coupling acetate oxidation to tetrachloroethylene dehalogenation. Appl. Environ. Microbiol. **62**:4108-4113.
27. **Krzycki, J., and J. G. Zeikus.** 1980. Quantification of corrinoids in methanogenic bacteria. Curr. Microbiol. **3**:243-245.
28. **Lettinga, G., A. F. M. van Velsen, S. W. Hobma, W. de Zeeuw, and A. Klapwijk.** 1980. Use of upflow anaerobic (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioeng. **22**:677-734.
29. **Long, J. L., H. D. Stensel, J. F. Ferguson, S. E. Strand, and J. E. Ongerth.** 1993. Anaerobic and aerobic treatment of chlorinated aliphatic compounds. J. Environ. Eng. - ASCE. **119**:300-320.
30. **Magnuson, J. K., R. V. Stern, J. M. Gossett, S. H. Zinder, and D. R. Burris.** 1998. Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. Appl. Environ. Microbiol. **64**:1270-1275.
31. **Maymogatell, X., Y. Chien, J. M. Gossett, and S. H. Zinder.** 1997. Isolation of a bacterium that

reductively dechlorinates tetrachloroethene to ethene. Sci. **276**:1568-1571.

32. **Mazumder, T. K., N. Nishio, S. Fukuzaki, and S. Nagai.** 1987. Production of extracellular vitamin B-12 compounds from methanol by *Methanosarcina barkeri*. Appl. Microbiol. Biotechnol. **26**:511-516.
33. **Middeldorp, P. J. M., M. A. van Aalst, H. H. M. Rijnaarts, F. J. M. Stams, H. F. de Kreuk, G. Schraa, and T. N. P. Bosma.** 1998. Stimulation of reductive dechlorination for in situ bioremediation of a soil contaminated with chlorinated ethenes. Water Sci. Technol. **37**:105-110.
34. **Parsons, F., P. R. Wood, and J. DeMarco.** 1984. Transformations of tetrachloroethene and trichloroethene in microcosms and groundwater. J. Amer. Water Works Assoc. **76**:56-59.
35. **Scholz Muramatsu, H., A. Neumann, M. Messmer, E. Moore, and G. Diekert.** 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Arch. Microbiol. **163**:48-56.
36. **Sharma, P. K., and P. L. McCarty.** 1996. Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenates tetrachloroethene to cis-1,2-dichloroethene. Appl. Environ. Microbiol. **62**:761-765.
37. **Skeen, R. S., J. W. Gao, and B. S. Hooker.** 1995. Kinetics of chlorinated ethylene dehalogenation under methanogenic conditions. Biotechnol. Bioeng. **48**:659-666.
38. **Terzenbach, D. P., and M. Blaut.** 1994. Transformation of tetrachloroethylene to trichloroethylene by homoacetogenic bacteria. FEMS Microbiol. Lett. **123**:213-218.
39. **Vogel, T. M., C. S. Criddle, and P. L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21**:722-736.
40. **Vogel, T. M., and P. L. McCarty.** 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinylchloride, and carbondioxide under methanogenic conditions. Appl. Environ. Microbiol. **49**:1080-1083.

7

Transformation of chlorinated aromatic compounds by unadapted methanogenic granular sludge

The dechlorinating ability of methanogenic granular sludge was investigated with sludge from an upflow anaerobic sludge blanket (UASB) reactor fed with methanol, a mixture of volatile fatty acids (VFA), or sucrose. Only the sludge which was cultivated with methanol as the primary substrate was able to dechlorinate chlorobenzenes. Hexa-, penta-, tetra- and trichlorobenzenes were transformed at low rates. Usually, one chlorine atom was removed. Pentachlorophenol (PCP) was readily transformed to an unknown product by sludge from the methanol fed reactor, whereas polychlorinated biphenyls (PCBs) were not dechlorinated within 45 days of incubation.

Introduction

Chlorinated aromatic compounds (chlorinated benzenes, chlorophenols, and polychlorinated biphenyls (PCBs)) are important industrial pollutants commonly used as solvent, pesticide, flame retardant, wood preservative, and/or disinfectant [1,31]. The biodegradability under different redox conditions is dependent on the amount of chlorine atoms in the molecule. Hexa- down to dichlorinated benzenes were biodegraded under anaerobic conditions by mixed cultures enriched from different sources [7,9,11,30,36], in soil [41], and by sewage sludge [15]. The anaerobic degradation of pentachlorophenol (PCP) and lower chlorinated phenols by pure and mixed cultures has been extensively studied [5,12,24,29,32,34,38] and the chlorophenols were also shown to be degraded by adapted granular sludge from upflow anaerobic sludge blanket (UASB) reactors [13,19,33,43]. PCBs were found to be dechlorinated by different anaerobic sediment cultures, and different dechlorination pathways were identified [2,6,17,37,39,40].

In this chapter, the dechlorinating capacity of unadapted granular sludge was investigated. Granular sludge is highly enriched with methanogenic and acetogenic bacteria containing metallo-cofactors such as vitamin B_{12} and cofactor F_{430}. These cofactors can catalyze the dechlorination of chlorinated aromatic compounds *in vitro* [3,18,42]. Therefore, unadapted granular sludge may be able to dechlorinate chlorobenzenes, PCP, and PCBs without any prior adaptation. By autoclaving the sludge (thus eliminating all specific enzymatic activity) we tried to distinguish between aspecific (chemical and cofactor-catalyzed) and enzymatic dechlorination.

Materials and methods

Chemicals. Benzene, chlorobenzene (CB), 1,3- and 1,4-dichlorobenzene (13DCB and 14DCB), 1,2,4,-trichlorobenzene (124TCB), 1,2,3,4,-tetrachlorobenzene (1234TeCB), pentachlorobenzene (QCB), and pentachlorophenol (PCP) were obtained from E. Merck, Amsterdam, The Netherlands. 12DCB, 135TCB, 1235TeCB, 1245TeCB, and hexachlorobenzene (HCB) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands), and 123TCB from Janssen Chimica ('s Hertogenbosch, The Netherlands). The PCB congeners were obtained from Ultra Scientific (Kingstown, USA) or Promochem Gmbh (Wesel, Germany).

Granular sludge. The granular sludge was grown in three UASB reactors with methanol, a VFA mixture (acetate, propionate, and butyrate) or sucrose as the main carbon source. Originally, they had been inoculated with granular sludge from a full scale UASB-reactor treating sugar-beet refinery

wastewater (CSM, Breda, The Netherlands) and were operated as described earlier [14] for at least one year prior to sludge sampling. The sludges were washed two times with demineralized water and one time with basal medium [22] to remove residual soluble substrate before use in the batch experiments. When appropriate, the sludge granules were crushed prior to the experiment by pressing the sludge suspension through sterile needles with decreasing diameter (smallest needle: Microlance 3, 25G5/8, 0.5x16 mm). The methanogenic activity of the sludge was not affected by this treatment.

Batch experiments. Granular sludge was transferred to 120 ml serum flasks containing 40 ml basal medium (unless stated otherwise), as described earlier by Holliger [22], but without the addition of the fermented yeast-extract. The pH in the batches was 7.2-7.3. The appropriate primary substrate was added weekly at 0.5 to 1.0 g COD l^{-1}. The batches were incubated statically at 30°C in the dark unless stated otherwise. The possible loss of the chlorinated compound due to leaking through the stoppers or chemical reactions by medium components was checked in separate batches with sterile medium without any sludge addition. All experiments were carried out in triplicate unless stated otherwise.

Abiotic involvement of the cofactors. The abiotic involvement of cofactors was tested by autoclaving the granular sludge, thus inactivating all microbial activity. The granular sludge was autoclaved in basal medium for 90 minutes at 120°C three days prior to the start of the experiment and again for 30 minutes on the day the experiment was started.

Transformation of chlorinated benzenes. The transformation of all chlorobenzenes was tested separately in a qualitative experiment with 4 grams (wet weight) of sludge from the reactors fed with methanol, VFA or sucrose. The experiment was carried out in singular batches. The chlorinated benzenes were added dissolved in acetone (final concentration of the chlorinated benzenes was approximately 5 mg l^{-1}). The added amount of acetone was not toxic for the methanogenic bacteria. Primary substrate (0.3 to 0.5 g COD l^{-1}) was added to the batches weekly.

To quantify the HCB transformation a second experiment was carried out with sludge from the reactor fed with methanol in 26-ml incubation tubes. HCB dissolved in acetone was transferred to these tubes, after which the acetone was evaporated. The HCB precipitates in the tubes were dissolved in 8 ml of sterile basal medium (final nominal HCB concentration in the liquid phase 0.2 mM). The tubes were closed with viton stoppers (Maag Technic AG, Dübendorf, Switzerland) and alucaps and the headspace in the tubes was exchanged for N_2/CO_2 (80/20 v/v, pressure 1.3 bar). The tubes were incubated at 30°C for 3 days to maximize the amount of HCB dissolved in the basal medium. Afterwards the crushed living or autoclaved sludge was added (3.1 g VSS l^{-1}). The tubes were incubated statically in the dark at 30°C. Primary substrate was added weekly (10 mM methanol = 0.5 g COD l^{-1}). For each measurement three tubes were sacrificed. The loss of HCB through physical processes was checked in separate tubes containing basal medium without sludge.

Transformation of PCP. The transformation of PCP was tested with 4 grams (wet weight) of sludge (VSS content of batches: 3.5 g VSS l^{-1}) from the reactor fed with methanol in 120-ml batches containing 40-ml basal medium. PCP was added as a solution in water (final concentration approximately 1, 5 or 10 mg l^{-1}).

Transformation of PCBs and Aroclors. The transformation of PCBs and Aroclors (technical PCB mixtures) was tested with 2 grams (wet weight) of crushed sludge from the reactor fed with methanol in 120-ml batches containing 40-ml basal medium (VSS content of batches: 2.4 g VSS l^{-1}). The PCBs and Aroclors were added dissolved in acetone (final concentration approximately 5 mg l^{-1}). The added amount of acetone was not toxic for the methanogenic bacteria.

Analytical methods. The concentration of the chlorinated benzenes in the batches was determined in the liquid phase after extraction of 1 ml sample with 1 ml hexane/acetone mixture (4/1 v/v). The PCB and Aroclor concentrations were determined after extraction of 2 ml sample with 2 ml hexane/acetone (4/1) mixture). The extraction-mixtures were sonified for 15 minutes before further extraction for 24 hour in an end-over-end-shaker. The chlorinated benzene, PCB and Aroclor concentrations were determined in the hexane extracts with a GC/MS method. The samples (1µl) were injected splitless on a Hewlett Packard 5890 series II GC equipped with a HP 5 column (30 m x 0.25 mm, d_f 0.25µm) connected with a MS detector. Mass spectra were detected using a SIM procedure. The carrier gas was helium (inlet pressure 21 kPa). For the chlorinated benzene concentration (down to dichlorobenzenes) the temperature of the injector and detector were 250 and 280°C, respectively. The oven was operated with the following temperature program: 60°C (3 minutes), and 5°C $minute^{-1}$ increase in temperature to 180°C. Chlorobenzene and benzene a Chrompack Sil5CB (25 m x 0.32 mm, d_f 1.2 µm) connected with a MS detector. The temperature of the injector and detector were 250 and 280°C, respectively. The oven was operated with the following temperature program: 40°C (6 minutes), and 5°C $minute^{-1}$ increase in temperature to 180°C. For the PCB and the Aroclor determination injector and detector temperature were 225 and 250°C, respectively. For the PCB analysis the oven was heated with the following temperature program: 70°C (2 minutes), 10°C $minute^{-1}$ increase to 250°C; for the Aroclor analysis: 70°C (1 minute), 10°C $minute^{-1}$ to 150°C, 150°C (10 minutes), 3.5°C $minute^{-1}$ to 300°C. External standards were used.

The PCP concentrations was determined in 1 ml liquid samples (after 5 minutes centrifugation at 13000 rpm) and acidification with 20 µl 1.2 M HCl. A TSP AS 3000-HPLC was used to determine the concentration with a ChromSep (100 x 3.0 mm) ChromSpher Pesticides column connected to a LKB 2158 Uvicord SD UV detector (206 nm). The retention time of PCP was 4.0 to 4.5 minutes. The column was eluted with a mixture of acetonitril and 0.1% H_3PO_4 (1/1 v/v) which was pumped through the column at 1 ml $minute^{-1}$. After every run the column was equilibrated with 100% acetonitril for 6 minutes.

The COD for methanol, VFA, and sucrose solutions was determined according to standard methods [4]. COD conversion factors (g g^{-1}) utilized were 1.07, 1.50, 1.07, 1.52, and 1.82 for

sucrose, methanol, acetate, propionate, and butyrate, respectively. The VSS content of the sludge was determined by subtracting the ash-content from the dry weight after incubating the sludge overnight at 105°C. The ash-content was determined after heating the dry sludge at 600°C for 90 minutes.

Results

Transformation of chlorinated benzenes. The transformation of chlorinated benzenes was tested in a qualitative experiment with sludge from the reactors fed with methanol, VFA or sucrose. All chlorinated benzenes were persistent in the presence of sludge from the sucrose fed reactor. In contrast the higher chlorinated benzenes were degraded in the presence of sludge from the reactors fed with methanol or VFA (Table 7.1). Clearly, a long lag phase was required before the dechlorination of HCB by the VFA grown sludge started, while the methanol sludge was capable of transforming HCB immediately after exposure. Penta-, tetra-, and two of the trichlorobenzenes are dechlorinated after a longer lag phase. The length of the lag phase increased and the amount of product formed decreased with a decreasing number of chlorine atoms in the molecule.

Table 7.1 Transformation of chlorinated benzenes by sludge from the reactors fed with methanol and VFA. Predominant products are printed in boldface.

Compound	Methanol			VFA		
	Product(s)	Day[a]	Amount formed[b]	Product(s)	Day[a]	Amount formed[b]
HCB	**QCB**	0	18.4	**QCB**	63	4.0
QCB	**1,2,4,5-TeCB**	28	9.5	**1,2,4,5-TeCB**	105	1.1
	1,2,3,4-TeCB	28	6.3	1,2,3,5-TeCB	105	tr
	1,2,3,5-TeCB	28	4.0	1,2,3,4-TeCB	119	tr
	1,2,4-TCB	98	tr			
1,2,4,5-TeCB	**1,2,4-TCB**	28	9.9	**1,2,4-TCB**	105	tr
1,2,3,4-TeCB	1,2,4-TCB	28	9.1	1,2,4-TCB	105	tr
1,2,3,5-TeCB	1,2,4-TCB	28	8.8	1,2,4-TCB	105	tr
1,2,4-TCB	**1,4-DCB**	42	2.9	**1,4-DCB**	119	tr
1,3,5-TCB	1,3-TCB	42	2.8	1,3-DCB	119	tr
1,2,3-TCB	ND[c]			ND		
1,2-DCB	ND			ND		
1,3-DCB	ND			ND		
1,4-DCB	ND			ND		
CB	ND			ND		
Benzene	ND			ND		

[a] Lag phase before transformation

[b] Amount of product as percentage of (substrate + product) after 126 and 119 days of incubation for the methanol and VFA fed sludge, respectively. tr = amount formed <1%

[c] ND = not detected

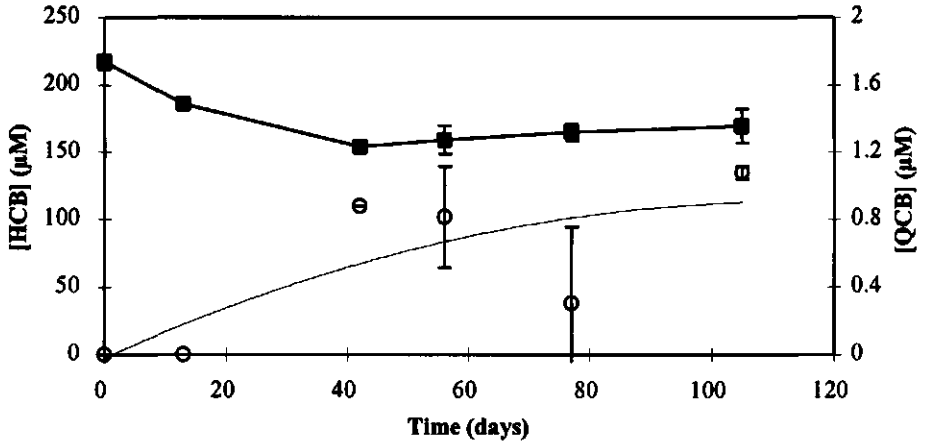


Figure 7.1 Formation of QCB (○) from HCB (■) by living sludge from the reactor fed with methanol.

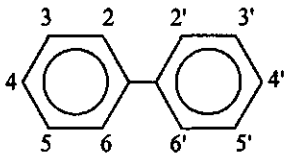
During the incubations, typically only one chlorine atom was removed. The amount of product, which was formed, was relatively low in all cases. The chlorine atoms in the para-position seem to be preferentially removed. 1,2,3-TCB, DCB, CB, and benzene were not observed to be dechlorinated by any of the sludges within the period of incubation.

In the quantitative experiment, the HCB dechlorination was investigated with the methanol grown sludge. The only product found was QCB which was formed in small amounts at very low rates of approximately 5 nmol g^{-1} VSS day^{-1} (Fig. 7.1). The HCB concentration in time was the same for the different treatments (data not shown). Also, in the blanks without sludge or with autoclaved sludge, the HCB concentration dropped significantly. However, formation of QCB was not observed.

Transformation of PCP. The dechlorination of PCP was investigated with sludge from the UASB reactor fed with methanol at 1.0, 5.0 and 10.0 mg l^{-1} PCP. PCP was depleted in the presence of living sludge at the three concentrations applied although the methane formation was severely inhibited at 5.0 and 10.0 mg l^{-1}. The 50% inhibitory concentration (IC_{50}) of methanogenic activity with methanol caused by PCP was found to be around 4 mg l^{-1} (data not shown). The mean normalized pseudo first order rate constant was 0.26 day^{-1} g^{-1} VSS and the initial rate of 190 to 210 nmol g^{-1} VSS day^{-1}. At the two highest concentrations of PCP tested, the formation of an unidentified degradation product could also be observed (retention time 1.9 minutes). PCP was not depleted in the presence of autoclaved sludge or in blanks containing medium without sludge.

Transformation of PCBs and Aroclors. The transformation of six highly chlorinated PCBs with six or seven chlorine atoms per molecule an a mixture containing six PCBs (Table 7.2) was investigated with methanogenic sludge from the reactor fed with methanol. This sludge was not able to significantly degrade the PCBs within 45 days of incubation. Also, two technical mixtures of PCBs with relatively high amounts of chlorine (Aroclor 1242 and 1260 containing 42 and 60% w/w chlorine respectively) were not transformed. The PCBs and Aroclors were also persistent in the presence of autoclaved anaerobic sludge and in blanks without sludge.

Table 7.2 PCB compounds tested with sludge form the reactor fed with methanol.

IUPAC nr.	Conformation chlorine atoms
	
PCB 136	22'33'66'
PCB 138	22'344'5'
PCB 153	22'44'55
PCB 156	233'44'5'
PCB 170	22'33'44'5
PCB 180	22'344'55'
Mixture containing:	
PCB 28	244'
PCB 52	22'55'
PCB 101	23'44'5
PCB 138	22'344'5'
PCB 153	22'44'55'
PCB 180	22'344'55'

Discussion

Aromatic chlorinated compounds were not readily biotransformed by unadapted granular sludge. The conversion of chlorinated benzenes in the experiments described here were limited to the removal of one chlorine atom, and PCBs were not significantly transformed at all within 45 days by living sludge from the methanol fed reactor. None of the aromatic chlorinated compounds tested were dechlorinated in the presence of autoclaved sludge, suggesting that the transformations that did take place were biologically mediated. Cofactors like vitamin B_{12} are known to transform PCP [18,42], chlorinated benzenes [3,18], and PCBs

[3] *in vitro* in the presence of a strong reductant (e.g., Ti(III)citrate). However, the redox conditions in the incubations with autoclaved sludge may not have been reduced enough [Smith, 1994 #1088. The results taken as a whole suggest that the dechlorination of chloroaromatics involves more specialized enzyme systems as was suggested earlier [35].

PCP was shown to be removed in the batch experiments, but unfortunately the product formed could not be identified. PCP was previously shown to be readily degradable in UASB reactors with a variety of primary substrates. A mixture of VFA and methanol [27,43], glucose [19,21], and phenol [13] supported dechlorination and/or mineralization. Mineralization of PCP to methane and CO_2 was also observed when PCP was applied as the sole carbon and energy source in the influent of an anaerobic reactor [28]. Although a considerable amount of PCP may become adsorbed to anaerobic sludge [25,26] it is highly likely that PCP was also dechlorinated in our experiments. The depletion rates measured in our research are approximately 10 to 275 times lower than the rates found by other researchers with adapted sludge [20,43].

To our knowledge to ability of unadapted granular sludge to degrade chlorobenzenes was not investigated before. The results here indicate that there is a significant, albeit minor constitutive activity towards dechlorinating chlorobenzenes in methanol grown granular sludge. Previously, mixed cultures from contaminated sediments were used in experiments [8,23,30,36]. Fathepure and Vogel [16] used anaerobic sewage sludge for their experiments and found that depending on the primary substrate used, HCB was dechlorinated to QCB (glucose), 1234TeCB (methanol), or DCB (acetate). In our experiments with methanol as a primary substrate, only small amounts of QCB were formed. Likewise, the other chlorobenzenes tested were only transformed by the removal of one chlorine atom. Extrapolating the results observed with individual chlorobenzenes, we found with the methanol grown sludge, the dechlorination of HCB would ultimately proceed via the pathway depicted in Fig. 7.2. This pathway is similar to the dechlorination pattern found in *in vitro* experiments with vitamin B_{12} [3,18]. We also found mainly the removal of the chlorine atom at the para position, whereas in other cases the meta-situated chlorine atom seems to be preferentially removed [8,11,15,23].

The carbon balance in both experiments is not complete. One of the possibilities is the formation of coupling products or the replacement of a chlorine atom by a methylsulphide group, as was demonstrated for PCBs in contaminated sediment [10]. The formation of coupling products is highly unlikely given the high oxidation state of chlorine, and the formation of methylated PCBs or HCB has never been observed with pure or mixed cultures.

The product formation rate of 5 nmol QCB g^{-1} VSS day^{-1} is lower than the rate of 380 µmol g^{-1} VSS day^{-1} observed in sewage sludge originally fed with industrial wastewater [15]. This again indicates that a microbial population which is enriched on chlorinated aromatic compounds is more suitable for remedial activities.

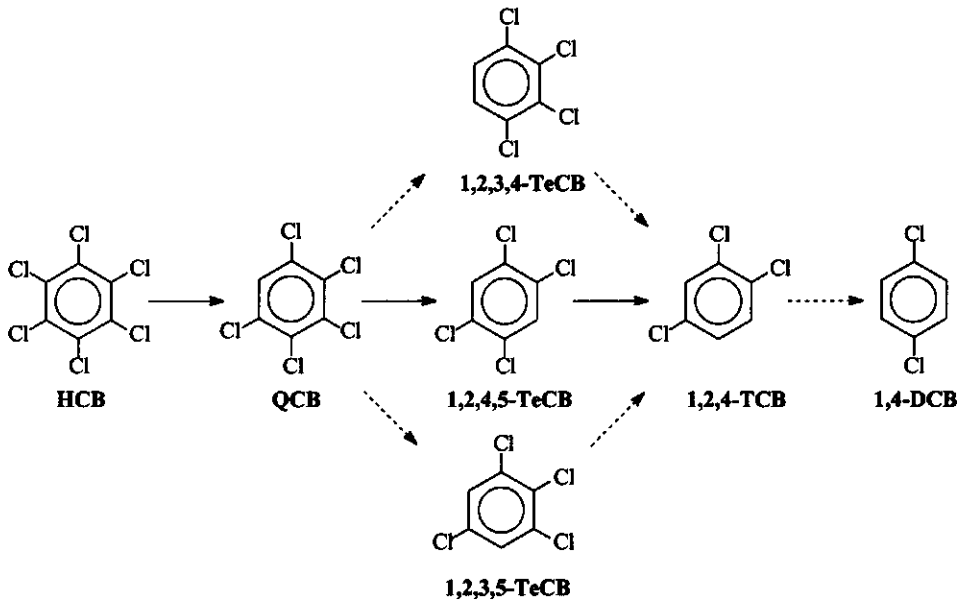


Figure 7.2 Proposed degradation pathway of hexachlorobenzene after prolonged incubation periods with sludge grown in the reactor fed with methanol or VFA.

Acknowledgments

This research was supported by a grant (project 92014) from the Innovative Research Program on Environmental Biotechnology (IOP-Milieubiotechnologie) of the Ministries of Economic Affairs and Housing, Physical Planning, and Environment.

References

1. **Agteren, M. H. van, S. Keuning, and D. B. Janssen.** 1998. Handbook on biodegradation and biological treatment of hazardous organic compounds, vol. 2. Kluwer Academic Publishers, Dordrecht.
2. **Alder, A. C., M. M. Haggblom, S. R. Oppenhelmer, and L. Y. Young.** 1993. Reductive dechlorination of polychlorinated biphenyls in anaerobic sediments. Environ. Sci. Technol. **27**:530-538.
3. **Assaf-Anid, N., L. Nies, and T. M. Vogel.** 1992. Reductive dechlorination of a polychlorinated biphenyl congener and hexachlorobenzene by vitamin B_{12}. Appl. Environ. Microbiol. **58**:1057-1060.

4. **American Public Health Association.** 1985. Standard methods for examination of water and wastewater, 16th ed. APHA, Washington DC.
5. **Beaudet, R., M. J. Levesque, R. Villemur, M. Lanthier, M. Chenier, F. Lepine, and J. G. Bisaillon.** 1998. Anaerobic biodegradation of pentachlorophenol in a contaminated soil inoculated with a methanogenic consortium or with *Desulfitobacterium frappieri* strain PCP-1. Appl. Microbiol. Biotechnol. **50:**135-141.
6. **Bedard, D. L., S. C. Bunnell, and L. A. Smullen.** 1996. Stimulation of microbial para-dechlorination of polychlorinated biphenyls that have persisted in Housatonic river sediments for decades. Environ. Sci. Technol. **30:**687-694.
7. **Beurskens, J. E. M., C. G. C. Dekker, J. Jonkhoff, and L. Pompstra.** 1993. Microbial dechlorination of hexachlorobenzene in a sedimentation area of the rhine river. Biogeochem. **19:**61-81.
8. **Beurskens, J. E. M., C. G. C. Dekker, H. Van den Heuvel, M. Swart, and J. De Wolf.** 1994. Dechlorination of chlorinated benzenes by an anaerobic microbial consortium that selectively mediates the thermodynamic most favorable reactions. Environ. Sci. Technol. **28:**701-706.
9. **Bosma, T. N. P., J. R. van der Meer, G. Schraa, M. E. Tros, and A. J. B. Zehnder.** 1988. Reductive dechlorination of all trichloro- and dichlorobenzenes. FEMS Microbiol. Ecol. **53:**223-229.
10. **Buser, H.-R., and M. D. Muller.** 1986. Methylthio metabolites of polychlorobiphenyls identified in sediment samples from two lakes in Switzerland. Environ. Sci. Technol. **20:**730-735.
11. **Chang, B. V., C. J. Su, and S. Y. Yuan.** 1998. Microbial hexachlorobenzene dechlorination under three reducing conditions. Chemosphere. **36:**2721-2730.
12. **Cole, J. R., A. L. Cascarelli, W. W. Mohn, and J. M. Tiedje.** 1994. Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol. Appl. Environ. Microbiol. **60:**3536-3542.
13. **Duff, S. J. B., K. J. Kennedy, and A. J. Brady.** 1995. Treatment of dilute phenol PCP wastewaters using the upflow anaerobic sludge blanket (UASB) reactor. Wat. Res. **29:**645-651.
14. **Eekert, M. H. A. van, T. J. Schröder, A. J. M. Stams, G. Schraa, and J. A. Field.** 1998. Degradation and fate of carbon tetrachloride in unadapted methanogenic granular sludge. Appl. Environ. Microbiol. **64:**2350-2356.
15. **Fathepure, B. Z., J. M. Tiedje, and S. A. Boyd.** 1988. Reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in anaerobic sewage sludge. Appl. Environ. Microbiol. **54:**327-330.
16. **Fathepure, B. Z., and T. M. Vogel.** 1991. Complete degradation of polychlorinated hydrocarbons by a two-stage biofilm reactor. Appl. Environ. Microbiol. **57:**3418-3422.
17. **Fish, K. M., and J. M. Principe.** 1994. Biotransformation of aroclor 1242 in Hudson River test tube microcosms. Appl. Environ. Microbiol. **60:**4289-4296.
18. **Gantzer, C. J., and L. P. Wackett.** 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. Environ. Sci. Technol. **25:**715-722.
19. **Hendriksen, H. V., and B. K. Ahring.** 1993. Anaerobic dechlorination of pentachlorophenol in fixed-film and upflow anaerobic sludge blanket reactors using different inocula. Biodegradation. **3:**399-408.
20. **Hendriksen, H. V., and B. K. Ahring.** 1992. Metabolism and kinetics of pentachlorophenol transformation in anaerobic granular sludge. Appl. Microbiol. Biotechnol. **37:**662-666.
21. **Hendriksen, H. V., S. Larsen, and B. K. Ahring.** 1992. Influence of a supplemental carbon source on anaerobic dechlorination of pentachlorphenol in granular sludge. Appl. Environ. Microbiol. **58:**365-370.
22. **Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder.** 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. **59:**2991-2997.

23. **Holliger, H. C.** Reductive dehalogenation by anaerobic bacteria. 1992. Ph.D. Thesis. Agricultural University, Wageningen.
24. **Juteau, P., R. Beaudet, G. Mcsween, F. Lepine, S. Milot, and J. G. Bisaillon.** 1995. Anaerobic biodegradation of pentachlorophenol by a methanogenic consortium. Appl. Microbiol. Biotechnol. **44:**218-224.
25. **Kennedy, K. J., J. Lu, and W. W. Mohn.** 1992. Biosorption of chlorophenols to anaerobic granular sludge. Water Res. **26:**1085-1092.
26. **Kennedy, K. J., and T. T. Pham.** 1995. Effect of anaerobic sludge source and condition on biosorption of PCP. Water Res. **29:**2360-2366.
27. **Kennes, C., W.-M. Wu, L. Bhatnagar, and J. G. Zeikus.** 1996. Anaerobic dechlorination and mineralization of pentachlorophenol and 2,4,6-trichlorophenol by methanogenic pentachlorophenol-degrading granules. Appl. Microbiol. Biotechnol. **44:**801-806.
28. **Krumme, M. L., and S. A. Boyd.** 1988. Reductive dechlorination of chlorinated phenols in anaerobic upflow bioreactors. Wat. Res. **22:**171-177.
29. **Madsen, T., and J. Aamand.** 1991. Effects of sulfuroxy anions on degradation pentachlorophenol by a methanogenic enrichment culture. Appl. Environ. Microbiol. **57:**2453-2458.
30. **Masunaga, S., S. Susarla, and Y. Yonezawa.** 1996. Dechlorination of chlorobenzenes in anaerobic estuarine sediment. Water Sci. Technol. **33:**173-180.
31. **McAllister, K. A., H. Lee, and J. T. Trevors.** 1996. Microbial degradation of pentachlorophenol. Biodegradation. **7:**1-40.
32. **Mikesell, M. D., and S. A. Boyd.** 1986. Complete reductive dechlorination and mineralization of pentachlorophenol by anaerobic microorganisms. Appl. Environ. Microbiol. **52:**861-865.
33. **Mohn, W. W., and K. J. Kennedy.** 1992. Limited degradation of chlorophenols by anaerobic sludge granules. Appl. Environ. Microbiol. **58:**2131-2136.
34. **Mohn, W. W., and K. J. Kennedy.** 1992. Reductive dehalogenation of chlorophenols by *Desulfomonile tiedjei* strain DCB-1. Appl. Environ. Microbiol. **58:**1367-1370.
35. **Mohn, W. W., and J. M. Tiedje.** 1992. Microbial reductive dehalogenation. Microbiol. Rev. **56:**482-507.
36. **Nowak, J., N. H. Kirsch, W. Hegemann, and H. J. Stan.** 1996. Total reductive dechlorination of chlorobenzenes to benzene by a methanogenic mixed culture enriched from Saale river sediment. Appl. Microbiol. Biotechnol. **45:**700-709.
37. **Oford, G. D., J. A. Puhakka, and J. F. Ferguson.** 1994. Reductive dechlorination of aroclor 1254 by marine sediment cultures. Environ. Sci. Technol. **28:**2286-2294.
38. **Puhakka, J. A., R. P. Herwig, P. M. Koro, G. V. Wolfe, and J. F. Ferguson.** 1995. Biodegradation of chlorophenols by mixed and pure cultures from a fluidized-bed reactor. Appl. Microbiol. Biotechnol. **42:**951-957.
39. **Quensen, I. J. F., S. A. Boyd, and J. M. Tiedje.** 1990. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. Appl. Environ. Microbiol. **56:**2360-2369.
40. **Quensen, I. J. F., J. M. Tiedje, and S. A. Boyd.** 1988. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. Science. **242:**752-754.
41. **Rosenbrock, P., R. Martens, F. Buscot, and J. C. Munch.** 1997. Initiation of [Cl 36]hexachlorobenzene dechlorination in three different soils under artificially induced anaerobic conditions. Appl. Microbiol. Biotechnol. **48:**115-120.
42. **Smith, M. H., and S. L. Woods.** 1994. Regiospecificity of chlorophenol reductive dechlorination by vitamin B_{12}s. Appl. Environ. Microbiol. **60:**4111-4115.
43. **Wu, W., L. Bhatnagar, and J. G. Zeikus.** 1993. Performance of anaerobic granules for degradation of pentachlorophenol. Appl. Environ. Microbiol. **59:**389-397.

8

Summary, discussion and concluding remarks

Introduction

Chlorinated hydrocarbons are an important group of contaminants found in sediments, wastewaters, groundwater, soil and off-gases. Many of them are found on the EPA list of priority pollutants. Highly chlorinated aliphatic and aromatic hydrocarbons are only biodegradable under anaerobic conditions [51]. A (primary) transformation step in the conversion of chlorinated compounds under anaerobic conditions is reductive dechlorination. This (initial) dechlorination can be carried out by specific halorespiring bacteria or by acetogenic and/or methanogenic bacteria which dechlorinate via aspecific reactions.

The research described in this thesis evaluated the aspecific dechlorinating ability of unadapted acetogenic and methanogenic bacteria in granular sludge from upflow anaerobic sludge blanket (UASB) reactors. Granular sludge has a high biomass content and is highly enriched with acetogenic and methanogenic bacteria [31]. The sludges were grown with methanol, a mixture of volatile fatty acids or sucrose as the main carbon source. By autoclaving the sludge, we tried to distinguish between abiotic and enzymatic reactions (Fig. 8.1). Abiotic reactions are either bulk chemical reactions of medium components or reactions catalyzed by cofactors or redox mediators of (dead) cells. Enzymatic reactions are mediated by aspecific cometabolic enzymes or they involve specific enzyme systems of halorespiring bacteria.

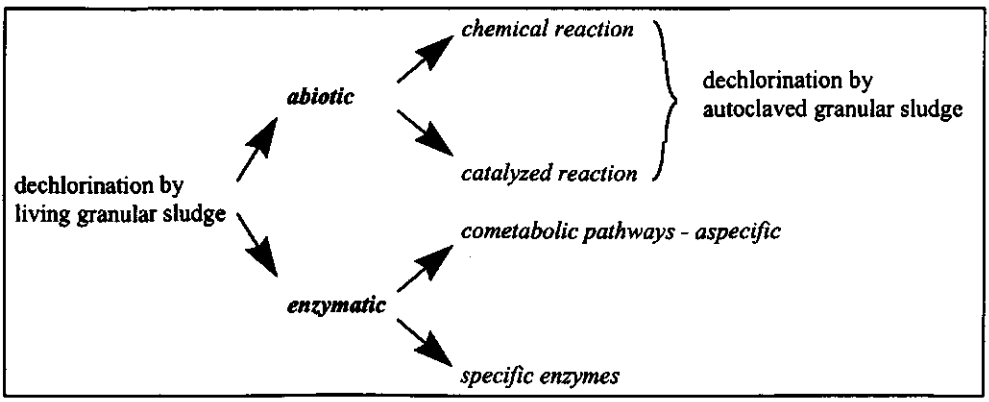


Figure 8.1 Abiotic and enzymatic mechanisms of dechlorination

Transformation of chlorinated compounds by methanogenic granular sludge

The granular methanogenic sludges evaluated in this study were able to transform a variety of chlorinated compounds (Table 8.1). Although the three sludges were different in

microbial composition as was evidenced by visual observations, by molecular ecological methods [25,43], and by their variable vitamin B_{12} content (Chapter 2), there were no major differences in the dechlorinating activity among the three sludges. Because aspecific dechlorinating activity is widely distributed among different groups of (facultative) anaerobic bacteria (Chapter 1), the microbial composition of the granular sludge may only be of minor importance. Also, the dechlorinating activity of methanogenic granular sludge is partially of abiotic nature, thus independent of the microbes present. Another reason for the similarity in the dechlorinating activity and rate among the sludges may have been the high biomass content used in the experiments. In that case, other factors like the diffusion of the chlorinated compounds from the gas into the medium, will have been determining the rate.

Abiotic dechlorination. Abiotic reactions play an important role in the transformation of chlorinated alkanes as evidenced by the dechlorinating capacity of autoclaved sludge (Table 8.1). Our research showed that a substantial amount of the higher chlorinated methanes and chlorinated ethanes was degraded in the presence of autoclaved sludge (Chapters 2 and 4). This dechlorination was relatively fast compared to the rates observed with living sludge (Fig. 8.2). CT and CF were transformed via net substitution reactions with CS_2 as the main product formed. DCM and MC were not dechlorinated by autoclaved sludge. The chlorinated ethanes were degraded via dichloroelimination and dehydrochlorination reactions to lower chlorinated ethenes. This dechlorinating activity is attributed to abiotic reactions, which are at least partially mediated by biologically generated cofactors like vitamin B_{12} and cofactor F_{430} present in the sludge. Given the high vitamin B_{12} content of the sludges and the ability of this cofactor to dechlorinate *in vitro*, it seems likely that cofactors play a significant role in the dechlorination by granular sludge. Organic molecules, e.g., hydroquinon-like structures [13,44], which are also able to function as redox mediator, may also be present in autoclaved sludge. These molecules could also be involved as mediators in the dechlorination process. It has been observed that the dechlorination of HCA or CT can be considerably increased through the presence of humic like structures such as juglone [44,45], anthrahydroquinone compounds [13] or compounds like bipyridine [7]. The reducing equivalents necessary for dechlorination may have been provided by reduced sludge components. Metals and sulfur contents of the sludges in g g^{-1} TSS were Fe 1.0-2.6; Co 0.015-0.268; Ni 0.22-0.42; S 9.3-11.1.

The abiotic nature of the reactions was stressed by the fact that the CT dechlorination by autoclaved sludge showed increasing rates with increasing temperatures beyond the biological optimum (Chapter 3). The dechlorination rates of the compounds tested were always higher in the presence of autoclaved sludge compared to medium containing no sludge. Together, these results point towards the sludge as the source of the dechlorination mediator. Highly chlorinated ethanes were also partially dechlorinated to lower chlorinated ethenes in medium without sludge (Chapter 4, Table 8.1), indicating the involvement of pure chemical reactions in the dechlorination observed. The other chlorinated compounds tested were not significantly degraded in medium without sludge.

In contrast with the chloromethanes and chloroethanes, no transformation via abiotic reactions was observed for β-HCH, the chloroethenes and the chlorinated aromatic compounds (Table 8.1). Slow transformation compared to chloromethanes and the higher chloroethanes only occurred if living sludge was present (Fig. 8.2). The inability of the autoclaved sludge to transform β-HCH, chlorinated ethenes and chlorinated aromatics is probably due to the lack of strong enough electron donors as was suggested for β-HCH (Chapter 5). The dechlorination rate of CT (Chapter 2) by autoclaved sludge was enhanced by the addition of extra reducing equivalents from Ti(III)citrate, while β-HCH was only transformed by autoclaved sludge after the addition of Ti(III)citrate (data not shown). In most experiments, the addition of an electron donor was omitted in experiments with autoclaved sludge.

In *in vitro* experiments, PCP was only dechlorinated by vitamin B_{12s} (Co(I)) and not by vitamin B_{12r} (Co(II)) [49]. Also, carbon tetrachloride was transformed to other (more reduced) products in the presence of vitamin B_{12} Co(I) as compared to dechlorination by Co(II) [32]. From this it may be concluded that the redox conditions are also of influence on the occurrence of abiotic reactions mediated by transition metal containing cofactors. In the experiments with the chlorinated ethenes and aromatic compounds, the redox potential may have been too high to sustain the dechlorination by the cofactors in the presence of autoclaved sludge.

Enzymatic dechlorination. Chlorinated alkanes and cycloalkanes were completely or substantially degraded to lower- or non-chlorinated end products by living sludge without prior adaptation (Fig. 8.2). Different dechlorination reactions depending on the nature of the chlorinated compounds were used by the consortium (Table 8.1). Chlorinated methanes were degraded mainly via oxidative and substitutive reactions to non-halogenated compounds like

CO_2 (Chapter 2). The formation of CS_2, which was the main product of dechlorination by autoclaved sludge, was not observed in the presence of living sludge. Either the degradation pathway by living sludge does not involve CS_2 as an intermediate, or CS_2 is converted very easily by living sludge. Higher (hexa-, penta-, and tetra-)chloroethanes were dechlorinated via dichloroelimination and reductive hydrogenolysis to lower chlorinated ethenes and ethanes (Chapter 4). Dichloroelimination took place mainly when chlorine atoms were present at both carbon atoms, and was a relatively fast process. 111TCA and the DCAs were degraded primarily via reductive hydrogenolysis.

β-HCH, a compound which is considered to be relatively stable under anaerobic conditions, was easily degraded by unadapted methanogenic granular sludge to benzene and chlorobenzene (Chapter 5). The amount of benzene formed was mostly around three times the amount of chlorobenzene. This is of interest because the formation of benzene is believed to be a biological process as opposed to the abiotic formation of chlorobenzene. Transformation of β-HCH by an enrichment culture, which was previously isolated in our laboratory resulted in three times more chlorobenzene than benzene [37]. The reason for this difference in benzene and chlorobenzene production is as yet unclear. Maximizing the benzene production may be important in light of a complete anaerobic transformation of (β-)HCH under anaerobic conditions, since mineralisation of chlorobenzene under anaerobic conditions has never been observed.

The degradation of chlorinated alkenes (tetrachloroethene and lower chlorinated ethenes) by unadapted consortia proceeded at low rates (Chapter 6) and the dechlorination pathway observed was very unusual. TeCE was dechlorinated via 11DCE, while cDCE is the normal intermediate or endproduct observed in the dechlorination of TeCE by anaerobic bacteria, e.g., [6,24]. This pathway was assumed to occur because, the granular sludge used in our experiments was unable to reductively dechlorinate cDCE and tDCE, while still the formation of VC was observed during the degradation of TCE.

Although not all of the products which could possibly have been formed, were actually measured, it seems reasonable to state that, except for the degradation of chlorinated methanes, reductive dechlorination (reductive hydrogenolysis and dichloroelimination) is the most commonly used mechanism for chlorine removal by granular methanogenic sludge (Table 8.1).

Acetogenic and methanogenic bacteria have been found to degrade chlorinated compounds cometabolically (Table 1.4, Chapter 1). The cofactor containing enzymes are believed to play a role in this dechlorination process as was shown for the degradation of

12DCA in pure cultures of methanogenic bacteria [28]. Additionally, some bacteria are known for their ability to use chlorinated compounds as an energy source. *Dehalobacterium formicoaceticum* and *Acetobacterium dehalogenans* (strain MC) are able to convert DCM and MC, respectively. DCM can even be used as a carbon source [33,36]. DCM and MC are the only chlorinated alkanes known so far, that are degraded via such pathways under anaerobic conditions. Also, it has been postulated that (γ-)HCH may act as an alternative electron acceptor of the Strickland reaction, thus associating reductive dechlorination to ATP synthesis [42]. Methanogenic and acetogenic bacteria in our granular sludge may have been degrading the chlorinated alkanes and alkenes via any of the pathways mentioned above. This would require the presence of functional enzymes, which would explain the lack of dechlorination of lower chlorinated compounds and β-HCH by autoclaved sludge. The instantaneous degradation of chlorinated alkanes and alkenes is probably due to the activity of cometabolic pathways, involving enzymes that are constitutively present. The rates we found with chlorinated ethenes are low compared to the rates reported for pure cultures of specifically dechlorinating bacteria, which also points towards cometabolic pathways. In general, dechlorination rates are higher when chlorinated compounds are degraded via specific (halorespiring) pathways.

It is known that chlorinated aromatics can be converted by anaerobic sludge in long term reactor experiments or with sludge which has been exposed to e.g., industrial wastewater [20,24,54]. Clearly, in those cases, the anaerobic sludge population may have been enriched with specifically dechlorinating bacteria. The sludge in our experiments had not been priorily exposed to chlorinated compounds. Given the low transformation rates of the chlorinated aromatic compounds (Fig. 8.2), it is highly likely that the transformation of such compounds requires more specific enzymes than the ones present in unadapted granular sludge. Apparently, the number of specialized halorespiring bacteria was very low in the sludges used in our experiments. Therefore, halorespiration, a process in which dechlorination is coupled to ATP formation, as such seems to be a minor mechanism by which the chlorinated compounds are removed by our granular sludge.

Table 8.1 Summary of the degradation potential of unadapted granular sludge

Compound		Sludge	
		Autoclaved	Living
Chlorinated methanes	CCl_4, $CHCl_3$	RH[a] to dichloromethane, O/S to CS_2	RH to methylchloride, O/S to CO_2
	CH_2Cl_2, CH_3Cl	no degradation	RH to methylchloride, O/S to CO_2
Chlorinated ethanes	C_2Cl_6[b], C_2HCl_5[b], $C_2H_2Cl_4$[b]	D or H to lower chlorinated ethenes	RH and/or D or H to lower chlorinated ethenes
	112-$C_2H_3Cl_3$	D or H to vinylchloride	RH and/or D or H to lower chlorinated ethenes and ethanes
	111-$C_2H_3Cl_3$, $C_2H_4Cl_2$	no degradation	RH and/or D or H to lower chlorinated ethenes and ethanes
Hexachlorocyclohexane	$C_6H_6Cl_6$	no degradation	D and/or H to chlorobenzene and benzene
Chlorinated ethenes	C_2Cl_4	no degradation	RH to lower chlorinated ethenes (down to VC)
PCP	C_6Cl_5OH	no degradation	RH to lower chlorinated phenols
Chlorinated benzenes	C_6Cl_6	no degradation	dechlorination of traces HCB to lower chlorinated benzenes
PCBs[c] and Aroclors		no degradation	no degradation

[a] RH=reductive hydrogenolysis, O/S=oxidative/substitutive, D=dichloroelimination, H=dehydrochlorination (see Chapter 1)
[b] Hexa-, penta-, and tetrachlorinated ethanes were also dechlorinated in medium without sludge added
[c] PCBs tested are listed in table 7.2

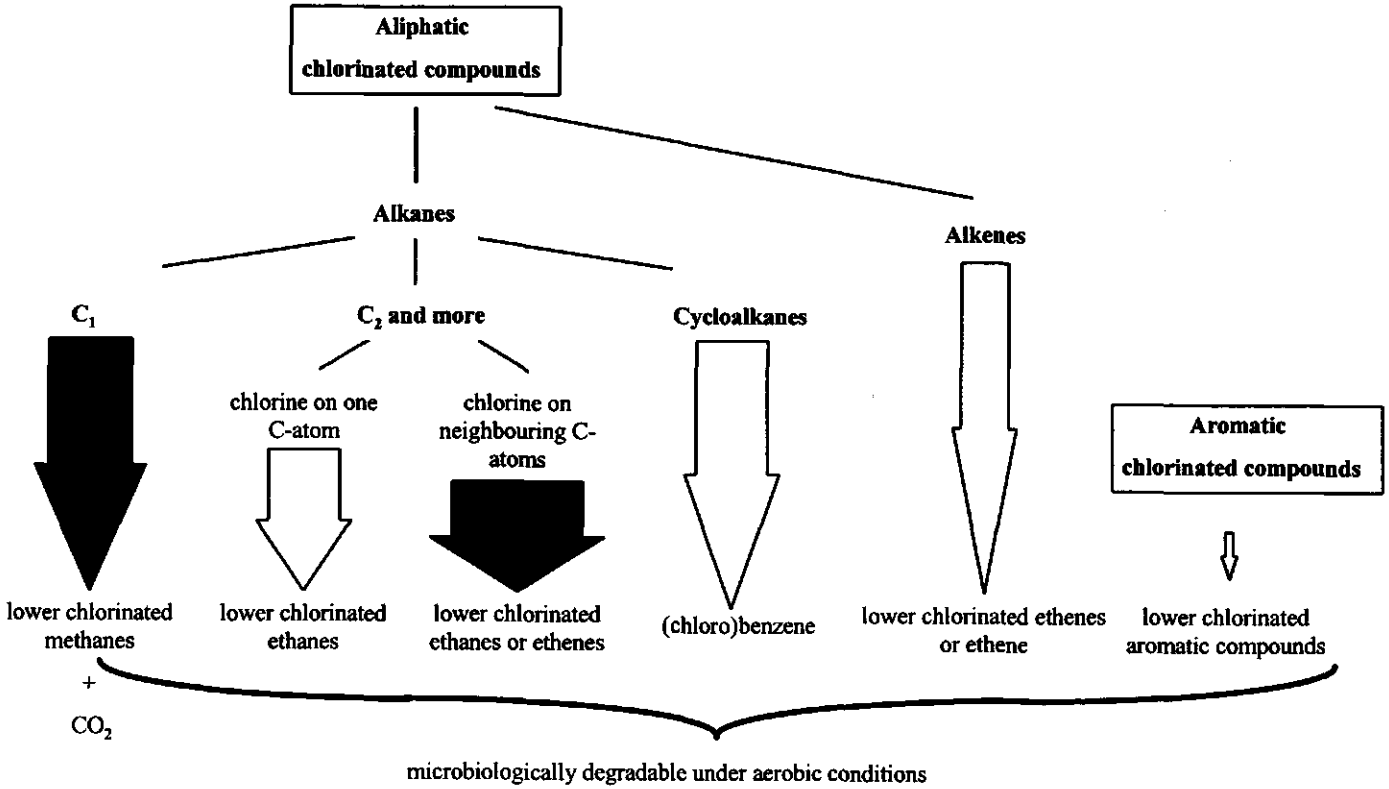


Figure 8.2 Dechlorination of chlorinated compounds by methanogenic granular sludge. Black arrows indicate dechlorination by abiotic reactions. The relative transformation rate is indicated by the thickness of the arrows.

Dechlorination rates found with unadapted granular sludge

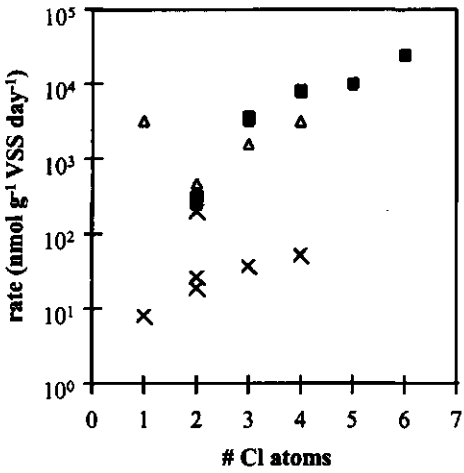


Figure 8.3 Dechlorination rates for different groups of chlorinated methanes (▲), chlorinated ethanes (■), and chlorinated ethenes (x) by living sludge from the methanol-fed reactor.

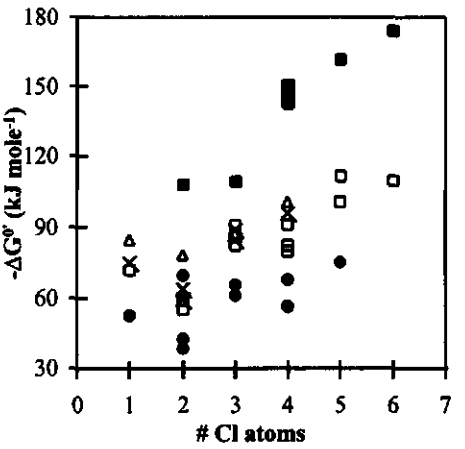


Figure 8.4 Gibbs free energy changes of dechlorination half reaction ($\Delta G^{0'}$ kJ mole^{-1}) for the reductive hydrogenolysis of chlorinated methanes (▲), chlorinated ethanes (□), and chlorinated ethenes (x), as well as dichloroelimination (■) and dehydrochlorination of chlorinated ethanes (●).

Structure - dechlorination rate. We found that the dechlorination rates of chlorinated methanes, ethanes, and ethenes by living sludge increased with the number of chlorine atoms in the molecule (Fig. 8.3). This is in agreement with results found and predicted before [51]. There is also a relationship between the Gibbs Free energy change of the dechlorination reactions ($\Delta G^{0'}$) and the number of chlorine atoms in the molecule (Chapter 4) as is depicted in Figure 8.4 for the half-reactions of the dechlorination of chlorinated methanes, ethanes and ethenes. Although the Gibbs free energy changes are merely indicators for the likelihood of a reaction to take place, a more negative $\Delta G^{0'}$ could mean a preferential use of the specific compound compared to others. This was found for chlorinated ethanes (Chapter 4) and chlorinated benzenes and PCBs [3,14]. Still, this assumption only holds when the reaction conditions are optimal for all the compounds tested. The reaction rates measured for chlorinated alkanes were clearly higher than the rates measured for chlorinated ethenes. The Gibbs free energy changes are also higher for the chlorinated ethanes, and to a lesser extent chloromethanes, than for the chloroethenes. The chlorinated aromatic compounds are dechlorinated very slowly. This however, cannot be related to the $\Delta G^{0'}$ for the reactions (-140 to -170 kJ/mole), but was to be expected since chlorinated aromatics are believed to be transformed by specific enzymes [39].

Microbial composition - dechlorination rate. The dechlorination rates found in this research are in most cases significantly lower than the rates obtained for the degradation of chlorinated compounds by other researchers for pure cultures or with adapted sludge. Those adapted sludges were enriched with specific dechlorinating bacteria, and this would normally result in higher conversion rates. The lower rates compared to the pure cultures of methanogenic and acetogenic bacteria, could be caused by the vitamin B_{12} content of the sludges. The vitamin B_{12} content of the sludges was up to 10 times lower than the concentrations found by other researchers for pure cultures of acetogenic and methanogenic bacteria [22] and could be due to the presence of non corrinoid containing biomass in the sludge which "dilutes" the dechlorinating activity and thus the biomass specific dechlorination rate.

Although the dechlorination rates are relatively low compared to those obtained with pure cultures, the use of unadapted granular sludge still has advantages. E.g., in the case of the dechlorination of chloroethenes, a 10^3 and 10^6 fold lower rate was found for unadapted sludge compared to pure cultures of cometabolically dechlorinating bacteria [16-18,50] and specific dechlorinators [27,46,48], respectively. Usually however, only one chlorine atom is removed via cometabolic pathways and up to two chlorine atoms via specific dechlorination. The only exception so far is *Dehalococcoides ethenogenes*, which is able to transform TeCE to VC (and ethene) [35]. On the other hand, adapted sludge dechlorinated TeCE about 10^3 times faster than the sludge in our experiments. Generally, the end product of such a transformation is DCE or VC [11,12 229,19,24]. Since our granular sludge was able to transform TeCE into minor amounts of VC, it seems clear that such an inoculum may be suitable for the start-up of a TeCE transforming reactor. The (initial) limitations in dechlorination rates compared to pure cultures of aspecific or halorespiring dechlorinating bacteria are compensated by the more complete dechlorination.

Anaerobic transformation of chlorinated compounds in contaminated soil

This research shows the dechlorinating potential of acetogenic and methanogenic bacteria. These bacteria which are present mainly in anaerobic environments, may have been partly responsible for the variety of products which can be found at sites contaminated with chlorinated compounds. Biological remediation at such a site can be stimulated by creating anaerobic conditions and although specialized bacteria may not be present, anaerobic acetogenic and methanogenic bacteria can make a substantial contribution to the transformation of pollutants. We observed this in soil contaminated with β-HCH (Chapter 5). The microorganisms present in the

soil were inactive, until the soil was transferred to anaerobic medium. A low redox potential and the addition of an electron donor were essential to initiate dechlorination. The β-HCH seemed to be readily available once the soil was moisturized. Obviously, this does not necessarily mean, that specific halorespiring bacteria were not present in the soil. In another experiment (data not shown), we observed the dechlorination of freshly spiked QCB in sediment by the microorganisms indigenously present. Clearly, microbial activity may be ubiquitously present in contaminated soil. This is evidenced by numerous cases of indigenous microbial activity in contaminated soil and sediment, which have been reported in the past few years, e.g., [1,2,4,21,30,34,38,47,53] (Chapter 5).

In most cases, the indigenous bacteria will have to be stimulated. This can be done by improving the environmental conditions or by adding supplements which are known to stimulate dechlorination. The addition of an electron donor and/or sulfide stimulated the dechlorination of β-HCH in contaminated soil (Chapter 5) and e.g., the dechlorination of PCE contaminated sediment [23,47]. The addition of micronutrients like cobalt and nickel (both present as active centers in vitamin B_{12} and cofactor F_{430}, respectively) may also enhance the dechlorination. Such a measure may only be suitable for treatment of wastewaters and less for the remediation of contaminated soils, because the dosage of such trace elements can best be controlled via the addition to the influent of wastewater treatment systems. The addition of trace elements *in situ* to contaminated soil may lead to immediate precipitation, which may leave the metals unavailable for the microorganisms. The addition to contaminated soil of vitamin B_{12} as such (instead of stimulating the production by adding cobalt) has been proposed as a way of enhancing in situ remediation of contaminated soils [40], because it was found that the presence of vitamin B_{12} was essential for the dechlorination of TeCE by pure cultures of specific dehalogenators [35].

The application of granular sludge to stimulate the environmental conditions for reductive dechlorination in contaminated soil may be considered. Active methanogenic granular sludge has the ability to lower the redox potential significantly, which usually is beneficial for the indigenous dechlorinating bacteria. As a consequence, the granular sludge after addition may simply serve as an electron donor for the indigenous microorganisms. Granular sludge could also function as a source for vitamin B_{12} in the soil. It has also been found that humic acid structures can act as electron acceptors in the microbial oxidation of lower chlorinated ethenes under anaerobic conditions [5]. Other redox mediators (e.g., organic matter in anaerobic sludge) may act in a similar way, thus enhancing *in situ* dechlorination.

Concluding remarks and future perspectives

The results obtained in this thesis suggest a broad spectrum applicability of methanogenic bacterial consortia for the degradation or transformation of halogenated compounds without prior adaptation. Chlorinated (cyclo)alkanes are degraded to a large extent. An important part of the total dechlorinating capacity of the sludge towards chlorinated methanes and chlorinated ethanes is of abiotic origin. The importance of such abiotic conversions was also stressed by other researchers, e.g., [8,52]. Because of the abiotic nature of the reactions, anaerobic granular sludge may be used for the bioremediation of wastestreams that are contaminated with a mixture of chlorinated compounds, e.g., groundwater and wastewater. The (degradation) products formed in the dechlorination process, i.e., lower chlorinated alkanes and alkenes, and (chloro)benzene in case of HCH, are biodegradable under aerobic conditions. This makes the application of granular sludge a suitable first step in the sequential anaerobic-aerobic treatment of wastestreams contaminated with highly chlorinated compounds. Chlorinated aromatic compounds are less easily degraded by the unadapted sludges, but after an adaptation period presumably needed to enrich for a suitable microbial population, polychlorinated aromatic compounds may be (partially) dechlorinated.

By applying methanogenic consortia in the bioremediation of contaminated wastestreams, an overall reduction of the risk for public health can be achieved. Gratuitously occurring reactions result in a fast reduction of the chlorinated compounds. This however, may be disadvantageous, if the dechlorination reactions are incomplete (e.g., TeCE to VC), because the occurrence of such aspecific reactions can not easily be avoided. The products formed by the methanogenic consortia are usually more mobile than their parent compounds, which could lead to a higher susceptibility for (aerobic) biodegradation. This could be important in the case of remediation of contaminants that tend to adsorb to solid particles, which renders them less available for microbial transformation. Obviously, the higher mobility of dechlorination products can also be a draw-back, when dechlorination takes place in soil and the environmental conditions are not suitable for the (complete) mineralisation of the products. However, because granular sludge is relatively easy to handle it may proof useful in the application of *in situ* barriers as a (first) anaerobic stage in the remediation of contaminated groundwater.

Contaminated soils often contain many bacteria, that are already able to perform remediation, provided that the environmental conditions are favorable. In that case, the addition of granular sludge does not seem to be necessary for the degradation of the chlorinated pollutants. In that case, it is more important that the environmental conditons are made optimal for microbiological dechlorination. The presence of a methanogenic bacterial consortium can lead to

e.g., a decrease of the redox-potential, thus favoring the environmental conditions for other, more specialized dechlorinating bacteria, as well as maintaining anaerobic conditions for prolonged periods of time.

The chlorinated compounds which are not easily degraded by the unadapted consortia used in this study have been shown by other researchers to be dechlorinated in long-term experiments. Tetrachloroethene [11,24] and PCP [15,26,54] containing wastewaters were successfully treated in UASB systems and HCB was found to be degraded by sewage sludge, which priorily had been fed with 40% industrial wastewater [20]. PCP degrading granules were successfully used as an starting material for the construction of a PCB dechlorinating consortium [41]. The inoculation of (sterile) granules from UASB reactors with pure cultures of halorespiring bacteria has also been proven to be successful in the dechlorination of TeCE [9,29] and PCP [10].

Another application of granular sludge may be in the transformation of nitro- and azoaromatic compounds in wastewater of e.g., the textile and (petro)chemical industries, since the first step in the anaerobic (bio)transformation of these compounds is a reduction as well. Also, it may be an interesting possibility to apply granular sludge by making use of the presence of facultative anaerobic or microaerophilic bacteria in the sludge. These bacteria can degrade the persistent lower chlorinated products under higher redox-conditions. Recently, we have shown that naturally occurring facultative anaerobic or microaerophilic bacteria in methanogenic sludge, are able, once they are supplied with oxygen, to degrade chlorobenzene and benzene which were formed during the degradation of HCH (preliminary results, data not shown). The transformation products were not identified. The responsible microorganism was isolated and appeared to be a *Pseudomonas* sp., which degraded (chloro)benzene cometabolically.

Altogether, the broad spectrum applicability of unadapted methanogenic granular sludge may make it a useful first "tool" in the (in situ) bioremediation of contaminated environments.

References

1. **Alder, A. C., M. M. Haggblom, S. R. Oppenhelmer, and L. Y. Young.** 1993. Reductive dechlorination of polychlorinated biphenyls in anaerobic sediments. Environ. Sci.Technol. **27**:530-538.
2. **Beurskens, J. E. M., C. G. C. Dekker, J. Jonkhoff, and L. Pompstra.** 1993. Microbial dechlorination of hexachlorobenzene in a sedimentation area of the rhine river. Biogeochem. **19**:61-81.
3. **Beurskens, J. E. M., C. G. C. Dekker, H. van den Heuvel, M. Swart, and J. De Wolf.** 1994. Dechlorination of chlorinated benzenes by an anaerobic microbial consortium that selectively mediates the thermodynamic most favorable reactions. Environ. Sci. Technol. **28**:701-706.
4. **Bradley, P. M., and F. H. Chapelle.** 1997. Kinetics of DCE and VC mineralization under methanogenic and Fe(III) reducing conditions. Environ. Sci. Technol. **31**:2692-2696.

5. **Bradley, P. M., F. H. Chapelle, and D. R. Lovely.** 1998. Humics acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. Appl. Environ. Microbiol. **64:**3102-3105.
6. **Bruin, W. P. d., M. J. J. Kotterman, M. A. Posthumus, G. Schraa, and A. J. B. Zehnder.** 1992. Complete biological reductive transformation of tetrachloroethene to ethane. Appl. Environ. Microbiol. **58:**1996-2000.
7. **Butler, E. C., and K. F. Hayes.** 1998. Effects of solution composition and pH on the reductive dechlorination of hexachloroethane by iron sulfide. Environ. Sci. Technol. **32:**1276-1284.
8. **Chen, C., J. A. Puhakka, and J. F. Ferguson.** 1996. Transformations of 1,1,2,2-tetrachloroethane under methanogenic conditions. Environ. Sci. Technol. **30:**542-547.
9. **Christiansen, N., and B. K. Ahring.** 1996. Introduction of a *de novo* bioremediation activity into anaerobic granular sludge using the dechlorinating bacterium DCB-2. Antonie van Leeuwenhoek. **69:**61-66.
10. **Christiansen, N., and B. K. Ahring.** 1994. Introduction of bioremediation ability into granular sludge. Wat. Sci. Technol. **29:**189-193.
11. **Christiansen, N., S. R. Christensen, E. Arvin, and B. K. Ahring.** 1997. Transformation of tetrachloroethene in an upflow anaerobic sludge blanket reactor. Appl. Microbiol. Biotechnol. **47:**91-94.
12. **Chu, K. H., and W. J. Jewell.** 1994. Treatment of tetrachloroethylene with anaerobic attached film process. J. Environ. Eng. - ASCE. **120:**58-71.
13. **Curtis, G. P., and M. Reinhard.** 1994. Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthrahydroquinone disulfonate and humic acid. Environ. Sci. Technol. **28:**2393-2401.
14. **Dolfing, J., and J. E. M. Beurskens.** 1995. The microbial logic and environmental significance of reductive dehalogenation, p. 143-206. *In* J. G. Jones (ed.), Adv. Microb. Ecol., vol. 14. Plenum Press, NewYork.
15. **Duff, S. J. B., K. J. Kennedy, and A. J. Brady.** 1995. Treatment of dilute phenol PCP wastewaters using the upflow anaerobic sludge blanket (UASB) reactor. Wat. Res. **29:**645-651.
16. **Egli, C., T. Tschan, R. Scholtz, A. M. Cook, and T. Leisinger.** 1988. Transformation of tetrachloromethane to dichloromethane and carbon dioxide by *Acetobacterium woodii*. Appl. Environ. Microbiol. **54:**2819-24.
17. **Fathepure, B. Z., and S. A. Boyd.** 1988. Reductive dechlorination of perchloroethylene and the role of methanogens. FEMS Microbiol. Lett. **49:**149-156.
18. **Fathepure, B. Z., J. P. Nengu, and S. A. Boyd.** 1987. Anaerobic bacteria that dechlorinate perchloroethene. Appl. Environ. Microbiol. **53:**2671-2674.
19. **Fathepure, B. Z., and J. M. Tiedje.** 1994. Reductive dechlorination of tetrachloroethylene by a chlorobenzoate-enriched biofilm reactor. Environ. Sci. Technol. **28:**746-752.
20. **Fathepure, B. Z., J. M. Tiedje, and S. A. Boyd.** 1988. Reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in anaerobic sewage sludge. Appl. Environ. Microbiol. **54:**327-330.
21. **Fish, K. M., and J. M. Principe.** 1994. Biotransformation of aroclor 1242 in Hudson River test tube microcosms. Appl. Environ. Microbiol. **60:**4289-4296.
22. **Florencio, L.** The fate of methanol in anaerobic bioreactors. 1994. Ph.D. Agricultural University, Wageningen.
23. **Gibson, S. A., and G. W. Sewell.** 1992. Stimulation of reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by addition of short-chain acids or alcohols. Appl. Environ. Microbiol. **58:**1392-1393.
24. **Guiot, S. R., X. Kuang, C. Beaulieu, A. Corriveau, and J. Hawari.** Anaerobic and aerobic/anaerobic treatment for tetrachloroethylene (PCE). 1995. Proceedings of the Third International In Situ and On-Site Bioreclamation Symposium, 4 - Bioremediation of chlorinated solvents, San Diego. 297-305.

25. **Harmsen, H. J. M., H. M. P. Kengen, A. D. L. Akkermans, A. J. M. Stams, and W. M. de Vos.** 1996. Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by in situ hybridization using 16s rRNA-based oligonucleotide probes. Appl. Environ. Microbiol. **62:**1656-1663.
26. **Hendriksen, H. V., and B. K. Ahring.** 1993. Anaerobic dechlorination of pentachlorophenol in fixed-film and upflow anaerobic sludge blanket ractors using different inocula. Biodegradation. **3:**399-408.
27. **Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder.** 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. **59:**2991-2997.
28. **Holliger, C., G. Schraa, E. Stupperich, A. J. M. Stams, and A. J. Zehnder.** 1992. Evidence for the involvement of corrinoids and factor F_{430} in the reductive dechlorination of 1,2-dichloroethane by *Methanosarcina barkeri*. J. Bacteriol. **174:**4427-4434.
29. **Hörber, C., N. Christiansen, E. Arvin, and B. K. Ahring.** 1998. Improved dechlorinating performance of upflow anaerobic sludge blanket reactors by incorporation of *Dehalospirillum multivorans* into granular sludge. Appl. Environ. Microbiol. **64:**1860-1863.
30. **Kleopfer, R. D., D. M. Easley, B. B. Haas, T. G. Deihl, D. E. Jackson, and C. J. Wurray.** 1985. Anaerobic degradation of trichloroethylene in soil. Environ. Sci. Technol. **19:**277-280.
31. **Lettinga, G., A. F. M. van Velsen, S. W. Hobma, W. de Zeeuw, and A. Klapwijk.** 1980. Use of upflow anaerobic (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol. Bioeng. **22:**677-734.
32. **Lewis, T. A., M. J. Morra, and P. D. Brown.** 1996. Comparative product analysis of carbon tetrachloride dehalogenation catalyzed by cobalt corrins in the presence of thiol or titanium (III) reducing agents. Environ. Sci. Technol. **30:**292-300.
33. **Mägli, A., M. Wendt, and T. Leisinger.** 1996. Isolation and characterization of *Dehalobacterium formicoaceticum* ge. nov. sp. nov., a strictly anaerobic bacterium utilizing dichloromethane as source of carbon and energy. Arch. Microbiol. **166:**101-108.
34. **Masunaga, S., S. Susarla, J. L. Gundersen, and Y. Yonezawa.** 1996. Pathway and rate of chlorophenol transformation in anaerobic estuarine sediment. Environ. Sci. Technol. **30:**1253-1260.
35. **Maymogatell, X., Y. Chien, J. M. Gossett, and S. H. Zinder.** 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Sci. **276:**1568-1571.
36. **Messmer, M., G. Wohlfarth, and G. Diekert.** 1993. Methyl chloride metabolism of the strictly anaerobic, methyl chloride-utilizing homoacetogen strain MC. Arch. Microbiol. **160:**383-387.
37. **Middeldorp, P. J. M., M. Jaspers, A. J. B. Zehnder, and G. Schraa.** 1996. Biotransformation of alpha-, beta-, gamma-, and delta-hexachlorocyclohexane under methanogenic conditions. Environ. Sci. Technol. **30:**2345-2349.
38. **Middeldorp, P. J. M., M. A. van Aalst, H. H. M. Rijnaarts, F. J. M. Stams, H. F. de Kreuk, G. Schraa, and T. N. P. Bosma.** 1998. Stimulation of reductive dechlorination for in situ bioremediation of a soil contaminated with chlorinated ethenes. Water Sci. Technol. **37:**105-110.
39. **Mohn, W. W., and J. M. Tiedje.** 1992. Microbial reductive dehalogenation. Microbiol. Rev. **56:**482-507.
40. **Morse, J. J., B. C. Alleman, J. M. Gossett, S. H. Zinder, D. E. Fennell, G. W. Sewell, and C. M. Vogel.** 1997. A treatability test for evaluating the potential applicability of the reductive biological in situ treatmet technology (RABITT) to remediate chloroethenes. Draft Technical Protocol Environmental Security Technology Certification Program.
41. **Natarajan, M. R., W. M. Wu, J. Nye, H. Wang, L. Bhatnagar, and M. K. Jain.** 1996. Dechlorination of polychlorinated biphenyl congeners by an anaerobic microbial consortium. Appl. Microbiol. Biotechnol. **46:**673-677.
42. **Ohisa, N., N. Kurihara, and M. Nakajima.** 1982. ATP synthesis associated with the conversion of hexachlorocyclohexane related compounds. Arch. Microbiol. **131:**330-333.

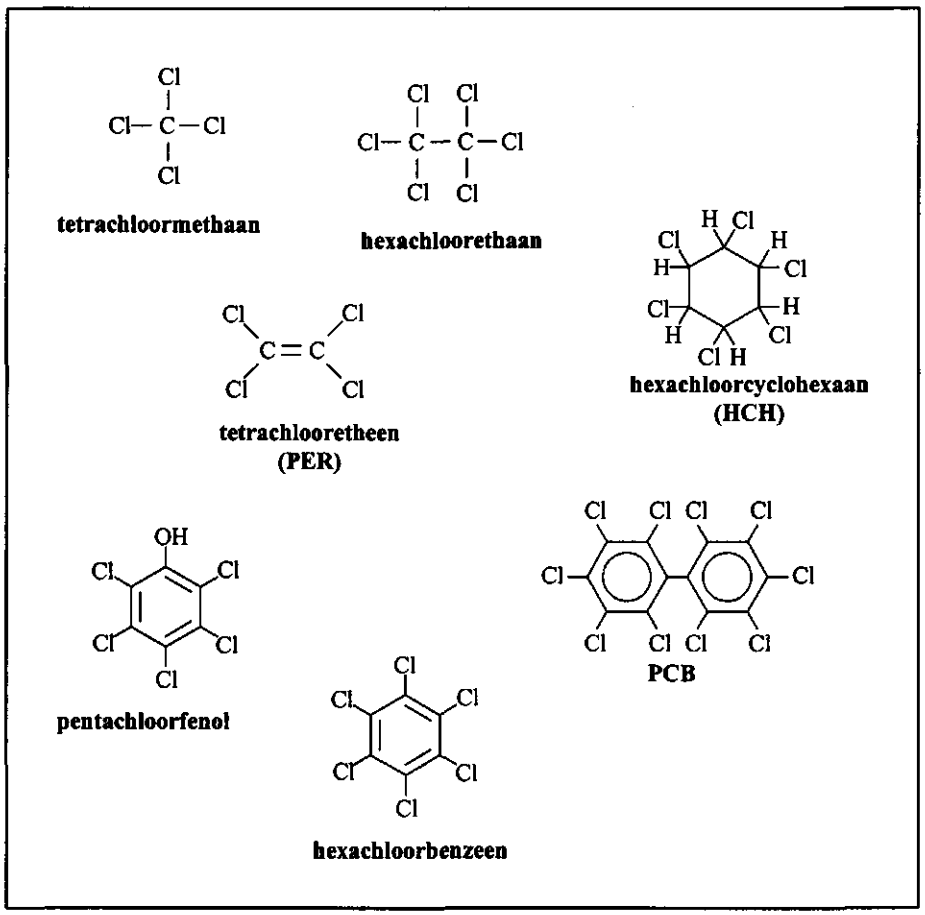
43. **Oude Elferink, S. J. W. H., A. Visser, L. Hulshoff Pol, and A. J. M. Stams.** 1994. Sulfate reduction in methanogenic bioreactors. FEMS Microbiol. Rev. **15**:119-136.
44. **Perlinger, J. A., W. Angst, and R. P. Schwarzenbach.** 1996. Kinetics of the reduction of hexachloroethane by juglone in solutions containing hydrogen. Environ. Sci. Technol. **30**:3408-3417.
45. **Perlinger, J. A., J. Buschmann, W. Angst, and R. P. Schwarzenbach.** 1998. Iron porphyrin and mercaptojuglone mediated reduction of polyhalogenated methanes and ethanes in homogeneous aqueous solution. Environ. Sci. Technol. **32**:2431-2437.
46. **Scholz Muramatsu, H., A. Neumann, M. Messmer, E. Moore, and G. Diekert.** 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Arch. Microbiol. **163**:48-56.
47. **Sewell, G. W., and S. A. Gibson.** 1991. Stimulation of the reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by the addition of toluene. Environ.Sci.Technol. **25**:982-984.
48. **Sharma, P. K., and P. L. McCarty.** 1996. Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenates tetrachloroethene to cis-1,2-dichloroethene. Appl. Environ. Microbiol. **62**:761-765.
49. **Smith, M. H., and S. L. Woods.** 1994. Regiospecificity of chlorophenol reductive dechlorination by vitamin B_{12}s. Appl. Environ. Microbiol. **60**:4111-4115.
50. **Terzenbach, D. P., and M. Blaut.** 1994. Transformation of tetrachloroethylene to trichloroethylene by homoacetogenic bacteria. FEMS Microbiol. Lett. **123**:213-218.
51. **Vogel, T. M., C. S. Criddle, and P. L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21**:722-736.
52. **Vogel, T. M., and P. L. McCarty.** 1987. Abiotic and biotic transformations of 1,1,1-trichloroethane under methanogenic conditions. Environ. Sci. Tech. **21**:1208-1213.
53. **Wilson, B. H., G. B. Smith, and J. F. Rees.** 1986. Biotransformations of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenis aquifer material: a microcosm study. Environ. Sci Technol. **20**:997-1002.
54. **Wu, W., L. Bhatnagar, and J. G. Zeikus.** 1993. Performance of anaerobic granules for degradation of pentachlorophenol. Appl. Environ. Microbiol. **59**:389-397.

9

Samenvatting

Introductie

Gechloreerde koolwaterstoffen (voorbeelden zijn weergegeven in Figuur 9.1) worden veelvuldig gebruikt als oplos- en ontvettingsmiddel in bijvoorbeeld de metaalindustrie en in stomerijen (chloormethanen, chloorethanen en chloorethenen), als fungicide (pentachloorfenol) of ter bescherming van zaden (hexachloorbenzeen). Daarnaast kunnen ze worden toegepast als vlamvertrager in transformatorhuisjes (PCBs) of gevormd worden als bijprodukt tijdens de produktie van pesticiden (bijv. β-HCH bij de produktie van lindaan (γ-HCH)). Als gevolg van (onzorgvuldig) menselijk handelen kunnen de gechloreerde koolwaterstoffen in sediment en bodem, afvalwater, grondwater en afgassen terecht komen, en vormen zo een bedreiging voor het milieu en de volksgezondheid.



Figuur 9.1 Voorbeelden van gechloreerde koolwaterstoffen.

De laatste jaren is veel onderzoek verricht naar de mogelijke reiniging van verontreinigde afvalstromen. Hierbij wordt steeds meer aandacht besteed aan het gebruik van (micro)biologische methoden naast de meer conventionele methoden, zoals bijvoorbeeld verbranden. Bij deze microbiologische methoden worden bacteriën en ander micro-organismen gebruikt die, soms na toediening van een extra voedingsbron, de gechloreerde verbindingen omzetten in produkten, die meestal minder schadelijk zijn.

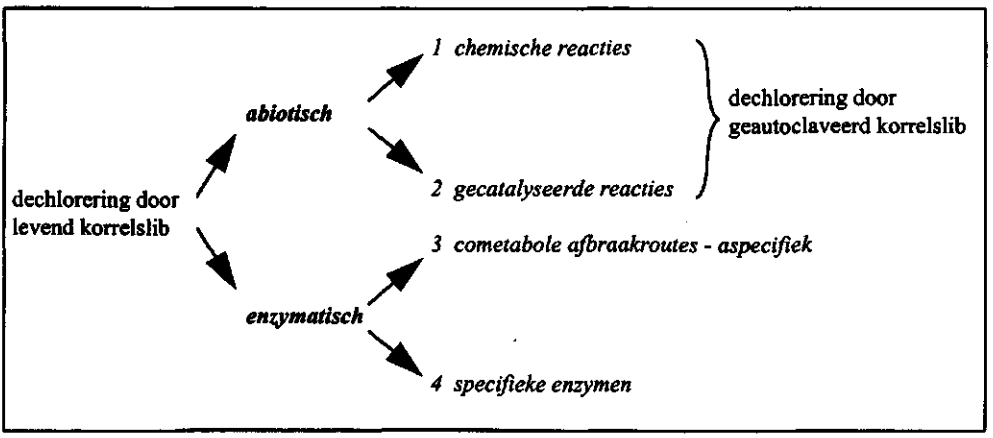
Gechloreerde koolwaterstoffen met een hoog chloorgehalte zijn in het algemeen alleen biologisch afbreekbaar onder anaërobe (zuurstofloze) condities. Een van de (eerste) stappen bij deze omzetting is reductieve dechlorering, waarbij chlooratomen afgesplitst worden (en vaak worden vervangen door waterstofatomen). Dit resulteert meestal in de vorming van stoffen die minder milieubelastend zijn. Reductieve dechlorering kan worden uitgevoerd door zogenaamde specifiek halorespirerende bacteriën die speciaal voor de omzetting van de chloorhoudende verbindingen enzymen aanmaken en uit deze omzetting energie halen voor hun groei. De omzettingssnelheid van gechloreerde koolwaterstoffen door dit soort bacteriën is over het algemeen hoog, doch het enzym kan vaak slechts een klein aantal verbindingen omzetten.

Anaërobe bacteriën, die normaal groeien op een niet chloorhoudend substraat zijn soms toch in staat dechloreringsreacties uit te voeren. In dit geval zetten zij de gechloreerde verbinding bij toeval om met de (delen van) enzymen die in de bacteriën aanwezig zijn om het "normale" groei-substraat om te zetten. Omdat dit "normale" substraat essentieel is voor de groei van de micro-organismen wordt het ook wel aangeduid als primair substraat. De dechloreringssnelheid is in dit geval laag omdat de gechloreerde koolwaterstof en het enzym elkaar bij toeval treffen. Daar staat tegenover dat de enzymen die hierbij betrokken zijn niet erg specifiek zijn en daarom een breed scala van gechloreerde koolwaterstoffen kan worden gedechloreerd. We spreken daarom ook wel van aspecifieke cometabole dechlorering.

Het onderzoek dat in dit proefschrift wordt beschreven evalueert de aspecifieke dechlorerende activiteit van anaërobe bacteriën in mengpopulaties die nog niet eerder waren blootgesteld aan gechloreerde koolwaterstoffen. Er zijn dus nog geen speciale (specifiek dechlorerende) enzymen aanwezig. Als modelsysteem voor de bacteriepopulaties is gekozen voor de anaërobe bacteriën die aanwezig zijn in korrelslib uit UASB (Upflow Anaerobic Sludge Blanket) reactoren. Dit zijn anaërobe, opwaarts doorstroomde systemen die wereldwijd worden gebruikt om industrieel afvalwater te zuiveren. Hierbij worden vervuilende stoffen in het afvalwater omgezet naar methaan, een gas dat kan worden hergebruikt, bijvoorbeeld voor verwarmingsdoeleinden. Door het toepassen van een bepaald

(hydraulisch) regime in de reactoren worden korrels (korrelslib) gevormd, die voornamelijk bestaan uit acetogene (acetaatvormende) en methanogene (methaanvormende) bacteriën. Het slib dat in dit onderzoek werd gebruikt is gekweekt in drie reactoren, die werden gevoed met methanol, een mengsel van vluchtige vetzuren of sucrose. Door deze substraten gedurende langere tijd toe te dienen ontstaan verschillende bacteriepopulaties die model staan voor populaties in "echte" grootschalige reactoren.

In dit onderzoek moest duidelijk worden in hoeverre eerder genoemde acetogene en methanogene bacteriën in korrelslib (maar ook in andere milieus, zoals vervuilde grond) in staat zijn gechloreerde koolwaterstoffen om te zetten en welke produkten hierbij worden gevormd. Bij de afbraak van de verbindingen is verder nagegaan of de omzetting werd uitgevoerd via cometabole reacties. Door het slib te autoclaveren, hebben we getracht een onderscheid te maken tussen abiotische en enzymatische reacties (Fig. 9.2). Abiotische reacties zijn chemische reacties van bulkchemicaliën in het medium (nr. 1 in Fig. 9.2) of reacties die worden gekatalyseerd door cofactoren en/of redoxmediatoren van (dode) cellen (nr. 2 in Fig. 9.2). Cofactoren vormen het actieve centrum van onder andere de enzymen die de aspecifieke cometabole dechloreringsreacties uitvoeren. Ze zijn hitte-stabiel en kunnen vaak ook op zichzelf een dechloreringsreactie uitvoeren. Dechlorerende activiteit door geautoclaveerd slib zou daarom kunnen wijzen op het voorkomen van cometabolische aspecifieke reacties in levend slib, omdat in geautoclaveerd slib de cofactoren (die onderdeel uitmaken van de cometabole enzymen) nog actief zijn.



Figuur 9.2 Abiotische en enzymatische dechloreringsmechanismen.

Voorbeelden van belangrijke cofactoren zijn vitamine B_{12} en cofactor F_{430}, die betrokken zijn bij algemeen voorkomende processen in anaërobe bacteriën, zoals acetaat- en methaanvorming. Naast cofactoren kunnen er ook andere zogenaamde redoxmediatoren aanwezig zijn in de anaërobe bacteriën. De redoxmediatoren zijn verbindingen die gemakkelijk electronen kunnen opnemen en weer afstaan. Enzymatische reacties (Fig. 9.2) worden gekatalyseerd door complete aspecifieke cometabolische enzymen (nr. 3 in Fig. 9.2) of door specifieke enzymsystemen (nr. 4 in Fig. 9.2) van halorespirerende bacteriën. Deze laatste enzymsystemen kunnen alleen in levend korrelslib worden aangetoond omdat de specificiteit wordt bepaald door het hitte-labiele enzymgedeelte dat het gechloreerde substraat "herkent".

Wanneer er geen afbraak van gechloreerde verbindingen in aanwezigheid van geautoclaveerd slib wordt waargenomen, sluit dit het voorkomen van aspecifieke cometabole dechlorering niet uit. In zo'n geval kan het simpelweg zo zijn dat de aanwezigheid van hele (intacte) enzymen noodzakelijk is voor de omzetting. De dechloring van zo'n verbinding door levend slib moet dan wel instantaan (zonder adaptatie of lange lag-phases) plaatsvinden (aangenomen dat de condities geschikt zijn voor dechloreringsreacties).

De omzetting van gechloreerde verbindingen door methanogeen korrelslib

De drie methanogene korrelslibben die in dit onderzoek werden gebruikt waren in staat een grote diversiteit aan gechloreerde verbindingen om te zetten (Tabel 8.1, Fig. 8.2). Ondanks het feit dat de slibben een verschillende samenstelling hadden waren de verschillen in dechlorerende activiteit minimaal. Eén van de mogelijke oorzaken hiervoor is het feit dat aspecifieke dechlorerende activiteit bij verschillende groepen anaërobe bacteriën kan worden aangetroffen (Hoofdtuk 1), waardoor de samenstelling van de slibben van minder groot belang wordt.

Aangezien een substantieel gedeelte van de hoger gechloreerde alkanen (met name chloormethanen en chloorethanen) werd afgebroken in de aanwezigheid van geautoclaveerd slib, is het duidelijk dat abiotische omzettingen een belangrijke rol spelen bij de omzetting van deze gechloreerde verbindingen (Hoofdstukken 2 en 4) (Tabel 8.1). De abiotische dechlorering verliep met relatief hoge snelheid vergeleken met de dechlorering die met levend slib werd waargenomen (Fig. 8.2). In aanwezigheid van levend slib werden chloormethanen volledig gedechloreerd naar CO_2 (Hoofdstuk 2). Hoger gechloreerde (hexa-, penta-, and tetra-) chloorethanen werden naar lager gechloreerde ethanen en ethenen omgezet

(Hoofdstuk 4). Ook lager gechloreerde ethanen werden afgebroken, zij het met een lage snelheid. De tri- en dichloorethanen werden echter niet omgezet door geautoclaveerd slib. In dat geval was de aanwezigheid van hele enzymsystemen dus vereist voor de dechlorering. Verhoging van de concentratie van de gechloreerde verbinding en de biomassa hebben een positief effect op de omzettingssnelheid van tetrachloormethaan (Hoofdstuk 3).

In tegenstelling tot de chloormethanen en -ethanen, werd de omzetting van β-HCH, de chloorethenen en de gechloreerde aromatische verbindingen niet waargenomen in aanwezigheid van geautoclaveerd slib (Tabel 8.1). β-HCH, een verbinding die verondersteld wordt relatief persistent te zijn onder anaërobe omstandigheden, werd echter wel gemakkelijk afgebroken door de levende ongeadapteerde methanogene korrelslibben. Hierbij worden benzeen en chloorbenzeen gevormd (Hoofdstuk 5). De afbraak van gechloreerde alkenen (tetrachlooretheen en lager gechloreerde ethenen) door ongeadapteerde consortia verliep bij lage snelheid (Hoofdstuk 6) en via een ongewone dechloreringsroute. Terwijl bij de afbraak van tetrachlooretheen door reincultures en de meeste mengcultures normaliter *cis*-1,2-dichlooretheen wordt gevormd (hetgeen dan soms verder wordt omgezet tot vinylchloride en etheen), werd door korrelslib voornamelijk 1,1-dichlooretheen gevormd dat snel werd omgezet naar vinylchloride.

Het slib in onze experimenten was zoals reeds vermeld niet vooraf blootgesteld aan gechloreerde koolwaterstoffen. Omdat de gechloreerde aromatische verbindingen niet of nauwelijks werden omgezet (Fig. 8.2), is het waarschijnlijk dat de omzetting van dit soort verbindingen meer specifieke enzymen vereist dan de enzymen die reeds in dit niet geadapteerde korrelslib aanwezig zijn. Blijkbaar was het aantal gespecialiseerde halorespirerende bacteriën in de slibben erg laag.

Anaërobe omzetting van gechloreerde verbindingen in vervuilde grond

Uit de experimenten met het korrelslib is gebleken dat acetogene en methanogene bacteriën een redelijk dechlorerend vermogen hebben. Deze bacteriën, die ook aanwezig zijn in andere anaërobe milieus, zouden gedeeltelijk verantwoordelijk kunnen zijn voor de verscheidenheid aan produkten die kunnen worden aangetroffen op lokaties die vervuild zijn met gechloreerde koolwaterstoffen. Het is echter niet onwaarschijnlijk dat in vervuilde grond meer gespecialiseerde bacteriën (tevens) verantwoordelijk zijn voor de dechlorering, aangezien de vervuiling vaak al gedurende langere tijd aanwezig is en er dus voldoende tijd is geweest voor de ontwikkeling van een geschikte microbiële populatie. Vaak zijn de redoxomstandigheden

echter niet optimaal voor dechlorering. Biologische "reiniging" van zo'n lokatie (en de ontwikkeling van een gespecialiseerde bacteriële populatie) kan dan worden gestimuleerd door de ontwikkeling van anaërobe condities door bijvoorbeeld de toevoeging van korrelslib of het stimuleren van methanogene activiteit *in situ*, aangezien hierdoor de redoxpotentiaal aanzienlijk verlaagd kan worden.

In het geval dat gespecialiseerde bacteriën niet aanwezig zijn, zouden acetogene en methanogene bacteriën echter toch een substantiële bijdrage kunnen leveren aan de omzetting van gechloreerde koolwaterstoffen. Dit is onder andere gebleken uit onderzoek met β-HCH in vervuilde grond (Hoofdstuk 5). De micro-organismen in deze grond waren niet actief, totdat de grond werd overgebracht naar een anaëroob medium met een voedingsbron, waarna wel dechlorerende activiteit werd waargenomen. Natuurlijk hoeft dit niet noodzakelijkerwijs te beteken dat er geen specifieke dechlorerende bacteriën aanwezig waren in de grond. Wel wordt duidelijk dat microbiële dechlorerende activiteit alom aanwezig kan zijn in vervuilde grond. Immers, in de afgelopen jaren zijn talrijke gevallen van dechlorerende activiteit door de natuurlijke microbiële populatie in vervuilde grond en sedimenten waargenomen.

Conclusies en toekomstperspectieven

De resultaten die zijn verkregen in dit proefschrift suggereren een brede toepasbaarheid van methanogene bacteriële consortia zoals korrelslib, voor de omzetting en in sommige gevallen volledige afbraak van gechloreerde verbindingen zonder voorafgaande adaptatie. Gechloreerde (cyclo)alkanen worden in hoge mate afgebroken. In het geval van gechloreerde methanen en gechloreerde ethanen verloopt een belangrijk deel van de totale dechlorerende capaciteit van het slib via abiotische reacties. De lager gechloreerde (afbraak)produkten die worden gevormd in het dechloreringsproces zijn veelal verder biologisch afbreekbaar onder aërobe (zuurstofhoudende) omstandigheden (Fig. 8.2). Toepassing van korrelslib is dus een geschikte eerste stap in de sequentiële anaërobe-aërobe behandeling van afvalstromen die zijn vervuild met hoger gechloreerde koolwaterstoffen. Gechloreerde aromatische verbindingen worden minder gemakkelijk afgebroken dan gechloreerde (cyclo)alkanen. Na een adaptatieperiode, die vermoedelijk nodig is voor ophoping van een geschikte microbiële populatie, zouden misschien zelfs meervoudig gechloreerde aromatische verbindingen (in ieder geval gedeeltelijk) kunnen worden gedechloreerd.

Door de toepassing van methanogene consortia in de bioremediatie van vervuilde afvalstromen, kan een reductie van het risico voor de volksgezondheid worden bereikt. De "toevallig voorkomende" reacties in korrelslib resulteren in een reductie van gechloreerde koolwaterstoffen. Dit zou echter ook nadelig kunnen zijn als de dechloreringsreacties niet volledig verlopen en accumulatie van toxische (eind)produkten optreedt. Een voorbeeld hiervan is de dechlorering van tetrachlooretheen (PER), dat veel wordt gebruikt in stomerijen. Hierbij ontstaat vinylchloride (VC) dat een meer carcinogene en veel vluchtiger stof is dan PER. Een tweede probleem kan zijn dat de produkten die worden gevormd tijdens de anaërobe afbraak vaak minder goed aan de bodem adsorberen dan hun substraten. Enerzijds kan dit voordelig zijn omdat op deze manier de biobeschikbaarheid wordt vergroot en de volgende, aërobe stap kan worden versneld. Anderzijds kan de hogere mobiliteit van de dechloreringsprodukten natuurlijk ook nadelig zijn als de dechlorering plaatsvindt in de bodem en de milieucondities niet geschikt zijn voor de (volledige) mineralisatie van de produkten. De produkten zullen zich dan met het grondwater in een groter gebied verspreiden.

Door de abiotische en aspecifieke aard van de reacties kan anaëroob korrelslib worden gebruikt voor de remediatie van afvalstromen die zijn vervuild met een mengsel van gechloreerde koolwaterstoffen, bijvoorbeeld grondwater en afvalwater. Omdat korrelslib relatief gemakkelijk hanteerbaar is, zou het ook nuttig kunnen zijn voor de toepassing van *in situ* reiniging van vervuild grondwater. Als de milieucondities gunstig zijn, en de vervuilde grond de geschikte bacteriën, die in staat zijn remediatie uit te voeren bevat, zal de toevoeging van korrelslib in principe niet noodzakelijk zijn om verdergaande afbraak van gechloreerde koolwaterstoffen te bewerkstelligen. De aanwezigheid van een methanogeen consortium zoals korrelslib, kan wel leiden tot een snellere vorming van methanogene condities, waardoor ook de milieucondities voor gespecialiseerde dechlorerende bacteriën verbeteren.

Een andere toepassing van korrelslib zou kunnen liggen in de omzetting van nitro- en azoaromatische verbindingen in afvalwater van de textiel en petrochemische industrie, omdat de eerste stap in de anaërobe afbraak van dat soort verbindingen ook een reductie is. Daarnaast zou gebruik gemaakt kunnen worden gemaakt van de aanwezigheid van facultatief anaërobe (kunnen in de aanwezigheid van zuurstof functioneren) of microaërofiele (hebben een lage zuurstofconcentratie nodig) bacteriën in het slib. Deze bacteriën kunnen persistente lager gechloreerde produkten in aanwezigheid van zuurstof afbreken. Recentelijk hebben wij aangetoond dat natuurlijk voorkomende facultatief anaërobe en microaërofiele bacteriën in methanogeen slib in staat zijn om chloorbenzeen en benzeen dat gevormd werd in de afbraak

van HCH, af te breken zodra ze zijn voorzien van zuurstof. De omzettingsprodukten werden (nog) niet nader geïdentificeerd.

Samenvattend kan gesteld worden dat ongeadapteerd methanogeen korrelslib een brede toepasbaarheid heeft en bruikbaar gereedschap is voor de (in situ) bioremediatie van vervuilde milieucompartimenten, zoals afvalwater, grondwater en bodem.

Notations

Acronym	Compound
A	ethane
Aroclor	technical mixture of PCB
c	cis
c/t	cis and trans
CA	chloroethane
CB	chlorobenzene
CF	chloroform
CT	carbon tetrachloride
DCA	dichloroethane
11DCA	1,1-dichloroethane
12DCA	1,2-dichloroethane
DCCH	dichlorocyclohexadiene
DCE	dichloroethene
11DCE	1,1-dichloroethene
cDCE	*cis*-1,2-dichloroethene
tDCE	*trans*-1,2-dichloroethene
DCM	dichloromethane
E	ethene
HCA	hexachloroethane
HCB	hexachlorobenzene

Acronym	Compound
HCH	hexachlorocyclohexane
MC	methyl chloride
PCA	pentachloroethane
PCB	polychlorinated biphenyls
PCE	tetrachloroethene
PCP	pentachlorophenol
QCB	pentachlorobenzene
TCA	trichloroethane
111TCA	1,1,1-trichloroethane
112TCA	1,1,2-trichloroethane
TCE	trichloroethene
TeCA	tetrachloroethane
TeCE	tetrachloroethene
1112TeCA	1,1,1,2-tetrachloroethane
1122TeCA	1,1,2,2-tetrachloroethane
TeCCH	tetrachlorocyclohexene
UASB	upflow anaerobic sludge blanket
VC	vinylchloride
VFA	volatile fatty acids
VSS	volatile suspended solids

Curriculum vitae

Miriam Henrica Augusta van Eekert werd te 's Hertogenbosch geboren op 25 mei 1966. In 1984 behaalde zij haar Atheneum B diploma op het St. Bonifatiuscollege te Utrecht. Daarna ging zij Moleculaire Wetenschappen aan de Landbouwhogeschool Wageningen studeren. De afstudeeronderzoeken vonden plaats op de vakgroepen Waterzuivering (anaërobe zuivering van afvalwater uit de papier- en pulpindustrie) en Microbiologie (afbraak van aromatische verbindingen door gisten en schimmels). Voor haar stage verbleef ze gedurende 8 maanden in Indonesië (toepassing van anaërobe zuivering voor de behandeling van zogenaamd "zwart" en "grijs" afvalwater). In 1990 studeerde zij af.
In 1991 werd zij aangesteld als wetenschappelijk medewerker op het toenmalige Nederlands Instituut voor Koolhydraat Onderzoek (NIKO) TNO (inmiddels behorend bij TNO Voeding) te Groningen op de afdeling microbiologie en waterzuivering.
Vanaf 1993 was zij als AIO verbonden aan de vakgroepen Microbiologie en Milieutechnologie van de Landbouwuniversiteit Wageningen (LUW). Het onderzoek dat ze in de periode 1993-1997 uitvoerde is beschreven in dit proefschrift. Daarna is ze bij het Laboratorium voor Microbiologie van de LUW betrokken geweest bij kortdurende (literatuur)onderzoek op het gebied van bodemreiniging en de *in situ* ruiming van landmijnen.
Momenteel is zij (sinds 1-1-99) in Apeldoorn werkzaam bij TNO Milieu, Energie en Procesinnovatie (MEP) op de afdeling Milieubiotechnologie (MBT).