NOBIS 97-4-04
SELECTION AND VALIDATION OF A PRACTICAL PROTOCOL ANAEROBIC DECHLORINATION

Phase 1: Inventory methods and work visit Cornell University USA

drs. J.J. van der Waarde (Bioclear b.v)
ir. M.H.A. van Eekert (Landbouwuniversiteit Wageningen)

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Bioclear b.v.(drs. J.J. van der Waarde, 050-5718455)
Landbouwuniversiteit Wageningen (ir. M.H.A. van Eekert)

Uitgever
CUR/NOBIS, Gouda

Samenvatting
In het kader van NOBIS is een inventarisatie gemaakt van de protocollen die door diverse onderzoeksgroepen binnen en buiten het NOBIS-raamwerk worden gebruikt om de natuurlijke afbraak van gechloreerde verbindingen (met name PCE en lager gechloreerde ethenen) in vervuilde grond vast te stellen. In dit kader is eveneens een werkbezoek gebracht aan Cornell University in Ithaca, NY (USA) alwaar diverse onderzoeksgroepen zich bezighouden met dezelfde probleemstelling. Er zijn richtlijnen opgesteld voor de uitvoering van batchstudies naar de dechlorerende activiteit in vervuilde bodem.

Trefwoorden

Gecontroleerde termen:	**Vrije trefwoorden:**
anaerobic, biodegradability, chloroethenes, protocol, laboratory research	dechlorination, feasibility study, VOCs

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Abstract
In the framework of NOBIS an inventory was made of protocols used by different research groups for the assessment of dechlorinating activity (of PCE and lower chlorinated ethenes) in batch studies. A visit was made to different research groups at Cornell University, Ithaca, NY (USA) to get more insight in the methods those groups are using to determine the dechlorinating activity in contaminated soil. A guideline for performing batch studies to assess the dechlorinating activity in contaminated soil was developed.

Keywords

Controlled terms:	**Uncontrolled terms:**
anaerobic, biodegradability, chloroethenes, protocol, laboratory research	dechlorination, feasibility study, VOCs

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PREFACE

In many cases anaerobic batch tests are used to assess the dechlorinating activity of micro-organisms present in contaminated soil. If dechlorinating activity is present biological in situ remediation of the soil may be a suitable alternative compared to more conventional methods like pump and treat methods. The set-up of such experiments may influence the outcome and thus the decision made on whether or not (stimulated) natural attenuation is a suitable remediation alternative in the given situation.

Different research groups working within the NOBIS framework use their own method to determine the microbial dechlorinating activity in contaminated soil. Therefore in the project 'Selection and validation of a practical protocol anaerobic dechlorination' an attempt is made to set general guidelines for the performance of such batch tests. This report describes the work carried out in phase 1 of the project. Different batch test protocols used by different research groups working on the anaerobic dechlorination of chlorinated ethenes are evaluated and discussed.

Also, this report describes a visit made to Cornell University (Ithaca, NY, USA) where research groups are also working on the assessment and monitoring of dechlorination of chloroethenes. We would like to thank the people at Cornell University for their hospitality, and their willlingness to share and exchange information. Our special thanks go to Donna Fennel, for sharing her expert judgement on anaerobic microcosms and bagles with us.

Based on the evaluation of the different protocols and the knowledge gained at Cornell, a guideline for performing batch feasibility studies was proposed.

April 1999

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SAMENVATTING

Selection and validation of a practical protocol anaerobic dechlorination

Het ongecontroleerde weglekken en lozen van gechloreerde ethenen heeft geleid tot een wijd verspreide vervuiling van de bodem. Naast meer conventionele behandelingsmethoden, zoals 'pump and treat' methoden, zijn anaërobe biologische behandelingsmethoden ontwikkeld om deze vervuilende stoffen te verwijderen.

Normaliter worden anaërobe batchtests gebruikt om de mogelijkheid van in situ biologische behandelingsmethoden vast te stellen. Verscheidene laboratoria voeren deze batchtests uit of zijn anderszins betrokken bij het onderzoek naar de anaërobe dechlorering van PCE. Er is echter nog geen algemene richtlijn met betrekking tot de uitvoering van anaërobe batchtests beschikbaar.

De protocollen (media en algemene opzet) die momenteel worden gebruikt door de onderzoeksgroepen, die betrokken zijn bij de afbraak van chloorethenen, worden in dit rapport geëvalueerd en bediscussieerd. Er zijn geen belangrijke verschillen in de diverse protocollen gevonden.

Er is eveneens een werkbezoek gebracht aan Cornell University (Ithaca, NY, USA). In het laboratorium van de School of Civil and Environmental Engineering van prof. J.M. Gossett wordt een protocol gebruikt om (het effect van toevoeging van e-donor op de) natuurlijke afbraak van chloorethenen in de bodem vast te stellen. Om meer inzicht in de door hen gebruikte methoden te krijgen zijn prof. J.M. Gossett en dr. D.E. Fennell, naast prof. E.L. Madsen en prof. S.H. Zinder, geïnterviewd. Daarnaast is er een gesprek geweest met dr. E. Lutz (Du Pont Company) over de gebruikte methoden bij het laboratorium- en pilot-scale-onderzoek naar versnelde dechlorering van chloorethenen op de Dover Air Force Base.

De in de literatuur gevonden gegevens met betrekking tot de gebruikte protocollen tezamen met de informatie verkregen op Cornell University hebben geleid tot een richtlijn voor een protocol voor het vaststellen van anaërobe dechlorering. Dit protocol is bediscussieerd met NOBIS-specialisten op het gebied van anaërobe dechlorering. Deze (eerste) fase van het project heeft geresulteerd in de selectie van belangrijke parameters in het protocol, die in een latere (tweede) fase van het project zullen worden gevalideerd.

SUMMARY

Selection and validation of a practical protocol anaerobic dechlorination

Uncontrolled leakage and spills of chlorinated ethenes have led to a widespread contamination of the subsurface. In addition to conventional treatment methods, like pump and treat methods, anaerobic biological treatment methods to remove these contaminants have been developed.

Usually, anaerobic batch tests are used to assess the possibility of *in situ* anaerobic biological methods for treatment of the contaminated plume. Different laboratories carry out these microcosm tests or are otherwise involved in the research dealing with anaerobic dechlorination of PCE. However, there is no general guideline available for carrying out these dechlorination experiments.

The protocols (media and general set-up) currently used by the research groups involved in chloroethene degradation are evaluated and discussed in this report. No major differences were found in the protocols used by the different laboratories investigated.

Also a visit was made to Cornell University (Ithaca, NY, USA). In the laboratory of the School of Civil and Environmental Engineering of Prof. J.M. Gossett a protocol is used to assess the (effect of stimulation of electron donor on) natural attenuation in contaminated soil. Prof. J.M. Gossett, and Dr. D.E. Fennell were interviewed to get more insight in the methods used and the rationale behind it, and other investigators at Cornell working in this field like Prof. E.L. Madsen and Prof. S.H. Zinder, about their points of view concerning natural attenuation. Furthermore, Dr. E. Lutz from Du Pont Company was interviewed about laboratory and pilot scale research on accelerated dechlorination of chlorinated ethenes at the Dover Air Force Base.

Together the information obtained in literature and at Cornell University led to the proposal and selection of a practical protocol for anaerobic dechlorination which was discussed with NOBIS specialists in the field of anaerobic dechlorination. This (first) phase of the project resulted in the selection of important parameters in the protocol which in a later (second) phase of the project will be validated.

NOTATIONS

BOD	biological oxygen demand
BTEX	benzene, toluene, ethylbenzene, xylene
cis-DCE	*cis*-1,2-dichloroethene
DO	dissolved oxygen
e-donor	electron donor
NOBIS	Nederlands Onderzoeksprogramma Biotechnologische In-situ Sanering (Dutch Research programme In-Situ Bioremediation)
PCE	tetrachloroethene
RTDF	Remediation Technologies Development Forum
TCE	trichloroethene
trans-DCE	*trans*-1,2-dichloroethene
VC	vinylchloride
VOC	volatile organic compounds
TOC	total organic carbon
VFA	volatile fatty acids
YE	yeast extract

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Soil contamination with chlorinated ethenes (VOC) has led to a widespread occurrence of groundwater contamination in The Netherlands and abroad. The conventional treatment for these contaminated plumes consists of a pump and treat method. This approach can be effective in reducing the risk of spreading contaminants. However in practice, pump and treat methods are operated over long periods (decades) without significant reduction of contaminant levels. There is a tendency to shift towards biological methods to treat or manage these plumes, either by using the naturally occurring biodegradation processes in a natural attenuation approach or by applying active in situ anaerobic dechlorination.

When applying biological processes, it is important to know the biodegradation potential of the soil at a particular site. In addition to groundwater characterization (e.g., CUR/NOBIS report 97-4-01 [25]) batch studies are performed to demonstrate the feasibility of a biological treatment at a particular site contaminated with VOCs.

Within NOBIS, it was felt that the procedures that are followed when performing batch or microcosms studies are important for the outcome of such experiments.

Different methods appear to be used in different projects, both within NOBIS projects and in other projects in The Netherlands and abroad. The aim of this study is to make an inventory of the different methods that are used for performing anaerobic batch or microcosm studies by the leading laboratories and research institutes working on anaerobic dechlorination and to identify critical steps in the performance of these feasibility studies. These results will be used to come to a guideline for performing batch studies to determine the potential for anaerobic dechlorination of chlorinated ethenes in soil. In this first phase of the study, an inventory of different methods was made and Cornell University (Ithaca NY, USA) was visited. In a following phase (phase 2) the proposed protocol will be tested and verified in a practical research phase.

1.2 Practical approach

A visit was made to Cornell University to the Section of Microbiology and the School of Civil and Environmental Engineering. Interviews were held with Prof. J.M. Gossett, Dr. D.E. Fennell, Prof. E.L Madsen, and Prof. S.H. Zinder (see chapter 2). Donna Fennell showed the methods that are used at Cornell to perform anaerobic microcosm studies and allowed the practical participation of Van Eekert and Van der Waarde in preparing and monitoring these microcosms. A telephone interview was held with Dr. E. Lutz from Du Pont company about laboratory and pilot scale research on accelerated anaerobic dechlorination of VOC at the Dover Air Force Base (see chapter 2).

Van Eekert and Van der Waarde gave a presentation to students and staff of Cornell on research projects on anaerobic dechlorination at the Wageningen Agricultural University and in NOBIS.

Additionally, methods for performing anaerobic batch incubations to determine the potential for anaerobic dechlorination of chlorinated ethenes were retrieved from published literature and from NOBIS documents or directly from NOBIS participants (see chapter 3). Methods from the following laboratories were screened: Wageningen Agricultural University, Groningen State

University, Cornell University, Universität Stuttgart, ETH/EAWAG, Michigan State University (Fathepure, Tiedje), University of Massachusetts, EPA, TNO and Bioclear.

Finally, a discussion session was held in The Netherlands with NOBIS specialists in the field of anaerobic dechlorination of chlorinated ethenes. As a result of this discussion a practical protocol for batch studies to determine the anaerobic dechlorination potential was selected to verify in the following laboratory phase of this project (to be held).

CHAPTER 2

INTERVIEWS MADE DURING VISIT AT CORNELL UNIVERSITY

2.1 Interview with Prof. Jim M. Gossett (21-09-98)

School of Civil and Environmental Engineering, Cornell University

The discussion with Prof. Gossett focussed on the use of microcosms studies and monitoring of batch and field studies. By developing a model for the stimulation of natural attenuation, Gossett hopes to be able to predict what will happen in the field without having to do elaborate batch studies.

One of the main problems concerning the bottle studies is the choice of the microcosm sampling place. It is very expensive to sample soil for microcosms all over the plume. Therefore, a choice for the best sampling points has to be made. Where the microcosm is sampled is the result of an educated guess based on the information which is available. It is best to sample at spots where daughter products are already present. Another problem is the incomplete dechlorination of PCE leading to the accumulation of VC. Also, the effect of the presence or other electron acceptors like sulfate, and the influence of cosubstrate still remains unclear. Some micro-organisms need the presence of sulfate for dechlorination whereas others will preferentially use sulfate (or other electron acceptors) instead of VOCs.

According to Gossett it is important to follow both the fate of the chlorinated ethenes as well as the electron donor. Therefore next to VOCs, VFA and methane should be measured, if possible. These parameters are measured in the batch studies and are used to generate data for the biomass formation in a model describing anaerobic dechlorination [14]. However, whether there is a need to measure these parameters in field experiments is not clear. In batch studies, the Cornell research group measures both electron donor depletion and the production of fermentation products to determine the fate of the electrons in the microcosms. Electron donor depletion that is not accounted for by dechlorination product formation is assumed to be channeled through other reduction processes like sulfate reduction. In this way, it is possible to estimate the amount of sulfate reduction which is taking place without monitoring the sulfate or sulfide concentration. The determination of the sulfate concentration may be difficult since sulfate may not be bio-available (precipitated salts) and the determination is not very quantitative. Methane is measured in batch studies and could be a useful tool in field studies to determine the fate of the electron donor. H_2 is never dominant in microcosms and in the field the measurement of H_2 concentrations will probably not give very detailed information about the relevant biodegradation processes. The threshold values for H_2 for dechlorinators in the field are probably about as high as for sulfate reducers.

The choice on the nature of the electron donor that is added is based on the 'slow hydrogen releasing compounds' theory. The group of Gossett carries out the batch studies with lactate, butyrate, or a mixture of lactate and benzoate, which in all cases gives both a fast and a slow hydrogen release during fermentation. Eventually, the choice for the electron donor which is used in the field is mainly directed by acceptance by the regulatory authorities. Whenever an electron donor also has its use as, e.g., a food additive, it will be much easier to get approval for injection of the compound for in situ treatment. It is extremely difficult in the USA to get approval for the application of natural attenuation in the field as a remediation alternative.

Besides the nature of the microcosm and the electron donor added, the actual set-up of the batch studies may also have an effect on the outcome of the test. The presence of a headspace in the microcosm incubations could influence the biodegradation kinetics because it may function

as a reservoir of H_2 and VOC. According to Gossett, the liquid/solids ratio should not have a significant effect on biodegradation kinetics. Other research groups (e.g., EPA, Bioclear) however, use a low liquid/solids ratio to mimic the field situation.

Future research of the group of Gossett will continue to focus on dechlorination of VOCs. One of the points of interest is the role of VC and cis-DCE in the dechlorination. From other research it is known that it is possible to degrade these compounds in e.g., Fe-reducing conditions either by oxidation or reduction.

Another point of interest is to assess the existing electron donor level in the field. It should be possible to develop a test in which the electron donor level can be measured, e.g., similar to a BOD test but faster, or by measuring stable isotopes. Furthermore, this type of research will give more information on the sustainability of natural attenuation.

Prof. Gossett stated that he is interested in working on the dechlorination of VOCs in cooperation with the NOBIS program or with individuals working within the program.

2.2 Interview with Dr. Donna E. Fennell (21-09-98)

School of Civil and Environmental Engineering, Cornell University

Dr. Fennell first described two projects which are currently being carried out at the School of Civil and Environmental Engineering of Cornell University. Both projects deal with the assessment of natural attenuation at contaminated sites with the goal to improve the bioremediation at the specific sites by stimulating dechlorinating activity via the addition of electron donors.

One project involves a site (Facility 1381) at Cape Canaveral which is contaminated with TCE. Originally, an air sparging/soil vapor extraction system was installed to remediate the contamination. However, this system was removed from the site (results of the treatment process are as yet unknown). The site was chosen for several reasons, i.e. the TCE concentration, which was significantly higher than the detection limit, and the presence of DCE and VC which indicated microbial dechlorinating activity in situ. So far, soil samples have been taken twice using the Geoprobe as a sampling device. An objective of the project is to make 'people' realize that *in situ* reductive dechlorination is completely different from BTEX bioremediation. In this project TCE dechlorination is being monitored and the main factors that are driving the dechlorination process are identified. The main focus will be on the role of the electron donor in the dechlorination process.

The results of batch and the field experiments are modeled and the results obtained in the lab and the field are compared to see whether they confirm each other. To do this a practical model (STELLA) was constructed [14] and two approaches were used to verify the model. The first approach involves application of known fundamental kinetic parameters (K_s and conversion rates determined by others) for dechlorination and other relevant anaerobic biological processes. The model is run to see whether it can explain the results found in the serum bottle tests. By varying the biomass concentration of the different microbial groups, thus fitting the model to the experimental data, the composition of the biomass is determined. The second approach involves the initial determination of the biomass composition via molecular methods. The rate constants which are found experimentally are put into the model together with the biomass composition and the kinetic parameters are determined. These kinetic data are compared to the known kinetic data of pure cultures.

The kinetic model will be implied in RT3D. The objective of the project is to see whether serum bottle and field test verify each other. Therefore, a test protocol to perform microcosm studies is

currently under investigation. When the serum bottle and field test give the same results, the elaborate laboratory tests may prove to be unnecessary in the future.

The second site which is being studied is a site at a former military airport in Plattsburg. The soil is contaminated with jet-fuel and chlorinated solvents. Extremely high concentrations of dichlorobenzene have been found. The site has been monitored with H_2 measurements using a Reduction Gas Detector (RGD) by P.M. Bradley and F.H. Chapelle.

The interview dealt mainly with the practical matters involved in the analysis of the anaerobic dechlorinating activity in batch assays.

1. Is it better to monitor one batch over time or should you do multiple incubations and sacrifice sampling?

In this research project, the experiments are carried out with triplicate incubations which are monitored in time via headspace analysis of the chlorinated ethenes, H_2, and methane. The concentration of the electron donor and its fermentation products in time are determined via analysis of 0.2 ml of the liquid phase.

The experiments are not significantly influenced by the multiple withdrawal of the liquid sample. Whenever the remaining volume of the liquid in the bottles becomes too small, fresh groundwater is added to the bottles. This procedure is carried out based on personal experience. The method in which bottles are being sacrificed for analysis is not being used because you may run out of bottles in case of slow reaction rates.

Good reproducibility is found for the chlorinated ethene analysis. Similar results are obtained in triplicate bottles for e.g., the loss of TCE due to sampling. Nevertheless, small differences have been found among triplicate incubations for the electron donor concentration and the formation of fermentation products.

Fennell expects to have no problems with the chlorinated ethenes mass balance while working with microcosms. With dilute aqueous solutions mass balances were within 99 %. Furthermore, obtaining a complete mass balance has a low priority in these experiments.

2. Is it better to perform static headspace or extractive analysis?

Currently, extractive analysis is not being used at Cornell University due to the extensive amount of bottles which would be necessary for such an experiment.

3. Should sulfide be added in the batch studies?

Sulfide is not being added in the serum bottle tests for two reasons:

- The microcosm medium should be as simple as possible to facilitate later approval of the technique by the regulatory authorities. E.g., the addition of lactate and propionate are easily accepted, whereas the addition of butyrate will be much more difficult to get approved.
- The donor itself should do the job of lowering the redox potential.

Sulfide is also not added to bottles without electron donor present. Sulfide could be added to the bottles without electron donor to ascertain a equally low redox potential in batches without donor compared to batches with electron donor present. Nevertheless, in the experiments carried out at Cornell sulfide is not added, because the main interest is to show the effect of the addition of the electron donors on the dechlorination processes.

4. What is the effect of residual H_2 present due to glovebox procedure?

The glovebox is operated at a concentration of 1 - 1.5 % of H_2 in the atmosphere. The residual H_2 in the headspace of the microcosm may be as high as 10 % of the total amount of H_2

generated in the microcosm experiments. Currently, microcosm tests are run with 50 grams of soil in 50 grams of groundwater in a 160-ml serum bottle, resulting in a fairly large headspace volume. It is expected that the protocol will be changed to 50 grams of soil in 100 grams of groundwater which would result in a much smaller headspace. In this way the influence of the presence of H_2 in the headspace could be reduced.

5. Is there a better redox indicator than resazurin?

Clearly, resazurin is not the ideal redox indicator because there is no clear relation between the presence of oxygen and the color of the indicator. However, at this moment there is no redox indicator known which could replace resazurin, because many are either toxic or easily biodegradable. Possibly, methyl viologen could be a candidate.

6. Do you analyze for substrate consumption as well?

Since there is a close relationship between dechlorination and electron donor conversion, everything is analyzed, including electron donor utilization and the formation of fermentation products.

7. Do you add N/P to batches/columns/field studies? If yes, only once or in repetitive additions?

The microcosms are not amended with extra nitrogen or phosphorus. However, yeast extract (YE, 20 mg/l) and vitamin B_{12} (0.05 mg/l) are routinely added at the beginning of the experiment. These additions do contain N and P. Electron donor is only added 2 to 3 times in the course of an experiment depending on the presence of the slow H_2 releasing compounds like propionate. Whenever compounds like propionate are still present at significant amounts after the depletion of the originally present electron donor, the addition of new electron donor is postponed. The effect of addition of lactate as the electron donor is investigated in the presence of yeast extract and/or vitamin B_{12}, butyrate is added separately and benzoate is dosed together with lactate. Vitamin B_{12} will also be added in the field studies.

8. If you prepare experiments, you may lose VOC. Do you spike VOC to the incubations and in which concentrations? Why?

Initially, the experiment is set up with 33 identical bottles, which are allowed to equilibrate for 24 h under gentle shaking. After an equilibrium is reached, 10 of the bottles are analyzed. If the TCE concentration is high enough and the concentration of the lower chlorinated ethenes is low, the experiment is carried out without further treatment. In the case of a high VC concentration, all bottles are purged and spiked with TCE. TCE is added whenever it is completely converted. The amount of TCE spiked is comparable to concentrations found in the field (approximately 15 µmol TCE total mass per bottle). This concentration was chosen because it is easy to measure (including the products). In the Cape Canaveral field studies TCE concentrations are 10 - 50 µmol/bottle.

Only the parent compounds are being tested. No bottles are incubated with only cis-DCE or VC. This may be interesting for characterization of distinctive paths of the plume.

9. Why is fermented YE important, which component is crucial for dechlorination?

The fermented YE is prepared by incubating YE with a 10 % inoculum of the 'high PCE culture'. In case of the electron donor research extra care has to be taken with the addition of (fermented) YE to microcosms. The addition of (a low concentration) of YE has an influence on the dechlorination because YE is a 'slow H_2 releasing compound'. Also, the effect of the addition of YE at a high concentration (200 mg/l) is investigated. In this case YE serves as a complex electron donor (shot gun effect) comparable to molasses or compost extract.

The addition of molasses would be admitted (you can eat it) in the USA, whereas the approval for the addition of compost extract would be much more difficult.

The nature of the YE component which stimulates the dechlorination has not been identified yet.

10. Which ratio of VOC: e-donor do you use, is there a guideline available?
The ratio e-donor:VOC is approximately 100:1. The electron donor is added in excess. The ratios are based on the amount of H_2 produced from the electron donor. The addition of too much electron donor could have a negative influence on the dechlorination process, because the non-dechlorinators could benefit from the high electron donor concentrations compared to the dechlorinating bacteria that benefit from low H_2 concentrations. Also, the electron donor should last long enough in the field after injection without being consumed by other non-dechlorinating bacteria. This is another reason to add slow H_2 releasing donors. By adding too high amounts of electron donor one could also generate too high concentrations of fermentation products, like propionate which could be toxic at a certain pH. This may however be beneficial to prevent clogging of the injection wells by fermenting biomass.

11. Is groundwater a good alternative for soil in batch studies?
There is no experience with the use of groundwater instead of soil in batch experiments. This would be worth investigating.

12. Do you always use groundwater or tap/demi water in soil laboratory studies?
In all experiments groundwater is used. Sometimes groundwater is simulated.

13. What is the influence of field sampling on the results of batch studies?
The sample collection which is currently being used at Cornell seems to be good (Geoprobe method). The permeability of the core is unknown and should be investigated. Sometimes groundwater is pumped up with a peristaltic pump in a jar, which is overflown for a certain period of time and then capped without air bubbles. This also seems to be a good method, but still the groundwater sometimes is aerobic when investigated in the laboratory. It is not clear whether this is due to the sampling or to the circumstances in the field *in situ*.

2.3 Interview with Prof. Eugene L. Madsen (22-09-98)

Section of Microbiology, Division of Biological Sciences, Cornell University

"If you go to a site, be logical"

Prof. Eugene Madsen is currently involved in the Commitee on Intrinsic Remediation. This Committee, which is chaired by Bruce E. Rittmann, has Perry McCarty and Frank Chapelle among its members. Its task is to evaluate the existing protocols for determining the feasibility of intrinsic bioremediation which have been put together by various organizations and industries. The Committee has been put together to prevent the abuse of intrinsic bioremediation.

In the evaluation process certain criteria are used to evaluate the protocols which are available:

- make sure the local community is involved;
- be sure to look at 'cause and effect';
- sustainability of the process.

It's Madsen's opinion that only in 20 % of the cases intrinsic bioremediation may be a successful method to remediate chlorinated solvents.

The interview focussed on molecular monitoring of bioremediation.

According to Madsen, molecular ecological methods are not a suitable tool to monitor natural attenuation. There are still no structural genes found, which encode for dechlorinating activity, nor is there a molecular probe available for the enzymes.

Another microscopic approach which involves e.g., staining the activity, which could be related to gene levels would be more promising. However, such a method is not available at the moment. This approach would be suitable for compounds like toluene. The (five) enzymes and genes involved in the degradation of toluene have been characterized completely.

Molecular ecological methods are not taken into account in the evaluation of the protocols, but models are.

At this moment modeling is the big controversy in the USA. Models are being used in the wrong way to prove that transformation of compounds is taking place, while there are not enough data points. The only site at which molecular ecology was used to monitor the dechlorination process, was at the Savannah River site which was polluted with TCE. Greg Saylor and coworkers carried out this research, but due to the low number of data points the molecular monitoring was not conclusive.

1. What is the use of molecular monitoring?

Until you have full understanding of the processes involved, you cannot work with (taxonomic) 16S rRNA methods. If you would work with this method, there would have to be the right (negative) controls outside the plume.

You could also use the TEAP (Terminal Electron Accepting Process) method. If a site is methanogenic you can be pretty sure that there are dechlorinators present. Then MPN counts or molecular ecology methods can be used. Another possibility is the use of a biomarker (e.g., methanogen specific cofactor; this method was developed by Jo Suflita).

2. Do you have a protocol to extract DNA from contaminated soil?

"DNA extraction is like baking cookies". Everyone uses their own method. Madsen does not have a fixed and verified protocol for DNA extraction from contaminated soils. A former PhD student from Madsen has tested a range of different protocols with varying result. Most methods were suitable to quantitatively detect methanotrophic (aerobic!) bacteria in sediments, the target organisms of that study. The group of Madsen may use the 'Ultra Clean Soil DNA' kit, a new commercial kit. No molecular ecology work was performed at Madsen's laboratory at the time of the visit.

In general, Madsen feels that molecular techniques are a valid tool for monitoring natural attenuation provided the right calibrations are made. Future techniques for monitoring intrinsic bioremediation may be: stable isotope measurements (C12/C13), key gene signatures (molecular analyses), and Donna Fennell’s approach (batch incubations, modeling).

2.4 Interview with Prof. Stephen H. Zinder (22-09-98)

Section of Microbiology, Division of Biological Sciences, Cornell University

In the laboratory of Prof. Zinder *Dehalococcoides ethenogenes* (strain 195) was isolated from an enrichment culture initially developed in the laboratory of Prof. Gossett in the School of Civil and Environmental Engineering. This bacterium is able to grow with PCE as a terminal electron acceptor. PCE is dechlorinated to VC and ethene.

Strain 195 grows poorly (more difficult than *Methanosarcina barkeri*) and is difficult to maintain in culture in the laboratory. The organism has very specific nutrient requirements. Zinder collects the discharge from the anaerobic reactors of Donna Fennell, spins down the cells and debris and uses an extract to supplement the growth medium. However, this does not always work. It is unknown which components in the culture liquid are essential.

The interview focussed on microbiological aspects of batch tests to assess anaerobic dechlorination.

1. Which cosubstrates give good dechlorination?

Components that give a continuous and slow release of H_2 are probably most suitable for dechlorination. That is probably why addition of yeast extract works so well. Optimal e-donor selection may be site dependent.

2. What is the ecological relevance of organisms like *Dehalococcoides ethenogenes* in contaminated environments?

The organism was isolated from an anaerobic waste water treatment plant in Ithaca. The plant has been restructured and was inoculated with the original sludge. Strain 195 cannot be detected in the sludge anymore, probably because nowadays the influent waste water is much cleaner and no VOCs are released anymore.

The 16S sequence of the organism has been compared with available sequences from many sources. It appeared that the sequence is closely related to the green non-sulfur bacteria. Closer is a sequence that originates from hot thermal Obsedian pool in Yellowstone Park. A 89 % similarity is present with sequences derived from soil at a nuclear power plant. Closest of all is a sequence from an anaerobic reactor treating PCBs. There appears to be a 99 % similarity with sequences derived from enrichment cultures grown on benzoate and PCE by Du Pont, but these data are not available. In conclusion, the highest similarity seems to be available with environmental sequences; pure cultures do not match the sequence. Strain 195 may belong to a new and poorly described group of organisms. Their ecological relevance is unknown, as long as nobody goes back to the natural environment and quantifies these organisms. So far, molecular detection showed no results in the Ithaca WWTP and in contaminated soils. However, the organism is present in the enrichment cultures from which it was originally isolated and in the anaerobic reactors from Donna Fennell.

Organisms that convert PCE to DCE are probably quite common and the process may not be highly specific for many organisms. McCarthy isolated an *Enterobacter* from a contaminated site that could dechlorinate PCE but found that *Enterobacter* strains from culture collections, that had not seen PCE before, could dechlorinate just as well. The next step from cis-DCE to VC may be more strain specific. Strain 195 will grow on a range of organics with DCE as the terminal electron acceptor yielding ethene. However, anaerobic growth on VC by strain 195 has never been demonstrated by Zinder or others.

In microcosms from contaminated soil at Dover AFB, dechlorination stopped at DCE. After addition of an enrichment culture that could dechlorinate all the way to ethene, dechlorination

proceeded to ethene in the microcosms as well (see interview with Dr. Lutz, section 2.5). In this case, the microbiology was clearly limiting the process. In other cases the availability of a suitable electron donor or the geochemistry may be limiting. We do not know enough about the factors that determine success or failure in anaerobic dechlorination on contaminated sites.

At Cape Canaveral, sampling sites where selected on the basis of the presence of intermediate concentrations of parent (TCE) compound. Little product (DCE) was present in these samples, and no dechlorination was found in microcosms. A repeated sampling at locations with relatively high concentrations of products (cis-DCE, VC) was performed. The results of these batch studies are not yet available.

3. What is the relation between long term and short term studies?

If cis-DCE or VC are found at a site one does not know whether this is fresh or old. Elucidation of the hydrology of the site is difficult and you do not know the speed of transportation of the products. So, there is little evidence that the process is still active. We do not know whether microcosms are representative for in situ activity.

No differences in dechlorination efficiency were observed in long term studies with enrichments containing strain 195 as a function of different types of electron donor, but this was probably caused by the YE that was present. In all microcosms, YE is added and this serves as a slow release e-donor and improves dechlorination. Electron donor dechlorination efficiency may well be site specific. At some sites, ethanol is anaerobically converted to propionate that serves as a slow release e-donor while ethanol itself is a fast release e-donor that may not support long term (sustainable) dechlorination.

4. When do (naturally occurring) organic compounds start functioning as e-acceptors (oxidation of cis-DCE and VC)?

Vinylchloride is often not found beyond the methanogenic and sulfate-reducing plume, although it is far more mobile than the other chlorinated ethenes. This indicates that VC is anaerobically degraded under these conditions, which could be both reductive, yielding ethene, or oxidative, yielding CO_2. This is probably site dependent. There are many processes that can convert VC, we do not know which processes are dominant. But oxidative removal of VC under anoxic or anaerobic (methanogenic or sulfate-reducing) conditions may be important in situ.

5. Do you have a protocol to extract DNA from contaminated soil?

We have applied FISH detection of the pure culture of strain 195 and are starting on PCR in contaminated environments and microcosm studies. DNA extraction is crucial, and Ghiorse for example showed that DAPI staining of samples that had been extracted for DNA with a certain protocol still showed intact bacterial cells with DNA. We do not have an optimized method for soil DNA extraction yet but bead beating is probably going to be essential. To improve the quality of your DNA you could perform an electrophoretic clean-up, but this is highly laborious.

6. What is the relation between H_2 production, dechlorination, methanogenesis and ratio and concentrations of e-donor and PCE?

The positive effect of methanol on PCE dechlorination in earlier microcosm incubations was probably caused by the growth of methanogens in the incubation, leading to formation of vitamin B_{12} that is essential for strain 195. Methanogens are easily outcompeted by dechlorinating micro-organisms on the basis of slow e-release. With sulfate reducers, we do not know which process is dominant in the natural contaminated environment and how microbial competition between these processes takes place. Dechlorinating micro-organisms may well be sulfate-reducing, but strain 195 is not.

7. When do VOCs become toxic to different classes of micro-organisms?
Toxicity of cis-DCE was probably caused by impurities (chloroform) in the pure product from the supplier. Some strains can grow in PCE saturated medium, strain 195 however cannot.

8. Are all dichloroethenes biodegraded by strain 195?
Strain 195 produces both 1,1-DCE and cis-DCE, and both are dechlorinated. However, small amounts of trans-DCE that are produced, are not further dechlorinated. This is probably caused by the fact that two adjacent Cl-atoms on different C-atoms are more easily removed than Cl-atoms that are spatially separated (like with trans-DCE).

9. What is the effect of temperature on dechlorination?
Strain 195 does not grow below 13 °C. For microcosms and environmental samples, we do not know what the temperature effect is.

10. Is groundwater a good alternative for soil in batch studies?
No, in general bacteria will adhere to soil and the groundwater will contain very little biomass.

2.5 Interview with Dr. Ed Lutz (25-09-98)

Du Pont Company

Du Pont is member of the US Remediation Technologies Development Forum (RTDF), a collaborative research program with 7 companies and 3 governmental organizations. Du Pont has been leading a natural attenuation and stimulated anaerobic dechlorination research project at Dover Air Force Base, Dover, Delaware (see CUR/NOBIS report 96.900 [28]).

1. Did you perform microcosm studies with material from the site and what did you learn from these studies?
The laboratories from four participating companies have performed anaerobic microcosm studies. Many ('thousands') of studies were performed, and the results are in the process of being written down, a report may be available next year. Although different methods were used, in principle most studies gave comparable results. Every method may have its own merits and specific application. One of the goals of this project was to determine optimal techniques for performing feasibility studies and recommendations will be made in the final report. So far, only the method with sealed glass capillaries has not been taken further by any of the consortium members, since the method is laborious and showed no improvement over the other methods.

2. How did you use the results of the laboratory studies in the pilot study?
We did two things: determine the natural attenuation of trichloroethene on the site and design a pilot for stimulated dechlorination.

The hydrogeology of the site was determined using cone penetrometer and geoprobe sampling and soil analyses. The data from the wells at the site did not fit the conceptual model that was prepared for the site. Based on the analytical results, sampling points for soil microcosms were selected. Selection criteria included: soil from most active part of the site (high in daughter products), samples from moderately active zone (little daughter products), sample down gradient from source (parent compound but no daughter products) and a reference from a not contaminated part of the site.

All the active microcosms degraded TCE to cis-DCE, but in all the ('thousands') of microcosms that were performed only one or two showed VC production. Data from the site seemed to indicate that cis-DCE and VC were disappearing from parts of the plume that were slightly aerobic. Therefore new samples were taken and incubated aerobically, showing aerobic oxidation of cis-DCE and VC. TCE was persistent in these incubations.

Three pilot studies were designed:

- Natural attenuation of the plume, which consisted of the installation of more monitoring wells.
- Accelerated dechlorination. The location was based on early well data on a site with some products (cis-DCE, VC) and high TCE concentrations, and the site was easily accessible.
- Cometabolic bioventing (see CUR/NOBIS report 96.900 [28]). The site was selected on the presence of TCE in the vadose zone via soil gas analysis.

The batch studies were intended to select the most suitable e-donor. A wide range of donors have been tested in RTDF research, including lactate, butyrate, molasses, compost, chicken manure and others. In the Dover RTDF study only chemical e-donors (lactate, acetate, butyrate etc.) were tested. In all microcosms, dechlorination stopped at cis-DCE. Lactate appeared to give the fastest dechlorination of TCE and was therefore chosen as e-donor in the following column study. These column studies were operated for a year with lactate and confirmed the results from the microcosm studies in that dechlorination of TCE stopped at cis-DCE.

Despite these somewhat disappointing results, a small scale pilot study was started in which lactate and nutrients (YE, ammonia and phosphorous) were added to a single well. Monitoring of well water composition in this well during 6 months showed no evidence of dechlorination of TCE. Two months later, 8 months after addition of lactate to the well, the well started to yield cis-DCE. A pilot was designed and installed and lactate, YE and nutrients were added to the injection wells and water was recirculated through the pilot. This system started showing cis-DCE formation but after 9 months of operation, still no VC or ethene was found. A mixed culture from a different site that did show complete dechlorination of TCE was added to the column studies and indeed dechlorination proceeded to VC and ethene in these columns. This mixed culture was also added to the injection wells and 60 days later VC and ethene was found in the pilot.

At the moment, the extraction well of the pilot contains 300 - 500 microgram TCE/L, which is injected in the injection well with amendments. After 4 feet in the pilot all the TCE is gone and dechlorination products are found.

3. Do you think the e-donor selection should be site specific?
Yes, in the Dover case we tested a range of 4 - 5 donors. Yeast extract supported dechlorination but resulted in a lot of biomass formation that probably did not dechlorinate. Now, lactate is the main substrate with minor amounts of YE added as complex source of nutrients.

4. Did you have problems with fouling in the pilot?
Yes, lots of troubles. We had biomass growth into the substrate feeding lines, which we overcame by adding the donor and nutrients via separate tubing that release at the bottom of the injection well. First we treated fouling by physical means, using pumps to surge and clean the well. This worked only for three months. Than we added H_2O_2, which worked fine in the beginning but seemed to become less effective after repeated treatments. Than we started using chlorax and acids and pumping out the released biomass to disinfect the well, but this also only worked for so long. A proprietary method with pressurized CO_2 was tested, but again this method failed in the long run. Now, we disinfect the wells with H_2O_2 every 1 - 2 weeks, regardless of fouling problems, and this seems to prevent the formation of fouling.

5. How much donor do you add?
At first we added 100 mg/l lactate. When we saw no dechlorination after 4 months, we increased this to 200 mg/L. This resulted in dechlorination of TCE to cis-DCE but we also started to have biofouling problems. We have decreased the feed via 100 mg/L to 50 mg/l now and dechlorination remains constant and biofouling is under control.

6. What type of monitoring is performed?

We mainly analyze for VOCs and ethene. Volatile fatty acids were analyzed a few times and H_2 measurements were carried out on three occasions. The H_2 analysis gave non-conclusive results about the processes in the plume and we do not trust that they can help us in the pilot. We routinely analyze for VOCs to monitor the dechlorination and TOC to monitor the substrate consumption. Analyses in the pilot are performed every 10 days.

7. Have you tried to model the pilot?

That is one of our goals and prior to the pilot we did a tracer study to characterize the pilot. The model now gives an appropriate description of the process and we have derived some *k*-values.

8. Do you perform molecular analyses to describe the microbial community on the site?

The department of energy (DOE) has done some work on microbial community analyses but no real molecular work was done on the site. No sampling was performed after bioaugmentation.

9. What does redox tell you about the dechlorination process?

In the plume at the site, the dissolved oxygen levels were relatively high (2 mg/L) and redox values did not correspond with DO levels. In the pilot, addition of substrate resulted in a lowering of the redox to low levels (-200 mV) and depletion of oxygen. Otherwise, redox is not used as a monitoring parameter.

10. Is full scale remediation planned at Dover?

Yes, the Air Force will take over the pilot and are designing a full scale remediation of the site.

11. Would you consider feasibility studies for other sites that are contaminated with VOC?

Yes. You should look at the native organisms using microcosms to see whether full dechlorination will occur. At Dover, the microcosm studies, column studies and pilot studies were all consistent with each other in that they showed dechlorination of TCE to cis-DCE. If the microcosm is negative with respect to complete dechlorination, you could consider bioaugmentation. You should make sure that the process works in the laboratory before you go and implement the process in the field.

12. Would the RTDF be interested in a continuation of exchange of results between NOBIS and RTDF?

Knowledge exchange is one of the goals of RTDF and Martin Bell from ICI has mentioned NOBIS to the RTDF members. I would appreciate further collaboration and I am very interested to hear about the results of the coming NOBIS field studies on enhanced anaerobic dechlorination.

CHAPTER 3

PROTOCOLS FOR LABORATORY STUDIES OF VOC DECHLORINATION (WITH PURE AND MIXED CULTURES AND IN MICROCOSM STUDIES)

3.1 Introduction

This chapter summarizes and evaluates the incubation conditions used by different research groups for the assessment of VOC dechlorination by pure and mixed cultures in batch and column studies (protocols A to G) and the incubation conditions used for microcosm studies with soil or groundwater (protocols H to Q). For each of the protocols, the medium used is reported (see appendices 1 and 3) together with the general set-up of the experiments (see appendices 2 and 4).

The research groups listed are those, which are (internationally) well known for their work on the (reductive) dechlorination of chlorinated ethenes and/or are working on PCE/TCE dechlorination within the NOBIS program. Most of the research groups working with protocols A up to G managed to obtain a pure culture of dechlorinating bacteria.

Firstly, the protocols described in literature and results obtained are briefly described in section 3.2. More information is listed in the appendices 1 up to 4 and in the literature cited. General features of the media and protocols used are evaluated in chapter 4.

3.2 Description of dechlorination protocols of different research groups

The protocols were analyzed for medium composition, technical experimental lay-out and experimental conditions. Data from different experimental protocols are presented in tables 1a, 1b, 1c and 2 (pure culture studies - see appendices 1 and 2) and 3a, 3b, 3c and 4 (soil incubations - see appendices 3 and 4). The protocols are designated A through Q and the letters in the descriptions below correspond with the letters in the tables.

Pure culture studies or column studies with freshly spiked PCE

A[1]: Institut für Mikrobiologie, Universität Stuttgart, Germany (A. Neumann, G. Diekert).
Dehalospirillum multivorans was isolated from activated sludge on pyruvate and PCE. This bacterium is able to transform PCE (300 µM) to cis-DCE, with pyruvate (40 mM), lactate (40 mM), hydrogen, or formate as the carbon and/or energy source. Acetate can serve as a carbon source. PCE concentrations higher than 300 µM inhibited dechlorination. The presence of fumarate or sulfur inhibited the PCE dechlorination, whereas nitrate and sulfate had no significant effect [24].

B: Institute of Microbiology, Swiss Federal Institute of Technology, ETH-Zentrum, Zürich, Switzerland (A. Wild, T. Leisinger).
Batch and bioreactor studies were carried out and ultimately resulted in the isolation of strain TEA. Strain TEA is capable of degrading PCE to cis-DCE and is strongly related to *Dehalobacter restrictus* (protocol C). The original enrichment culture obtained in the reactor studies was able to convert PCE to ethene and originated from a dichloromethane degrading culture. The medium used in the reactor study is listed under protocol B. The growth medium used for the pure culture was slightly adjusted from the reactor medium: 55 mM carbonate was used as a buffer, 1.0 mM Ti(III)citrate as a reductant and 2.8 bar H_2/CO_2 (80 %/20 %) was

[1] Capitals refer to the protocols in the tables and the appendices.

present in the gas phase. 5 % (v/v) of filter sterilized spent medium was also added as a source of growth factors. The reactor was run on TCE (55 µM) and glucose (2 mM).
Strain TEA was able to use H_2 as the electron donor and acetate and/or CO_2 as a carbon source. The strain did not grow fermentatively on glucose, pyruvate, lactate, acetate or formate in the presence of PCE or TCE [30, 31].

C: Laboratory of Microbiology, Agricultural University Wageningen, Wageningen, The Netherlands (C. Holliger, G. Schraa, A.J.B. Zehnder).
Dehalobacter restrictus was isolated from an anaerobic packed bed column originally packed with anaerobic sediment (River Rhine) and anaerobic granular sludge. This packed bed column transformed PCE to ethane and was fed with a mineral medium as described previously, which is not listed in the tables 1a, 1b, 1c and 2 [6]. *Dehalobacter restrictus* was routinely grown in more complex medium which is listed under protocol C. This strain (also known as PER-K23) is able to transform PCE to cis DCE, with hydrogen, formate, or lactate as the electron donor with PCE or TCE as the electron acceptor. The bacterium did not grow with oxygen, nitrate, nitrite, sulfate, sulfite, thiosulfite, S(0) or CO_2 as the electron acceptor. Organic electron acceptors, e.g., fumarate or chlorinated ethanes, DCEs, or VC were also not utilized. CO_2 or components originating from fermented yeast extract served as the carbon source [19].

D: School of Civil and Environmental Engineering and Department of Microbiology, Cornell University, Ithaca, NY, USA (J.M. Gossett, S.H. Zinder).
Dehalococcoides ethenogenes strain 195 was isolated from an enrichment culture obtained from digested sludge from the Ithaca wastewater plant. The strain is able to completely dechlorinate PCE to ethene with H_2, methanol, or butyrate as the electron donor. PCE:electron donor ratios were typically around 1:2-3 (mole/mole). The medium described in protocol D is the basal medium used for routinely growing the enrichment culture containing strain 195 [16] supplemented with a vitamin solution previously described [1, 21]. Ultimately, it was found that growth of strain 195 required the presence of acetate (2 mM), vitamin B_{12} (50 µg/l) and 25 % (v/v) anaerobic digester sludge supernatant [20].

E: Department of Microbiology, University of Groningen, Groningen, The Netherlands (J. Gerritse, J.C. Gottschal).
Desulfitobacterium strain PCE1 was isolated from an PCE dechlorinating enrichment culture obtained from contaminated soil. The enrichment culture was able to dechlorinate PCE completely. Strain PCE1 is able to degrade PCE to TCE and small amounts of cis- and trans-DCE. Several chlorinated phenols, 3-chloro-4-hydroxy-phenylacetate (Cl-OHPA), sulfite, thiosulfate or fumarate could also serve as electron acceptors. Sulfate, nitrate, and nitrite could not. Lactate, pyruvate, butyrate, formate, succinate, or ethanol serve as electron donors. Lactate was used as the carbon source. Pyruvate could be used fermentatively. The strain was routinely grown on lactate or pyruvate and PCE. Usually 10 mM PCE was dechlorinated in the presence of 20 - 50 mM electron donor. H_2 inhibited the reductive dechlorination by the enrichment culture [17].

F and G: Department of Crop and Soil Sciences and Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan, USA (J.M. Tiedje). Two media were used:

F: The RAMM (Revised Anaerobic Mineral Medium) was originally developed for determining the anaerobic biodegradation potential [27]. *Desulfomonile tiedje*, a 3-chlorobenzoate (3-CB) dechlorinating micro-organism was able to reduce PCE to TCE and cis- and trans-DCE in

the presence of 3-CB in modified RAMM-medium (protocol F). In a typical experiment, PCE was supplied at concentrations of 50 µM and a pyruvate concentration of 20 mM [7, 11, 29].

G: The PREM (Pre-Reduced Enriched Methanogenic Medium)-medium (protocol G1) was used for batch studies to assess the dechlorinating activity of several strains of pure cultures, like *Methanosarcina*, *Clostridium*, and *Desulfovibrio* sp. with methanol and acetate, glucose, and pyruvate as their respective electron donors. Whereas the acetoclastic methanogens, the *Clostridium*, and the *Desulfovibrio* were not dechlorinating PCE in significant amounts, the methanogenic bacteria fed with methanol dechlorinated PCE to TCE. PCE was supplied at 6 µM and the electron donors at 25 to 50 mM [2, 8, 10]. A modified PREM medium (protocol G2) was used in anaerobic column studies. PCE (0.3 to 0.6 µM) was converted (to TCE and) lower chlorinated ethenes with glucose, methanol or acetate (all at a concentration of 5.6 mM) [12].

Microcosm studies with contaminated soil

H: Laboratory studies with flow-through soil columns carried out at Wageningen Agricultural University in close cooperation with BioSoil R&D and TNO Institute for Environmental Sciences.
The optimal conditions for in situ biodegradation of PCE and TCE by the indigenous microbial population were determined. The columns were run with groundwater/PCE/TCE and/or compost extract or with artificial groundwater (composition under protocol H in table 3a, 3b and 3c) with PCE and defined electron donors. The groundwater concentration was 120 and 530 µM of PCE and TCE, respectively (among other chlorinated compounds). In artificial groundwater, the PCE concentration was kept at 50 µM and the electron donor concentration at 800 mg TOC/l. Complete dechlorination of PCE to ethene was only observed in the native groundwater column fed with compost extract. The other electron donors only sustained dechlorination to TCE and cis-DCE [22].

K: U.S. Geological Survey, Stephenson Center, Colombia, South Carolina, USA (P.M. Bradley, F.H. Chapelle), Department of Microbiology, University of Massachusetts, Amherst, Massachusetts, USA (D.R. Lovely).
The degradation of DCE and VC was studied with ^{14}C labeled DCE and VC in anaerobic microcosms under methanogenic and iron-reducing conditions. Microcosm studies were carried out in 20-ml serum flasks with 10 g of water saturated sediment (moisture content 25 %). Methanogenic conditions were induced by exchanging the headspace with helium gas. Methanogenic conditions were monitored by following the methane formation [4]. In other experiments the effect of the presence of humic acids as electron acceptor on the oxidation of VC and DCE was investigated. The presence of humic acids was beneficial for DCE and VC oxidation by microcosms [5].

L: U.S. Environmental protection agency (G.W. Sewell), NSI Technology Services Corporation (S.A. Gibson).
Anaerobic microcosm studies to investigate the PCE dechlorination with toluene as the electron donor. Microcosm studies were carried out in 160-ml serum flasks with 50 g water saturated sediment. Phosphate buffer was added together with sodium sulfide and resazurin as the redox indicator. PCE was spiked at a concentration of 36 µM. Toluene was a suitable source of reducing equivalents for the reductive dechlorination of chloroethenes under anaerobic conditions [18, 26].

M: Michigan State University, East Lansing, Michigan, USA (B.Z. Fathepure).
Large scale in situ batch studies were carried out in the Gulf Coast region of the United States. Pilot study with bioreactors ('biocells', volume 500 l) that were placed directly in the

contaminated sediment. The autochthonous contamination consisted of a mixture of chlorinated methanes, chlorinated ethanes, and chlorinated ethenes. Some of the cylinders (0.61 x 4.57 m) were covered to protect against rain fall, others were equipped with an air tight headspace sampling port. 0.2 % casaminoacids and/or 0.2 % sodiumacetate were added as the electron donors, together with sodium (mono- en di equimolar) phosphate as the source of phosphorus (C:P 100:1). No extra nitrogen was used in addition to the casaminoacid. The nutrients were added as concentrated solutions in a small volume of pond water. In some cases (1 - 5 % v/v) activated sludge or anaerobic sludge from a domestic wastewater treatment system was added. In that case, the sludge/sediment was separated from the water by a physical (bentonite or sand) layer. For headspace sampling, the biocells were closed 24 h prior to sampling. The headspace gas was collected for 24 h in Tedlar bags and analyzed in the lab. Analysis of the sediment and the waterphase was carried out after 2 and 4 weeks after the start of the experiment [13].

N: University of Groningen, Groningen, The Netherlands.
Soil slurry batch experiments with lactate as the electron donor to assess the dechlorination rate in soil contaminated with VOCs. Dechlorination seemed to be dependent on soil type and less on contamination history.

O: TNO Institute for Environmental Sciences, Energy Research and Process Innovation, Apeldoorn, The Netherlands.
In microcosm batch and column studies, PCE was converted via cis-DCE to VC and ethene with compost extract or methanol as the electron donor. H_2 did not sustain dechlorination. The presence of nitrate inhibited the dechlorination of PCE, whereas sulfate had no effect [3].

P: School of Civil and Environmental Engineering, Cornell University, Ithaca, New York, USA (J.M. Gossett, D.E. Fennell).
Microcosm studies to evaluate the effect of the addition of electron donors on the dechlorinating activity in soil. Electron donors used are lactate (3 mM), butyrate (3 mM), and a mixture of lactate and benzoate (1.5 mM each). Also, YE is used as a complex electron donor (200 mg/l). For every contaminated soil, the effect of each of these electron donors is investigated. YE and vitamin B_{12} are added to stimulate dechlorinating activity (nutritional requirements of strain 195). Whenever the concentration of lower chlorinated ethenes at the beginning of the experiment is too high, the headspace of the bottle is flushed with oxygen free N_2/CO_2 (70 %/30 %) and TCE is spiked at a concentration of 10 - 50 µM. Results from the microcosm experiments will be correlated to results obtained in field studies. In the microcosms the electron balance is determined. Lactate, butyrate and benzoate are chosen as electron donors, because the conversion of these compounds will result in an initial fast H_2 release followed by a slow H_2 production at low concentrations for a prolonged period. This may be beneficial for the dechlorination of chlorinated ethenes. The electron donor is respiked whenever the slow hydrogen releasing compound (e.g., propionate in the case of lactate addition) is completely converted [23].

Q: Bioclear Environmental Biotechnology, Groningen, The Netherlands.
Microcosm studies in batch tests. Batch tests are carried out in 250-ml serum flasks with 150 g of contaminated soil and a saturating amount of groundwater. VOCs are spiked after microcosm preparation based on VOC concentrations at the site. Nutrients are usually added to the microcosms together with sulfide and resazurin. E-donors are added in a e-donor: VOC ratio or 1-100:1 (mole/mole); depending on VOC concentrations.

CHAPTER 4

DISCUSSION METHODS

4.1 Selection sampling sites

Site selection for soil material for the microcosm is important. At the Cape Canaveral site, no dechlorination occurred in the microcosms although 20 meter from the sampling point VC was detected in situ. At the Dover site, a range of sampling points were used, including active and non-active zones and non-contaminated references. The best place for sampling depends on the research question (e.g., whether it is possible to achieve complete dechlorination in soil material from the contaminated site or whether at a specific place on the site a bioscreen would be effective). In most cases, it is probably best to sample in the most active zone where both parent and daughter products are found at the site. The substrate that is added should be the parent compound (PCE, TCE). In the plume where mainly daughter products are present, further dechlorination of these compounds may be analyzed by adding cis-DCE or VC. This is not done by the US laboratories, but was considered to be an interesting alternative. Dechlorination of lower chlorinated compounds may however be dependent on the presence of higher chlorinated compounds. This should be investigated further.

The type of soil seems to influence the anaerobic dechlorination potential. Sandy soils may be less effective than soils containing high amounts of organic carbon. The nature of the organic carbon van vary, e.g., peat, organics from a leaking sewer or co-contaminants (BTEX). If different soil types are contaminated at a site, each soil type should be tested for its biodegradation (dechlorinating) potential.

4.2 Anaerobic handling

Soil is usually sampled in plastic (Geoprobe) or metal cores that are driven into the soil. These cores are capped and transported to the laboratory where they are put into an anaerobic glovebox or anaerobic chambers or bags. At Groningen University, soil is handled under a continuous stream of nitrogen and oxygen is removed from the final incubation by replacement of the headspace through a series of vacuum/pressurizing steps with N_2/CO_2 atmosphere. Most other laboratories use anaerobic gloveboxes or glovebags (sometimes in combination with the aforementioned headspace replacement system). Soil handling in anaerobic gloveboxes is performed aseptically at Cornell to prevent microbial contamination of the incubations with e.g. dechlorinating micro-organisms. Anaerobic gloveboxes have the advantage that no oxygen is present in the atmosphere, but H_2 may be introduced into the microcosm, thus influencing the dechlorination process. Glovebags are simple, cheap, and can be operated aseptically. Handling of material outside an anoxic environment may be detrimental to strictly anaerobic micro-organisms but the work at Groningen State University seems to indicate that this is not a problem when handling soil.

4.3 Microcosm set-up (see tables 1 to 4)

Macronutrients, trace elements and vitamins

The main (obvious) difference between pure/mixed culture and microcosm studies is in the composition of the medium used. The media listed used for pure culture studies are complex and rich in nutrients, trace elements and vitamins, whereas the microcosm studies usually are not. These additions are apparently dictated by the strict nutritional demands of the respective pure cultures which have been isolated. Although the media used in the different studies appear to be largely different, basic trends are clear.

For pure/mixed culture studies usually a phosphate and/or carbonate buffer is used, and these compounds also often serve as a phosphorus and/or nitrogen source. In most cases an ammoniumsalt is added as a source of nitrogen. Magnesium and calcium (salts) are present in mM ranges in all media. Sodium salts are added in media that were originally developed for sulfate-reducing bacteria. KOH is only added in one protocol (E). Most protocols use sulfide in a range of 1 - 5 mM as a medium bulk reductant and to remove traces of oxygen. Only the RAMM medium (protocol F) contains cystein and dithionite as the main reducing agents. Resazurin is used at concentrations up to 2 mg/l as a redox indicator. In all cases (except protocol F) (fermented) yeast extract is applied to ensure the presence of all the essential nutrients to the pure and enriched cultures.

Microcosm studies with contaminated soil often have limited macronutrient additions. Protocol H, in which the addition of groundwater is mimiced, is an exception to this rule. In the protocols L, N, O, and Q a limited range of macronutrients is routinely added. Only in three protocols, sulfide is used as a reducing agent as is the case with resazurin as the redox indicator. Yeast extract is dosed in two cases (protocols N and P) at concentrations of 20 or 100 mg/l.

Every pure/mixed culture medium listed in protocols A to G-2 contains a variety of trace elements and vitamins although the concentrations used may vary significantly. With the microcosm studies only two protocols (and protocol H in which trace elements are occasionally added) use trace elements, and only in one case the whole range of vitamins was applied. Only protocol P specifically requires the addition of vitamin B_{12} to the microcosms.

Additions

The choice of e-donor is in principle open, but in the US there is a tendency to use food additives to facilitate regulatory approval. There is consensus among the researchers that there is no preferred substrate that could be applied to any site, and substrate selection should be based on site specific microcosm studies. However, both in the pure/mixed culture protocols (A to G) and the microcosm study protocols (H to Q) lactate is commonly used. The concentrations applied are usually in the mM range, thus 100 to 1000 times higher than the VOC concentration. More complex electron donors like compost extract and yeast extract are also used. The ratio of e-donor to VOC is 100:1 (mole/mole) in most studies. At Cornell University, feeding of the microcosms with new e-donor is timed to the depletion of the VFAs produced from the original e-donor. Most often the chlorinated ethenes are spiked at the start of the incubation. In some cases the transformation of the originally present VOCs is investigated. The spiked VOC concentration is usually in the 10 - 100 µM range. In microcosm studies the (spiked) VOC concentration is often kept within the concentration range found at the contaminated site, which can be much higher (up to saturation). The VOC concentration should in principle be relevant to site specific conditions and impose no difficulties on the analytical procedures.

pH and temperature

In all cases, the dechlorination is measured in the pH range of 7 to 7.5. For the pure/mixed culture studies the pH is maintained at this level by adding strong buffers, while for the microcosm studies the use of a buffer is confined to one protocol (O). In two cases (protocols L and P), the alkalinity of the contaminated soil and groundwater was routinely measured. Protocol P prescribes the addition of bicarbonate when the alkalinity is lower than 50 meq/l, the other protocols assume sufficient buffering capacity. Alkalinity is added whenever the headspace gas contains CO_2. For the pure culture studies, the headspace gas is either N_2/CO_2 or H_2/CO_2, the latter being the case when hydrogen is used as an electron donor.

There are large variations (10 - 35 ºC) in the incubation temperatures applied in the protocols, but the majority of the protocols suggest that the experiments are carried out in the dark under stationary conditions.

Incubation conditions
The total volume of serum bottles and the volume of the liquid phase differs among the protocols. However, it can be stated that headspaces in dechlorination experiments are usually large compared to the volume of the liquid phase (with the exception of protocols K and O). Furthermore, there are equally big differences in the amount of soil used in the microcosm experiments and the resulting soil/liquid ratios.

The test bottles are usually closed with crimp caps and the septa used are either made of Teflon-lined butylrubber or viton rubber. The medium is usually prepared in an anaerobic hood or glove-bag to maintain anaerobic conditions.

Replicates and controls
The number of replicates that is used by most researchers varies between duplicate or triplicate microcosms. The average amount of blanks was 1 on two (living) test bottles. The USAF protocol prescribes a range of incubations, including 4 different e-donors, two types of non-amended controls and a sterile control. The control should be sterilized (by autoclaving). Most researchers use a control that is sterilized at least twice on separate days, in order to avoid microbial growth from spores. Addition of $HgCl_2$ to the control incubation may be effective in preventing microbial contamination caused by the sampling of the incubation. However, the omission of $HgCl_2$ from this control allows the detection of these possible infections, which is important when working with pure cultures.

Monitoring
In all reviewed research projects dealing with stimulated anaerobic dechorination of VOCs, monitoring of the biodegradation process includes analysis of VOCs and ethene.

At Cornell University and at Wageningen Agricultural University, in addition to VOCs and daughter products of dechlorination, microcosm studies are monitored for methane, VFAs and H_2. Especially propionate and butyrate seem to serve as a slow release H_2 donor that may be important to sustain dechlorination. At Dover, VFAs and H_2 have been measured, but H_2 does not seem to give conclusive information and VFA analysis has been replaced by TOC analysis. VFA analysis is important to be able to better understand the dechlorination process and therefore forms an integral part of the monitoring at Cornell and Wageningen. When the aim of the research is to demonstrate the feasibility of anaerobic dechlorination in soil material, monitoring of VOCs and daughter product formation may be sufficient. VFA analysis can be useful to determine the relation between substrate consumption and dechlorination and to optimize the feeding strategy. This could be performed in microcosm studies but probably better in column studies. In the field, substrate consumption can be monitored by VFA analysis but possibly TOC analysis is sufficient for this purpose. There is no consensus about the status of H_2 measurements. Many scientists are skeptical about the relevance and applicability of the analysis in the field. For enhanced anaerobic dechlorination, it may not add new information. Furthermore, the method is highly laborious and needs specialized personnel.

CHAPTER 5

GUIDELINE FOR PERFORMING BATCH FEASIBILITY STUDIES FOR ANAEROBIC DECHLORINATION OF VOCS

Following are guidelines for the set-up of anaerobic experiments which are carried out to get information about the dechlorinating activity at sites contaminated with chlorinated ethenes. Some recommendations are made concerning the selection of sampling points, sampling and treatment of the sample at the contaminated site. Others are dealing with the set-up of microcosm studies, incubation conditions and analyses. Not every parameter which can be varied in such experiments is discussed. Some parameters have no significant impact on the outcome of the results of the studies. E.g., the incubation temperature could be set anywhere between 10 and 37 ºC, and a higher temperature will only increase reaction rates but will not influence the 'verdict' on whether the dechlorination takes place at a certain site or not.

Sampling points

- The position of the sampling point is dependent on the research question, on the type of soil, and the profile of the pollution.
  - at sites with only one type of soil present: one sample;
  - if more than one soil type is present on the site: one sample per soil type.
- When considering full scale stimulated anaerobic dechlorination, a sample should be taken on a spot where daughter products are present, i.e., the zone with dechlorinating activity. The sample should be taken in the active zone with the largest range of products, i.e., both PCE/TCE and lower chlorinated ethenes. This increases the chances of positive results, and decreases the chance that a site is deselected on the basis of a negative feasibility study while the site does have the potential for dechlorination.
- When considering a bioscreen approach for (parts of) the plume at the contaminated site with only lower chlorinated ethene daughter products, a separate sample should be taken, which is then tested for activity with the lower chlorinated ethene as the main contaminant
- Sterile controls should be performed for each soil type.
- Sampling soil to obtain 'no-activity-blanks' (e.g. a non-contaminated control) is not necessary.

Sampling

- Undisturbed core sampling is performed with hollow tube e.g. Geoprobe or 'Akkerman steekbus' that should fit in anaerobic glovebox/bag.
- The gas volume in the sampling core should be minimized by, e.g., filling the tube with groundwater or by using core tubes than can be adjusted in length in such a way that there is no longer a headspace present. The cores should be capped and carefully taped to prevent leakage of liquids or gasses (in and out).
- Core samples should be kept cool and preferable stored in an anaerobic jar or under water during transport and storage.

Treatment of the soil sample

- Soil samples should be handled in an anaerobic glove compartment/chamber or an anaerobic bag. When such devices are not available the core should be manipulated under N_2 flushing.
- The hydrogen content of the atmosphere in the anaerobic compartment should be as low as possible. In any case, the H_2 concentration (amount of electron equivalents) should be much lower than the electron donor concentration (in electron equivalents) applied in the microcosm studies. If not, the head space should be exchanged to remove H_2.

Set-up of microcosm studies

- The experiments are carried out in glass serum flasks with a viton lined gas tight inlay septum or stopper.
- Groundwater from the sampling site should be used as the liquid phase/medium (no NAPLs present). If no groundwater is available, demineralised water should be used. The soil:liquid ratio should be 1:1 (v/w), but whenever possible less water than soil should be used.
- The headspace should be kept as small as possible to prevent the headspace from acting as a storage volume for H_2. However, the headspace volume should be large enough to take samples for the analysis of the chlorinated ethene concentration, to 'store' the methane which could be produced (to avoid the build-up of high pressures in the bottle) or to allow addition of extractant in case of extractive analyses.
- N- and P-source should be added to ensure sufficient supply of nutrients (not in batches that mimic 'natural attenuation').
- The pH of the microcosms should be in the range of pH 6 to 8 with a buffer capacity high enough to prevent acidification of the microcosms. A good approach is to determine the alkalinity of the microcosms at the beginning of each experiment. When the alkalinity is lower than 50 meq/l, enough $NaHCO_3$ is applied to achieve that level in the microcosm liquid phase. When pH fluctuations beyond pH 6 - 8 are expected, e.g., acidification of organic material, sufficient buffer should be added. In batches that mimic 'natural attenuation' no pH adjustments are done.
- Dependent on the presence of a phosphate or carbonate buffer the headspace should contain N_2 or N_2/CO_2, respectively.
- Lactate could be used as an electron donor, to cover the fast (initial conversion of lactate to acetate and propionate) and slow (conversion of propionate) hydrogen releasing compounds. A microcosm study with a more complex substrate like yeast extract, molasses or compost extract could give additional information about the dechlorinating activity of the soil under study and the feasibility to use these compounds in full scale bioremediation.
- The ratio electron donor: VOC should be around 100:1 on a molar basis. Research is needed to determine a minimum level of e-donor required to stimulate anaerobic dechlorination and to determine possible negative effects of a high e-donor: VOC ratio.
- The concentration of the VOC applied in the microcosm study should be equal to a relevant concentration found at the contaminated site or at a concentration high enough to sustain adequate analysis of both the mother and daughter compounds.
- Living microcosms should be tested at least in duplicate for each of the conditions applied. The dead controls can be carried out in singular tests. The dead controls should be autoclaved two times for 1 h with at least 2 days in between. The autoclaved control can act as a control for sterile conditions during sampling. $HgCl_2$ or NaN_3 can be added to maintain the autoclaved control sterile.

Incubation conditions

- The microcosms should be incubated for 6 months or until the formation of ethene is detected.
- Incubation should be carried out in the dark, statically.
- Incubation temperature is between 10 - 30 ºC, depending on research question.

Analyses

- PCE, TCE, all DCE isomers, VC, ethene, and if possible ethane.
- The determination of volatile fatty acids (VFA, up to C_5) or alternatively TOC is recommended to give information on the electron balance in the system. For the same reason methane could be measured.

CHAPTER 6

RESEARCH TOPICS

Bioaugmentation of a contaminated site was found to be successful at Dover Air Force Base (see interview with Dr. Ed Lutz, section 2.5). Therefore, it seems advisable that more attention should be paid to this phenomenon in batch experiments in the future.

In a further experimental program (phase 2 of the project) the following parameters can be investigated using a research protocol as described above:

1. The effect of the absolute and relative concentrations e-donor and VOC in the incubation on the outcome of the feasibility study.
   The concentration of PCE at a contaminated site can vary from 10 to over 100,000 µg/l. It is unknown whether a PCE concentration of 100 µM (approximately 16,400 µg/l) is representative for the biodegradation potential at a site. If a site specific PCE concentration and a fixed e-donor: VOC ratio is used, the donor concentration can vary widely as well. It is unknown what the effect of high or low e-donor concentrations is on the outcome of dechlorination studies. This needs to be investigated using a dechlorination study with varying concentration and ratios of e-donor and VOC.
2. Relation between dechlorination and substrate levels as determined with VFA and TOC analyses.
   It is unknown how VFA and TOC analyses can be used to monitor the availability of the e-donor to fuel the dechlorination process. This can be tested by monitoring VFA and TOC in addition to VOC levels during the batch incubations.
3. Reproducibility of batch incubations.
   Batch incubations are carried out in triplicate to determine the reproducibility of the incubations and to determine the necessity of multiple incubations when performing feasibility studies.
4. Effect of nature of spiked VOC on dechlorination (PCE, TCE, cis-DCE or VC).
   It is unknown whether the capacity of micro-organisms in a contaminated soil to degrade, e.g., cis-DCE can be determined by addition of PCE or whether strictly cis-DCE should be applied in the batch incubations.
5. Effect of sampling location in the plume on the outcome of the batch study.
   In some cases daughter products (in the case of chlorinated ethenes TCE and lower chlorinated) are not present at a specific part of a contaminated site, while at the same site at some distance only the daughter products are present. In those cases the choice of the sampling location could strongly influence the outcome of the batch study.
6. Necessity of sulfide and resazurine additions.
   The addition of sulfide could strongly influence the outcome of a batch study by lowering the redox potential beyond values found in the field. This could enhance or inhibit the dechlorination processes, and therefore influence the outcome of the research.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH IN PHASE 2 OF THE PROJECT

Conclusions

- Anaerobic conditions should be maintained during sampling and storage of soil samples.
- Anaerobic handling of soil samples in a glovebox, glovebag or by exchanging gas phases is used by all reviewed laboratories.
- Technical experiment lay-out and experimental procedures are largely comparable between the reviewed laboratories, no crucial imperfections were found.
- Microcosm studies appear to be essential to predict the feasibility of complete dechlorination at a given site.
- Substrate (e-donor) selection should be based on site specific laboratory research.
- The design of the batch experiment is dependent on the specific research question.
- A guideline for performing batch feasibility studies for anaerobic dechlorination of VOCs was developed.

Further research in phase 2 of the project

1. The effect of the absolute and relative concentrations e-donor and VOC in the incubation on the outcome of the feasibility study.
2. Relation between dechlorination and substrate levels as determined with VFA and TOC analyses.
3. Reproducibility of batch incubations.
4. Effect of nature of spiked VOC on dechlorination (PCE, TCE, cis-DCE or VC).
5. Effect of sampling location in the plume on the outcome of the batch study.
6. Necessity of sulfide and resazurine additions.

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APPENDIX 1

PROTOCOLS FOR PURE AND MIXED CULTURES; MEDIUM COMPOSITION

Table 1a. Macronutrients.

compound	Mw		protocol							
			A	B	C	D	E	F	G	G-2
Na_2HPO_4	120.0	mM		11.8	1.3					
KH_2PO_4	136.1	mM	1.5	4.8	4.8	0.4		1.5	3.3	3.3
K_2HPO_4	174.2	mM				0.4			2.6	5.2
$NH_4H_2PO_4$	115	mM					2.5			
$(NH_4)_2HPO_4$	128	mM								
$NaHCO_3$	84.0	mM	30.0[a]		44.4	59.5	28.6	30		
Na_2CO_3	106.0	mM							3.0	
NH_4HCO_3	79.1	mM			5.6				37.9	
NH_4Cl	53.5	mM	4.7			3.7		5.6		8.4
NH_4Br	97.9	mM		1.0						
NH_4NO_3	80	mM								
$(NH_4)_2SO_4$	132.1	mM							3.4	
NaCl	58.6	mM	17.1					17.1	15.4	15.4
KCl	74.6	mM	6.7					4.0		
$MgCl_2$	59.8	mM	2.0		0.6	1.0		29.9		
$MgBr_2$	184.1	mM		1.0						
$MgSO_4$	120.4	mM					0.4		0.9	1.7
$CaCl_2$	111.1	mM	1.0		0.75			0.1	0.8	0.1
$CaBr_2$	199.9	mM		0.4						
$Ca(NO_3)_2$	164.1	mM					0.2			
KOH	56.1	mM					17.8			
Na_2SO_4	142.0	mM	0.5							
Na_2S	78	mM	2.0[b]	5.2	1.0	2.1	0.90		1.0/3.8	
$Na_2S_2O_4$	174.2	mM						0.1		
cysteine	121.0	mM						1.0	1.6/2.5	
resazurin		mg l^{-1}		1	0.5	1	0.4		½	
yeast extract		g l^{-1}	2	0.5	0.4(ferm)	0.05[c]	0.1		1	

[a] autoclaved separately under CO_2
[b] autoclaved separately under N_2
[c] later 0.025 g l^{-1} fermented yeast extract (e.g., [15])

Table 1b. Trace elements.

compound	Mw		protocol							
			A	B	C	D	E	F	G	G-2
HCl 25 %		ml l^{-1}	0.02		1 conc			0.01		
EDTA	372.2	µM		12.7	1.34		2.69			
NTA	194.0	µM							77.3	
HEPES (Na-salt)		mM						10		
$FeSO_4$	151.9	µM	7.20				7.20		75.6	
$FeCl_2$	126.8	µM		10.06	10.06	503		7.54		40.4
$ZnCl_2$	136.3	µM	1.03	0.51	0.51	7.33		0.51		1.47
$ZnSO_4$	161.5	µM					0.35		3.47	
$MnCl_2$	125.9	µM	1.01	0.62	0.51	5.05	0.15	0.51		
$MnSO_4$	151.0	µM							29.6	
$CaCl_2$	111.1	µM	1.18			18.0				
$CuCl_2$	134.5	µM	0.02	0.01	0.02		0.06	0.01		1.17
$CuSO_4$	159.6	µM							0.40	
$CoCl_2$	129.9	µM		0.80	0.80	7.14	0.84	0.80	4.20	4.2
$NiCl_2$	129.7	µM	0.20	0.10	0.10	2.10	0.08	0.10		
$NiSO_4$	154.8	µM								0.08
$AlCl_3$	133.5	µM			0.04					1.66
$AlKSO_4$	162.2	µM							0.26	
$Al_2(SO_4)_3$	342.3	µM								
H_3BO_3	61.8	µM	0.20	0.10	0.10	3.07	4.85	0.10	1.62	6.47
Na_2MoO_4	242.0	µM	0.03	0.02	0.02	0.83	0.12	0.15	0.41	0.83
$(NH_4)_6MoO_4$	811.3	µM								
Na_2SeO_3	172.9	µM	0.02	0.02			0.10	0.02		
Na_2Wo_4	293.9	µM	0.01	0.01			0.1	0.02		

Table 1c. Vitamins.

compound	Mw		protocol							
			A	B	C	D2	E	F	G	G-2
p-aminobenzoate (Na-salt)[d]	159.1	µg l^{-1}	46.5	46.5	250	250	100		50	50
biotin (vitamin H)	244.3	µg l^{-1}	10	10	50	100	20		20	50
folic acid·$2H_2O$	477.4	µg l^{-1}			20	100	50	50	20	20
lipic acid	206.3	µg l^{-1}			20	250	50	50	50	
nicotic acid		µg l^{-1}	100	100		250			50	50
nicotinamide		µg l^{-1}			550		200	500		
1,4-naphtoquinone		µg l^{-1}						200		
hemin		µg l^{-1}						50		
pantothenate[e]	218.2	µg l^{-1}	42.3	42.3	226	211	100			
pyridoxamine[f]		µg l^{-1}	150	150			500			
pyridoxine[f] (vit. B_6)	169.2	µg l^{-1}			100	500			100	100
thiamine[f] (vit. B_1)	337.3	µg l^{-1}	80	80	100	250	200		50	50
cyanocobalamin (vit. B_{12})	1355.4	µg l^{-1}	50	50	50	5	100		5	5
riboflavin (vit. B_2)	376.4	µg l^{-1}			50	250	100	50	50	50

[d] also added as an acid
[e] added as calcium- or sodium salt
[f] added in conjunction with HCl

APPENDIX 2

PROTOCOLS FOR PURE AND MIXED CULTURES; INCUBATION SET-UP

Table 2. Incubation set-up.

parameter	protocol			
	A	B	C	D
pH	7.2 - 7.4/7.5		7.0 - 7.2	7.0 - 7.5
gas phase	80 % N_2, 20 % CO_2 (150 kPa)	80 % N_2, 20 % CO_2 (2.8 bar)	80 % N_2, 20 % CO_2 (150 kPa)	70 % N_2, 30 % CO_2
T ºC	25	20	30 - 37	35
incubation[g]	D 300 rpm	S, D	S, D 120 rpm	S, L
volume ml (total/liquid)	35/24	300/200	120/20	160/100
septum[h]	T-l-B	V	V, cr	T-l-B, cr
inoculation	1 ml fresh	20	1 %	2 - 10 v%
blank[k]			A	2A (30')
medium-preparation[l]			AH + GP	AH (N_2), F
spike	300 µM	55 µM TCE	1 mmol	10 - 150 µmol
electron donor	up to 80 mM (e.g. lactate, pyruvate)	2 mM glucose	20 - 50 mM (e.g., lactate, H_2, formate)	up to several mM (e.g., butyrate, methanol, H_2)
sampling[m]	L	G + L (Cl^-)	G + L (Cl^-)	G

Table 2. continued.

parameter	protocol			
	E	F	G	G-2
pH	7.2	7.5	6.6 - 6.9[n]	id.
gas phase	80 % N_2, 20 % CO_2	95 % N_2, 5 % CO_2	60 % N_2, 40 % CO_2, or 100 % N_2	-
T ºC	37	37	35, 37[p]	22
incubation[g]		D	5 %[o], 2 %[p]	
volume ml (total/liquid)			25/10[o] 160/50[p]	245
septum[h]	B + V	T-l-B, cr	T-l-B, cr[p]	T
inoculation				-
blank[k]			A	-
medium-preparation[l]	Hungate tubes, F	F	F (N_2)[p]	-
spike	1-20 mM	50 µM	6 µM	3-6 µM
electron donor	20 mM (e.g., lactate, pyruvate)	20 Mm (e.g., pyruvate)	20 - 50 mM (e.g., methanol, acetate)	5.6 mM (e.g., methanol, glucose, acetate)
sampling[m]	G + L (Cl^-)	G	O	L (in/out)

[g] D = dark, S = stationary, L = liquid in contact with septum
[h] T = teflon, l = lined, B = butylrubber, V = viton, cr = crimp cap
[k] blank: autoclaved (A), $HgCl_2$ of NaN_3
[l] medium is prepared in anaerobic hood (AH), gas phase is exchanged with gas changing device (GP), gas phase is changed by flushing (F)
[m] sampling: L = liquid phase, G = gas phase, O = sampling by sacrificing bottles
[n] Fathepure adjusts to pH 7.0 [8]
[o] data Fathepure [8]
[p] data Fathepure [9]

APPENDIX 3

PROTOCOLS FOR MICROCOSM STUDIES; MEDIUM COMPOSITION

Table 3a. Macronutrients.

compound	Mw		protocol							
			H	K	L	M	N	O	P	Q
Na_2HPO_4	120.0	mM	0.45					5.22		
KH_2PO_4	136.1	mM	0.15					5.14		[s]
K_2HPO_4	174.2	mM								
$NH_4H_2PO_4$	115	mM								
$(NH_4)_2HPO_4$	128	mM			5					
$NaK(NH_4)PO_4$	176.1	mM					25			
$NaHCO_3$	84.0	mM	1.0				30		[r]	
Na_2CO_3	106.0	mM								
NH_4HCO_3	79.1	mM								
NH_4Cl	53.5	mM	1.0							[s]
NH_4Br	97.9	mM								
NH_4NO_3	80	mM						12.5		
$(NH_4)_2SO_4$	132.1	mM								
NaCl	58.6	mM	0.12							
KCl	74.6	mM								
$MgCl_2$	59.8	mM	0.05							
$MgBr_2$	184.1	mM								
$MgSO_4$	120.4	mM						0.41		
$CaCl_2$	111.1	mM	(6)							
$CaBr_2$	199.9	mM								
$Ca(NO_3)_2$	164.1	mM						0.21		
KOH	56.1	mM					18			
Na_2SO_4	142.0	mM	0.06 (+ 1.0)							
Na_2S	78	mM			1.0		2.6			0.4
$Na_2S_2O_4$	174.2	mM								
cysteine	121.0	mM								
resazurin		mg l^{-1}			1				<1	5
yeast extract		g l^{-1}					0.1		0.02	

[r] if the alkalinity of the microcosm is below 0.05 eq/l, then $NaHCO_3$ should be added to achieve that level
[s] the C:N:P ratio applied is 250:100:10

Table 3b. Trace elements.

compound	Mw		protocol							
			H	K	L	M	N	O	P	Q
HCl 25 %		ml l^{-1}	(1 conc)							
EDTA	372.2	µM	(1.34)				2.69	17.1		
NTA	194.0	µM								
HEPES (Na-salt)		mM								
$FeSO_4$	151.9	µM					0.72	101		
$FeCl_2$	126.8	µM	(10.06)							
$ZnCl_2$	136.3	µM	(0.51)							
$ZnSO_4$	161.5	µM					0.35	0.04		
$MnCl_2$	125.9	µM	20				0.15	0.01		
$MnSO_4$	151.0	µM								
$CaCl_2$	111.1	µM								
$CuCl_2$	134.5	µM	(0.02)				0.062			
$CuSO_4$	159.6	µM						0.30		
$CoCl_2$	129.9	µM	(0.80)				1.68	0.10		
$NiCl_2$	129.7	µM	(0.10)				0.08	0.02		
$NiSO_4$	154.8	µM								
$AlCl_3$	133.5	µM	(0.04)							
$AlKSO_4$	162.2	µM								
$Al_2(SO_4)_3$	342.3	µM						0.005		
H_3BO_3	61.8	µM	(0.10)				4.85	8.10		
Na_2MoO_4	242.0	µM	(0.02)				0.11			
$(NH_4)_6MoO_4$	811.3	µM						0.001		
Na_2SeO_3	172.9	µM					0.11			
Na_2Wo_4	293.9	µM					0.10			

Table 3c. Vitamins.

compound	Mw		protocol							
			H	K	L	M	N	O	P	Q
p-aminobenzoate (Na-salt)[d]	159.1	µg l^{-1}					100			
biotin (vitamin H)	244.3	µg l^{-1}					20			
folic acid·$2H_2O$	477.4	µg l^{-1}					50			
lipic acid	206.3	µg l^{-1}					50			
nicotic acid		µg l^{-1}								
nicotinamide		µg l^{-1}					200			
1,4-naphtoquinone		µg l^{-1}								
hemin		µg l^{-1}								
pantothenate[e]	218.2	µg l^{-1}					100			
pyridoxamine[f]		µg l^{-1}					500			
pyridoxine[f] (vit. B_6)	169.2	µg l^{-1}								
thiamine[f] (vit. B_1)	337.3	µg l^{-1}					200			
cyanocobalamin (vit. B_{12})	1355.4	µg l^{-1}					100		50	
riboflavin (vit. B_2)	376.4	µg l^{-1}					100			

[d] also added as an acid
[e] added as calcium- or sodium salt
[f] added in conjunction with HCl

APPENDIX 4

PROTOCOLS FOR MICROCOSM STUDIES; INCUBATION SET-UP

Table 4. Incubation set-up.

parameter	protocol			
	H	K	L	M
pH		7.1 ± 0.3	7.4[s]	-
gas phase	100 % N_2 (flushing)	100 % He	-	-
T ºC	20	ambient (22 ºC)	20	ambient
incubation[g]		D	S, D	S, in situ
volume (total/liquid) ml		20/1 or 30/0	160[t]	500 L!
septum[h]		T-l-B	T + B, cr	T
inoculation g		10[v] or 20[v]	50[v]	
blank[k]		1A (1h) per 2 living	2 - 4A per 3 - 4 living	NS, NA
medium-preparation[l]			AH (also incubation)	-
spike	50 µM PCE	^{14}C-labeled	36 µM PCE	-
electron donor	various, 800 mg TOC/l (e.g., compost extract, methanol)		108 µM toluene	casamino-acids/acetate
VOC/donor mole/mole	1:500		1:3	
sampling[m]	L inf/eff	G (VOC) + G ($^{14}CO_2$ and CH_4)	2 mL L	S (VOC) + L (VOC, DO, redox, etc) + G (bags, VOC)

Table 4. continued.

parameter	protocol			
	N	O	P	Q
pH	6.8 ± 0.2	± 7?	6 - 8	6 - 8
gas phase	80 % N_2, 20 % CO_2	N_2/CO_2?	70 % N_2, 30 % CO_2[y]	N_2
T ºC	30		20 - 24	10 - 30
incubation[g]			S, D	S, D
volume (total/liquid) ml	30/5	110/100[u]	160/50[u] no compost extract	250, variable
septum[h]	B + V		T-l-B, cr	B + V, sc
inoculation g	5 cm^3	1	50 (dry)	150[v]
blank[k]	A	A (2 x 1h)[w]	3A (2 x 1h) per 3 living	A + $HgCl_2$ + NaN_3
medium-preparation[l]	GP		AH N_2/H_2 (1 - 3% H_2)	GB (N_2)
spike	PCE (100 µM)	PCE (7.5 µM)	TCE (10 - 50 µm)	if necessary
electron donor	lactate (10 mM)	compost extract, methanol (2.5 mM)	all electron donors are tested at 3 mM (e.g., lactate, propionate, benzoate, YE)	site dependent concentrations, variable donors
VOC/donor mole/mole	1:100	1:333	1:100	1:1-100
sampling[m]	G (VOC)	G (VOC)	G (VOC) + L (ferm. prod.) 1/week	G + O

[g] D = dark, S = stationary
[h] T = teflon, l = lined, B = butylrubber, V = viton, sc = screw cap, cr = crimp cap
[k] blank: autoclaved (A), $HgCl_2$ of NaN_3, no substrate (NS), no amendments (NA)
[l] medium is prepared in anaerobic hood (AH), anaerobic glovebag (GB), gas phase is exchanged with gas changing device (GP)
[m] sampling: L = liquid phase, G = gas phase, S = solid (sediment) phase, O = sampling by sacrificing bottles
[s] 326 $CaCO_3$ equivalents
[t] bottle completely filled with groundwater
[u] groundwater (in some cases (protocol O, TNO) together with compost extract)
[v] water saturated, or groundwater saturated (protocol Q, Bioclear)
[w] autoclaved controls in 110-ml bottles with 30 grams of soil
[y] purging only if lower chlorinated ethene levels are too high and fresh TCE has to be spiked