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Anaerobic transformation of chlorinated hydrocarbons In a packed-bed reactor

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INTRODUCTION

Chlorinated aliphatic hydrocarbons (CAHs) belong to the most frequently found contaminants in soil and groundwater. Most of these contaminations result from careless handling during production, transport, application of CAHs and unproper waste disposal. Although emission of CAHs by mankind has been strongly reduced over the last decade, large amounts of CAHs are still being released into air and (ground)water. Table 1.1 lists the origin and emission of CAHs in the Netherlands in 1995. In addition to an anthropogenic origin, contamination of soil and groundwater with trace levels of CAHs may also originate from natural sources. The main sources of natural production of CAHs such as chloromethane, chloroform and perchloroethylene are marine algae, fungi and volcanic eruptions (Gribble 1994, Hoekstra and de Leer 1995). Emissions of CAHs from natural origin are primarily released into the atmosphere. Due to deposition these emissions also result in contamination of soil.

Table 1.1 *Emission and the source of emission of chlorinated aliphatic hydrocarbons in the Netherlands in 1995.*

Chlorinated hydrocarbon	**Abbreviation**	**Application**[a]	**Emission**[a] **(ton/yr)**	
			air	water
vinylchloride	VC	PVC production	87.1	0
trichloroethene	TCE	solvent, dry cleaning	991	0.73
perchloroethene	PCE	metal degreasing	2520	0.41
1,2-dichloroethane	1,2-DCA	PVC production	156	6.46
1,1,1-trichloroethane	TCA	metal degreasing	1120	0.29
dichloromethane	DCM	rubber-, medicine industry,	2640	--
chloroform	CF	solvent	29.9	3.19
carbon tetrachloride	CT	solvent, fiber industry	131	0.88
		solvent, extraction		

[a] source: Ministry of Housing, Spatial Planning and the Environment (1997)

Soils contaminated with CAHs are a potential threat to man, flora and fauna because CAHs are toxic and persistent or can be transformed into (more) hazardous products. Many governments have recognized the necessity of soil remediation and have introduced regulations for soil cleanup. The Dutch government introduced their first legislation, the Soil Cleanup Act, in 1983, which was replaced by the Soil Protection Act in 1995 (Ministry of Housing, Spatial Planning and Environment, 1995). Nowadays, the urgency and necessity of soil

cleanup is based on the No Observed Effect Concentration and the Admissible Daily Intake of contaminants, expressed in Intervention Values. Whenever Intervention Values are exceeded, the use of soil is assumed to present a risk to man, flora or fauna. On the basis of dispersion rates and exposure risks, the urgency of remediation is established, and depending on the location and its use, a remediation plan must be made (Ministry of Housing, Spatial Planning and Environment 1995).

Three different remediation technologies are available for remediation of soil contaminated with CAHs.

- *First*, technologies that only prevent dispersion of the contaminant and aim at risk-reduction. Examples are excavation and storage in safe deposits, and physical isolation.
- *Second*, technologies such as stripping and extraction that aim at removing the contaminants from the soil and concentrate them. During extraction, the contaminated soil is rinsed with chemicals, while stripping involves the evaporation of contaminants using air or steam, followed by adsorption on activated carbon. Both methods shift the contamination from one environmental compartment to another and thus transfer but not solve the problem. Moreover, with extraction large amounts of chemicals have to be removed after cleanup and the residue concentrations of the contaminant usually are rather high (van den Berg et al. 1992).
- *Third*, we distinguish remediation technologies which not only aim at the removal but also at the destruction of the contaminants, like thermal processing and bioremediation. During thermal cleaning the soil is heated and the contaminant is burned or immobilized (in the case of heavy metals). The residual concentrations after the burning of CAHs usually are very low but the technology is expensive, the quality of the soil residue is low, and during burning of CAHs there is a real chance that dioxins are formed.

Another fairly new technology for soil cleanup is bioremediation, the biological degradation of contaminants by (micro)organisms or higher organisms like trees and plants (phytoremediation). In the early 80s it was first recognized that CAHs, previously considered non-biodegradable, are susceptible to biological degradation (Brunner et al. 1980, Rittmann and McCarty 1980, Bouwer et al. 1981, Bouwer and McCarty 1983a, 1983b). Many researchers have since studied the biological transformation of CAHs. Often, microbial dechlorination processes are divided into aerobic and anaerobic processes. In aerobic processes oxygen serves as an electron acceptor, whereas under anaerobic conditions other electron acceptors such as iron(III), nitrate, sulfate, carbon

dioxide or the CAHs themselves are used. This introduction will focus on anaerobic dechlorination processes since many contaminated sites in the Netherlands and in large parts of Europe are depleted of oxygen. Moreover, the transformation of highly chlorinated aliphatic hydrocarbons such as carbon tetrachloride and 1,1,1-trichloroethane has only been described under anaerobic conditions and is not likely to occur in the presence of oxygen.

Thermodynamics of anaerobic biotransformation

Chemical reactions are controlled both by thermodynamics, i.e. the change of the Gibbs free energy ($\Delta G^{0'}$) and by kinetic aspects, i.e. the free energy of activation ($\Delta G^{\ddagger}$). A reaction can only proceed if it is energetically favorable, i.e. has an overall negative $\Delta G^{0'}$. Table 1.2 shows the Gibbs free energy change and redox potential difference for all major half-reactions of the reduction of chlorinated methanes, ethanes and ethenes and electron acceptors such as sulfate, nitrate and carbon dioxide. These data show that under standard conditions ($[H^+]=10^{-7}$ M; $[Cl^-]=10^{-3}$ M; T=25°C) the reductive dechlorination of nearly all CAHs is an energetically favorable (exergonic) reaction. Many dechlorination reactions potentially yield more energy than other naturally occurring redox processes such as sulfate reduction, or carbon dioxide reduction during methanogenesis. This implies that biological reductive dechlorination processes may occur in nature.

The rate of a thermodynamically feasible reaction is controlled by the free energy of activation ($\Delta G^{\ddagger}$) according to equation I (Solomons 1996).

$$k = k_0 e^{-\Delta G^{\ddagger}/RT} \qquad \text{(I)}$$

In this equation, k is the rate constant (s^{-1}), k_0 is the absolute rate constant (s^{-1}), which equals the rate at which all transition states proceed to products, R is the gas constant (8.31 $J \cdot mol^{-1} \cdot K^{-1}$) and T is the temperature (K). Because the relationship between k and the $\Delta G^{\ddagger}$ ($kJ \cdot mol^{-1}$) is exponential, a reaction with a lower $\Delta G^{\ddagger}$ will occur much faster than a reaction with a higher one (Solomons 1996).

Table 1.2 *Half reactions of reductive transformations of chlorinated aliphatic hydrocarbons and conventional electron acceptors.*

Electron acceptor	Half-reaction of reductive transformations		$\Delta G^{0'}$/electron (kJ)[a]	E_0' (mV)[b]
O_2	**$O_2 + 4H^+ + 4e^-$**	**$\rightarrow 2H_2O$**	**- 78.7**	**816**
1,2-DCA	$CH_2Cl\text{-}CH_2Cl + 2e^-$	$\rightarrow C_2H_4 + 2Cl^-$	- 71.3	739
CT	$CCl_4 + H^+ + 2e^-$	$\rightarrow CHCl_3 + Cl^-$	- 65.0	674
MnO_2	**$MnO_2 + HCO_3^- + 3H^+ + 2e^-$**	**$\rightarrow MnCO_3 + 4H_2O$**	**- 58.9**	**610**
PCE	$C_2Cl_4 + \ + H^+ + 2e^-$	$\rightarrow C_2HCl_3 + Cl^-$	- 55.3	574
TCA	$C_2H_3Cl_3 + H^+ + 2e^-$	$\rightarrow C_2H_4Cl_2 + Cl^-$	- 54.1	561
CF	$CHCl_3 + H^+ + 2e^-$	$\rightarrow CH_2Cl_2 + Cl^-$	- 54.0	560
TCE	$C_2HCl_3 + + H^+ + 2e^-$	$\rightarrow$ *cis* $\text{-}C_2H_2Cl_2 + Cl^-$	- 53.0	550
TCE	$C_2HCl_3 + \ H^+ + 2e^-$	$\rightarrow$ *trans* $\text{-}C_2H_2Cl_2 + Cl^-$	- 50.9	528
TCE	$C_2HCl_3 + H^+ + 2e^-$	$\rightarrow 1,1\text{-}C_2H_2Cl_2 + Cl^-$	- 50.8	527
DCM	$CH_2Cl_2 + H^+ + 2e^-$	$\rightarrow CH_3Cl + Cl^-$	- 47.5	492
CM	$CH_3Cl + H^+ + 2e^-$	$\rightarrow CH_4 + Cl^-$	- 45.2	468
CA	$C_2H_5Cl + H^+ + 2e^-$	$\rightarrow C_2H_6 + Cl^-$	- 44.5	461
VC	$C_2H_3Cl + 2H^+ + e^-$	$\rightarrow C_2H_4 + Cl^-$	- 43.4	450
NO_3^-	**$NO_3^- + 2H^+ + 2e^-$**	**$\rightarrow NO_2^- + H_2O$**	**- 41.7**	**432**
1,1-DCE	$(1,1\text{-})C_2H_2Cl_2 + H^+ + 2e^-$	$\rightarrow C_2H_3Cl + Cl^-$	- 40.5	420
trans-DCE	*trans* $\text{-}C_2H_2Cl_2 + H^+ + 2e^-$	$\rightarrow C_2H_3Cl + Cl^-$	- 40.4	419
cis-DCE	*cis*$\text{--}C_2H_2Cl_2 + H^+ + 2e^-$	$\rightarrow C_2H_3Cl + Cl^-$	- 38.3	397
DCA	$C_2H_4Cl_2 + H^+ + 2e^-$	$\rightarrow C_2H_5Cl + Cl^-$	- 38.3	397
1,2-DCA	$CH_2Cl\text{-}CH_2Cl + H^+ + 2e^-$	$\rightarrow C_2H_5Cl + Cl^-$	- 36.2	375
$Fe(OH)_3$	**$Fe(OH)_3 + 3H^+ + e^-$**	**$\rightarrow Fe^{2+} + 3H_2O$**	**- 11.4**	**118**
SO_4^{2-}	**$SO_4^{2-} + 9H^+ + 8e^-$**	**$\rightarrow HS^- + 4H_2O$**	**+ 20.9**	**- 217**
HCO_3^-	**$HCO_3^- + 9H^+ + 8e^-$**	**$\rightarrow CH_4 + 3H_2O$**	**+ 23.0**	**- 238**

[a] Calculated on the basis of data from Thauer et al. (1977), Dolfing and Janssen (1994) and Vogel et al. (1987), assuming the following conditions: H^+, 10^{-7} M; Cl^-, 10^{-3} M.
[b] The redox potentials were calculated from $\Delta G^{0'}$ according to Thauer et al. (1977) with the relationship $E_0' = (\Delta G^{0'}/-nF)$. n= number of electron equivalents transferred= 1, F= Faraday's constant (96.487 kJ/volt-equivalent)

The $\Delta G^{\ddagger}$ is equal to the difference in free energy between the transition state ($G_S^{\ddagger}$) and the substrate (G_S) according to equation II (Stryer 1995), and depends on the kinetics of bond and solvent reorganization during a reaction. During a reaction, bonds are stretched or compressed, bond angles are changed, and torsional movements are made causing a change in free energy (Perlinger 1994).

$$\Delta G^{\ddagger} = G_{S\ddagger} - G_S \qquad \text{(II)}$$

The $\Delta G^{\ddagger}$ is independent of the $\Delta G^{0'}$, which means that even if a (dehalogenation) reaction is energetically favorable (negative $\Delta G^{0'}$), the reaction rate can be very low as a result of a high activation energy. The $\Delta G^{\ddagger}$ of dechlorination reactions could explain why many reductive dechlorination reactions are not found in nature, despite of favorable Gibbs free energies. Enzymes may decrease the $\Delta G^{\ddagger}$ in microorganisms by stabilizing the transition state ($G_{S\ddagger}$) or by allowing a different reaction pathway with a lower $\Delta G^{\ddagger}$. Up to now, nearly no information is available about the $\Delta G^{\ddagger}$ of dechlorination reactions. However, even though the $\Delta G^{\ddagger}$ is independent of the $\Delta G^{0'}$, several relationships between the $\Delta G^{\ddagger}$ and the $\Delta G^{0'}$ have been described (Perlinger 1994). These relationships usually describe a negative non-linear correlation between the $\Delta G^{\ddagger}$ and the $\Delta G^{0'}$. This means that the $\Delta G^{\ddagger}$ decreases when the $\Delta G^{0'}$ of a reaction decreases (becomes more negative).

Metabolic versus cometabolic transformation

The previous section provides evidence that most anaerobic dechlorination reactions are energetically favorable (exergonic) processes. Microorganisms that catalyze dechlorination reactions can either couple these transformations to their metabolism and benefit from the energy released during the transformation of CAHs or may carry out dechlorination reactions according to a cometabolic process. In a **cometabolic process**, dechlorination is not coupled to growth and is a form of gratuitous metabolism carried out by enzymes or cofactors which normally catalyze other reactions. The microorganism involved has no apparent benefit from a cometabolic transformation (Janssen and de Koning 1995). Under anaerobic conditions, enzyme systems such as iron-sulfur clusters, cobalamins (Krone et al. 1989a, 1991), factor F_{430} (Krone et al. 1989b, Gantzer and Wackett 1991) or hematin (Klecka and Gonsior 1984) can take part in side reactions that yield cometabolic transformation of CAHs. All of these enzyme systems contain redox-active metal centers and are usually referred to as transition-metal cofactors. In anaerobic bacteria, transition-metal cofactors have a biological role as electron carriers. Since electron transfer by these transition-metal cofactors is not very specific, a wide range of CAHs can be transformed. Cometabolic dechlorination by transition metal cofactors usually yields reductive dechlorination (Gantzer and Wackett 1991).

In a **metabolic dechlorination process** microorganisms couple dechlorination to energy conservation and thus use the CAHs as a source of metabolic energy. Three different mechanisms can be distinguished where microbial transformation of CAHs is coupled to energy conservation under anoxic conditions. First, CAHs can be transformed via oxidative conversions and act as an electron donor and source of organic carbon (primary substrate). The presence of an electron acceptor such as iron (III), nitrate, sulfate or carbon dioxide is required in these reactions. While oxidative dechlorination of CAHs is a common process under aerobic conditions (Hartmans et al. 1986, Brunner et al. 1980, Hartmans et al. 1985, Janssen et al. 1985), under anoxic conditions it is only known for DCM (Kohler-Staub et al. 1995). DCM was oxidized under nitrate reducing conditions with nitrate serving as electron acceptor.

Second, in metabolic reductive dechlorination processes, the chlorinated hydrocarbon serves as a terminal electron acceptor for anaerobic microorganisms. When this reaction is coupled to growth, the process is termed (de)halorespiration (Holliger and Schumacher 1994). *Desulfomonile tiedje* was the first organism reported to carry out (de)halorespiration (Dolfing 1990, Mohn and Tiedje 1990). This bacterium couples reductive dechlorination of 3-chlorobenzoate to growth. Over the last five years, several microorganisms have been isolated that can carry out respiratory reductive dechlorination of perchloroethylene (Scholz-Muramatsu et al. 1995, Gerritse et al. 1996, Krumholz et al. 1996, Wild et al. 1996, Maymo-Gatell et al. 1997, Holliger et al. 1998). Most of these organisms reduce perchloroethylene via trichloroethene to *cis*-1,2-dichloroethene.

The third known mechanism for metabolic dechlorination is fermentation. In the case of fermentative conversions, the chlorinated hydrocarbon serves as an electron donor, electron acceptor and carbon source. Both chloromethane and dichloromethane are known to be fermented by homoacetogenic bacteria to acetic acid (Mägli et al. 1996, Traunecker et al. 1991). Both are energetically very favorable processes. The $\Delta G^{0'}$ value for chloromethane conversion to acetic acid (-105 kJ/mole) is nearly twice as negative as that for methanol fermentation (-53 kJ/mole) (Traunecker et al. 1991). The $\Delta G^{0'}$ for dichloromethane fermentation to acetic acid (-259 kJ/mole) is even lower than for chloromethane fermentation.

Metabolic dechlorinations are coupled to energy conservation and thus are beneficial to the dechlorinating microorganisms. In contrast, microorganisms involved in cometabolic dechlorinations have no apparent benefit from these transformations and therefore have no driving force for carrying out these reactions. Often, cometabolic transformations even present a disadvantage for the

microorganism since the input of reducing equivalents cost energy or because toxic transformation products are formed (Janssen and de Koning 1995).

Factors affecting anaerobic biotransformation of CAHs

Anaerobic microbial dechlorination processes are catalyzed by anaerobic bacteria that produce enzymes or cofactors that react with chlorinated substrates. The presence and activity of these organisms is influenced by environmental conditions, such as temperature, pH and the availability of electron donors and acceptors. These will be discussed in the following paragraphs.

Electron acceptors

Anaerobic reductive dechlorinations have been encountered in the presence of all conventional electron acceptors (CO_2, SO_4^{2-}, Fe^{3+}, NO_3^-, Mn^{4+}, O_2). The highest transformation rates are usually found at low redox potentials i.e. under carbon dioxide reducing and sulfate reducing conditions. For metabolic dechlorination processes this can be understood on the basis of the redox potential coupled to these electron acceptor conditions. By changing the predominant electron acceptor from nitrate to sulfate or carbonate, the redox potential decreases and reductive dechlorination becomes energetically more favorable compared to the reduction of the conventional electron acceptors (Vogel et al. 1987; Table 1.2). Also upon a decrease of the redox potential in the environment a higher number of microorganisms may be present which contain strongly reduced electron carriers (transition-metal cofactors) such as ferredoxins (E_0'=-398 mV; Stryer 1995) and vitamin B_{12} (E_0'= -530 to -560 mV; Chiu and Reinhard 1996). These transition-metal cofactors readily react with CAHs. As a result, the rate of cometabolic dechlorinations usually increases with a decrease of the redox potential.

Besides the effects of the availability of electron acceptors on the redox potential, they can also directly inhibit cometabolic reductive dechlorination of chlorinated hydrocarbons as a result of either toxicity or of competition for available electrons. The concept of competition for available electron donor is discussed below.

Electron donors

All bacterial reductive dehalogenation processes depend on the presence of an electron donor, unless the halogenated compound is converted by fermentation like dichloromethane by *Dehalobacterium formicoaceticum* (Mägli et al. 1996) or by phototrophic growth on CAHs such as the growth of *Rhodopseudomonas palustris* on 3-chlorobenzoate and 3-bromobenzoate (van der Woude et al. 1994). Consequently, CAHs compete with other electron acceptors such as sulfate and nitrate for available electron donor(s). Competition for available electrons can occur either within a single dechlorinating microorganism or between dechlorinating and other non-dechlorinating species. *Shewanella putrefaciens* 200, able to respire anaerobically using NO_3^-, NO_2^-, Fe(III) and trimethylamine oxide, is capable of cometabolic dehalogenation of carbon tetrachloride to chloroform and other unidentified products (Picardal et al. 1993). Whether dechlorination occurs depends on the kinetics of electron transfer to the electron acceptor used. For example, when oxygen is present no transformation of carbon tetrachloride occurs. The rate of electron transfer to oxygen is very high and all electrons are directed towards oxygen (Picardal et al. 1995). A similar mechanism is probably involved in the transformation of carbon tetrachloride by *Escherichia coli* K-12, a microorganism that is able to transform carbon tetrachloride under different electron acceptor conditions (Criddle et al. 1990b).

Competition for electron donors may also occur between dechlorinating and other non-dechlorinating microorganisms (Kohring et al. 1989, Kuhn et al. 1990, Fennell et al. 1997). Whether dechlorination occurs mainly depends on the affinity of the dechlorinating population for available electron donor compared to that of the other population(s) present. For metabolic dechlorinations, the dechlorinating population can only compete successfully with other microorganisms if dechlorination provides enough energy for maintenance and growth. With cometabolic transformations dechlorination has no apparent benefit for the organism, often energy is even lost in the process. Therefore, cometabolic processes usually only occur in the presence of a surplus of electron donor. Doong and Wu (1996) found that the rate of carbon tetrachloride and 1,1,1-trichloroethane transformation increased with an increase in electron donor concentration. However, there are almost no reports on electron donor concentrations and their effect on cometabolic biotransformation of chlorinated hydrocarbons.

Because of competition the affinity of dechlorinating populations for electron donors has a major impact on biological dechlorination (Freedman and Gossett 1989, Bagley and Gossett 1990, Lewis and Crawford 1993, Petrovskis et

al. 1994). The availability of an electron for which dechlorinating microorganisms have a (much) higher affinity than non-dechlorinating anaerobes greatly enhances dechlorination. Most (de)halorespiring microorganisms known can use H_2 as an electron donor and have a higher affinity for H_2 than methanogens or sulfate-reducing microorganisms (Chapelle 1996). Therefore dechlorinating microorganisms have a competitive advantage over other microorganisms at low levels of H_2. Smatlak et al. (1996) reported that the performance of dechlorination is linked to the H_2 concentration. On-site or *in-situ* application of H_2 as an electron donor may not be desirable because of the risk of explosions. Instead, electron donors which represent a slow and steady source of low levels of H_2, such as butyrate, benzoate and propionate (Gossett et al. 1996) or complex substrates like, humic acid, compost extract or BTEX (Fennell et al. 1997) could be used. Further research is needed on the *in-situ* application of slow H_2-releasing compounds and on the availability of the H_2 released for the dehalorespiring microorganisms.

Temperature

Most dechlorinating microorganisms described to date have optimum growth temperatures between 30 and 40°C and an activity range between 15°C and 50°C (Scholz-Muramatsu et al. 1995, Gerritse et al. 1996, Mägli et al. 1996). Temperatures at natural habitats are usually below 15°C which means that *in-situ* or on-site dechlorination would occur at much lower rates or would involve other organisms. For example aerobic transformation of 1,4-dichlorobenzene by *Alcaligenes* sp. strain 175 was nearly completely inhibited when the temperature was changed from 20°C to 10°C (van der Meer et al. 1992). However, dechlorinating microorganisms can have significant dechlorination activities at 10°C which suggests that in natural habitats these microorganisms are better adjusted to lower temperatures than dechlorinating microorganisms enriched at room temperature or higher in the laboratory (de Bruin et al. 1992).

Alkalinity (pH)

Most dechlorinating microorganisms tolerate a pH between pH 5.0 and 9.0 with an optimum pH range between 6.0 and 8.0 (Mägli et al. 1996, Gerritse et al. 1996, Scholtz-Muramatsu et al. 1995, Traunecker et al. 1991). Usually, contaminated groundwaters have a pH within this range, causing no problem for biotransformation in the field. However, many exceptions exist like systems containing high concentrations of humic acid, sulfide or carbonate creating

relatively low or high pH. Biotransformation rates in these systems can be considerably lower.

Nutrients

Nutrients like phosphorus, nitrogen, trace metals and vitamins are required for growth of microorganisms. Specific requirements may be involved in dechlorination reactions by microorganisms, exemplified by the transformation of CAHs by transition metal complexes (Gantzer and Wackett 1991). Lewis and Crawford (1993) found that traces of copper are required for CT transformation by *Pseudomonas* sp. strain KC because copper plays a role in the synthesis of the (unknown) components involved in carbon tetrachloride transformation. Nutrients usually are present in soils and sediments in sufficient concentration but low bioavailability or competition for these nutrients may limit biodehalogenation. Addition of nutrients could enhance biotransformation but almost no information is available on this subject (Mohn and Tiedje 1992).

When the concentration of certain nutrients is too high, biotransformation of CAHs can be inhibited. Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrifying conditions was completely inhibited by even low concentrations (< 10 µM) of iron, cobalt, copper and vanadium (Tatara et al. 1993, Lewis and Crawford 1993, Criddle et al. 1990a). The degree of inhibition depended on the concentration of the nutrients and the pH of the medium. The latter is a result of the change in solubility of metal ions at different pH values. Inhibition of dehalogenation by copper is also known for aerobic cometabolic transformation of CAHs by methanotrophs. In the presence of copper at concentrations as low as 4.8 µM, transformation was nearly completely inhibited (Oldenhuis et al. 1989, Henry and Grbic-Galic 1990). This is caused by the two different classes of methane monooxygenases (MMO) most methanotrophs can produce. When sufficient copper is available, a membrane-bound copper-containing MMO (pMMO) is produced with a high affinity for methane (natural substrate), but a narrow substrate range. Under copper limitation, a soluble MMO (sMMO) is produced with a low affinity for methane but a very broad substrate range. As a result of this broad substrate range methanotrophs expressing sMMO can degrade, besides methane, many chlorinated hydrocarbons.

Biotransformation of chlorinated aliphatic hydrocarbons

In the following paragraphs, the present knowledge on the anaerobic biotransformation of chlorinated methanes, chlorinated ethanes and chlorinated ethenes, will be discussed.

Reaction mechanisms

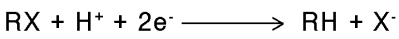
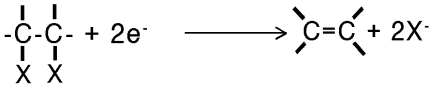
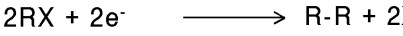
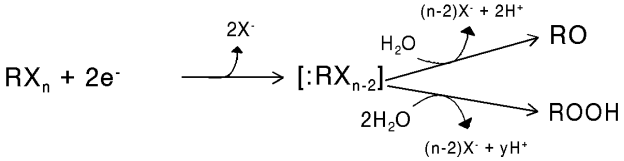
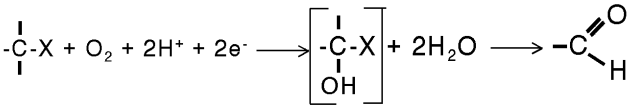
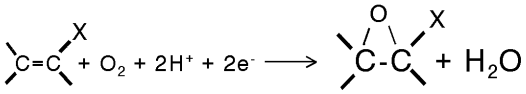
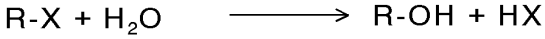
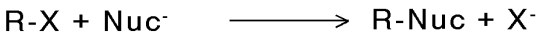
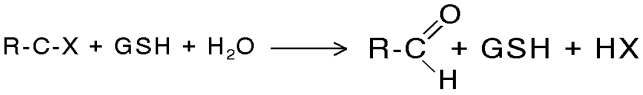
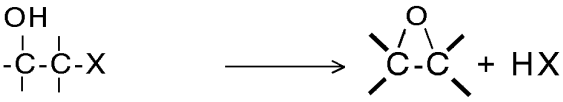
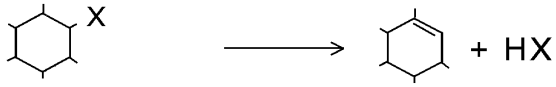
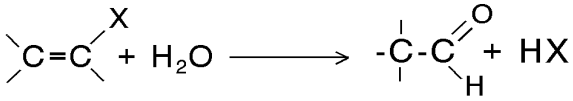
Several different mechanisms have been described for the transformation of halogenated compounds including reduction, oxidation, substitution, hydration and dehydrohalogenation (Vogel et al. 1987, Fetzner and Lingens 1994) (see Table 1.3). The main difference between these reaction mechanisms is in the transfer of electrons. Both reduction and oxidation are electron dependent reactions (redox-reactions) and need either the input of an external electron acceptor or electron donor. This is in contrast with substitution, dehydrohalogenation and hydration reactions. During the course of these reactions, the oxidation state of the reacting molecule does not change and therefore no input of an external electron donor or electron acceptor is needed (non-redox reactions).

Dechlorination by means of oxidation, substitution or hydratation has only been found for aerobic bacteria. Biotransformation of CAHs under **anaerobic conditions** is mainly restricted to four reaction types. These reactions will be discussed in detail. The first are reductive transformations that require the presence of an electron donor. With **hydrogenolysis**, the halogen substituent is replaced by hydrogen, probably according to a nucleophilic substitution mechanism. This type of reaction is the one which occurs most frequently. **Dihalo-elimination** of CAHs is a reductive elimination of two chlorine substituents to form an alkene and is only found with chlorinated alkanes. The third type of reductive transformation is **hydrolytic reduction**, a two electron reduction that is followed by reaction with water, which leads to an oxygenated product. An example is the transformation of carbon tetrachloride to formic acid or carbon monoxide (Criddle and McCarty 1991).

The fourth important reaction which often occurs under anaerobic conditions, **dehydrohalogenation**, does not involve the transfer of electrons and therefore does not directly depend on the presence of either an electron donor or electron acceptor. Dehydrohalogenation involves the elimination of a halogen from one carbon atom followed by the elimination of a hydrogen atom from an

adjacent carbon. This results in the formation of an additional bond between the two carbon atoms (Holliger 1992).

Table 1.3 *Abiotic and biotic reactions of chlorinated aliphatic hydrocarbons (Vogel et al. 1987, Holliger 1992, Fetzner and Lingens 1994).*

Reactions	Mechanisms
Electron transfer dependent reactions	
1. Reduction	
a. hydrogenolysis	
b. dihaloelimination	
c. coupling	
d. hydrolytic reduction	
2. Oxidation	
a. α-hydroxylation	
b. epoxidation	
Electron transfer independent reactions	
3. Substitution	
a. hydrolysis	
b. conjugation	
c. thiolytic dehalogenation	
d. intramolecular substitution	
4. Dehydrohalogenation	
5. Hydration	

Chlorinated methanes

Hydrogenolysis. Reductive dechlorination by hydrogenolysis is an energetically favorable process for all chlorinated methanes (Table 1.2). Chlorinated methanes potentially even are better electron acceptors than carbon dioxide, sulfate, iron(III) and nitrate. Indeed, CT is also a better electron acceptor than manganese(IV). Only under oxygen reducing conditions, hydrogenolytic reductive dechlorination of chlorinated methanes is not preferred.

As expected from the $\Delta G^{0'}$ (Table 1.2), chlorinated methanes resist reductive dechlorination under aerobic conditions. However, under methanogenic (Egli et al. 1987, 1990), acetogenic (Egli et al. 1988, 1990), sulfate reducing (Egli et al. 1987, 1988, 1990, Bouwer and Wright 1988), iron reducing (Picardal et al. 1993, Petrovskis et al. 1994), and nitrate reducing conditions (Bouwer and McCarty 1983b, Criddle et al. 1990a, 1990b) hydrogenolytic reductive dechlorination of CT to chloroform (CF), dichloromethane (DCM) and chloromethane (CM) (Fig. 1.1) was described, both for pure and for mixed cultures. Reduction of DCM to CM has only been described to occur under methanogenic and acetogenic conditions at very low rates (Egli et al. 1988, Mikesell and Boyd 1990). All of these reductive transformations are cometabolic aspecific reactions catalyzed by biologically synthesized transition metal complexes such as vitamin B12 (Stromeyer et al. 1992, Chiu and Reinhard 1996), coenzyme F_{430} (Krone et al. 1989b, Gantzer and Wackett 1991) or corrinoids (Krone et al. 1989a, 1991). The rate of dechlorination by these complexes decreases with each reductive step. This may be caused by an increase of the $\Delta G^{\ddagger}$, related to a decrease of the $\Delta G^{0'}$ with each reductive step (Table 1.2 and see ‘Thermodynamics of anaerobic biotransformation’, this chapter)

Hydrolytic reduction. Besides CF and DCM, carbon dioxide is a main product of CT transformation under methanogenic, acetogenic and nitrate-reducing conditions (Bouwer and McCarty 1983a 1983b, Bouwer and Wright 1988, Criddle et al. 1990a 1990b, Egli et al. 1987 1988). The pathway of CT mineralization is not yet clear, but dechlorination through hydrolytic reduction (Table 1.3) could explain the observed formation of CO_2 from CT. It is generally agreed that the first step of CT transformation according to this pathway is a one-electron reduction to give a trichloromethyl radical and a chloride ion (Fig. 1.1). Dechlorination of the trichloromethyl radical via a second electron transfer step leads to the formation of a dichlorocarbene, which hydrolyzes either to formic acid or carbon monoxide (Krone et al. 1991, Criddle and McCarty 1991). Both these products can be

biologically oxidized to CO_2. Transformation of CT to carbon monoxide has been demonstrated in acetogenic bacteria (Stromeyer et al. 1992, Hashham et al. 1995). Conversion of CT to CO_2 via carbon monoxide by cobalamins in the presence of a strong reducing agent (Krone et al. 1991, Stromeyer et al. 1992, Chiu and Reinhard 1996) suggest that cobalamins are involved in the mineralization of CT to CO_2.

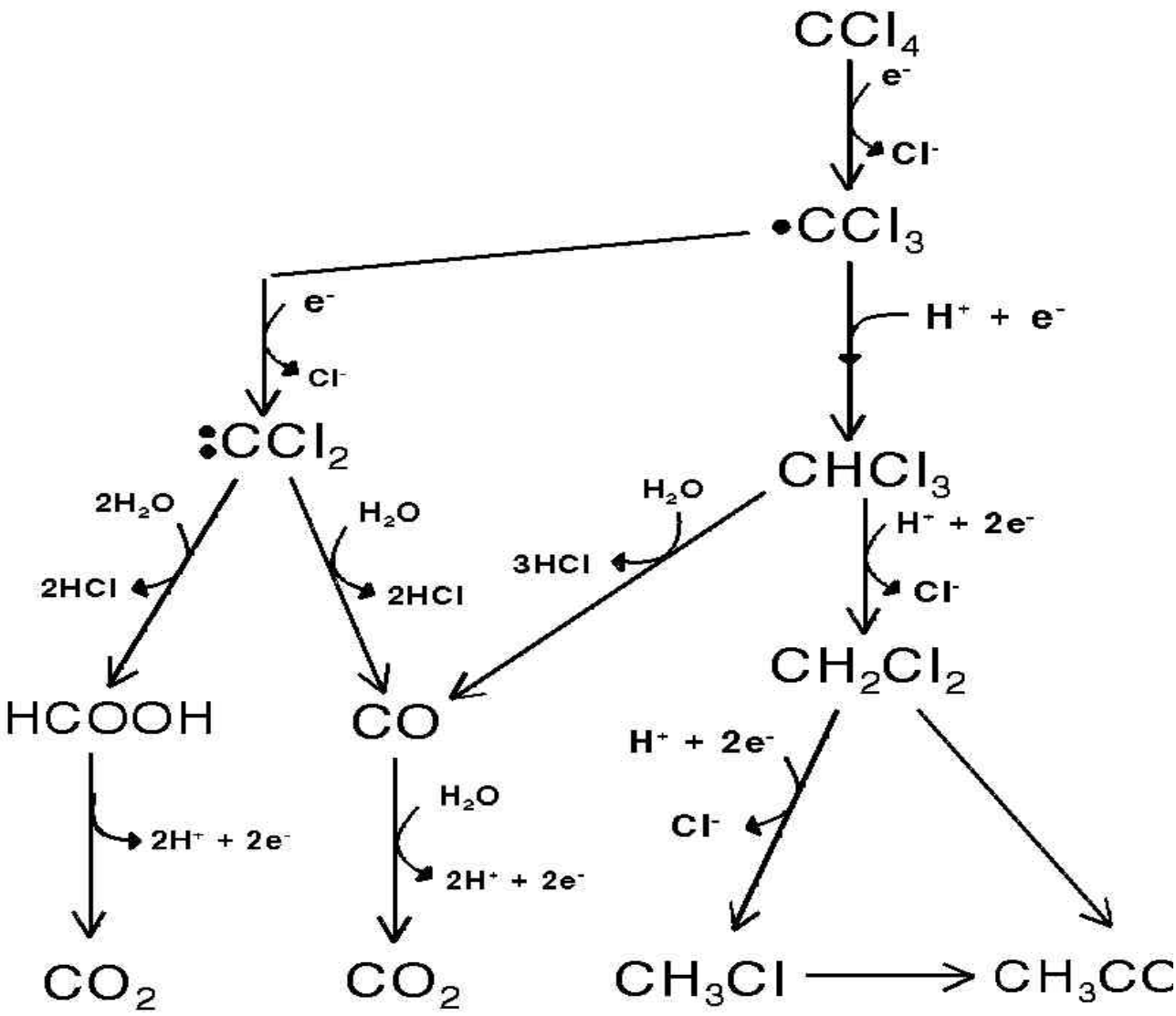


Figure 1.1 *Proposed pathways for the biological transformation of chlorinated methanes.*

Indeed, Hashham et al. (1995) found that CT transformation to carbon monoxide by an anaerobic enrichment culture was enhanced by the presence of cobalamin homologues. This anaerobic enrichment culture also transformed CT to formic

acid, but formic acid only accounted for about 5% of the products formed. Formic acid was also found as a product of CT transformation by *Pseudomonas* sp. strain KC (Dybas et al. 1995), an organism that mainly transforms CT to CO_2. Formic acid was not an intermediate in the pathway of CT transformation to CO_2 by this strain suggesting that another mechanism was involved in CT transformation to CO_2. CT transformation by *Pseudomonas* sp. strain KC is an intracellular as well as an extracellular process, while other dechlorination reactions reported are intracellular (Krone et al. 1989, Stromeyer et al. 1992, Neumann et al. 1996) or membrane associated (Mohn and Tiedje 1992, Schumacher et al. 1997, Miller et al. 1998) processes. The mechanism of CT transformation by strain KC is not yet clear but the results suggested that metal containing cofactors, excreted by strain KC, are involved in the extracellular transformation of CT, similar to the intracellular transformation of CT to carbon monoxide, which also involves these metal containing cofactors (Krone et al. 1989, Stromeyer et al. 1992). The cofactors excreted by *Pseudomonas* sp. strain KC are normally involved in the reduction of iron, explaining the inhibition of CT transformation in the presence of iron.

According to the overall electron balance of hydrolytic reduction of CT to CO_2, either via formic acid or carbon monoxide, no net electrons, thus no addition of an electron donor or electron acceptor is needed (Fig 1.1). The overall reaction is also energetically very favorable with a $\Delta G^{0'}$ of -619.7 kJ. This indicates that CT could theoretically serve as a growth substrate. Up to now only cometabolic transformations of CT to carbon dioxide have been reported, however.

Hydrolysis. Transformation of CF to CO_2 has been found under methanogenic (Egli et al. 1990, Mikesell and Boyd 1990, Bagley and Gossett 1995) and acetogenic (Egli et al. 1988 1990) conditions. Becker and Freedman (1994) found transformation of ^{14}CF to ^{14}CO and $^{14}CO_2$ in a methanogenic enrichment culture. They suggest that oxidation of CF to CO_2 proceeds via net hydrolysis to carbon monoxide (Fig. 1.1). carbon monoxide can be oxidized to CO_2. Although theoretically CF can serve as a growth substrate via its net hydrolysis to carbon monoxide followed by oxidation to CO_2 ($\Delta G^{0'}$= -489.8 kJ/overall reaction), only cometabolic transformations of CF to CO_2 have been described.

Hydrolytic dehalogenation was also found for DCM. Under nitrate reducing conditions DCM was hydrolyzed to formaldehyde by *Hyphomicrobium* sp. strain DM2 (Kohler-Staub et al. 1995). The dehalogenase involved uses glutathione as a cofactor, and is in fact a GSH s-transferase. Formaldehyde then serves as the growth substrate. Nitrate serves as terminal electron acceptor and is reduced to

nitrite. The observed growth rate (μ) of *Hyphomicrobium* sp. strain DM2 with DCM under denitrification conditions was 0.015 [h^{-1}].

Fermentative dechlorination. Both DCM (Freedman and Gossett 1991, Stromeyer et al. 1991) and CM (Traunecker et al. 1991) can be fermented under anaerobic conditions and serve as a growth substrate for microorganisms. From the mixed culture of Stromeyer et al. (1991), *Dehalobacterium formicoaceticum* was isolated (Mägli et al. 1996). This organism ferments DCM to formic acid plus acetic acid (in a molar ratio of 2:1), biomass, and traces of pyruvate. DCM was transformed at a rate of 0.098 μmol·min^{-1}·mg $protein^{-1}$. The pathway of DCM transformation is not yet clear, but methylene tetrahydrofolate is probably an important intermediate (Mägli et al. 1996). The pathway of CM fermentation to acetic acid by the strictly anaerobic homoacetogenic strain MC (Traunecker et al. 1991, Meßmer et al. 1993) very much resembles the proposed pathway of DCM transformation in *D. formicoaceticum* and also involves methylene tetrahydrofolate. This suggests that the mechanism of CM fermentation is similar to the mechanism of DCM fermentation.

Chlorinated ethanes

The transformation of chlorinated ethanes under laboratory conditions has not been studied in detail. The known biological transformations of some important chloroethanes for different redox conditions are shown in Figure 1.2.

Hydrogenolysis. Transformation of chlorinated ethanes by hydrogenolytic reductive dechlorination with hydrogen as the electron donor are energetically favorable processes (Table 1.2). The $\Delta G^{0'}$ of the half reactions of reductive dechlorination indicate that chlorinated ethanes potentially are better electron acceptors than carbon dioxide, sulfate and iron(III). 1,1,1-Trichloroethane (TCA) and chloroethane (CA) potentially are also better electron acceptors than nitrate.

So far, hydrogenolysis of TCA has only been reported under sulfate- and carbon dioxide reducing conditions, both in continuous flow systems (Bouwer and McCarty 1983a, Bouwer and Wright 1988, Cobb and Bouwer 1991, Vogel and McCarty 1987a, Wrenn and Rittmann 1996) and by pure cultures of *Methanobacterium thermoautotrophicum*, *Desulfobacterium autotrophicum*, *Acetobacterium woodii* and *Clostridium* sp. strain TCAIIB (Egli et al. 1987, 1988, Gälli and McCarty 1989). Reduction of TCA in these cases is a cometabolic

reaction catalyzed by transition-metal cofactors like cobalamins and coenzyme F_{430}, and primarily leads to the formation of 1,1-dichloroethane (DCA). Although hydrogenolysis of DCA to CA is energetically very favorable under sulfate- and carbon dioxide reducing conditions (Table 1.2), further reduction of DCA to CA has only been reported under methanogenic conditions and usually accounts for a minor percentage (< 5%) of the transformation products (Vogel and McCarty 1987a).

There is only one report about the hydrogenolysis of CA. Transformation of CA by *Methanosarcina barkeri* led to the formation of ethane (Holliger 1992). *M. barkeri* also reductively dechlorinated 1,2-dichloroethane (1,2-DCA) to form CA. Transformation of 1,2-DCA by *M. barkeri* was a cometabolic reaction that was catalyzed by corrinoids and cofactor F_{430} (Holliger 1992).

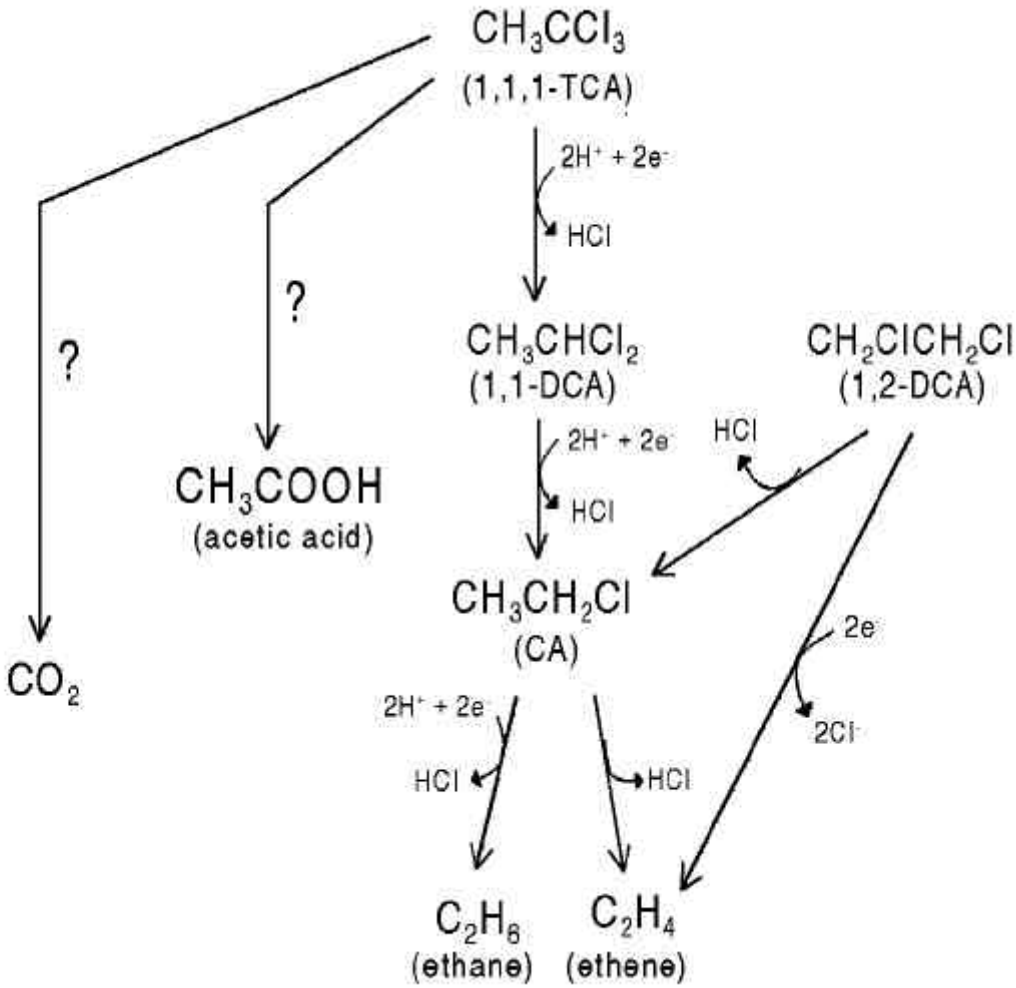


Figure 1.2 *Proposed pathways for the biological transformation of chlorinated ethanes.*

Dihaloelimination. 1,2-DCA can also be reduced by dihaloelimination. Dihaloelimination of 1,2-DCA to ethene is one of the most favorable reductive dechlorination reaction with a $\Delta G^{0'}$ of -71.3 kJ/electron. Theoretically, this transformation can occur at all redox conditions except oxygen reducing conditions (Table 1.2), but under laboratory conditions dihaloelimination has only been described for methanogenic and acetogenic bacteria as a cometabolic process (Belay and Daniels 1987, Egli et al. 1987, Holliger 1992, Wild et al. 1995).

Other reactions. Besides hydrogenolysis to DCA and CA, TCA can be completely dechlorinated to carbon dioxide or acetic acid under carbon dioxide reducing conditions via as yet unknown pathways (Vogel and McCarty 1987a, Gälli and McCarty 1989). Acetic acid and carbon dioxide were only minor products (<10%) of TCA transformation. TCA was mainly transformed to DCA.

Chlorinated ethenes

Hydrogenolysis. The Gibbs free energy of hydrogenolytic reductive dechlorination of chlorinated ethenes varies between -38.3 kJ/mole electrons and -55.3 kJ/mole electrons (Table 1.2). According to these $\Delta G^{0'}$ values, chlorinated ethenes potentially are better electron acceptors than carbon dioxide, sulfate and iron(III). Hydrogenolysis of perchloroethylene (PCE), trichloroethylene (TCE) and vinylchloride (VC) is theoretically also favorable under nitrate reducing conditions.

Complete stepwise reductive dechlorination of PCE to successively TCE, *cis*-1,2-dichloroethene (*cis*-DCE), vinylchloride (VC) and ethene (Fig. 1.3) in continuous flow systems has only been described under methanogenic and acetogenic conditions (Vogel and McCarty 1985, Bagley and Gossett 1990, de Bruin et al. 1992, Wild et al. 1995). Besides *cis*-DCE, *trans*-1,2-dichloroethene (*trans*-DCE) and 1,1-dichloroethene (1,1-DCE) were occasionally found as products of TCE transformation, but only accounted for a minor percentage of TCE trnasformed (Gerritse et al. 1995). Under sulfate reducing conditions PCE was transformed to *cis*-DCE (Bagley and Gossett 1990), whilst no transformation occurred under denitrifying (Bouwer and Wright 1988, Bae et al. 1990) and aerobic conditions (Bouwer and McCarty 1982, 1985).

Pure cultures of methanogens (Fathepure and Boyd 1988, Egli et al. 1987) and acetogens (Egli et al. 1988) are able to dechlorinate PCE in the presence of an excess of electron donor and electron acceptor, using a cometabolic reaction.

The dechlorination rate of these cultures are much lower compared to those obtained in continuous flow systems (de Bruin et al. 1992, Gerritse et al. 1995, Wild et al. 1995). This suggests that methanogens and acetogens were not the bacteria responsible for the greater part of PCE transformation in continuous flow systems. Indeed, several microorganisms have been isolated with much higher dechlorination rates for PCE (Table 1.4). These organisms are able to utilize PCE as an electron acceptor and couple the reductive dechlorination of PCE to energy conservation ((de)halorespiration; Table 1.4). A detailed description of this process is given by Holliger and Schumacher (1994) and recently by Maymo-Gatell et al. (1997).

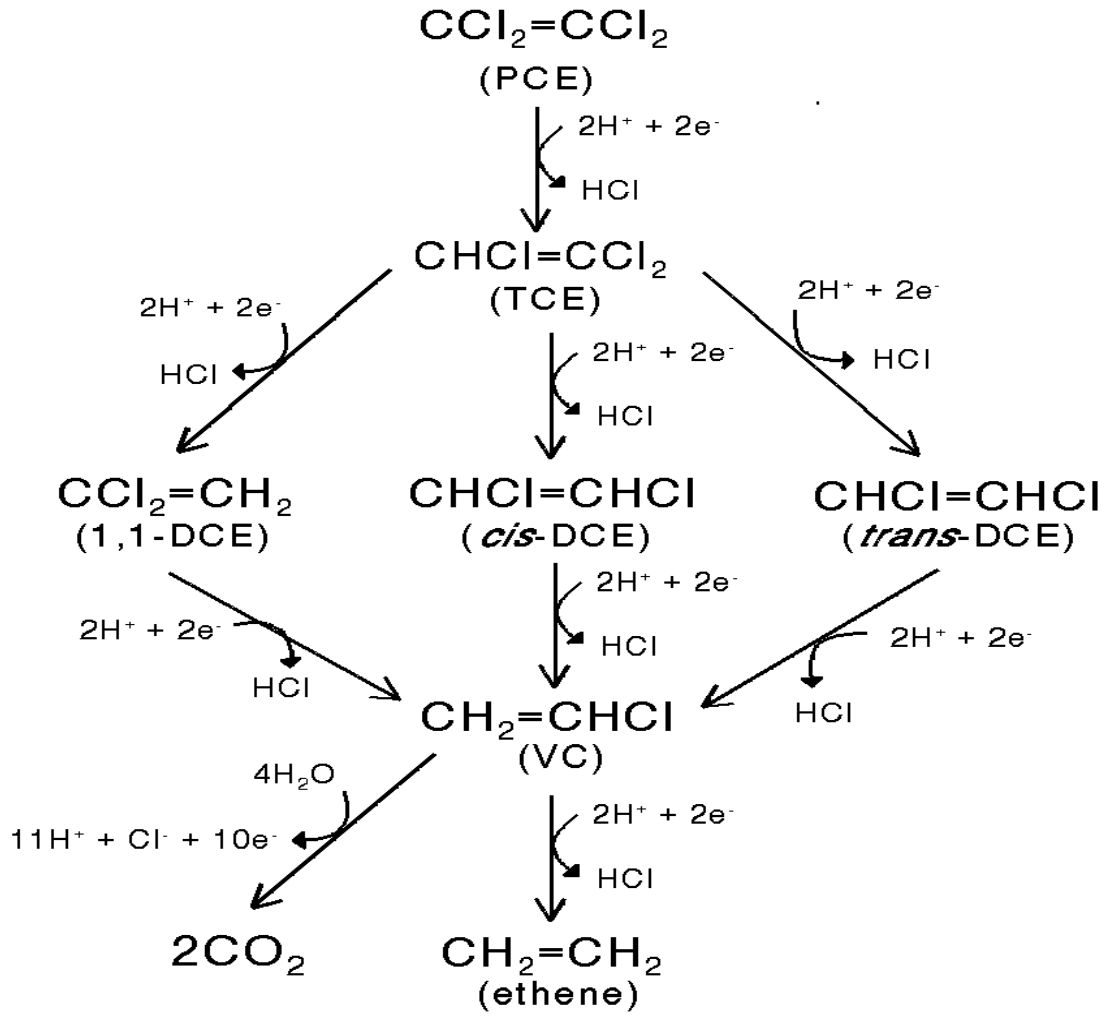


Figure 1.3 *Proposed pathways for the biological transformation of chlorinated ethenes.*

Most but not all (de)halorespiring microorganisms can use H_2 as electron donor. Maymo-Gatell et al. (1995) suggested that in anaerobic mixed cultures, methanogens and acetogens provide the H_2 necessary for respiratory dehalogenating bacteria through the transformation of other electron donors. This explains why inhibition of acetogens and methanogens often affects PCE dechlorination (Distefano et al. 1992, Freedman and Gossett 1989) although these bacteria are not directly responsible for dechlorination. Besides methanogens and acetogens, sulfate reducers and fermentors probably are also involved in the production of H_2, utilized by dehalorespiring species. The end product of PCE transformation by most respiratory dehalogenating bacteria is *cis*-DCE (Table 1.4). This suggests that in continuous flow systems with complete PCE dechlorination, other bacteria are involved in transformation of *cis*-DCE and VC to ethene. De Bruin and Schraa (personal communication) obtained an enrichment culture from a PCE dechlorinating packed-bed reactor that is able to dechlorinate cis-DCE to ethene in the presence of an electron donor. Rosner et al. (1997) described an anaerobic mixed culture that reduced VC to ethene with a maximum VC transformation rate of 28 nmol·min^{-1}·mg of protein^{-1}.

Only recently, *Dehalococcoides ethenogenes* strain 195 has been isolated, an organism able to completely dechlorinate PCE to ethene (Máymo-Gatell 1997; Table 1.4). The degradation of PCE to VC occurred by (de)halorespiration, whereas further transformation of VC to ethene is a cometabolic reaction. The intermediates in the pathway act as substrates for this cometabolic transformation. This demonstrates that one microorganism can achieve complete dechlorination of PCE.

Table 1.4 *Bacteria capable of (de)halorespiration of PCE*

Bacterium	**Product**	**Electron donors**	**Dechlorination rate (nmol Cl^-·min^{-1}·mg protein^{-1})**	**Reference**
Dehalobacter restrictus strain PER-K23	*cis*-1,2-DCE	H_2	330	1
Dehalospirillum multivorans	*cis*-1,2-DCE	H_2, pyruvate, lactate, ethanol, formic acid	50	2
Desulfitobacterium strain PCE1	TCE, *cis*-1,2-DCE	lactate, propionate, pyruvate, butyrate, ethanol etc.	310	3
Dehalococcus ethenogenes strain 195	ethene	H_2	69	4
Desulfuromonas chloroethenica	*cis*-1,2-DCE	acetate, pyruvate	--	5,6
strain TEA	*cis*-1,2-DCE	H_2	--	7

[a] per mg dry weight; [b] 1= Holliger et al. (1998), 2= Scholz-Muramatsu (1995), 3= Gerritse et al. (1996), 4= Maymo-Gatell et al. (1997), 5= Krumholtz et al. (1996), 6=Krumholtz (1997), 7=Wild et al. (1996).

Other reactions. Besides hydrogenolytic reductive dechlorination, other dechlorination mechanisms have not been observed for PCE, TCE, 1,1-DCE, and *trans*-DCE under anaerobic conditions. Only for VC, oxidation to CO_2 was found in an anaerobic aquifer microcosm with Fe(III) serving as the terminal electron acceptor according to reaction I (Bradley and Chapelle 1996, 1997).

$$C_2H_3Cl + 10Fe^{3+} + 4H_2O \rightarrow 2CO_2 + 10Fe^{2+} + 11H^+ + Cl^- \qquad (I)$$

Oxidation of VC with Fe(III) is an energetically very favorable reaction with an overall $\Delta G^{0'}$ of -1213 kJ/reaction. The rate of VC oxidation in the aquifer microcosm was enhanced when the bioavailability of Fe(III) was increased by adding chelated Fe(III) (as Fe-EDTA).

Abiotic transformation of chlorinated aliphatic hydrocarbons

Except biological transformation, most CAHs can undergo abiotic transformation. In the presence of (hydrogen) sulfide, CT can be transformed to CS_2, $CHCl_3$, and CO_2. The rate of CT transformation depends both on sulfide concentration and pH (Curtis and Reinhard 1994, Hashham et al. 1995), and can be catalyzed by many different reductants such as corrinoids (Hashham et al. 1995), humic acid, hematine (Curtis and Reinhard 1994) and the minerals biotite, vermiculite (Kriegman-King and Reinhard 1992) and pyrite (Kriegman-King and Reinhard 1994).

The reduction of 1,2-DCA is also catalyzed by HS^-. Barbash and Reinhard (1989) reported a half-life of 23 years in a solution of 1.0 mM of total sulfide and 0.05 M of phosphate buffer (pH=7; T=15°C) compared to a half-life of 310 years in distilled water. The main end products of 1,2-DCA reduction are unknown, but vinyl chloride accounted for less than 1.9% of the products formed.

TCA can undergo both abiotic dihaloelimination and hydrolysis. Dihaloelimination of TCA leads to 1,1-dichloroethene. The half-life for this transformation at 20°C is about 17 years (Vogel and McCarty 1987b). TCA can also undergo abiotic hydrolysis to acetic acid. No half-life for this transformation has been documented as yet, but Vogel and McCarty (1987a) calculated a half-

life of about 3.5 years at 20°C from experimental abiotic TCA transformation data. The mechanism of abiotic TCA transformation is not clear.

Finally, abiotic hydrolysis of CA leads to the formation of ethanol. Vogel and McCarty (1987a) estimated a half-life of 1.9 years.

Although the rate of abiotic transformations usually are much lower than for biotic transformations, abiotic transformations may play an important role at contaminated sites with only low levels of microbial activity (McNab and Narasimhan 1994).

OUTLINE OF THIS THESIS

Thermodynamically spoken, under anaerobic conditions, nearly all CAHs are better electron acceptors than naturally occurring electron acceptors such as carbon dioxide, sulfate and nitrate (Table 1.2). This suggests that in the presence of nearly all electron acceptors, CAHs can act as electron acceptor and complete biological reductive dechlorination may occur. In addition, reductive dechlorination reactions could theoretically serve as a source for energy conservation and growth. So far, the occurrence and extent of reductive dechlorination has only been described for a limited number of redox conditions. Coupling of dechlorination of CAHs to energy conservation and growth has only been described for the fermentation of dichloromethane and chloromethane and the reduction of PCE. All other biological dechlorination reactions described are cometabolic fortuitous processes, which do not seem to be coupled to growth.

The aim of this thesis was to obtain more insight in anaerobic biological dechlorination of CAHs under different environmental conditions, especially in relation to the electron acceptor and electron donor present. The effect of the presence of naturally occurring electron acceptors, such as sulfate and nitrate, and the presence or absence of different electron donors on the (extent of) biological dechlorination was studied. Furthermore, the nature of the organisms and the mechanisms involved in the transformation of different CAHs, studied under the same conditions, will be discussed.

Chapter 2 describes the complete biological dechlorination of carbon tetrachloride under carbon dioxide reducing conditions without addition of an external electron donor. The pathway and the mechanism of this process are discussed. Carbon tetrachloride transformation was also studied at sulfate reducing conditions (chapter 3). Chapter 4 describes the cometabolic reductive dechlorination of 1,1,1-trichloroethane by methanogens. The extent of cometabolic 1,1,1-trichloroethane dechlorination by methanogens in relation to the presence of different electron acceptors and electron donors and different electron donor and electron acceptor concentrations are discussed in chapter 5. The effect of different electron acceptors on dechlorination was also determined for dichloromethane fermentation by a mixed population (chapter 6). Finally, in chapter 7, the results are discussed in relation to present knowledge on biological dechlorination processes. Furthermore, the application of biological dechlorination processes for remediation purposes, based on current knowledge on this subject, is discussed.

Chapter 2

Metabolic transformation of carbon tetrachloride in an anaerobic packed-bed reactor

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Dick. B. Janssen and Wim Harder

Submitted for publication

Abstract

Carbon tetrachloride (52 µM) was biodegraded for more than 72% in an anaerobic packed-bed reactor without addition of an external electron donor. The chlorine mass balance demonstrated that all carbon tetrachloride transformed was completely dechlorinated. Chloroform and dichloromethane were sometimes also found as transformation products, but neither accumulated to significant levels in comparison to the amount of carbon tetrachloride transformed. Transformation of carbon tetrachloride in the absence of an added electron donor suggests that carbon tetrachloride itself may serve as electron donor. The pathway of carbon tetrachloride transformation is not clear; it may be dehalogenated by hydrolytic reduction to carbon monoxide or formic acid which are electron demanding transformations. Carbon monoxide or formic acid may be further utilized and serve as electron donor. For the complete dechlorination according to this pathway neither an additional electron donor nor an electron acceptor would then be needed. Vancomycin, an inhibitor of Gram-positive eubacteria, severely inhibited carbon tetrachloride transformation in batch incubations with an enrichment culture from the reactor, indicating that Gram-positive eubacteria were involved in carbon tetrachloride transformation. Batch experiments with bromoethanesulfonic acid, used to inhibit methanogens, and molybdate, an inhibitor of sulfate reduction in sulfate reducing bacteria, demonstrated that neither methanogens nor sulfate reducers were involved in the complete dechlorination of carbon tetrachloride.

Abbreviations: CT - carbon tetrachloride; CF - chloroform; DCM - dichloromethane

Introduction

Carbon tetrachloride is a common water pollutant due to widespread use as an industrial solvent. CT resists aerobic biotransformation, but several authors have described transformation of CT under anaerobic conditions in both continuous flow studies (Bae et al. 1990, Bouwer and McCarty 1983a, Bouwer and Wright 1988, Cobb and Bouwer 1991, Petersen et al. 1994) and batch culture studies (Bouwer and McCarty 1983b, Criddle et al. 1990, Egli et al. 1987, 1988, Gälli and McCarty 1989, Picardal et al. 1995). In all of these studies, CT transformation was described as a cometabolic process. During cometabolic transformation, dehalogenation is not coupled to energy conservation and occurs due to a fortuitous reaction with certain enzymes or coenzymes (Holliger and Schumacher 1994). Cometabolic dehalogenation processes require both an external electron donor and electron acceptor to support growth of the active organisms.

Another microbial process for dehalogenation is dehalorespiration. In this process reductive dehalogenation is coupled to energy conservation. The halogenated compound serves as a terminal electron acceptor and only an external electron donor is required (Holliger and Schumacher 1994, Máymo-Gatell et al. 1997, Mohn and Tiedje 1990). Theoretically, CT can also serve as a terminal electron acceptor since the redox potential of redox couples for CT transformation are positive and higher than the redox potential for couples of common electron acceptors such as nitrate, sulfate or carbon dioxide (Vogel et al. 1987).

A third anaerobic dehalogenation process, described for dichloromethane (Mägli et al. 1996) and chloromethane (Traunecker et al. 1991), is fermentation. With fermentative dehalogenations, the halogenated compound serves both as electron donor and electron acceptor. Fermentations thus do not require the input of an external electron donor or electron acceptor.

The aim of this work was to investigate the requirement of CT transformation for electron donors and electron acceptors. CT transformation was studied in an anaerobic continuous-flow packed-bed reactor at different electron donor (acetate) concentrations and in the absence of an additional electron donor and electron acceptor. To obtain more information about the pathway and products of CT transformation, ^{13}C-CT transformation was studied. Finally, the role of methanogenic, acetogenic and sulfate reducing microorganisms in CT transformation is discussed.

Material and methods

Packed-bed reactor studies. The experiments were performed in an upflow packed-bed reactor (glass, height 32 cm, inside diameter 4.42 cm, volume 492 ml) (Figure 2.1) packed with polyurethane foam (PUR) particles (5×5×6 mm, Bayer BV, Mijdrecht, the Netherlands) mixed with digested sludge (20 v/v%) from the wastewater treatment plant Kralingseveer (Rotterdam, the Netherlands). The packed-bed reactor was wrapped with aluminum foil to prevent growth of phototrophs.

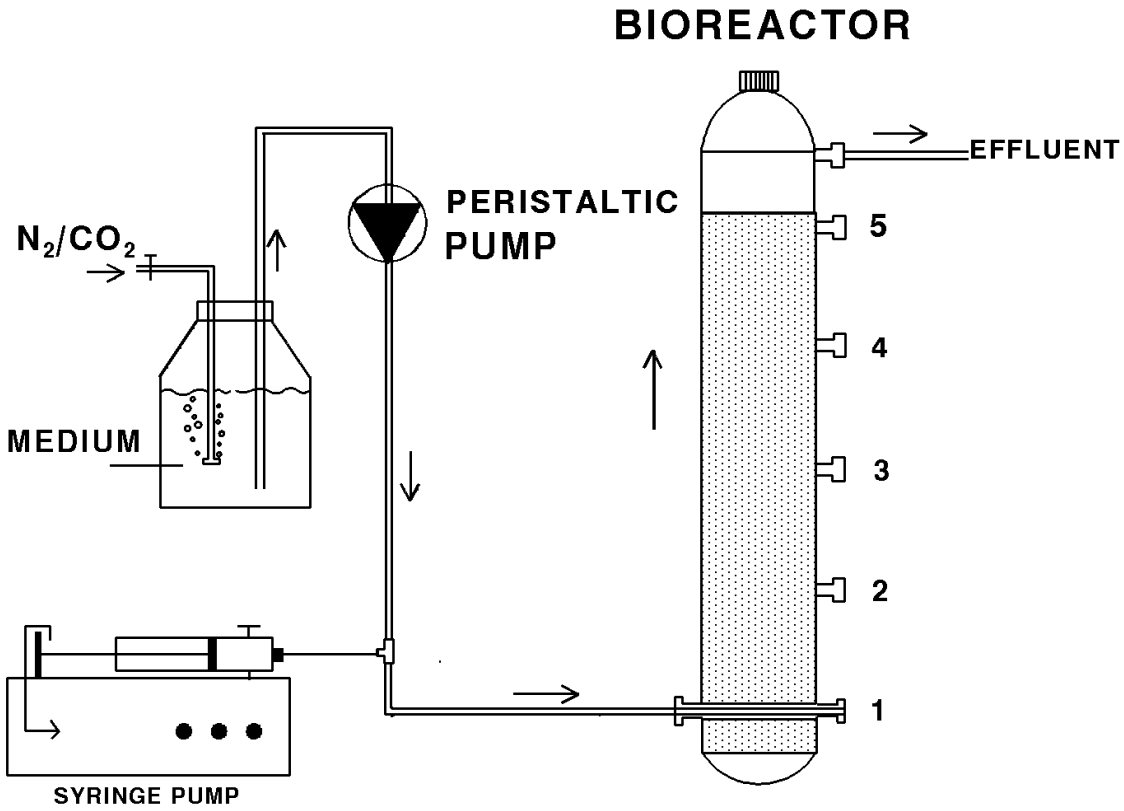

Figure 2.1 *Schematic presentation of packed-bed reactor*

The packed-bed reactor was continuously fed with an anaerobic non-sterile mineral medium containing (mg/l) K_2HPO_4 (8), KH_2PO_4 (3.6), $NaHCO_3$ (40), NH_4Cl (26.6), $MgCl_2 \cdot 6H_2O$ (101.6), $CaCl_2 \cdot 2H_2O$ (62.6), resazurine (1). From a trace element solution, 0.125 ml/l were added. The trace element solution contained (mg/l) $FeSO_4 \cdot 7H_2O$ (2800), H_3BO_3 (50), $Al_2(SO_4)_3 \cdot 16H_2O$ (118.3), $MnCl_2 \cdot 4H_2O$

(50), $CuSO_4 \cdot 5H_2O$ (92.8), EDTA (500), $ZnCl_2$ (50), $(NH_4)_6Mo_7O_{27} \cdot H_2O$ (50), $CoCl_2$ (27.3), $NiCl_2 \cdot 6H_2O$ (91.6), 1 ml HCl (37%). The medium was continuously purged with a mixture of N_2 and CO_2 (99.5%/0.5%) (Hoek Loos BV, Dieren, the Netherlands) to remove all oxygen.

The medium (pH 7.3 ±0.2) was pumped into the packed-bed reactor by means of a peristaltic pump with marprene tubing (Watson Marlow, England). All other tubing was either viton or teflon. TCA, acetate and Na_2S (42 µM, to maintain reducing conditions) were added to the medium as a concentrated solution at the influent of the packed-bed reactor with a syringe pump. The hydraulic retention time in the packed-bed was 24 h. All experiments were carried out at 25°C.

^{13}C-carbon tetrachloride studies. Experiments with ^{13}C-CT were done in the packed-bed reactor under the same conditions as described above. ^{13}C-CT (56 µM), together with acetate (30 µM) and Na_2S (42 µM), was added to the medium from a concentrated stock solution at the influent of the reactor with a syringe pump. After two weeks of adaptation, daily samples (5 ml) were taken at the influent and effluent (40 cm) of the reactor and at two sample ports at different heights along the reactor (20 cm, 30 cm) for a period of 20 days. Samples were frozen until used.

Immediately after the 20 daily samples taken at the same sample port were pooled, the pH was adjusted to a value below the pKa (4.77) of acetic acid. Each sample was saturated with NaCl and extracted with diethylether (1:1). The extract was concentrated by a factor 10 and analyzed.

Batch culture studies. Batch incubations were done using an anaerobic minimal medium containing (per liter of demineralized water) 80.1 mg $(NH_4)_2HPO_4$, 200 mg $MgSO_4 \cdot 7H_2O$, 1 mg resazurine and 5 ml trace element solution. The medium was purged with a mixture of CO_2 and N_2 (0.5:99.5 v/v%, 700 ml/min) for 45 minutes. After purging, $Na_2S \cdot 9H_2O$ (91 mg/l) and $NaHCO_3$ (100 mg/l) were added.

The medium was transferred to 120 ml bottles (brown glass) in an anaerobic glove-box. Each bottle contained 60 ml of medium and was closed with teflon-lined butyl rubber stoppers and aluminum crimp seals. After sterilization, CT (6.3 µM) and acetate (100 µM) were added as concentrated solutions. All cultures were inoculated with 2 ml of the liquid phase taken from the reactor. The cultures were incubated on a shaker (100 rpm) in a canted position (90°) at 25°C and analyzed regularly for chlorinated compounds, acetate, sulfate, CO_2, CO, CH_4 and chloride. In sterile control batches there was no transformation of CT and CH_3COOH (data not shown).

To investigate the role of methanogens, sulfate reducing bacteria, and

Gram-positive eubacteria in the transformation of CT, inhibitors were added to some batch cultures at t=0. Vancomycin (0.14 mM), 2-bromoethane sulfonic acid (BES; 6 mM), and molybdate (2 mM) were used as inhibitors.

Analytical methods. CT, CF, DCM and chloromethane were quantified by headspace gas chromatography using a headspace sampler. Liquid samples (100-1000 µl) were injected in 10 ml headspace autosampler vials with Teflon-lined butyl rubber stoppers and aluminum crimp seals. The final volume was adjusted to 2 ml with demineralized water. The vials were analyzed using a Hewlett-Packard 19395A headspace autosampler connected to a HRGC 5300 Carlo Erba gas chromatograph equipped with an electron capture detector and a CP-Sil 5CB column (length 25 m, inner diameter 0.53 µm, film thickness 2 µm, Chrompack, the Netherlands). Helium (16 ml/min) served as a carrier gas for the headspace sampler. The gas chromatograph had the following settings: injection temperature, 200°C; oven temperature, 35°C; detector temperature, 300°C. The flow rate of the carrier gas (helium) in the column was 20 ml/min. The detector make-up gas was nitrogen. The detector signal was processed with the Mosaic Chromatography data system (Chrompack, Bergen op Zoom, the Netherlands). A four point curve was used for calibration.

Carbon dioxide and methane concentrations were routinely analyzed on a Varian 3700 Gas chromatograph and flame ionization detector after separation on a Carboplot P7 column (length 12.5 m, inner diameter 0.53 µm, film thickness 25 µm, Chrompack, Bergen op Zoom, the Netherlands) and reduction of CO_2 and CO by a methanizer at 400°C (Varian, Houten, the Netherlands). The carrier gas was helium (40 ml/min). Injector, oven and detector temperatures were set at 280, 50 and 280°C, respectively. Liquid samples (2 ml) were injected in 10 ml headspace autosampler vials with Teflon-lined butyl rubber stoppers and aluminum crimp seals and equilibrated at 80°C for 45 min. An amount of 50 µl of the headspace was injected into the GC by hand with a 100 µl Hamilton gas and liquid-tight syringe. A four point calibration curve was used for quantification.

Samples from ^{13}C-CT experiments were analyzed for peaks of molecular weights of 60 (^{12}C-CH_3COOH), 61 and 62 (^{13}C-CH_3COOH) with a gas chromatograph equipped with a mass selective detector (Hewlett Packard, Wilmington, USA) in the selected-ion-monitoring (SIM) mode. The presence of ^{13}C-CH_3COOH besides ^{12}C-CH_3COOH in was demonstrated from an increase in the 61/60 amu isotopic intensity ratio (AII-ratio). The natural ratio between ^{13}C and ^{12}C is 1.1%. This means that unlabeled CH_3COOH will give a peak in the mass spectrum at 60 amu (^{12}C-CH_3COOH) and a smaller one at 61 amu (^{13}C-CH_3COOH). The intensity of the peak at 61 amu will be about 1.1% of the peak at

60 amu. An increase in this intensity ratio demonstrates the presence of ^{13}C-CH_3COOH in a sample. The detection limit for unlabeled ^{12}C-CH_3COOH was 1.67 µM.

Sulfate and chloride were determined by ion chromatography. Liquid samples were centrifuged (14,000 g for 10 min) and injected in 2 ml screw cap vials with Teflon-lined silicone liners. The vials were sampled (50 µl) with a Marathon-XT autosampler (Spark Holland, Emmen, the Netherlands) and injected into a Dionex DX-100 ion chromatograph (Dionex, Breda, the Netherlands) equipped with a conductivity detector, thermal stabilizer and ASRS suppressor. Sulfate was separated on an IONPAC AG9-SC guard column and IONPAC AG9-SC anion column (Dionex, Breda, the Netherlands). $NaHCO_3$ (1.7 mM)/Na_2CO_3 (1.8 mM) was used as eluent at a flow rate of 2 ml/min. The detector signal was processed with the Maestro Chromatography data system (Chrompack, Bergen op Zoom, the Netherlands). A 10 point calibration curve was used.

Acetate concentrations were determined with an enzymatic test-combination (Boehringer, Mannheim, Germany) based on the formation of NADH (Boehringer Mannheim GmbH Biochemica 1989). NADH formation was measured by the increase in absorbance at 340 nm on a JASCO 7800 UV/VIS spectrophotometer.

Chemicals. All chemicals were obtained from commercial supplies. Carbon tetrachloride was obtained from Baker. Chloroform and dichloromethane were purchased from Rathburn. ^{13}C-carbon tetrachloride was purchased from C.N. Schmidt BV (Amsterdam, the Netherlands) as a 99% pure solution. Vancomycin and sodium molybdate were purchased from Sigma. Chloromethane and 2-bromo-ethanesulfonic acid was obtained from Aldrich. Calibration gases were obtained from AGA (carbon dioxide, methane).

Results

Transformation of CT was studied in a packed-bed reactor under conditions of simultaneous methanogenesis and sulfate reduction to investigate the rate and extent of biological mineralization. Acetate (1 mM) served as a substrate. First the packed-bed reactor was operated without CT. Acetate was completely utilized by methanogenic and sulfate reducing bacteria. Methane production (0.85 mM) indicated that 85% of the added acetate was converted by methanogens. Methanogenic bacteria use acetate both as electron acceptor and donor and convert it to methane and carbon dioxide. The presence of methanogens was confirmed by fluorescence microscopy (Doddema and Vogels 1978). Sulfate reducing bacteria utilized 15% of the added acetate for the complete reduction of all available sulfate (0.15 mM).

Four days after CT (1.3 µM) was first added to the influent, complete transformation of CT occurred. Most of the CT transformation (> 85%) took place within the first 10 cm of the packed-bed reactor. Chloroform (0.30 µM) was found as a transformation product at the first sample port. Further along the reactor, chloroform (CF) was completely degraded.

Since CT is often found in contaminated groundwaters at higher concentrations, CT transformation in the packed-bed reactor was studied at different concentrations. The CT concentration in the reactor was increased stepwise to 60 µM over a period of 15 weeks. Up to a concentration of 40 µM CT was completely transformed. Besides CF (< 0.9 µM), dichloromethane (< 0.8 µM) was found as a transformation product in the effluent of the reactor. At a CT concentration of 60 µM, low concentrations of CT (0.9 ±0.2 µM) were detected in the effluent of the reactor showing that CT was transformed for more than 98%.
Acetate (1 mM) was completely metabolized up to a CT concentration of 40 µM. Sulfate reducing microorganisms accounted for about 15% of the removal of added acetate as calculated from the amount of sulfate removed, while methanogens converted about 85% to methane according to the amount of methane produced. When the CT concentration was further increased to 60 µM, acetate utilization decreased until only about 65% of the added acetate was metabolized (Table 2.1). Sulfate reducing bacteria still utilized 0.15 mM of the added acetate for the complete reduction of sulfate. Methane production by methanogens decreased from 0.85 mM to about 0.55 mM.

Effect of the acetate concentration on carbon tetrachloride transformation

To determine which acetate concentration is sufficient for complete transformation of CT, the acetate concentration was decreased stepwise from 1000 µM to 0 µM at an influent CT concentration of about 60 µM.

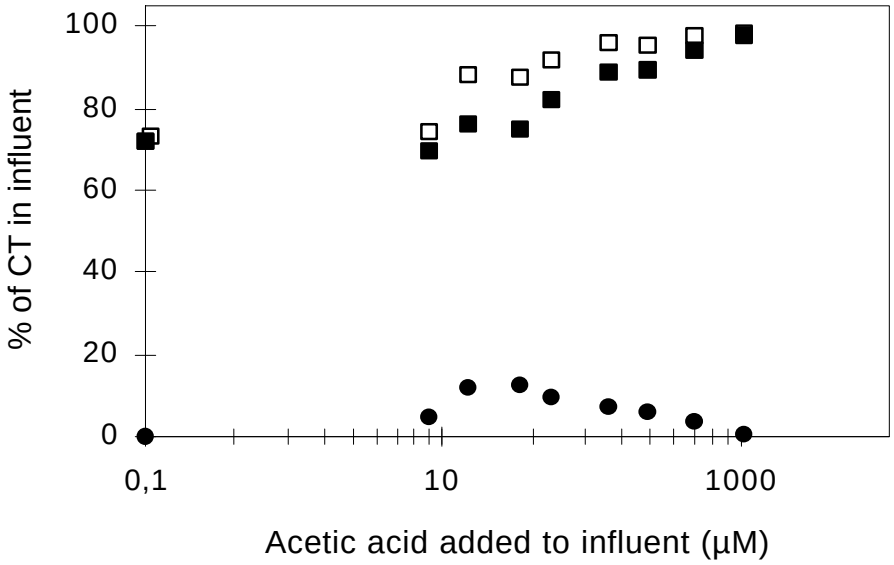

Figure 2.2 *Transformation of carbon tetrachloride in an anaerobic packed-bed reactor at different acetate concentrations. Symbols: CCl_4 transformed (ÿ); CCl_4 mineralized (■); $CHCl_3$ in effluent (●).*

Down to an acetate concentration of 30 µM, the amount of CT transformed slowly decreased (Fig. 2.2). This was caused by a decrease in CT mineralization. The amount of CT converted to CF increased. The concentration of dichloromethane (DCM) in the effluent of the reactor was always low (<0.6 µM) and further decreased during the course of the experiment until DCM was no longer found as a transformation product. At acetate concentrations below 30 µM, the percentage of CT mineralized no longer decreased and remained constant at about 73% of the CT added.

Carbon tetrachloride transformation in the absence of acetate

The continuous absence of acetate in the influent of the reactor had no significant effect on CT transformation. After 115 days still 37.5 µM (72%) of CT was degraded (Fig. 2.3), mainly to non-chlorinated products as indicated by the chloride production (141 µM). CF was no longer found as a transformation product.

Transformation of CT in the absence of acetate suggested that the transformation was a biological fermentation process, since no other electron donor was present. The complete dechlorination of CT in the reactor was assumeb not to be an abiotic (chemical) conversion for two reasons. First, in a sterile control reactor no transformation of CT was detected. This indicated that hydrolysis of CT to CO_2 (Fig. 2.4, pathway 1), as described by Kriegman-King and Reinhard (1992), was not a significant process in our reactor. Secondly, removal of sulfide, a potential chemical reductant of CT (Fig. 2.4, pathway 5) (Curtis and Reinhard 1994, Kriegman-King and Reinhard 1992), from the influent of the reactor had no significant effect on the transformation of CT (Table 2.1).

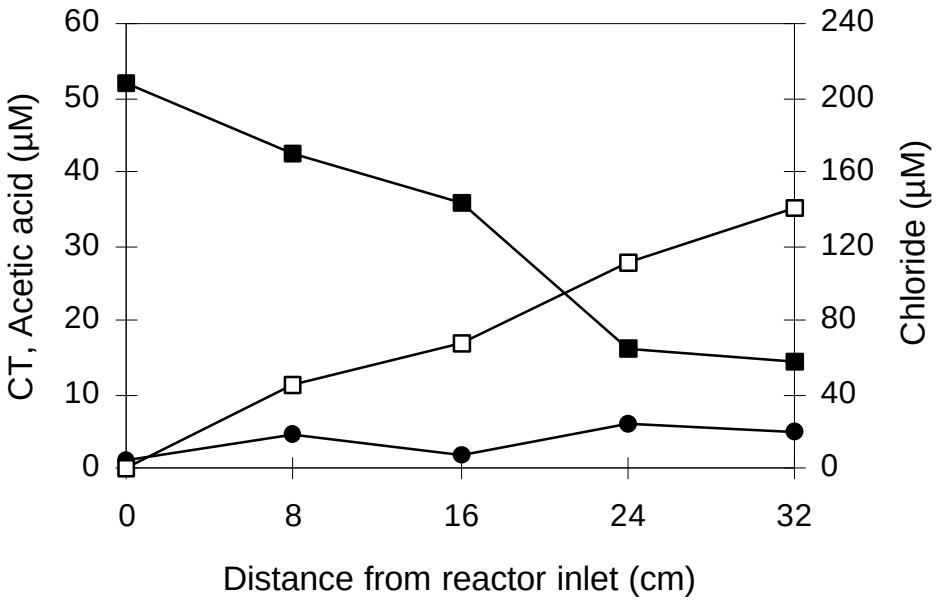

Figure 2.3 *Transformation of carbon tetrachloride in an anaerobic packed-bed reactor without addition of an electron donor. Symbols: CCl_4 (■); Cl^- (ÿ); CH_3COOH (●).*

Mass balance for carbon tetrachloride transformation

For a better understanding of the mechanism of microbial CT transformation in the reactor, mass balances were determined at different acetate concentrations. Table 2.1 shows mass balances of the packed-bed reactor at nine different acetate concentrations. The mass balance at each acetate concentration is the average of at least two steady states. A steady state was characterized by a constant degree of CT removal for a time period of at least 14 days.

With a decrease in acetate concentration, the production of methane proportionally decreased until no methane production occurred. Sulfate was nearly completely reduced down to an acetate concentration of 132 µM. At acetate concentrations below 132 µM, sulfate reduction decreased to zero. Since CT transformation still occurred, these results suggest that neither methanogenic nor sulfate reducing bacteria were involved in CT transformation.

Table 2.1 *Mass balance of an anaerobic carbon tetrachloride degrading packed-bed reactor.*

Influent (µM)		**(Influent - effluent) (µM)**							
CH_3COOH	CCl_4	CCl_4	$CHCl_3$	CH_2Cl_2	Cl^-	CH_3COOH	SO_4^{2-}	CH_4	CO_2
1030	60.3	59.4	-0.3	-0.2	--[a]	630	128	-570	-420
484	56.6	55.3	-2.1	-0.2	--	319	134	-268	-224
237	56.3	53.9	-3.5	-0.1	--	160	105	-71	-149
132	61.3	58.8	-4.3	<[b]	--	101	110	-30	-115
53	59.1	54.2	-5.7	-0.5	204	46	46	- 3	-73
33	55.8	49.0	-7.2	<	157	15	11	<	--
15	53.5	47.3	-6.5	<	161	8	--	-1	--
8	59.4	44.1	-2.9	<	178	6	<	<	--
0	51.9	37.5	<	<	141	-4	<	<	--
0[c]	53.5	37.3	<	<	143	-5	<	<	--

[a] --, not determined; [b] <, below detection limit; [c] no Na_2S (42 µM) added to the influent of the reactor

When acetate was no longer added to the influent of the reactor, acetate was still found at different heights in the reactor (Fig. 2.3). A net acetate production of 4 µM up to 20 µM was also measured at acetate concentrations in the influent of the reactor between 8 µM and 250 µM (data not shown). Acetate production usually indicates activity by acetogenic bacteria. Besides CT added to the influent, dead organic material (DOM) present in the reactor, is the only possible carbon source which could be present in the reactor and which

theoretically could serve as a substrate for acetogens. Utilization of DOM by acetogens has never been reported, but Egli et al. (1988) found transformation of ^{14}C-CT to $^{14}CH_3COOH$ by *Acetobacterium woodii*. $^{14}CH_3COOH$ accounted for 38% of the products formed. To demonstrate transformation of CT to CH_3COOH in our reactor we carried out experiments with ^{13}C labeled carbon tetrachloride (^{13}C-CT).

Transformation of ^{13}C-carbon tetrachloride

The transformation of ^{13}C-CT was studied at a concentration of unlabeled ^{12}C-CH_3COOH in the influent of the reactor of 30 µM. Addition of unlabeled ^{12}C-CH_3COOH to the influent of the reactor was necessary because the concentration of ^{13}C-CH_3COOH was determined from the 61/60 amu isotopic intensity ratio (AII-ratio) of unlabeled ^{12}C-CH_3COOH (see Material and methods). Unlabeled ^{12}C-CH_3COOH was added to the influent at a low concentration to prevent interference of the peak of unlabeled ^{12}C-CH_3COOH with the peak of ^{13}C-CH_3COOH in the mass spectrum.

When ^{13}C-CT (60 µM) was added, the conversion of CT did not alter. During the 35 days of the experiment, about 80% of the CT added was transformed. CF and DCM were not found as transformation products. The liquid samples taken at the influent and effluent of the reactor and at sample ports at 20 and 30 cm from the inlet of the reactor, were analyzed for $^{13}CH_3COOH$ and $^{12}CH_3COOH$. The results are shown in Table 2.2.

Table 2.2 *Transformation of ^{13}C-labeled carbon tetrachloride in an anaerobic packed-bed reactor.*

Sample port	Distance from reactor inlet (cm)	CT (µM)	Cl^- (µM)	^{12}C-CH_3COOH (µM)	^{13}C-CH_3COOH (µM)	61/60 AII-ratio[a] (%)
1	0 (=influent)	56.2	0	33	<[b]	1.3 ±0.1
3	20	33.6	ND[c]	28	0.1	1.6 ±0.1
4	30	18.6	ND	1.7	<	1.6 ±0.1
5	40 (=effluent)	15.2	157	<	<	--[d]
-	standard[e]	-	-	33.3	-	1.3 ±0.1

[a] AII-ratio = amu isotopic intensity ratio; [b] <, below detection limit; [c] ND= not determined; [d]cannot be determined; [e] standard = 33.3 µM (2.0 mg/l) unlabeled CH_3COOH.

The 61/60 amu ratio of the standard of unlabeled ^{12}C-CH_3COOH (33.3 µM) was 1.3 ±0.1% . This ratio differs from the theoretical ratio of 1.1%. This is

probably caused by an interference of the solvent used for extraction with the intensity of molecular mass 60 and seemed to have no effect on the determination of $^{13}C\text{-}CH_3COOH$.

At the influent of the reactor, 33 µM of unlabeled acetate was found. The AII-ratio was similar to the standard, indicating that no additional $^{13}C\text{-}CH_3COOH$ is present. At sample ports 3 and 4, part of the added acetate was utilized, and an increase in the AII-ratio from 1.3 ± 0.1% to 1.6 ± 0.1% was detected. This increase indicated that $^{13}C\text{-}CH_3COOH$ was a product of CT transformation in the reactor. From the increase in the AII-ratio and the unlabeled $^{12}C\text{-}CH_3COOH$ (28 µM) concentration, a concentration of $^{13}C\text{-}CH_3COOH$ of 0.1 µM was calculated for sample port 3, resulting from ^{13}C-CT transformation. The $^{12}C\text{-}CH_3COOH$ concentration of sample port 4 is too low to calculate a reliable $^{13}C\text{-}CH_3COOH$ concentration. These very low concentrations of $^{13}C\text{-}CH_3COOH$ in the reactor were probably caused by an immediate utilization of $^{13}C\text{-}CH_3COOH$ formed. Besides $^{12}C\text{-}CH_3COOH$ added (30 µM) to the influent of the reactor and $^{13}C\text{-}CH_3COOH$ formed, no other electron donor is known to be available in the reactor.

Effect of inhibitors on carbon tetrachloride transformation

To determine whether acetogenic bacteria could be involved in the transformation of CT (6.3 µM), batch experiments with an enrichment culture obtained from the reactor were performed in the presence of vancomycin (0.14 mM). Vancomycin, an inhibitor of cell wall synthesis in Gram-positive eubacteria (Distefano et al. 1992) and used to inhibit acetogenic bacteria, nearly completely inhibited transformation of CT for 14 days (Table 2.3). In the absence of vancomycin, transformation of CT started immediately. These results indicated that Gram-positive bacteria, possibly acetogens, were in some way involved in the transformation of CT.

At acetate concentrations in the reactor below 53 µM, no methane production occurred while about 74% of the CT added to the influent of the reactor was removed. This suggested that methanogenic bacteria were not involved in the transformation of CT. This was confirmed by batch experiments in the presence of 2-bromoethane sulfonic acid (Bres; 6 mM). Bres, an inhibitor of methanogenesis (Distefano et al. 1992), only reduced the transformation of CT by about 10%, whereas methane production was completely inhibited (Table 2.3). Sulfate reducing bacteria also were not involved in CT transformation since molybdate, a specific inhibitor of sulfate reducing bacteria (Smith and Klug 1981) only reduced the transformation of CT in batch cultures with about 15% while

sulfate reduction was completely inhibited. Only the transformation of CT to CF was partially inhibited by molybdate. This suggested that sulfate reducing bacteria could be involved in the reductive transformation of CT to CF as described previously (Chapter 3, Egli et al. 1987).

Table 2.3 *Effect of inhibitors on transformation of carbon tetrachloride.*

Inhibitor[3]	CCl_4 degraded (µM)	$CHCl_3$ formed (µM)	CH_3COOH degraded (µM)	CH_4 formed (µM)
None	6.29 (100%)	0.69	37.5	36.5
Bres[2]	5.30 (88%)	0.69	33.8	<
Vancomycin	2.31 (38%)	<[a]	6.5	14.0
Molybdate	5.42 (86%)	0.29	35.5	35.8

[a] below detection limit

Discussion

Here we report complete biological dechlorination of CT (37.5 µM) in an anaerobic packed-bed reactor without addition of an electron donor. These results suggest that CT served as an electron donor in the reactor. It is also likely that CT served as an electron acceptor since CT is a much stronger electron acceptor than carbon dioxide, the only other potential electron acceptor present in the reactor.

Table 2.4 *Gibbs free energy changes ($G^{0'}$) of carbon tetrachloride transformation.*

Reaction		$\Delta G^{0'}$ (kJ/reaction)[a]
1. $CCl_4 + H_2O + 2e^-$	$\rightarrow CO + 2H^+ + 4Cl^-$	- 528.3
2. $CCl_4 + 2H_2O + 2e^-$	$\rightarrow HCOOH + 2H^+ + 4Cl^-$	- 544.8
3. $CO + H_2O$	$\rightarrow CO_2 + 2H^+ + 2e^-$	- 91.4
4. $HCOOH$	$\rightarrow CO_2 + 2H^+ + 2e^-$	- 74.9
5. $CO + ½H_2O$	$\rightarrow ¼CH_3COOH + ½CO_2$	- 69.5
6. $HCOOH$	$\rightarrow ¼CH_3COOH + ½CO_2 + ½H_2O$	- 53.0
7. $¼CH_3COOH + ½H_2O$	$\rightarrow ½CO_2 + 2H^+ + 2e^-$	- 51.9
8. **$CCl_4 + 2H_2O$**	**$\rightarrow CO_2 + 4H^+ + 4Cl^-$**	**- 619.7**

[a] Calculated on the basis of data from Thauer et al. (1977) and Dolfing and Janssen (1994), assuming the following conditions: H^+, 10^{-7} M; Cl^-, 10^{-3} M.

Two possible pathways for the microbial transformation of CT can be distinguished where CT serves as electron donor as well as electron acceptor (fermentation). Both pathways start with a 2-electron reduction of CT, which formally leads to the formation of dichlorocarbene (Fig. 2.4, pathway 3 and 4).

Dichlorocarbene is further transformed to either CO or HCOOH through substitutive dehalogenation. In a productive pathway, these products will be formed enzymatically without release of the chemically reactive carbene as an intermediate. CO and HCOOH can be further utilized and serve as electron donor and source of carbon resulting in the formation of carbon dioxide (Fig. 2.4, Table 2.4). Overall, for the complete dechlorination of CT to CO_2 no net electrons, thus no addition of an electron donor or electron acceptor would be needed. Fermentation of CT to CO_2 via HCOOH or CO are energetically very favorable reactions with an overall $\Delta G^{0'}$ of -619.7 kJ/reaction.

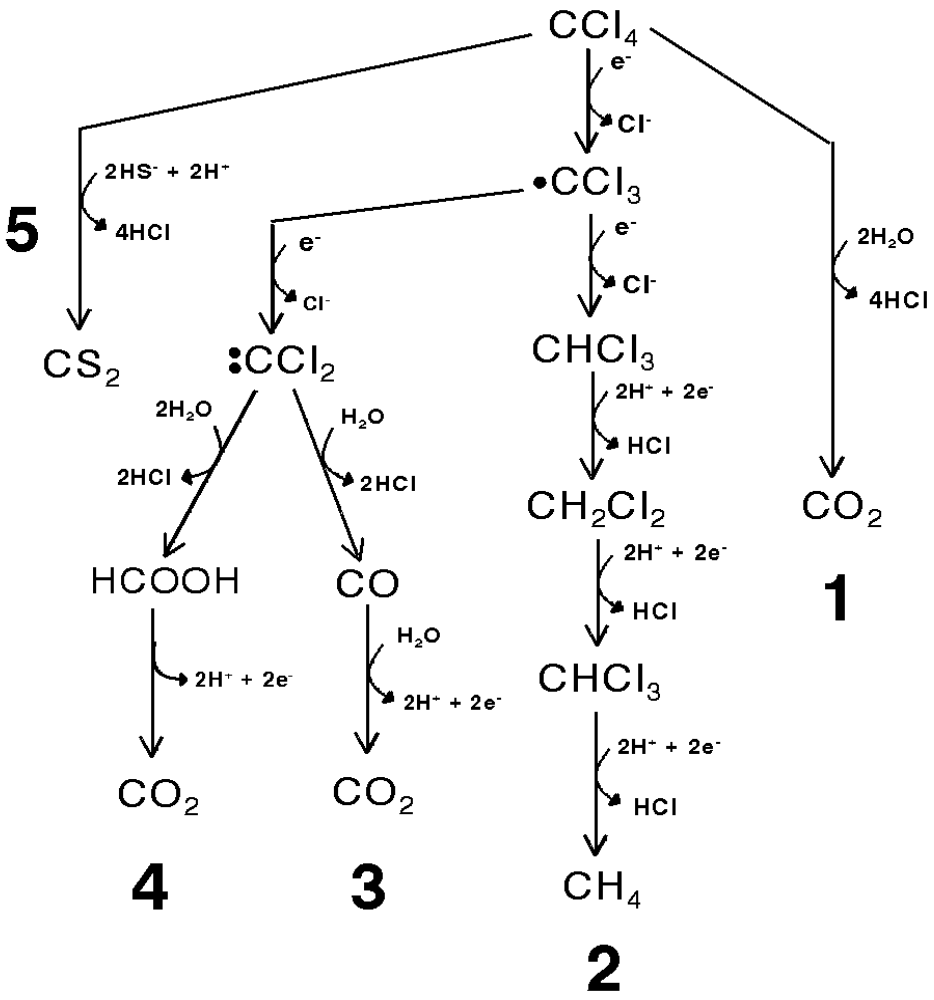

Figure 2.4 *Proposed pathways for the anaerobic transformation of carbon tetrachloride. (deduced from Criddle et al. 1991).*

Transformation of CT to HCOOH has never been reported but CT transformation to CO has been demonstrated in acetogenic bacteria (Hashham et al. 1995, Stromeyer et al. 1992). The mechanism of this conversion is not yet clear, but Egli et al. (1988, 1990) proposed that CT transformation may involve

the acetyl-CoA pathway and that cobamides could play a role. There are several reports about the stimulation of CT transformation in the presence of cobamides (Chiu and Reinhard 1996, Hashham et al. 1995, Stromeyer et al. 1992). It is not clear whether CT was degraded via HCOOH or CO in our reactor. Both the transformation of CT to CO and the transformation of CT to HCOOH are energetically very favorable reactions (Table 2.4). The samples of the ^{13}C-CT experiment were screened for ^{12}C- and ^{13}C-HCOOH but neither of these compounds were detected in any of the samples, indicating that less than 2.2 µM was present (detection limit). We were unable to detect for ^{12}CO and ^{13}CO since the mass of ^{12}CO is similar to that of N_2.

The observed formation of ^{13}C-CH_3COOH from ^{13}C-CT indicated that CH_3COOH was a (minor) product of CT transformation in the reactor. Formation of CH_3COOH could result from the utilization of CO or HCOOH by acetogens (Ljungdahl 1986) (Table 2.4). CH_3COOH, when formed, again could be oxidized and serve as an electron donor in the reactor, yielding 8 electrons per CH_3COOH oxidized (Table 2.4). Batch experiments with vancomycin strongly suggested that acetogens could be involved in CT transformation in our reactor.

Only isolation of the CT degrading microorganism can give a definitive answer whether acetogenic bacteria or a new physiological type of microorganism were involved in CT transformation in our open microbial system.

Chapter 3

Transformation of carbon tetrachloride under sulfate reducing conditions

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Biodegradation (1998) **8:** 429-436

Abstract

The removal of carbon tetrachloride under sulfate reducing conditions was studied in an anaerobic packed-bed reactor. Carbon tetrachloride, up to a concentration of 30 mM, was completely converted. Chloroform and dichloromethane were the main transformation products, but part of the carbon tetrachloride was also completely dechlorinated to unknown products. Gram-positive sulfate-reducing bacteria were involved in the reductive dechlorination of carbon tetrachloride to chloroform and dichloromethane since both molybdate, an inhibitor of sulfate reduction, and vancomycin, an inhibitor of gram-positive bacteria completely inhibited carbon tetrachloride transformation. Carbon tetrachloride transformation by these bacteria was a cometabolic process and depended on the input of an electron donor and electron acceptor (sulfate). The rate of carbon tetrachloride transformation by sulfate reducing bacteria depended on the type of electron donor present. A transformation rate of 5.1 $nmol \cdot ml^{-1} \cdot h^{-1}$ was found with ethanol as electron donor. At carbon tetrachloride concentrations higher than 18 mM, sulfate reduction and reductive dechlorination of carbon tetrachloride decreased and complete inhibition was observed at a carbon tetrachloride concentration of 56.6 µM. It is not clear what type of microorganisms were involved in the observed partial complete dechlorination of carbon tetrachloride. Sulfate reducing bacteria probably did not play a role since inhibition of these bacteria with molybdate had no effect on the complete dechlorination of carbon tetrachloride.

Abbreviations: CT - carbon tetrachloride; CF - chloroform; DCM - dichloromethane

Introduction

Carbon tetrachloride (CT) is a toxic and carcinogenic compound of great environmental concern, as it is often found as a contaminant in groundwater and soil. Bioremediation is an attractive clean-up technique for sites contaminated with CT, provided complete dechlorination occurs and no toxic metabolites accumulate. Whether complete dechlorination takes place depends on the environmental conditions, with the redox potential as the most important factor. CT resists aerobic biodegradation, but under anaerobic conditions it has been shown that in the presence of several electron acceptors, except sulfate, CT can be completely dechlorinated (Bouwer and McCarty 1983a, Gälli and McCarty 1989, Criddle et al. 1990, Picardal et al. 1995). CT transformation under sulfate reducing conditions in continuous flow reactors has been described (Bouwer and Wright 1988, Cobb and Bouwer 1991) but the products of CT transformation are unknown. In pure cultures of *Desulfobacterium autotrophicum*, CT was for more than 70% transformed to chloroform (CF) and dichloromethane (DCM) (Egli et al. 1987, 1988), but unidentified water-soluble products were also detected (Egli et al. 1990). Accumulation of the metabolites CF and DCM is undesirable because, like CT, both compounds are toxic and carcinogenic.

Since sulfate is often present in groundwater, the aim of this study was to determine the products of CT transformation under sulfate reducing conditions. CT transformation in the presence of sulfate was studied in an anaerobic packed-bed reactor. The role of sulfate reducing bacteria in the transformation of CT, the toxicity of CT and the effect of different electron donors on CT transformation have been investigated. Furthermore, we established how complete dechlorination of CT can be achieved in groundwater containing high sulfate concentrations.

Material and methods

Packed-bed reactor studies. These experiments were performed in an upflow packed-bed reactor (glass; height 40 cm; inside diameter 4.6 cm; volume 665 ml) (Chapter 2, Figure 2.1) packed with polyurethane foam (PUR) particles (5x5x6 mm, Bayer B.V., Mijdrecht, the Netherlands) mixed with digested sludge (20 v/v%) from the wastewater treatment plant Kralingseveer (Rotterdam, the Netherlands). The packed-bed reactor was wrapped with aluminum foil to prevent growth of phototrophs.

The reactor was continuously fed with the anaerobic non-sterile mineral medium described in chapter 2. The medium was continuously purged with oxygen free N_2/CO_2 (99.5%/0.5%) to remove all oxygen. The medium (pH 7.3±0.2) was pumped into the reactor by means of a peristaltic pump with marprene tubing. All other tubing was either viton or teflon. CT, acetate and Na_2S (41.8 µM, to maintain reducing conditions) were added to the medium as a concentrated solution at the entrance of the reactor with a syringe pump. The hydraulic retention time in the packed-bed was 24 h. All experiments were carried out at 25°C.

Batch culture studies. Experiments were done with a minimal anaerobic medium that contained (per liter of demineralized water) 80.1 mg $(NH_4)_2HPO_4$, 200 mg $MgSO_4 \cdot 7H_2O$, 1 mg resazurine and 5 ml trace element solution (Chapter 2). The medium was purged with oxygen free N_2/CO_2 (99.5%/0.5%; 700 ml/min) for 45 min. After purging, $Na_2S \cdot 9H_2O$ (91 mg/l) and $NaHCO_3$ (100 mg/l) were added.

The medium was transferred to 120 ml bottles (brown glass) in an anaerobic glove-box. Each bottle contained 60 ml of medium and was closed with teflon-lined butyl rubber stoppers and aluminum crimp seals. After sterilization, CT (11µM) and an electron donor (1 mM) were added form concentrated solutions. All cultures were inoculated with 2 ml of an enrichment culture from the packed-bed reactor. The cultures were incubated on a shaker (100 rpm) in a canted position (90°) at 25°C and analyzed regularly for chlorinated compounds, electron donor, sulfate and chloride. Sterile batches were used to test for abiotic losses.

To investigate the role of sulfate reducing microorganisms in the degradation of CT, inhibitors were added to some of the batch cultures at t=0. Vancomycin (0.14 mM), 2-bromoethane sulfonic acid (Bres; 6 mM), molybdate (2 mM) and H_2O_2 (5.8 mM) were used as inhibitors. The effect of sulfate on CT transformation was also tested in batch cultures. $MgSO_4 \cdot 7H_2O$ was replaced by $Mg(CH_3COO)_2 \cdot 4H_2O$ to remove all sulfate.

Analytical methods. CT, CF, DCM and chloromethane were quantified by headspace gas chromatography. Liquid samples (100-1000 µl) were injected in 10 ml headspace autosampler vials with teflon-lined butyl rubber stoppers and aluminum crimp seals. The final volume was adjusted to 2 ml with demineralized water. The vials were analyzed using a Hewlett Packard 19395A headspace sampler connected to a gas chromatograph equipped with an electron capture detector and a CP-Sil 5CB reactor (Chapter 2). Calibration samples were analyzed according to the same method to adjust for air/water partitioning. A four-

point curve was used for calibration.

Carbon dioxide, carbon monoxide and methane concentrations were determined after separation on a Carboplot P7 column using a gas chromatograph equipped with FID and a methanizer (Chapter 2). Liquid samples (2 ml) from the packed-bed reactor were injected in 10 ml headspace autosampler vials with teflon-lined butyl rubber stoppers and aluminum crimp seals and equilibrated at 80°C for 45 min. An amount of 50 ml of the headspace was injected into the GC by hand with a 100 ml Hamilton gas and liquid-tight syringe. For batch cultures, 50 ml of the headspace was injected into the GC. A four point calibration curve was used for quantification.

Sulfate and chloride were determined after separation on an IONPAC AG9-SC guard column and IONPAC AG9-SC anion column (Dionex, Breda, the Netherlands) (Chapter 2) with an ion chromatograph equipped with a conductivity detector, thermal stabilizer and suppressor (Dionex, Breda, the Netherlands).

Acetate concentrations were determined with an enzymatic test-combination (Boehringer, Mannheim, Germany).

Chemicals. All chemicals were obtained from commercial suppliers. CT was obtained from Baker. CF and DCM were purchased from Rathburn. Vancomycin and sodium molybdate were purchased from Sigma. Chloromethane and 2-Bromoethanesulfonic acid were obtained from Aldrich. Calibration gases (carbon dioxide, carbon monoxide, methane) were obtained from AGA.

Results

Although the biotransformation of CT has been studied extensively, little information is available on the products of CT transformation and the physiological factors affecting CT transformation under sulfate reducing conditions, especially in continuous flow systems. Here, CT transformation in the presence of sulfate (0.51 mM) was studied in an anaerobic packed-bed reactor, inoculated with digested sludge. Acetate (1 mM) served as an electron donor.

After 3 weeks of operation, sulfate reducing bacteria utilized 55% of the added acetate for complete reduction of sulfate. Acetate was not further utilized by other bacteria. CT (2.5 µM), which was then added to the influent of the reactor, was transformed without delay. CF (1.3 µM) and DCM (0.44 µM) were found as main transformation products (Table 3.1). No further transformation to chloromethane was detected. Part of CT (0.77 µM) was converted to unknown

products. In a sterile control packed-bed reactor, which was run under the same conditions except for the inoculation with digested sludge, no transformation or loss of CT occurred. This suggests that CT transformation is a biological process rather than an abiotic chemical transformation caused by reaction with sulfide or other reducing compounds present in the reactor.

Table 3.1 *Carbon tetrachloride (2.5 µM) transformation in a sulfate reducing packed-bed reactor at different sulfate concentrations.*

	Substrate utilization			**CT transformation**		
SO_4^{2-} influent (µM)	CH_3COOH utilization (µM)	SO_4^{2-} reduction (µM)	CH_4 production (µM)	CT conversion (%)	CF formation (%)	DCM formation (%)
510	622	511	0	100	51.8	15.9
510	999	350	672	100	0	0
5000	974	963	0	100	46.0	13.1

After complete reduction of sulfate (0.51 mM) in the reactor, about 0.4 mM of acetate was still available for conversion by non sulfate reducing microorganisms. After 23 weeks of operation, methane was detected in the packed-bed reactor, indicating that methanogens had started to grow. When acetate was completely utilized, 0.67 mM of methane was produced in the reactor. Sulfate reduction had decreased to 0.35 mM (Table 3.1). The development of a methanogenic population had a profound effect on CT transformation. Instead of reductive dechlorination to CF and DCM, CT was completely dechlorinated. Under methanogenic conditions more than 99% of the added CT was mineralized.

In a subsequent experiment, the sulfate concentration in the influent of the reactor was increased from 1 mM to 5 mM (Table 3.1). When a new steady state was reached, sulfate reducing bacteria utilized all available acetate for the reduction of 0.96 mM of sulfate. Methane production no longer occurred. CT was still completely transformed but CF and DCM again were found as main transformation products.

These results demonstrate that the products of CT transformation depend on the microbial population present in the reactor. The sulfate reducing population mainly transformed CT to CF and DCM. When methanogens were present in the reactor, CT was completely dechlorinated. CF and DCM were not found as intermediates in the effluent nor at several sample ports at different heights of the reactor (data not shown). Complete dechlorination of CT under strictly sulfate reducing conditions by the microbial population in the reactor apparently is not

possible. CT transformation under these conditions was studied further to obtain a better understanding of the factors affecting CT transformation and the microorganisms involved in this process.

CT transformation at different concentrations

The performance of the reactor was studied in a CT concentration range from 2.5 µM to 56.6 µM. Under the starting conditions used (CT 2.5 µM, acetate 1 mM, SO_4^{2-} 5 mM), CT was completely transformed, primarily to CF and DCM via reductive dechlorination (Table 3.2). Part of CT (41%) was converted to unknown products. Nearly all available acetate was utilized by sulfate reducing bacteria for the reduction of 0.96 mM sulfate.

Table 3.2 *Transformation of carbon tetrachloride in anaerobic packed-bed reactor under sulfate reducing conditions at different carbon tetrachloride concentrations.*

CT influent (µM)	CT effluent (µM)	CF effluent (µM)	DCM effluent (µM)	Cl^- formation (µM)	SO_4^{2-} reduced (µM)	CH_3COOH utilized (µM)
2.5	0 (0%)[1]	1.5 (46%)	0.3 (13%)	--[2]	960	970
11.8	0 (0%)	4.6 (39%)	2.5 (21%)	--	900	1000
20.9	0 (0%)	7.2 (34%)	1.6 (8%)	--	980	1010
29.6	0 (0%)	12.3 (42%)	1.0 (3%)	--	970	910
56.6	23.5 (42%)	<0.1 (0%)	<0.1 (0%)	114	40	310

[1] % of CT in influent; [2] not determined

Up to a concentration of 29.6 µM, CT was mainly transformed to CF and DCM. Part of the CT was converted to unknown products. At CT concentrations higher than 11.8 µM, reductive dechlorination of CT to DCM was partially inhibited and the percentage of CT transformed to unknown products increased. At the highest CT concentration tested (56.6 µM), reductive dechlorination of CT to CF and DCM was completely inhibited, and about 23.5 µM of CT remained in the effluent of the reactor. According to the amount of chloride formed (Table 3.2) up to 28.5 µM of the added CT was completely dechlorinated.

Sulfate reduction was nearly completely inhibited at a CT concentration of 56.6 µM. This inhibition coincided with the inhibition of reductive dechlorination to CF and DCM. This is a further indication that sulfate reducing bacteria are probably involved in the reductive dechlorination of CT to CF and DCM. Other

bacteria probably play a role in the complete dechlorination of CT since significant dechlorination of it still occurred when sulfate reduction was nearly completely inhibited.

The reactor was run for 30 weeks at a CT concentration of about 57 µM. The removal of CT, measured by the production of chloride, did not change and remained at about 50%. Sulfate reduction and methane production were not detected in the reactor.

Role of sulfate reducing bacteria in CT transformation

To determine the role of sulfate reducing bacteria in CT transformation, specific inhibitors were added to an enrichment culture from the packed-bed reactor. This CT transforming enrichment culture was obtained at a CT concentration in the reactor of 18.2 µM, using the liquid phase of the packed-bed reactor as an inoculum. Ethanol (1 mM) served as an electron donor.

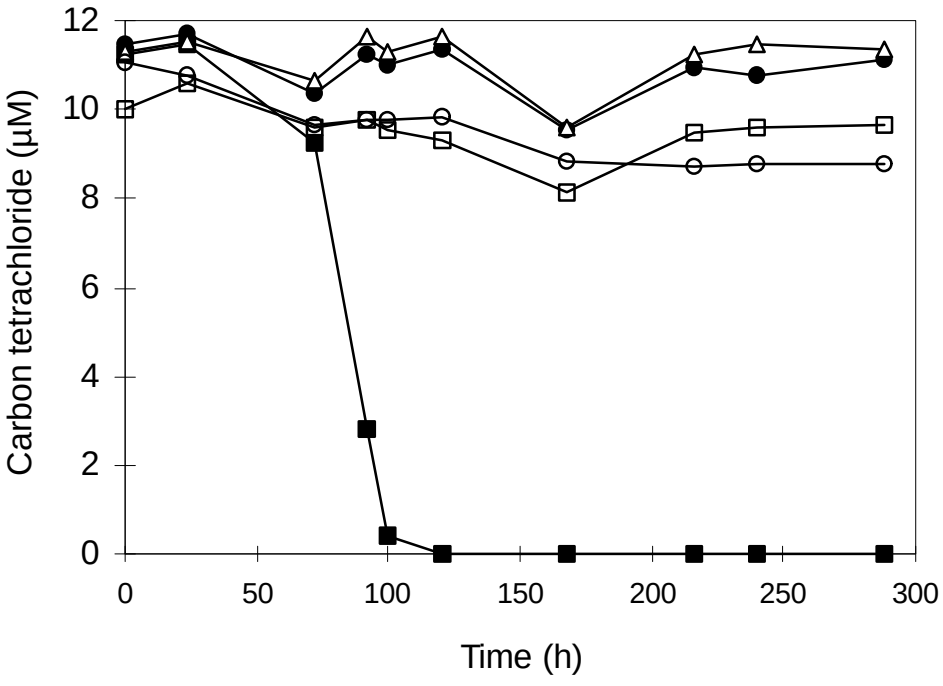

Figure 3.1 *Effect of inhibitors on the transformation of carbon tetrachloride (11 µM) by a sulfate reducing mixed culture in a minimal medium. Ethanol (1 mM) served as an electron donor. symbols: none ( ); molybdate (●); vancomycin* (∆); *H_2O_2 (❍); no sulfate (*□)

In the absence of inhibitors, CT (11.3 µM) was completely transformed within 115 hours at a maximum CT transformation rate of 5.1 nmol·ml^{-1} ·h^{-1} (Fig. 3.1). CF (9.4 µM) was found as the only chlorinated transformation product. Part of CT (1.9 µM) was transformed to unknown products. Ethanol was completely utilized for the reduction of 0.49 mM sulfate according to the following reactions.

$$CH_3CH_2OH + \tfrac{1}{2}SO_4^{2-} \rightarrow CH_3COO^- + \tfrac{1}{2}HS^- + \tfrac{1}{2}H^+ + H_2O$$
$$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$$

$$\mathbf{CH_3CH_2OH + 1\tfrac{1}{2}SO_4^{2-} \rightarrow 2HCO_3^- + 1\tfrac{1}{2}HS^- + \tfrac{1}{2}H^+ + H_2O}$$

DCM was not found as a transformation product during this experiment, probably because of the short duration of the incubations (13 days). In other long-term batch culture experiments up to 27% of the CF formed was further transformed to DCM but only after 4 weeks of incubation. DCM was not transformed by the sulfate reducing mixed culture (data not shown).

Molybdate, an inhibitor of sulfate reduction in sulfate reducing bacteria (Smith and Klug 1981) and vancomycin, an inhibitor of cell wall synthesis in gram positive eubacteria (Distefano et al. 1992), completely inhibited CT transformation (Fig. 3.1) and sulfate reduction. This demonstrated that gram-positive sulfate reducing bacteria were involved in the reductive dechlorination of CT to CF. Reductive dechlorination by these bacteria is a cometabolic process since no CT transformation occurred in the absence of an electron donor (ethanol) or a suitable electron acceptor (sulfate). Inhibition of CT transformation by hydrogen peroxide suggested that CT transformation also depended on the input of reducing equivalents.

Toxicity of CT for sulfate reducing bacteria

At a CT concentration in the packed-bed reactor of 56.6 µM, sulfate reduction was nearly completely inhibited. This suggests that CT is toxic to sulfate reducing bacteria. The toxicity level of CT for the sulfate reducing population in the packed-bed reactor was determined in batch cultures. Ethanol (2 mM) served as an electron donor.

In the absence of CT, ethanol was utilized by the sulfate reducing population to nearly complete reduction of all available sulfate (0.81 MM) (Fig. 3.2). Up to a CT concentration of 18.2 µM, sulfate reduction did not significantly change. However, at higher CT concentrations the amount of sulfate reduced

rapidly decreased and at a CT concentration of 38.2 µM, sulfate reduction was nearly completely inhibited. This results in an toxicity level of CT for the sulfate reducing population of about 38 µM. Egli et al. (1988) described inhibition of the autotrophic growth of *Desulfobacterium autotrophicum* at a CT concentration of 80 µM.

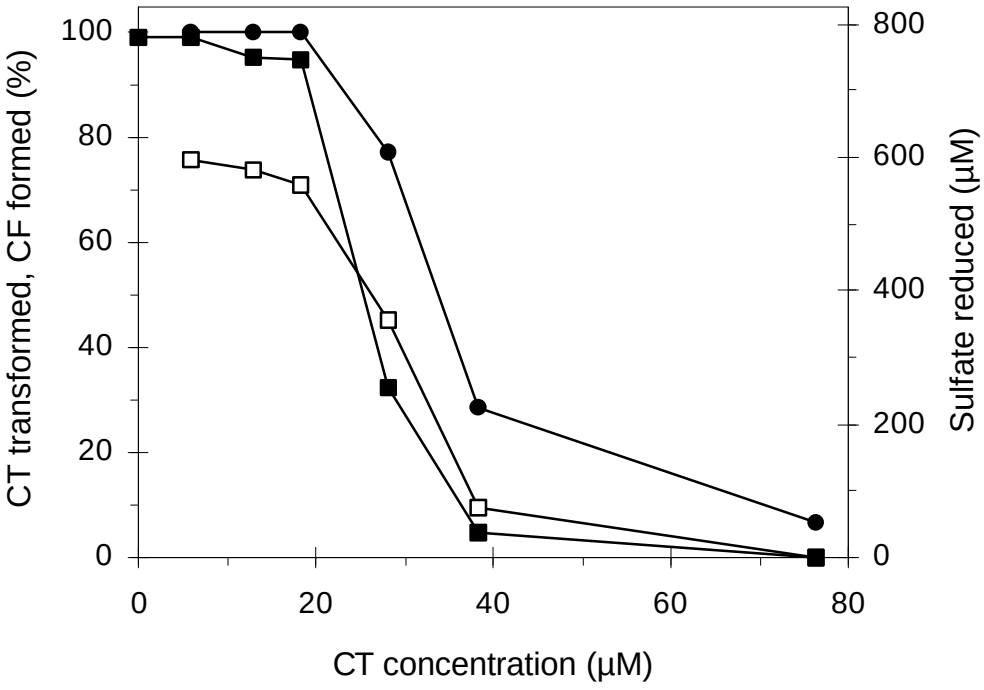

Figure 3.2 *Effect of the carbon tetrachloride concentration on carbon tetrachloride transformation by a sulfate reducing mixed culture in a minimal medium. Ethanol (1 mM) served as an electron donor. The batch cultures were analyzed after 27 days. Symbols: % of CT transformed (■); % of CT transformed to CF (❑); concentration of sulfate reduced (●).*

Since sulfate reduction became inhibited at CT concentrations above 20 µM, the amount of CT transformed to CF also decreased. At the highest CT concentration tested (76.3 µM), no sulfate reduction and thus no reductive dechlorination of CT to CF occurred.

Effect of different electron donors on CT transformation by sulfate reducing bacteria

The rate and the products of cometabolic transformation of chlorinated hydrocarbons often depend on the type of electron donor that is available (Mikesell and Boyd 1990, Lewis and Crawford 1993). To determine whether this also applies for CT transformation by the sulfate reducing enrichment culture from the packed-bed reactor, different electron donors were tested (Table 3.3;Fig. 3.3).

Table 3.3 *Transformation of carbon tetrachloride by a sulfate reducing mixed batch culture in the presence of different electron donors. The initial concentration of the different electron donors was 1 mM.*

Electron donor	$\Delta G^{0'}$ [1] (kJ/mol)	t=0 CT (µM)	t=13 days CT (µM)	t=13 days CF (µM)	R_{max} [2] (nmol ·ml^{-1} ·h^{-1})	SO_4^{2-} reduced (mM)
H_2/CO_2	- 9.5	10.1	2.2 (22%)[3]	5.2 (51%)[4]	1.83	0.049
formate	- 36.7	10.9	2.4 (22%)	6.5 (60%)	1.72	0.246
methanol	- 73.0	10.3	10.3 (100%)	<0.1 (0%)	0	0.003
acetate	- 47.6	9.7	<0.1 (0%)	6.6 (68%)	2.03	0.369
ethanol	- 66.4	10.4	<0.1 (0%)	9.4 (90%)	5.09	0.486
lactate	- 80.0	10.3	7.9 (77%)	2.1 (20%)	0.21	0.333
propionate	- 37.7	10.1	7.2 (71%)	3.4 (34%)	0.15	0.494

[1] $\Delta G^{0'}$ values are taken from Thauer et al. (1977);[2] R_{max} = maximum observed rate of CT transformation; [3] percentage of CT remaining after 13 days; [4] percentage of CT transformed to CF after 13 days

With all electron donors tested, except methanol, both sulfate reduction and CT transformation to chloroform occurred. Methane production was not found. The concentration of CT transformed seems unrelated to either the amount of sulfate reduced or the energy yield per mole of electron donor (Table 3.3).

Only with acetate or ethanol as electron donors complete CT transformation occurred. The maximum observed rate of CT transformation (R_{max}), which was calculated from the rate of CT disappearance in the batch cultures (Fig. 3.3), was 3 times higher with ethanol then with acetate (Table 3.3). However, with ethanol CT was nearly completely (>90%) transformed to CF, while with acetate only 68% was transformed to CF and 32% was transformed to unidentified products. This corresponds with our findings in the packed-bed reactor. With all other electron donors tested, the percentage of CT transformed to unidentified products was below 27%.

For application of microbial CT transformation under sulfate reducing conditions, complete dechlorination of CT is to be preferred over the formation and accumulation of CF or DCM. Acetate therefore appeared a more promising electron donor than ethanol, although complete dechlorination of CT under sulfate reducing conditions without the formation of CF is not likely to occur.

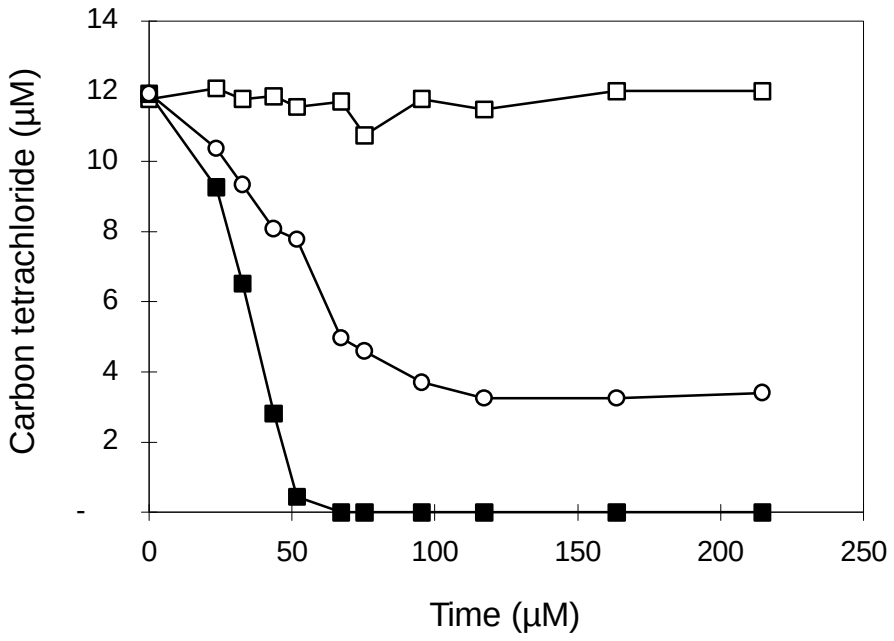

Figure 3.3 *Transformation of carbon tetrachloride (12 µM) by a sulfate reducing mixed culture with different electron donors (1 mM) in a minimal medium. Symbols: methanol (□); hydrogen (❍); ethanol ( ).*

Discussion

Bioremediation processes can only be applied for clean-up purposes at CT contaminated sites if complete mineralization can be established. Complete dechlorination of CT without the formation of CF or DCM does not appear to be possible by the sulfate reducing population in our reactor. The main pathway of CT transformation by sulfate reducing bacteria was found to be reductive dechlorination to CF and DCM (Fig. 3.4). Both CF and DCM accumulated and were not further dechlorinated to chloromethane or methane. The accumulation of CF and DCM probably resulted from a decrease in the redox potential for reductive dechlorination with each reductive step making the transformation less favorable. Reductive dechlorination of DCM to chloromethane and methane has

been described but only under methanogenic and acetogenic conditions and at very low transformation rates (Egli et al. 1988, Mikesell and Boyd 1990). Besides reductive dechlorination, DCM can also be fermented by anaerobic bacteria, a process which supports growth (Mägli et al. 1996). We did not find evidence for fermentation of DCM in our experiments. This could be a result of the toxicity of CF, the other product of CT transformation, to DCM utilizing bacteria (Mägli et al. 1996). Inhibition of DCM transformation is probably not caused by sulfate. When all sulfate present in the reactor was completely reduced, DCM transformation still did not occur (Table 3.1).

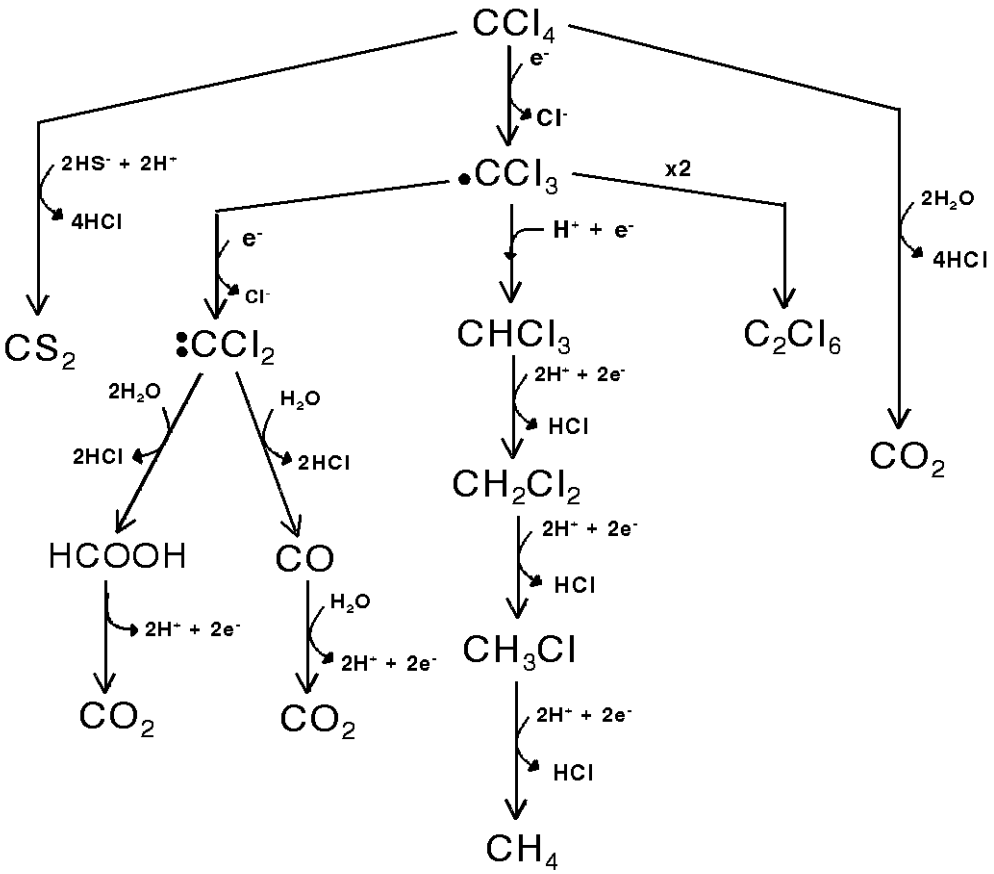

Figure 3.4 *Proposed pathways for the anaerobic transformation of carbon tetrachloride (deduced from Criddle et al. 1991).*

Part of CT transformed in the reactor was converted to unknown products. Hexachloroethane, besides CF and DCM the only other reported chlorinated product of CT transformation under anerobic conditions (Criddle et al. 1991, Fig. 3.4), was not found as a product of CT transformation. The formation of chloride

at a CT concentration of 56.6 µM (Table 3.2) suggested that CT was completely dechlorinated. Two main products for complete CT dechlorination under anaerobic conditions have been described, carbon disulfide and carbon dioxide.

In the presence of sulfide, CT can be transformed to CS_2 (Fig. 3.4; Kriegman-King and Reinhard 1992, Curtis and Reinhard 1994). The reaction of CT with sulfide is an abiotic transformation which can be catalyzed by corrinoids, present in anaerobic bacteria (Hashham et al. 1995). CT transformation to CS_2 probably did not occur in the reactor. At a CT concentration of 56.6 µM, sulfate reduction, and thus the formation of sulfide, were completely inhibited in the reactor (Table 3.2) while CT transformation to unknown products still occurred. Removal of sulfide (42 µM) from the influent of the reactor, added to maintain reducing conditions, also had no significant effect on the transformation of CT (data not shown).

Carbon dioxide can be formed as a result of both biotic (Bouwer and Mccarty 1983a, 1983b, Egli et al. 1988, 1990, Criddle et al. 1990) and abiotic (Kriegman-King and Reinhard 1992) transformation of CT. The pathway of biological CT transformation to CO_2 is not yet clear. It is generally agreed that the first step is a one electron reduction of CT to give a trichloromethyl radical and a chlorine ion (Criddle and McCarty 1991). A second electron transfer step would lead to the formation of dichlorocarbene, which hydrolyzes either to formic acid or carbon monoxide (Fig. 3.4). Transformation of CT to formic acid has never been reported but carbon monoxide formation from CT has been demonstrated (Krone et al. 1991, Stromeyer et al. 1992, Hashham et al. 1995, Chiu and Reinhard 1996). Both formic acid and carbon monoxide, when formed, can be biologically oxidized to CO_2. Biological transformation of CT to CO_2 in the reactor is possible. Abiotic hydrolysis of CT to CO_2 probably did not occur since in a sterile control reactor no transformation of CT was detected.

Although CT was mainly transformed to CF and DCM in the presence of sulfate in the reactor, under two different conditions CT transformation without the formation of CT and DCM occurred. First, at CT concentrations higher than 40 µM, when sulfate reduction (and methane production) was completely inhibited. Secondly, when besides sulfate reducing bacteria also methanogens were present in the packed-bed reactor. Under methanogenic conditions, CT is readily mineralized (Bouwer and McCarty 1983a, Egli et al. 1987, Chapter 2).

These results suggested that inhibition of sulfate reduction and/or stimulation of methanogenic activity can lead to complete CT mineralization. This could be used for the bioremediation of CT contaminated groundwaters containing high sulfate concentrations. Stimulation of methanogenic activity at sites with high sulfate concentrations could be achieved by the addition of an excess of electron donor

resulting in simultaneous sulfate reduction and methanogenesis.

Besides stimulation of methanogenic activity, the development of acetogenic activity or denitrification could possibly also lead to CT mineralization. Mineralization of CT to CO_2 is also the predominant transformation pathway under these electron accepting conditions (Bouwer and Mccarty 1983b, Egli et al. 1988, Criddle et al. 1990). CT transformation under iron reducing conditions has only been studied by Picardal et al. (1993). They found CF as the only transformation product of CT transformation in *Shewanella putrefaciens* 200.

Although complete dechlorination of CT by the sulfate reducing bacteria in our reactor did not occur without simultaneous formation of CF and DCM, there are possibilities to achieve complete anaerobic biological dechlorination of CT present in groundwater that also contains high sulfate concentrations. More information is needed on the costs and the possibilities for application of these techniques to assess whether bioremediation of contaminated groundwater is an attractive option that can compete with other techniques such as activated carbon absorption, extraction or stripping.

Chapter 4

Transformation of 1,1,1-trichloroethane in an anaerobic packed-bed reactor at various concentrations of 1,1,1-trichloroethane, acetate and sulfate

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Abstract

Biotransformation of 1,1,1-trichloroethane (TCA) was observed in an anaerobic packed-bed reactor under conditions of both sulfate reduction and methanogenesis. Acetate (1 mM) served as an electron donor. TCA was completely converted up to the highest investigated concentration of 10 µM. 1,1-Dichloroethane and chloroethane were found as main transformation products. A fraction of the TCA was completely dechlorinated via an unknown pathway. The rate of TCA transformation and the transformation products formed depended on the concentrations of TCA, acetate and sulfate. With an increase in sulfate- and TCA concentrations and a decrease in acetate concentration, the degree of TCA dechlorination decreased. Both packed-bed reactor studies and batch experiments with bromoethanesulfonic acid (BES), an inhibitor of methanogenesis, demonstrated the involvement of methanogens in TCA transformation. Batch experiments with molybdate showed that sulfate reducing bacteria in the packed-bed reactor were also able to transform TCA. However, packed-bed reactor experiments indicated that sulfate reducers only had a minor contribution to overall TCA transformation in the packed-bed reactor.

Abbreviations: TCA - 1,1,1-trichloroethane; DCA - 1,1-dichloroethane; CA - chloroethane

Introduction

1,1,1-Trichloroethane (TCA) is an ubiquitous contaminant in groundwater, mainly due to accidental spills in industrial processes. Because TCA is toxic for both man and animals, remediation of contaminated sites is necessary. Microbial remediation could be an attractive clean-up technique if rapid degradation can be achieved. However, TCA is one of the chlorinated aliphatic hydrocarbons that are difficult to degrade biologically. So far, dechlorination of TCA has not been described under aerobic or denitrifying conditions. Only Oldenhuis et al. (1989) reported partial conversion of TCA to trichloroethanol by *Methylosinus trichosporium* OB3b under aerobic conditions. Transformation of TCA in anaerobic continuous flow systems has been reported under both sulfate reducing and methanogenic conditions (Bouwer and McCarty 1983a, Bouwer and Wright 1988, Cobb and Bouwer 1991, Vogel and McCarty 1987a, Wrenn and Rittmann 1996). Transformation only occurred after a long acclimatization period (10-12 weeks) and was only investigated at TCA concentrations below 1 µM. Also the involvement of methanogenic and/or sulfate reducing bacteria in TCA transformation was not established.

1,1-Dichloroethane (DCA) was found as the main product of TCA biotransformation (Egli et al. 1987, 1988, Gälli and McCarty 1989, Gälli and McCarty 1989a, Parsons et al. 1985, Vogel and McCarty 1987a) but conversion to chloroethane (CA) (Parsons and Lage 1985, Vogel and McCarty 1987a) and complete dechlorination to CO_2 (Vogel and McCarty 1987a), acetic acid (Gälli and McCarty 1989) and unknown products (Gälli and McCarty 1989) was also detected. Transformation of TCA to DCA and CA occurs through reductive dechlorination. The pathway of complete TCA dechlorination is not yet clear.

The aim of this study was to explore the potential of complete TCA dechlorination in an anaerobic packed-bed reactor under methanogenic conditions of concentrations as high as 1.3 mg/l (10 µM). For the application of TCA biotransformation in the treatment of contaminated groundwaters it is important to obtain more information about the effect of important process parameters on TCA transformation. Therefore, the effects of varying sulfate and acetate (primary substrate) concentrations on the transformation of TCA were investigated. For a better understanding of these effects, the role of methanogenic and sulfate reducing bacteria in the transformation of TCA was studied.

Material and methods

Packed-bed reactor studies. The experiments were performed in an upflow packed-bed reactor (glass, height 32 cm, inside diameter 4.42 cm, volume 492 ml) (Chapter 2, Fig. 2.1) packed with polyurethane foam (PUR) particles (5×5×6 mm, Bayer BV, Mijdrecht, the Netherlands) mixed with digested sludge (20 v/v%) from the wastewater treatment plant Kralingseveer (Rotterdam, the Netherlands). The packed-bed reactor was wrapped with aluminum foil to prevent growth of phototrophs.

The packed-bed reactor was continuously fed with an anaerobic non-sterile mineral medium containing (mg/l) K_2HPO_4 (8), KH_2PO_4 (3.6), $NaHCO_3$ (40), NH_4Cl (26.6), $MgCl_2 \cdot 6H_2O$ (101.6), $CaCl_2 \cdot 2H_2O$ (62.6), resazurine (1). From a trace element solution, 0.125 ml/l were added. The trace element solution contained (mg/l) $FeSO_4 \cdot 7H_2O$ (2800), H_3BO_3 (50), $Al_2(SO_4)_3 \cdot 16H_2O$ (118.3), $MnCl_2 \cdot 4H_2O$ (50), $CuSO_4 \cdot 5H_2O$ (92.8), EDTA (500), $ZnCl_2$ (50), $(NH_4)_6Mo_7O_{27} \cdot H_2O$ (50), $CoCl_2$ (27.3), $NiCl_2 \cdot 6H_2O$ (91.6), 1 ml HCl (37%). The medium was continuously purged with a mixture of N_2 and CO_2 (99.5%/0.5%) (Hoek Loos BV, Dieren, the Netherlands) to remove all oxygen.

The medium (pH 7.3 ±0.2) was pumped into the packed-bed reactor by means of a peristaltic pump with marprene tubing (Watson Marlow, England). All other tubing was either viton or Teflon. TCA, acetate and Na_2S (42 µM, to maintain reducing conditions) were added to the medium as a concentrated solution at the influent of the packed-bed reactor with a syringe pump. The hydraulic retention time in the packed-bed was 24 h. All experiments were carried out at 25°C.

Batch culture studies. Experiments were done with the following medium (g/l): KH_2PO_4 (0.43), $Na_2HPO_4 \cdot 2H_2O$ (0.53), $CaCl_2 \cdot 2H_2O$ (0.12), $MgSO_4 \cdot 7H_2O$ (0.13), NH_4Cl (0.3) and resazurine (0.0005). The medium also contained 1 ml of trace element solution (see above) and 1 ml of a vitamin solution. The vitamin solution contained (mg/l): biotin (2), folic acid (2), riboflavin (5), thiamin (5), cyanocobalamin (5), nicotinamide (5), *p*-aminobenzoic acid (5).

The medium was purged with a mixture of CO_2 and N_2 (0.5:99.5 v/v%, 700 ml/min) for 30 min. After 15 min, $Na_2S \cdot 9H_2O$ (67 mg/l) and $NaHCO_3$ (100 mg/l) were added. The medium was transferred to 120 ml bottles (brown glass) in an anaerobic glove-box. Each bottle contained 60 ml of medium and was closed with Teflon-lined butyl rubber stoppers and aluminum crimp seals. TCA (5.8 µM) and acetate (1 mM) were added as concentrated solutions.

All batch cultures were inoculated with 2 ml of the liquid phase taken from the packed-bed reactor (1 ml of liquid phase from sample port 2 and 1 ml from sample port 3; Chapter 2, Fig. 2.1). The cultures were incubated on a shaker (100 rpm) in a canted position (90°) at 25°C. In sterile control batch cultures, there was no transformation of TCA and acetate (data not shown).

Inhibitor studies. To investigate the role of methanogenic, acetogenic and sulfate reducing microorganisms in the transformation of TCA, three inhibitors were used. After complete transformation of the first amount of added TCA, vancomycin (0.14 mM), 2-bromoethanesulfonic acid (BES 6 mM) or molybdate (2 mM) were added to the batch cultures together with TCA (5.8 µM) and acetate (0.5 mM).

Analytical methods. TCA, DCA and CA were quantified by headspace gas chromatography. Liquid samples (100-1000 µl) were injected in 10 ml headspace autosampler vials with teflon-lined butyl rubber stoppers and aluminum crimp seals. The final volume was adjusted to 2 ml with demineralized water. The vials were analyzed using a Hewlett Packard 19395A headspace sampler connected to a gas chromatograph equipped with an electron capture detector and a CP-Sil 5CB reactor (Chapter 2). Calibration samples were analyzed according to the same method to adjust for air/water partitioning. A four-point curve was used for quantification.

Carbon dioxide, carbon monoxide and methane concentrations were determined after separation on a Carboplot P7 column using a gas chromatograph equipped with FID and a methanizer (Chapter 2). Liquid samples (2 ml) from the packed-bed reactor were injected in 10 ml headspace autosampler vials with teflon-lined butyl rubber stoppers and aluminum crimp seals and equilibrated at 80°C for 45 min. An amount of 50 µl of the headspace was injected into the GC by hand with a 100 µl Hamilton gas and liquid-tight syringe. For batch cultures, 50 µl of the headspace was injected into the GC. A four point calibration curve was used for quantification.

Sulfate was determined after separation on an IONPAC AG9-SC guard column and IONPAC AG9-SC anion column (Dionex, Breda, the Netherlands) (Chapter 2) with an ion chromatograph equipped with a conductivity detector, thermal stabilizer and suppressor (Dionex, Breda, the Netherlands).

Acetate concentrations were determined with an enzymatic test-combination (Boehringer Mannheim, Germany).

Chemicals. All chemicals were obtained from commercial companies. TCA and CA were obtained from Fluka. DCA and sodium acetate were obtained from Janssen Chimica. Vancomycin and sodium molybdate were purchased from Sigma. 2-Bromoethanesulfonic acid was obtained from Aldrich. Calibration gases were obtained from AGA (carbon dioxide, methane).

Results

Transformation of TCA in an anaerobic packed-bed reactor inoculated with digested sludge was studied to establish the possibilities of complete TCA dechlorination under methanogenic conditions. Before the addition of TCA, the packed-bed reactor was operated for two weeks with acetate (1 mM) as the sole substrate. After two weeks, 75% of the added acetate were utilized. Methane production (0.62 mM) indicated that 62% of the acetate added were converted by methanogens. The presence of methanogens was confirmed by fluorescence microscopy according to the method described by Doddema and Vogels (1978). Sulfate reducing bacteria utilized 13% of the added acetate for the complete reduction of all available sulfate (0.13 mM). Although no sulfate was added, about 0.13 mM sulfate was present in the influent of the packed-bed reactor, probably due to (microbial) oxidation of sulfide by traces of oxygen in the influent.

When TCA (0.75 µM) was added to the packed-bed reactor it took 7 days until complete breakthrough of TCA. This period of time is a result of initial sorption of TCA to PUR, the carrier material of the packed-bed reactor. This sorption was taken into account when collecting steady state samples. A steady state was characterized by a constant degree of removal of TCA for a time period of 14 days after at least 7 hydraulic detention times.

Seven days after complete breakthrough of TCA, TCA transformation started and DCA was found as the only transformation product. Two days later, CA was also detected. During the next twenty days, the concentration of DCA in the effluent decreased until TCA was completely recovered as CA. Subsequently, the concentration of CA in the effluent of the bioreactor also decreased. In a steady state 62.5% of the added TCA were recovered as CA (Fig. 4.1A) while 38.5% were converted to unknown products. These results indicate that part of the TCA was probably mineralized, or completely converted to ethane by reductive dechlorination.

Transformation at different 1,1,1-trichloroethane concentrations

The transformation of TCA in the packed-bed reactor was studied at a concentration range from 1.3 µM to 10 µM under starting conditions. At all concentrations tested, TCA was completely transformed to DCA, CA and unknown products (Fig. 4.1A). Up to a TCA concentration of 2.5 µM, CA was found as the only chlorinated transformation product. At higher TCA concentrations, also DCA was detected. With an increase in TCA concentration the percentage of TCA recovered as chlorinated intermediates (=TOTout) also increased, while the percentage of TCA transformed to unknown products decreased. At the highest concentration tested, TCA (10 µM) was nearly completely converted to the dichloro and monochloro derivatives.

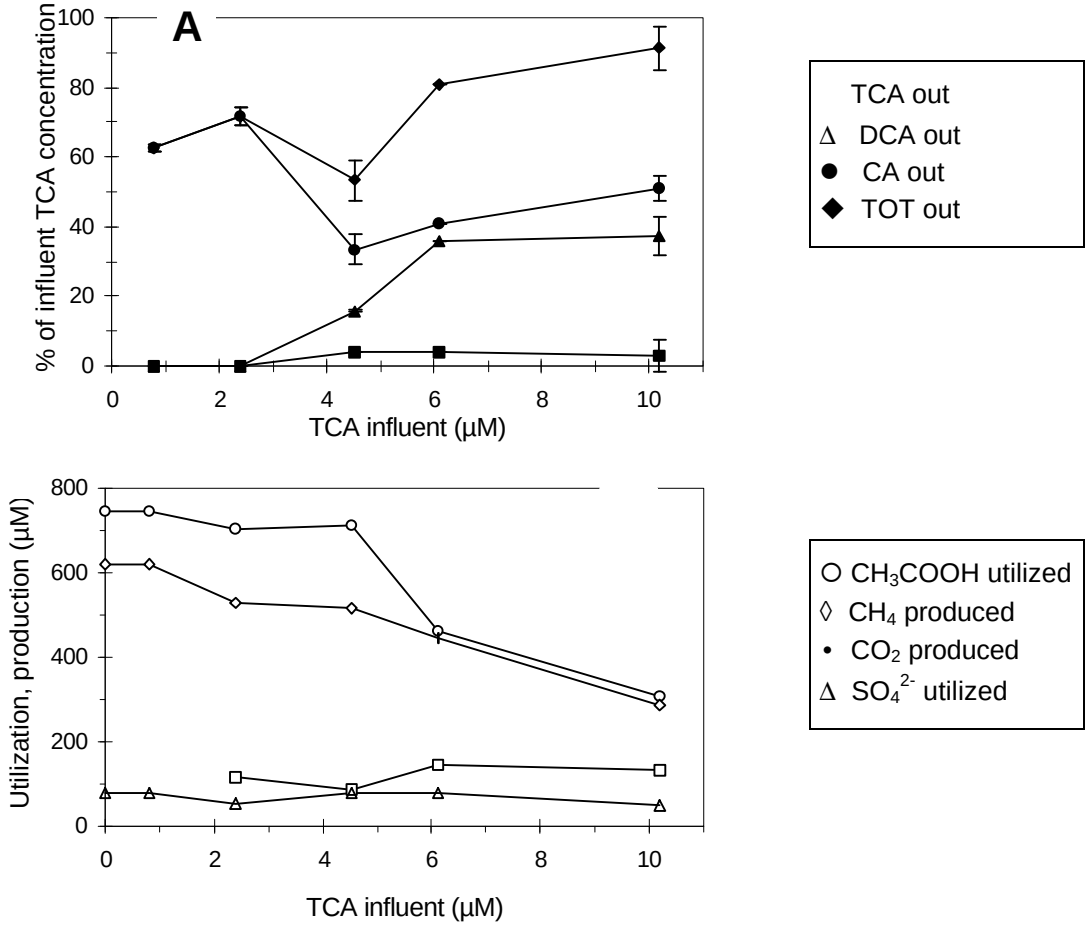

Figure 4.1. *Effect of 1,1,1-trichloroethane concentration on its transformation (**A**) and the substrate conversion (**B**) in an anaerobic packed-bed reactor. Chlorinated ethanes in effluent of reactor are expressed as the percentage of TCA in the influent. Error bars represent standard deviations on 2-5 measurements taken within 7 days. TOT out = (TCA out + DCA out + CA out).*

Up to a TCA concentration of 4.5 µM, acetate (1 mM) was utilized for about 75% by both methanogenic and sulfate reducing bacteria (Fig. 4.1B). Methane was produced (0.529 mM) and sulfate (0.06 mM) completely reduced. At higher TCA concentrations, sulfate was still completely removed, but less acetate was metabolized. This was caused by a decrease in methanogenic activity, as evident from a decrease in methane production. These results indicate that methanogenic activity is probably inhibited by TCA at concentrations higher than 4.5 µM. This is close to the inhibition level between 6 µM and 15 µM that was reported by Vargas and Ahlert (1987) for semi-batch culture studies with a mixed anaerobic culture.

Effect of sulfate concentration on the transformation of 1,1,1-trichloroethane

The effect of the sulfate concentration on TCA removal was tested by increasing the sulfate content of the influent of the packed-bed reactor in 4 steps from 0.06 mM to 0.95 mM. At the starting conditions, TCA (2.5 µM) was completely degraded to CA and unknown products (Fig. 4.2A). 70% of the added acetate (1 mM) were utilized. Sulfate reducing bacteria utilized 0.06 mM and methanogenic bacteria converted 0.61 mM to methane (Fig. 4.2B).

Up to a concentration of 0.33 mM, sulfate had no significant effect on the transformation of TCA (Fig. 4.2A). At higher concentrations, sulfate clearly influenced the transformation of TCA. DCA again was found as a transformation product and the concentration of CA in the effluent of the packed-bed reactor decreased, indicating that TCA transformation became less complete.

At a sulfate concentration of 0.95 mM, the degree of TCA removal decreased rapidly. After 7 days of operation at a sulfate concentration of 0.95 mM, only 56.5% of the added TCA were transformed. DCA (0.72 µM) was found as the only transformation product. No formation of CA occurred. To prevent complete loss of TCA transformation capacity, the sulfate concentration was decreased to the original concentration of 0.06 mM before a steady state was reached.

During the increase in sulfate concentration in the influent of the packed-bed reactor, the amount of sulfate reduced increased from 0.06 mM at an influent sulfate concentration of 0.060 mM to 0.85 mM at an influent sulfate concentration of 0.95 mM (Fig. 4.2B). At the same time, methane production by methanogenic bacteria decreased from 0.61 mM to 0.09 mM. These results indicate that sulfate reducing bacteria were not involved in the transformation of TCA. Methanogenic bacteria probably play a role in the transformation of TCA since the decrease of TCA transformation coincided with the decrease of methane production by

methanogenic bacteria.

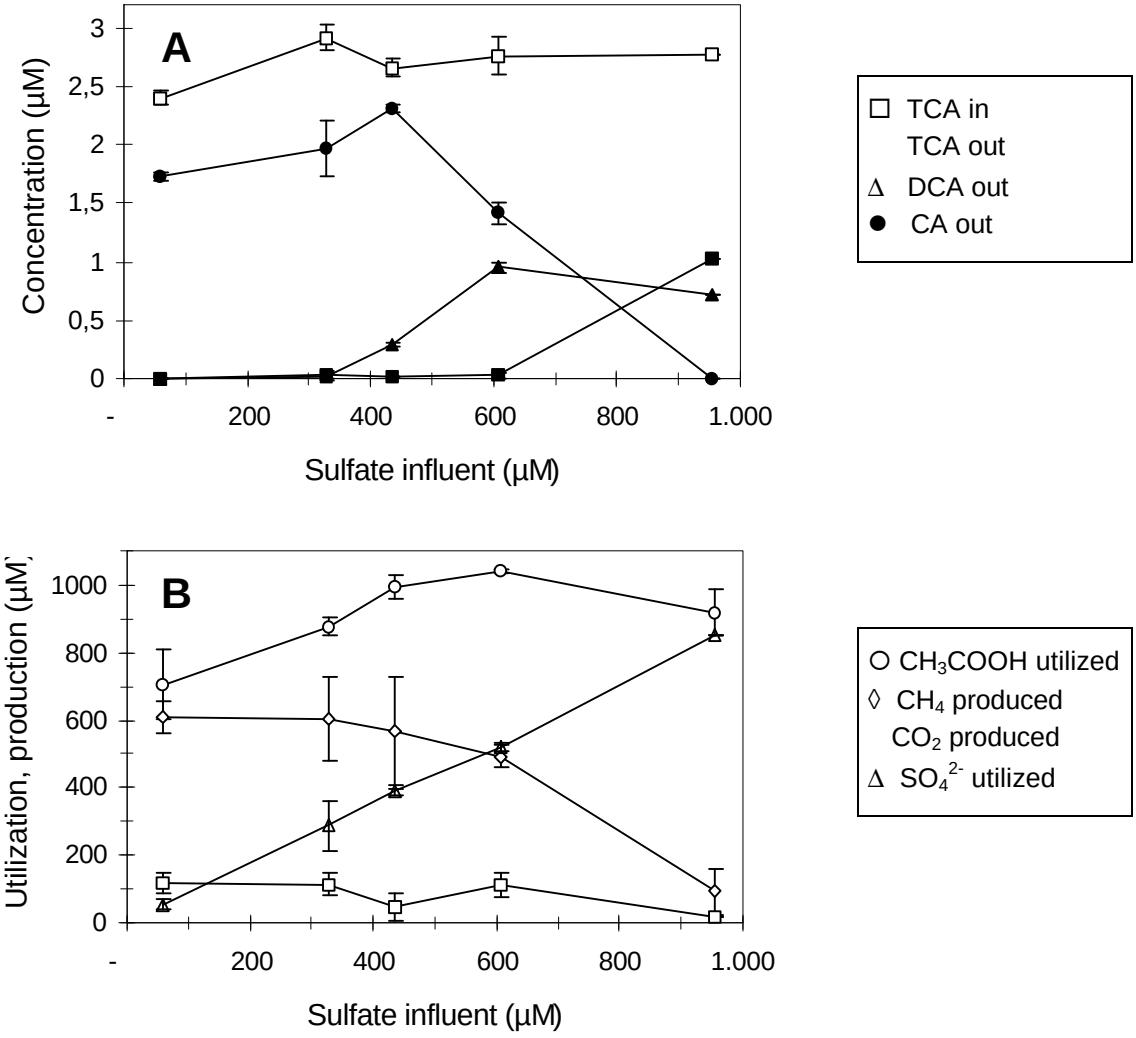

Figure 4.2 *Effect of sulfate concentration on the transformation of 1,1,1-trichloroethane (**A**) and acetate conversion (**B**) in an anaerobic packed-bed reactor. Error bars represent standard deviations on 2-5 measurements taken within 7 days.*

Effect of acetate concentration on 1,1,1-trichloroethane transformation

The effect of the electron donor concentration on TCA transformation was tested in a range from 0.25 mM to 1 mM. The packed-bed reactor was first operated under starting conditions at a TCA concentration of 10 µM. TCA was mainly converted to DCA (3.8 µM) and CA (5.2 µM) (Fig. 4.3A). About 10% were converted to unknown products. Acetate (1 mM) was utilized for 23% by

methanogens and sulfate reducing bacteria (Fig. 4.3B).

In the range studied, the acetate concentration did not have a significant effect on TCA transformation (Fig. 4.3A). TCA transformation remained complete at all times. However, the acetate concentration had a profound effect on the transformation products formed. At lower acetate concentrations, less TCA was converted to CA while the concentration of DCA in the effluent of the packed-bed reactor increased. The changes in the transformation products formed coincided with a decrease in methanogenic activity (Fig. 4.3B). Again, this indicates that methanogenic bacteria were involved in the transformation of TCA. Sulfate reduction (0.06 mM) did not change and remained complete at all times.

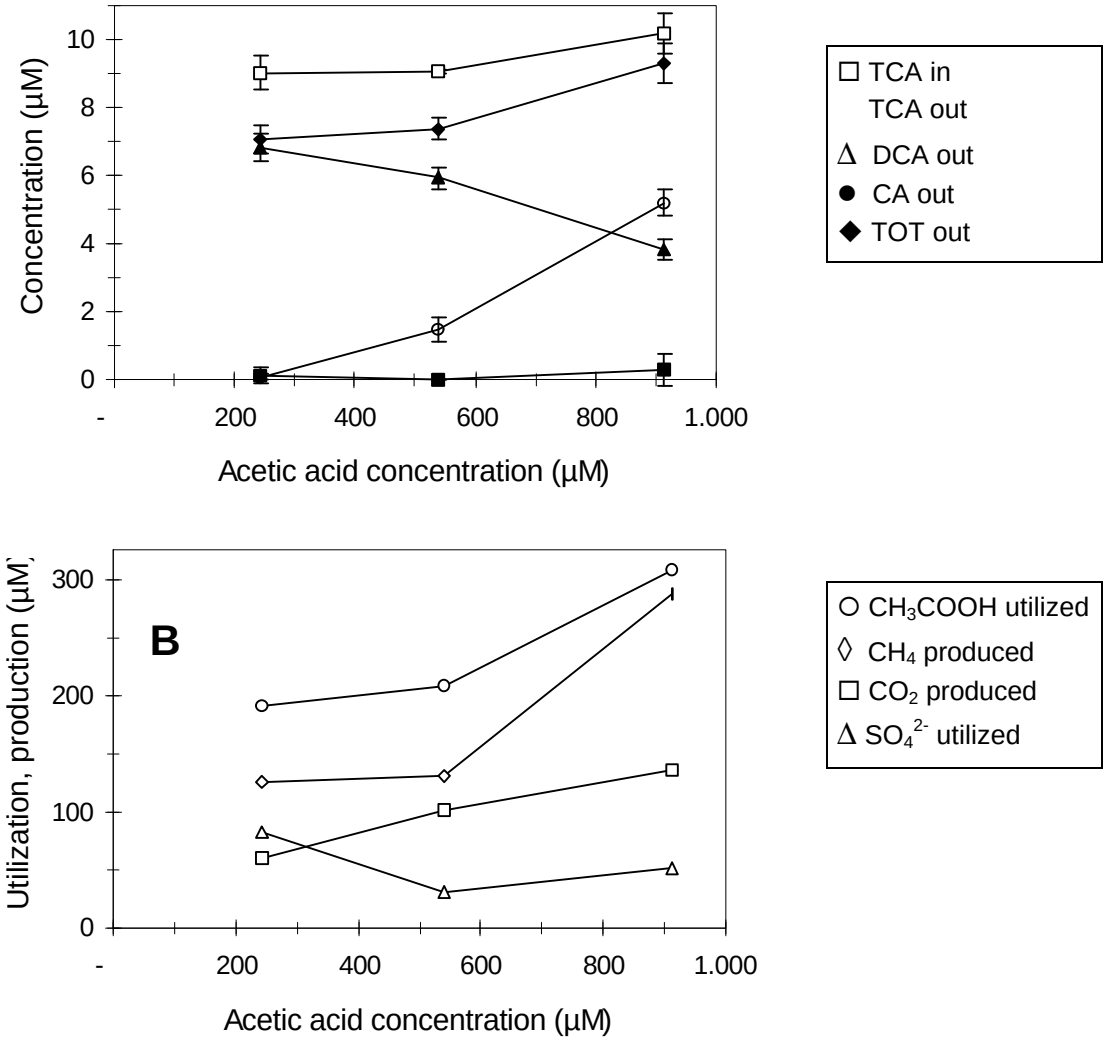

Figure 4.3 *Effect of acetate concentration on 1,1,1-trichloroethane transformation (**A**) and acetate conversion (**B**) in anaerobic packed-bed reactor. TOT out = (TCA out + DCA out + CA out)*

Transformation of 1,1,1-trichloroethane in batch cultures

The results presented above indicate that methanogenic bacteria and sulfate reducing bacteria were both present in the packed-bed reactor. The involvement in TCA transformation of these two bacterial groups and of acetogenic bacteria was investigated by adding specific inhibitors to batch cultures. First, a TCA-degrading microbial population was cultivated in the absence of inhibitors. The cultures were inoculated with liquid from the packed-bed reactor. In all batch cultures TCA transformation started within one week. After 23 days, TCA (5.83 µM) was completely converted. Acetate (1 mM) was utilized for 55-60% and converted to about 580 µM of methane. When TCA was completely transformed, it was added again together with acetate (0.5 mM). At the same time specific inhibitors were added (Table 4.1).

Table 4.1 *Effect of inhibitors on the transformation of 1,1,1-trichloroethane in batch cultures. Disappearance is marked as - and formation is marked as + (TCA, 1,1,1-trichloroethane; DCA, 1,1-dichloroethane; UP, unknown products; BES, 2-bromoethanesulfonic acid).*

Inhibitors	**Chlorinated hydrocarbons**			**Substrate conversion**		
	TCA (µM)	DCA (µM)	UP (µM)	CH_3COOH (µM)	CH_4 (µM)	SO_4^{2-} (µM)
None	-5.8	+4.0	+1.8	-350	+135	- 15.0
Molybdate	-2.5	+2.2	+0.3	-167	+147	- 16.7
Vancomycin	-5.0	+3.7	+1.3	-183	+157	- 6.7
BES	0	0	0	0	+ 20	+ 33.3

2-Bromoethanesulfonic acid (BES), an inhibitor of methanogenesis (Distefano et al. 1992), completely inhibited TCA transformation and methane production. This confirms the findings in the packed-bed reactor that methanogenic bacteria were involved in the transformation of TCA.

In the presence of molybdate, an inhibitor of sulfate reduction (Smith and Klug 1981), TCA transformation was partly inhibited. Molybdate also had an effect on the ratio of transformation products that were formed. More TCA was converted to DCA (87% versus 71% in the absence of inhibitors). This means that sulfate reducing bacteria could be involved in TCA transformation, although no significant reduction of sulfate was detected in any of the batch cultures.

Vancomycin is an inhibitor of cell wall synthesis in gram-positive eubacteria and was used to inhibit acetogenic bacteria (Distefano et al. 1992). Vancomycin did not affect the transformation of TCA. This means that acetogenic bacteria as

described by Egli et al. (1988) and Clostridia, as described by Gälli and McCarty (1989a, 1989b), were not responsible for the TCA transformation observed in our packed-bed reactor.

Discussion

In this paper, TCA transformation in an anaerobic packed-bed reactor in which both sulfate reduction and methanogenesis occurred is described. In the range (0.75 - 10 µM) studied, TCA was degraded for over 95% under standard conditions (0.1 mM sulfate and 1 mM acetate). DCA and CA were found as main transformation products. Part of the TCA was converted to unknown non-chlorinated products. This is the first report of TCA transformation in a continuous flow reactor with CA as main transformation product (>90%). Vogel and McCarty (1987a) also described CA as a transformation product in a continuous-flow reactor, but CA only accounted for 5% of the transformation products formed, whereas over 90% were transformed to DCA. We observed that up to 90% of added TCA were transformed to CA.

The results of the batch experiments with inhibitors and of the packed-bed reactor studies suggest that methanogenic bacteria are involved in TCA transformation. In the packed-bed reactor, both higher sulfate concentrations and lower acetate concentrations inhibited TCA transformation. This inhibition coincided with a decrease in methanogenic activity. Inhibition of TCA transformation by sulfate has not been reported before, but there are several reports about inhibition of dechlorination of other chlorinated compounds by sulfate (Suflita et al. 1988, Kuhn et al. 1990, Sharak Genther et al. 1989, Kohring et al. 1989, Gibson and Suflita 1986). In all cases, the inhibition of dechlorination activity was caused by the inhibition of methanogenic activity. Cobb and Bouwer (1991) reported no effect of sulfate on the transformation of TCA in a biofilm reactor. However, sulfate was only added at very low concentrations (0.1 mM) at an acetate concentration (electron donor) of 1 mM. Under these conditions, no inhibition of TCA transformation was observed in our packed-bed reactor, since methanogenesis was not suppressed. Wrenn and Rittmann (1996) also reported no effect of sulfate on TCA transformation in a methanogenic biofilm reactor even at a sulfate/formate ratio of 1. However, in these experiments no time for adaptive changes in the microbial population was allowed.

Batch experiments with molybdate indicated that sulfate reducing bacteria in the packed-bed reactor could also transform TCA, as observed before by Egli

et al. (1987) for *Desulfobacterium autotrophicum*. However, both the effect of the acetate and sulfate concentration on TCA transformation showed that sulfate reducing bacteria only accounted for a minor percentage of TCA transformation in the packed-bed reactor.

Transformation of TCA in the packed-bed reactor mainly occurred through reductive dechlorination to DCA and CA (Fig. 4.4). The percentage of TCA transformed to CA depended on the methanogenic activity. With an increase in methanogenic activity, the extent of TCA dechlorination also increased. This suggests that TCA transformation by methanogens in the packed-bed reactor is a cometabolic process with no benefit for the organisms.

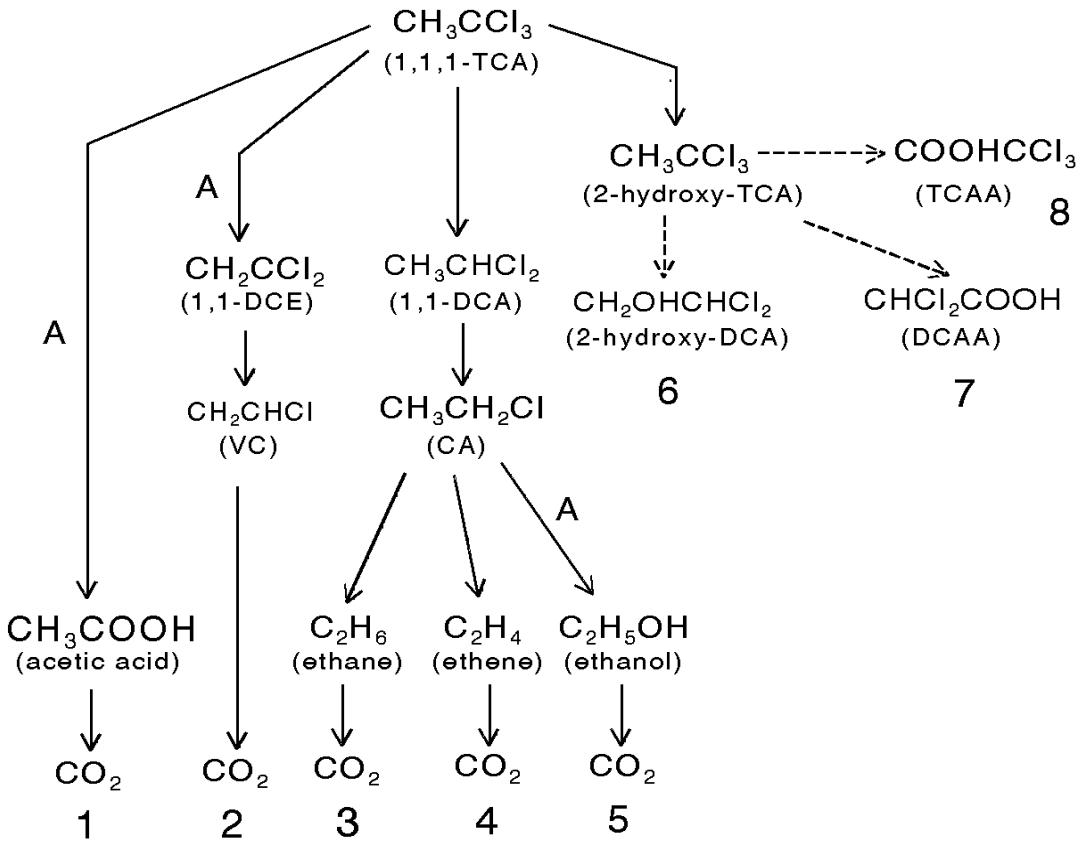

Figure 4.4 *Pathways for the transformation of 1,1,1-trichloroethane (deduced from Vogel and McCarty 1987). Transformations that have been reported are shown with solid lines. Proposed pathways are shown with dotted lines. Abiotic transformations are marked A. TCA, 1,1,1-trichloroethane; DCA, 1,1-dichloroethane; CA, chloroethane; VC, vinylchloride; DCE, 1,1-dichloroethene.*

Part of the TCA was converted to unknown products. For the transformation of TCA to other products than DCA and CA there are two

possibilities: either CA, when formed, was further converted or TCA was transformed via other initial reactions (Fig. 4.4). CA can undergo both biotic transformation to ethene or ethane (pathway 3 & 4, Belay and Daniels 1987, Vogel and McCarty 1987a) and abiotic transformation to ethanol (pathway 5, Vogel and McCarty 1987b). Three pathways have been described for the transformation of TCA via other initial reactions. First, TCA can be converted to non-volatile halocarbons according to pathway 6, 7 and 8 (Gälli and McCarty 1989). We did occasionally analyze for halogenated acetic acids but never detected any of these compounds. The second pathway has been described by Gälli and McCarty (1989a). They found transformation of TCA to acetic acid by a *Clostridium* sp (pathway 1). Finally TCA can undergo abiotic transformation to 1,1-dichloroethene (pathway 2) which can be further degraded (Gälli and McCarty 1989a, Vogel and McCarty 1987a, Vogel and McCarty 1987b). We never detected any 1,1-dichloroethene in the packed-bed reactor as expected since the first-order rate coefficient for abiotic 1,1-dichloroethene formation is only 0.0024 d^{-1} (Gälli and McCarty 1989a). Our results suggest that part of the TCA in the packed-bed reactor was converted to non-chlorinated products. It is not yet clear via which pathway complete dechlorination of TCA occurred and to which products.

Our results suggest that TCA removal by methanogens is a feasible option, provided that sulfate can be removed and a sufficient amount of suitable electron donor is added. Complete dechlorination occurred, but usually DCA and CA accumulated as undesirable transformation products. Sequential anaerobic/aerobic transformation of TCA now seems a feasible option for complete mineralization of TCA since both DCA and CA can be degraded under aerobic conditions (Oldenhuis et al. 1989, Scholtz et al. 1987). DCA transformation under oxic conditions is much slower than CA transformation and appears to be a cometabolic process (Vogel et al. 1987, McCarty and Semprini 1994). Therefore, a packed-bed reactor that would completely transform TCA to CA and not form any DCA is of great interest. Further research will focus on the possibilities of complete transformation of TCA to CA under methanogenic conditions and the mechanism of this transformation in methanogens.

Chapter 5

Complete transformation of 1,1,1-trichloroethane to chloroethane by a methanogenic mixed population

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Abstract

A methanogenic mixed population in a packed-bed reactor completely transformed 1,1,1-trichloroethane (10 µM) to chloroethane by a cometabolic process. Chloroethane was not further transformed. Acetate and methanol served as electron donors. Complete transformation of 1,1,1-trichloroethane to chloroethane only occurred when sufficient electron donor was fed into the reactor. Otherwise, besides chloroethane also 1,1-dichloroethane was found as a product. The products of 1,1,1-trichloroethane transformation also depended on the type of electron donor present. With acetate, the degree of dechlorination was higher, i.e. more 1,1,1-trichloroethane was transformed to chloroethane than with methanol. In an enrichment culture obtained from the reactor contents, 1,1,1-trichloroethane was only transformed to 1,1-dichloroethane and was not further metabolized. Methanol, acetate, formate, ethanol, 2-propanol, trimethylamine and H_2, but not dimethylamine and methylamine, served as electron donors for 1,1,1-trichloroethane transformation by this enrichment culture. Both nitrate and nitrite inhibited 1,1,1-trichloroethane transformation; while nitrate completely inhibited 1,1,1-trichloroethane dechlorination, some conversion did occur in the presence of nitrite. The product(s) of this conversion remain unknown, since no chlorinated hydrocarbons were detected.

Abbreviations: TCA - 1,1,1-trichloroethane; DCA - 1,1-dichloroethane; CA - chloroethane

Introduction

The toxic solvent 1,1,1-trichloroethane (TCA) is often encountered as a contaminant in soil and groundwater. The evidence available to date indicates that it can only be biodegraded at a significant rate under anaerobic conditions. Complete mineralization of TCA has been described (Vogel and McCarty 1987, Gälli and McCarty 1989, Chapter 4), but usually 1,1-dichloroethane (DCA) and chloroethane (CA) are found as main transformation products (Parsons et al. 1985, Gälli and McCarty 1989, Vogel and McCarty 1987, Chapter 4).

Bioremediation can only be considered as a useful remediation technique for TCA-contaminated sites if complete dechlorination can be achieved. Sequential anaerobic/aerobic transformation of TCA seems a feasible option since both DCA and CA, products of anaerobic TCA transformation, can be degraded under aerobic conditions (Oldenhuis et al. 1989, Scholtz et al. 1987). DCA transformation under oxic conditions is much slower than CA transformation and appears to be a cometabolic process (Vogel et al. 1987, McCarty and Semprini 1994). Therefore, complete transformation of TCA to CA under anaerobic conditions without formation of DCA is of interest.

Previous studies showed that the ratio of DCA to CA, as products of TCA transformation by a methanogenic population in a packed-bed reactor, depended on the electron donor concentration in the reactor feed and was inhibited by sulfate (Chapter 4). The aim of this study was to obtain complete transformation of TCA to CA in this reactor and establish how it is influenced by process conditions. Therefore, the transformation of TCA and it's products were studied at different electron donor concentrations in the reactor feed. The effect of the type of electron donor on the products of TCA transformation was also investigated. Furthermore, the effects of the electron acceptors nitrate and nitrite on TCA transformation is described. Finally the effect of the pH and the temperature, both important process parameters, is discussed.

Material and methods

Packed-bed reactor studies. The experiments were performed in an upflow packed-bed column (glass; height 32 cm; inside diameter 4.42 cm; volume 492 ml) (Chapter 2) packed with polyurethane foam (PUR) particles (5x5x6 mm, Bayer B.V., Mijdrecht, the Netherlands) which were mixed with digested sludge (20 v/v%) from the wastewater treatment plant Kralingseveer (Rotterdam, the Nether-

lands). The packed-bed reactor was wrapped with aluminum foil to prevent growth of phototrophs.

The column was continuously fed with an anaerobic non-sterile phosphate and bicarbonate buffered mineral medium (Chapter 2). The medium was continuously purged with oxygen free N_2/CO_2 (99.5%/0.5%) to remove oxygen. The medium (pH 7.3 ±0.2) was pumped into the column by means of a peristaltic pump with marprene tubing. All other tubing was either viton or teflon. TCA, Na_2S (41.8 µM, to maintain reducing conditions), acetate and/or methanol were added to the medium as a concentrated solution at the influent of the column with a syringe pump. The medium contained no sulfate, but about 20 µM sulfate tended to be present in the influent of the reactor, probably due to oxidation of sulfide by oxygen permeating the tubing. The hydraulic retention time in the reactor was 24 h. All experiments were carried out at 25°C.

Batch culture studies. Batch culture studies were done with enrichment cultures which were obtained from the TCA transforming packed-bed reactor according to the method described previously (Chapter 2).

Effect of nitrate and nitrite: TCA (5 µM), acetate (1 mM) and methanol (1 mM) were added to the batch cultures as concentrated solutions. After inoculation, nitrate (0, 240, 490, 950 and 2,010 µM) or nitrite (0, 80, 187, 397 and 1,092 µM) was added as a concentrated solution. The batch cultures were analyzed daily for chlorinated ethanes, methane, carbon dioxide, sulfate, nitrate and nitrite.

Effect of temperature and pH: After addition of TCA (5 µM), acetate (1 mM) and methanol (1 mM), the batch cultures were inoculated and incubated at different temperatures (11.1; 20.5; 25.0; 30.0; 37.3 and 44.0°C). The cultures were analyzed daily for chlorinated ethanes.

The effect of the pH (6.71; 6.82; 7.26; 7.45; 7.54; 7.87 and 8.17) on TCA transformation (5 µM) was tested in batch cultures with 50 ml of medium and 10 ml of different buffer solutions to obtain the different pH-values. After addition of acetate (1 mM) and methanol (1 mM) as electron donors, the batch cultures were inoculated. The cultures were analyzed daily for chlorinated ethanes.

Different electron donors: To last the effect of different electron donors on TCA transformation, sulfate was omitted from the medium and replaced by $MgCl_2$. TCA (4.0 µM) and all electron donors tested were added as concentrated solutions. H_2 was added to the headspace with a gastight syringe. The final concentration of all electron donors was 1.0 mM. The cultures were analyzed regularly for chlorinated ethanes, methane, carbon dioxide and electron donor.

Analytical methods. TCA, DCA and CA were quantified by headspace gas

chromatography. Liquid samples (100-1,000 µl) were injected in 10 ml headspace autosampler vials closed with teflon-lined butyl rubber stoppers and aluminum crimp seals. The final volume was adjusted to 2 ml with demineralized water. The vials were analyzed using a Hewlett Packard 19395A headspace sampler connected to a gas chromatograph equipped with an ECD and a CP-Sil 5CB column (Chapter 2). Calibration samples were analyzed according to the same method to adjust for air/water partition. A four point curve was used for calibration.

Carbon dioxide and methane concentrations were determined after separation on a Carboplot P7 column using a gas chromatograph equipped with FID and a methanizer (Chapter 2). For the reactor, liquid samples (2 ml) were injected in 10 ml headspace autosampler vials closed with teflon-lined butyl rubber stoppers and aluminum crimp seals and equilibrated at 80°C for 45 min. A volume of 50 µl of the headspace was injected into the GC by hand with a 100 µl Hamilton gas and liquid-tight syringe. For batch cultures, 50 µl of the headspace was injected into the GC. A four point calibration curve was used for quantification.

Sulfate, nitrate and nitrite were determined after separation using an IONPAC AG9-SC guard column and IONPAC AG9-SC anion column (Dionex, Breda, the Netherlands) on an ion chromatograph equipped with a conductivity detector, thermal stabilizer, and ASRS suppressor (Chapter 2).

Methanol was quantified by gas chromatography. Liquid samples were centrifuged (10,000 g for 10 min) and injected in 2 ml screw-cap vials with teflon-lined silicone liners. The vials were sampled (10 µl) with a Chrompack CP 9010 liquid sampler and analyzed on a Chrompack 9001 gas chromatograph (Chrompack, Bergen op Zoom, the Netherlands) equipped with a split injector (split ratio 1:10), a Chrompack CP-Poraplot Q column (length 25 m, inner diameter 0.32 mm, film thickness 10 µm) and an FID detector. Helium served as a carrier gas (0.8 ml/min). The GC had the following settings: injection temperature, 250°C; oven temperature, 100°C; detection temperature, 275°C. The detector signal was processed with the Maestro Chromatography Data System (Chrompack, Bergen op Zoom, the Netherlands). A five point calibration curve was used for quantification.

Acetate concentrations were determined using an enzymatic test-combination (Boehringer, Mannheim, Germany).

Results

Electron donor concentration

Previous studies showed that cometabolic transformation of TCA (10 µM) by a methanogenic population was possible in an anaerobic packed-bed reactor (Chapter 4). DCA and CA were found as transformation products in the reactor effluent. To determine whether TCA could be completely transformed to CA in the reactor, TCA transformation was studied at different electron donor concentrations.

At the starting conditions, TCA (10.4 µM) was completely transformed to DCA (2.4 µM) and CA (8.0 µM). Acetate (0.97 mM) and methanol (0.69 mM), serving as electron donors, were completely converted. Methane production (0.9 mM) indicated that part of acetate and methanol added was converted by methanogens. Sulfate reducing bacteria utilized part of the available electron donor for the reduction of all available sulfate (20 µM).

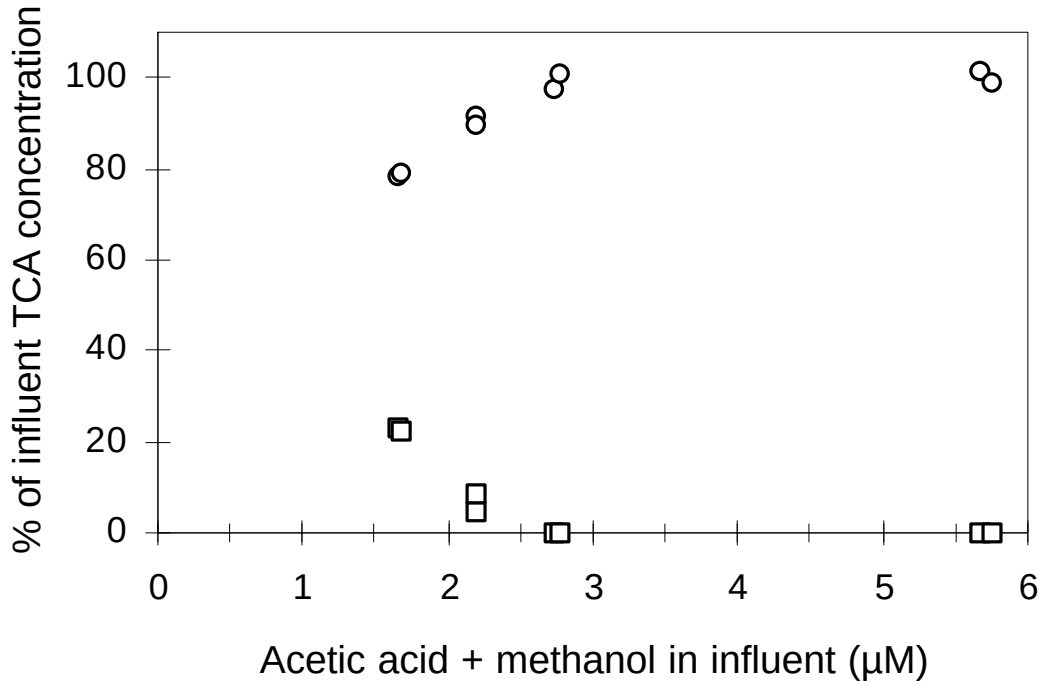

Figure 5.1 *Effect of the electron donor concentration on the transformation of 1,1,1-trichloroethane by methanogens in a packed-bed reactor. Chlorinated ethanes in the effluent are expressed as the percentage of 1,1,1-trichloroethane in the influent. The concentration of CH_3OH in the influent of the reactor is 0.69 mM. The concentration of CH_3COOH varies between 1 and 5 mM. Symbols: 1,1-dichloroethane (□); chloroethane (○).*

With an increase of the acetate concentration from 0.97 mM to 1.5 mM, leaving the concentration of methanol (0.69 mM) unchanged, the proportion of TCA that was transformed to CA increased, and less DCA was found as a transformation product (Fig. 5.1). At an acetate concentration of 2.1 mM, TCA was completely transformed to CA. DCA was no longer detected as a transformation product. CA was not further transformed. Even when the acetate concentration was increased from 2.1 mM to 5.0 mM, all TCA transformed was recovered as CA in the effluent of the reactor. The maximum dechlorination rate of $9.6 \cdot 10^{-3}$ $kg \cdot m^{-3} \cdot d^{-1}$ that was calculated for TCA transformation in the reactor is similar to the transformation rates for TCA described previously (Bouwer and McCarty 1983, Bouwer and Wright 1988). Both acetate and methanol were utilized for more than 97% by the methanogenic population in the reactor at all concentrations tested.

Different electron donors

The difference between methanol and acetate as electron donors for TCA transformation in the reactor was examined. In the presence of acetate (1.56 mM), TCA (10.4 µM) was completely transformed to DCA (2.5 µM) and CA (8.1 µM) (Table 5.1).

Table 5.1 *Transformation of different electron donors in a TCA (10 µM) transforming packed-bed reactor.*

Electron donor	**CH_3COOH (µM)**	**CH_3OH (µM)**
Influent		
CH_3COOH	1562	0
CH_3OH	0	1529
TCA	10.4	9.8
Effluent		
TCA transformed	10.4	9.8
DCA formed	2.4	7.8
CA formed	8.0	1.7
CH_3COOH utilized	1562	--
CH_3OH utilized	--[a]	1529
CH_4 formed	990	759
CO_2 formed	909	203
SO_4^{2-} reduced	<2	<2
ratio $[CH_{4\ m}]/[CH_{4\ th}]$[b]	0.63	0.66

[a] not determined; [b] ratio of the measured CH_4 production ($CH_{4\ m}$) and the theoretically expected CH_4 production ($CH_{4\ th}$).

Acetate was completely utilized, mainly by methanogens as indicated by methane production (0.99 mM). Sulfate reduction did not occur. Replacement of acetate by methanol (1.53 mM) as electron donor in the reactor had a profound effect on TCA transformation. When a steady state was reached, TCA was still completely transformed but TCA was no longer primarily transformed to CA (1.7 µM) but to DCA (7.8 µM). The production of methane decreased (Table 5.1) as predicted from the higher [methane]/[electron donor] ratio with acetate as an electron donor (reaction I) compared to that obtained with methanol (reaction II).

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad (I)$$

$$CH_3OH \rightarrow 0.75CH_4 + 0.25CO_2 + 0.5H_2O \qquad (II)$$

A theoretical $[CH_{4\ acetate}]/[CH_{4\ methanol}]$ of 1/0.75 = 1.33 can be calculated from the stoichiometry of these reactions. This ratio is close to the observed ratio of 990/759 = 1.3 in the reactor.

The results indicated that acetate was a more suitable electron donor than methanol for transformation of TCA by methanogens as the degree of dechlorination in the reactor was much better, i.e. more TCA was transformed to CA with acetate as an electron donor than with methanol as an electron donor.

Table 5.2 *Effect of different electron donors (1 mM) on the transformation of TCA by an enrichment culture from a TCA transforming packed-bed reactor.*

Electron donor	**t= 48 days**			
	TCA transformed		**DCA formed**	
	(µM)	**(%)**	**(µM)**	**(%)**
formic acid	3.87	(100%)[a]	3.74	(97%)[b]
acetic acid	3.94	(100%)	3.63	(92%)
methanol	0.90	(23%)	1.09	(121%)
ethanol	4.01	(100%)	4.15	(103%)
2-propanol	4.04	(100%)	3.66	(91%)
methylamine	0.24	(6%)	< 0.01	(0%)
dimethylamine	0.21	(5%)	< 0.01	(0%)
trimethylamine	1.16	(30%)	1.07	(92%)
H_2/CO_2	3.76	(100%)	3.74	(99%)
none	< 0.01	(0%)	< 0.01	(0%)

[a] percentage of TCA transformed after 48 days; [b] percentage of TCA transformed to DCA after 48 days

To determine whether other electron donors supported cometabolic transformation of TCA by methanogens, an enrichment culture from the reactor was used (Table 5.2). This enrichment culture converted TCA to DCA in a batch culture using acetate as substrate. DCA was not further transformed. With all electron donors tested except methylamine and dimethylamine, significant transformation of TCA was observed (Table 5.2). DCA was detected as the only product of TCA transformation with a recovery of DCA higher than 91%. Both trimethylamine and methanol were poor electron donors for TCA transformation by this enrichment culture since it was only partially transformed.

Effect of nitrate and nitrite on 1,1,1-trichloroethane transformation

Previous studies on TCA transformation (Chapter 4) showed that sulfate -an electron acceptor often found in groundwater- has an effect on (the products of) TCA transformation. Therefore we studied the effect of two other naturally occurring electron acceptors - nitrate and nitrite- using an enrichment culture from the reactor at nitrate concentrations between 0 mM and 2.01 mM and nitrite concentrations between 0 mM and 1.09 mM. Acetate (1 mM) and methanol (1 mM) served as electron donors.

In the absence of nitrate or nitrite, 1.63 µM of TCA was completely transformed to DCA (Table 5.3). No other transformation products were found. Acetate and methanol were utilized by methanogens and sulfate-reducing bacteria as indicated by the production of methane (0.47 mM) and the reduction of all available sulfate (0.48 mM) (Table 5.3).

When nitrate or nitrite were present in the batch cultures, besides methane production and sulfate reduction, nitrate reduction (Table 5.3) and nitrite reduction (Table 5.4) to N_2 occurred, respectively. Both nitrate and nitrite inhibited the reduction of TCA to DCA in enrichment cultures (Tables 5.3 and 5.4). The inhibition by nitrite was much stronger than inhibition by nitrate. At nitrate concentrations over 490 µM, conversion of TCA to DCA was only partially inhibited while at a nitrite concentration of 119 µM, TCA transformation still occurred but DCA nor CA were found as products. The inhibition of the conversion of TCA to DCA coincided with a decrease or complete inhibition of methane production and sulfate reduction in the batch cultures. Since previous studies showed that methanogens were probably involved in this conversion (Chapter 4), inhibition of TCA conversion probably resulted from a decrease in methanogenic activity.

Table 5.3 *Effect of nitrate on the transformation of 1,1,1-trichloroethane by an enrichment culture from a 1,1,1-trichloroethane transforming packed-bed reactor*

	t = 39 days						
NO_3^- (mM)	TCA transformed (µM)	DCA formed (µM)	CH_3COOH utilized (mM)	CH_3OH utilized (mM)	NO_3^- reduced (mM)	SO_4^{2-} reduced (mM)	CH_4 formed (mM)
0	1.63	1.62	0.75	1.13	< 0.01	0.48	0.47
0.24	1.52	1.42	0.86	1.07	0.23	0.44	0.44
0.49	1.52	1.63	0.89	1.24	0.48	0.42	0.44
0.95	0.71	0.71	0.84	0.96	0.95	0.01	0.16
2.01	0.17	0.10	0.92	1.06	2.01	< 0.01	0.11

Table 5.4 *Effect of nitrite on the transformation of 1,1,1-trichloroethane by an enrichment culture from a 1,1,1-trichloroethane transforming packed-bed reactor*

	t = 37 days						
NO_2^- (mM)	TCA transformed (µM)	DCA formed (µM)	CH_3COOH utilized (mM)	CH_3OH utilized (mM)	NO_2^- reduced (mM)	SO_4^{2-} reduced (mM)	CH_4 formed (mM)
0	1.63	1.62	0.75	1.05	< 0.01	0.48	0.47
0.08	1.74	0.55	0.95	1.15	0.08	0.45	0.43
0.19	1.24	< 0.01	0.72	< 0.05	0.19	< 0.01	0.06
0.40	1.19	< 0.01	0.57	< 0.05	0.40	< 0.01	< 0.01
1.09	1.17	< 0.01	0.96	< 0.05	1.09	< 0.01	0.03

Effect of pH and temperature on TCA transformation

The effect of two other important environmental conditions, namely pH and temperature, on the transformation of TCA was determined using the enrichment cultures isolated from the reactor. The rate of DCA production, the only product of TCA transformation, was used as a measure for the dechlorination activity.

Transformation of TCA to DCA by the methanogenic population was observed at temperatures between 11°C and 44°C (Fig. 5.2A), with an optimum between 26°C and 33°C. Temperatures in groundwater usually are between 10°C and 15°C. This means that for this mixed population the rate of *in situ* or on-site biotransformation of TCA could be significantly reduced. However, it has been reported that dechlorinating microorganisms can adapt to temperatures below 15°C without a significant effect on the kinetics of dechlorination (de Bruin et al. 1992).

TCA transformation by the enrichment culture occurred between pH 6.7 and 8.5 with an optimum between pH 7.4 and 7.6 (Fig. 5.2B). Usually, contaminated groundwaters have a pH within this range, so that no problems are expected for *in situ* and on-site biotransformation. However, there may be exceptions like systems with high concentrations of humic acid, sulfide or carbonate creating extremely high or low pH's (Wilson et al. 1996) where the transformation rates may be considerably lower.

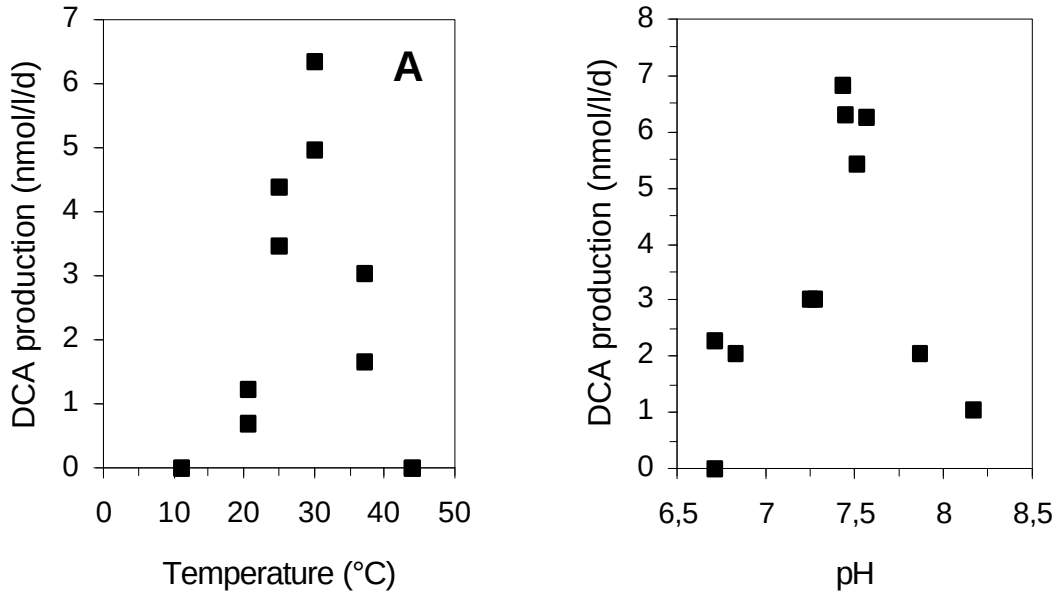

Figure 5.2 *Effect of temperature (**A**) and pH (**B**) on the production of DCA from TCA by an enrichment culture from a TCA transforming packed-bed reactor. Acetate (1 mM) and methanol (1 mM) served as electron donors. A: pH=7.5 B: T=25°C.*

Discussion

Previously we found CA as main product of TCA transformation in a packed-bed reactor (Chapter 4), while other studies reported DCA as main transformation product of biotic TCA transformation (Egli et al. 1987, Gälli and McCarty 1989) and sometimes also traces of CA (Vogel and McCarty 1987). This chapter described the complete biological cometabolic transformation of TCA to CA and the conditions necessary for this complete transformation to CA.

In general, the rate of cometabolic dechlorination processes increases with an increase in electron donor concentration (Doong and Wu 1996, Wrenn and Rittmann 1996). Here we report that not only the rate of dechlorination, but also the nature of the products depend on the electron donor concentration.

By extrapolation of the data presented in Fig. 5.1 it can be calculated that an electron donor concentration (acetate + methanol) of 2.75 mM is necessary for complete transformation of TCA to CA under these conditions. The corresponding molar ratio of [acetate + methanol] to [TCA] of about 275 (2750/10) confirmed that TCA transformation was a cometabolic process. This ratio is within the range of 100 to 1,000 found for other cometabolic transformations of chlorinated compounds (Bouwer and McCarty 1983, Vogel and McCarty 1987, Bouwer and Wright 1988).

Besides on the electron donor concentration, the microbial transformation of TCA also depended on the type of electron donor present. Methylamine and dimethylamine did not support TCA transformation by an enrichment culture from the reactor, while methanol and trimethylamine were very poor electron donors compared to others such as acetate and H_2. The final transformation products of TCA also depended on the type of electron donor present. With acetate as an electron donor, the degree of dechlorination in the reactor was much better, i.e. more TCA was transformed to CA than with methanol as an electron donor. The effect of different electron donors on the products of biological dechlorination has never been described, but there are several reports of the effect of different electron donors on (the rate of) microbial dechlorination (Bagley and Gossett 1990, Holliger 1992, Lewis and Crawford 1993, Petrovskis et al. 1994, Doong et al. 1996, Wrenn and Rittmann 1996).

Under appropriate conditions, CA was the end product of TCA transformation in the reactor. Reduction of CA to ethane was not found although it has been described that CA can be reduced to ethane by cell suspensions of *Methanosarcina barkeri* with a dechlorination rate of 0.58 mmole·mole CH_4^{-1} in the presence of acetate as an electron donor (Holliger 1992). The pseudo-first order rate constant for abiotic hydrolysis of CA to ethanol of 0.0010 d^{-1} (Vogel and McCarty, 1987) indicated that no significant amount of CA could be transformed to ethanol in the reactor as the hydraulic retention time in the reactor was only 24 h.

Both nitrate and nitrite inhibited reductive dechlorination of TCA to DCA in enrichment cultures from the reactor. The decrease in methanogenic activity in the presence of nitrate was a result of the competition for the available electron donor between the TCA-transforming methanogens and nitrate-reducers or sulfate-reducers (competitive inhibition) since both acetate and methanol were nearly completely utilized at all nitrate concentrations. Competitive inhibition of the TCA transforming methanogens could be prevented by adding excess electron donor. The decrease in methanogenic activity in the presence of nitrite was not a result of competition for the available substrate, but was caused by the toxicity of nitrite. At a nitrite concentration of 0.19 mM or higher, sufficient

substrate was available for nitrite reduction as well as sulfate reduction and methane production but only nitrite reduction occurred (Table 5.4). Nitrite is often found to be toxic for microorganisms due to its inhibitory effects on electron carriers (Stouthamer 1988). Electron carriers such as cobalamins (Krone et al. 1989a, 1991) or factor F_{430} (Krone et al. 1989b, Gantzer and Wackett 1991) often are involved in the cometabolic transformation of chlorinated hydrocarbons under anaerobic conditions.

Although TCA transformation to DCA was completely inhibited at nitrite concentrations above 80 µM, about 1.2 µM of TCA was still transformed (Table 5.4). The products of this transformation are unknown but no chlorinated hydrocarbons were found as transformation products. Others also described complete dechlorination of TCA to CO_2 (Vogel and McCarty 1987, Gälli and McCarty 1989), acetic acid and other unknown products (Gälli and McCarty 1989), but these products only accounted for a minor percentage of TCA transformed. Methanogens and sulfate reducing bacteria were probably not involved in this transformation of TCA in the presence of nitrite. While methane production and sulfate reduction no longer occurred, TCA was still transformed. It is not clear whether nitrite reducing bacteria play a role in this transformation of TCA to non-chlorinated products.

Fast and complete biological dechlorination of TCA is only likely to occur in a sequential anaerobic/aerobic process. Here we described the conditions necessary for the anaerobic process: complete transformation of TCA to CA by a methanogenic mixed population without the formation of DCA. CA, when formed can be further dechlorinated under aerobic conditions (Keuning et al. 1985, Scholtz et al. 1987). Whether TCA was completely transformed to CA depended both on the electron donor concentration and the type of electron donor present in the reactor. Further research should focus on the development and optimization of a sequential anaerobic/aerobic reactor system for TCA mineralization.

Chapter 6

Dichloromethane utilization in a packed-bed reactor in the presence of different electron acceptors

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submitted for publication

Abstract

Dichloromethane, added as sole source of carbon and energy, was utilized by microorganisms in a packed-bed reactor under carbon dioxide-, sulfate-, nitrate- and nitrite- reducing conditions. Only in the presence of nitrite (4 mM), the transformation of dichloromethane was partly inhibited. The maximum transformation rate for dichloromethane under carbon dioxide reducing conditions was 1.25 $kg \cdot m^{-3} \cdot d^{-1}$. Carbon dioxide, acetate and formate were detected as (intermediate) products of dichloromethane transformation in the reactor, indicating that it was a fermentative process. Both acetate and formate, when formed, were further utilized. The type of microorganisms that utilized formate and acetate depended on the electron acceptor present in the reactor. When carbon dioxide was the only electron acceptor available, acetate and formate were utilized by methanogens as indicated by methane production. When sulfate, nitrate or nitrite were present in the reactor, acetate and formate were utilized by sulfate-, nitrate- or nitrite- reducing microorganisms, respectively. Inhibition of methanogens with 2-bromoethane sulfonic acid or of sulfate reduction with molybdate had no effect on the utilization of dichloromethane in enrichment cultures from the reactor. Also the presence of nitrate or nitrite was not necessary for the transformation of dichloromethane. These results suggested that neither methanogens nor sulfate-, nitrate- and nitrite- reducers were involved in the transformation of dichloromethane but that these organisms only utilized acetate and formate, the products of dichloromethane fermentation in the reactor.

Abbreviations: DCM - dichloromethane; CM - chloromethane

Introduction

Dichloromethane (DCM) and chloromethane are the only chlorinated hydrocarbons of which it is known that they can serve as growth substrate for aerobic (Brunner et al. 1980, Lapat-Polasko et al. 1984, Kohler-Staub et al. 1986, Bader and Leisinger 1994) as well as anaerobic (Freedman and Gossett 1991, Mägli et al. 1996) microorganisms. Both aerobic (Rittmann and McCarty 1980, Gälli and Leisinger 1985, Stucki 1990) and anaerobic (Stromeyer et al. 1991, Winkelbauer and Kohler 1991) microbial transformation of DCM have been exploited in bioreactors for wastewater and groundwater treatment. Since aerobic transformation is a much faster process than its anaerobic counterpart (Leisinger et al. 1994), it is favored for practical applications. Anaerobic treatment would be an attractive option when besides DCM other chlorinated hydrocarbons, like carbon tetrachloride and tetrachloroethene, are present that cannot be treated under aerobic conditions. Moreover, anaerobic processes have the advantage that little biomass is produced, that no oxygen must be introduced into the system, and that less energy is needed for operation of the reactor (Stromeyer et al. 1991).

Little is known about the effect of different primary electron acceptors -such as sulfate and nitrate- on the anaerobic transformation of DCM. This information is important in view of the application of anaerobic biotransformation of DCM for in situ and on-site biological groundwater remediation at contaminated sites under different or even multiple electron acceptor conditions. In this study we examined the transformation of DCM in an anaerobic packed-bed reactor under anaerobic conditions. The maximum transformation capacity of the reactor and the mass balance of DCM transformation under these conditions are presented. Furthermore the effect of primary electron acceptors on the anaerobic transformation of DCM were examined by adding different concentrations of sulfate, nitrate and nitrite to the reactor influent.

Material and methods

Packed-bed reactor studies. The experiments were performed in an anaerobic upflow packed-bed reactor (glass, height 32 cm, inside diameter 4.42 cm, volume 492 ml) (Chapter 2, Fig. 2.1) packed with polyurethane foam (PUR) particles (5×5×6 mm, Bayer BV, Mijdrecht, the Netherlands) mixed with digested sludge (20

v/v%) from the wastewater treatment plant Kralingseveer (Rotterdam, the Netherlands). The packed-bed reactor was wrapped with aluminum foil to prevent growth of phototrophs.

The packed-bed reactor was continuously fed with an anaerobic non-sterile mineral medium containing (mg/l) K_2HPO_4 (8), KH_2PO_4 (3.6), $NaHCO_3$ (40), NH_4Cl (26.6), $MgCl_2 \cdot 6H_2O$ (101.6), $CaCl_2 \cdot 2H_2O$ (62.6), resazurine (1). From a trace element solution, 0.125 ml/l were added. The trace element solution contained (mg/l) $FeSO_4 \cdot 7H_2O$ (2800), H_3BO_3 (50), $Al_2(SO_4)_3 \cdot 16H_2O$ (118.3), $MnCl_2 \cdot 4H_2O$ (50), $CuSO_4 \cdot 5H_2O$ (92.8), EDTA (500), $ZnCl_2$ (50), $(NH_4)_6Mo_7O_{27} \cdot H_2O$ (50), $CoCl_2$ (27.3), $NiCl_2 \cdot 6H_2O$ (91.6), 1 ml HCl (37%). The medium was continuously purged with a mixture of N_2 and CO_2 (99.5%/0.5%) (Hoek Loos BV, Dieren, the Netherlands) to remove all oxygen.

The medium (pH 7.3 ±0.2) was pumped into the packed-bed reactor by means of a peristaltic pump with marprene tubing (Watson Marlow, England). All other tubing was either viton or teflon. TCA, acetate and Na_2S (42 mM, to maintain reducing conditions) were added to the medium as a concentrated solution at the influent of the packed-bed reactor with a syringe pump. The hydraulic retention time in the packed-bed was 24 h. All experiments were carried out at 25°C.

Batch culture studies. Batch culture studies were started to obtain an enrichment culture from the reactor. Four different media were tested for their ability to support DCM transformation. Besides the medium that was also used for the reactor (*medium 1*) and the effluent of the reactor (*medium 2*), two other media were tested. *Medium 3:* (in g/l of demineralized water) KH_2PO_4 (0.43), $Na_2HPO_4 \cdot 2H_2O$ (0.53), NH_4Cl (0.3), $CaCl_2 \cdot 2H_2O$ (0.12), $MgSO_4 \cdot 7H_2O$ (0.13), resazurine (0.0005). The medium also contained (per liter) 1 ml of trace element solution (de Best et al. 1997) and 1 ml of a vitamin solution. The vitamin solution contained (mg/l): biotin (2), folic acid (2), riboflavin (5), thiamine (5), cyanocobalamin (5), nicotinamide (5), *p*-aminobenzoic acid (5). *Medium 4:* (in g/l of demineralized water) $(NH_4)_2HPO_4$ (0.080), $MgSO_4 \cdot 7H_2O$ (0.20), resazurine (0.001) and 5 ml trace element solution (see above). The different media were purged with a mixture of CO_2 and N_2 (0.5%/99.5%, 700 ml/min) for 45 min. After purging, $Na_2S \cdot 9H_2O$ (67 mg/l) and $NaHCO_3$ (100 mg/l) were added to media 2 and 3. The media were transferred to 120 ml or 250 ml bottles in an anaerobic glovebox. Bottles (120 ml) contained 60 ml of medium and were closed with teflon-lined butyl rubber stoppers and aluminum crimp seals. Bottles of 250 ml contained 180 ml of medium and were closed with viton stoppers and a aluminum screw cap. After sterilization the batch cultures were inoculated and DCM (48 µM) was added from a concentrated stock solution. The cultures were incubated on a

shaker (100 rpm) at 25°C in the dark and analyzed regularly for chlorinated hydrocarbons.

Analytical methods. Dichloromethane and chloromethane were quantified by headspace gas chromatography. Liquid samples (100-1,000 µl) were injected in 10 ml headspace autosampler vials with teflon-lined butyl rubber stoppers and aluminum crimp seals. The final volume was adjusted to 2 ml with demineralized water. The vials were analyzed using a Hewlett Packard 19395A headspace sampler connected to gas chromatograph equipped with an ECD and a CP-Sil 5CB column (Chapter 2). Calibration samples were analyzed according to the same method to adjust for air/water partitioning. A four point curve was used.

Carbon dioxide and methane concentrations were determined after separation on a Carboplot P7 column in a gas chromatograph equipped with an FID and a methanizer (Chapter 2). For samples from the reactor, liquid samples (2 ml) were injected in 10 ml headspace autosampler vials with teflon-lined butyl rubber stoppers and aluminum crimp seals and equilibrated at 80°C for 45 min. A volume of 50 ml of the headspace was injected into the GC by hand with a 100 ml Hamilton gas- and liquid-tight syringe. For batch cultures, 50 ml of the headspace was injected into the GC. A four point calibration curve was used for quantification.

Sulfate, nitrate, nitrite and chloride were determined after separation on an IONPAC AG9-SC guard column and IONPAC AG9-SC anion column (Dionex, Breda, the Netherlands) using an ion chromatograph equipped with a conductivity detector, thermal stabilizer and ASRS suppressor (Chapter 2).
Acetate and formate concentrations were determined with an enzymatic test combination (Boehringer, Mannheim, Germany).

Chemicals. All chemicals were obtained from commercial companies. DCM and chloromethane were obtained from Sigma-Aldrich. Sodium acetate was obtained from Janssen Chimica. Sodium sulfate and potassium nitrate were purchased at J.T. Baker. Sodium nitrite was purchased from Merck. Calibration gases were obtained from AGA (carbon dioxide, methane).

Results

Transformation of dichloromethane in an anaerobic packed-bed reactor

The transformation of DCM (25 µM) was studied in an anaerobic packed-bed reactor, inoculated with digested sludge. Acetate (0.83 mM) served as an electron donor. After a lag period of 12 days, during which all DCM added was recovered in the effluent, DCM was completely transformed in the reactor whilst simultaneous methanogenesis and sulfate reduction occurred (Table 6.1). Chloromethane or other chlorinated hydrocarbons were not found as transformation products. Acetate was completely utilized. The occurrence of methane production (741 µM) and sulfate reduction (54 µM) indicated that both methanogenic and sulfate-reducing bacteria were responsible for acetate removal.

Table 6.1 *Effect of acetate concentration on dichloromethane (25 µM) transformation in an anaerobic packed-bed reactor.*

Influent (µM)		**Effluent** (µM)			
CH_2Cl_2	CH_3COOH	CH_2Cl_2 transformed	CH_3COOH transformed	SO_4^{2-} reduced	CH_4 formed
22.3	834	22.3	806	54	741
27.8	0	27.3	0	45	23

Omitting acetate from the influent of the reactor resulted in a slow decrease of methane production and sulfate reduction since acetate served as growth substrate for both methanogenic bacteria and sulfate-reducing bacteria. After three weeks still some methane production (23 µM) and sulfate reduction (45 µM) occurred. This could be due to conversion of organic material still present in the reactor and originating from the inoculum (digested sludge). The removal of acetate from the influent of the reactor had no effect on the transformation of DCM (Table 6.1). This indicated that DCM served as a growth substrate for microorganisms, as described before for anaerobic bacteria (Freedman and Gossett 1991, Mägli et al. 1996).

Transformation capacity and mass balance

To determine the volumetric transformation capacity of the reactor, the concentration of DCM in the influent was increased stepwise, starting at 390 µM, until DCM was no longer completely transformed and appeared in the effluent of the reactor. Besides DCM no other substrate was added to the reactor. Carbon dioxide was the only potential electron acceptor present.

Up to a concentration of 2.1 mM, DCM was completely transformed in the reactor (Table 6.2). Only at a concentration of 19 mM, DCM was detected in the effluent of the reactor. At this concentration, a volumetric DCM transformation capacity of 0.31 $kg \cdot m^{-3} \cdot d^{-1}$ could be calculated for the reactor. However, DCM transformation only occurred in the first 25% of the volume of the reactor. For this part of the reactor, a transformation capacity of 1.25 $kg \cdot m^{-3} \cdot d^{-1}$ was calculated.

Table 6.2 *Effect of dichloromethane concentration on utilization of dichloromethane in an anaerobic packed-bed reactor.*

Influent (µM)	**Effluent (µM)**						**Recovery (%)**	
CH_2Cl_2	CH_2Cl_2 transformed	Cl^- formed	CH_3COOH formed	HCOOH formed	CH_4 formed	CO_2 formed	C	Cl^-
390	390	--[a]	<4	<4	80	--	--	--
573	573	1110	14	<4	130	474	110	97
1151	1151	2319	90	<4	234	787	104	102
2115	2096	3980	141	<4	446	1455	104	95
19000	3756	6833	370	82	<5	1482	61	91

[a] not determined

At DCM concentrations higher than 390 µM, a chlorine and carbon balance could be determined (Table 6.2). Between 91% and 102% of the chlorine appeared as chloride, indicating that DCM was completely dechlorinated. Carbon dioxide, methane and acetic acid were detected as products of DCM transformation. At a DCM concentration of 19 mM, formic acid was also found as a transformation product. The closed mass balance for carbon at the four lower DCM concentrations tested indicated that no other products were formed. At the highest DCM concentration, however, the recovery of carbon was poor because part of the gas produced did not dissolve but left the reactor as small gas bubbles. The volume of these gas bubbles and their composition were not measured and thus not included in the data presented in Table 6.2.

At DCM concentrations up to 2.1 mM, part of acetate formed in the first half of the reactor was utilized again at the upper half of the reactor (data not shown). Acetate was probably utilized by methanogens, as indicated by the production of methane. The presence of methanogens was confirmed by fluorescence microscopy (Doddema and Vogels 1978).

Reaction I shows the overall transformation of DCM to methane, resulting in a [$DCM_{transformed}$ /CH_4 formed]-ratio of 2. From the data presented in Table 6.2, an average [$DCM_{transformed}$ /CH_4 formed]-ratio of 4.1 was calculated for the reactor. This ratio indicates that DCM was not completely transformed to methane. Part of DCM was probably transformed to carbon dioxide by other bacteria, as indicated by the production of carbon dioxide (Table 6.2)

$$2CH_2Cl_2 + 2H_2O \rightarrow CO_2 + CH_4 + 4H^+ + 4Cl^- \qquad (I)$$

At the highest DCM concentration tested (19 mM) both acetate and formic acid, when formed, were not transformed by methanogens or other bacteria. This was probably due to an inhibitory effect of DCM and a decrease of the pH of the medium from 7.3 to 6.1, which resulted from HCl formation. As a result, acetate and formic acid accumulated.

Effect of sulfate on dichloromethane transformation

Sulfate is a potential electron acceptor that is often found in groundwater at DCM contaminated sites. To determine the effect of sulfate on DCM transformation, sulfate was added to the influent of the reactor. Two different sulfate concentrations were examined (Table 6.3).

At the starting conditions, DCM (573 µM) was completely dechlorinated. Carbon dioxide and methane were found as main transformation products but part of DCM was also converted to acetate. Sulfate reduction did not occur.

Addition of sulfate had no effect on the transformation of DCM. At both sulfate concentrations tested (0.76 and 4.1 mM), DCM was completely dechlorinated according to the chlorine mass balance (Table 6.3). Methane production was completely inhibited when higher sulfate concentrations were present in the influent of the reactor. This inhibition of methanogenesis had no effect on the transformation of DCM, indicating that methanogens were not involved in this transformation.

Table 6.3 *Effect of sulfate, nitrate and nitrite on the transformation of dichloromethane in an anaerobic packed-bed reactor.*

Influent (µM)			**Effluent (µM)**								**Recovery (%)**	
electron acceptor		CH_2Cl_2	CH_2Cl_2	Cl^-	CH_3COOH	SO_4^{2-}	NO_3^-	NO_2^-	CH_4	CO_2	C	Cl^-
added	concentration		transformed[1]	formed	formed	reduced[1]	reduced[1]	reduced[1]	formed	formed		
None	0	573	573	1110	14	<2	<2	<5	130	474	110	97
SO_4^{2-}	763	531	531	986	<4	153	<2	<5	<5	--[2]	--	93
	4109	581	579	1196	<4	138	<2	<5	<5	561	97	103
NO_3^-	3050	560	560	1222	<4	<2	660	<5	<5	436	78	109
NO_2^-	998	617	605	1144	<4	<2	<2	989	<5	--	--	95
	3953	611	359[3]	670	<4	<2	<2	1284	<5	--	--	93

[1] based on the concentration in the influent minus the concentration in the effluent, [2] not determined, [3] a steady state was not yet reached

Instead of methane production, an average of 146 µM of sulfate was reduced at both sulfate concentrations tested. Reaction II shows that transformation of 555 µM of DCM (average) provides enough electrons for the reduction of 278 µM of sulfate. The production of carbon dioxide (Table 6.3) indicated that part of DCM was oxidized to carbon dioxide by other microorganisms. Acetate was only found as a product of DCM transformation at several sample ports at different heights of the reactor (data not shown). No acetate was detected in the effluent of the reactor indicating that acetate, when formed, was utilized in the reactor.

$$2CH_2Cl_2 + SO_4^{2-} \rightarrow 2CO_2 + HS^- + 3H^+ + 4Cl^- \qquad (II)$$

When sulfate was removed from the influent, and sulfate reduction thus no longer could occur, methane production was detected again in the reactor after 5 days. DCM transformation still was complete.

Effect of nitrate on dichloromethane transformation

Nitrate is another primary electron acceptor which frequently occurs in groundwater. The effect of nitrate (3 mM) on the transformation of DCM in the reactor was examined (Fig. 6.1). Before nitrate was added (day 1-10), DCM (570 µM) was completely dechlorinated according to the chlorine mass balance (Table 6.3). Carbon dioxide (474 µM) and methane (130 µM) were found as products of DCM transformation. Part of DCM was converted to acetate (15 µM).

The transformation of DCM was only partially and temporarily inhibited by the presence of nitrate. When an excess of nitrate (3 mM) was added to the influent of the reactor at day 10, the concentration of DCM detected in the effluent of the reactor rapidly increased until it stabilized at about 155 µM after 7 days. Next, DCM transformation recovered and 100 days after nitrate was first added to the reactor, DCM was again completely dechlorinated.

When nitrate was present in the influent of the reactor, methane production by methanogens was completely inhibited. Instead of a methanogenic population, a nitrate reducing bacterial population developed in the reactor within 14 days after nitrate was first added. At first, part of nitrate was reduced to nitrite but when a steady state was reached after 150 days, nitrite was no longer detected in the effluent of the reactor while 660 µM of nitrate was reduced (Table 6.3). Nitrate can either be reduced to NO, N_2O, N_2 or NH_4^+ (Stouthamer 1988). The electron balance of DCM oxidation to CO_2 and nitrate reduction to NO, N_2O, N_2 or NH_4^+

showed that transformation of 560 µM of DCM (Table 6.3) does not provide enough electrons for the reduction of all the nitrate to NH_4^+, N_2 or N_2O. This indicated that nitrate may be reduced to NO according to (overall) reaction III. For the reduction of 660 µM of NO_3^- to NO, oxidation of 495 µM DCM to CO_2 would be needed.

$$3CH_2Cl_2 + 4NO_3^- \rightarrow 4NO + 3CO_2 + 2H_2O + 2H^+ + 6Cl^- \qquad \text{(III)}$$

Acetate was only found as a product of DCM transformation at several sample ports at different heights of the reactor (data not shown). No acetate was detected in the effluent of the reactor indicating that acetate, when formed, was utilized in the reactor.

Two weeks after nitrate was omitted from the influent of the reactor, methane (131 µM) and carbon dioxide (450 µM) again were found as products of DCM transformation. DCM was still completely dechlorinated.

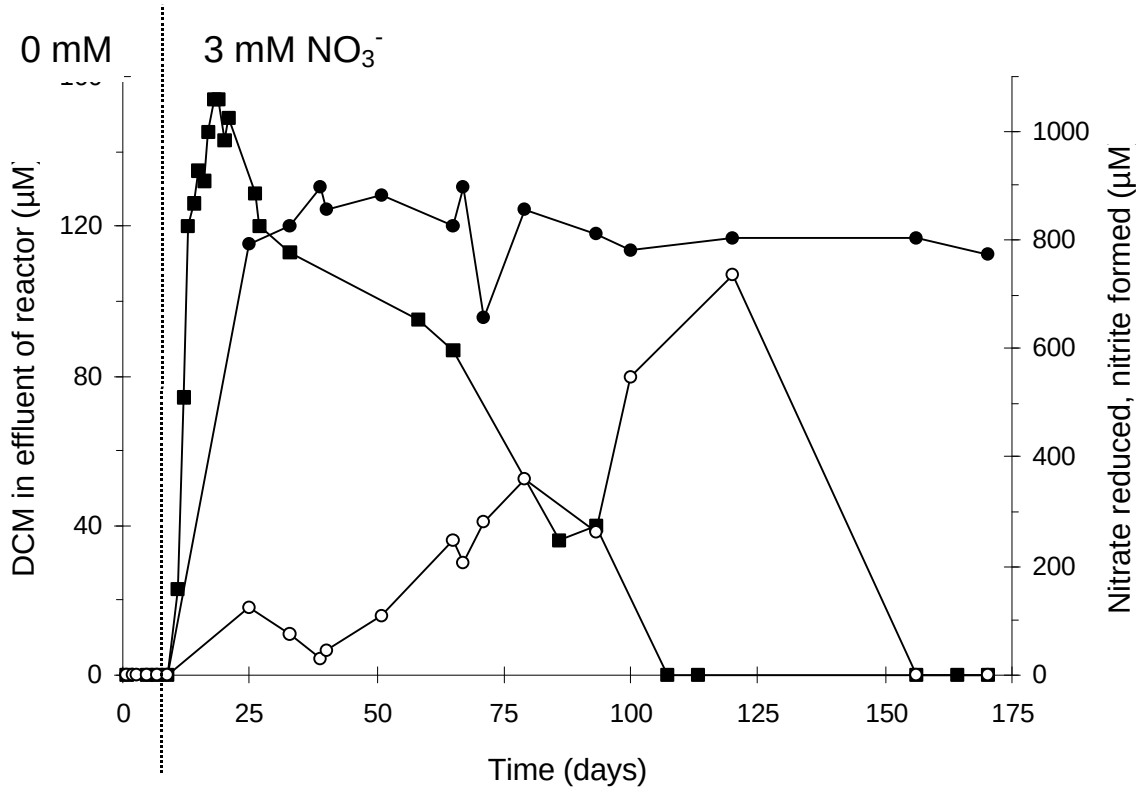

Figure 6.1 *Effect of nitrate (3 mM) on the transformation of dichloromethane (570 µM) in a packed-bed reactor. Most of the dichloromethane is removed; the remaining concentration in the effluent of the reactor is plotted. Symbols: DCM in the effluent of the reactor ( ); nitrate reduced (●); nitrite formed (❍).*

Effect of nitrite on dichloromethane transformation

The temporary inhibition of DCM transformation by nitrate could be a result of the formation of nitrite. Nitrite is often found to be toxic for microorganisms (Tiedje 1988). To verify this hypothesis, nitrite was added to the influent of the reactor at two different concentrations (Fig. 6.2).

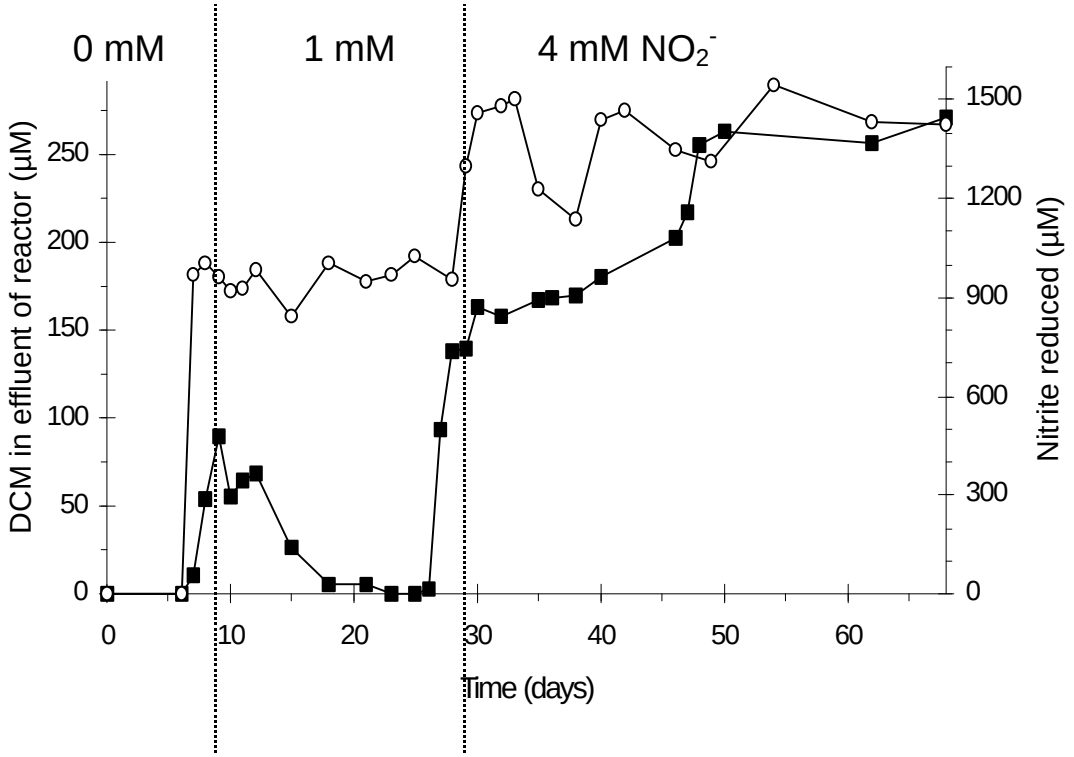

Figure 6.2 *Effect of nitrite on the transformation of dichloromethane (± 600 µM) in a packed-bed reactor. Symbols: DCM in the effluent of the reactor ( ); nitrite reduced (❍).*

Before addition of nitrite, DCM (550 µM) was completely dechlorinated under methanogenic conditions as indicated by the production of methane (131 µM). Addition of nitrite (1 mM) at day 6 had little effect on the transformation of DCM. The concentration of DCM in the effluent of the reactor increased to a concentration of 90 µM, but within 12 days DCM again was completely dechlorinated according to the formation of chloride (Table 6.3). Methane production no longer occurred, but 989 µM of nitrite was reduced. The electron balance indicated that nitrite was reduced to N_2O according to reaction IV. For this reduction, oxidation of 495 µM DCM to carbon dioxide would be needed. About 605 µM of DCM was oxidized in the reactor.

$$CH_2Cl_2 + 2NO_2^- \rightarrow N_2O + CO_2 + H_2O + 2Cl^- \qquad (IV)$$

At day 26, the concentration of nitrite was increased to 4 mM (Fig. 6.2). Nitrite reduction increased to an average of 1284 µM (607 µmoles·l^{-1}·d^{-1}) and about 2.7 mM of nitrite appeared in the effluent of the reactor. The presence of nitrite in the influent clearly affected DCM transformation. After a fast initial inhibition the concentration of DCM in the effluent of the reactor slowly increased further until after 68 days only 359 µM of DCM was transformed (no steady state). According to the formation of chloride (Table 6.3), all of the DCM which was transformed was completely dechlorinated. The electron balance indicated that NO_2^- was reduced to NO instead of N_2O according to the following overall reaction:

$$CH_2Cl_2 + 4NO_2^- + 2H^+ \rightarrow 4NO + CO_2 + 2H_2O + 2Cl^- \qquad (V)$$

Before a steady state was reached, nitrite was omitted from the influent of the reactor. Within 15 days, DCM again was completely dechlorinated. Methane production (130 µM) indicated that methanogens had survived and were active in the reactor.

Dichloromethane utilizing enrichment culture

Anaerobic enrichments were started in 250 ml bottles using different media. The batch cultures were inoculated with 4 ml of liquid phase from the packed-bed reactor. DCM (48 µM) was added as single substrate.

A DCM transforming enrichment culture could only be obtained using sterilized effluent from the reactor as medium. This indicated that a growth factor was required that was produced/present in the reactor. Specific nutritional requirements have been reported for several respiratory-dehalogenating bacteria (Shelton and Tiedje 1984, Holliger 1992, Mägli et al. 1995).

Within 10 days DCM (48 µM) was completely transformed (Fig. 6.3). Chloromethane was not detected as a transformation product indicating that DCM was completely dechlorinated. Repeated additions of DCM were also dechlorinated without a significant lag period (Fig. 6.3). Growth was observed by light microscopy. A mixed population containing at least two different microorganisms had developed. In control bottles without an inoculum from the

reactor or a sterilized inoculum, no DCM transformation occurred and losses were limited to 5% after 100 days.

Addition of 2-bromoethane sulfonic acid (BES 6 mM), an inhibitor of methanogenesis, or molybdate (2 mM), an inhibitor of sulfate reduction in sulfate-reducing bacteria, to a DCM degrading enrichment culture had no effect on DCM transformation. This indicated that neither methanogenic nor sulfate-reducing bacteria were involved in DCM transformation. Vancomycin (0.07 mM), an inhibitor of cell wall synthesis in gram positive eubacteria, completely inhibited DCM transformation. These results indicated that gram positive bacteria, like acetogens or *Clostridium* species, were involved in the transformation of DCM.

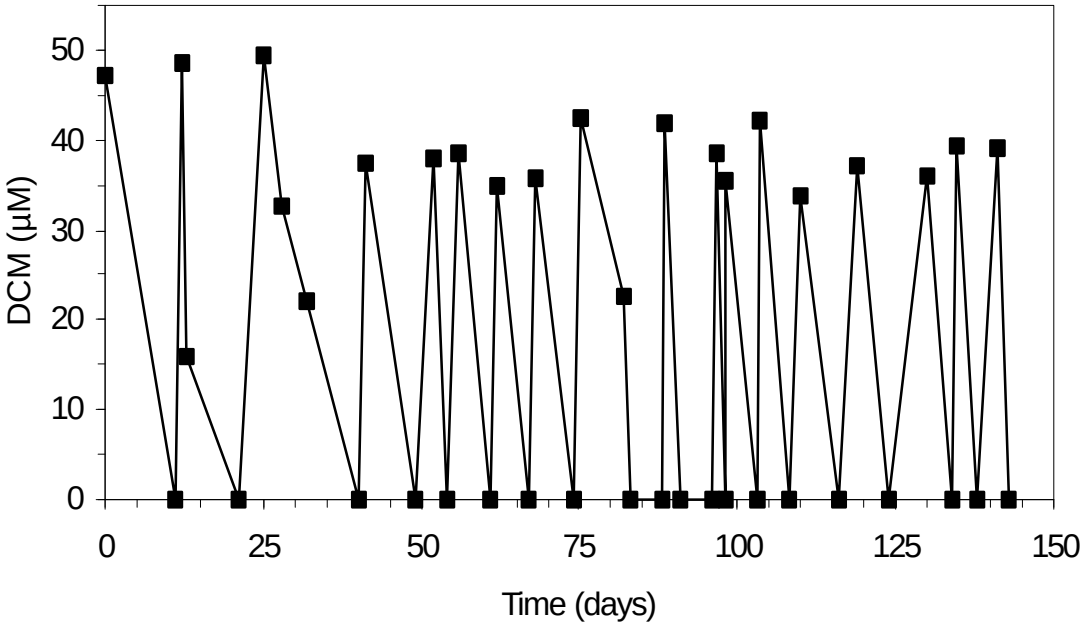

Figure 6.3 *Transformation of dichloromethane by an anaerobic mixed culture. DCM was added as single substrate. The sterilized effluent of a DCM degrading packed-bed reactor served as medium. Symbols: dichloromethane ( ).*

After anaerobic transfer from the enrichment cultures to fresh effluent medium, the ability to grow under anaerobic conditions on halogenated hydrocarbons other than DCM was tested. Growth was only observed on DCM and on dibromomethane (1 mM). Tetrachloromethane (1 mM), chloroform (1 mM), tetrachloroethene (1 mM), trichloroethene (1 mM), *cis*-1,2-dichloroethene (0.5 mM), *trans*-1,2-dichloroethene (0.5 mM), 1,1-dichloroethene (0.5 mM), 1,1,1-trichloroethane (1 mM), 1,1-dichloroethane (1 mM), 1,2-dichoroethane (1 mM) and chloroethane (0.5 mM) were not transformed, nor was growth observed

microscopically.

Discussion

Here we report microbial utilization of DCM in a packed-bed reactor under carbon dioxide-, sulfate-, nitrate- and nitrite-reducing conditions. Anaerobic utilization of DCM in a reactor has been described previously, but only under carbon dioxide reducing conditions (Freedman and Gossett 1991, Stromeyer et al. 1991) as a fermentative process, and under nitrate reducing conditions (Kohler-Staub et al. 1995). Utilization of DCM under denitrification conditions was not a fermentative process. Instead, *Hyphomicrobium* sp. Strain DM2 coupled the reduction of nitrate to nitrite to the hydrolysis of DCM to formaldehyde, which then served as the growth substrate.

Carbon dioxide and acetate were found as (intermediate) products of DCM transformation in our reactor under all redox conditions that were examined. Under carbon dioxide reducing conditions, also formate was found as a product of DCM transformation. These products are similar to the products of DCM transformation by *Dehalobacterium formicoaceticum*, a microorganism that was isolated from a DCM utilizing fixed-bed reactor (Stromeyer et al. 1991). *D. formicoaceticum* is a DCM fermenting microorganism that is able transform DCM to acetate and formate using DCM as sole substrate for growth (Mägli et al. 1996). These results suggested that DCM transformation under carbon dioxide-, sulfate-, nitrate- and nitrite-reducing conditions in the reactor was also a fermentative process. So far, fermentation of chlorinated aliphatic hydrocarbons has been limited to DCM and chloromethane (Traunecker et al. 1993, Mägli et al. 1996). Both with DCM and chloromethane fermentation, acetate and formate were found as (intermediate) products of transformation.

Acetate and formate, when formed, were partly or completely utilized in the reactor. The type of microorganism that utilized acetate and formate depended on the predominating electron acceptor condition in the reactor. If carbon dioxide was the only electron acceptor available, acetate and formate were utilized by methanogens as indicated by methane production. When sulfate, nitrate or nitrite were present in the reactor, sulfate-, nitrate- and nitrite- reducing microorganisms, respectively, developed in the reactor and utilized acetate and formate. Methanogens and sulfate reducers were not involved in the transformation of DCM. Inhibition of methanogens with 2-bromoethane sulfonic acid or sulfate-reduction with molybdate had no effect on the utilization of dichloromethane in

enrichment cultures from the reactor. Nitrate- and nitrite reducers were probably also not involved in DCM transformation in the reactor since the presence of nitrate or nitrite was not necessary for DCM fermentation.

The maximum DCM elimination rate of the reactor under carbon dioxide reducing conditions, calculated for the first 25% of the volume, was 1.25 kg $DCM \cdot m^{-3} \cdot d^{-1}$. This rate is much higher than the rate observed for the fermentative transformation of DCM by a mixed culture in a fixed-bed reactor under carbon dioxide reducing conditions (Stromeyer et al. 1991) or the rate observed for DCM transformation by *Hyphomicrobium* sp. strain DM2 under denitrification conditions in a fed batch culture (Kohler-Staub et al. 1995). However, it is still about 10 times lower than the elimination rate Stucki (1990) described for the removal of DCM in an aerobic fluidized bed reactor (12 kg $DCM \cdot m^{-3} \cdot d^{-1}$).

Although aerobic transformation is much faster, the anaerobic process described in this paper has the advantage that DCM can be transformed with either carbon dioxide, sulfate, nitrate, nitrite as predominant electron acceptor in the reactor. Only the presence of 4 mM of nitrite inhibited DCM transformation for a longer period of time. This inhibition could be caused by the toxicity of nitrite or NO, the expected product of nitrite reduction in the reactor, for the DCM fermenting microorganisms. Both nitrite and NO are often found to be toxic for microorganisms (Rowe et al. 1979, Tiedje 1988, Stouthamer 1988). Transformation of DCM by *Hyphomicrobium* sp. strain DM2 was also inhibited by nitrite depending on the concentration of nitrite (Kohler-Staub et al. 1995). At a nitrite concentration of 5 mM, DCM transformation was completely inhibited whereas partial inhibition of DCM transformation was observed at nitrite concentrations between 0.1 mM and 4 mM. This is in line with the partial inhibition of DCM transformation at 4 mM nitrite observed in our reactor.

Conclusions

Dichloromethane, added as sole source of carbon and energy, can be utilized by microorganisms in a packed-bed reactor under carbon dioxide-, sulfate-, nitrate- and nitrite- reducing conditions. Carbon dioxide, acetate and formate were detected as (intermediate) products of dichloromethane transformation in the reactor, indicating that it was a fermentative process. This was confirmed by batch experiments with an enrichment culture from the reactor. The ability to transform DCM with different electron acceptors, i.e. under different redox conditions, is important in view of practical applications of in situ and on-site bioremediation of

contaminated sites. Different redox conditions occur at different sites and often redox conditions may differ at one contaminated site (Chapelle 1996, Nipshagen et al. 1997). Therefore, a practical process which transforms DCM at different redox conditions can be of great importance. The maximum elimination rate of 1.25 kg·DCM·m^{-3}·d^{-1} detected for anaerobic DCM transformation in our reactor can also compete with transformation of DCM under aerobic conditions.

Chapter 7

Discussion

Fermentative dechlorination processes

The data presented in chapter 2 and 6 of this thesis show that both carbon tetrachloride and dichloromethane can be biodegraded in an anaerobic packed-bed reactor in the absence of an added electron donor or electron acceptor. These results suggest that both compounds are being fermented. Fermentation is an ATP-regenerating metabolic process in which part of the substrate is oxidized and serves as an electron donor, and part of the substrate is reduced and serves as an electron acceptor for the microorganisms involved in these transformations. As yet, fermentation of chlorinated aliphatic hydrocarbons has only been described for dichloromethane (Mägli et al. 1996) and chloromethane (Traunecker et al. 1991). Fermentation of carbon tetrachloride has never been reported.

Carbon dioxide was found as the main transformation product of carbon tetrachloride transformation (Chapter 2). Several hypothetical pathways are possible where carbon tetrachloride serves as an electron donor as well as electron acceptor (fermentation) with the formation of carbon dioxide as the end product (Fig. 7.1). The most likely pathway is a hydrolytic reduction. This pathway has been described extensively in chapter 2, and involves a 2-electron reduction to dichlorocarbene, followed by substitutive dehalogenation to either formic acid or carbon monoxide. Both formic acid and carbon monoxide are further oxidized to carbon dioxide.

Carbon tetrachloride can also be converted to carbon dioxide by way of chloroform as intermediate. There are three hypothetical pathways for the transformation of chloroform to carbon dioxide (Fig. 7.1):

- transformation via dichlorocarbene as described above;
- transformation through monochlorocarbene. Hydrolysis of monochlorocarbene would lead to formaldehyde, which could be oxidized to CO (Becker and Freedman 1994). CO can be further oxidized to carbon dioxide (Ljungdahl 1986);
- net hydrolysis to carbon monoxide, which can be further oxidized to carbon dioxide.

Transformation of carbon tetrachloride via chloroform probably does not occur in the reactor used. In an enrichment culture from the reactor, chloroform transformation is very poor and dichloromethane has been found as the main transformation product (data not shown).

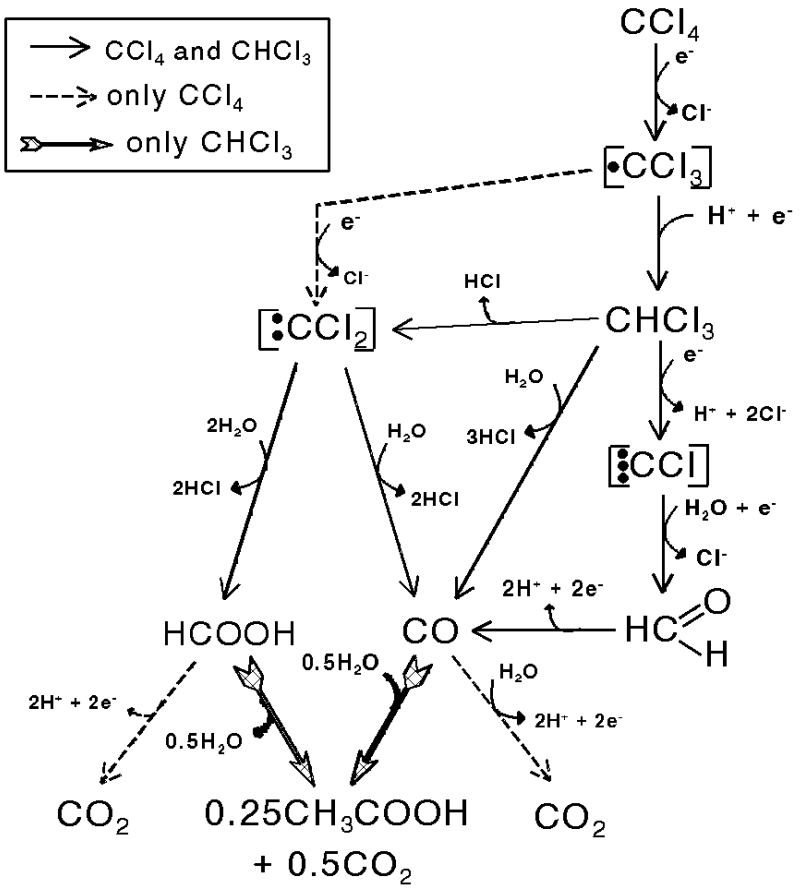

Figure 7.1 *Hypothetical pathways for the fermentation of carbon tetrachloride (CCl_4) and chloroform ($CHCl_3$). The arrow style determines whether carbon tetrachloride, chloroform or both can be fermented according to this pathway (see box). [ ] = unstable intermediate.*

The transformation of carbon tetrachloride to carbon dioxide via either one of these pathways is energetically very favorable with an overall $\Delta G^{0'}$ of -619.7 kJ/mole (Table 7.1), indicating that fermentative growth on carbon tetrachloride is possible. However, up to now we were unable to demonstrate growth on carbon tetrachloride in the reactor or obtain an enrichment culture from the reactor which could ferment carbon tetrachloride for more than one month. Only the isolation of the carbon tetrachloride transforming microorganism from the reactor can give a definitive answer whether carbon tetrachloride transformation indeed is a fermentative process.

In enrichment cultures obtained from the dichloromethane transforming reactor, growth on dichloromethane as a single substrate was observed (Chapter 6). This indicated that the transformation of dichloromethane indeed is a fermentative process. Acetic acid, formic acid and carbon dioxide are found as

products of DCM fermentation in our reactor. These products are similar to the products of chloromethane fermentation by strain MC and dichloromethane fermentation by *Dehalobacterium formicoaceticum*. Fermentation of chloromethane by stain MC leads to the formation of acetic acid, whereas *D. formicoaceticum* ferments dichloromethane to acetic acid and formic acid, at a molar ratio of 2:1. This could mean that the pathway of transformation in our reactor is similar to those described for strain MC (Meßmer et al. 1993, 1996) and *D. formicoaceticum* (Mägli et al. 1996), which are very much alike (Fig. 7.2). In the first step, the methyl group of chloromethane or dichloromethane is transferred to tetrahydrofolate (THF). The methylated THF is either converted to formic acid or carbon dioxide in the oxidative branch or reduced to the methyl group of acetate in the reductive branch of the fermentation pathway.

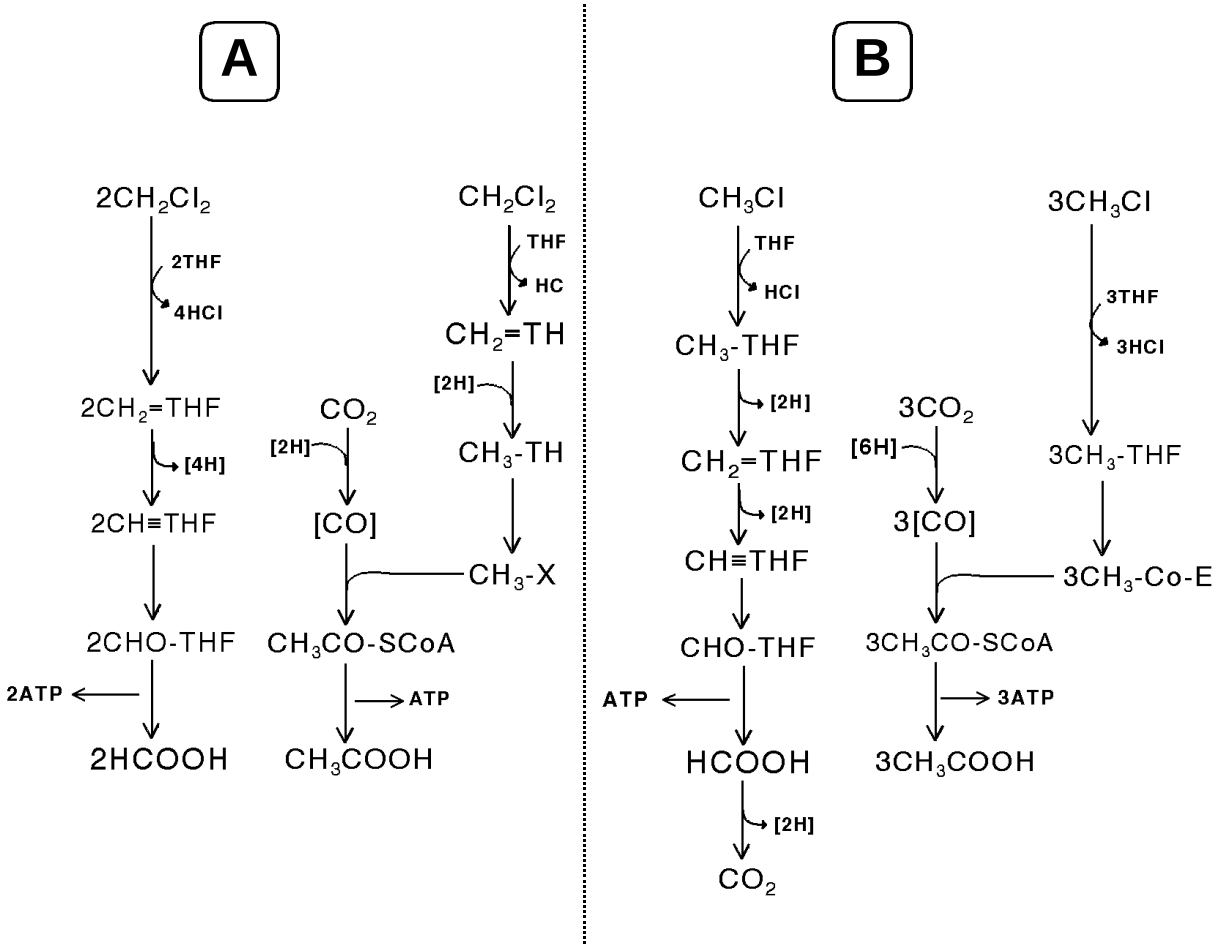

Figure 7.2 ***A.*** *Scheme of dichloromethane fermentation by Dehalobacterium formicoaceticum (deduced from Mägli et al. 1996).* ***B.*** *Scheme of chloromethane fermentation by strain MC (deduced from Meßmer et al. 1993). THF = tetrahydrofolate; CH_3CO-SCoA = acetyl-CoA; X = methyl carrier such as a corrinoid enzyme; CH_3-Co-E = corrinoid enzyme.*

As yet, only about the pathway of chloromethane fermentation some information is available (Meßmer et al. 1996). The pathway of dichloromethane fermentation by *D. formicoaceticum* (Fig. 7.2) is still purely hypothetical, while no information is available on the pathway of dichloromethane fermentation in our reactor. Further research on this is needed. Besides fermentative dechlorination of dichloromethane, chloromethane and carbon tetrachloride, other halogenated hydrocarbons can also be subject to fermentation. Fermentation of dibromomethane and bromomethane can proceed via the same mechanism as for dichloromethane and chloromethane. Indeed, the DCM-fermenting population described in chapter 6 not only grows on dichloromethane, but also on dibromomethane as sole source of carbon and energy. *D. formicoaceticum* does not grow on dibromomethane, but transforms it only at low concentrations, whilst growing on dichloromethane. Strain MC was not able to ferment bromomethane (Traunecker et al. 1991). Mägli et al. (1996) theorized that dibromomethane was the original substrate of *D. formicoaceticum*, since dibromomethane is much more common in nature than dichloromethane in view of it's production by marine algae (Gribble 1994). The ability to grow on dibromomethane was apparently lost because *D. formicoaceticum* had been grown with dichloromethane for hundreds of generations.

Chloroform and 1,1,1-trichloroethane can also be fermented. The hypothetical pathways for the fermentation of chloroform are similar to those described for carbon tetrachloride (this paragraph, Fig. 7.1), with one major difference: formic acid or carbon monoxide, which occur as intermediates, are not oxidized to carbon dioxide but fermented to acetic acid and carbon dioxide. The transformation of chloroform via dichlorocarbene is not very likely since a carbon-hydrogen bond would be broken first, instead of a weaker carbon-chlorine bond. Up to now there is no evidence for growth of bacteria on chloroform. However, Becker and Freedman (1994) describe chloroform transformation to carbon dioxide via carbon monoxide in a methanogenic enrichment culture that had not received other organic compounds for over eight months. The rate of chloroform transformation had not declined during that period.

1,1,1-Trichloroethane is susceptible to hydrolytic reduction, which leads to the formation of acetic acid. So far, only cometabolic transformation of 1,1,1-trichloroethane to acetic acid has been reported (see §7.2). Gälli and McCarty (1989) detected acetic acid as a minor product (6%) of biotic 1,1,1-trichloroethane transformation by *Clostridium* sp. strain TCAIIB but the mechanism of this transformation is unknown. Others also reported acetic acid formation from 1,1,1-trichloroethane, but always as a result of abiotic hydrolysis (Dilling et al. 1975, Maybey and Robertson 1978).

Table 7.1 shows that all fermentative dechlorination reactions described above are energetically very favorable. The question remains why, as yet, fermentative dechlorination has only been found for dichloromethane (Chapter 6, Mägli et al. 1996), dibromomethane (Chapter 6), chloromethane (Traunecker et al. 1991), and possibly for carbon tetrachloride (Chapter 2). The development of two completely different dechlorinating populations in a packed-bed reactor inoculated with the same material, but operated under different environmental conditions (Chapter 2 and 3), indicate that environmental conditions could be an important parameter determining whether fermentative dechlorination develops as the dominant process in a microbial system.

Table 7.1 *Overall reaction and Gibbs free energy of fermentative dechlorinations of chlorinated aliphatic hydrocarbons.*

Compound[1]	**Reaction**		**$\Delta G^{0'}$ (kJ)**
CT	$CCl_4 + 2H_2O$	$\rightarrow CO_2 + 4HCl$	- 619.7
CF	$4CHCl_3 + 6H_2O$	$\rightarrow CH_3COOH + 2CO_2 + 12HCl$	-416.6
DCM	$3CH_2Cl_2 + CO_2 + 4H_2O$	$\rightarrow CH_3COOH + 2HCOOH + 6HCl$	-685.2
CM	$4CH_3Cl + 2CO_2 + 2H_2O$	$\rightarrow 3CH_3COOH + 4HCl$	-455.5
TCA	$CH_3CCl_3 + 2H_2O$	$\rightarrow CH_3COOH + 4HCl$	-379.5

[1] CT carbon tetrachloride; CF chloroform; DCM dichloromethane; CM chloromethane; TCA 1,1,1-trichloroethane

Fermentative dechlorination processes have a great potential for application for *in situ* and on-site soil remediation under anoxic conditions, mainly because of three reasons:

- Fermentative processes do not depend on the availability of other electron donors or other electron acceptors;
- the high rate of transformation;
- the nature of the transformation products formed.

These will be discussed next.

Availability of other electron donors or electron acceptors

Fermentative processes do not depend on the availability of other electron donors (Chapter 6, Mägli et al. 1996, Traunecker et al. 1991) or other electron acceptors such as CO_2, SO_4^{2-}, NO_3^-, NO_2^- (Chapter 6, Mägli et al. 1996), as long as the conditions remain anoxic. As a result, fermentative processes usually are more stable than cometabolic or respiratory dechlorination processes. Cometabolic dechlorination processes depend on the presence of both a suitable electron donor and electron acceptor (Chapter 3, 4, 5), while the rate and products of

halorespirative processes depend on the presence of a suitable electron donor (Scholz-Muramatsu et al. 1995, Gerritse et al. 1996, Holliger et al. 1993, Maymó-Gatell et al. 1997). Since the chlorinated hydrocarbon serves as a terminal electron acceptor for halorespiring microorganisms, the presence of another electron acceptor is not needed. Several dehalorespiring microorganisms can also use other electron acceptors besides chlorinated hydrocarbons (Gerritse et al. 1996, Scholz-Muramatsu et al. 1995). In these cases, the presence of other electron acceptors can inhibit halorespiration as a result of competition for available electron donor.

Transformation rate

We have calculated a volumetric elimination rate of 1.25 $kg \cdot m^{-3} \cdot d^{-1}$ for the fermentation of dichloromethane in the reactor. Although this rate is still about ten times lower than the elimination rate of 12 $kg \cdot m^{-3} \cdot d^{-1}$ found for the removal of dichloromethane in an aerobic fluidized bed reactor (Stücki 1990), it is much higher than the transformation rate of cometabolic and respiratory dechlorination processes (Table 7.2). Winkelbauer and Kohler (1991) showed that at these rates, fermentation can be applied for fast and complete on-site remediation. In a fixed-bed reactor dichloromethane was eliminated from groundwater contaminated by material from paint wastes by means of fermentation. The elimination capacity of their reactor was about 0.07 $kg \cdot m^{-3} \cdot d^{-1}$.

The maximum dechlorination rate of $9.1 \cdot 10^{-3}$ $kg^{-1} \cdot m^{-3} \cdot d^{-1}$ that was calculated for the supposed fermentation of carbon tetrachloride in the reactor (Chapter 2) is much lower than for dichloromethane fermentation (Table 7.2). This suggests that the transformation of carbon tetrachloride in the reactor is not a fermentative metabolic process. However, the lower transformation rates can also be caused by the toxicity of carbon tetrachloride for the carbon tetrachloride transforming microbial population. In general, chlorinated hydrocarbons are known for their ability to inhibit anaerobic microbial processes (Chapter 3, Stuckey et al. 1980, Vargas and Ahlert 1987, Renard et al. 1993, Hughes and Parkin 1996, 1996a). Carbon tetrachloride transformation in our reactor is severely inhibited at concentrations higher than 70 µM. At a carbon tetrachloride concentration in the influent of 100 µM, about 20 µM of carbon tetrachloride remained in the effluent of the reactor (data not shown). The effect of lower carbon tetrachloride concentrations on the rate of transformation should be the subject of further studies. Besides the toxicity, the slow rate of carbon tetrachloride transformation can also be due to unfavorable environmental conditions in the reactor, such as the pH, the redox potential, or an unfavorable (=high) activation energy $\Delta G^{\ddagger}$ of carbon tetrachloride transformation (Chapter 1).

Table 7.2 *Anaerobic biotransformation of chlorinated aliphatic hydrocarbons in continuous flow fixed-bed reactors.*

Compound[1]	Conditions[2]	Mechanism[3]	C_{in} (µM)	Main product(s)[1]	Rate ($g \cdot m^{-3} \cdot d^{-1}$)	Reference
CT	co_2	fe	60	CO_2	9.1	Chapter 2
	su	co	30	CF, CO_2	7.8	Chapter 3
	me	co	0.11	--[5]	0.011	Bouwer and McCarty (1983)
	su	co	0.25	--	0.14	Bouwer and Wright (1988)
	su/dn	co	0.52	--	0.023	Cobb and Bouwer (1991)
TCA	me	co	10	CA	9.6	Chapter 5
	me	co	0.19	--	0.0079	Bouwer and McCarty (1983)
	su	co	0.44	--	0.083	Bouwer and Wright (1988)
	su/dn	co	0.76	--	0.021	Cobb and Bouwer (1991)
DCM	co_2	fe	19000	CO_2	1250	Chapter 6
	co_2	fe	105	CO_2	70	Stromeyer et al. (1991)
	dn	me[4]	4000	CO_2	120	Kohler-Staub et al. (1995)
PCE	co_2	hr/co	9	ethane	0.008	de Bruin et al. (1992)
	co_2	hr	40-70	*cis*-DCE	0.011	Gerritse et al. (1995)
	me	co	0.11	--	0.004	Bouwer and McCarty (1983)
	co_2	co	50	ethene	3.6	Wild et al. (1995)

[1] CT carbon tetrachloride; TCA 1,1,1-trichloroethane; DCM dichloromethane; PCE tetrachloroethene; CF chloroform; CA chloroethane; *cis*-DCE *cis* -1,2-dichloroethene; [2] co_2 carbon dioxide reducing; me methanogenic; su sulfate reducing; dn nitrate reducing; [3] co cometabolic; fe fermentation; me metabolic; hr halorespiration; [4] dichloromethane acts as an electron donor, nitrate as an electron acceptor; [5] -- not detected.

The nature of transformation products formed

With respiratory and cometabolic dechlorination processes usually dechlorination is incomplete, and mono- and/or dichlorinated compounds are formed that may often be more toxic than the original compounds. Fermentative dechlorinations result in complete dechlorination to innocuous products, mainly acetic acid. Acetic acid, when formed, can serve as an electron donor for the reduction of other xenobiotics. Indeed, Lehmicke et al. (1997) found that acetic acid, which resulted from the *in situ* fermentation of dichloromethane in a shallow aquifer beneath a chemical transfer facility in Oregon (USA), served as an electron donor for the reductive dechlorination of chlorinated ethenes.

A possible disadvantage of fermentative dechlorination processes is the narrow substrate range that has been detected to date for the microorganisms carrying out fermentative dechlorinations. The few microorganisms described up to now can only dechlorinate one or two chlorinated hydrocarbons. This also applies for

(de)halorespiring microorganisms (Mohn and Tiedje 1986, Holliger 1992, Scholz-Muramatsu 1995, Gerritse et al. 1996). In contrast, microorganisms that carry out cometabolic dechlorinations usually have a wide substrate range because the transition-metal cofactors involved in these transformations are not very specific (Chapter 1). A wide substrate range can be of importance at sites that are contaminated with different chlorinated hydrocarbons, as is often the case (Cox et al. 1997, Lehmicke et al. 1997, Lorah et al. 1997, Armstrong et al 1997).

Sequential anaerobic/aerobic processes

Under anaerobic conditions chlorinated methanes can be fermented, and transformation of chlorinated ethenes (except vinyl chloride) can occur by means of respiratory dechlorination. For chlorinated ethanes, however, only cometabolic transformations have been described (Egli et al. 1987, 1988, Gälli and McCarty 1989a, Vogel and McCarty 1987a). We have also only found cometabolic transformation of 1,1,1-trichloroethane in the anaerobic packed-bed reactor (Chapter 4 and 5). The degree and rate of this cometabolic dechlorination can be improved, i.e. more 1,1,1-trichloroethane was transformed to chloroethane and less to 1,1-dichloroethane, by adding a suitable electron donor at a sufficient concentration. Previously, a correlation between the dechlorinating activity and the amount of methane formed was described (Fathepure and Boyd 1988a, Baek and Jaffé 1989, Mikesell and Boyd 1990, Holliger 1992). Figure 7.3, derived from the data presented in Chapter 4 and 5, shows that for the cometabolic transformation of 1,1,1-trichloroethane, the products of transformation also correlated with the amount of methane formed. This probably results from the competition between 1,1,1-trichloroethane and 1,1-dichloroethane for the same electrons in one microorganism.

Chloroethane accumulates as the end product of 1,1,1-trichloroethane transformation in the reactor. Even when an excess of electron donor was added to the reactor, no transformation of chloroethane occurred (Chapter 5, Fig. 7.3). The reason for this accumulation is not clear since the reduction of chloroethane to ethane is energetically more favorable and has a higher redox potential than the reduction of 1,1-dichloroethane to chloroethane (Chapter 1; Table 1.3). In the case of 1,1,1-trichloroethane, at this time a sequential anaerobic/aerobic process seems the most feasible option for complete mineralization. In the first anaerobic reduction step, TCA is transformed to CA which can be further mineralized in a second aerobic oxidation step, as discussed in Chapter 4. Sequential

anaerobic/aerobic biodegradation of 1,1,1-trichloroethane to ethane has previously been demonstrated in a mixed anaerobic/aerobic aquifer (Cox et al. 1995, Edwards et al. 1997). Future investigations should focus on the development and optimization of a sequential anaerobic/aerobic column or reactor system for 1,1,1-trichloroethane mineralization.

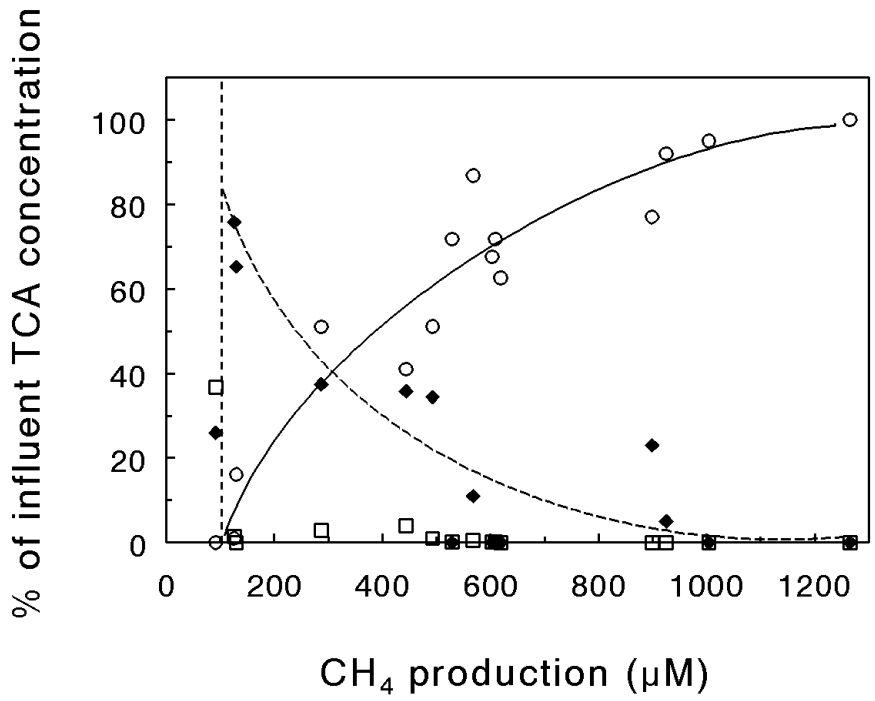

Figure 7.3 *Correlation between the products of 1,1,1-trichloroethane transformation by methanogenic population in a packed-bed reactor and the amount of methane formed by this population. The products of 1,1,1-trichloroethane transformation are expressed as the percentage of 1,1,1-trichloroethane in the influent of the reactor. Acetic acid and methanol served as electron donors (different concentrations). Symbols: 1,1,1-trichloroethane (□); 1,1-dichloroethane (◆); chloroethane (❍)*

Based on laboratory experiments, sequential anaerobic/aerobic transformation has also been suggested as a solution for the complete mineralization of several other halogenated compounds such as chloroform (Fathepure and Vogel 1991), β-hexachlorocyclohexane (Middeldorp 1997), polychlorinated biphenyls (PCBs) (Anid and Vogel 1991, Middeldorp 1997), polychlorinated benzenes (Fathepure and Vogel 1991, Beurskens 1995, Middeldorp 1997), chlorophenols (Campos-Velarde et al. 1997) and DDT (Field et al. 1995) which can only be degraded under anaerobic conditions. Similar to the reduction of 1,1,1-trichloroethane (Chapter 4 and 5) the rate of anaerobic reduction of these compounds decreases with each reductive step and

monochloro and dichloro derivatives tend to accumulate, which can be further mineralized in an additional aerobic step. As yet, *in situ* sequential mineralization of these halogenated compounds has only been demonstrated for PCB's (Harkness et al. 1993).

A sequential anaerobic/aerobic process is also feasible for treatment of sites contaminated with tetrachloroethene. Although complete dechlorination of tetrachloroethene was demonstrated in continuous flow reactors (Table 7.1) as well as in pure culture (Máymo-Gatell et al. 1997), accumulation of *cis*-1,2-dichloroethene and vinyl chloride occurs at high loading rates. This makes a change to a much faster aerobic process attractive, since both *cis*-1,2-dichloroethene and vinyl chloride can be cometabolized by aerobic bacteria at high rates. Indeed, fast and complete dechlorination of tetrachloroethene has been described in sequential anaerobic/aerobic reactor systems (Fathepure and Vogel 1991, Gerritse et al. 1995, 1997, Pon and Semprini 1997, Saberiyan et al. 1997). It has also been successfully applied for remediation of soils contaminated with tetrachloroethene (Beeman 1995, Edwards and Cox 1997, Spuij et al. 1997).

Although sequential dechlorination processes are an attractive option for soil and groundwater remediation, one step remediation processes, when available, are to be preferred. Sequential processes usually cost more and the technology is more complicated because of the necessary switch from anaerobic to aerobic conditions that can cause problems (Bouwer 1994). Another possibility could be an integrated oxidative and reductive process in a single reactor, using gel beads with aerobic outer layer containing aerobic degraders, and an anaerobic inner layer containing anaerobic degraders (dos Santos et al. 1996). With such a system simultaneous nitrification and denitrification could be achieved in a single air-lift loop reactor at high rates. It has also potential for sequential anaerobic/aerobic degradation of xenobiotics.

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SUMMARY

Chlorinated aliphatic hydrocarbons belong to the most frequently found contaminants in soil. Because of their toxicity and persistence, they are a potential threat to human health and the diversity of ecosystems. For the cleanup of soils contaminated with these compounds, biological degradation by microorganisms can be an attractive option provided complete dechlorination occurs and no toxic metabolites accumulate.

Although biological transformation of chlorinated aliphatic hydrocarbons has been subject of many investigations, still a better understanding of these processes is required. The aim of this thesis was to obtain more insight in the anaerobic microbial dechlorination of carbon tetrachloride, 1,1,1-trichloroethane and dichloromethane under different environmental conditions. The effect of the presence or absence of different electron donors and of naturally occurring electron acceptors, such as sulfate and nitrate, on the (extend of) biological dechlorination was studied, using a continuously operated packed-bed reactor. The microbial transformation of carbon tetrachloride, 1,1,1-trichloroethane and dichloromethane was studied in separate (non-sterile) packed-bed reactors under (initially) methanogenic conditions. The transformation of carbon tetrachloride was also studied under sulfate-reducing conditions.

Both carbon tetrachloride and dichloromethane were transformed by microorganisms in the reactor without addition of another electron donor or electron acceptor. This indicated that carbon tetrachloride and dichloromethane served as electron donor as well as electron acceptor for the microorganisms involved in their transformation and that their transformation was probably a fermentative metabolic process.

The chlorine mass balance of a reactor showing 72% removal of 52 µM carbon tetrachloride demonstrated that all carbon tetrachloride transformed was completely dechlorinated, probably to carbon dioxide. Chloroform and dichloromethane were sometimes also found as transformation products, but neither accumulated to significant levels in comparison to the amount of carbon tetrachloride transformed. The pathway of carbon tetrachloride transformation is not clear; it may be dehalogenated by hydrolytic reduction to carbon monoxide or formic acid which are electron demanding transformations. Carbon monoxide or formic acid may be further utilized and serve as electron donor. For complete dechlorination according to this pathway neither an additional electron donor nor an electron acceptor would then be needed. Transformation of carbon tetrachloride to carbon dioxide via carbon monoxide or via formic acid are

thermodynamically very favourable reactions with an overall $\Delta G^{0'}$ of -619.7 kJ/mole.

If dichloromethane was added to a packed-bed reactor, it was still completely transformed at an influent concentration of 2.1 mM with a transformation rate of about 0.73 $kg \cdot m^{-3} \cdot d^{-1}$. Carbon dioxide, acetate and formate were detected as (intermediate) products of dichloromethane transformation. Both acetate and formate were further utilized by methanogens as evidenced by methane production. Dichloromethane was also fermented in the presence of either sulfate, nitrate or nitrite. Only in the presence of nitrite (4 mM), the transformation of dichloromethane was partly inhibited. When sulfate, nitrate or nitrite were present in the reactor influent, acetate and formate were not utilized by methanogens but by sulfate-, nitrate- or nitrite- reducing microorganisms, respectively.

Vancomycin, an inhibitor of gram positive eubacteria, severely inhibited both carbon tetrachloride and dichloromethane transformation in enrichment cultures obtained from both reactors, indicating that gram positive eubacteria were involved in their transformation. Inhibition of methanogens with 2-bromoethane sulfonic acid (BES) or of sulfate reduction with molybdate had no effect on carbon tetrachloride or dichloromethane transformation. These results suggested that neither methanogens nor sulfate-reducing bacteria were involved in the transformation of carbon tetrachloride or dichloromethane.

Carbon tetrachloride transformation was also studied under sulfate-reducing conditions. Instead of being fermented, carbon tetrachloride was transformed in the reactor according to a cometabolic process. Chloroform and dichloromethane were found as main transformation products, but part of the carbon tetrachloride was also completely dechlorinated to unknown products. Batch culture experiments with an enrichment culture from the reactor indicated that gram-positive sulfate-reducing bacteria were involved in the reductive transformation of carbon tetrachloride since both molybdate and vancomycin completely inhibited carbon tetrachloride transformation to chloroform and dichloromethane. The rate of carbon tetrachloride transformation by the enrichment culture depended on the type of electron donor present. The highest transformation rate of 5.1 $nmol \cdot ml^{-1} \cdot h^{-1}$ was found with ethanol as electron donor. At carbon tetrachloride concentrations higher than 18 µM, reductive dechlorination of carbon tetrachloride by the sulfate-reducing population was severely inhibited and complete inhibition was observed at a carbon tetrachloride concentration of 57 µM.

Transformation of 1,1,1-trichloroethane in the reactor was also a cometabolic process, and involved a methanogenic population, as indicated by

packed-bed reactor studies as well as batch experiments with BES. 1,1,1-Trichloroethane was completely transformed up to the highest investigated concentration of 10 µM. At concentrations lower than 6 µM, part of 1,1,1-trichloroethane was completely dechlorinated via an unknown pathway. However, at all other conditions the methanogenic population in the reactor transformed 1,1,1-trichloroethane to 1,1-dichloroethane and/or chloroethane. Chloroethane was not further transformed.

The ratio of 1,1-dichloroethane to chloroethane detected in the effluent of the reactor depended on the 1,1,1-trichloroethane as well as the electron donor concentration. With a decrease in the 1,1,1-trichloroethane concentration and an increase in the electron donor concentration, the degree of TCA dechlorination increased i.e. more 1,1,1-trichloroethane was transformed to chloroethane. Complete transformation of 1,1,1-trichloroethane to chloroethane only occurred when sufficient electron donor was fed into the reactor. Otherwise, besides chloroethane also 1,1-dichloroethane was found as a product.

The transformation of 1,1,1-trichloroethane and the products formed also depended on the type of electron donor present. With acetate, the degree of dechlorination in the reactor was higher than with methanol. In an enrichment culture obtained from the reactor contents methanol, acetate, formate, ethanol, 2-propanol, trimethylamine and H_2, but not dimethylamine and methylamine, served as electron donors for 1,1,1-trichloroethane transformation. 1,1,1-Trichloroethane was only transformed to 1,1-dichloroethane by this enrichment culture and was not further metabolized.

The presence of sulfate, nitrate, or nitrite partly or completely inhibited transformation of 1,1,1-trichloroethane, dependent on the concentration of the electron acceptor. This inhibition was a result of competition for available electron donor between the 1,1,1-trichloroethane transforming methanogenic population and either sulfate-, nitrate- or nitrite- reducing bacteria. Nitrite was also toxic for the 1,1,1-trichloroethane transforming microorganisms.

In conclusion, this thesis showed that fermentative dechlorination of dichloromethane has great potential for soil clean-up, mainly because fermentation of dichloromethane does not depend on the presence of (other) electron donors or electron acceptors, the high rates of transformation and because of the complete dechlorination to harmless products. The complete dechlorination of carbon tetrachloride in the reactor without addition of another electron donor or electron acceptor is also very promising and appears to be a fermentative dechlorination process. However, since only little is known about the pathway and mechanism of this transformation process, further research on this is needed before application seems feasible. The most feasible option for complete

dechlorination of 1,1,1-trichloroethane is a sequential anaerobic/aerobic cometabolic process. In the first anaerobic step, 1,1,1-trichloroethane is transformed to chloroethane. Chloroethane can be further transformed to carbon dioxide in a second aerobic step. This thesis described the conditions necessary for complete anaerobic transformation of 1,1,1-trichloroethane to chloroethane.

SAMENVATTING

Gechloreerde alifatische koolwaterstoffen zijn toxische en veelal persistente verbindingen die vaak als verontreiniging in de bodem worden aangetroffen. Ze vormen hier een potentieel gevaar voor de gezondheid van mens, flora en fauna. Een aantrekkelijke optie voor de sanering van bodems die verontreinigd zijn met deze verbindingen is biologische afbraak door micro-organismen. Voorwaarde is dan wel dat volledige dechlorering optreedt en er geen accumulatie van toxische metabolieten plaatsvindt.

De afgelopen tien jaar is veel onderzoek uitgevoerd naar de biologische afbraak van gechloreerde alifatische koolwaterstoffen en de processen die daarbij een rol spelen. Er zijn echter nog veel onduidelijkheden. Het onderzoek dat beschreven is in dit proefschrift heeft tot doel een beter inzicht te krijgen in de anaërobe microbiële afbraak van tetrachloormethaan, 1,1,1-trichloorethaan en dichloormethaan onder verschillende omgevingscondities. Het effect van de aan- of afwezigheid van verschillende electrondonoren of van nature voorkomende electronacceptoren zoals sulfaat en nitraat op (de mate van) biologische afbraak van deze drie verbindingen is onderzocht. De microbiële afbraak van tetrachloormethaan, 1,1,1-trichloorethaan en dichloormethaan is bestudeerd in afzonderlijke niet steriele gepakt-bed reactoren onder in eerste instantie methanogene condities. Daarnaast is de afbraak van tetrachloormethaan ook bestudeerd onder sulfaatreducerende omstandigheden.

Zowel tetrachloormethaan als dichloormethaan werden volledig gedechloreerd door micro-organismen in de reactor, zonder toevoeging van een andere electrondonor of electronacceptor. Dit wijst er op dat zowel de omzetting van tetrachloormethaan als van dichloormethaan een fermentatief metabolisch proces is waarbij beide verbindingen zowel als electrondonor als electronacceptor fungeren voor de micro-organismen die betrokken zijn bij deze afbraak.

Tetrachloormethaan werd bij een influentconcentratie van 52 µM voor meer dan 72% volledig gedechloreerd, waarschijnlijk naar kooldioxide. Chloroform en dichloormethaan werden een enkele keer als afbraakproducten gevonden, maar accumuleerden niet in de reactor en maakten nooit meer dan één procent uit van de totale hoeveelheid tetrachloormethaan die werd omgezet. De afbraakroute voor de afbraak van tetrachloormethaan is nog niet duidelijk. De meest waarschijnlijke route is hydrolytische reductie naar koolmonoxide of mierezuur, beide reacties waarbij tetrachloormethaan als electronacceptor fungeert. Koolmonoxide of mierezuur kunnen vervolgens worden omgezet naar kooldioxide en fungeren daarbij als electrondonor. Voor de volledige dechlorering van tetrachloormethaan volgens deze afbraakroute is geen (andere) electrondonor of

electronacceptor nodig. Zowel de afbraak van tetrachloormethaan naar kooldioxide via koolmonoxide als via mierezuur is een energetisch zeer aantrekkelijke reactie met een overall $\Delta G^{0'}$ van -619.7 kJ per reactie.

Dichloormethaan werd onder methanogene condities bij een influent concentratie van 2.1 mM nog steeds volledig gedechloreerd in de reactor. De afbraaksnelheid bij deze concentratie bedroeg 0.73 $kg \cdot m^{-3} \cdot d^{-1}$. Kooldioxide, acetaat en formiaat werden als afbraakproducten gevonden. Acetaat en formiaat werden beide verder omgezet naar methaan en kooldioxide door de methanogene populatie in de reactor. Fermentatie van dichloormethaan trad ook op in aanwezigheid van sulfaat, nitraat of nitriet. Alleen in aanwezigheid van nitriet (4 mM) werd de fermentatie van dichloormethaan gedeeltelijk geremd. Acetaat en formiaat, de afbraakproducten van dichloormethaan, werden in aanwezigheid van sulfaat, nitraat of nitriet niet omgezet door methanogenen, maar door respectievelijk sulfaat-, nitraat- of nitrietreducerende micro-organismen.

Vancomycine, een specifieke remmer van gram-positieve eubacteriën, had een sterk remmende werking op de afbraak van zowel tetrachloormethaan als dichloormethaan in ophopingscultures uit reactoren waarin tetrachloormethaan en dichloormethaan werden afgebroken. Dit betekent dat gram-positieve eubacteriën waarschijnlijk betrokken zijn bij de omzetting van deze verbindingen. Methanogenen en sulfaatreducerende micro-organismen zijn niet verantwoordelijk voor de afbraak van tetrachloormethaan of dichloormethaan. Inhibitie van methanogenen met 2-bromoethaan-sulfonzuur (BES) of sulfaatreducerende micro-organismen met molybdaat, had geen effect op de afbraak van tetrachloormethaan of dichloormethaan.

De afbraak van tetrachloormethaan is ook bestudeerd onder sulfaatreducerende condities. Onder deze condities werd tetrachloormethaan in de reactor omgezet volgens een cometabolisch proces met chloroform en dichloormethaan als belangrijkste afbraakproducten. Een deel werd volledig gedechloreerd naar onbekende producten. In een ophopingsculture uit de reactor werd de reductieve dechlorering van tetrachloormethaan naar chloroform en dichloormethaan volledig geremd door zowel molybdaat als vancomycine. Hieruit blijkt dat gram-positieve sulfaatreducerende micro-organismen betrokken zijn bij deze omzetting. De afbraaksnelheid van tetrachloormethaan in de ophopingsculture is afhankelijk van de beschikbare electrondonor. De hoogste afbraaksnelheid (5.1 $nmol \cdot ml^{-1} \cdot h^{-1}$) werd waargenomen in aanwezigheid van ethanol als electron donor. Bij tetrachloormethaanconcentraties hoger dan 18 µM werd de reductieve dechlorering van tetrachloormethaan sterk geremd. Bij concentraties hoger dan 57 µM vond er geen reductieve dechlorering van tetrachloormethaan plaats.

De afbraak van 1,1,1-trichloorethaan in een gepakt-bed reactor onder methanogene condities was eveneens een cometabolisch proces. Batchexperimenten met BES toonden aan dat methanogenen betrokken waren bij deze omzetting. 1,1,1-Trichloorethaan werd volledig omgezet tot de hoogst bestudeerde concentratie van 10 µM. Bij concentraties lager dan 6 µM werd een deel volledig gedechloreerd via een nog onbekende afbraakroute. Onder alle andere omstandigheden werd 1,1,1-trichloorethaan door de methanogene populatie in de reactor omgezet naar 1,1-dichloorethaan en/of chloorethaan. Chloorethaan werd niet verder afgebroken.

De ratio van 1,1-dichloorethaan en chloorethaan die in het effluent van de reactor als afbraakproducten werd gevonden is afhankelijk van zowel de concentratie 1,1,1-trichloorethaan als van de electrondonorconcentratie. De mate van dechlorering neemt toe, hetgeen wil zeggen dat er meer chloorethaan als eindproduct wordt gevonden en minder 1,1-dichloorethaan bij een afname van de concentratie 1,1,1-trichloorethaan en een toename van de electrondonorconcentratie. Volledige omzetting van 1,1,1-trichloorethaan naar chloorethaan trad alleen op als de electrondonorconcentratie in het influent van de reactor hoog genoeg is. Was dit niet het geval, dan werd er naast chloorethaan ook 1,1-dichloorethaan als afbraakproduct gevonden in het effluent van de reactor.

De afbraak van 1,1,1-trichloorethaan en afbraakproducten die worden gevonden waren ook afhankelijk van het type electrondonor dat aanwezig was. In de reactor werd in aanwezigheid van acetaat als electrondonor meer 1,1,1-trichloorethaan omgezet naar chloorethaan dan in aanwezigheid van methanol als electrondonor. In batchexperimenten met een ophopingsculture uit de reactor fungeerden methanol, acetaat, formiaat, ethanol, 2-propanol, trimethylamine en waterstof als electron donor voor de afbraak van 1,1,1-trichloorethaan. Er vond geen afbraak van 1,1,1-trichloorethaan plaats in aanwezigheid van dimethylamine en methylamine. 1,1,1-Trichloorethaan werd door de ophopingsculture bij alle experimenten slechts omgezet naar 1,1-dichloorethaan. 1,1-Dichloorethaan werd niet verder omgezet.

De afbraak van 1,1,1-trichloorethaan werd volledig of gedeeltelijk geremd in aanwezigheid van sulfaat, nitraat of nitriet. De mate van remming is afhankelijk van de concentratie van deze electronacceptoren en is het gevolg van de competitie voor beschikbare electrondonoren tussen de micro-organismen die 1,1,1-trichloorethaan afbraken enerzijds en sulfaat-, nitraat- of nitriet-reducerende micro-organismen anderzijds. Nitriet is daarnaast ook toxisch voor de micro-organismen die 1,1,1-trichloorethaan afbreken.

Concluderend heeft dit proefschrift aangetoond dat de fermentatie van dichloormethaan in de reactor een veelbelovend proces is voor de sanering van bodem en grondwater. Dit heeft te maken met de hoge afbraaksnelheden, de volledige omzetting naar onschadelijke producten en het gegeven dat de fermentatie van dichloormethaan niet afhankelijk is van de aanwezigheid van (andere) electrondonoren of electronacceptoren. De volledige dechlorering van tetrachloormethaan zonder toevoeging van (andere) electrondonoren of electronacceptoren is eveneens veelbelovend. Mogelijk hebben we hier ook te maken met een fermentatieve dechlorering. Nader onderzoek naar de afbraakroute en het mechanisme van dit biologische proces zal dit moeten bevestigen. Voor de volledige dechlorering van 1,1,1-trichloorethaan komt een sequentieel anaëroob/aëroob cometabolisch proces in aanmerking. In de eerste anaërobe stap wordt 1,1,1-trichloorethaan omgezet naar chloorethaan. Chloorethaan kan in de tweede aërobe stap verder worden omgezet naar kooldioxide. Dit proefschrift beschrijft de condities die leiden tot de volledige anaërobe omzetting van 1,1,1-trichloorethaan naar chloorethaan.

NAWOORD

Tijdens mijn eerste afstudeeronderzoek, dat ik heb uitgevoerd bij de vakgroep Microbiologie in Wageningen (LUW), wist ik al snel dat ik na mijn afstuderen nog enkele jaren verder wilde in het onderzoek. Mijn voorkeur ging daarbij uit naar een promotieonderzoek met een grote mate van zelfstandigheid en een onderwerp waarbij de praktijk niet uit het oog werd verloren. De keuze voor een promotieonderzoek bij TNO lag voor mij dan ook voor de hand, en dat bleek achteraf een juiste keuze. Ik heb er bijna vijf jaar met veel plezier gewerkt.

Het is een cliché, maar tijdens die vijf jaar is het voor mij duidelijk geworden dat je, hoe zelfstandig je ook kunt, wilt en moet werken, een promotieonderzoek nooit alleen kan voltooien. Mijn dank gaat dan ook uit naar de volgende personen (en instanties):

- mijn promotores Wim Harder en Dick Janssen, en mijn begeleider Hans Doddema. Zonder hun kritische noten en aansporingen was dit proefschrift nooit in deze vorm tot stand gekomen;
- alle studenten die, vaak met erg veel enthousiasme, hebben geholpen bij de uitvoering van het vele experimentele werk. Bedankt (in volgorde van opkomst) André, Silvia, Esa, Joek, Annemieke, Hetty, Peter en André;
- de collega's van TNO. Na inspanning volgde vaak ook ontspanning in de vorm van squashen, cabaret, spelletjesavonden en borrels. De 'day after' staat mij niet altijd even helder meer voor de geest, en dat zegt genoeg;
- mijn ouders voor het negeren van alle studieadviezen, en daarmee voor hun vertrouwen in mij;
- het IOP-Milieubiotechnologie voor de financiering van het onderzoek.

Tot slot wil ik het woord richten tot Quinta en Pauline, op dit moment de twee belangrijkste personen in mijn leven. Quinta, je schreef al in het voorwoord van je proefschrift dat we in hetzelfde (promotie)schuitje zaten. Die van mij voer echter noodgedwongen veel vaker in de avonden, nachten en weekenden. Gelukkig maakte je daar (bijna) nooit een probleem van. Hopelijk behoort het ploeteren achter de computer nu allemaal tot het verleden en is er volop tijd voor de dingen die ons in de komende tijd te wachten staan (....). Pauline, je was en bent het zonnetje bij ons in huis. Tegenslagen en writer-blocks waren meestal snel vergeten als we samen door het huis gingen 'hennen' of 'boe doen' achter de koelkast. Hopelijk is je zonnige kijk op het leven een blijvertje.

Wageningen, 14 december

CURRICULUM VITAE

Jappe Hinco de Best was born in 1968 on July 17th in ‘s Hertogenbosch. He graduated from high school (VWO - Hertog Jan College in Valkenswaard) in 1986. From 1986 till 1992 he studied environmental biology at the Agricultural University of Wageningen. In 1992 he graduated as an agricultural engineer (MSc) in Biology.

In January 1993 he started as a PhD student. While employed at the department of Biochemistry of Groningen University he performed his research at the department of Environmental Biotechnology of TNO in Delft. During the last year of his PhD, besides performing his research, he also worked on a report about the intrinsic biodegradation of chlorinated solvents by order of TNO.

After finishing his experimental work in July 1997, he (re)wrote a report about temporary disposal sites for dredging material for RIZA in Lelystad. Since may 1st 1998 he is working as a consulting engineer at the department of soil of Grontmij Consulting engineers in Houten. His work is mainly focused on (contaminated) sediments and on dumping grounds.

Jappe de Best is married to Quinta Kools and they have a daughter, Pauline, born in 1996 on October 6th. They are expecting their second child in March 1999.