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INTRINSIC BIODEGRADATION OF CHLORINATED SOLVENTS: FROM THERMODYNAMICS TO FIELD

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Samenvatting
Natuurlijke afbraakprocessen leveren een belangrijke bijdrage aan de in situ verwijdering van chloorkoolwaterstoffen. Dit rapport presenteert een overzicht van de beschikbare kennis over dit process voor de belangrijkste chloorkoolwaterstoffen. Op basis van dit overzicht zijn de belangrijste kennishiaten voor de toepassing van in situ dechlorering geïdentificeerd en worden aanbevelingen voor toekomstig onderzoek gedaan.

Trefwoorden
Gecontroleerde termen:
biodegradation, chlorinated ethanes, chlorinated ethenes, chlorinated methanes, groundwater, subsurface pollution

Vrije trefwoorden:
intrinsinc bioremediation, natural attenuation

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Abstract
Natural degradation processes play a crucial role in the in situ removal of chlorinated compounds. This report presents a review of the available knowledge in this area for the most important chlorinated compounds. Based upon this review, the essential knowlegde gaps for the application of in situ dechlorination are identified and recommendations for future research are made.

Keywords
Controlled terms:
biodegradation, chlorinated ethanes, chlorinated ethenes, chlorinated methanes, groundwater, subsurface pollution

Uncontrolled terms:
intrinsic bioremediation, natural attenuation

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SAMENVATTING

Intrinsic biodegradation of chlorinated solvents: from thermodynamics to field

Natuurlijke afbraak kan een belangrijke bijdrage leveren bij de beheersing van de risico's van grondwaterverontreinigingen en is het voornaamste proces dat leidt tot 'natural attenuation', het stabiliseren van grondwaterpluimen als gevolg van natuurlijke verwijderingsprocessen. Een systematisch overzicht van mogelijke en van nature daadwerkelijk optredende afbraakprocessen ontbreekt tot op heden. Dit rapport geeft een dergelijk overzicht voor gechloreerde ethenen, ethanen en methanen. De gevolgde benadering wordt het best gekarakteriseerd als '*van de thermodynamica naar het veld*'. Op basis hiervan zijn de verschillende natuurlijke afbraakprocessen geïndentificeerd en wordt een typologie van intrinsieke dechlorering in verontreinigde bodem en grondwater voorgesteld.

Elementen in het vaststellen van intrinsieke dechlorering
Voor een snelle biologische dechlorering moet aan de volgende voorwaarden worden voldaan:

i. de dechlorering moet energetisch gunstig zijn;
ii. er moet sprake van zijn dat dechlorering energetisch voordeliger is voor de betrokken micro-organismen dan concurrerende reacties;
iii. de aanwezigheid van geadapteerde micro-organismen;
iv. voldoende hoge concentraties van de voornaamste reactanten, zoals elektronendonoren en elektronenacceptoren, of co-substraten.

Aan de hand van deze voorwaarden zijn de intrinsieke dechloreringsreacties van gechloreerde ethenen, ethanen en methanen geanalyseerd. De biologische standaard vrije energie is gebruikt voor een eerste thermodynamische evaluatie.

Biodegradatiereacties
Dechlorering kan verlopen via:

i. reductieve reacties;
ii. oxidatieve reacties;
iii. oxygenase gekatalyseerde reacties;
iv. niet-redox reacties.

Reducties en oxidaties zijn de belangrijkste intrinsieke dechloreringsreacties. Gedurende deze reacties vindt een overdracht van elektronen plaats, met andere woorden, elektronendonoren en elektronenacceptoren spelen een cruciale rol. Door oxygenase gekatalyseerde reacties zijn eveneens belangrijk. Daarbij wordt moleculaire zuurstof ingebouwd in het gechloreerde molecuul. Het gevormde product is meestal zeer reactief en breekt spontaan verder af.

Typologie van intrinsieke dechlorering
Veldgegevens van diverse studies uit binnen- en buitenland zijn gebruikt om een typologie van dechlorering op veldschaal te ontwerpen. De volgende typen zijn daarbij onderscheiden:

i. volledige reductieve dechlorering;
ii. onvolledige reductieve dechlorering;
iii. volledige sequentiële (eerst reductief dan oxidatief) dechlorering;
iv. onvolledige sequentiële dechlorering;
v. redox-onafhankelijke afbraak;
vi. geen afbraak.

Hieronder worden de resultaten beknopt weergegeven voor gechloreerde ethenen, ethanen, en methanen.

Gechloreerde ethenen: PER (per- of tetrachlooretheen), TRI (trichlooretheen), DCE (dichlooretheen) en VC (vinylchloride)

Reductieve dechlorering
Reductieve dechlorering levert voor alle gechloreerde ethenen energie op. De reactie kan daardoor een rol spelen in het energiemetabolisme van bacteriën: dehalorespiratie, waarbij de chloorethenen als terminale elektronenacceptor worden gebruikt. De energetische opbrengst van de reductieve dechlorering van DCE en VC is dermate laag, dat deze reacties onder bepaalde omstandigheden, bijvoorbeeld een lage waterstofspanning of onvoldoende koolstofbronnen, juist energie kosten. Reductieve dechloring van chloorethenen onder mangaanreducerende of aërobe condities is thermodynamisch ongunstig, daarentegen uitermate gunstig onder methanogene en sulfaat- en ijzerreducerende omstandigheden. In diverse laboratoriumstudies is dehalorespiratie, ademhaling met PER en TRI, onomstotelijk bewezen. Voor DCE en VC is co-metabolische dechlorering wel, maar dehalorespiratie (nog) niet aangetoond. Overigens worden deze DCE en VC lang niet altijd afgebroken in gereduceerde milieus. De waterstofconcentratie en de aard van de aanwezige koolstofbronnen blijken een sleutelrol te spelen bij de reductieve dechlorering van chloorethenen.

Oxidatieve reacties
De oxidatie van chloorethenen levert onder alle omstandigheden energie op, behalve de oxidatie van VC in sterk gereduceerde milieus. Oxidatieve reacties zouden derhalve de voorkeur genieten wanneer ze enzymatisch mogelijk zijn. Metabolische oxidaties van PER en TRI zijn echter tot dusver niet gevonden. Het lijkt erop dat er voor dit type reactie (nog) geen enzymen voorkomen in de natuur. Verschillende typen micro-organismen kunnen echter de lager gechloreerde ethenen oxideren. Onder anaërobe omstandigheden is alleen de oxidatie van VC overtuigend aangetoond, met Fe(III) als elektronenacceptor.

Oxygenase reacties
Oxygenases komen in diverse micro-organismen voor. Zij spelen een rol bij de koppeling van zuurstof aan alkanen, alkenen, methaan en allerlei aromatische verbindingen. Als zijreactie kunnen ook gechloreerde ethenen worden geoxideerd. Daarbij worden epoxiden gevormd. Deze reacties treden alleen onder aërobe omstandigheden op. Behalve PER, kunnen alle chloorethenen met behulp van oxygenases in epoxiden worden omgezet. De eerste stap in de afbraak van VC door aërobe organismen, die deze verbinding als koolstof- en energiebron gebruiken, wordt ook door een oxygenase gekatalyseerd.

Intrinsieke dechlorering op veldschaal
Volledige reductieve dechlorering (type i) van PER en TRI via *cis*-DCE en VC tot etheen, is in vele grondwaterpluimen gevonden. Belangrijke factoren die bijdragen tot volledige reductieve dechlorering zijn: een lage redoxpotentiaal, voldoende koolstofbronnen, bijvoorbeeld veen, lekkende riolen, BTEX en anaërobe afbraakproducten als fenolen en benzoaten. Waterstof speelt een sleutelrol bij de dechlorering op veel locaties. Onvolledige reductieve dechlorering (type ii) wordt eveneens vaak gevonden. Dit kan door vele factoren worden veroorzaakt, bijvoorbeeld:

a. onvoldoende koolstofbronnen;
b. koolstofbronnen van een verkeerde kwaliteit;
c. onjuiste waterstofspanning;
d. ontbreken van de juiste micro-organismen;
e. toxische effecten, met name bij een sterke accumulatie van *cis*-DCE.

Volledige sequentiële dechlorering (type iii) kan optreden wanneer de kern van de verontreiniging zich in sterk gereduceerd grondwater bevindt en er benedenstrooms weer opmenging met aëroob grondwater plaatsvindt. In een eerste stap worden PER en TRI gereduceerd tot *cis*-DCE, VC en etheen. Het overblijvende *cis*-DCE en VC worden vervolgens in het aërobe grondwater geoxideerd tot CO_2. Soms leidt de aanwezigheid van aromatische verbindingen en/of methaan in het verontreinigde grondwater tot co-metabolische omzetting van lager gechloreerde producten in het aërobe grondwater. Onvolledige sequentiële dechlorering (type iv) wordt meestal veroorzaakt door een niet volledige verwijdering van PER en TRI in het anaërobe deel van de pluim. Intrinsieke dechlorering blijft meestal achterwege (type vi) in sterk geoxideerd grondwater met een laag organisch koolstofgehalte.

Gechloreerde ethanen: TCA (trichloorethaan), DCA (dichloorethaan) en CA (chloorethaan)

Reductieve dechlorering
Reductieve dechlorering van chloorethanen levert onder alle omstandigheden energie op. Onder methanogene, sulfaatreducerende en ijzerreducerende condities kan omzetting leiden tot de afsplitsing van een chloride per reactiestap of er vindt een gelijktijdige afsplitsing van twee chloriden onder de vorming van een dubbele binding plaats. Onder denitrificerende omstandigheden levert alleen de stapsgewijze afsplitsing van chloride energie op. Reductieve dechlorering kost energie in aërobe milieus.

Oxidatieve reacties
Deze leveren doorgaans energie op, behalve in sterk gereduceerde milieus. Niettemin wordt oxidatieve afbraak van deze verbindingen alleen onder aërobe omstandigheden gevonden.

Oxygenase reacties
Oxygenases kunnen een atoom uit moleculaire zuurstof koppelen aan chloorethanen onder de vorming van een alcohol. Deze reacties kosten energie en treden op als zijreactie bij de oxidaties van onder andere methaan en andere alkanen en aromatische verbindingen die als primair substraat voor groei dienen.

Overige reacties
TCA kan worden gehydrolyseerd onder de vorming van azijnzuur. Dit is vermoedelijk een belangrijke reactie in de natuurlijke afbraak van TCA.

Intrinsieke dechlorering op veldschaal
Volledige reductieve dechlorering van 1,2-DCA is op 2 locaties gevonden. Op 1 locatie heersen anaërobe omstandigheden (ijzerreducerend tot methanogeen). De reductieve dechlorering van TCA is in veel gevallen onvolledig (type ii). TCA kan tevens abiotisch worden omgezet in 1,1-DCE, een betrekkelijk autonome reactie die een belangrijke rol in het natuurlijk afbraakproces speelt, evenals de omzetting van TCA in azijnzuur (zie boven). TCA kan ook volledig sequentieel worden omgezet (type iii).

Chloormethanen: CT (tetrachloormethaan), CF (chloroform), DCM (dichloormethaan) en CM (chloormethaan)

Reductieve dechlorering
Deze reacties leveren onder alle omstandigheden energie op. In feite zijn de chloorverbindingen vaak betere elektronenacceptoren dan de van nature aanwezige elektronenacceptoren, zoals carbonaat, sulfaat, Fe(III) en nitraat. In principe kan CT worden gedechloreerd tot methaan, via CF, DCM en CM. Tot dusverre zijn echter geen aanwijzingen gevonden voor een metabolische reductieve dechlorering.

Oxidatieve reacties
Deze leveren alleen energie op in aanwezigheid van zuurstof. DCM en CM kunnen onder deze omstandigheden als enige koolstof- en energiebron fungeren.

Oxygenase reacties
Behalve CT, kunnen alle chloormethanen oxygenase reacties ondergaan.

Overige reacties
Alle chloormethanen kunnen fermentatieve reacties ondergaan, waarbij CO en CO_2 kunnen ontstaan.

Intrinsieke dechlorering op veldschaal
Hierover is nauwelijks informatie beschikbaar.

Kennishiaten
1. Een gevoeligheidsanalyse van de thermodynamische gegevens ten aanzien van de actuele concentraties van de reactanten bij dechlorering zou kunnen leiden tot het identificeren van kritische parameters voor dechorering. Met behulp van de resultaten van een dergelijke analyse kunnen de actuele concentratieniveaus, van bijvoorbeeld waterstof, in het veld worden gekoppeld aan het dechloreringspatroon. Dit is van grote praktische betekenis. Deze gegevens zouden behulpzaam kunnen zijn voor het kwantificeren op veldschaal van de potentie voor reductieve dechlorering.
2. De rol van waterstof bij reductieve dechlorering is nog niet geheel duidelijk. Vaak wordt gesuggereerd dat waterstof de primaire elektronendonor is - en daarmee de motor - voor reductieve dechlorering. Een beter begrip van de rol van verschillende elektronendonoren is van groot belang voor de toepassing van reductieve dechlorering in de bodemsanering.
3. Oxidatieve omzettingen in licht gereduceerd grondwater (nitraat- en ijzerreductie) spelen potentieel een zeer grote rol in de volledige sequentiële dechlorering. In dat geval is de aanwezigheid van een aërobe zone in de pluim niet noodzakelijk om toch een volledige sequentiële dechlorering te bewerkstelligen. Er is nog nauwelijks kennis over dechlorering in licht gereduceerde milieus voorhanden.
4. Over het optreden van natuurlijke co-metabolische afbraak van lager gechloreerde componenten aan de aërobe rand van de pluim is weinig bekend. Dit proces zou een grote rol in natuurlijke afbraak kunnen spelen.
5. Nauwkeuriger en goedkopere methoden voor de karakterisering van de redoxomstandigheden ter plaatse zijn noodzakelijk. Het gaat daarbij met name om het in kaart brengen van de heterogeniteit in de ondergrond. Als gevolg van de bemonsteringsprocedures leveren klassieke chemische analysemethoden vooral een gemiddelde waarde. Moleculaire karakteriseringen van de microbiële populaties in de ondergrond geven in potentie een beeld van de heterogeniteit ter plaatse.
6. Een belangrijke vraag is hoe we van de situatie van 'iedere locatie een NA-project' naar een rendabele beoordeling van nieuwe locaties kunnen komen. Een belangrijke aanzet hiertoe is gegeven in het beslismodel dat is ontwikkeld in het kader van het NOBIS-project 'Natuurlijke afbraak'. Een systematische analyse van de kritsiche parameters uit diverse projecten met chloorkoolwaterstoflocaties, met behulp van statistische technieken en/of moderne technieken uit de informatietechnologie, zouden dit beslismodel nog verder kunnen aanscherpen.

SUMMARY

Intrinsic biodegradation of chlorinated solvents: from thermodynamics to field

Naturally occurring degradation can mitigate risks of groundwater contaminants substantially and generally is the most important process contributing to natural attenuation of contaminants. A systematic overview of possible and actually occurring intrinsic biodegradation processes does not exist. This report presents such an overview for chlorinated ethenes, ethanes and methanes. A '*from thermodynamics to field*' approach was used to identify distinct intrinsic biodegradation processes and to classify different types of intrinsic dechlorination behaviour in contaminated aquifers and soils. Knowledge gaps were identified that need to be resolved in order to promote a wide and safe application of intrinsic biodegradation of chlorinated compounds. The approach can easily be extended to other compounds.

Basic elements in the assessment of intrinsic biodegradation
Biodechlorination can occur at significant rates when the following conditions have been met:

i. the Gibbs energy of reaction is negative;
ii. a preference for the dechlorination exists over other reactions possible in the specific environment;
iii. micro-organisms with appropriate enzymes are present;
iv. substances that take part in the biodegradation reaction, such as electron donors, acceptors, or cosubstrates, are present in sufficient amounts.

This set of requirements was used in analysing the intrinsic dechlorination processes for chlorinated ethenes, ethanes and methanes. In this study, biological standard values of Gibbs energy were used for a first thermodynamic evaluation.

Biodegradation reactions
Dechlorination can proceed through:

i. reductive reactions;
ii. oxidative reactions,
iii. oxygenase mediated reactions;
iv. non-redox reactions.

Reductions and oxidations form the most important group of intrinsic dechlorination processes. These redox processes are based on electron transfer, i.e., electron donors and acceptors play an essential role. Oxygenase mediated reactions are also important. These reactions involve a reduction of molecular oxygen, which is built into the chlorinated molecule by the enzyme. The oxigenated product can then be further degraded, often by oxidative processes.

Types of intrinsic dechlorination
Field data reported in the literature and from recent studies performed in the USA and the Netherlands were used to identify and classify the various types of intrinsic dechlorination that can occur in the field. The following types of intrinsic dechlorination were identified:

i. complete reductive dechlorination;
ii. incomplete reductive dechlorination;
iii. complete sequential reductive-oxidative transformation;
iv. incomplete sequential reductive-oxidative degradation;

v. redox independent degradation;
vi. no degradation.

The following briefly reports the results obtained for chlorinated ethenes, ethanes and methanes.

Chlorinated ethenes: PCE (perchloroethylene), TCE (trichloroethylene), DCE (dichloroethylene) and VC (chloroethylene or vinyl chloride)

Reductive dechlorination
These reactions have a negative standard Gibbs energy for all the compounds considered. The reaction can support metabolic reductive dechlorination (also called dehalorespiration) of PCE and TCE. The energy yield from degradation of VC and especially of DCE, is that low that actual conditions can easily become unfavourable for dehalorespiration. Such an unfavourable condition can be a too low concentration of electron donor (hydrogen or an adequate organic compound). Reductive transformation of chlorinated ethenes is not favourable under manganese- and oxygen reducing conditions, and favourable under carbon dioxide, sulphate and iron reducing conditions. In laboratory studies, dehalorespiration of PCE and TCE has been demonstrated several times. For DCE and VC, metabolic conversion has only rarely been reported, whereas cometabolic reductive dechlorination or absence of reductive dechlorination has been more often found. Hydrogen pressure and the 'quality' of organic compounds serving as electron donor appear to be key factors influencing these processes.

Oxidative reactions
In all cases these reactions are highly exergonic, except for VC under sulphate reducing and methanogenic conditions. Hence, oxidative reactions are highly preferential when they are biochemically possible. To date no metabolic oxidative conversion of PCE and/or TCE has been reported. There seems to be no enzyme system to mediate these reactions. Different types of microbial enzymes can oxidise the other chloroethenes and often operate under aerobic conditions. Under anaerobic redox conditions only the oxidation of VC with Fe(III) serving as electron acceptor has been reported. The mechanism of this reaction is not yet understood.

Oxygenase mediated reactions
Oxygenases, present in many aerobic micro-organisms, transform choroethenes to chlorinated epoxides and require molecular oxygen. Hence, the reactions can only occur under aerobic conditions. All chlorinated ethenes except PCE can undergo oxygenase mediated epoxidation via a cometabolic process. The micro-organisms involved experience some energetic disadvantage by performing the reaction, but can generally mitigate this disadvantage by the high energy gain from the aerobic metabolic oxidation of the cosubstrates (methane, toluene, phenol, butane, ethene, etc.). VC and possibly also *cis*-DCE can be metabolically epoxidised and then serve as a sole carbon and energy source for microbial growth.

Field observations of various types of intrinsic dechlorination
Complete reductive dechlorination (type i) of PCE/TCE via *cis*-DCE and VC to ethene has been observed in (parts of) chlorinated solvent plumes at many sites in the US and in the Netherlands. Important factors supporting this process are a sufficiently low reducing condition and sufficient amounts of intrinsic electron donor such as natural organic matter, sewage or landfill leachate organics, BTEX, and anaerobic BTEX-degradation products like benzoate and phenol. Hydrogen is hypothesised to be the primary electron donor at a number of sites. Incomplete reductive dechlorination (type ii) has often been observed.

Several factors can cause this ineffective degradation, namely:

a. insufficient amount of electron donor;
b. inappropriate electron donor, i.e., producing too low or too high hydrogen levels to support DCE and/or VC reductive dechlorination;
c. absence of *cis*-DCE and VC dechlorinating micro-organisms;
d. toxicity effects, especially caused by accumulation of *cis*-DCE.

Complete sequential reductive-oxidative dechlorination (type iii) occurs by a sequence of anaerobic and aerobic processes along the flow-path of a contaminant plume. The anaerobic reductive dechlorination is followed by transformation of *cis*-DCE and VC via oxidation or epoxidation to carbon dioxide and chloride in the aerobic zone. These oxidations can be mediated by various processes. First of all, VC can be metabolically oxidised under aerobic or iron(III) reducing conditions. Second, they can be degraded cometabolically. Several cosubstrates produced in the anoxic zone (methane, ethene, or BTEX degradation products like benzoate and phenol) can be expected to support cometabolic conversion after entering the oxic zone. Indications for such type of intrinsic processes establishing stabile chlorinated ethene plumes have been found at several sites in the USA and at one site in the Netherlands. However, no solid proof and quantitative estimates were provided. Incomplete sequential reductive-oxidative dechlorination (type iv) often occurs through insufficient intrinsic degradation of the parent compounds PCE and TCE during passage through an anaerobic zone. Hence, the sequential intrinsic processes are then insufficiently protective, and stimulated bioremediation is required. No intrinsic degradation (type vi) is generally found in aerobic, low DOC aquifers contaminated with PCE and TCE. In these cases, the autochtonous micro-organisms can often be stimulated towards cometabolic epoxidation and a subsequent oxidation of the chlorinated ethenes by adding cosubstrate and oxygen.

Chlorinated ethanes: TCA (trichloroethane), DCA (dichloroethane) and CA (chloroethane)

Reductive dechlorination
Gibbs free energies of reactions are sufficiently negative to support metabolic dechlorination in all cases. Under carbon dioxide, sulphate reducing and iron(III) reducing conditions chlorinated ethanes can be transformed via either hydrogenolysis or dihalo-elimination. Under nitrate reducing conditions, only hydrogenolysis of TCA and CA is a preferred reaction. Dihalo-elimination of 1,2-DCA can occur under manganese reducing conditions. No reductive processes are preferential in aerobic systems. In the laboratory, dechlorination of 1,2-DCA to CA, ethene, and/or ethane, and of 1,1-DCA to CO_2 by methanogenic and/or acetogenic bacteria, has been observed. Recently, complete removal of 1,2-DCA in iron reducing/methanogenic columns and microcosms was demonstrated. Reductive dechlorination of TCA to CA has been reported under sulphate reducing, acetogenic and methanogenic conditions, via a cometabolic reaction catalysed by transition-metal complexes like cobalamins and coenzyme F_{430}. TCA can also be completely dechlorinated under anaerobic conditions.

Oxidative reactions
Under sulphate reducing and methanogenic conditions Gibbs free energies for transformation are slightly negative and the first oxidation step even costs energy. Under iron, nitrate, manganese or oxygen reducing conditions, the oxidation reactions are strongly exergonic and can in principle support microbial growth. Nevertheless, oxidative reactions of TCA, DCA and CA have only been reported under aerobic conditions.

Oxygenase mediated reactions
These reactions incorporate molecular oxygen into the chlorinated ethane under the formation of a chloroalcohol and require aerobic conditions. This process costs energy. This disadvantage can be compensated by the high energy gain from aerobic metabolic oxidation of the

cosubstrates (methane, toluene, phenol, butane, ethene, etc.) and the formed chloroalcohol. Cometabolic biotransformation of TCA, 1,1-DCA and 1,2-DCA has been reported.

Other reactions
Non-redox dehalogenations, combined with oxidative degradations are often reported. Dehalogenases catalyse CA and 1,2-DCA conversions in metabolic mineralisation. Chlorinated ethanes can also be abiotically transformed. Hydrolysis of TCA leads to acetic acid.

Field observations of various types of intrinsic dechlorination
Complete reductive dechlorination (type i) of 1,2-DCA has been demonstrated at two sites recently. In one case iron reducing/methanogenic conditions prevail. At the other site mixed conditions exist. Evidence for similar reductive processes in the field were found for TCA, DCA, and CA. Incomplete reductive dechlorination (type ii) has been found for TCA that was reductively dechlorinated to 1,1-DCA and CA, and sometimes to 1,1-DCE. Complete sequential reductive-oxidative dechlorination (type iii) was found for TCA. In the anaerobic zone, TCA is degraded to 1,1-DCA, CA, ethene and ethane. The degradation products were further degraded in the aerobic zone.

Chlorinated methanes: CT (tetrachloromethane or carbon tetrachloride), CF (trichloromethane or chloroform), DCM (dichloromethane) and CM (chloromethane)

Reductive dechlorination
Gibbs free energies of reactions are sufficiently negative to support metabolic conversions in all cases. Under carbon dioxide, sulphate, iron(III) and nitrate reducing conditions, the chlorinated methanes are better electron sinks than the natural electron acceptors. CT can also be reduced under manganese reducing conditions. Reduction of chlorinated methanes is not preferential under oxygen reducing conditions. CT can be hydrogenolytically dechlorinated to CF, DCM and CM under methanogenic, sulphate reducing, iron reducing and nitrate reducing conditions. Thus far, these transformations have been demonstrated to be cometabolic and aspecific, and catalysed by transition metal complexes. CT can also be mineralised to CO_2 but the pathway of CT mineralisation is not yet completely clear.

Oxidative reactions
Under all conditions except for oxygen and manganese reducing conditions, Gibbs free energies for transformation do not favour metabolic transformation and the first oxidation step costs significant amounts of energy. Under manganese and oxygen reducing conditions microbial growth is thermodynamically possible. Under aerobic conditions DCM and CM were found to serve as sole source of carbon and energy for microbial growth. DCM is converted to formaldehyde by specific DCM dehalogenases via thiolytic dehalogenation.

Oxygenase mediated reactions
Except for CT, all chlorinated methanes can undergo oxygenase mediated transformation under aerobic conditions.

Other reactions
Both DCM and CM have been reported to be fermentatively degraded.

Field observations of various types of intrinsic dechlorination
Nearly no information is available on the intrinsic bioremediation of chlorinated methanes. Fermentation of DCM to acetic acid by acetogenic micro-organisms in a shallow aquifer was reported once.
Knowledge gaps
The following major knowledge gaps still exist:

1. A thermodynamic analysis extended to actual Gibbs energies of transformation (and not only on standard Gibbs energies, as done in this report) is required to identify critical concentrations of important electron donors (hydrogen) and electron acceptors. The actual Gibbs Free energies can also be used to better couple the chemical conditions in the field with the possible transformations. This is of great practical importance; by monitoring e.g., in situ H_2-pressures the ability of an aquifer to support complete reductive dechlorination could be qualified.
2. The role of hydrogen and other electron donors in reductive dechlorination is not yet understood. In many cases hydrogen seems to be the primary electron donor in reductive dechlorination. Organic compounds can also function as the electron donor. Understanding the role of the possible electron donors is of prime importance for the application of intrinsic reductive dechlorination.
3. Oxidative (non-oxygenase) transformation under moderately reducing (iron- nitrate- and oxygen reducing) conditions is extremely important for natural attenuation/intrinsic bioremediation in sequential redox situations. These processes have been hardly investigated, neither at the fundamental, nor at the applied field level.
4. The occurrence of natural cometabolic oxygenase mediated processes at the edges of anaerobic chlorinated solvent plumes in aerobic aquifers is important for application of natural attenuation and insufficiently investigated.
5. More accurate and efficient methods for the assessment of *in situ* redox conditions are required. Molecular redox characterisation techniques may bring the answer.
6. An important problem is how to get from the situation of 'every site a natural attenuation research project' to a cost-effective assessment of natural attenuation and additional solutions at new sites. This can be achieved by collecting data on critical parameters as observed in current field investigations and by performing statistical or machine learning techniques (e.g. fuzzy logic).

In conclusion, the '*from thermodynamics to field*' approach as initiated in this project provides a track towards a solid science based understanding of the possibilities of intrinsic bioremediation and natural attenuation of chlorinated ethenes, ethanes and methanes. In addition to these compounds, the approach can be extended to other compounds.

CHAPTER 1

INTRODUCTION

World-wide, chlorinated hydrocarbons are among the most frequently occurring contaminants in soil and groundwater. These pollutants pose a potential threat to the well-being of man, flora and fauna. Hence, measures to control the risks associated with sites contaminated with these compounds are required. In many cases a fast clean-up by pump-and-treat or intensive *in situ* technologies is economically and/or technically not feasible. The use of naturally occurring intrinsic degradation processes (intrinsic bioremediation or natural attenuation) is becoming more and more an option for the restoration of contaminated sites. Natural attenuation is the naturally occurring reduction of contaminant concentration as a result of destructive or non-destructive biotic and abiotic processes and is defined by the U.S. Environmental Protection Agency's (EPA) office as: 'the biodegradation, dispersion, dilution, sorption, volatilisation, and/or chemical and biochemical stabilisation of contaminants to effectively reduce contaminant toxicity, mobility or volume to levels that are protective to human health and the ecosystem'. The most important mechanism of contaminant destruction during natural attenuation is microbially mediated degradation (intrinsic biodegradation).

In this report we focus on the intrinsic biodegradation of chlorinated hydrocarbons. Many microbial dechlorination processes have been demonstrated in the laboratory. Numerous field studies have indicated that a number of these dechlorination processes also takes place under natural conditions. Many field data on the natural attenuation of chlorinated hydrocarbons are currently being produced. No structural review of existing knowledge is available that combines the thermodynamic, geochemical and microbial aspects into a unifying framework. Such a framework would be a powerful tool to give a first assessment of the potential of intrinsic bioremediation at a site, given its biogeochemical and geohydrological characteristics. Moreover it can be used to identify knowledge gaps and future research needs.

The objective of this report is to produce such a unifying framework. The most important chlorinated contaminants were identified on the basis of their production and emission rates and their occurrence in soil and groundwater (see chapter 2). The basic elements in assessing the potential of intrinsic biodegradation of chlorinated hydrocarbons are discussed in chapter 3. Chapter 4 reviews the available information on the potential of intrinsic degradation of some important chlorinated hydrocarbons. In chapter 5, a scheme for classification of the type of intrinsic dechlorination in contaminated plumes is proposed. Chapter 6 presents the important gaps in knowledge of intrinsic degradation reactions, identified on the basis of the results, and the research needed for improvary and the application of natural attenuation of chlorinated hydrocarbons.

CHAPTER 2

IMPORTANT COMPOUNDS

The importance of a specific substance as a pollutant results from its production volume (e.g. in tons per year) and its use (see table 1). Compounds with high production volumes but a limited number of high volume applications will yield a limited number of - potentially highly contaminated - sites. Thus, 1,2-dichloroethane (1,2-DCA), an intermediate in the production of vinyl chloride (VC), is the chlorinated hydrocarbon in the highest volume world-wide, but is the main contaminant at only 3 sites in the Netherlands (see table 1). However, the size of the pollution at these sites is very large.

This contrasts with compounds which are produced in lesser amounts but have applications in a wide range of industrial and commercial activities. Examples of such compounds are perchloroethylene (PCE) and trichloroethylene (TCE). Both PCE and TCE are applied as metal degreasing agents in many different industrial processes, and are being used in dry-cleaning facilities. As a result, numerous sites are contaminated with these compounds, some with big loads, others with smaller loads. Moreover, large PCE and TCE contaminations exist at fire training facilities of (former) military bases.

The compounds like 1,1,1-TCA and the chlorinated methanes have similar uses as PCE and TCE but they are being used in smaller amounts. These compounds are often found together at contaminated sites.

Table 1. The most important chlorinated hydrocarbons: application, world production and emission rates and occurrence at contaminated sites in the Netherlands [23, 75].

chlorinated hydrocarbon	abbreviation	application	# sites NL	production (10^6 kg/yr)	emission (10^6 kg/yr)
perchloroethene	PCE	cleansing solvent (laundries)	1500[a]	1.1	1.1
trichloroethene[b]	TCE	cleansing solvent (metal, laundries)	1500[a]	0.6	0.6
1,2-dichloroethene[b]	1,2-DCE	production synthetics	1500[a]	0.2	0.002
vinyl chloride[b]	VC	PVC production	1500[a]	10.0	0.2
1,1,1-trichloroethane[c]	TCA	metal degreasing	500[a]	0.6	0.6
1,1-dichloroethane[c]	1,1-DCA	solvent	500[a]	0.5	?
chloroethane[c]	CA	solvent	500[a]	0.4	0.015
1,2-dichloroethane	1,2-DCA	PVC production	3	13.0	1.2
carbon tetrachloride[d]	CT	solvent, extraction agent	500[a]	1.0	0.05
chloroform[d]	CF	degreasing, solvent degreasing	500[a]	0.25	0.02
dichloromethane[d]	DCM	paint stripping, metal	500[a]	0.5	0.5
chloromethane[d]	CM	solvent extraction	500[a]	0.4	5.0

[a] Estimated on the basis of the number of laundries and metal factories which contribute the bulk of the contaminated sites. A more accurate estimate will appear in the final version of the report.

[b] PCE through VC often occur together because they belong to the PCE dechlorination pathway.

[c] TCA through CA often occur together because the they belong to the TCA dechlorination pathway.

[d] CT through CM often occur together because they belong to the CT dechlorination pathway.

CHAPTER 3

BASIC ELEMENTS IN THE ASSESSMENT OF INTRINSIC BIODEGRADATION

The objective of the current chapter is to outline a theoretical framework including thermodynamic, biochemical, and microbial aspects of natural attenuation. The focus is on intrinsic biodegradation of chlorinated hydrocarbons but the conceptual approach can be extended to other classes of compounds.

Any process, independent on its nature - biological or not - can only proceed if it is energetically favourable. Therefore, we first discuss the thermodynamic principles of the transformation process (see 3.1). Then, we discuss the possible reaction mechanisms of chlorinated hydrocarbons (see 3.2). The microbial aspects of the degradation of chlorinated hydrocarbons include several factors affecting the competition between microbial populations (see 3.3). Paragraph 3.4 outlines the *in situ* conditions important for the intrinsic degradation of chlorinated hydrocarbons.

3.1 Thermodynamics

Reactions can be classified into two categories: those that are controlled by equilibrium thermodynamics and those that are controlled by kinetics, i.e., limited by the activation energy [33].

Any process, independent of its nature - biological or not - can only proceed if it is energetically favourable. The energy yield of reactions can be estimated using basic thermodynamic laws, which are based on equilibrium considerations, as discussed in 3.1.1.

Sometimes reactions do not proceed in nature although they are energetically favourable. In such cases the process is limited by its activation energy: the energy input needed for the first step. The activation energy is then so high that it prevents a reaction to occur, despite the overall energy yield that would be achieved. One of the features of (enzymatic) catalysts is to reduce the activation energy. These kinetic aspects are discussed in 3.1.2. Many dechlorination reactions are redox reactions. The thermodynamics of redox reactions are discussed in 3.1.3.

3.1.1 *Equilibrium considerations*

Associated with each process is a change of the thermodynamic state of the system. The concept of *Gibbs free energy*, or simply, *free energy* (*G*), has proven very useful to describe the thermodynamic state of a system [33]. It combines the notions of energy (often expressed as enthalpy, *H*) and of entropy (*S*, a measure of the 'chaos' in a system), which together determine whether a reaction is energetically favourable or not (note that 'energetically favourable' in this context means: 'leads to a decrease of the Gibbs free energy of the system involved'). The Gibbs free energy is defined as:

$$G = H - TS \qquad (1)$$

where *T* is the absolute temperature (in K) and *G, H,* and *S* are expressed in kJ mol^{-1}. The usefulness of the Gibbs free energy is that it enables us to consider the overall result of enthalpy and entropy changes in a process.

The change of free energy (ΔG) during a (biochemical) reaction results from the changes in enthalpy and entropy and can be obtained from the difference in free energy between the substrates and the products involved in the reaction:

$$\Delta G = \Delta H - T\Delta S = G_{substrates} - G_{products} \quad (2)$$

A reaction can only proceed when ΔG has a negative value, i.e., when the reaction yields energy. The energy yield of a reaction is only determined by the energy difference between the parent compounds and the products. It is completely independent from the reaction pathway or the mechanism of transformation (see fig. 1A and B).

In physical chemistry, the free energy changes of a reaction are commonly expressed for standard conditions (all reactants at unit molar concentration, gases at 1 atmosphere, temperature at 298 K) in order to get a common reference base. The free energy changes of a biochemical reaction are normally given for the biochemical standard state. In contrast to the standard state used in physical chemistry, the biochemical standard state sets the hydrogen-ion concentration at 10^{-7} M, corresponding the physiological pH of about 7 [33]. Consequently, all reactions involving the uptake or liberation of hydrogen-ions will have a different change of free energy according to the biochemical standard conditions. ΔG^0 is therefore commonly replaced by $\Delta G^{0'}$ in discussing reactions involving biochemical or microbial conversions.

The actual free energy change from a biochemical or microbial reaction is obtained in several steps. First, the standard change in free energy has to be calculated from equation (2) using published values for the Gibbs energies of the products involved. Then, $\Delta G^{0'}$ has to be calculated according to:

$$\Delta G^{0'} = \Delta G^0 + xRT\ln(10^{-7}) \quad (3)$$

for reactions consuming x protons, and

$$\Delta G^{0'} = \Delta G^0 - xRT\ln(10^{-7}) \quad (4)$$

for reactions producing x protons.

Finally, ΔG is obtained by correcting $\Delta G^{0'}$ for the actual concentrations of the reactants and products:

$$\Delta G = \Delta G^{0'} - RT\ln(K') \quad (5)$$

where K' is the equilibrium constant for the reaction under consideration using the actual instead of the equilibrium concentrations of the reactants and products [33].

3.1.2 *Kinetic considerations*

Many reactions appear to be kinetically controlled: the rate of reaction is controlled by the activation energy instead of the equilibrium energy yield. When this is the case for one reaction in a chain reaction of an overall process, the whole process will appear to be kinetically controlled (see fig. 1A and B). Thus, it may occur that a reaction with a negative ΔG does not occur because of kinetic limitations.

Generally, a reaction proceeds because the reactants collide and form a so-called activated complex (the transition state) which has a high energy (see fig. 1A). The next step is the con-

version of the activated complex to the reaction products. Let us consider the reaction of one reactant (*S*) to one product (*P*) [186]:

$$S \Leftrightarrow S^{\ddagger} \Leftrightarrow P \tag{6}$$

where $S^{\ddagger}$ represents the transition state, which is the most unstable intermediate along the reaction. Note that this scheme is only valid for reactions with pseudo first order kinetics.

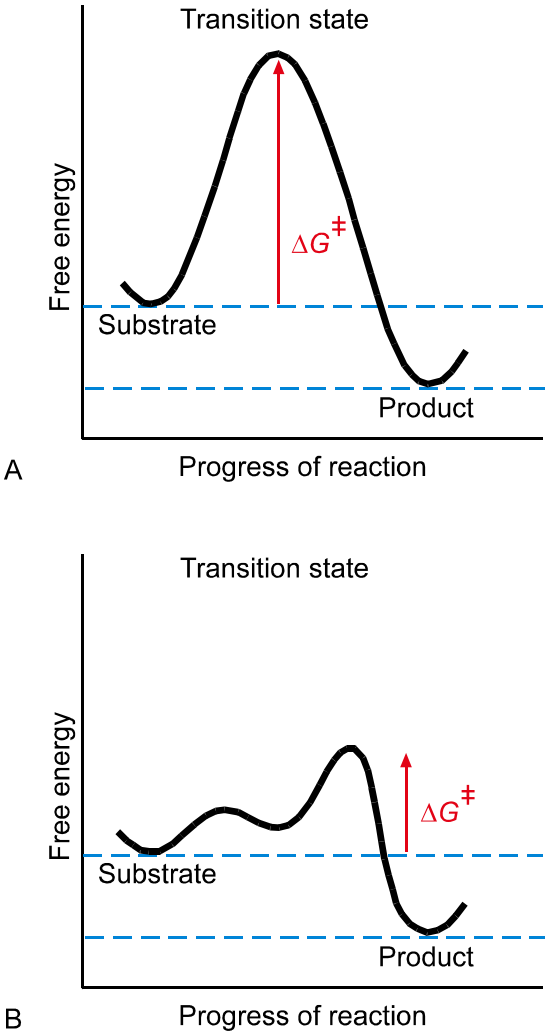

Fig. 1. Enzymes accelerate reactions by decreasing $\Delta G^{\ddagger}$, the free energy of activation. The free energy profiles of uncatalysed (A) and catalysed (B) reactions are compared (redrawn after [186]).

The free energy of activation ($\Delta G^{\ddagger}$) is equal to the difference in free energy between the transition state and the reactant. The rate of reaction (*V*) is proportional to the concentration of the activated complex $S^{\ddagger}$, which is in equilibrium with *S*. Hence, the reaction rate depends on $\Delta G^{\ddagger}$ [186]:

$$[S^{\ddagger}] = [S]e^{-\Delta G^{\ddagger}/RT} \tag{7}$$

$$V = k_1[S] = \frac{kT}{h} e^{-\Delta G^{\ddagger}/RT}[S] \tag{8}$$

where k is Boltzmann's constant, h is Planck's constant, and k_1 is the pseudo-first order reaction rate constant. From equation (8) it is obvious that reactions with a high value of $\Delta G^{\ddagger}$, i.e., a high activation energy, only proceed very slowly.

An important role of catalysts is that they decrease the activation energy by stabilising the transition state or by allowing a different reaction pathway with a lower activation energy (see fig. 1B). Thus, the rate of reaction increases without affecting the change in free energy from the reaction as a whole. In living systems, enzymes take over this role very effectively. Also all kinds of mineral surfaces are known to catalyse reactions that cannot proceed in their absence such as the reduction of nitroaromatic compounds by goethite [93] or the reductive dechlorination of chlorinated solvents by transition metal complexes [5, 15, 76, 170].

3.1.3 *Thermodynamics of redox reactions*

Many reactions of chlorinated hydrocarbons involve the transfer of electrons between reactants in so-called redox reactions. Many half reactions are listed in tabular form together with the standard reduction potential ($E^{0'}$) for these reactions, e.g. chlorinated compounds can be arranged in a sequence according to the redox potentials of the half reactions in the same way as the natural elements O_2, MnO_2, NO_3^-, $Fe(OH)_3$,SO_4^{2-} and HCO_3^- [198]. The half reactions of redox reactions are mostly listed in tables with their standard reduction potential under physiological conditions, $E^{0'}$ (V), e.g.:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \qquad E^{0'} = +0.816\ V \tag{9}$$

The free energy change of this half reaction can be calculated using the Nernst equation:

$$E^{0'} = -\Delta G^{0'}/(nF) = (RT\ln K)/(nF) \tag{10}$$

where n is the number of electrons involved in the reaction and F is the Faraday constant (C/mole). The free energy change of a complete redox reaction is simply obtained by summing up the free energy changes associated with each half reaction.

3.1.4 *Conclusion*

Thermodynamic and kinetic factors together determine whether and at what rate a chemical reaction can proceed, and hence, the degradability of chemical compounds in the environment. Since the transformation of most chlorinated solvents is highly exergonic for both oxidative and reductive transformations [52, 198] kinetics are most probably limiting their degradation in environmental systems.

3.2 **Reaction mechanisms**

The thermodynamic principles governing the occurrence and rate of biochemical reactions have been presented in the preceding paragraph. In the current paragraph we shall shortly review the reactions of chlorinated hydrocarbons as they have been observed in laboratory and in the field. We have included possible abiotic reactions in this review in order to get a complete overview although the focus of the later paragraphs will be on microbially mediated reactions.

Transformations of halogenated compounds have been reported to proceed via redox reactions involving electron transfer and via non-redox reactions, not involving electron transfer (see appendix A). The oxidation state of the reacting molecules changes during redox reactions, while they remain unchanged during non-redox reactions. The redox reactions are discussed in 3.2.1, the non-redox reactions in 3.2.2.

3.2.1 *Redox reactions*

Redox reactions involve reductive and oxidative transformations. Several mechanisms for the reductive transformation of chlorinated hydrocarbons can be distinguished:

i. *Hydrogenolysis*
   In this reaction, the halogen substituent is replaced by hydrogen. Examples are the sequential reductive dechlorination of trichlorobenzenes [19] and the sequential reductive dechlorination of chlorinated ethenes [48]. The reaction mechanism is most probably a nucleophilic substitution.
ii. *Dihalo-elimination*
   Dihalo-elimination is a reductive elimination of two halogens from two adjacent carbon atoms to form an additional bond between the carbon atoms involved in the reaction. Examples are the reduction of hexachloroethane to tetrachloroethene [167], the reduction of (*Z*)-1,1,2,3,4-pentachlorobutadiene to trichloro-1-buten-3-yn [15] and the reduction of trichloroethene to chloroacetylene and of 1,2-dichloroethene to acetylene [80].
iii. *Hydrolytic reduction*
   Hydrolytic reduction involves a two electron transfer to yield a carbenoid which spontaneously hydrolyses to give oxygenated products. Examples are the transformation of carbon tetrachloride to CO_2 [115] or formic acid and carbon monoxide [45] and the formation of acetate from 1,1,1-trichloroethane.
iv. *Coupling*
   Coupling can occur when free radicals are involved in the reductive reaction. Products of coupling reactions are mostly found as side products from other reactions, e.g. the formation of ethane from chloromethane.

These reductive transformations require the presence of electron donors like H_2, formate or other carbon sources. Organic pollutants like e.g. mineral oil or BTEX compounds can also potentially fulfil this role.

3.2.2 *Non-redox reactions*

Other dechlorination reactions do not involve the transfer of electrons and, hence, do not directly depend on the presence of electron acceptors or donors. We mention the following reaction mechanisms:

i. *Hydrolysis*
   During hydrolysis, the halogen substituent is replaced by a hydroxyl group which is derived from water. Hydrolytic dehalogenations have been described for aerobic conditions and are then mostly coupled to growth. Hydrolysis may also proceed abiotically however. Examples are the hydrolysis of 1,2-DCA and 1,2-DBA (1,2-dibromoethane) to give chloroethanol and bromoethanol respectively, and 1,2-ethanediol as the end products [9] and of the dihalo-methanes CH_2Cl_2, CH_2ClBr and CH_2Br_2 to yield the corresponding alcohols [133].
ii. *Thiolytic dehalogenation*
   The mechanism of thiolytic dehalogenation is similar to that of hydrolysis but the halogen is being replaced by gluthathion instead of OH. It has been reported for the microbially mediated, aerobic dechlorination of dichloromethane and is catalysed by DCM dehalogenases that use reduced glutathione as a cofactor [112]. This leads to the formation of an unstable *S*-chloromethyl glutathione conjugate which disintegrates to formaldehyde and hydrochloric acid. Formaldehyde can be used for biosynthesis or undergo oxidation for energy. Thiolytic dehalogenation may also proceed abiotically and has been described for 1,2-DCA and 1,2-DBA to give 1,2-ethanedithiol as the end product [9] and for the dihalo-methanes CH_2Cl_2, CH_2ClBr and CH_2Br_2 to yield the corresponding dithiols [169].

iii. *Dehydrohalogenation*

Dehydrohalogenation involves the elimination from one carbon atom accompanied by the elimination of an adjacent hydrogen leading to the formation of an additional bond between the carbon atoms [134]. Examples are the formation of 1,1-DCE from 1,1,1,-TCA [201], of PCE from pentachlorethane [168] and of TCE from 1,1,2,2-tetrachloroethane [40]. Dehydrodehalogenation can also be catalysed by enzymes as a detoxification reaction in house flies which are resistant to DDT [130, 131] or in a cometabolic fashion by methanogenic bacteria [97].

A very important mechanism for the dechlorination of chlorinated hydrocarbons is oxygenolytic dehalogenation or *epoxidation*. This reaction is catalysed by mono- and dioxygenases and results in the incorporation of one atom of oxygen into the substrate to form an epoxide. The aerobic mineralisation of vinyl chloride is an example of an oxidative transformation initiated by epoxidation [89]. Also the cometabolic transformation of chlorinated aliphatics by methanotrophs [86, 147] and denitrifying bacteria proceeds via epoxidation. The resulting epoxides are chemically unstable and react spontaneously with protein to give CO_2. For this reason, the cometabolic transformation is toxic to the organisms that are carrying them out. Epoxidation is an oxygen dependent reaction and, hence, can only occur under aerobic conditions. Oxidative dechlorination may occur as the initial step in the aerobic mineralisation of chlorinated compounds. The first step in the degradation of 4-chlorobenzoate e.g., was found to be the conversion to 4-hydroxybenzoate [84].

3.2.3 *Conclusion*

Chlorinated hydrocarbons may degrade through various reactions as described above (see appendix A). The most important group of these reactions - the redox reactions - involve the transfer of electrons, while other reactions - hydrolysis, thiolytic dehalogenation, dehydrohalogenation - do not. The availability of reactants, e.g. electron donors and acceptors, OH^-, HS^-, and thermodynamic principles as delineated in 3.1 together, determine whether or not a certain reaction will proceed under certain conditions.

3.3 **Microbiological aspects**

The fact that a reaction is thermodynamically possible, does not necessarily mean that micro-organisms develop that carry them out. Thus, chlorobenzene is very persistent in anoxic environments, although both its reduction to benzene and its oxidation to CO_2 would provide a micro-organism with enough energy for survival.

It makes a difference whether a micro-organism converts a chlorinated hydrocarbon for metabolic purposes or whether the dechlorination is just a side reaction that is carried out fortuitously. In the first case, the micro-organism depends on the reaction for its survival and in that case it may have to compete with other micro-organisms for common substrates. Cometabolic reactions just happen - mostly at a slow rate. It does not make them less useful though since a stimulation of the metabolic process that causes the cometabolic reaction may yield very effective remediation set-ups.

Biochemical catalysis may follow other routes than one would predict at first sight from the chemical reaction mechanisms. A well-known deviation of biochemical catalysis versus ordinary chemical reactions is a high compound-specificity of many biochemical reactions. Enzyme catalysis is known to be specific to optical enantiomers of a compound, e.g. the enantioselective metabolism of chiral 3-phenylbutyric acid, an intermediate of linear alkylbenzene degradation, by Rhodococcus rhodochrous PB1 [180]. Also, the patterns of microbially mediated reductive dechlorination of PCE [48] of chlorinated benzenes [19, 66], and of perchlorobutadiene [16]

appears to differ from the patterns observed in electrochemical experiments [80, 122, 159] or in non-living systems amended with cofactors or natural organic compounds [5, 6, 15, 80, 97].

Once an organism has evolved that can carry out a desired reaction, it must be able to survive under environmental conditions.

These aspects are discussed in this section. First, we discuss the difference between metabolic and cometabolic reactions (see 3.3.1) followed by a discussion of the factors that affect the competition between micro-organisms (see 3.3.2). Since the higher chlorinated compounds appear to be mainly transformed by reductive dechlorination, we shall focus 3.3.2 on this process and only discuss the option of oxidative transformation for the less chlorinated products.

3.3.1 *Metabolic and cometabolic reactions*

Many studies have shown that bacteria are capable of linking the breakdown of chlorinated compounds to their growth in a metabolic process [54, 55, 100, 175, 193]. In other cases bacteria transform the chlorinated hydrocarbons without having a clear benefit from the reaction: the so-called cometabolic reactions [65, 86, 120, 147]. The implications of the nature of metabolic and cometabolic reactions towards chlorinated hydrocarbons are discussed in some more detail below.

Metabolic transformation

In metabolic dechlorination processes, the energy released during the reaction is coupled to the micro-organisms' metabolism. The micro-organism uses the energy for maintenance, growth, nutrient uptake, and defence. In a metabolic process energy is gained by the transfer of electrons from an electron donor to a final electron acceptor. Micro-organisms can use chlorinated hydrocarbons to gain energy in three different ways: by oxidative and reductive transformation and by fermentative conversion where the chlorinated hydrocarbon serves as the electron donor and acceptor simultaneously.

In oxidative conversions, the chlorinated hydrocarbons act as an electron donor and as source of organic carbon (primary substrate). An electron acceptor is required in these reactions. The prevalent redox conditions determine which electron acceptor - O_2, NO_3^-, Fe^{3+}, Mn^{4+}, SO_4^{2-} or HCO_3^- - is available to oxidize the chlorinated hydrocarbon. Only little is known up to now about the possible oxidation of chlorinated hydrocarbons under anoxic conditions, e.g. the oxidation of VC and 1,2-DCA under iron reducing conditions [25] [24]. Oxidative transformations seem to be limited to compounds with a low number of chlorine atoms. In the presence of oxygen, mainly C1 and C2 chlorinated aliphatics have been found to serve as primary substrate, namely, CM, DCM, VC and 1,2-DCA [27, 88, 90, 103]. Also CT degradation has been shown to occur under denitifying conditions [74].

Chlorinated compounds can also be reductively dechlorinated and then serve as a terminal electron acceptor for micro-organisms in an anaerobic environment. When this reaction is coupled to the growth of the dechlorinating micro-organisms the process is called dehalorespiration. These dehalorespiring micro-organisms can have a selective advantage compared to other anaerobes that strictly depend on sulphate reduction or methanogenis because dehalorespiration potentially yields more energy than sulphate reduction or methanogenis. Desulfomonile tiedjei was the first dehalorespiring organism reported [51, 151], and couples the reductive dechlorination of 3-chlorobenzoate to its growth. The last three years several micro-organisms have been isolated that can carry out respiratory reductive dechlorination of PCE [78, 143, 174, 179]. Most of these organisms reduce PCE via TCE to *cis*-1,2-DCE and appear to be closely related. One organism is able to dechlorinate PCE to ethene. The last step - VC to ethene - was found to be a cometabolic reaction that only occurred in the presence of PCE or TCE [142].

An alternative mechanism for metabolic dechlorination are fermentative conversions. Fermentative reactions mostly occur in anoxic environments. CM and DCM are known to be fermented by homoacetogenic bacteria. The chlorinated hydrocarbons simultaneously serve as electron donor, electron acceptor and as carbon source for these bacteria and are converted to acetic acid and/or formic acid. Besides DCM or CM, these bacteria also need carbon dioxide as an additional electron acceptor [137, 192].

Cometabolic transformation
In cometabolic processes dechlorination is not coupled to growth and carried out by enzymes or cofactor-factors, which normally catalyse other reactions. The micro-organisms involved have no apparent benefit from a cometabolic transformation and sometimes energy is even lost because cometabolism needs production of enzymes. Again oxidative and reductive processes can be distinguished. Well known oxidative cometabolic processes are the aerobic transformations by mono-oxygenase and dioxygenase systems [102]. These systems have a wide substrate range and are able to transform most chlorinated hydrocarbons via epoxidation. Anaerobic micro-organisms possess transition metal complexes like ferredoxines, corrinoids and factor F_{430} that can catalyse reductive dechlorination [29, 30, 36, 76, 114, 115].

Metabolic versus cometabolic transformation
Both metabolic and cometabolic biotransformation can be applied for in situ remediation. Generally speaking, metabolic processes are more stable than cometabolic processes: the dechlorination is beneficial to the micro-organisms and helps the them to maintain their population size and their bioreactivity. In contrast, micro-organisms involved in cometabolic dechlorination have no benefit from these transformations and therefore have no driving force for carrying out these reactions. Moreover, they often must be supported by external substrates which can also be used by other micro-organisms, which tend to outcompete the dechlorinating micro-organisms. As a result, cometabolic transformations are often unstable processes. In case of aerobic cometabolic dechlorination, this instability may even be intensified due to the formation of epoxides that can cause enzyme inactivation or cell death [102, 194].

Hence, metabolic processes offer a good perspective for application in an intrinsic remedation set-up because they are stable and self-maintaining. The application of cometabolic processes is primarily useful in situations where metabolic processes are not feasible or insufficient under the condition that the primary process - e.g. methane or phenol oxidation - can easily be stimulated.

3.3.2 *Competition*
In field situations, micro-organisms carrying out dechlorination reactions have to compete with other micro-organisms for available electron donors and nutrients. This may prevent dechlorination from occurring although the reaction is thermodynamically favourable and known to be possible as demonstrated by previous lab and field studies. Especially for reductive dechlorination, several reports indicate inhibitory effects caused by the competition for available electron donors between the dehalogenating population and other micro-organisms [113, 116, 129, 162, 163, 177, 189, 214].

The competition between various groups of micro-organisms is probably the key to the understanding of the intrinsic degradation of chlorinated hydrocarbons by mixed populations, not only in the field situation but also in engineered biological treatment systems. The fact that the isolation of pure cultures living at the expense of reductive dechlorination has proven so difficult [68, 98, 150, 151], is only one piece of evidence, that we do not really know how to favour the population that can fully dechlorinate chlorinate hydrocarbons.

The following factors influence the competitiveness of anaerobic reductive dechlorination:

i. The ability of the microbial population to develop enzymatic systems for the breakdown of chlorinated hydrocarbons.
ii. The effectiveness of energy conservation from the dechlorination reaction coupled to the oxidation of an electron donor in comparison to competing metabolic reactions.
iii. Competition for electron donor.
iv. The possible need for a complex organic carbon source.

Evolution of dechlorination pathways
The discussion of evolutionary mechanisms leading to new enzyme machineries able to cope with anthropogenic compounds is not within the scope of this report. This field is very well studied for aerobic micro-organisms. We just want to briefly mention some aspects that are important to keep in mind.

Most transformation pathways for chlorinated hydrocarbons are modifications of existing pathways for the breakdown of naturally occurring analogues. The aerobic conversion of chlorinated aromatic compounds for example, involves a family of genes and enzymes of the so-called upper pathways [193] and the genes of the so-called lower pathway. The enzymes belonging to the upper pathways convert the chlorinated aromatic compounds to one common intermediate: a catechol (a dihydroxybenzene). Enzymes belonging to the lower pathway channel the resulting catechol into the common metabolic system. Thus, the transformation pathways of chlorinated aromatics converge into common, already existing metabolic routes. It appears that the genes belonging to the upper pathways have evolved from existing enzymes via slight modifications that change their substrate specificity [193]. Hence, the available range of metabolic capacities in a given system determines to a large extent the potential of a population to cope with new compounds. This knowledge can be used in the engineering of contaminated sites: The important idea behind bioaugmentation is to add a metabolic capacity that enables the microflora at a polluted site to develop the desired biotransformation pathway instead of adding micro-organisms that specifically transform the target compounds, a probably much more tedious task [166, 182].

The evolution of anaerobic dehalogenation pathways is less well studied. It is striking to note that most isolates of dehalorespiring bacteria appear to be new species, belonging to groups that were not known before, e.g. Desulfomonile tiedjei [151], Dehalospririllum [174], Dehalobacter restrictus [101, 176], and Desulfitobacterium [78]. Thus, there are not many clues to search for their ancestors let alone, the functioning of enzymes that were modified to yield dehalogenating enzymatic functions.

Effectiveness of energy conservation
A population can only effectively compete with other micro-organisms when it obtains enough energy for maintenance and growth. Generally, the energy released by the dehalogenation of strongly chlorinated molecules (e.g. PCE) is larger than for the other available electron acceptors (SO_4^{2-}, CO_2), thus providing a potentially strong means for the micro-organisms to compete effectively [198] (and chapter 4!). However, what matters in the competition between microbial populations is how much energy is being conserved, not how much energy is being released during dechlorination. The isolate Dehalobacter restrictus appears to be very ineffective at this point: it can only generate 1 ATP per mole of electrons transferred after the reduction of PCE to TCE or TCE to *cis*-DCE , corresponding to -31 kJ instead of -90 kJ, which would theoretically be possible [98]. Hence, this organism is wasting a lot of the energy resulting from dechlorination which - of course - is a drawback for its competitiveness. From this point of view it is interesting to note that it has not been possible yet to identify Dehalobacter in environmental samples using 16S RNA techniques [176]. The effectiveness of energy conservation in other isolated strains has not been investigated yet.

During cometabolic dechlorination, the energy resulting from dechlorination is not conserved for growth. Hence, cometabolic dechlorination completely depends on the metabolic reaction that renders cometabolic dechlorination possible.

Competition for electron donor

The affinity of the dechlorinating population for the (primary) electron donor, compared to the other population(s) present determines to a great extent whether dechlorination occurs in a natural environment [8, 73, 116, 129, 160]. Some of the isolates that couple the energy derived from dechlorination to their growth, appear to solely depend on molecular hydrogen as the electron donor [55, 68, 99]. It is not clear whether this is true for all dechlorinating organisms. Some isolates appear to be much more versatile with respect to electron donor requirement and can also grow with organic substrates as the electron donor [78, 142, 174].

An important primary electron donor in natural systems is H_2. Hydrogen comes available from hydrogenotrophic micro-organisms through the fermentation of more complex substrates like humic acids [67] or artificially added electron donors like alcohols or fatty acids. At least three known dehalorespiring micro-organisms, exclusively use H_2 as the electron donor [55, 68, 99]. The most recently isolated strain appears to have a much greater affinity for H_2 than methanogens or sulphate reducers [68]. Smatlack et al. [181] found that the performance of dechlorination was linked to the H_2 concentration present. Dechlorinating bacteria were shown to have a tenfold higher affinity (tenfold lower K_s) for H_2 than sulphate reducers and methanogens [68]. Micro-organisms exhibiting a high affinity for a substrate, have a competitive advantage compared to organisms with a lower affinity, when this substrate is present at low concentrations [87]. Hence, dechlorinating micro-organisms have a competitive advantage at low hydrogen concentrations. This hypothesis is supported by recent findings that electron donors that create a slow release and low levels of H_2 are the best cosubstrates to support complete reductive dechlorination [67, 68, 82]. Butyrate and propionate were compared with ethanol and lactate. Butyrate and propionate were slowly fermented resulting in a release of only low concentrations of H_2 and acetate, which favoured the complete dechlorination. In contrast, both ethanol and lactate were rapidly fermented, resulting in a rapid release of relatively high concentrations of H_2. Under these conditions, reductive dechlorination of PCE proceeded much slower and was incomplete. Besides butyrate and propionate, benzoate, natural organic material, BTEX or other low-reactive organics may be suitable substrates to support complete dehalorespiration.

Another group of dechlorinating strains does not completely depend on H_2 as electron donor, but can also use organic substrates instead [78, 174]. Hence, these strains may very well be better competitors at higher levels of H_2 compared to the organisms that we have discussed above. In this light, it is interesting to note that Desulfobacterium strain PCE1 - a strain that can use organic carbon sources as electron donor for the dechlorination of PCE - has been detected in environmental samples with the use 16S RNA probes [77].

Complex substrates

In many laboratory investigations it has been shown that complete reductive dechlorination can only be achieved after the addition of a complex organic substrate mixture like yeast extract [99], peat extract [49], rumen fluid [151] etc. The mechanism explaining these observations is not known. The solution may ly in both the complexity of the organic substrate and the presence of trace metals assisting the catalysis of dechlorination. Possibly, dechlorinating micro-organisms need a mixed diet for maintaining their physiological functions. Understanding this phenomenon may be of great significance to explain and predict intrinsic bioremediation processes and to further improve enhanced bioremediation techniques.

3.4 In situ conditions favourable for dechlorination

In this paragraph the most important conditions influencing the in situ degradation process of chlorinated hydrocarbons will be discussed. These factors are the following:

i. the *in situ* redox condition;
ii. the presence of sufficient supporting compounds like electron donors and acceptors, co-substrates and nutrients;
iii. the presence of dechlorinating catalysts (biotic and abiotic);
iv. physico-chemical parameters like pH (alkalinity) and temperature.

3.4.1 *In situ redox conditions*

The naturally occurring redox condition is a key factor determining the thermodynamics of intrinsic and stimulated dechlorination processes (see chapter 4). Low redox conditions are generally favourable for reductive processes. High redox conditions generally allow for oxidative degradation. An important feature influencing the redox status of an aquifer, is the oxidation of natural or anthropogenic organic substances. When degradable organic matter enters the sub-surface, the naturally available electron acceptors are subsequently used as electron acceptors for the oxidation [37]. The electron acceptors will be depleted in the order O_2, Mn(IV), NO_3^-, Fe(III), SO_4^{2-} and HCO_3^- when the electron donor supply continues. Thus, oxic circumstances (O_2) change into sub-oxic (Fe(III), NO_3^- and Mn(IV) reducing) and SO_4^{2-} reducing conditions, subsequently. Finally, methanogenesis will become the redox determining process. Oxic aquifers develop when the organic influx is absent or when oxygen rich water reenters the system at a sufficient rate. Hence, the redox condition in the field is determined by the organic load (the age) of the sediment, the organic influx (naturally or artificially), and the amounts of the various electron acceptors present in the sediment and the groundwater.

Anaerobic aquifers

Many of the industrialised areas in Western Europe are situated in the sedimentary basins created by the major rivers, i.e. the rivers Rhine, Schelde, Elbe, Weser, Po, etc. The superficial geologic formations in these regions are relatively young with high concentrations of organic matter. These shallow aquifers are strongly reduced (SO_4^{2-} reducing or methanogenic) as a result of the rapid O_2 and NO_3^- consumption during the downwards seeping of water in the overlying vadoze zone. Such environments have a redox condition that is sufficiently low for complete reductive dechlorination. In the Netherlands for example, evidence for the occurrence of the complete intrinsic dehalogenation of chlorinated compounds has been found at such sites (e.g. [14, 49, 155, 171]). Similar natural situations are likely to exist in other sedimentary regions, e.g. in the Northern parts of Germany and Italy. In more elevated regions farther away from the sedimentary basins, less reduced conditions like Fe(III) reducing, Mn(IV) reducing and NO_3^- reducing conditions predominate. Under such conditions, the reductive dehalogenation of chlorinated compounds as well as their oxidative dechlorination is thermodynamically possible (see chapter 4).

Aerobic aquifers

Industrialisation has also taken place in geologically older and more elevated regions, like large parts of the U.S.A and Central Europe. The groundwater in these regions contains mostly significant levels of oxygen, which renders the reductive dehalogenation of highly chlorinated compounds, like PCE and TCE, difficult. This mostly results in their persistence because this class of compounds seems not susceptible to oxidative transformations. However, reducing microzones supporting reductive dechlorination may still exist in some aerobic aquifers [64]. In contrast, the aerobic degradation of lower chlorinated molecules, like VC, is generally no problem [89].

Aquifers with mixed redox conditions

The redox chemistry of the groundwater below landfills and contaminated land is affected by the input of pollutants. Landfills are characterised by the presence of mixtures containing all kinds of chemicals including halogenated and non-halogenated organic compounds. The organic material leaching from the landfills is oxidized by the available electron acceptors creating a zonation with respect to the redox conditions. This effect has been demonstrated in aerobic aquifers below field waste depository sites in Denmark and the United Kingdom [37]. An example is the Grindsted landfill in Denmark [13].The leachate originating from this landfill is rich in dissolved organic carbon and has created extensive anaerobic zones in the plumes during the past years. The redox environments range from methanogenic, SO_4^{2-} reducing, Fe(III) reducing, Mn(IV) reducing, and NO_3^- reducing to aerobic. The most reduced zones have developed close to the leachate source and the aerobic zones were found at the edges of the plume where oxygenated groundwater is encountered (see fig. 2). The extension of the polluted plume depends on the buffering capacity towards redox reactions. When the aquifer material is rich in oxidised species such as Fe(III) oxides and Mn(IV) oxides, a substantial redox buffering is observed [96]. When the redox buffering capacity of the aquifer is low, the plume may migrate relatively unretarded and anaerobic redox zones may develop over long distances downgradient of the landfill. Similar phenomena have been found in chlorinated solvent sites like the Rademarkt site in the Netherlands [155] (see fig. 3) and at the Dover Air Force Base in the USA [63] Thus, a redox zonation has developed that is perfectly suitable to first reductively transform chlorinated compounds and then oxidatively mineralise the lower chlorinated products.

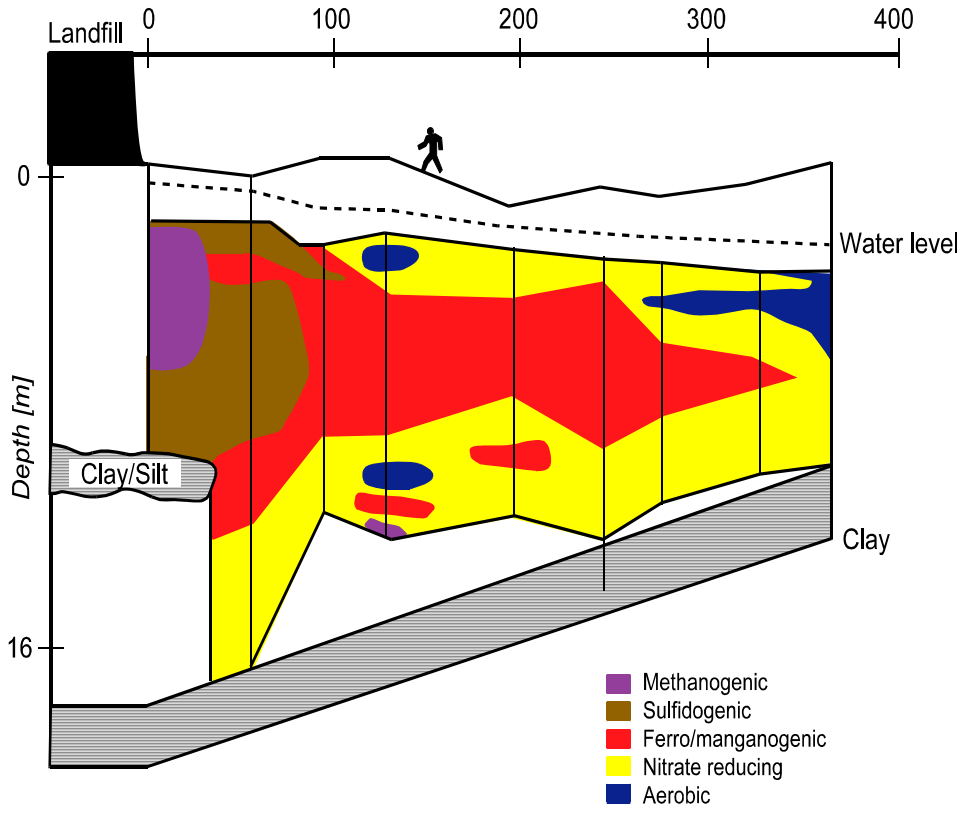

Fig. 2. Proposed redox zones along the main flow direction from the Grindsted Landfill (reproduced from Bjerg et al. [13]).

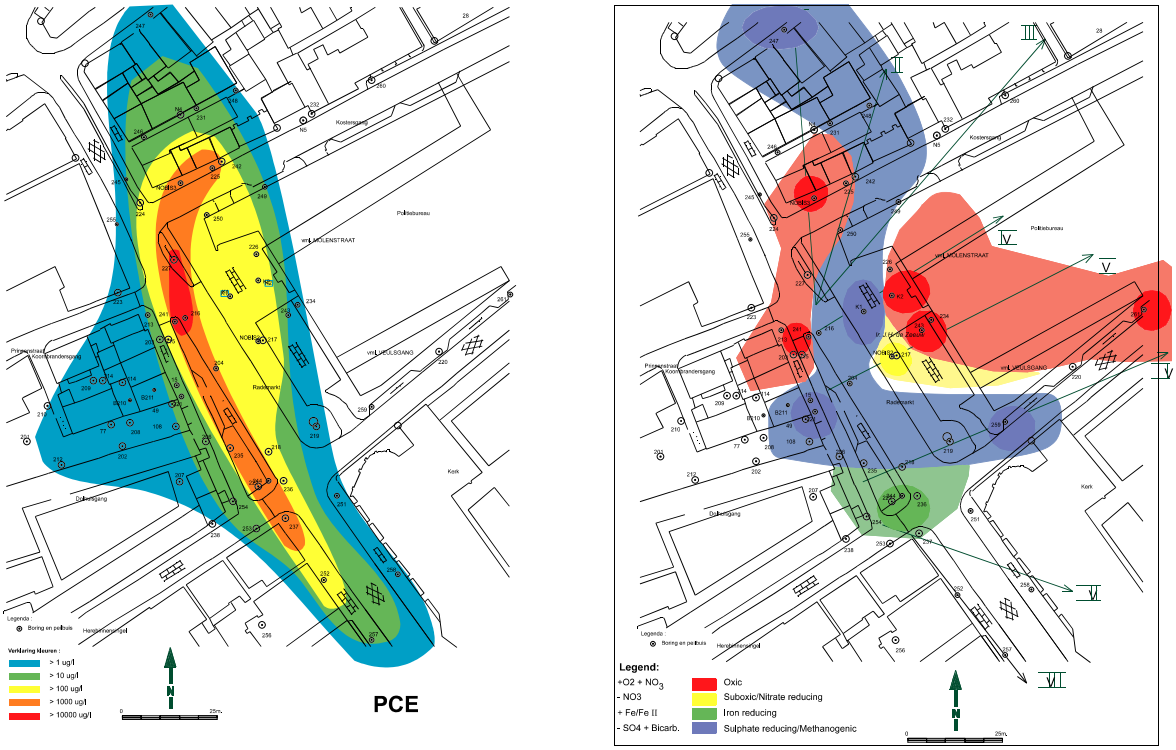

Fig. 3. A chlorinated solvent plume (a) (PCE shown) and a redox profile (b) at the Rademarkt site in the Netherlands. The redox zonation was caused by organic rich sewage water and BTEX, leaking from the sewer system into the unconfined, oxic contaminated aquifer.

Similar situations may indeed arise with chlorinated solvent plumes, when the pollution is accompanied by non-halogenated organics like grease, mineral oil or organic material from leaking sewer systems. As a matter of fact this appears to be often the case [18, 63, 155, 206].

3.4.2 *Presence of supporting compounds*

Electron donors for reductive dehalogention

All bacterial reductive dehalogenation processes depend on the presence of an external electron donor. One exception is conversion by fermentation, such as the transformation of DCM by *Dehalobacterium formicoaceticum* [137]. Electron donors include natural organic carbon, and anthropogenic carbon like BTEX and other fuel hydrocarbons, landfill leachate (rich in volatile organic acids [37]), and organic substances from leaking sewer systems [155].

Besides the electron donor concentration, the type of electron donor has a major impact on biological dechlorination [8, 73, 116, 129, 160]. An electron donor that can be preferentially used by dechlorinating micro-organisms could greatly improve the performance of enhanced and intrinsic biodegradation processes.

Hence, the presence of additional organic carbon sources in the plume and in the pristine groundwater affects the dehalogenation of chlorinated compounds in two ways:

i. The groundwater gets reduced due to the reaction with natural electron acceptors and a redox zonation develops as discussed in the previous paragraph.
ii. The additional organic carbon is used as the electron donor to reduce the chlorinated solvents under reducing conditions.

In the upgradient part of a TCE plume at Plattsburgh Air Force Base in New York, where BTEX is present, the amount of BTEX was insufficient to sustain complete dechlorination [206]. It appeared that in this situation a significant TCE degradation occurred as long as BTEX was present as cocontaminant. However, dechlorination significantly slowed down and possibly even ceased in the downgradient part of the plume where the BTEX has become exhausted but where sufficient amounts of natural organic matter are present (DOC > 15 mg/l). This suggests that in this case the native organic matter of this aquifer may not be of sufficient nutritional quality to support a reductively dechlorinating microbial community. Other studies have also indicated that aromatics can stimulate reductive dechlorination.

Cosubstrates for cometabolic transformation
Reductive cometabolic dechlorination can occur with virtually all chlorinated compounds [66, 97]. These reactions are catalysed by anaerobic micro-organisms that contain several cofactors that reduce the chlorinated compounds relatively easy. Examples of such cofactors are various types of cobalamins, Methyl-coenzyme M-reductase and factor F_{430} [5, 80, 100, 202]. However, cometabolic rates are generally too slow to account for the observed *in situ* degradation of chlorinated solvents in anaerobic groundwater.

Oxidative cometabolic conversion has been extensively studied. The following cosubstrates have been reported to be effective under aerobic conditions: methane, toluene, phenol, ethene, propane, butane, and isopropylbenzene [104, 107, 117, 191, 197]. Methane is produced in strongly reduced parts of the aquifers. When the aquifer has mixed redox conditions, the methane produced in the anaerobic zone can promote intrinsic cometabolic conversion upon entering an oxic zone contaminated with TCE, 1,2-DCE, or VC. Indications for such processes have been found in mixed-redox aquifers [63, 155]. Intrinsic cometabolic reactions with toluene or phenol may also occur. Such substrates can certainly be applied to enhance cometabolic transformations.

Nutrients
Minimal amounts of phosphorus, nitrogen, vitamins, and trace elements in groundwater are required to support intrinsic dechlorination processes. Usually these nutrients are present in soil and sediments but reduced bioavailability or competition for these nutrients may limit biodehalogenation. Supply of nutrients could enhance biotransformation. Iron, cobalt and copper caused complete inhibition of biotransformation [44, 129, 190]. 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 at different pH's. More research has to be done on this topic to get a better insight in the interactions. The possible consequences for intrinsic dehalogenation are probably as follows. When natural organic matter forms the major part of the electron donor for reductive dechlorination, sufficient amounts of nutrients are likely to become available upon the degradation of these compounds. When landfill leachate or sewer leakage forms the anthropogenic carbon source, no nutrient limitations are to be expected. Only in cases where large amounts of cocontaminants like BTEX and other fuel hydrocarbons are present, reductive dechlorination may become nutrient limited. Nutrient limitations of intrinsic oxidative processes have not been studied. Phosphorus and nitrogen are generally added when enhanced oxidative biodegradation is applied.

3.4.3 *Presence of micro-organisms and abiotic catalysts*
Dechlorination reactions of many chlorinated aliphatics are characterised by a negative $\Delta G^{0'}$ and thus are thermodynamically favourable. Nevertheless, these chlorinated compounds are sometimes not degraded in field situations where appropriate redox and other favourable conditions exist.

This recalcitrance can then only be explained by the absence of the appropriate microbial community, and which may be caused by one of the following factors:

i. *A too short adaptation time*
   Especially for bioreactions with a low selective pressure (i.e. the reductive degradation of the lower chlorinated compounds like VC) adaptation may require years [102].
ii. *Insufficient selective pressure*
   In situ factors, other than redox conditions, may inhibit the development of an appropriate microbial community. An example is a high *in situ* hydrogen pressure in a methanogenic sulphate lacking aquifer.
iii. *Substrates and trace elements*
   The absence of a complex organic mixture or an essential nutrient or spore element may be another cause for a lack of reductively dechlorinating micro-organisms. Inhibition by copper is also well-known with the cometabolic transformation of chlorinated hydrocarbons by methanotrophs which possess methane mono-oxygenase [95, 157].

3.4.4 *Other in situ conditions*

Besides the conditions mentioned in the previous paragraphs, other factors also influence biodegradation of chlorinated hydrocarbons. One such a factor is toxicity. Generally, chlorinated solvents are hydrophilic, therefore do not accumulate in biomembranes and do not disturb membrane functions. The major toxicity effects result from reactive toxic intermediates, such as epoxides formed by mono- and dioxygenases. Two other major factors, namely pH and temperature, are of importance and discussed in more detail below.

Temperature

Most dechlorinating laboratory microbial cultures and strains have optimum temperatures between 30 and 40 °C and an activity range between 5 and 50 °C [78, 137, 174]. Temperatures in natural geological habitats are in the lower range of this interval. At depths lower than 5 to 10 m, subsurface temperature is between 10 to 15 °C and is fairly constant [210]. Natural consortia have been demonstrated to have significant dechlorination activities at temperatures as low as 10 °C [48]. Probably, these autochtonous micro-organisms are better adjusted to these temperatures than the cultures obtained by enrichment at optimum temperatures in the laboratory.

Alkalinity (pH)

Microbial degradation of chlorinated hydrocarbons is also affected by the pH. Although most micro-organisms can tolerate a pH range of 5.0 to 9.0, bacteria generally prefer a neutral or slightly alkaline pH level, with an optimum pH range between 6.0 to 8.0 [78, 137, 174, 192, 210]. In general, areas contaminated by fuel hydrocarbons exhibit an elevated alkalinity compared to background values. This is expected because the microbially mediated reactions causing biodegradation of fuel hydrocarbons cause an increase in the total alkalinity of the system. Changes in alkalinity are most pronounced during aerobic respiration, denitrification, iron reduction, and sulphate reduction, and are less pronounced during methanogenesis [152]. Usually, contaminated groundwater's have a pH within the tolerated range for micro-organisms, causing no problem for biotransformation. Some soils have more extreme pH's, like systems with high concentrations of humic acid, sulfide or carbonate creating extremely high or low pH's. Biotransformation rates in these systems may be considerably lower. Dechlorination reactions generally produce hydrochloric acid. Conversion of high amounts of chlorinated chemicals can cause a significant drop in pH. In addition the short-chain aliphatic acid ions, produced during biodegradation of fuel hydrocarbons can contribute to the alkalinity in groundwater [209]. Whether these acidifications affect biotransformation, depends on the buffer capacity of the contaminated soil.

CHAPTER 4

INTRINSIC DECHLORINATION OF IMPORTANT COMPOUNDS

This chapter will review the information relevant for intrinsic biological transformations of chlorinated ethenes (see 4.1), chlorinated ethanes (see 4.2) and chlorinated methanes (see 4.3). The objective is to identify the field conditions i) under which intrinsic (and stimulated) dechlorination can be applied effectively, and ii) under which conditions these processes are limited and/or incomplete. The procedure consists of a number of steps.

Thermodynamic considerations and laboratory observations
The thermodynamic principles as outlined in 3.1 are used to determine favourable reductive, oxidative and other dechlorination reactions. In addition to the standard convention for calculation of $\Delta G^{0'}$, we assume the chloride concentration to be 10^{-3} M, to produce results that are compatible with previous free energy calculations [198]. Hence, in this report, $\Delta G^{0'}$ represents a physiological standard state with a chloride concentration of 10^{-3} M. We have used the $\Delta G^{0'}$-values as a screening tool to define thermodynamically favourable reactions. A simple calculation is given in appendix B. Reductive or oxidative transformations are defined as favourable under a specific *in situ* redox condition when the following requirements are met:

i. *Gibbs energy of transformation*
   The value of the actual ΔG for transformation, which is the sum of the ΔG-values of the electron donating and accepting half reactions, must be negative.
ii. *Preference for microbial process*
   An energetic advantage exists for micro-organisms to perform the dechlorinating reaction instead of the other redox processes that take place under the specific redox conditions. A first indication of a preference for dehalogenation can be deduced from the values of $\Delta G^{0'}$ for the redox half reactions involved; the actual preference depends on the values of the actual ΔG under field conditions.
iii. *Presence of appropriate microbial enzymes*
   The activation energy of the reaction can be lowered by an enzyme or (bio)catalyst to allow the reaction to proceed at a sufficient rate. Some reactions were shown not to proceed in the laboratory although they are thermodynamically feasible - e.g. aerobic conversion of PCE. However, other possible conversions were seldomly tried in the laboratory - e.g. oxidation of chlorinated compounds in suboxic environments. In such cases, one may not conclude that such a reaction is impossible.

Bioreactions under field conditions
Literature results for biodehalogenation under various in situ redox and other field conditions (see 3.4) are reviewed. A classification is proposed that distinguishes between various types of in situ dechlorination behaviour that can occur in contaminant plumes, namely:

i. complete reductive dechlorination;
ii. incomplete reductive dechlorination;
iii. complete sequential reductive-oxidative dechlorination;
iv. incomplete sequential reductive-oxidative dechlorination;
v. redox independent dechlorination;
vi. no dechlorination.

The key parameters that control these types of in situ dechlorination behaviour are identified and will be discussed and illustrated with field data.

4.1 Chlorinated ethenes

4.1.1 *Thermodynamic considerations and laboratory observations*

Chlorinated ethenes can be transformed by various reductive and/or oxidative reactions. The results of the thermodynamic analysis and literature review of laboratory results are shown in table 2 and are discussed below.

Reductive processes

Gibbs energy of transformation

For the chloroethenes, the values of $\Delta G^{0'}$ for the electron accepting half reactions vary between -55 kJ/mole electrons (PCE/TCE) and -38 kJ/mole electrons (*cis*-DCE). Using hydrogen as the electron donor ($\Delta G^{0'}$ = -40 kJ/mole electrons), which is realistic for many in situ environments [34, 83, 132], $\Delta G^{0'}$ of reductive transformation varies between -95 kJ/mole electrons (PCE/TCE) and -78 kJ/mole electrons (*cis*-DCE). Hence, requirement i) has been met for all the dechlorinating reactions shown (see appendix C). Metabolic dechlorination is considered to be favourable when the overall $\Delta G^{0'}$ of a reaction has a magnitude sufficient for the synthesis of at least one ATP per molecule transformed. An indicative value for this energetic threshold is -15 kJ per mole of chlorinated ethene transformed [94, 195]. This number is only indicative since it holds true for biological standard conditions whereas other values are valid for laboratory or in situ environments. $\Delta G^{0'}$'s of transformation of PCE and TCE are significantly more negative than the threshold, and appear to be favourable for dehalorespiration (metabolic reductive dechlorination). The $\Delta G^{0'}$-values for the lower chloroethenes are closer (DCE and VC) to this threshold. Hence, for these compounds, conditions can become more easily unfavourable for metabolic reductive conversion. The consequence of such unfavourable conditions is that biodehalogenation of PCE/TCE may stop with DCE and/or VC as end-products. Alternatively, the reaction may proceed by a cometabolic process when a cosubstrate is present that induces dechlorinating enzymes and provides the micro-organisms with energy and carbon source [141].

Preference of the process for micro-organisms

Preference for the dechlorinating reaction under a specific redox condition can be inferred from the half reactions. Preference for dechlorination exists when the $\Delta G^{0'}$-value for the electron accepting half-reaction with the chlorinated ethene is lower than for the half-reaction with the systems redox-determining electron acceptor, i.e. O_2, MnO_2, NO_3^-, $Fe(OH)_3$, SO_4^{2-}, or CO_2 (= HCO_3^-). Hence, reductive dechlorination reactions shown in appendix C are favourable for a specific redox condition when listed above the reductive half reaction of the corresponding natural electron acceptor. For example, the reductive dechlorination of PCE to TCE has a $\Delta G^{0'}$ of -55 kJ/mole electrons. This $\Delta G^{0'}$ is lower than the $\Delta G^{0'}$ of sulphate reduction (+20.9 kJ/mole electrons) indicating that micro-organisms can gain more energy from dechlorination than from sulphate reduction. In contrast, the value of $\Delta G^{0'}$ for reduction of PCE to TCE is higher (less negative) than that for manganese reduction (-59 kJ/mole electrons). Therefore, PCE reduction is not likely to occur under these conditions, because the micro-organisms prefer to reduce the manganese instead of the chlorohydrocarbon.

Summarising the results, reductive transformation of chlorinated ethenes is not favourable under manganese- and oxygen reducing conditions, and favourable under carbon dioxide, sulphate and iron reducing conditions. In the presence of nitrate, dehalogenation of PCE/TCE, VC and DCE is highly preferential, hardly preferred and unfavourable, respectively.

Appropriate microbial enzyme systems

The reductive reactions shown in appendix C have been reported in the literature indicating that microbial enzymes exist that can reduce the activation energy effectively. The degradation of chlorinated ethenes, especially of perchloroethylene (PCE) and trichloroethylene (TCE), is the most intensively researched area on biotransformation of chlorinated hydrocarbons. The only known transformation pathway for PCE under anaerobic conditions is a stepwise reductive de-

chlorination to successively TCE, *cis*-DCE, VC, ethene and ethane (see appendix F). *Trans*-DCE and 1,1-DCE sometimes were also found as transformation products of TCE transformation, but only accounted for a minor percentage [79]. Complete reductive dechlorination of PCE in continuous flow systems has only been described under methanogenic and acetogenic conditions [8, 48, 200, 208]. Under sulphate reducing conditions PCE was transformed to *cis*-DCE [8] while no transformation occurred under denitrifying and aerobic conditions. Transformation of PCE under iron reducing conditions has been reported in microcosm studies [1].

Table 2. Bacteria capable of dehalorespiration of PCE.

bacterium	product	electron donors	dechlorination rate (nmol Cl^-·min^{-1}·mg $protein^{-1}$)	reference
Dehalobacter restrictus	*cis*-1,2-DCE	H_2	330	[97]
Dehalospirillum multivorans	*cis*-1,2-DCE	H_2, pyruvate, lactate, ethanol, formate	50	[174]
Desulfitobacterium strain PCE1	*cis*-1,2-DCE	lactate, propionate, pyruvate, butyrate, ethanol etc.	310	[78]
Dehalococcus ethenogenes strain 195	ethene	H_2	--	[143]
Enterobacter agglomerans	*cis*-1,2-DCE	acetate	8.3[a]	[179]

[a] Per mg dry weight.

Pure cultures of methanogens [59, 66] and acetogens [61] are able to dechlorinate PCE according to a cometabolic reaction. However, the dechlorination rates of these cultures are much lower compared with those in continuous flow systems. This suggests that methanogens and acetogens were not the bacteria responsible for the greater part of PCE transformation in continuous flow systems.

Recently, several micro-organisms have been isolated that utilize PCE as electron acceptor and couple the reductive dechlorination of PCE to energy conservation and growth in a process called dehalorespiration (see table 2). A detailed description of this process is described by Holliger and Schumacher [100] and more recently by Máymo-Gatell et al. [141]. Most dehalorespiring micro-organisms can use H_2 as electron donor. Máymo-Gatell et al. [143] 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 the findings that inhibition of non-dechlorinating acetogens and methanogens often affects PCE dechlorination [50, 73]. The end product of PCE dehalorespiration by most isolated respiratory dehalogenating bacteria is *cis*-DCE [79, 100]. Recently, an organism was reported to completely reductively dechlorinate PCE down to ethene [141]. The degradation of PCE to VC occurred by dehalorespiration, whereas the transformation of VC to ethene occurred cometabolically, with the intermediates upstream in the pathway acting as the cosubstrates. This demonstrates that complete reductive conversion can be achieved by a single microbial culture. Nevertheless, in systems displaying complete PCE dechlorination, different bacteria degrading PCE to DCE and DCE to ethene/ethane appear to be involved in the process. De Bruin et al. [48] obtained an enrichment culture from a PCE dechlorinating packed-bed reactor that was able to dechlorinate *cis*-DCE to ethene in the presence of an electron donor.

Oxygenase mediated epoxidation

Gibbs energy of transformation

$\Delta G^{0'}$-values of the rate limiting step of this cometabolic process could not be established. These reductive reactions reduce the oxygen-molecule, not the chlorohydrocarbon. The oxygen is incorporated into the chlorinated ethene by an oxygenase under the formation of an epoxide. Thus, the chlorinated molecule becomes activated and disintegrates spontaneously to products which can be further metabolised. No thermodynamic data are available on this process, since the Gibbs energies of formation of the epoxides are not known. The oxygenation involves an energy input of two electrons per epoxide formed. Under standard conditions, this corresponds to at least an energy loss of 30 kJ per mole of epoxides, i.e. the energy associated with two ATP is needed.

Preference of the process for micro-organisms

The oxygenases transform choroethenes to chlorinated epoxides and require molecular oxygen. Hence, the reactions can only occur under aerobic conditions and not under any other redox condition. The micro-organisms involved experience some energetic disadvantage by performing the reaction. If the reaction proceeds cometabolically, these biodegradation processes are not very stable since other non-dechlorinating microbial consortia can compete for the same cosubstrate. On the other hand, the dechlorinating micro-organisms generally can mitigate this disadvantage to a great extent by the high energy gain from aerobic metabolic oxidation of the co-substrates (methane, toluene, phenol, butane, ethene, etc.).

Appropriate microbial enzymes

All chlorinated ethenes except PCE can undergo oxygenase mediated epoxidation under aerobic conditions (see appendix C and F). The first step, is generally a cometabolic epoxidation reaction, and can be catalysed both by mono-oxygenases [71, 139, 153, 157, 161, 164, 196, 202] and dioxygenases [154]. In addition to methane [104], toluene and phenol [104, 107, 117, 191, 197], new cosubstrates like ethene, ethane [74], butane, and propane [106] have been identified. The transformation rates of chlorinated ethenes strongly differ among different pure and mixed microbial cultures but usually the rates of epoxidation increase in the order of increasing instability of the epoxide formed, namely: TCE < DCE-isomers < VC.

Vinyl chloride (VC) and possibly also *cis*-DCE can be metabolically degraded and can serve as a sole carbon and energy source for microbial growth. Under aerobic conditions *Mycobacterium aurum* L1 transforms VC via a series of reactions to CO_2 with a growth rate of about 0.04 h^{-1} [88]. The initial step is an oxidation of VC to chlorooxirane catalysed by alkene mono-oxygenase. Indications have been found that *cis*-DCE can also be metabolically oxidised. The biodegradation mechanism has not been elucidated yet.

Epoxides are toxic and chemically non-stabile compounds. At high concentrations or loading rates, the formation of epoxides from chlorinated ethenes results in a decrease in viability of the degrading micro-organisms [194] and thus inhibits transformation of chlorinated ethenes. This form of self-intoxication should be taken into account when applying cometabolic in situ or on-site bioremediation. Field scale trials thus far indicated that self-intoxication can be prevented effectively [56, 81].

Oxidative processes

Gibbs energy of transformation

The $\Delta G^{0'}$-values for the oxidative half-reactions vary between -109 kJ/mole electrons (TCE) and -47 kJ/mole electrons (VC) (see appendix C). The $\Delta G^{0'}$-values for the electron acceptors range between +23 kJ/mole electrons (CO_2) and -79 kJ/mole electrons (O_2). The overall $\Delta G^{0'}$ for the reactions range between -188 kJ/mole electrons for PCE under aerobic conditions and

-24 kJ/mole electrons under methanogenic conditions. The $\Delta G^{0'}$ for oxidation of VC under sulphate reducing and methanogenic conditions is less negative than -31 kJ/mole electrons, the indicative threshold value for energy conservation. Hence, these oxidation reactions are probably metabolically not favourable. All other oxidative reactions shown are highly favourable from a thermodynamic point of view. This was also found for aerobic conditions by [53]. Epoxidation with water is a reaction that can occur in principle [198]. However a thermodynamic evaluation is not possible due to lack of data on the free energy of formation of the epoxides formed.

Preference of processes for micro-organisms
Oxidative reactions are in all cases highly exothermic, except for VC under sulphate reducing and methanogenic conditions. Hence, this high energy yield makes oxidative dechlorinations, when biochemically possible, in almost all cases highly preferential.

Appropriate microbial enzymes
To date no metabolic oxidative conversion of PCE and/or TCE has been reported. No enzyme can mediate these highly favourable reactions. Different types of microbial enzymes that can oxidise chloroethenes exist (see table 2). For other redox conditions, (nitrate and iron(III) reducing conditions), oxidative bioreactions have been reported. However, the types of catalysts or enzymes involved have not yet been identified.

Oxidative biodegradation of chloroethene under anaerobic redox conditions has been rarely reported (see appendix C and F). Only Bradley and Chapelle [25] found that VC could be oxidised to CO_2 in an anaerobic aquifer microcosm with Fe(III) serving as electron acceptor. The rate of this mineralisation can be enhanced by increasing the bioavailability of Fe(III). Other indications of metabolic oxidation under iron reducing conditions have been found [1].

4.1.2 *In situ transformations*

Complete reductive dechlorination
Complete reductive dechlorination of PCE/TCE via *cis*-DCE and VC to ethene (see appendix F) has been observed in (parts of) chlorinated solvent plumes at many sites in the US [11, 34, 206, 211] and in the Netherlands [49, 155]. This complete dechlorination pathway has only been found at sites that have sufficiently low (methanogenic/sulphate) reducing conditions and a sufficient amount of electron donor [70, 110, 138, 203, 211]. Several electron donors have been found to be capable of supporting complete reductive dechlorination, i.e. natural organic matter (NOM) [11, 28, 34, 49, 206, 211], sewage or landfill leachate organics [34, 38, 155], BTEX [11, 34, 110, 206, 211] and substrates that can be added to enhance intrinsic bioremediation (methanol, ethanol, actetate, lactate, proprionate, butyrate) [141].

Incomplete reductive dechlorination
Reductive dechlorination often has been observed to be incomplete, i.e., to result in accumulation of *cis*-DCE [42, 46, 57, 92] and/or VC. Several factors can cause this ineffective degradation, namely:

i. insufficient amount of electron donor;
ii. inappropriate electron donor, i.e., producing hydrogen levels too low or too high to support DCE and/or VC conversion [67, 68, 83];
iii. absence of *cis*-DCE and VC dechlorinating micro-organisms;
iv. toxicity effects, especially caused by accumulation of *cis*-DCE [92].

Complete sequential reductive-oxidative dechlorination
Another way to achieve complete intrinsic dechlorination of PCE and/or TCE is by a sequence of anaerobic and aerobic processes occurring along the flow-path of a contaminant plume. In the anaerobic zone with either methanogenic, sulphate reducing or iron(III) reducing conditions, PCE

and/or TCE are reductively dechlorinated to 1,2-DCE and/or VC. The anaerobic reductive dechlorination is followed by transformation of 1,2-DCE and VC via oxidation or epoxidation to carbon dioxide and chloride in the aerobic zone. These oxidations can be mediated by various processes. VC (and possibly also *cis*-DCE can be metabolically and cometabolically oxidized under aerobic [46, 92, 155] or iron(III) reducing conditions [1, 25]. Several cosubstrates produced in the anoxic zone (methane, ethene, or BTEX degradation products like benzoate and phenol) can be expected to support cometabolic conversion after entering the oxic zone. Indications for such type of intrinsic processes establishing stable chlorinated ethene plumes have been found at several sites [1, 92, 155, 206] Nevertheless, solid proof and quantitive estimates of the contribution of these oxidation and epoxidation processes to intrinsic remediation have not yet been reported.

At many sites in the USA, oxygen reducing conditions predominate. During contamination with chlorinated solvents, often other aerobically degradable contaminants like oil and BTEX also entered the aquifers, thus creating anoxic zones surrounded by an aerobic environment. Sequential anaerobic/aerobic situations and the associated intrinsic bioremediation processes of PCE and/or TCE have been reported [34, 41, 42, 145]. At all sites, PCE was reductively dechlorinated to 1,2-DCE and/or VC in the anaerobic zone. At a PCE contaminated site in Groningen, the Netherlands [155], under methanogenic and sulphate reducing conditions, PCE was biodegraded to *cis*-DCE, VC and ethene. In a downgradient oxygen reducing part of the plume concentrations of *cis*-DCE, VC and ET decreased rapidly, suggesting that further dechlorination took place. Stimulated sequential biodegradation has been successfully applied at a site in Breda, the Netherlands [183].

Incomplete sequential reductive-oxidative dechlorination
The above-mentioned sequential process can also be unsuccessful. One of the main problems is an insufficient intrinsic degradation of the parent compounds PCE and TCE during passage through an anaerobic zone. This is exemplified by the situation in another part of the plume at the Rademarkt Site, Groningen, the Netherlands [155]. Under methanogenic and sulphate reducing conditions PCE was partially biodegraded to VC and ethene. Downgradient, under oxygen reducing conditions, further oxidative degradation of vinyl chloride took place. In contrast, the concentrations of PCE, TCE, and *cis*-DCE did not significantly decrease in this zone. Even more downgradient another zone exists with methanogenic and sulphate reducing conditions. Here, again VC and ethene are produced out of PCE, TCE and *cis*-DCE. The vinyl chloride produced at the head of the plume presents a potential hazard to nearby located houses and shops. Hence, the sequential intrinsic processes in that part of the plume are insufficiently protective, and stimulated bioremediation is required.

No intrinsic dechlorination
No dechlorination of PCE and TCE occurs in aerobic, low DOC aquifers. To our knowledge there are no reports about high rates of naturally occurring oxidation of PCE and TCE at contaminated sites. Several reports are available on the stimulated oxidation of chlorinated ethenes using cometabolic biostimulation [81, 177]. This can be applied to TCE, DCE, and VC but not to PCE. The autochtonous micro-organisms can often be stimulated to cometabolically oxidize chlorinated ethenes by adding substrate and oxygen. In some cases, micro-organisms need to be added (bioaugmentation). Both injection of *Burkholderia (Pseudomonas) cepacia* G4 and *Methylosinus trichosporium* OB3b [56] resulted in oxidation of TCE at different sites. The main disadvantage of cometabolic bioaugmentation is the necessity of a continuous injection of dechlorinating micro-organisms because these organisms are not able to compete with natural occurring micro-organisms. Moreover, high amounts of cosubstrate are lost to the growth of the indigenous microflora. Nevertheless, in some cases this approach may be the only feasible solution.

4.2 Chlorinated ethanes

4.2.1 *Thermodynamic considerations and laboratory observations*

The results of the thermodynamic analysis and the literature review of oxidative, reductive and other chloroethane degradation processes shown in appendix D and G are discussed below.

Reductive processes

Gibbs energy of transformation

Reduction of chlorinated ethanes usually occurs via hydrogenolysis (see appendix A, reaction 1a). With hydrogenolysis of chlorinated ethanes, one chlorine substituent is replaced by hydrogen to form a lesser chlorinated ethane. 1,2-DCA can also be reduced via dihalo-elimination to form ethene, which is the most favourable reductive dechlorination reaction ($\Delta G^{0'}$ = -71.3 kJ/mole electrons). For the chloroethanes, the values of $\Delta G^{0'}$ for the electron accepting half reactions vary between this value and -36 kJ/mole electrons (1,2-DCA, hydrogenolysis). Using hydrogen as the electron donor ($\Delta G^{0'}$ = -40 kJ/mole electrons), $\Delta G^{0'}$ of reductive transformation varies between -111 kJ/mole electrons and -76 kJ/mole electrons. Hence, requirement i) has been met for all the dechlorinating reactions shown. In principal, metabolic dechlorination is considered to be possible in all cases, since the $\Delta G^{0'}$-values are more negative than the required minimum energy yield of -15 kJ per mole of chlorinated ethane.

Preference of the process for micro-organisms

Preference for the dechlorinating reaction under a specific redox condition can be inferred from the half reactions. Under carbon dioxide, sulphate reducing and iron(III) reducing conditions chlorinated ethanes can be transformed via either hydrogenolysis or dihalo-elimination (see appendix D). Under nitrate reducing conditions, only hydrogenolysis of TCA and CA is theoretically favourable. 1,2-DCA dihalo-elimination can occur under manganese reducing and more reduced conditions. No reductive processes are preferential in aerobic systems.

Appropriate microbial enzyme systems

The transformation of chlorinated ethanes under all redox conditions relevant for reductive dechlorination has not been studied as extensively as the chlorinated ethenes. The known abiotic and biotic transformations for different redox conditions are shown in appendix D. Dihalo-elimination of 1,2-DCA to ethene (the most favourable reductive transformation) can, according to thermodynamics, occur at all redox conditions except for oxygen reducing conditions. This process has been described for methanogenic and acetogenic bacteria as a cometabolic process [12, 59, 97, 208]. Besides dihalo-elimination Holliger also found hydrogenolysis of 1,2-DCA to CA [97]. CA was further transformed to ethane. Recently, Bosma et al. [17] found complete removal of 1,2-DCA (combined with transformation of VC) in iron reducing/methanogenic columns and in microcosms with sediment of a contaminated aquifer. Indications were found for involvement of iron in the degradation, and further research is underway to elucidate the mechanisms.

So far, reductive dechlorination of TCA has only been reported under sulphate reducing, acetogenic and methanogenic conditions both in continuous flow systems [20, 22, 39, 46, 198, 214] and in batch pure cultures of *Methanobacterium thermoautotrophicum*, *Desulfobacterium autotrophicum* , *Acetobacterium woodii* and *Clostridium* sp. strain TCAIIB [59, 61, 75]. Reduction of TCA is a cometabolic reaction catalysed by transition-metal complexes like cobalamins and coenzyme F_{430} and leads to the formation of DCA and CA. CA formation has only been reported under methanogenic conditions and usually accounts for a minor percentage (< 5 %) of the transformation products. De Best et al. [46] reported complete transformation of TCA to CA in a packed-bed reactor operated under methanogenic conditions. 1,1-DCA and CA can persist transiently but CA is very vulnerable to abiotic hydrolysis to give ethanol [108].There are only two reports about the anaerobic transformation of 1,1-DCA and CA. 1,1-DCA was partially

mineralised to CO_2 by a mixed methanogenic culture [198] while hydrogenolysis of CA by *Methanosarcina barkeri* led to the formation of ethane [97].

Besides reductive dechlorination to DCA and CA, TCA can also be completely dechlorinated under anaerobic conditions [46, 75, 198]. The pathway and products of complete anaerobic dechlorination are not yet clear but Vogel et al. [198] found that about 10 % of TCA degraded was transformed to CO_2

Oxygenase mediated hydroxylation

Gibbs energy of transformation

These reductive reactions reduce the oxygen-molecule, not the chlorohydrocarbon. The oxygen is incorporated into the chlorinated ethene by an oxygenase under the formation of an chloroalcohol. Thus, the chlorinated molecule becomes activated and can be further biologically oxidised. $\Delta G^{0'}$-values of the first cometabolic and rate limiting step are strongly negative, and therefore chemically favourable. Nevertheless, the reaction requires an investment of energy. The oxygenation involves an energy input of two electrons per chloroalcohol formed. Under standard conditions, this corresponds to at least an energy loss of 30 kJ per mole of chlorinated ethane, i.e. the energy of two ATP needed.

Preference of the process for micro-organisms

The oxygenases transform choroethanes to chloroalcohols and that requires molecular oxygen, i.e. aerobic conditions. The micro-organisms involved need to invest energy for performing the initial part of the degradation but can mitigate this disadvantage by the high energy gain from aerobic metabolic oxidation of the cosubstrates (methane, toluene, phenol, butane, ethene, etc.) and the formed chloroalcohol.

Appropriate microbial enzymes

Oldenhuis et al. [157] reported partial oxidative transformation of TCA and DCA by *Methylosinus trichosporium* OB3b under aerobic conditions. Both transformations are rather slow cometabolic reactions. Cometabolic biotransformation of 1,2-DCA has also been reported [31, 157].

Oxidative processes

Gibbs energy of transformation

The $\Delta G^{0'}$-values for the oxidative half-reactions vary between -80 kJ/mole electrons (TCA) and -38 kJ/mole electrons (see appendix D). The $\Delta G^{0'}$-values for the electron acceptors range between +23 kJ/mole electrons (CO_2) and -79 kJ/mole electrons (O_2). Hence, the overall $\Delta G^{0'}$ for the complete reactions range between -159 kJ/mole electrons (aerobic conditions/TCA) and -15 kJ/mole electrons (methanogenic conditions/CA). Under sulphate and methanogenic conditions $\Delta G^{0'}$'s for transformation are close to or less negative than the indicative threshold for metabolic transformation, that is -31 kJ per electron. Under these conditions, the first oxidation step by α-hydroxylation is energetically unfavourable since corresponding $\Delta G^{0'}$'s are between +16 kJ/mole electrons and +36 kJ/mole electrons. At less reduced conditions (iron, nitrate, manganese or oxygen reducing conditions), the oxidation reactions shown are strongly exothermic (requirement i), a result that is consistent with calculations performed for aerobic conditions by Dolfing and Janssen [53]. Moreover, the first hydroxylation step is hardly unfavourable to highly favourable, and therefore do not limit the reaction.

Preference of processes for micro-organisms

Overall oxidative reactions are not likely to occur under sulphate reducing and methanogenic conditions: the overall energy yield is insufficient for metabolic conversion and the first hydroxylation step is unfavourable. Under more oxidised conditions the oxidations are more preferential:

conditions for metabolic conversion have been met, an the first hydroxylation is not a limiting factor.

Appropriate microbial enzymes
The oxidative conversion of TCA, DCA and CA has only been reported under aerobic (oxygen reducing) conditions (see appendix D and G), although the thermodynamic analysis shows that these transformations are also favourable under iron-, nitrate and manganese reducing conditions (see appendix D).

Other reactions
Chlorinated ethanes can also be abiotically transformed. Hydrolysis of TCA leads to acetic acid. Vogel et al. [198] calculated a pseudo-first order rate of about 0.2 yr^{-1} at 20 °C from experimental abiotic TCA transformation data. TCA can also undergo abiotic transformation to 1,1-dichloroethene [199]. The pseudo-first-order rate constant for this transformation was reported as 0.04 yr^{-1} at 20 °C. This means that the half-life time for abiotic TCA transformation would be about 2.8 years which is much slower than can be achieved by biotic transformation under optimal laboratory conditions [46].

4.2.2 *In situ transformations*
Complete reductive dechlorination
Recently, Bosma et al. [17] found complete removal of 1,2-DCA in iron reducing/methanogenic columns and microcosms containing sediment from a contaminated aquifer. Indications were found for the involvement of iron in the degradation. At the site, high concentrations of ethene and ethane in the plume demonstrated intrinsic biodegradation to acceptable end products. An estimation of the dechlorination rates in the field and in laboratory systems indicated that enhanced intrinsic bioremediation may be appropriate for managing the plume at this site. Further research is currently performed to elucidate the biodegradation mechanisms and the appropriate way to stimulate these processes in the field. Evidence for similar reductive processes in the field (among other degradation pathways) were found by Lee et al. (124) for 1,2-DCA and by Lehmicke et al. [126, 127] for TCA, DCA, and CA. At these sites, a natural attenuation approach is considered.

Incomplete reductive dechlorination
Several sites have been described where TCA is reductively dechlorinated to 1,1-DCA and sometimes to traces of CA, usually under mixed redox conditions [4]. At a site in Ontario natural biodegradation occurred under mixed iron reducing, sulphate reducing and methanogenic conditions. Some chemical transformation of TCA to 1,1-DCE had occurred (0.4 mg/l). Reductive dechlorination was the main transformation mechanism, as indicated by a 1,1-DCA concentration of 7.2 mg/l, compared with a TCA concentration of 5.5 mg/l. Some CA was also present at the site (0.19 mg/l) indicating that part of the DCA formed was also further reduced [70].

Complete sequential reductive-oxidative dechlorination
According to the literature, no micro-organisms are known that can completely mineralise TCA under anaerobic conditions. Sequential anaerobic/aerobic transformation could be an attractive alternative for complete mineralisation of TCA since both DCA and CA can be biodegraded under aerobic conditions. Since oxidative transformation of DCA is a much slower than CA transformation and a cometabolic process, complete anaerobic transformation of TCA to CA in an anaerobic zone or reactor followed by mineralisation of CA in an aerobic zone or reactor seems the most feasible option for TCA mineralisation [46].

Indeed Cox et al. [41] found complete intrinsic biodegradation of TCA at a site in Sacramento, California. In the anaerobic zone, TCA is sequentially dechlorinated to ethene and ethane.

However, the rate of dechlorination is not sufficient to prevent transport of 1,1-DCA, and CA from the anaerobic zone to the aerobic zone. In the aerobic zone, CA is transformed to non-chlorinated products. Intrinsic biodegradation of TCA to non-chlorinated end products thus proceeds via two processes:

1. complete reductive dechlorination to ethene and ethane under anaerobic conditions;
2. reductive dechlorination to VC or CA under anaerobic conditions followed by biodegradation of VC and CA under aerobic conditions.

At a Gulf Coast site transformation of DCA was found. Although geochemical evaluation demonstrated that microbial community is composed out of oxygen, nitrate, sulphate , iron, manganese and carbon dioxide reducing micro-organisms, elevated methane concentrations indicated carbon dioxide as the major electron acceptor. Transformation products of DCA include 2-chloroethanol, ethanol, ethene and ethane. These products indicate that oxidative transformation (2-chloroethanol), reductive dechlorination (ethene and ethane) and chemical transformation (ethanol) occurs simultaneously [124].

4.3 Chlorinated methanes

4.3.1 *Thermodynamic considerations and laboratory observations*

The results of the thermodynamic analysis and the literature review of oxidative, reductive and other chloromethane degradation processes shown in appendix E and Appendix H are discussed below.

Reductive processes

Three reduction pathways of chlorinated methanes have been reported in the literature. An important reaction is hydrogenolysis (Appendix 1, reaction 1a) in which one chlorine substituent is replaced by hydrogen to form a lesser chlorinated methane. Another mechanism, coupling (see appendix A, reaction 1c), is generally a side reaction and therefore not discussed here (see chapter 3). A third process, hydrolytic reduction (see appendix A, reaction 1d), is a reduction completed by hydrolysis.

Gibbs energy of transformation

Reductive dechlorination by hydrogenolysis is most favourable for CT/CF ($\Delta G^{0'}$ = -65 kJ/mole electrons) and the least favourable for CM/methane ($\Delta G^{0'}$ = -45 kJ/mole electrons). Hydrolytic reduction is only possible for CT and is characterised by a $\Delta G^{0'}$ of -262 or -264 kJ/mole electrons, depending on the last step of the pathway. Using hydrogen as the electron donor ($\Delta G^{0'}$ = -40 kJ/mole electrons), $\Delta G^{0'}$ of reductive transformations by hydrogenolysis vary between -105 kJ/mole electrons and -85 kJ/mole electrons. For hydrolytic reduction, the Gibbs energy is even extremely negative, i.e. -300 kJ/mole electrons. Hence, all these dechlorinating reactions are thermodynamically favourable (requirement i) and can, in principal, allow for metabolic dechlorination ($\Delta G^{0'}$-values are far more negative than -15 kJ per mole of chlorinated methane).

Preference of the process for micro-organisms

Preference for the dechlorinating reaction under a specific redox condition can be inferred from the half reactions involved. Under carbon dioxide, sulphate, iron(III) and nitrate reducing conditions, the chlorinated methanes are better electron sinks than the natural electron acceptors (see appendix E). Reductive dechlorination is therefore a preferred reaction under these conditions (see appendix H). CT can also be reduced under manganese reducing conditions. Reduction of chlorinated methanes is not preferential under oxygen reducing conditions.

Appropriate microbial enzyme and other biocatalytic systems

Hydrogenolysis

Under anaerobic conditions CT can be successively dechlorinated to chloroform (CF), dichloromethane (DCM) and chloromethane (CM). There are numerous reports about the hydrogenolytic reductive dechlorination of CT to CF and DCM both by pure and by mixed cultures under methanogenic [58, 59, 60], acetogenic [60, 61] sulphate reducing [22, 59, 60, 61] iron reducing [162] and nitrate reducing conditions [21, 43, 44]. Under methanogenic and acetogenic conditions, sometimes also slow reduction of DCM to CM was found [58, 61, 149]. Thus far, these transformations have been demonstrated to be cometabolic and aspecific, and catalysed by biologically mediated transition metal complexes. The rate of dechlorination by these complexes decreases with each reductive step. Apparently, their catalytic effectiveness (i.e. the ability to lower the activation energy sufficiently) decreases with decreasing number of chlorine atoms.

Hydrolytic reduction

CT can also be mineralised to CO_2 [20, 21, 22, 43, 44, 59, 61]. The pathway of CT mineralisation is not yet clear, but evidence has been found for the following succeeding reaction steps [45] (see appendix A):

i. a one-electron reduction to trichloromethyl radical and a chlorine ion;
ii. dechlorination of the trichloromethyl radical via hydrolytic reduction to dichlorocarbene;
iii. hydrolysis to formate or carbon monoxide;
iv. oxidation to CO_2 by formate dehydrogenase and CO dehydrogenase, present in anaerobic bacteria.

Several studies indicate that cobalamins can take part in this pathway [36, 91, 115, 184]. Hashham et al. [91] found CO formation from CT in an anaerobic enrichment culture, grown on DCM, that received cobalamin homologues. Similar mechanisms have been proposed for the transformation of CF to CO_2 although the precise reaction steps and mechanisms have not been completely elucidated [7, 10, 60, 61, 149].

Oxygenase mediated hydroxylation

Gibbs energy of transformation

Like for the chlorinated ethanes, these cometabolic reductive reactions have strongly negative $\Delta G^{0'}$-values, and are therefore chemically favourable. Nevertheless, the reaction requires an investment of energy, i.e. 30 kJ per mole of chlorinated methane, i.e. the energy of two ATP needed.

Preference of the process for micro-organisms

The oxygenases transform choromethanes to chloroalcohols and that requires molecular oxygen, i.e. aerobic conditions. The micro-organisms can mitigate the need to invest energy by the aerobic metabolic oxidation of the cosubstrates (methane, toluene, phenol, butane, ethene, etc.) and the formed chloroalcohol.

Appropriate microbial enzymes

Except for CT, all chlorinated methanes can undergo oxygenase mediated transformation under aerobic conditions. CF can be oxygenised by toluene mono-oxygenase [146] or methane mono-oxygenase [2, 157] and leads, via a series of cometabolic reactions, to the formation of CO_2 and HCl. Cometabolic transformation of DCM and CM by mono-oxygenases [157, 158, 196] also leads to the complete dechlorination of these compounds.

Oxidative processes

Gibbs energy of transformation

The $\Delta G^{0'}$-values for the oxidative half-reactions vary between -245 kJ/mole electrons (CF) and -48 kJ/mole electrons (see appendix E). The $\Delta G^{0'}$-values for the electron acceptors range between +23 kJ/mole electrons (CO_2) and -79 kJ/mole electrons (O_2). Hence, the overall $\Delta G^{0'}$ for the complete reactions range between -314 kJ/mole electrons (aerobic conditions/CF) and -25 kJ/mole electrons (methanogenic conditions/CM). Hence, under all conditions, $\Delta G^{0'}$'s for transformation are less negative than the indicative threshold for metabolic transformation, that is -15 kJ per mole electrons. However, the first oxidation step by α-hydroxylation is energetically unfavourable under nitrate, iron, sulphate, and carbon dioxide reducing conditions ($\Delta G^{0'}$'s are between +31 kJ/mole electrons and +61 kJ/mole electrons). At less reduced conditions the oxidation reactions shown are thermodynamically not limited (MnO_2/$\Delta G^{0'}$'s are between -11 and -17 kJ/mole electrons) or exothermic (O_2/$\Delta G^{0'}$'s are between -31 and -37 kJ/mole electrons).

Preference of processes for micro-organisms

Overall oxidative reactions are not likely to occur under nitrate, iron and sulphate reducing and methanogenic conditions: the overall energy yield is sufficient for metabolic conversion but the first hydroxylation step is highly unfavourable. Under more oxidised conditions the oxidations are more preferential: conditions for metabolic conversion have been met, an the first hydroxylation is not a limiting factor.

Appropriate microbial enzymes

Until now there are no reports about the aerobic transformation of CT. DCM and CM can serve as sole carbon source for growth. Under aerobic conditions several pure cultures have been identified, all belonging to the facultative methylotrophic bacteria [27, 118, 120, 173, 188], that can utilise DCM as growth substrate. DCM is converted to formaldehyde by specific DCM dehalogenases via thiolytic dehalogenation, at maximum degradation rate of 0.29 µmol·min^{-1}·mg protein^{-1} [111]. CM can be utilised as a growth substrate by a strain of *Hyphomicrobium*. This strain oxidises CM with a stoichiometric release of chloride [90].

Other reactions

In addition to purely reduction or oxidation reactions, non-redox or combined oxidative-reductive dehalogenation pathways are possible.

Fermentative dehalogenation

Both DCM and CM can be utilised as a growth substrate under anaerobic conditions. Two mixed cultures have been described that utilise DCM under anaerobic conditions [72, 185]. Acetic acid and formic acid were found as transformation products, suggesting that DCM transformation is a fermentative process. The mixed culture of Stromeyer et al. [185] was further purified and characterised [26, 136] and recently a dichloromethane fermenting micro-organism was isolated with a proposed name of *Dehalobacterium formicoaceticum* [137]. This organism is able to convert dichloromethane to formate plus acetate (in a molar ratio of 2 : 1), biomass and traces of pyruvate at a transformation rate of 0.0982 µmol·min^{-1}·mg protein^{-1}. The pathway of DCM transformation is not yet clear but methylene tetrahydrofolate is probably an important intermediate. The pathway of CM fermentation to acetate and chloride by an anaerobic homoacetogen strain [148, 192] very much resembles the proposed pathway of DCM transformation in *Dehalobacterium formicoaceticum*. This suggest that a similar mechanism is involved.

4.3.2 *In situ transformations*

Nearly no information is available on the intrinsic bioremediation of chlorinated methanes. Only Lehmicke et al. [127] found fermentation of DCM to acetic acid by acetogenic micro-organisms in a shallow aquifer beneath a bulk chemical transfer facility on Oregon USA. DCM concentrations

in the source area decreased by an order of magnitude (from 2300 μg/l to 190 μg/l) and the distribution of DCM attenuates far more rapidly than predicted by its mobility in the site. Groundwater concentrations reduce to less than 1 μg/l within 100 meters from the source area. Acetic acid produced as a result of fermentation, could serve as an electron donor for the reductive dechlorination of other contaminants (PCE, TCE, TCA) present at the site.

Additional information is available on biotreatment of chloromethanes in wastewater. Aerobic and anaerobic biotransformation of DCM has been applied. In an aerobic fluidised-bed reactor, the DCM concentration in pharmaceutical wastewater was reduced from about 2000 μg/l in the feed to below 1 μg/l in the effluent at volumetric loading rates of 3 - 4 kg DCM/m^3 reactor·d^{-1} [187]. Under anaerobic conditions DCM contaminated groundwater was biodegraded in an active charcoal filter with a transformation rate of 0.03 kg DCM/m^3 reactor·d^{-1} [213]. This rate is about 100 times lower than aerobic transformation, consistent with the reported differences in DCM transformation rate between cultures of aerobic and anaerobic bacteria [128].

CHAPTER 5

PLUME CLASSIFICATION: COMPLETE AND LIMITED INTRINSIC DECHLORINATION

Intrinsic biodechlorination can occur completely or can be limited due to the in situ status of the various factors discussed above, i.e., the in situ redox condition, the presence of sufficient bio-process supporting compounds, the presence of dechlorinating micro-organisms or abiotic catalysts, and the value of other (physico-chemical) parameters like pH (alkalinity) and temperature. Thus, chlorinated solvent plumes can be classified according to the status of these parameters and the occurrence of complete or limited intrinsic dechlorination. Such a classification can be helpful in the identification of the critical bioremediation factors. This is an important step in the characterisation of chlorinated solvent plumes and for designing intrinsic or bio-stimulated remediation approaches. Moreover, such a classification can make a more generic comparison between plumes and identification of knowledge gaps possible.

Classification has already been applied to PCE/TCE plumes [205]. They proposed to distinguish between three types of plume behaviours:

1. Reductive dechlorination is supported by anthropogenic organic carbon, such as BTEX or landfill leachate.
2. Reductive dechlorination is supported by natural organic carbon.
3. Reductive dechlorination is not occurring. This is generally the case in aquifers that are characterised by low concentrations of native and/or anthropogenic carbon and by high dissolved oxygen concentrations.

This classification is already a very useful tool in assessing the status of a contaminated plume and the feasibility of a natural attenuation approach for a specific site [205]. However, it does not encompass all the bioprocesses and factors influencing intrinsic dechlorination as encountered in the field (see chapter 4). For example, complete and incomplete dechlorination are not being distinguished. Reductive intrinsic dechlorination of PCE and TCE often occurs, but is in many cases incomplete, leading to accumulation of lower chlorinated compounds. Another phenomenon not accounted for is the complete dechlorination by combination of reductive and oxidative processes in mixed redox systems. In chapter 4, a more complete scheme for plume classification has been proposed for the different groups of chlorinated compounds, namely:

i. complete reductive dechlorination;
ii. incomplete reductive dechlorination;
iii. complete sequential reductive-oxidative dechlorination;
iv. incomplete sequential reductive-oxidative dechlorination;
v. redox independent dechlorination;
vi. no dechlorination.

This scheme needs to be further developed in future, and may serve as a more generic knowledge base on intrinsic and other in situ biodehalogenation processes. Further more it can be extended to identification of the appropriate measures for enhancing intrinsic bioprocesses, for cases where intrinsic dechlorination is incomplete (ii and iv) or not occurring at all (vi).

In table 3 this classification has been applied to a selection of well documented sites. The cases selected could be classified with types i, ii, iii and iv. Involving more sites in the evaluation, classes v (non-redox dechlorination) and vi no dechlorination, will probably also be useful. Critical factors controlling the dechlorination behaviour, are also shown. The amount and quality

of electron donor for reductive dechlorination is critical, since it determines the occurrence and extent of reductive dechlorination. Cometabolic conversion, induced by anaerobic products like methane, ethene or non-degraded BTEX-compounds entering the aerobic fringe of the plume is likely to occur. However, in no case this was thoroughly investigated and quantified. The same holds for oxidative processes under less reduced (iron-, nitrate- and oxygen reducing) conditions. The protectiveness of a natural attenuation approach is also indicated and in a number of cases additional measures are required to protect down gradient receptors. The obvious method is to enhance the intrinsic biodegradation processes in a biostimulated zone.

Table 3. Classification of type of dechlorination occurring in chlorinated solvent plumes at a selection of well documented sites, critical factors controlling the dechlorination behaviour, first indications of the protectiveness of a natural attenuation approach and additional measures possibly required.

site	reference	dechlorination type[a]	down stream sections[b]	critical field parameters						natural attenuation protectiveness	additional measures considered
				chlorinated compounds degraded	compounds produced	redox condition[c]	e-donors/ cosubstrates[d]	microbial community[e]	other factors		
chlorinated ethenes:											
St. Joseph, US	[144, 178, 204, 211, 212]	i) complete red.	A1,2,3	TCE, DCE, VC	ET, ETA	Me/Su	NOM	?			
		ii) incompl. red.	B		DCE, VC	Me/Su	insufficient	?			
Picatinny Ars. US	[62, 140]	ii) incompl. red.	1	TCE, DCE	VC	Me/Su	not determined	?			
Maassluis, NL	[49]	i) complete red.		PCE, TCE, DCE, VC	ET, ETA	Me/Su	NOM/BTEX	addapted			full site clean-up required; enhanced anaerobic bioremediation considered
Groningen, NL	[155]	iii) compl. seq.	1A	PCE, TCE	DCE, VC, ET	Me/Su	NOM/BTEX but insufficient	addapted		probably	no further measures considered
			2A	DCE, VC, ET		Ox/Ni	not deterimined	not demonstr.		protective	
		iv) incompl. seq.	1B	PCE, TCE	DCE, VC, ET	Me/Su	NOM/BTEX	partially addapted		not	aerobic treatment zone at plume head
			2B	VC, ET		Ox/Ni	not determined	not demonstr.		protective	and anaerobic stimulation investigated
			3B	PCE, TCE, DCE	VC, ET	Me/Su	insufficient				
Plattsburgh, US	[206]	iii) compl. req.	1	TCE, DCE	VC, ET	Me/Su	NOM/BTEX	?		probably	
			2	VC, ET		Fe/Ni/Ox	not determined	not demonstr.		protective	
Cecil Field, US	[34]	iii) compl. req.	1	PCE,	DCE, VC	Me/Su	NOM/BTEX	present (1)		probably	
			2	DCE, VC		Fe/Ni/Ox	not determined	not demonstr.		protective	
chlorinated ethanes:											
Botlek, NL	[14]	i) compl. red.		1,2-DCA, VC	ET, ETA	Me/Su//Fe	not determined				
Sacramento, US	[41]	iii) compl. seq.	1	TCA	1,1-DCA, CA	Me/Su	?				
			2	VC, CA		Ox	?				
chlorinated methanes:											
Oregon, US	[127]	v) fermentation		DCM	acetate	Me/Su	fermentation			protective	

a) Dechlorination types, see text.
b) Numbers are for discrimination different plume sections.
c) Redox conditions: Me = methanogenic, Su = sulphate reducing, Fe = iron reducing, Ni = nitrate reducing, Ox = oxic, aerobic conditions.
d) Electron donors that stimulate reductive dechlorination and cosubstrates that can possible stimulate cometabolic oxygenation by mono-oxygenase carrying micro-organisms.
e) Presence of microbial community demonstrated with laboratory microcosms or dechlorinating column studies.

CHAPTER 6

KNOWLEDGE GAPS AND CONCLUSION

The approach 'from thermodynamics to field' provides a systematic way to understand which processes can occur and which not, and to classify dechlorination phenomena in the field. Moreover, such an approach helps to identify areas where our understanding of processes in the field is limited. The actual Gibb Free energies can also be used to better couple the chemical conditions in the field with possible transformations. The approach in this report was to identify potential dechlorination pathways on the basis of the release of Gibbs free energy during the reactions. Thus, we have obtained an array of potential dechlorination reactions that seem to be thermodynamically possible. As to be expected, not all of those have been observed either in the field or in the lab. The most important reason for that is the absence of adequate enzyme systems to attack certain classes of compounds. A well-known example is given by the perchlorinated compounds, e.g., PCE. Although full PCE mineralisation under aerobic conditions is thermodynamically feasible, it has been demonstrated to be non-degradable in the presence of oxygen.

Based upon the thermodynamic calculations and their interpretation we have identified the following areas for future research:

1. *Sensitivity analysis of actual Gibbs energies towards reactant concentration*
   A thermodynamic analysis extended to actual Gibbs energies of transformation (and not only on standard Gibbs energies, as done in this report) is required to identify critical concentrations of important electron donors (hydrogen) and electron acceptors. For example, such an analysis could reveal that under methanogenic conditions combined with a hydrogen concentration below a certain value, vinyl chloride cannot be metabolically reduced. This is of great practical importance; by monitoring, e.g., in situ H_2-pressures the ability of an aquifer to support complete reductive dechlorination could be qualified.
2. *Development of methods to assess actual redox processes in the subsoil*
   More accurate and efficient methods for the assessment of in situ redox conditions is required. On site hydrogen and methane measurements can already be applied. Molecular techniques to identify the presence and to quantify the number of methanogenic, sulphate reducing, iron reducing and nitrate reducing micro-organisms are likely to become a new and most efficient method to assess the microbiological redox status of groundwater and soils. Further optimising and testing currently available RNA techniques is of primary importance.
3. *Development of in situ H_2-measurement*
   The role of hydrogen and other electron donors in reductive dechlorination is not well understood. Hydrogen is often but not always the only key electron donor in reductive dechlorination steps. Research in our and other laboratories indicated that the anaerobic conversion of several compounds requires electron donors other then hydrogen. Hence, a purely 'hydrogen based' concept of reductive dechlorination is too simple. This is supported by the finding that many reductively dechlorinating micro-organisms need a complex mixture of organic substrates for their survival. Also the stimulation of reductive dechlorination by aromatic compounds in electron donor rich environments is not understood. The role of natural organic carbon is poorly understood: it may act as electron donor and as source of trace elements which are necessary to sustain dechlorination.

4. *Assessment of oxidative transformation pathways in suboxic environments*
   Oxidative (non-mono-oxygenase) dechlorination under moderately reducing (iron- nitrate- and oxygen reducing) conditions may be of extreme importance for natural attenuation/intrinsic bioremediation in sequential-redox situations. This process is hard to detect at the field scale because the end product is CO_2. It also got little attention in laboratory research thus far.
5. *Assessment of the importance of cometabolic transformation in intrinsic degradation processes*
   The occurrence of natural cometabolic mono-oxygenase mediated processes at the edges of anaerobic chlorinated solvent plumes in aerobic aquifers is important for application of natural attenuation. Again, the process is difficult to assess in the field. The nature of the organic compounds present may help to find a suitable cometabolic process. In presence of methane, for example, one may want to stimulate a methanotrophic process, while a toluene oxygenase would be preferred in presence of toluene.
6. *A systematic evaluation of current field data*
   An important problem is how to get from the situation of 'every site a natural attenuation research project' to a cost-effective assessment of natural attenuation and additional solutions at new sites. This can be achieved by collecting data on critical parameters as observed in current field investigations and by performing statistical analyses on these data or by applying artificial intelligence tools. Thus, a matured classification system can be generated that can serve as a tool to predict the dechlorination behaviour and the possibility for a natural attenuation approach at newly investigated sites on the basis of limited but critical information.

In conclusion, the 'Intrinsic dechlorination: from thermodynamics to field' approach as initiated in this project provides a track towards a solid science based understanding of the possibilities of intrinsic bioremediation and natural attenuation of chlorinated ethenes, ethanes and methanes. In addition to these compounds, the approach can be extended to other compounds like oil-related compounds (BTEX and MTBE), hexachlorocyclohexanes, PAH's, chloroaromatics (chlorinated benzenes, chlorophenols, PCB's), pesticides (i.e HCH, 2,4-D), and explosives related compounds (TNT, DNT, etc.). Research efforts aimed at resolving the six above-mentioned knowledge gaps form an important next step towards safe and wider application of natural attenuation approaches at contaminated sites.

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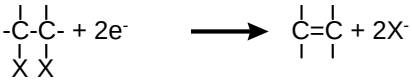
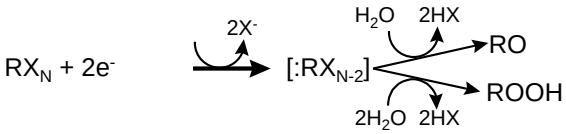
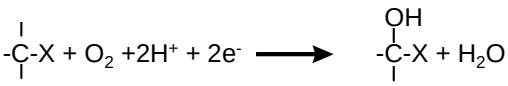
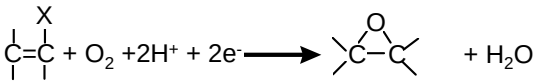
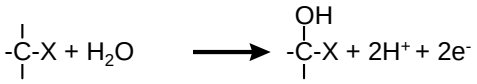
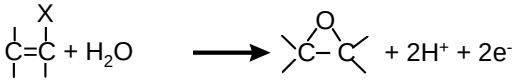
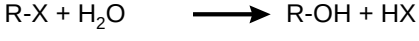
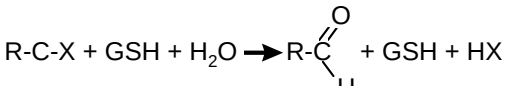
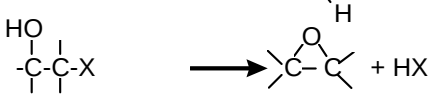
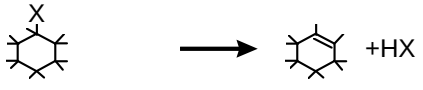
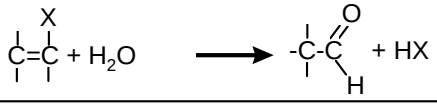
APPENDIX A

ABIOTIC AND BIOTIC REACTIONS

Abiotic and biotic reactions of halogenated aliphatic compounds [45, 69, 198].

Reactions	Mechanism
Electron dependent reactions	
1. Reduction	
a. **hydrogenolysis**	
b. **dihalo-elimination**	
c. **coupling**	
d. **hydrolytic reduction**	
2. Monooxygenase reactions	
a. **a-hydroxylation (with oxygen)**	
b. **epoxidation (with oxygen)**	
3. Oxidation	
a. **a-hydroxylation (with water)**	
b. **epoxidation (with water)**	
Electron independent reactions	
4. Substitution	
a. **hydrolysis (=hydrolytic dehalogenation)**	
b. **conjugation**	
c. **thiolytic dehalogenation**	
d. **intramolecular substitution**	
4. Dehydrohalogenation	
5. Hydratation	

APPENDIX B

CALCULATION OF FREE ENERGY CHANGES

Calculation of free energy changes ($\Delta G^{0'}$) for a given half-reaction

Reaction: $RX + H^+ + 2e^- \rightarrow RH + X^-$

Formula: $\Delta G^0_f\ (aq) = \Sigma\ \Delta G^0_f\ (aq)_{products} - \Sigma\ \Delta G^0_f\ (aq)_{reactants}$

Correction for non-standard conditions

For mCl^-: $\Delta G^{0'} = \Delta G^0_f + RT \ln [Cl^-]^m$

For mH^+: $\Delta G^{0'} = \Delta G^0_f + RT \ln [H^+]^m$

Calculation of free energy per electrons transferred

Formula: $\Delta G^{0'}/e^- = \Delta G^{0'}/n$ [kJ (mole electrons)$^{-1}$]

R	= universal gas constant	[8.3 x 10^{-3} kJ·mole^{-1}·K^{-1}]
T	= temperature	[298 K]
$[H^+]$	= concentration H^+	[pH = 7, 10^{-7} moles]
$[Cl^-]$	= concentration Cl^-	[10^{-3} moles]
n	= number of reacting moles	
e	= electron equivalents transferred	

Example: $C_2Cl_4 + H^+ + 2e^- \rightarrow C_2HCl_3 + Cl^-$

$\Delta G^{0'}_f\ (C_2Cl_4)$	= 27.6	kJ/mole
$\Delta G^{0'}_f\ (H^+)$	= 0 - 39.9 = -39.9	kJ/mole
$\Delta G^{0'}_f\ (C_2HCl_3)$	= 25.4	kJ/mole
$\Delta G^{0'}_f\ (Cl^-)$	= -131.3 - 17.1 = -148.4	kJ/mole

Calculation: $\Delta G^0_f = (25.4 + -148.4) - (27.6 + -39.9) = -110.5$ kJ/mole

Correction for Cl^- and H^+ concentration [10^{-3} resp. 10^{-7}]:

$\Delta G^{0'} = -133.5 + RT \ln [10^{-3}]^1 + RT \ln [10^{-7}]^1 = -110.7$ kJ/mole

so:

$\Delta G^{0'}/e^- = -55.3$ kJ/mole electron

APPENDIX C

BIODEGRADATION PROCESSES OF CHLORINATED ETHENES

Table C1. Biodegradation processes of chlorinated ethenes; biochemically feasible reductive and oxidative half-reactions, free energies ($\Delta G^{0'}$) of the half-reactions and literature references of laboratory and field observations; A = acetogenic, M = methanogenic, S = sulphate reducing, I = iron reducing, N = nitrate reducing, Mn = manganese reducing, O = oxygen reducing conditions.

mechanism	chlorinated ethene	half-reaction	$\Delta G^{0'}$ (kJ/mole electrons)	laboratory references	field references
reduction		**$O_2 + 4H^+ + 4e^-$ → $2H_2O$**	**- 78.7**		
		$MnO_2 + HCO_3^- + 3H^+ + 2e^-$ → $MnCO_3 + 2H_2O$	**- 58.9**	[8, 48, 59, 60, 66, 200, 208]; [78, 143, 174, 179]; [8] (S);	
	PCE	$C_2Cl_4 + H^+ + 2e^-$ → $C_2HCl_3 + Cl^-$	- 55.3		[121, 138] (M); [125, 127] (S); [125] (I)
	TCE	$C_2HCl_3 + H^+ + 2e^-$ → (*cis*-)$C_2H_2Cl_2 + Cl^-$	- 53.0	[8, 48, 73, 200, 208] (M); [8] (S)	[41, 125, 165, 204] (M); [85, 123] (S); [70, 121] (I)
	TCE	$C_2HCl_3 + H^+ + 2e^-$ → (*trans*-)$C_2H_2Cl_2 + Cl^-$	- 50.9	[9, 207] (M)	[41, 165, 204]; [85] (S)
	TCE	$C_2HCl_3 + H^+ + 2e^-$ → (1,1-)$C_2H_2Cl_2 + Cl^-$	- 50.8	[8, 48, 73, 200, 208] (M)	[165, 204] (M); [127] (A); [85] (S)
	VC	$C_2H_3Cl + H^+ + 2e^-$ → $C_2H_4 + Cl^-$	- 43.4		[41, 165, 204] (M); [127] (A); [121] (S); [121] (I)
		$NO_3^- + 2H^+ + 2e^-$ → $NO_2^- + H_2O$	**- 41.7**		
	1,1-DCE	(1,1-)$C_2H_2Cl_2 + H^+ + 2e^-$ → $C_2H_3Cl + Cl^-$	- 40.5		[165, 204] (M); [127] (A)
	trans-DCE	(*trans*-)$C_2H_2Cl_2 + H^+ + 2e^-$ → $C_2H_3Cl + Cl^-$	- 40.4	[8, 48, 200, 208]	[41, 165, 204] (M); [85] (S)
	cis-DCE	(*cis*-)$C_2H_2Cl_2 + H^+ + 2e^-$ → $C_2H_3Cl + Cl^-$	- 38.3		[41, 121, 165, 204] (M); [85, 121] (S); [70, 121] (I)
		$Fe(OH)_3 + 3H^+ + e^-$ → $Fe^{2+} + 3H_2O$	**- 11.4**		
		$SO_4^{2-} + 9H^+ + 8e^-$ → $HS^- + 4H_2O$	**+ 20.9**		
		$HCO_3^- + 9H^+ + 8e^-$ → $CH_4 + 3H_2O$	**+ 23.0**		
oxygenase reactions	TCE	$C_2HCl_3 + O_2 + 2H^+ + 2e^-$ → $2C_2HCl_3O + H_2O$	unknown	[71, 153, 154, 164, 196, 202] (O)	[35, 42] (O) (O_2 → CO_2)
	trans-DCE	(*trans*-)$C_2H_2Cl_2 + O_2 + 2H^+ + 2e^-$ → (*trans*-)$C_2H_2Cl_2O + H_2O$	unknown	[71, 139, 156, 196, 202] (O)	
	cis-DCE	(*cis*-)$C_2H_2Cl_2 + O_2 + 2H^+ + 2e^-$ → (*cis*-)$C_2H_2Cl_2O + H_2O$	unknown	[71, 139, 156, 161, 196, 202] (O)	
	1,1-DCE	(1,1-)$C_2H_2Cl_2 + O_2 + 2H^+ + 2e^-$ → (1,1-)$C_2H_2Cl_2O + H_2O$	unknown	[71, 139, 156, 161, 196, 202] (O)	
	VC	$C_2H_3Cl + O_2 + 2H^+ + 2e^-$ → $C_2H_3ClO + H_2O$	unknown	[71, 90, 139, 156, 161, 196, 202] (O)	
oxidation		**H_2 → $2H^+ + 2^{e-}$**	**- 39.9**		
non-aerobic oxidation	TCE	$C_2HCl_3 + 4H_2O$ → $2CO_2 + 9H^+ + 3Cl^- + 6e^-$	- 108.8		
	cis-DCE	(*cis*-)$C_2H_2Cl_2 + 4H_2O$ → $2CO_2 + 10H^+ + 2Cl^- + 8e^-$	- 68.9		
	1,1-DCE	(1,1-)$C_2H_2Cl_2 + 4H_2O$ → $2CO_2 + 10H^+ + 2Cl^- + 8e^-$	- 68.9		
	trans-DCE	(*trans*-)$C_2H_2Cl_2 + 4H_2O$ → $2CO_2 + 10H^+ + 2Cl^- + 8e^-$	- 67.3		
	VC	$C_2H_3Cl + 4H_2O$ → $2CO_2 + 11H^+ + Cl^- + 10e^-$	- 47.0		
epoxidation with water	TCE	$C_2HCl_3 + H_2O$ → $C_2HCl_3O + 2H^+ + 2e^-$	unknown		
	cis-DCE	(*cis*-)$C_2H_2Cl_2 + H_2O$ → (*cis*-)$C_2H_2Cl_2O + 2H^+ + 2e^-$	unknown		
	trans-DCE	(*trans*-)$C_2H_2Cl_2 + H_2O$ → (*trans*-)$C_2H_2Cl_2O + 2H^+ + 2e^-$	unknown		
	(1,1-)DCE	(1,1-)$C_2H_2Cl_2 + H_2O$ → (1,1-)$C_2H_2Cl_2O + 2H^+ + 2e^-$	unknown		
	VC	$C_2H_3Cl + H_2O$ → $C_2H_3Cl_3O + 2H^+ + 2e^-$	unknown		

APPENDIX D

BIODEGRADATION PROCESSES OF CHLORINATED ETHANES

Table D1. Biodegradation processes of chlorinated ethanes; biochemically feasible reductive, oxidative and other half-reactions, free energies ($\Delta G^{0'}$) of the half-reactions and literature references of laboratory and field observations; A = acetogenic, M = methanogenic, S = sulphate reducing, I = iron reducing, N = nitrate reducing, Mn = manganese reducing, O = oxygen reducing conditions.

mechanism	chlorinated ethanes	half-reaction	$\Delta G^{0'}$ (kJ/ mole electrons)	laboratory references	field references
reduction		**$O_2 + 4H^+ + 4e^-$ → $2H_2O$**	**- 78.7**		
	1,2-DCA	$CH_2Cl\text{-}CH_2Cl + 2e^-$ → $C_2H_4 + 2Cl^-$	- 71.3	[12, 59, 97, 208] (M)	
		$MnO_2 + HCO_3^- + 3H^+ + 2e^-$ → $MnCO_3 + 2H_2O$	**- 58.9**	[20, 39, 47, 59, 61, 75, 199] (M); [22, 39, 59, 214] (S)	
	TCA	$C_2H_3Cl_3 + H^+ + 2^{e-}$ → $C_2H_4Cl_2 + Cl^-$	- 54.1	[97] (M)	[41, 70] (M); [70] (I) [70] (S); [127] (A)
	CA	$C_2H_5Cl + H^+ + 2e^-$ → $C_2H_6 + Cl^-$	- 44.5		[41] (M); [127] (A);
		$NO_3^- + 2H^+ + 2e^-$ → $NO_2^- + H_2O$	**- 41.7**		
	DCA	$C_2H_4Cl_2 + H^+ + 2^{e-}$ → $C_2H_5Cl + Cl^-$	- 38.3	[47, 199] (M)	[70] (M); [70] (I) [70] (S); [127] (A)
	1,2-DCA	$CH_2Cl\text{-}CH_2Cl + H^+ + 2e^-$ → $C_2H_5Cl + Cl^-$	- 36.2	[97] (M)	[41] (M)
		$Fe(OH)_3 + 3H^+ + e^-$ → $Fe^{2+} + 3H_2O$	**- 11.4**		
		$SO_4^{2-} + 9H^+ + 8e^-$ → $HS^- + 4H_2O$	**+ 20.9**		
		$HCO_3^- + 9H^+ + 8e^-$ → $CH_4 + 3H_2O$	**+ 23.0**		
oxygenase reactions	CA	$C_2H_5Cl + O_2 + 2H^+ + 2e^-$ → $C_2H_4ClOH + H_2O$	- 152.3	[105, 172] (O)	
	TCA	$C_2H_3Cl_3 + O_2 + 2H^+ + 2e^-$ → $C_2H_2Cl_3OH + H_2O$	- 142.9	[172] (O)	
	DCA	$C_2H_4Cl_2 + O_2 + 2H^+ + 2e^-$ → $C_2H_3Cl_2OH + H_2O$	- 141.4	[172] (O)	
	1,2-DCA	$CH_2Cl\text{-}CH_2Cl + O_2 + 2H^+ + 2e^-$ → $CH_2Cl\text{-}CHClOH + H_2O$	- 134.4		
oxidation		**H_2 → $2H^+ + 2e^-$**	**- 39.9**		
non-aerobic oxidation	TCA	$C_2H_3Cl_3 + 4H_2O$ → $2CO_2 + 11H^+ + 3Cl^- + 8e^-$	- 79.8		
	DCA	$C_2H_4Cl_2 + 4H_2O$ → $2CO_2 + 12H^+ + 2Cl^- + 10e^-$	- 53.6		
	1,2-DCA	$CH_2Cl\text{-}CH_2Cl + 4H_2O$ → $2CO_2 + 12H^+ + 2Cl^- + 10e^-$	- 52.6		
	CA	$C_2H_5Cl + 4H_2O$ → $2CO_2 + 13H^+ + Cl^- + 12e^-$	- 37.8		
α-hydroxy-lation	CA	$C_2H_5Cl + H_2O$ → $C_2H_4ClOH + 2H^+ + 2e^-$	- 4.9		
	TCA	$C_2H_3Cl_3 + H_2O$ → $C_2H_2Cl_3OH + 2H^+ + 2e^-$	+ 4.5		
	DCA	$C_2H_4Cl_2 + H_2O$ → $C_2H_3Cl_2OH + 2H^+ + 2e^-$	+ 6.1		
	1,2-DCA	$CH_2Cl\text{-}CH_2Cl + H_2O$ → $CH_2Cl\text{-}CHClOH + 2H^+ + 2e^-$	+ 13.1		
other	TCA	$C_2H_3Cl_3 + H_2O$ → $CH_3COHCl_2 + Cl^- + H^+$	- 96.1	[199]	
	CA	$C_2H_5Cl + H_2O$ → $C_2H_5OH + H^+ + Cl^-$	- 95.3	[199]	
	TCA	$C_2H_3Cl_3$ → $C_2H_2Cl_2 + H^+ + Cl^-$	- 87.1	[201]	
	1,2-DCA	$CH_2Cl\text{-}CH_2Cl + H_2O$ → $CH_2OHCH_2CL + H^+ + Cl^-$	- 82.2	[32, 103, 157, 207] (O)	
	DCA	$C_2H_4Cl_2$ → $C_2H_3Cl + H^+ + Cl^-$	- 59.9		[17]
	1,2-DCA	$CH_2Cl\text{-}CH_2Cl$ → $C_2H_3Cl + H^+ + Cl^-$	- 55.7	[9]	

APPENDIX E

BIODEGRADATION PROCESSES OF CHLORINATED METHANES

Table E1. Biodegradation processes of chlorinated methanes; biochemically feasible reductive, oxidative and other half-reactions, free energies ($\Delta G^{0'}$) of the half-reactions and literature references of laboratory and field observations; A = acetogenic, M = methanogenic, S = sulphate reducing, I = iron reducing, N = nitrate reducing, Mn = manganese reducing, O = oxygen reducing conditions.

mechanism	chlorinated methane	half-reaction	$\Delta G^{0'}$ (kJ/mole electrons)	laboratory references	field references
reduction		**$O_2 + 4H^+ + 4e^-$ ➜ $2H_2O$**	**- 78.7**	[59, 60, 61] (M); [22, 59, 60, 61] (S); [162] (I); [20, 43, 44] (N)	
	CT	$CCl_4 + H^+ + 2e^-$ ➜ $CHCl_3 + Cl^-$	- 65.0		
		$MnO_2 + HCO_3^- + 3H^+ + 2e^-$ ➜ $MnCO_3 + 2H_2O$	**- 58.9**		
	CF	$CHCl_3 + H^+ + 2e^-$ ➜ $CH_2Cl_2 + Cl^-$	- 54.0	[59, 60, 61] (M); [22, 59, 60, 61] (S)	[123] (I)
	DCM	$CH_2Cl_2 + H^+ + 2e^-$ ➜ $CH_3Cl + Cl^-$	- 47.5	[51, 61, 149] [61, 149] (M)	
	CM	$CH_3Cl + H^+ + 2e^-$ ➜ $CH_4 + Cl^-$	- 45.2		
		$NO_3^- + 2H^+ + 2e^-$ ➜ $NO_2^- + H_2O$	**- 41.7**		
		$Fe(OH)_3 + 3H^+ + e^-$ ➜ $Fe^{2+} + 3H_2O$	**- 11.4**		
		$SO_4^{2-} + 9H^+ + 8e^-$ ➜ $HS^- + 4H_2O$	**+ 20.9**		
		$HCO_3^- + 9H^+ + 8e^-$ ➜ $CH_4 + 3H_2O$	**+ 23.0**		
oxygenase reactions	CF	$CHCl_3 + O_2 + 2H^+ + 2e^-$ ➜ $CCl_3OH + H_2O$	- 146.5	[3, 146, 157] (O)	
	DCM	$CH_2Cl_2 + O_2 + 2H^+ + 2e^-$ ➜ $CHCl_2OH + H_2O$	- 144.4	[157, 158, 196] (O)	
	CM	$CH_3Cl + O_2 + 2H^+ + 2e^-$ ➜ $CH_2ClOH + H_2O$	- 143.5	[90] (O)	
oxidation		**H_2 ➜ $2H^+ + 2e^-$**	**- 39.9**		
non-aerobic oxidation	CF	$CHCl_3 + 2H_2O$ ➜ $CO_2 + 3Cl^- + 5H^+ + 2e^-$	- 244.9		
	DCM	$CH_2Cl_2 + 2H_2O$ ➜ $CO_2 + 2Cl^- + 6H^+ + 4e^-$	- 95.4		
	CM	$CH_3Cl + 2H_2O$ ➜ $CO_2 + Cl^- + 7H^+ + 6e^-$	- 47.8		
α-hydroxylation	CF	$CHCl_3 + H_2O$ ➜ $CCl_3OH + 2H^+ + 2e^-$	+ 0.95		
	DCM	$CH_2Cl_2 + H_2O$ ➜ $CHCl_2OH + 2H^+ + 2e^-$	+ 3.05		
	CM	$CH_3Cl + H_2O$ ➜ $CH_2ClOH + 2H^+ + 2e^-$	+ 3.90		
other	DCM	$CH_2Cl_2 + H_2O$ ➜ $HCOH + 2H^+ + 2Cl^-$	- 203.8	[27, 109, 111, 118, 119, 172, 188] (O); [20, 59, 61] (M); [21, 58] (N)	
fermentation	DCM	$CH_2Cl_2 + H_2O$ ➜ $\frac{1}{2} CH_3COO^- + 2\frac{1}{2}H^+ + 2Cl^-$		[26, 185] (M)	[127] (A)
	DCM	$CH_2Cl_2 + 2H_2O$ ➜ $HCOO^- + 4H^+ + 2Cl^- + 2e^-$		[72] (M)	
	CM	$CH_3Cl + H_2O$ ➜ $\frac{1}{2} CH_3COO^- + 3H^+ + Cl^- + 2e^-$		[148, 192] (M)	
	DCM	$CH_2Cl_2 + CO_2 + H_2O + 2e^-$ ➜ $\frac{1}{2} CH_3COO^- + HCOO + 1{,}5H^+ + 2Cl^-$		[135, 136, 137] (M)	[127] (A)

APPENDIX F

BIODEGRADATION PROCESSES OF CHLORINATED ETHENES UNDER REDUCING CONDITIONS

Biodegradation processes (reductive and oxidative dechlorination and other reactions) of chlorinated ethenes under methanogenic, sulhpate, iron, nitrate, manganese and oxygen reducing conditions:

i. biochemically feasible reactions (white lines);
ii. biochemically feasible reactions together with observed laboratory reactions (dashed black lines);
iii. biochemically feasible reactions together with observed laboratory reactions and observed field reactions (black lines).

In case reaction products are observed in the field and not in the laboratory black lines are marked with a star.

First step reaction products are given between brackets.

APPENDIX G

BIODEGRADATION PROCESSES OF CHLORINATED ETHANES UNDER REDUCING CONDITIONS

Biodegradation processes (reductive and oxidative dechlorination and other reactions) of chlorinated ethanes under methanogenic, sulphate, iron, nitrate, manganese and oxygen reducing conditions:

i. biochemically feasible reactions (white lines);
ii. biochemically feasible reactions together with observed laboratory reactions (dashed black lines);
iii. biochemically feasible reactions together with observed laboratory reactions and observed field reactions (black lines).

In case reaction products are observed in the field and not in the laboratory black lines are marked with a star.

First step reaction products are given between brackets.

APPENDIX H

BIODEGRADATION PROCESSES OF CHLORINATED METHANES UNDER REDUCING CONDITIONS

Biodegradation processes (reductive and oxidative dechlorination and other reactions) of chlorinated methanes under methanogenic, sulphate, iron, nitrate, manganese and oxygen reducing conditions:

i. biochemically feasible reactions (white lines);
ii. biochemically feasible reactions together with observed laboratory reactions (dashed black lines);
iii. biochemically feasible reactions together with observed laboratory reactions and observed field reactions (black lines).

In case reaction products are observed in the field and not in the laboratory black lines are marked with a star.

First step reaction products are given between brackets.

For convenience, the transformation of CT to CO_2 is included as an oxidation, although the reaction is not oxidative.