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

Phase 2: Validation of selected parameters in batch experiments

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Phase 2: Validation of selected parameters in batch experiments

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Samenvatting
Om de mogelijkheid van in situ biologische behandelingsmethoden vast te stellen, worden anaërobe batchproeven gebruikt. Verscheidende laboratoria voeren deze batchproeven uit of zijn anderszins betrokken bij het onderzoek naar de anaërobe dechlorering van bijvoorbeeld tetrachlooretheen (PER). De uitkomst van deze batchproeven kan echter worden beïnvloed door de wijze van uitvoering. In dit project is daarom een protocol opgesteld voor het uitvoeren van anaërobe batchproeven.
In de eerste fase van het project is een voorlopig protocol opgesteld en zijn een aantal belangrijke parameters in het protocol geselecteerd, die in de tweede fase van het project gevalideerd zijn. Hiervoor is een aantal batchproeven uitgevoerd, waarin de invloed van de in de eerste fase geselecteerde parameters op de anaërobe dechlorering is onderzocht. De resultaten van deze experimenten zijn beschreven in dit rapport. Op basis van de resultaten van dit project is een richtlijn gegeven voor het uitvoeren van anaërobe batchproeven, zodat de resultaten van de batchproeven betrouwbaar zijn en gebruikt kunnen worden om een beslissing te nemen over de haalbaarheid van een (gestimuleerde) biologische saneringsvariant voor de onderzochte locatie.

Trefwoorden
Gecontroleerde termen:
anaerobic, biodegradation, chloroethenes, laboratory research

Vrije trefwoorden:
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Abstract
To determine the feasibility of biological treatment technologies for contaminated soils microcosm studies are used. Several research groups perform these microcosm studies, for instance to investigate the anaerobic dechlorination of chlorinated ethenes (perchloroethene, PCE). The set-up of these experiments may influence the outcome and thus the decision made on whether or not (stimulated) natural attenuation is a suitable remediation alternative for the site in question. Therefore, in this project a guideline for performing anaerobic microcosm experiments was formulated.
The first phase of this project has resulted in a preliminary guideline and the selection of several important parameters, that are validated in the second phase of this project (this report). For this purpose batch experiments were performed in which the influence of the selected parameters on the anaerobic dechlorination was investigated. The results of these experiments are described in this report. The guideline presented in chapter 6 gives the prerequisites for performing these microcosms studies, so the outcome of the microcosm experiments can be used to determine the possibilities for a (stimulated) bioremediation approach for the investigated contaminated site.

Keywords
Controlled terms:
anaerobic, biodegradation, chloroethenes, laboratory research

Uncontrolled terms:
dechlorination, feasibility study, protocol

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PREFACE

To determine the feasibility of biological treatment technologies for contaminated sites, microcosm studies are used. These microcosm studies are performed by several research groups, for instance to investigate the anaerobic dechlorination of chlorinated ethenes (perchloroethene, PCE). The set-up of these experiments may influence the outcome and thus the decision made on whether or not (stimulated) natural attenuation is a suitable remediation alternative for the site in question. However, a general guideline concerning the performance of these batch experiments is not yet available. Therefore, in this project a guideline was formulated, based on literature, the results of an inventory of different methods used in different studies and the results of a practical research phase.

In the first phase of this project, different protocols for microcosm studies used by several research groups working on anaerobic dechlorination were evaluated and discussed. Also, a work visit was made to Cornell University (Ithaca, NY, USA) where research groups are working on the assessment and monitoring of dechlorination of chlorinated ethenes. The results of the first phase are described in the report 'Selection and validation of a practical protocol anaerobic dechlorination - Phase 1: Inventory methods and work visit Cornell University USA' [Van der Waarde and Van Eekert, 1999]. Based on the evaluation and the work visit to Cornell University a guideline for performing anaerobic contaminated microcosm studies was proposed. Also, several recommendations were made for further research in phase 2 of the project.

In the second phase of the project, described in this report, several parameters that are thought to be of importance on the outcome of contaminated microcosm studies were investigated in a practical research phase. Based on the results of the first and second phase of this project a guideline for performing anaerobic microcosm studies was formulated. This guideline describes the set-up of anaerobic microcosm experiments and contains the most important factors that have to be considered when performing microcosm studies.

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CONTENTS

SAMENVATTING

Selection and validation of a practical protocol for anaerobic dechlorination

Het ongecontroleerd weglekken en lozen van gechloreerde ethenen heeft geleid tot een wijd verspreide vervuiling van de bodem. Naast conventionele behandelingstechnieken zijn anaërobe biologische technieken ontwikkeld om deze verontreinigingen te verwijderen.

Om de mogelijkheid van anaërobe in situ biologische behandelingsmethoden voor verontreinigde locaties vast te stellen, worden batchproeven gebruikt. Verscheidende laboratoria voeren deze batchproeven uit of zijn anderszins betrokken bij het onderzoek naar de anaërobe dechlorering van bijvoorbeeld tetrachlooretheen (PER). Er is echter nog geen algemene richtlijn met betrekking tot de uitvoering van anaërobe batchproeven beschikbaar. In dit project is daarom op basis van literatuurgegevens, informatie verkregen tijdens een werkbezoek aan Cornell University, USA en uitgevoerde batchproeven een protocol opgesteld voor het vaststellen van anaërobe dechlorering.

In de eerste fase van het project zijn de protocollen (onder andere de gebruikte media en algemene opzet), die momenteel worden gebruikt door internationale onderzoeksgroepen en die beschreven zijn in de literatuur, geëvalueerd en bediscussieerd. Tevens is een werkbezoek gebracht aan Cornell University. De in de literatuur verzamelde gegevens, tezamen met de informatie verkregen op Cornell University, hebben geleid tot een richtlijn voor een protocol voor het vaststellen van anaërobe dechlorering. De resultaten van de eerste fase zijn beschreven in het CUR/NOBIS-rapport 97-4-04 'Selection and validation of a practical protocol anaerobic dechlorination - Phase 1: Inventory methods and work visit Cornell University USA' [Van der Waarde en Van Eekert, 1999].

De eerste fase van het project heeft geresulteerd in de selectie van een aantal belangrijke parameters in het protocol, die in de tweede fase van het project gevalideerd zijn. Hiervoor is een aantal batchproeven uitgevoerd, waarin de invloed van de in de eerste fase geselecteerde parameters op de anaërobe dechlorering is onderzocht.

Het biodegradatiepotentieel in verontreinigde grond van een locatie verontreinigd met gechloreerde ethenen (voornamelijk perchlooretheen (PER) en *cis*-dichlooretheen (*cis*-DCE)) is onderzocht door anaërobe batchproeven uit te voeren volgens de protocollen van Wageningen Universiteit, Laboratorium voor Microbiologie en Bioclear. Deze protocollen voldoen aan de in de eerste fase opgestelde richtlijn, maar verschillen op een aantal belangrijke punten van elkaar. In dit onderzoek zijn onderstaande parameters gevarieerd en is de invloed van deze parameters op de uitkomst van de batchproeven vastgesteld. Daarnaast zijn diverse algemene aspecten van de gebruikte protocollen onderzocht:

- verlies van gechloreerde verbindingen door lekkage door de gebruikte septa (onderzocht tijdens incubatie van de batches rechtop en omgekeerd);
- reproduceerbaarheid van de batchexperimenten;
- effecten van autoclaveren;
- absolute en relatieve concentratie van elektronendonor en gechloreerde ethenen;
- relatie tussen dechlorering en elektronendonorconcentratie bepaald met VFA-analyses (volatile fatty acids oftewel vluchtige vetzuren);
- type van de toegevoegde component (PER of *cis*-DCE);
- type elektronendonor (melasse, een mengsel van vluchtige vetzuren in compostpercolaat, lactaat).

De voor het laboratoriumonderzoek gebruikte protocollen zijn beide geschikt voor het uitvoeren van anaërobe batchexperimenten. Gedurende een incubatieperiode van 12 weken is geen lekkage opgetreden van de gechloreerde ethenen. Batches kunnen zowel rechtop als omgekeerd worden geïncubeerd, en de geteste afsluitingen (dikke viton stop en crimp cap of schroefdop met een viton inleg) van de batches voldoen goed. De reproduceerbaarheid van de triplo incubaties was goed, de standaarddeviatie van de concentraties gechloreerde ethenen in de triplo batches bedroeg over het algemeen minder dan 10 %.

De initiële concentratie PER of *cis*-DCE had geen toxisch effect op de dechlorering tijdens de incubatieperiode binnen de onderzochte range tot 100.000 µg/l (PER) of 60.000 µg/l (*cis*-DCE). Complete dechlorering trad op binnen 6 weken in de range tussen 8.000 µg/l en 16.000 µg/l voor PER en 5.000 µg/l en 10.000 µg/l voor *cis*-DCE. De dechlorering in de batches gespiked met 100.000 µg/l PER of 60.000 µg/l *cis*-DCE was wel gelimiteerd. Na 16 weken werd in deze batchproeven hoofdzakelijk vinylchloride (VC) en een kleine hoeveelheid etheen gemeten. Dit werd waarschijnlijk veroorzaakt door de relatief lage concentratie melasse (160 mg/l) ten opzichte van de concentratie PER of *cis*-DCE. Dit werd bevestigd door de resultaten van de batches met een initiële concentratie van 16.000 µg/l PER en 80 mg/l, waarin dechlorering van PER optrad tot gelijke hoeveelheden VC en etheen in 16 weken. De exact benodigde hoeveelheid kon echter niet worden vastgesteld, omdat niet duidelijk is in hoeverre van nature aanwezig organisch materiaal gebruikt is voor de dechlorering. Aanbevolen wordt de benodigde hoeveelheid te berekenen voor complete reductie van de concentratie aan gechloreerde ethenen en de concentratie alternatieve elektronenacceptoren (zoals nitraat en sulfaat) en deze hoeveelheid in een vijfvoudige overmaat toe te voegen.

PER en *cis*-DCE werden beide geheel gedechloreerd tot etheen als deze componenten afzonderlijk aan de verontreinigde grond werden toegevoegd. Dit wijst erop dat batchexperimenten ook kunnen worden uitgevoerd met tussenproducten van de anaërobe afbraak van PER. Grondmonsters stroomafwaarts van de kern van de verontreiniging kunnen alleen verontreinigd zijn met deze tussenproducten. Aanbevolen wordt het biodegradatiepotentieel in batchexperimenten vast te stellen met de meest voorkomende gechloreerde verbinding (zoals *cis*-DCE) als verontreiniging in een concentratie, zoals die in het veld is gemeten.

In de batchexperimenten, waaraan verschillende elektronendonoren werden toegevoegd, werd geen significant verschil in dechlorering van PER vastgesteld. Binnen 6 weken trad complete dechlorering van PER op tot etheen met melasse, een mengsel van vluchtige vetzuren in compostpercolaat of lactaat als elektronendonor. Het type elektronendonor had in deze grond dus geen effect op de dechlorering. Aanbevolen wordt om in batchproeven, waarin het effect van een elektronendonor op de dechlorering wordt onderzocht, een elektronendonor te gebruiken waarmee ook in het veld kan worden gewerkt (acceptatie van de overheid, niet toxisch, goedkoop) en voldoende nutriënten (stikstof en fosfaat) toe te voegen.

Batchexperimenten zijn een geschikte onderzoeksmethode om het optreden van dechloreringsprocessen op een locatie vast te stellen. Voor het uitvoeren van de batchproeven is op basis van de resultaten van de eerste en tweede fase van het project een protocol opgesteld om de potentie voor dechlorering vast te stellen in verontreinigde grond. Dit protocol is gevalideerd en omvat richtlijnen voor de monstername van verontreinigde grond en grondwater, de opzet van de batchexperimenten, aanbevelingen over het type gechloreerde verbinding en elektronendonor en parameters die gemeten dienen te worden om het proces te volgen. Het ontwikkelde protocol zal worden gepubliceerd op de SKB-website www.skb.nl.

SUMMARY

Selection and validation of a practical protocol for anaerobic dechlorination

During the last years there has been a shift from conventional treatments (like pump and treat) towards *in situ* biological methods to remediate groundwater contaminated with chlorinated ethenes. For the application of biological processes, it is important to know the biodegradation potential of the soil at a particular site. The feasibility of a biological treatment (natural attenuation or enhanced dechlorination) at a site contaminated with chlorinated ethenes can be demonstrated by groundwater characterization and microcosm studies.

Research groups are working with different protocols for the set-up of microcosm studies. The procedures used are expected to determine the outcome of such experiments to a large extent and can therefore influence the decision whether or not (stimulated) biological dechlorination is a suitable remediation technique. In general the outcome of the microcosm study should give information on whether or not degradation of chlorinated ethenes takes place under certain (environmental) conditions.

A NOBIS project was carried out to examine and to develop general guidelines for the performance of anaerobic microcosms. A number of protocols used by research groups working on the anaerobic dechlorination of chlorinated ethenes was evaluated and discussed. Critical steps of these feasibility studies were identified and the influence of these steps on the dechlorination process was determined in laboratory experiments. The project has resulted in guidelines for performing microcosm studies to determine the dechlorination potential of contaminated soil.

The first phase of the project consisted of an inventory of different methods based on published literature, NOBIS documents and directly from NOBIS participants. Also, three scientists in the field of dechlorination were visited at Cornell University, USA. The results of this phase have been reported in the CUR/NOBIS report 97-4-04 'Selection and validation of a practical protocol anaerobic dechlorination - Phase 1: Inventory methods and work visit Cornell University USA' [Van der Waarde and Van Eekert, 1999]. In the second phase of the project several parameters have been experimentally investigated. Two practical protocols have been selected to be tested and verified.

The biodegradation potential in contaminated soil from a site contaminated with chlorinated ethenes, mainly PCE (tetrachloroethene) and *cis*-DCE, (*cis*-1,2-dichloroethene), was determined by performing microcosm studies in triplicate according to the protocols used by two research laboratories (the Laboratory of Microbiology of Wageningen University, and Bioclear). These protocols comply with the guideline proposed in phase 1, but differ at some important points. In this research the following parameters were varied and their influence on the outcome of the performed microcosm studies was determined. Several general aspects of the used protocols were also tested:

- possible loss of the chlorinated compounds via leakage through the used septa (investigated during incubation of the flasks straight up or upside down);
- reproducibility of the batch incubations;
- effects of autoclaving;
- absolute and relative concentrations of electron donor and chlorinated ethene in the incubation;
- relation between dechlorination and electron donor concentrations as determined with VFA analyses (volatile fatty acids);
- type of the spiked chlorinated ethene (PCE or *cis*-DCE) on dechlorination;

- type of electron donor (molasses, a volatile fatty acid (VFA) mixture in compost percolate, lactate) used for the dechlorination.

Both the protocols used in the practical research phase are suitable for anaerobic microcosm studies. No leakage of chlorinated ethenes occurred through the tested septa (thick viton stopper and crimp cap or screw cap with viton lined gas tight inlay septum) during an incubation period of 12 weeks. The results of the leakage test were not influenced by incubation straight up or upside down or by autoclaving. The reproducibility of the triplicate incubations was good, generally the standard deviation between the measured concentrations of chlorinated ethenes was less than 10 %.

The initial concentration of PCE or *cis*-DCE had no (toxic) effect on the dechlorination in the measured time period. Complete dechlorination occurred within 6 weeks in the range between 8,000 µg/l and 16,000 µg/l for PCE and 5,000 µg/l and 10,000 µg/l for *cis*-DCE. The dechlorination in the microcosms spiked with 100,000 µg/l PCE or 60,000 µg/l *cis*-DCE was limited, since after 16 weeks only VC and small amounts of ethene were detected. Presumably, this was caused because of the relatively low concentration of molasses (160 mg/l) compared to the concentration of PCE or *cis*-DCE. This was confirmed by the results of microcosms with an initial concentration of 16,000 µg/l of PCE and 80 mg/l of molasses that showed dechlorination to equal amounts of VC and ethene within 16 weeks. However, the exact amount of electron donor necessary for complete dechlorination could not be established, because it was not clear to what extent organic matter in the soil sample was used for biological dechlorination. Therefore it is recommended to calculate the amount of electron donor needed for complete reduction of the amount of chlorinated ethenes and alternative electron acceptors (nitrate and sulphate), and to add a five-fold excess of the calculated amount of electron donor.

Both PCE and *cis*-DCE were completely dechlorinated if they were individually spiked to the contaminated soil, indicating that microcosm studies may also be carried out with daughter products. Soil samples taken down gradient from the source zone may only contain lower chlorinated ethenes like *cis*-DCE and the dechlorination potential may therefore better be tested with the lower chlorinated *cis*-DCE as contaminant. It is recommended to determine the biodegradation potential in microcosm experiments by using the prevalent chlorinated ethene as main contaminant with an initial concentration as measured in the field.

No significant difference between the dechlorination of PCE was observed in the microcosms spiked with different electron donors. Complete dechlorination to ethene occurred within 6 weeks with molasses, a mixture of volatile fatty acids in compost percolate or lactate as electron donor. Microcosms in which the effect of an electron donor on dechlorination is investigated, should therefore be spiked with an electron donor which can or will be used in a stimulated bioremediation of a contaminated site (accepted by the regulatory authorities, non-toxic, relatively cheap and available in large quantities) and with sufficient nutrients (N and P).

The results of this project show that microcosm studies are a useful tool to provide evidence for the occurrence of dechlorination processes in the field. A protocol has been developed to determine the dechlorination potential of contaminated soil in anaerobic microcosm studies. This protocol has been validated and includes technical set-up, type and concentrations of chlorinated ethenes and carbon sources and monitoring tools. The protocol is suitable for general application and allows variation of several parameters, depending on research questions or site specific properties. The protocol will be published on the SKB website www.skb.nl.

CHAPTER 1

INTRODUCTION

1.1 Background

The use of chlorinated solvents by several branches of industry has led to a widespread contamination of soil with chlorinated compounds (like perchloroethene (PCE)). Several physical-chemical techniques are available to remediate these contaminated sites. However, during the last years there has been a shift from these conventional treatments (like pump and treat) towards *in situ* biological methods as an alternative for remediation of groundwater contaminated with chlorinated ethenes, whether or not in combination with conventional techniques to remove pure product or high levels of chlorinated ethenes.

A biological treatment of a contaminated site can consist of monitoring the naturally occurring biodegradation processes in a natural attenuation approach. By monitoring these processes it becomes clear if this approach is sufficient to protect potential receptors. Applying active in situ anaerobic dechlorination by adding a suitable electron donor and/or nutrients to the contaminated aquifer is also possible. In both cases it is important to determine the biodegradation potential for chlorinated ethenes of the soil on a contaminated site. To demonstrate the feasibility of a biological treatment at a site contaminated with chlorinated ethenes, groundwater characterizations in the field and microcosm experiments can be performed.

In scientific research and in bioremediation practice different methods to perform microcosm experiments are used. The procedures that are followed to perform batch experiments are important for the outcome of these experiments. The aim of this study is to develop a guideline for performing microcosm studies, which are used to determine the potential for anaerobic dechlorination of chlorinated ethenes in soil.

In the first phase of this project an inventory of the methods that are used for performing batch experiments by the laboratories and research institutes working on anaerobic dechlorination was made and three scientists in the field of dechlorination were visited at Cornell University, USA. The critical steps in the performance of these feasibility studies were identified. Based on the results a guideline for performing batch experiments was made. The results of the first phase are reported in the CUR/NOBIS report 97-4-04 'Selection and validation of a practical protocol anaerobic dechlorination - Phase 1: Inventory methods and work visit Cornell University USA' [Van der Waarde and Van Eekert, 1999].

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 and based on the results of the first phase two practical protocols were selected to be tested and verified in the following laboratory phase of this project. The results of this laboratory research are presented in this report.

1.2 Practical approach

The biodegradation potential for a location contaminated with chlorinated ethenes (mainly PCE (perchloroethene) and *cis*-DCE (*cis*-dichloroethene)) under different conditions was determined by performing batch experiments according to the protocols used by two research laboratories (Wageningen University and Bioclear). These protocols were selected because they comply with the guideline proposed in the first phase of this project [Van der Waarde and Van Eekert, 1999]. However, these protocols differ at some important points, like soil/liquid ratio, size of the batches

and used septa. In this research several parameters were varied and their influence on the outcome of the performed batch experiments was determined. The following parameters were tested:

- effect of the absolute and relative concentrations of electron donor and clorinated ethenes in the incubation;
- relation between dechlorination and electron donor concentration as determined with volatile fatty acid (VFA) analyses;
- effect of the type of the spiked chlorinated ethene on dechlorination (PCE or *cis*-DCE);
- effect of the type of electron donor used for the dechlorination.

Several general aspects of the used protocols were also tested:

- possible loss of the chlorinated compounds via leakage through the used septa (investigated during incubation of the flasks straight up or upside down);
- reproducibility of the batch incubations;
- effects of autoclaving.

CHAPTER 2

SELECTED PROTOCOLS FOR LABORATORY STUDY TO DEMONSTRATE DECHLORINATION

The protocols used to construct the microcosms are described in the following paragraphs. The complete description of the used protocols is given in the CUR/NOBIS report 'Selection and validation of a practical protocol anaerobic dechlorination - Phase 1: Inventory methods and work visit Cornell University USA' [Van der Waarde and Van Eekert, 1999].

2.1 Wageningen University, Laboratory of Microbiology, Wageningen, The Netherlands

In an anaerobic glove box the contaminated soil is homogenized to obtain a representative soil sample. The atmosphere in the glove box consists of nitrogen with a small amount of hydrogen. The hydrogen is used for the (catalysed) reduction of traces of oxygen to water in the atmosphere of the glove box. Microcosm studies are performed in 117 ml serum flasks with 20 g (dry weight) of the homogenized soil sample and 40 ml of liquid phase (consisting of anaerobic groundwater from the contaminated site and stock solutions of sulphide, resazurin and, if necessary, stock solutions of chlorinated ethene and electron donor). The microcosms are constructed in the anaerobic glove box. To prevent acidification of the microcosms a phosphate buffer is added (KH_2PO_4; 4.8 mM and Na_2HPO_4; 1.3 mM) to the groundwater. The microcosms are sealed with a viton stopper and an aluminium crimp cap. Sulphide ($Na_2S.9H_2O$) is added in a concentration of 1 mM (32 mg/l S^{2-}) to remove any traces of oxygen in the microcosms. Resazurin (0.45 mg/l) is added as a redox indicator.

To remove any traces of hydrogen in the headspace of the microcosms the headspace is exchanged with a mixture of nitrogen and carbon dioxide (80/20 % v/v).

To correct for abiotic losses of the contaminants a sterilized control batch experiment is performed. To sterilize the microcosms the microcosms are autoclaved three times for 20 minutes at 121 ºC at daily intervals to avoid microbial growth from spores.

Chlorinated ethenes are spiked after microcosm preparation, based on chlorinated ethene concentrations at the site. In microcosm studies that mimic natural conditions no further additions are made. To investigate the effect of addition of a suitable electron donor the tested electron donor is added in a electron donor:chlorinated ethene ratio ranging from 1:1 to 100:1 (mole/mole), depending on chlorinated ethene concentrations. Based on the used electron donor the nutrients N and P are added according to a ratio of C:N:P of 250:10:5 (on weight basis).

The microcosms are incubated statically in the dark for 4 to 6 months or until the formation of significant amounts of ethene takes place. The incubation temperature is between 10 and 30 ºC, depending on the research question. The dechlorination of chlorinated ethenes is determined by regular analyses for PCE, TCE, *cis*-DCE and *trans*-DCE, VC, ethene and ethane. Methane is also measured in the microcosms.

2.2 Bioclear Environmental Biotechnology, Groningen, The Netherlands

Under anaerobic conditions (glove bag with a nitrogen atmosphere with less than 0.1 % oxygen) a homogenized soil sample is prepared. Microcosm studies are carried out in 315 ml serum flasks with 150 g of the homogenized contaminated soil sample and a small amount of groundwater from the contaminated site (enough to obtain a water-saturated slurry). The microcosms

are sealed with an aluminium screw cap with a viton lined gas tight inlay septum. Sulphide (31 mg/l S^{2-}) is added to remove any traces of oxygen in the microcosms. Resazurin (5 mg/l) is added as a redox indicator.

To correct for abiotic losses of the contaminants a sterilized control batch experiment is performed. To sterilize the microcosms they are autoclaved for 1 hour at 121 ºC. After autoclaving mercury chloride ($HgCl_2$, 250 mg/l) and sodium azide (NaN_3, 500 mg/l) are added to maintain sterile conditions during the experiment. This control batch experiment is constructed the same way as the living microcosms except the use of sulphide to avoid precipitation of mercury with sulphide.

Chlorinated ethenes are spiked after microcosm preparation based on chlorinated ethene concentrations at the site. In microcosm studies that mimic natural conditions no further additions are made. In microcosm studies that are performed to investigate the effect of addition of a suitable electron donor, the tested electron donor is added in an electron donor:chlorinated ethene ratio ranging from 1:1 to 100:1 (mole/mole), depending on concentrations of chlorinated ethenes and alternative electron acceptors (like nitrate or sulphate). Based on the used electron donor the nutrients N and P are added according to a ratio of C:N:P of 250:10:5 (on weight basis).

The microcosms are incubated statically in the dark for 4 to 6 months or until the formation of significant amounts of ethene takes place. The incubation temperature is between 10 and 30 ºC, depending on the research question. The dechlorination of chlorinated ethenes is determined by regular analyses for PCE, TCE, *cis*-DCE and *trans*-DCE, VC, ethene and ethane. Methane is also measured in the microcosms.

CHAPTER 3

EXPERIMENTAL SET-UP

3.1 Soil samples

In the microcosm experiments, soil originating from a contaminated site in the northern part of The Netherlands was used. The aquifer at the site is contaminated with PCE and TCE (up to 1,700 µg/l and 3,200 µg/l, respectively) due to the activities of a dry-cleaning company. High concentrations of *cis*-DCE and VC (up to 11,000 µg/l and 300 µg/l, respectively), anaerobic intermediates in the dechlorination of PCE, have also been detected. This is a strong indication that anaerobic dechlorination of PCE by the indigenous bacterial population already occurs. Also, in previous microcosm experiments with soil material from this site we found complete dechlorination of PCE to ethene within 88 days under intrinsic or lactate stimulated conditions.

Soil samples were taken anaerobically with 'Akkerman' core samplers and were transported to the laboratory under cooled conditions. The soil material consisted mainly of clay. Some peat was also present. The used soil samples were homogenized under anaerobic conditions. The dry weight percentage of the soil sample was 70.9 %; the organic fraction was 3.4 % (in percent of the determined dry weight percentage). Groundwater samples were taken anaerobically from a monitoring well in the neighbourhood of the sampling point for soil material and were transported in completely filled glass flasks. Until construction of the microcosms the soil and groundwater samples were stored under anaerobic conditions at 10 ºC in the dark.

3.2 Construction of the microcosms

The microcosms used to perform the experiments were constructed as described in chapter 2. Each experiment was performed in triplicate to investigate the reproducibility of the experiments. Microcosms according to the protocol used by Wageningen University are referred to as WA-microcosms, microcosms according to the Bioclear protocol are coded BC-microcosms. After the necessary additions, the microcosms were incubated statically, straight up, in the dark at room temperature.

Depending on the research question, different concentrations of chlorinated ethenes or electron donor were added, different electron donors were used or the type of the spiked chlorinated ethene was changed. The set-up of the performed experiments is given in paragraph 3.4.

3.3 Analyses

To follow the course of the dechlorination of chlorinated ethenes to less chlorinated compounds or, eventually, ethene or ethane, analysis for chlorinated ethenes, ethene and ethane were performed. Methane was also measured. Substrate levels were determined by volatile fatty acid (VFA) analysis. Analysis for chlorinated ethenes, ethene, ethane, methane and VFAs were performed at the beginning of the experiment and after 4, 8 and 12 weeks of incubation.

Chlorinated ethenes
The concentrations of chlorinated ethenes present in the microcosms were quantified by headspace analyses. PCE, TCE, *cis*-DCE and *trans*-DCE were separated and detected by means of gas chromatography and a mass spectrometry detector (GC-MS). VC, ethene, ethane and methane were separated and detected by means of gas chromatography and a flame ionisation detector (GC-FID). Quantification was done by using external standards.

Volatile fatty acids
The concentration of volatile fatty acids (acetate (C_2), propionate (C_3), butyrate (C_4) and valerate (C_5)) was determined by extracting 1 ml of liquid from the microcosms. This sample was centrifuged for 5 minutes at 10,000 rpm. The supernatant was acidified to a pH below 2 with a 3 % formic acid solution and transferred to a 2 ml crimp seal vial. Separation and detection of the volatile fatty acids was performed by means of GC-FID. Quantification of the concentration of volatile fatty acids was done by using external standards.

3.4 Performed experiments

3.4.1 *Leakage test*
One of the possible explanations for decreasing concentrations of chlorinated ethenes without an increase in the concentrations of daughter products is leakage through the septa of the microcosms. To investigate the possible leakage of chlorinated ethenes in the microcosms used for the experiments described below, flasks used for the microcosms were incubated partially filled with water. The components spiked to these flasks were VC and ethene. Analysis for VC, ethene, ethane and methane were performed regularly at the beginning of the experiment and after 3, 6, 9 and 12 weeks of incubation.

Glass serum flasks (117 ml) used for the Wageningen University microcosms were filled with 40 ml of anaerobic demineralized water. The serum flasks were closed with a viton stopper and aluminium crimp cap. The flasks were spiked with 40 µl VC and 40 µl ethene. The flasks were incubated upside down and statically in the dark at room temperature. The experiment was performed in triplicate.

Glass flasks (315 ml) used for the Bioclear microcosms were filled with 150 ml of anaerobic demineralized water. The serum flasks were closed with an aluminium screw cap and a viton lined butyl rubber septum. The flasks were spiked with 100 µl VC and 100 µl ethene. Three flasks were incubated upside down, three flasks were incubated straight up. Also, in this experiment the effect of autoclaving the flasks was investigated by autoclaving one bottle of the triplicates for 1 hour at 121 ºC before spiking VC and ethene. The flasks were incubated statically in the dark at room temperature.

To sterilize the batches $HgCl_2$ (250 mg/l) and NaN_3 (500 mg/l) were added as a stock solution in anaerobic water. No sulphide was added. All actions were performed in an anaerobic glove bag.

3.4.2 *Initial concentration of chlorinated ethenes (experiment 1*)
To investigate the effect of the initial concentration of chlorinated ethenes on the dechlorination process, different concentrations of PCE were spiked to the microcosms. The effect of the initial concentration of PCE was determined using the protocols of Wageningen University and Bioclear.

Microcosms were constructed according to the protocols described in chapter 2. Molasses was used as electron donor. The amount of nitrogen and phosphorous already present in molasses was determined and extra nutrients were added according to a C:N:P ratio of 250:10:5. The additions to the different microcosms are summarized in table 1.

The dechlorination of PCE was followed by periodical analyses for chlorinated ethenes and ethene and ethane. Methane was also measured during incubation. Volatile fatty acids were not measured.

Table 1. Set-up experiment 1.

batch (code)	concentration PCE		concentration of molasses (mg/l)	ratio electron donor: chlorinated ethene (w/w)
	(µg/l)	(µmoles/batch)		
WA-PCE-1	8,000	1.96	160	20
WA-PCE-2	16,000	3.92	160	10
WA-PCE-3	100,000	24.13	160	1.6
BC-PCE-1	8,000	4.22	160	20
BC-PCE-4	16,000	8.44	160	10
BC-PCE-2	100,000	48.25	160	1.6

WA-PCE-1 and WA-PCE-2 were spiked with respectively 2.5 and 5 ml of a 130 mg/l stock solution of PCE in anaerobic groundwater from the location. WA-PCE-3 was spiked with 2.5 µl pure PCE. BC-PCE-1 and BC-PCE-4 were spiked with respectively 5 and 10 ml of a 140 mg/l stock solution of PCE in anaerobic groundwater from the location. BC-PCE-2 was spiked with 5 µl pure PCE.

Molasses was spiked as a solution in anaerobic demineralized water to a total concentration of 160 mg/l in the liquid phase of the microcosms.

3.4.3 *Type of the spiked chlorinated ethene (experiment 2)*

The effect of the type of the spiked chlorinated ethene on the dechlorination was investigated by spiking *cis*-DCE instead of PCE. This experiment was performed using the protocol of Wageningen University. To investigate the effect of the spiked concentration of *cis*-DCE the concentration was also varied in this experiment. Molasses, supplemented with the necessary nutrients, was used as electron donor. The experimental set-up is given in table 2.

The dechlorination of PCE was followed by periodical analyses for chlorinated ethenes and ethene and ethane. Methane was also measured during incubation. VFAs were not measured during incubation.

Table 2. Set-up experiment 2.

batch (code)	concentration *cis*-DCE		concentration of molasses (mg/l)	ratio electron donor: chlorinated ethene (w/w)
	(µg/l)	(µmoles/batch)		
WA-cDCE-1	5,000	2.06	160	32
WA-cDCE-2	10,000	4.13	160	16
WA-cDCE-3	60,000	24.78	160	2.7

The first analysis results of the microcosms constructed according to the protocol of Wageningen University for experiment 1 showed the presence of *cis*-DCE in the microcosms at a concentration of about 2 to 3 µmoles/batch. These concentrations of *cis*-DCE were already present in the used soil sample. The microcosms constructed to investigate the dechlorination with an initial concentration of 5,000 µg/l *cis*-DCE were therefore not spiked with *cis*-DCE. The microcosms that should have an initial concentration of 10,000 µg/l and 60,000 µg/l *cis*-DCE were spiked with the intended amount of *cis*-DCE (respectively as a stock solution of *cis*-DCE in anaerobic groundwater (80 mg/l) and as pure *cis*-DCE).

3.4.4 *Type of electron donor (experiment 3)*

The effect of the type of electron donor used to stimulate anaerobic dechlorination of PCE was determined by spiking three different electron donors to the microcosms. To construct the microcosms the Bioclear protocol was used.

The electron donors used in this experiment were molasses, a mixture of VFAs in compost percolate and lactate. The compost percolate that was used originated from a composting facility in Wijster, The Netherlands. According to an analysis for volatile fatty acids, only low concentrations of acetate were present. Therefore, acetate, propionate, butyrate and valerate were added to the compost percolate. The set-up of experiment 3 is given in table 3.

The initial concentration of molasses in the microcosms was 160 mg/l. This concentration was converted to the amount of electrons delivered when the molasses would be completely degraded. For this calculation it was assumed that molasses consists of 50 % sugar ($C_6H_{12}O_6$). The VFA mixture in compost percolate or lactate was added in similar concentrations (based on amount of electrons). Additional nutrients were added according to a C:N:P ratio of 250:10:5. A correction was made for the nutrients already present in the used electron donors.

The dechlorination of PCE was followed by periodical analyses for chlorinated ethenes and ethene and ethane. Methane and volatile fatty acids were also measured during incubation.

Table 3. Set-up experiment 3.

batch (code)	concentration PCE (µg/l)	electron donor used
BC-PCE-4	16,000	molasses
BC-PCE-6	16,000	VFAs in compost percolate
BC-PCE-7	16,000	lactate

3.4.5 *Initial concentration of electron donor (experiment 4)*

To investigate the effect of different initial concentrations of electron donor (molasses), different concentrations of molasses were spiked to the microcosms constructed according to the protocols of Wageningen University and Bioclear. Nutrients were added according to a C:N:P ratio of 250:10:5. The additions made to the microcosms in this experiment are summarized in table 4.

The dechlorination of PCE was followed by periodical analyses for chlorinated ethenes and the harmless end products ethene and ethane. Methane and volatile fatty acids were also measured during incubation.

Table 4. Set-up experiment 4.

batch (code)	concentration PCE		concentration of molasses (mg/l)	ratio electron donor: chlorinated ethene (w/w)
	(µg/l)	(µmoles/batch)		
WA-PCE-4	16,000	8.44	80	5
WA-PCE-2	16,000	8.44	160	10
WA-PCE-5	16,000	8.44	1,600	100
BC-PCE-3	16,000	8.44	80	5
BC-PCE-4	16,000	8.44	160	10
BC-PCE-5	16,000	8.44	1,600	100

3.4.6 *Abiotic control experiments*

To correct for possible abiotic losses an abiotic control experiment for the microcosms constructed according to Bioclear and Wageningen University protocol was performed. The abiotic control experiments were performed according to the set-up given in table 5.

Table 5. Set-up abiotic control experiments.

batch (code)	concentration PCE		concentration *cis*-DCE	
	(µg/l)	(µmoles/batch)	(µg/l)	(µmoles/batch)
BC-abio	16,000	8.44	10,000	4.13
WA-abio	16,000	8.44	10,000	4.13

The batches were sterilized according to the guidelines described in the used protocols. No electron donor was added. PCE and *cis*-DCE were added as stock solutions in anaerobic groundwater.

The losses of PCE and/or *cis*-DCE were followed by analyses for chlorinated ethenes and ethene and ethane at the beginning and after 12 weeks of incubation. Methane was also measured during incubation.

3.5 **Calculations**

To compare the results of the dechlorination in the microcosms spiked with different (concentrations of) chlorinated ethenes or electron donor, the degree of dechlorination was calculated for each sampling round. The degree of dechlorination represents the amount of chlorine that is removed from the original contamination. The degree of dechlorination is calculated based on the results of the analyses for chlorinated ethenes, ethene and ethane according to the following equation:

$$\frac{[TCE] + 2[DCEs] + 3[VC] + 4[ethene] + 4[ethane]}{4 \cdot ([PCE] + [TCE] + [DCEs] + [VC] + [ethene] + [ethane])} \cdot 100\ \%$$

The degree of dechlorination can vary from 0 % (only PCE present) to 100 % (only ethene or ethane present).

CHAPTER 4

RESULTS

The results of the performed experiments are given in this chapter. Each experiment is discussed in a separate paragraph. In this chapter the results of the calculated degree of dechlorination of experiments 1 to 4 are given in figures, calculated according to the method described in chapter 3. The analysis results are presented in figures (see appendix A to appendix D). In these figures the concentrations of *trans*-DCE are not given; this compound was measured but was not formed in concentrations above the detection limit. Values given in the figures in this chapter represent the average of triplicate incubations. The error bars indicate the standard deviations based on triplicate incubations.

4.1 Leakage test

The results of the leakage test for the flasks constructed according to Wageningen University protocol are given in figure 1. The concentrations of VC and ethene during the leakage test are given in µg/l in the water phase, assuming that the total mass of VC and ethene is dissolved (sum total mass in gas phase and sum total mass in liquid phase, given as mass per unit liquid).

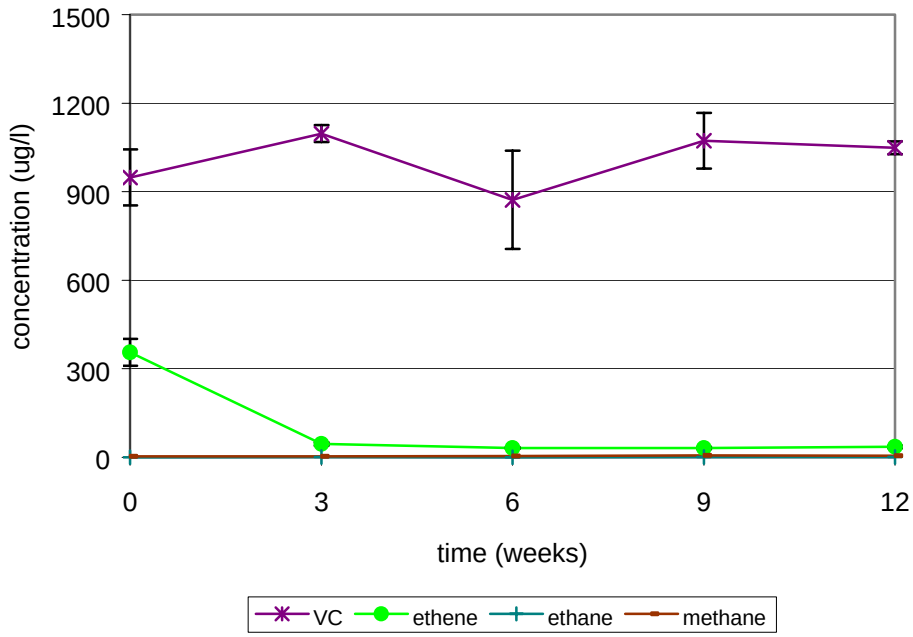

Fig. 1. Analysis results leakage test Wageningen University flasks, incubation upside down.

The (spiked) amount of VC did not decrease during the incubation period. Leakage of VC did not occur. However, the amount of ethene spiked to the flasks decreased during the first three weeks of incubation with a factor 10 from about 400 µg/l to 40 µg/l. After three weeks of incubation the concentration of ethene did not decrease anymore. Ethane and methane (not spiked to the flasks) were measured at low concentrations during the experiment. No significant changes of the measured concentration of ethane or methane occurred.

The results of the leakage test for the flasks constructed according to the protocol of Bioclear are given in figure 2 (incubation upside down) and figure 3 (incubation straight up).

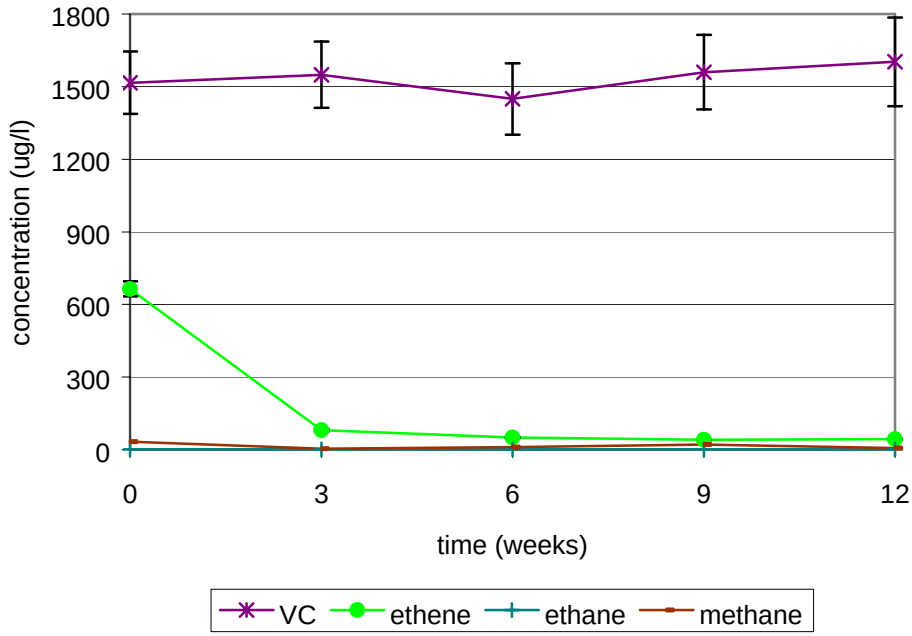

Fig. 2. Analysis results leakage test Bioclear flasks, incubation upside down.

During the incubation period of 12 weeks the concentration of VC did not decrease. No leakage of VC occurred. The concentration of ethene did decrease with a factor 10 during the first 3 weeks of incubation. These results are comparable with the results of the Wageningen University flasks.

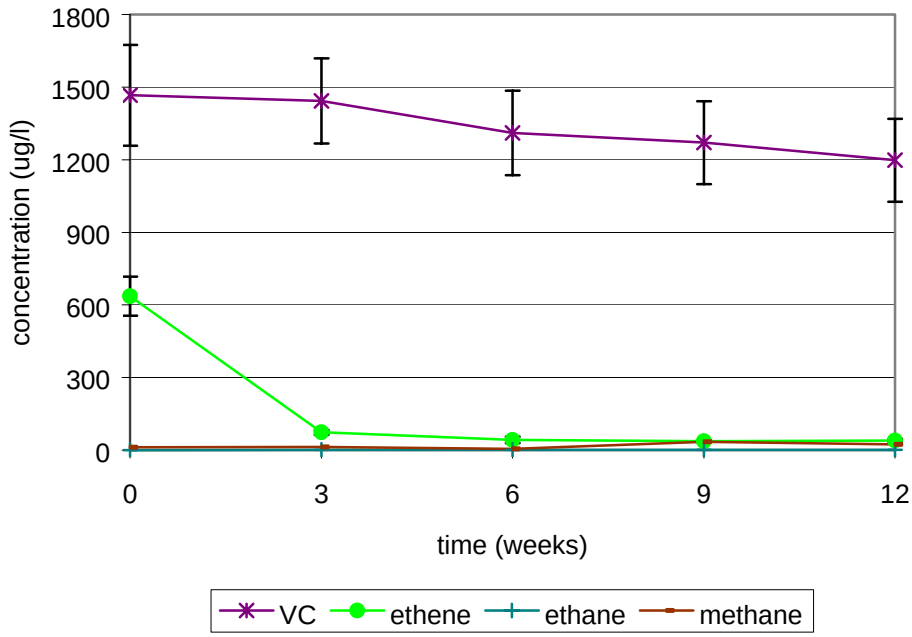

Fig. 3. Analysis results leakage test Bioclear flasks, incubation straight up.

During incubation no significant decrease of the spiked concentration of VC occurred. However, also in these flasks a decrease of the ethene concentration (with a factor 10) was observed.

Autoclaving the flasks did not influence the outcome of the experiment; the flasks that were autoclaved showed the same results as the flasks that were not autoclaved.

4.2 Initial concentration of chlorinated ethenes (experiment 1)

In this experiment the influence of the initial concentration of PCE on the anaerobic dechlorination of chlorinated ethenes was investigated. This experiment was performed using the protocols of both Wageningen University and Bioclear. The results of the Wageningen University microcosms with an initial concentration of 100,000 µg/l PCE are given in figure 4.

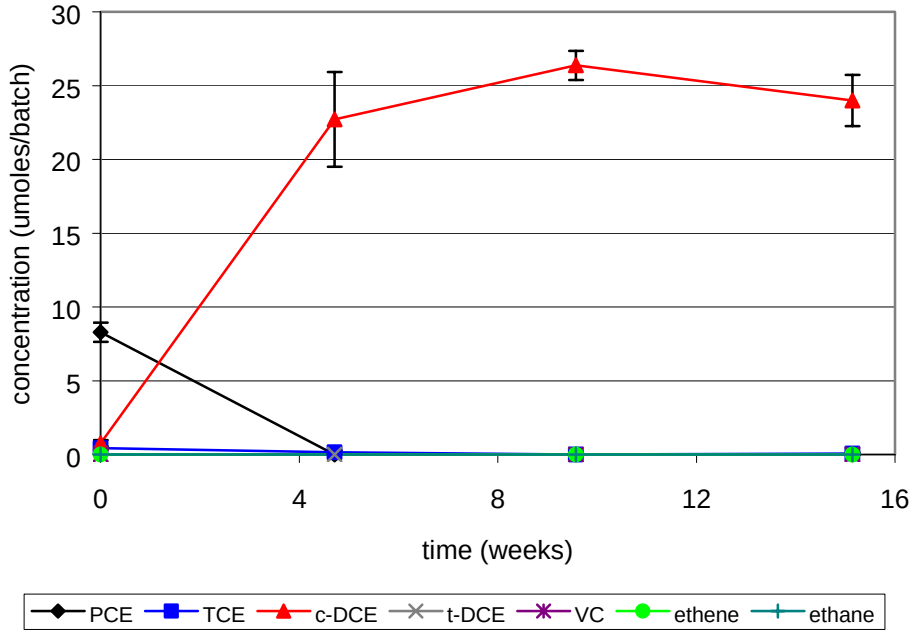

Fig. 4. Dechlorination of PCE in a Wageningen University microcosm spiked with 100,000 µg/l PCE.

Within 5 weeks dechlorination of the spiked PCE to *cis*-DCE occurred. However, no further dechlorination was observed during the incubation period of 15 weeks. The concentration of *cis*-DCE remained constant after 5 weeks of incubation and no formation of VC, ethene or ethane was observed within the incubation period of 15 weeks. The results of the other microcosms spiked with PCE showed similar results; dechlorination of the spiked PCE to *cis*-DCE occurred within 5 weeks but no VC, ethene or ethane was formed. Based on these results and the results of the previously performed microcosm experiments with soil samples from this site (complete dechlorination to ethene within 88 days) it was concluded that the used soil material was no longer capable to dechlorinate PCE completely. This experiment was therefore finished and soil material of a different site was used to repeat experiment 1 and to perform experiment 2, 3 and 4.

For the continuation of the microcosm experiments soil originating from a contaminated site in the western part of The Netherlands was used. The aquifer at the site is mainly contaminated with PCE and TCE (600 mg/kg dry weight and 60,000 mg/kg dry weight, respectively) due to the activities of a galvanizing company. In the source zone pure product is present. High concentrations of *cis*-DCE (40,000 µg/l) and VC (10,000 µg/l), anaerobic intermediates in the dechlorination of PCE, are also present. This is a strong indication that anaerobic dechlorination of PCE and TCE by the indigenous bacterial population already occurs.

Soil samples were taken anaerobically with 'Akkerman' core samplers and were transported to the laboratory under cooled conditions. The soil material consisted mainly of clay and peat. The used soil samples were homogenized under anaerobic conditions. The dry weight percentage of the soil sample was 77.1 %; the organic fraction was 3.8 % (in percent of the determined dry weight percentage). Groundwater samples were taken anaerobically from a monitoring well in the neighbourhood of the sampling point for soil material and were transported in completely filled glass flasks. Until construction of the microcosms the soil and groundwater samples were stored under water at 10 ºC in the dark.

4.3 Initial concentration of chlorinated ethenes (experiment 1, repeated)

The influence of the initial concentration of PCE on the anaerobic dechlorination of chlorinated ethenes was investigated by repeating experiment 1 with soil material from a different site (see above). This experiment was performed using the protocols of both Wageningen University and Bioclear. The analysis results are given in appendix A.

4.3.1 *Results of microcosms Wageningen University*

In the microcosms constructed according to the protocol used by Wageningen University and spiked with 8,000 µg/l or 16,000 µg/l PCE, PCE was completely dechlorinated to ethene within 6 weeks of incubation. The dechlorination of PCE in the microcosms spiked with 8,000 µg/l is given in figure 5.

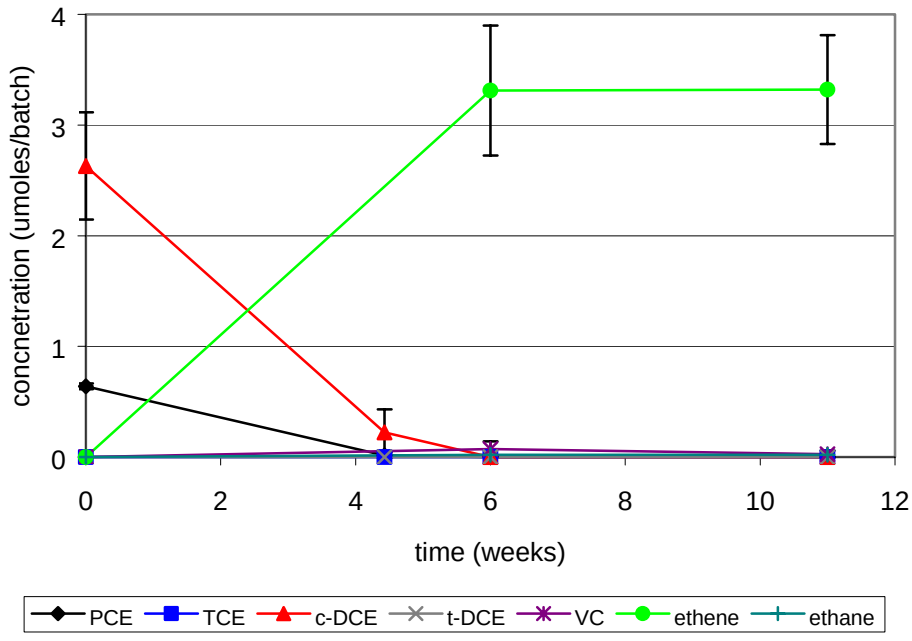

Fig. 5. Dechlorination of PCE in the Wageningen University microcosms spiked with 8,000 µg/l PCE.

Based on the analysis results the degree of dechlorination was calculated for each point in time (according to the equation given in chapter 3). In figure 6 the results of the Wageningen University microcosms for experiment 1 are summarized by calculating the course of the degree of dechlorination during the incubation period.

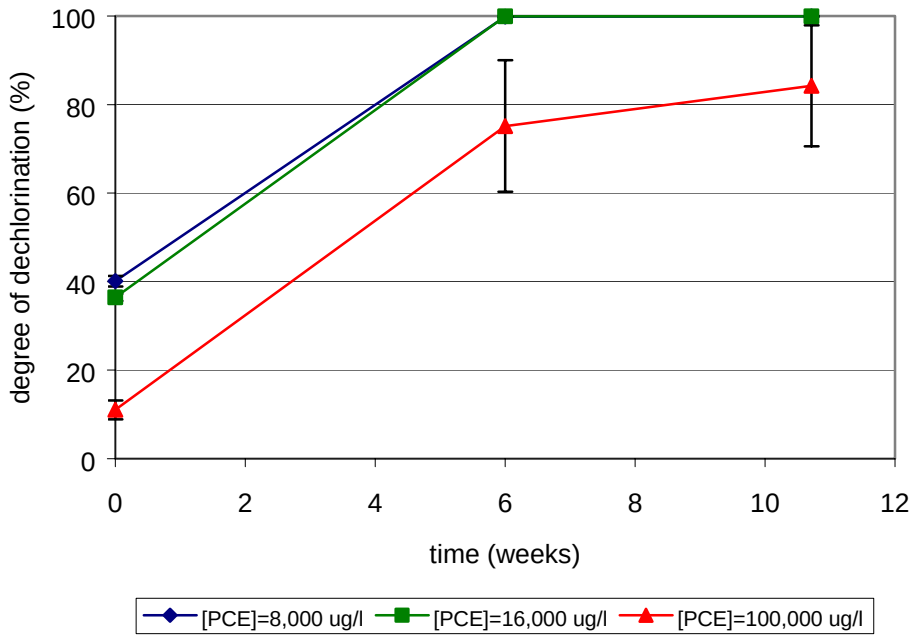

Fig. 6. Effect of initial PCE concentration on dechlorination in Wageningen University microcosm experiments.

Complete dechlorination occurred in the microcosms spiked with 8,000 µg/l PCE and 16,000 µg/l PCE; the degree of dechlorination after 6 weeks of incubation is 100 %. No significant difference could be observed in dechlorination in the microcosms spiked with 8,000 µg/l PCE or 16,000 µg/l PCE. In the microcosms spiked with 100,000 µg/l PCE is dechlorinated to mainly VC within 11 weeks. VC was dechlorinated to ethene at very low rates. The average degree of dechlorination increases form 11 % at the beginning of the experiment (mainly PCE present) to 84 % after 11 weeks of incubation (VC and ethene present).

The standard deviation between the triplicate microcosms with 100,000 µg/l PCE is relatively high because in one of the triplicate incubations spiked with 100,000 µg/l PCE, complete dechlorination to ethene occurred within 11 weeks. However, the recovered amount of ethenes after 11 weeks does not correspond to the spiked amount of PCE and the amount of *cis*-DCE already present in the soil.

The microcosms spiked with 100,000 µg/l PCE were analysed again after 16 weeks of incubation. Unfortunately, the results of the analysis for chlorinated ethenes after 16 weeks of incubation could not be interpreted because of problems with the analysis equipment.

4.3.2 *Results of microcosms Bioclear*

In the microcosms constructed according to the protocol of Bioclear a rapid dechlorination of PCE occurred. After 4 weeks of incubation no PCE, TCE or *cis*-DCE could be detected in the microcosms spiked with 8,000 µg/l PCE or 16,000 µg/l PCE. Therefore the second analysis was advanced. This second analysis after 6 weeks of incubation showed a complete dechlorination of the chlorinated ethenes to ethene. This was confirmed by an analysis after 8 weeks. In figure 7 the results of the Bioclear microcosms for experiment 1 are summarized (by calculating the degree of dechlorination for each time point).

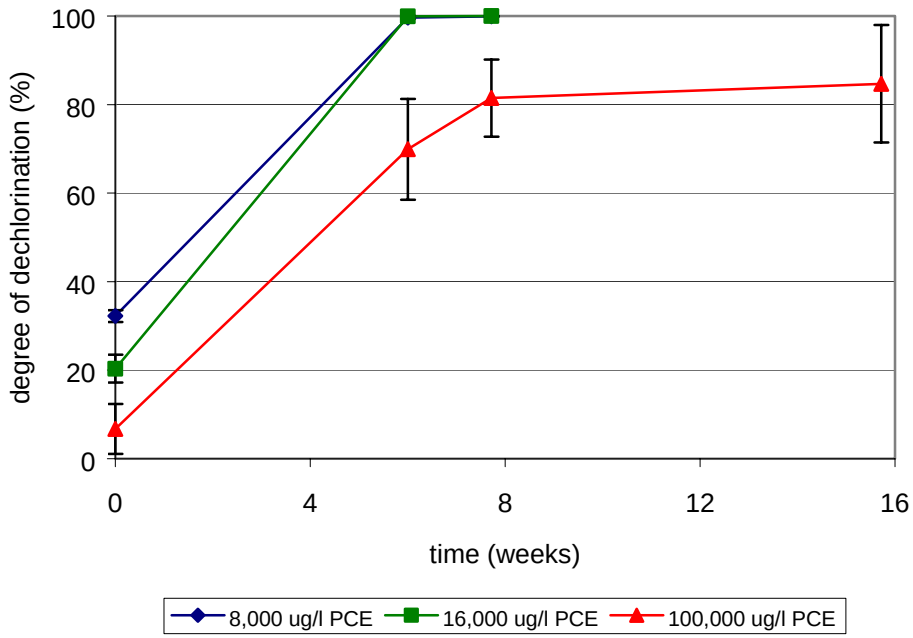

Fig. 7. Effect of initial PCE concentration on dechlorination in Bioclear microcosm experiments.

No significant difference could be detected in dechlorination of chlorinated ethenes with an initial concentration of 8,000 or 16,000 µg/l PCE. In these microcosms, complete dechlorination of PCE occurred within 6 weeks.

In the microcosms spiked with 100,000 µg/l, PCE was dechlorinated to (equal amounts of) *cis*-DCE and VC within 4 weeks. After 4 weeks further dechlorination occured to VC; after 8 weeks no *cis*-DCE was present in the headspace of the microcosms. However, VC was not dechlorinated to ethene within 16 weeks of incubation.

In one of the triplicate incubations with 100,000 µg/l PCE spiked complete dechlorination to ethene occurred within 16 weeks. However, after 16 weeks only 30 µmoles of total ethenes was measured. This does not correspond to the spiked amount of PCE and the amount of *cis*-DCE already present in the soil. Also, in the other two microcosms of the triplicate incubations significantly higher amounts of total ethenes were measured after 16 weeks.

4.4 Type of the spiked chlorinated ethene (experiment 2)

The results of the microcosms spiked with different concentrations of *cis*-DCE are given in figure 8. The microcosms were constructed according to the protocol used by Wageningen University. The analysis results are given in appendix B.

Complete dechlorination of *cis*-DCE to ethene was observed within 14 weeks in the microcosms spiked with 5,000 µg/l or 10,000 µg/l *cis*-DCE. No significant differences were found in dechlorination for the microcosms spiked with 5,000 µg/l *cis*-DCE or 10,000 µg/l *cis*-DCE. In the microcosms spiked with 60,000 µg/l, *cis*-DCE was partially dechlorinated to VC within 9 weeks. In one of the triplicate incubations ethene was also detected. However, after 9 weeks VC was still present in this microcosm. In the other two microcosms no ethene was detected. Unfortunately, the results of the last sampling round for the microcosms spiked with 60,000 µg/l *cis*-DCE (after 14 weeks of incubation) were not interpretable because of problems with the analysis equipment.

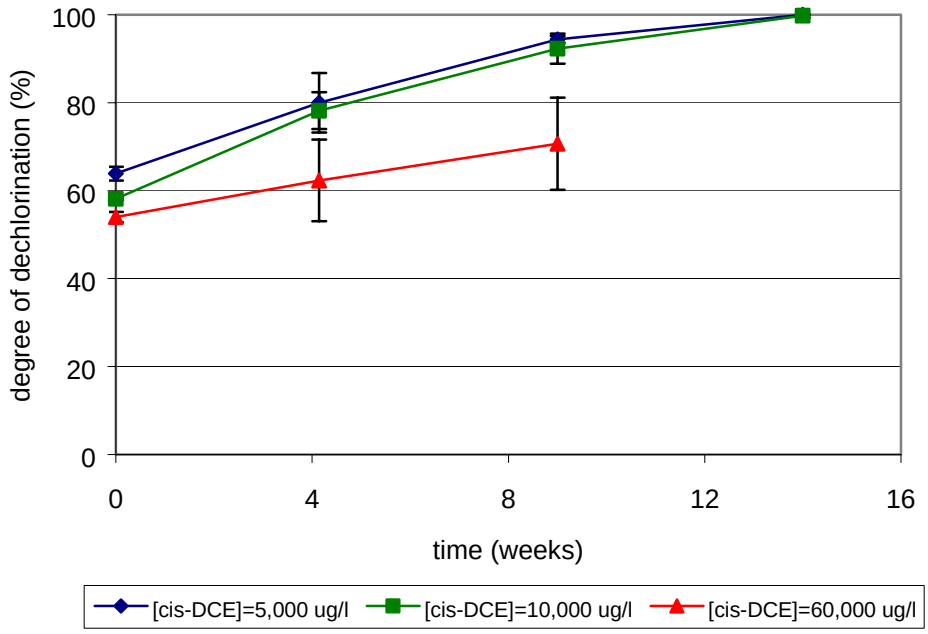

Fig. 8. Effect of initial *cis*-DCE concentration on dechlorination in Wageningen University microcosm experiments.

4.5 Type of electron donor (experiment 3)

To investigate the effect of the used electron donor on the dechlorination of PCE three different electron donors were added to the microcosms. The tested electron donors were molasses, a mixture of VFAs in compost percolate and lactate. The microcosms were constructed according to the Bioclear protocol and spiked with 16,000 µg/l PCE. The analysis results are given in appendix C.

The course of the dechlorination in the microcosms spiked with different electron donors is given in figure 9. In most microcosms, complete dechlorination occurred within 6 weeks. In general, no significant difference in dechlorination in the microcosms spiked with different electron donors was observed.

After 6 and 8 weeks of incubation a relatively high standard deviation of the analyses results of the microcosms spiked with lactate was observed (see appendix C).

This is caused by the results of one of the triplicate incubations. In this microcosm (microcosm 7c), VC was still present after 8 weeks and therefore these microcosms were analysed again after 16 weeks. Complete dechlorination in microcosm 7c was observed within 16 weeks. The decrease of ethene in microcosm 7a (see appendix C) is probably caused by a leaking septum. This was confirmed by the colour of the liquid phase. Introduction of oxygen to this microcosm caused oxidation of reduced iron (Fe(II)) to insoluble, brown-coloured Fe(III) (Fe-oxides).

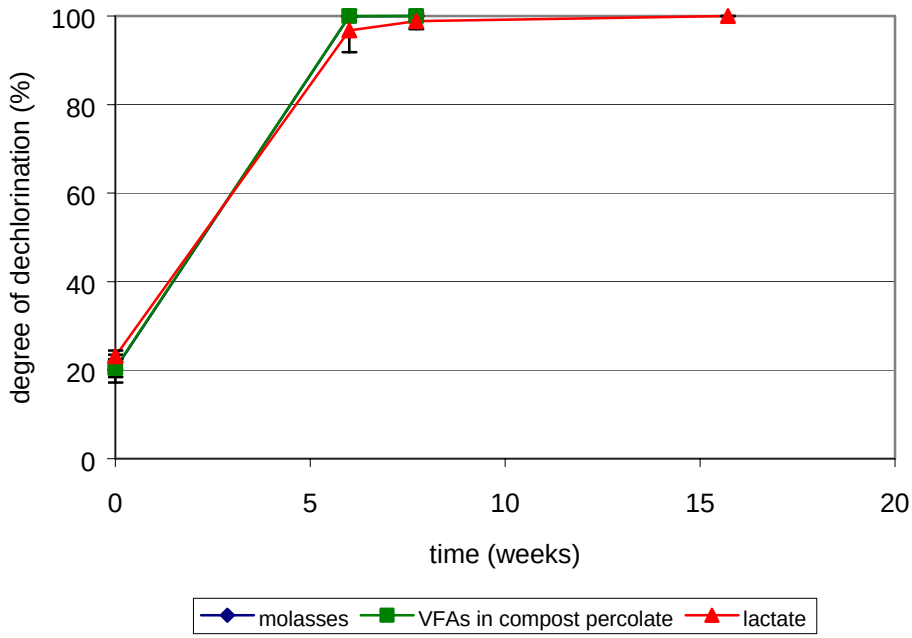

Fig. 9. Effect of electron donor on dechlorination in Bioclear microcosm experiments.

The results of the VFA and methane analysis for the microcosms spiked with different electron donors are given in figure 10 to 12.

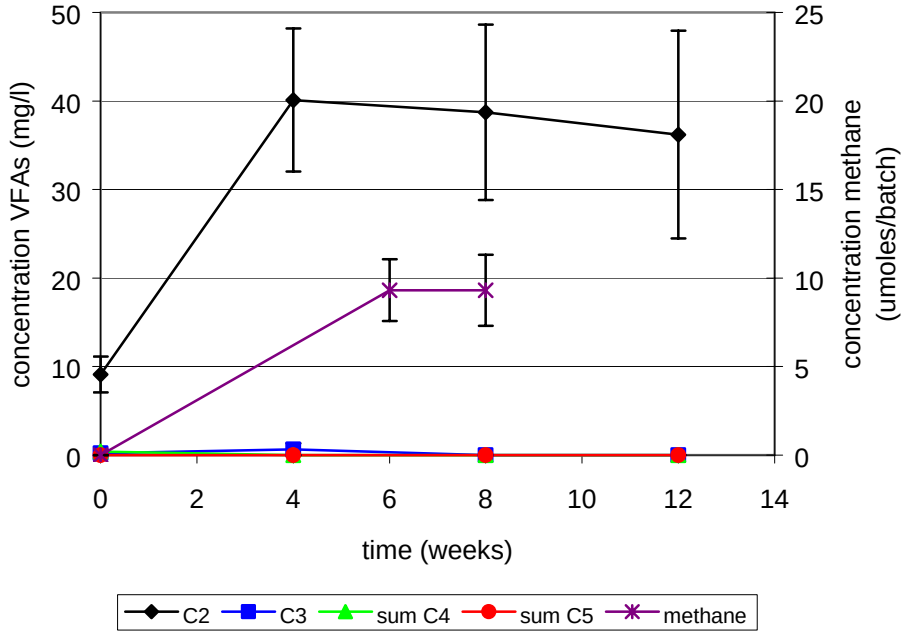

Fig. 10. Substrate levels and end products in the microcosms spiked with molasses.

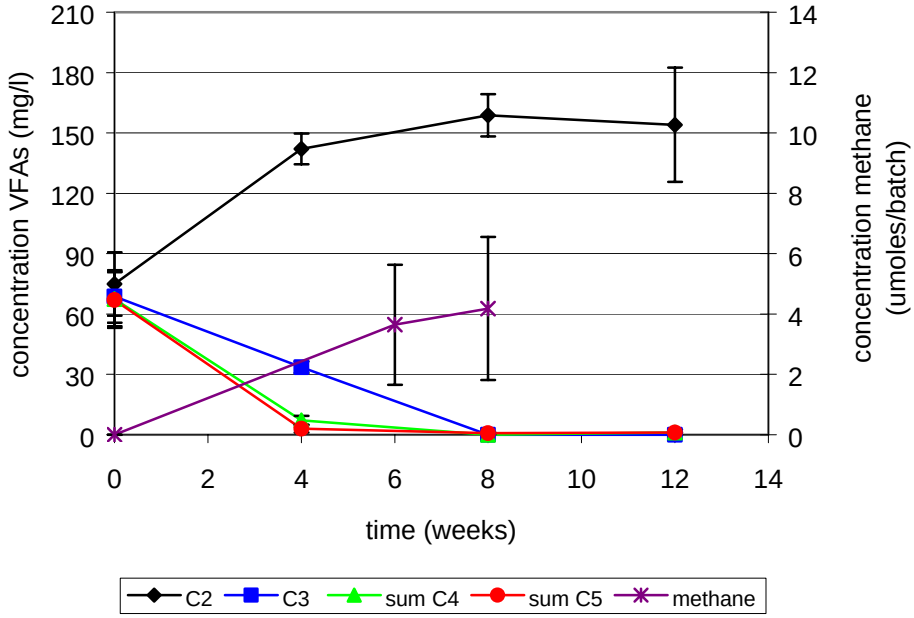

Fig. 11. Substrate levels and end products in the microcosms spiked with VFAs in compost percolate.

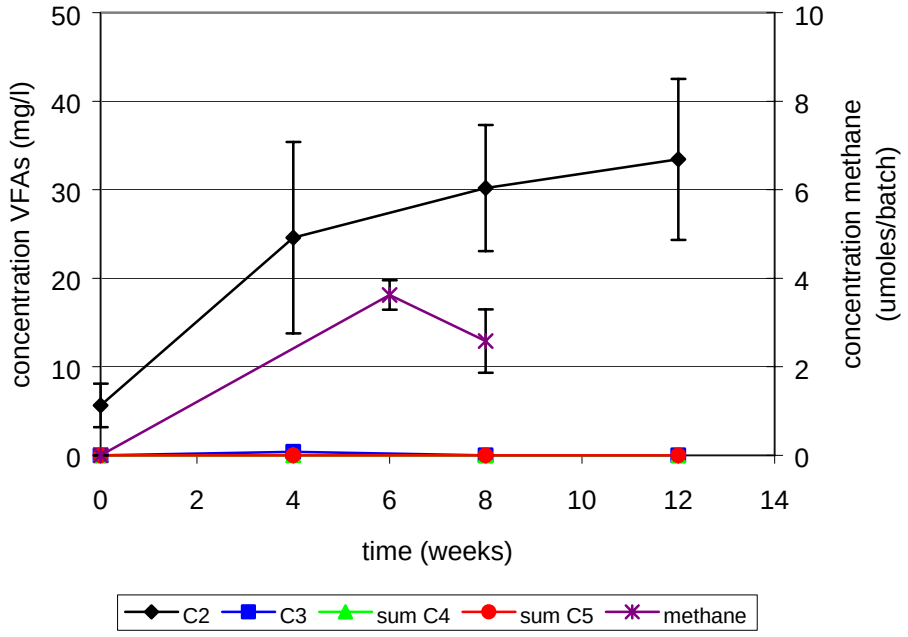

Fig. 12. Substrate levels and end products in the microcosms spiked with lactate.

Molasses and lactate were degraded to acetate and small amounts of propionate. No C_4 or C_5 fatty acids were found (analysed as i-C_4 and n-C_4, two isomers of butyrate and b-C_5 and n-C_5, two isomers of valerate). After 4 weeks of incubation the concentration of acetate did not change significantly, indicating a rapid degradation of molasses and lactate to acetate. In the microcosms spiked with a mixture of VFAs in compost percolate propionate, butyrate and valerate were rapidly degraded to acetate. After 4 weeks of incubation the concentration of acetate remained constant; no significant degradation of acetate was observed within 12 weeks of incubation.

4.6 Initial concentration of electron donor (experiment 4)

The effect of the initial concentration of electron donor (molasses) on the dechlorination process of PCE was determined by spiking molasses in different concentrations to microcosms constructed according to the protocols of Wageningen University and Bioclear. The analysis results are given in appendix D.

4.6.1 *Results of microcosms Wageningen University*

The course of the dechlorination in the microcosms with different concentrations of molasses and constructed according to the protocol used by Wageningen University are given in figure 13.

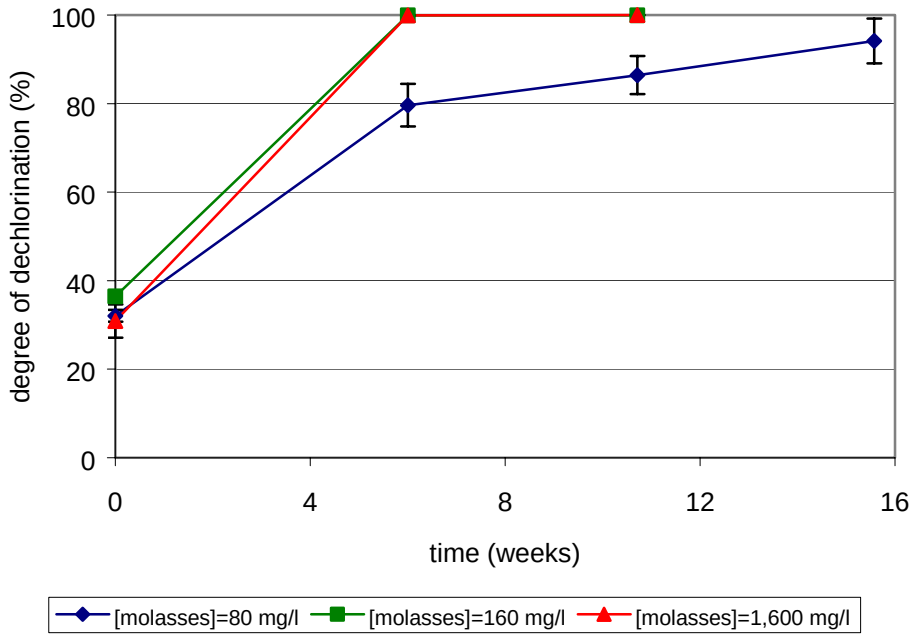

Fig. 13. Effect of initial electron donor concentration (molasses) on dechlorination in Wageningen University microcosm experiments

In the microcosms constructed according to the protocol of Wageningen University no significant effect of the initial concentration of molasses on the dechlorination of chlorinated ethenes could be detected between the microcosms spiked with 160 mg/l or 1,600 mg/l molasses. Dechlorination of PCE to ethene occurred within 6 weeks. However, the dechlorination process in the microcosms spiked with 80 mg/l molasses occurred significantly slower. In these microcosms PCE was dechlorinated to mainly VC and small amounts of ethene within 11 weeks. An extra analysis after 16 weeks was performed, but the dechlorination was only completed within this period in one of the triplicate incubations. In the other two microcosms VC was still present.

In the microcosms constructed according to the protocol of Wageningen University VFAs and methane were analysed during incubation. The results are given in figures 14 to 16 (microcosms with an initial concentration of molasses of 80 mg/l, 160 mg/l and 1,600 mg/l, respectively).

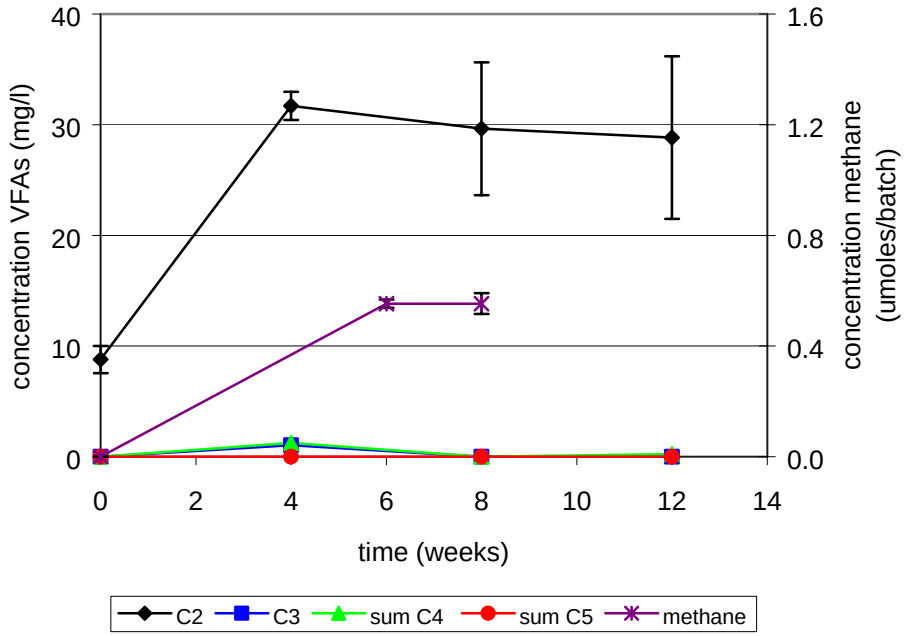

Fig. 14. Concentrations of VFAs and methane in the microcosms with an initial concentration of 80 mg/l molasses.

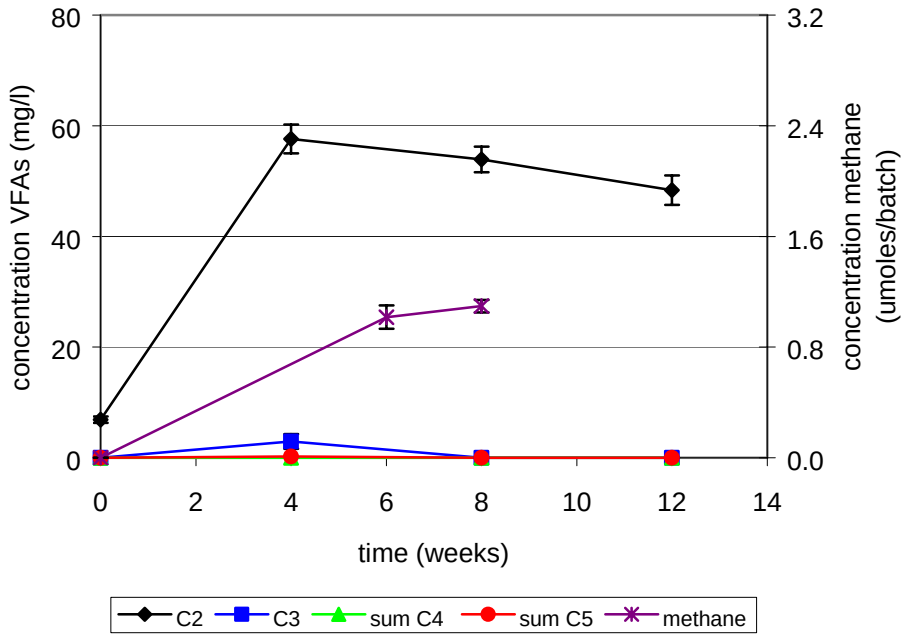

Fig. 15. Concentrations of VFAs and methane in the microcosms with an initial concentration of 160 mg/l molasses.

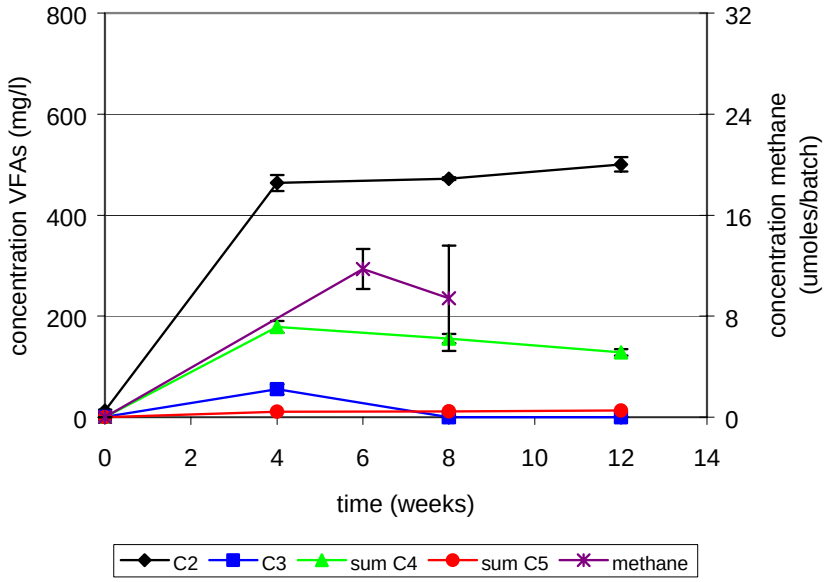

Fig. 16. Concentrations of VFAs and methane in the microcosms with an initial concentration of 1,600 mg/l molasses.

During the first 4 weeks of incubation molasses is degraded to mainly acetate (C_2) and methane. After 4 weeks the concentration of acetate and methane remains constant over the remaining of the incubation period. In the microcosms spiked with 80 or 160 mg/l molasses propionate (C_3), butyrate (given as the sum of i-C_4 and n-C_4, two isomers of butyrate) and valerate (sum of b-C_5 and n-C_5) were detected in relatively low concentrations during the incubation period. In the microcosm spiked with 1,600 mg/l molasses butyrate was detected in higher concentrations.

4.6.2 *Results of microcosms Bioclear*

Based on the analysis results of the Bioclear microcosms with different concentrations of molasses, the degree of dechlorination was calculated according to the method described in chapter 3. The course of the dechlorination process is given in figure 17.

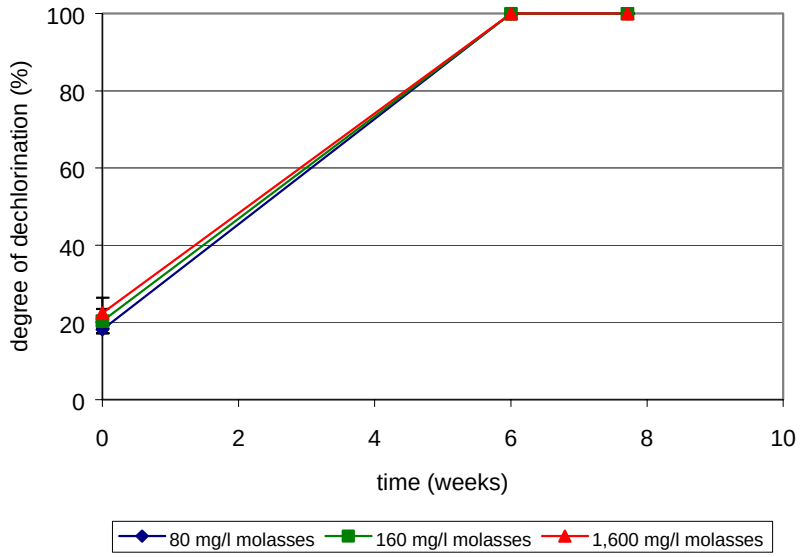

Fig. 17. Effect of initial electron donor concentration (molasses) on dechlorination in Bioclear microcosm experiments.

Complete dechlorination of PCE (and *cis*-DCE already present in the soil) to ethene occurred within 6 weeks. No significant difference in dechlorination in the microcosms spiked with different concentrations of molasses were observed.

4.7 **Abiotic control experiments**

To correct for possible abiotic losses an abiotic control experiment for the microcosms constructed according to Bioclear and Wageningen University protocol was performed. The results of these abiotic control experiments are given in table 6.

Table 6. Results abiotic control experiments.

microcosm (code)	component	results t = 0 weeks (µmoles/batch)	results t = 16 weeks (µmoles/batch)
WA-abio	PCE	2.5 ± 0.5	2.0 ± 0.2
	TCE	0	0
	cis-DCE	3.5 ± 0.2	4.5 ± 0.3
	trans-DCE	0.1 ± 0.0	0.1 ± 0.0
	VC	n.a.[1)]	< d.l.[2)]
	ethene	n.a.	< d.l.
	ethane	n.a.	< d.l.
BC-abio	PCE	4.3 ± 0.9	2.4 ± 2.3
	TCE	0	0
	cis-DCE	6.88 ± 1.5	3.3 ± 2.3
	trans-DCE	0.23 ± 0.0	0
	VC	n.a.	< d.l.
	ethene	n.a.	< d.l.
	ethane	n.a.	< d.l.

1) not analysed
2) concentration below the detection limit

The high standard deviations for the results of PCE and *cis*-DCE of the microcosms constructed according to Bioclear protocol are caused by one erratic analysis result of the triplicate incubation. If this result is not taken into account no significant differences in the concentrations at the beginning and after 16 weeks are found.

During the incubation period of 16 weeks no dechlorination of PCE or *cis*-DCE occurred in the microcosms constructed according to the protocols of Wageningen University and Bioclear. The concentrations remained constant. Also, no dechlorination products like VC or ethene were found. No abiotic losses of chlorinated ethenes occurred during the incubation period. Therefore the results of the biotic microcosms were not corrected for abiotic losses.

CHAPTER 5

DISCUSSION

The results presented in chapter 4 are discussed in this chapter. In each paragraph a research question given in the introduction is discussed.

5.1 Leakage test

The flasks constructed according to Wageningen University and Bioclear protocol are gas tight. No decrease of VC occurred in the flasks during an incubation period of 12 weeks. The results are not significantly influenced by incubation straight up or upside down or by autoclaving.

The concentration ethene decreased during the first three weeks of incubation. After three weeks the concentration of ethene did not decrease anymore during the rest of the incubation period. The decrease in concentration was comparable in all the performed experiments (Wageningen University upside down, Bioclear upside down and straight up); the concentration ethene decreased by a factor 10 during the first three weeks of incubation. In most microcosm experiments performed with contaminated soil complete dechlorination of PCE and *cis*-DCE occurred within 6 weeks. After this complete dechlorination, the concentration of ethene remained constant in the microcosms for a period of at least 10 weeks (experiment 3, Bioclear microcosms and experiment 4, Wageningen University microcosms). No sound explanation was found for the decreasing concentration of ethene. The decreasing concentrations of ethene in the flasks used for the leakage experiment could be caused by a chemical reaction in the flasks, possibly by a reaction between ethene and NaN_3 and/or $HgCl_2$.

5.2 Effect of absolute and relative concentrations of electron donor and chlorinated ethene

The initial concentration of chlorinated ethenes (PCE and *cis*-DCE) had no effect on the dechlorination rate in the range between 8,000 µg/l and 16,000 µg/l PCE or 5,000 µg/l and 10,000 µg/l *cis*-DCE. Complete dechlorination occurred in the microcosms spiked with these concentrations of PCE or *cis*-DCE. However, since dechlorination was already complete at the time of the first sampling round, possible differences in dechlorination rates could have occurred in the first few weeks of the experiment.

No complete dechlorination was observed in the microcosms spiked with 100,000 µg/l PCE or 60,000 µg/l *cis*-DCE. However, dechlorination to VC and small amounts of ethene was observed, so no toxicity effects of the high chlorinated ethene concentration occurred. The dechlorination of chlorinated ethenes in the microcosms spiked with 100,000 µg/l PCE and 60,000 µg/l *cis*-DCE is probably limited by relatively low concentrations of electron donor (ratio electron donor:PCE 1.6:1 and ratio electron donor:*cis*-DCE 2.7:1 on weight basis). This is confirmed by the results of the WA microcosms with an initial concentration of molasses of 80 mg/l (ratio electron donor:chlorinated ethene 5:1 on weight basis). In these microcosms the dechlorination process occurred significantly slower compared to the microcosms with higher concentrations of molasses.

The results of one of the microcosms of the triplicate incubations (Bioclear protocol microcosm 2a and WA microcosm 3a; see appendix A), in which complete dechlorination of high concentrations of PCE did occur, could be caused by losses of PCE during the spiking. Insufficient spiking of PCE may not have resulted in a low electron donor:chlorinated ethene ratio. This may also be an explanation for the low amounts of total ethenes measured.

With the used soil sample, a ratio electron donor:chlorinated ethene of 10:1 on weight basis (or 13:1 on electron equivalent basis) is sufficient to dechlorinate the chlorinated ethenes that were present in the beginning of the experiment. However, other electron donors that were already present in the soil, like organic matter, may have contributed to anaerobic dechlorination. The soil was a peaty clay, containing high concentrations of organic matter. Also, after extractive analysis of a homogenized soil sample it appeared that the soil material used in the experiments was also contaminated with small amounts of BTEX (benzene, toluene, ethylbenzene and xylenes), up to 60 mg/kg dry weight (sum BTEX). This could have had an effect on the dechlorination of chlorinated ethenes. It is assumed that anaerobic degradation products of BTEX can support the dechlorination process. The degradation products can be used directly as an electron donor for the reductive dechlorination or the degradation of BTEX reduces the conditions in the contaminated aquifer (use of for instance sulphate) and therefore stimulates the reductive dechlorination. These effects cannot be excluded since no control experiment was performed without addition of an electron donor. Therefore, it is advised to calculate the amount of electron donor needed for complete reduction of the amount of chlorinated ethenes and alternative electron acceptors (nitrate and sulphate), and to add a five-fold excess of the calculated amount of electron donor.

5.3 Effect of electron donor concentration as determined with VFA analysis

In the microcosms spiked with different concentrations of molasses fermentation of molasses occurred in the first 4 weeks of the incubation. The main products were acetate and methane. After 4 weeks the concentrations of acetate and methane did not increase significantly; indicating a relatively slow degradation of acetate. Apparently the reducing equivalents that were produced in the first weeks of the incubation were sufficient to drive complete dechlorination. This was accompanied by fatty acid (C_3, C_4 and C_5) degradation and acetate production, suggesting that dechlorination was indeed coupled to degradation of the higher fatty acids and not to acetate degradation. This however cannot be proven since acetate production may have occurred simultaneously.

Analysis of VFAs can provide additional information to estimate the amount of electron donor that is used for reductive dechlorination and the amount that is used for other reduction processes like sulphate reduction. In practice, the amount of electron donor necessary for complete removal of the chlorinated ethenes present at a contaminated site should be calculated based on the load of chlorinated ethenes and alternative electron donors (i.e. nitrate, Fe(III) and sulphate) that are present in the (incoming) ground water or soil matrix. To demonstrate the occurrence of intrinsic biological dechlorination and the possibility to stimulate the dechlorination in microcosm studies it is sufficient to analyse for PCE and daughter products and, possibly, methane. VFA analysis could in principle be used as a monitoring instrument to guide electron donor addition. In this experiment this was not possible since all higher VFAs and chlorinated ethenes had been transformed before the first monitoring round. Further research with samples from different sites should show whether dechlorination is indeed coupled to higher VFA degradation, thus allowing monitoring and control of electron donor addition based on VFA analysis.

5.4 Effect of the type of the chlorinated ethene spiked (PCE or *cis*-DCE)

In the microcosms complete degradation of the spiked PCE or *cis*-DCE was demonstrated. The used soil sample originated from the source zone of the contaminated area. In the source PCE and daughter products were present. If spiking is necessary, it is recommended to spike the microcosms with the highest chlorinated ethene present in the soil sample. Soil samples down gradient from the source zone may only contain lower chlorinated ethenes like *cis*-DCE. To investigate the dechlorination potential in these soil samples the microcosms should be spiked

with *cis*-DCE. For instance, when considering a bioscreen approach for the plume at a contaminated site with only lower chlorinated ethenes (daughter products of PCE and/or TCE) a sample should be taken near the proposed location of the bioscreen. This sample should be tested for the activity to dechlorinate lower chlorinated ethenes with the lower chlorinated ethene as the main contaminant.

5.5 Effect of the type of electron donor used for the dechlorination

No significant difference between the dechlorination in the microcosms spiked with different electron donors was observed. Complete dechlorination occurred within 6 weeks with molasses, a VFA mixture in compost percolate or lactate as electron donor (two out of three microcosms). Unfortunately, it appeared that the proposed analysis scheme was not appropriate to follow the course of the dechlorination of chlorinated ethenes to ethene. The calculated dechlorination rate constants are based on too few analysis results to detect possible differences in dechlorination in the microcosms spiked with different electron donors.

The choice for the electron donor and nutrients (N and P) that will be used in the field is mainly determined by acceptance by the regulatory authorities, the price and the availability of the electron donor and the possibility to add the electron donor to the contaminated aquifer. Whenever an electron donor also has its use as a, for instance, food additive it will be much easier to get approval for injection of the electron donor for in situ treatment. Electron donors like molasses are non-toxic, relatively cheap and available in large quantities and are therefore usable as electron donor in the field.

Microcosms should therefore be spiked with an electron donor which can or will be used in a stimulated bioremediation of a contaminated site. Based on this research, no preferences can be given to any of the tested electron donor substrates since complete dechlorination of PCE to ethene occurred with every electron donor tested.

5.6 Reproducibility of batch incubations

In general, the standard deviation between the measured concentration of chlorinated ethenes in the triplicate incubations is 10 % or less. The reproducibility of the performed experiments is good, apart from some exceptions. These exceptions could be caused by losses during the spiking of the chlorinated ethene or artefacts during analysis. Therefore, single sample experiments still have the risk of failure so triplicate (or at least duplicate) experiments from a single sample are strongly recommended.

5.7 Procedural and analytical aspects

In general it can be concluded that microcosm experiments provide a clear documentation of the occurrence of dechlorination of chlorinated ethenes in the field. If the microcosms are properly designed, implemented and interpreted the results can be used in a qualitative way to illustrate the important processes that control the fate of the chlorinated ethenes at contaminated sites. There are some minor concerns when performing anaerobic dechlorination tests as described below.

5.7.1 *Analysis*

The concentration of higher chlorinated ethenes (PCE or *cis*-DCE) in the headspace measured at the beginning of the experiment does not correspond to the spiked amount of PCE or *cis*-DCE because of sorption. Therefore it is recommended to perform extractive analysis for PCE, TCE and DCE at the beginning and at the end of the experiment.

5.7.2 *Analysis scheme*

A simple linear time scale was chosen to investigate the dechlorination of the chlorinated ethenes. These time scales are most likely to give interpretable results because lag phases frequently occur and the rate of dechlorination after the lag phase is often high. Based on the first results the analysis scheme can be adapted to the course of the dechlorination. Unfortunately, the proposed analysis scheme (analysis at the beginning and after 4, 8 and 12 weeks of incubation) was not adequate to determine any differences in dechlorination rates between the different microcosms. For most experiments however, a linear time scheme is representative for the actual dechlorination rates and appropriate to accurately monitor the biodegradation process. A suitable time scheme would be analyses at the beginning of the experiment and after 4, 8, 12, 16 and 24 weeks of incubation. Based on the results of the first few analysis this time scheme can be adapted, if necessary. To gain more insight in the dechlorination process in the first weeks of the incubation it is recommended to include a (qualitative) analysis after 2 weeks of incubation.

5.7.3 *Practicability of the used protocols*

In this practical research phase two protocols were used. Both protocols are suitable for performing anaerobic dechlorination experiments. No significant differences in dechlorinating potential were observed. However, depending on the research question, one protocol could be preferred over the other.

The Wageningen University protocol is preferred in experiments where many parameters are measured over a longer period. The use of thick viton stoppers makes it possible to monitor the microcosms for a long period without leakage, even when many sampling rounds for different parameters are performed. However, because of the thick stoppers it is more difficult to obtain a headspace or liquid sample from the microcosms. Also, because the microcosms are constructed in an anaerobic glove box with a small amount of H_2 present in the atmosphere, H_2 may be introduced into the microcosms, thus influencing the dechlorination process. Therefore the headspace has to be exchanged with a headspace replacement system.

The Bioclear protocol is preferred in more field oriented microcosm experiments. The microcosms are constructed in anaerobic glove bags that are simple, cheap, time-efficient and easy to work with. The glove bags are disposable, which is an advantage in the case of heavily polluted or clayey soil material. Because of the aluminium screw caps with viton lined septa it is easy to obtain headspace or liquid samples. Also, in the case of extractive analysis for higher chlorinated ethenes it is possible to add methanol to the microcosms (sampling by sacrificing microcosms).

CHAPTER 6

GUIDELINE FOR PERFORMING ANAEROBIC MICROCOSM EXPERIMENTS

A guideline is presented for the set-up of anaerobic experiments that are carried out to get information about the dechlorinating activity at sites contaminated with chlorinated ethenes. The former guideline given in the NOBIS-report 'Selection and validation of a practical protocol anaerobic dechlorination - Phase 1: Inventory methods and work visit Cornell University USA' [Van der Waarde and Van Eekert, 1999] has been revised based on the results of the microcosm experiments in this research project.

This guideline describes the set-up of anaerobic microcosm experiments and contains the most important factors that should be considered when performing microcosm studies and is suitable for general application.

Some recommendations have been made concerning the selection of sampling points, sampling and treatment of the sample at the contaminated site. Others deal with the set-up of microcosm studies, incubation conditions and analyses. Not all parameters that can be varied in such experiments are discussed. Some parameters have no significant impact on the outcome of the results of the studies. The incubation temperature e.g., can be chosen anywhere between 10 and 37 ºC, an increase in temperature will increase reaction rates but will not influence the 'verdict' on whether the dechlorination takes place at a certain site or not.

Sampling points

- The location of the sampling points is dependent on the research question, on the type of soil, and the profile of the pollution:
  - at sites with one dominant type of soil present: one sample;
  - if more than one soil type is present: one sample per soil type.
- When full-scale stimulated anaerobic dechlorination is considered, samples have to be taken on spots where daughter products are present, i.e., the zone with dechlorinating activity. The sample has to 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 a bioscreen approach for (parts of) the plume at the contaminated site with only lower chlorinated ethene daughter products is considered, a separate sample has to be taken, which has to be tested for activity with the lower chlorinated ethene as the main contaminant.
- Sterile controls have to 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 has to be performed with a hollow tube e.g. Geoprobe or 'Akkerman steekbus' that has to fit in anaerobic glove box/bag.
- The gas volume in the sampling core has to 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 have to be capped and carefully taped to prevent leakage of liquids or gasses (in and out).
- Core samples must be kept cool and preferable stored in an anaerobic jar or under water during transport and storage.

Treatment of the soil sample

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

Set-up of microcosm studies

- The experiments have to be carried out in glass serum flasks with a viton lined gas tight inlay septum or stopper.
- Groundwater from the sampling site has to be used as the liquid phase/medium (no NAPLs present). If no groundwater is available, it has to be replaced by demineralized water. The soil:liquid ratio has to be be 1:1 (w/w), but whenever possible less water than soil has to be be used.
- The headspace has to be kept as small as possible to prevent the headspace from acting as a storage volume for H_2. However, the headspace volume must be large enough to take samples for the analysis of the chlorinated ethene concentration, to 'store' methane which could be produced or to allow addition of extractant in case of extractive analyses.
- A N- and P-source has to be added to ensure sufficient supply of nutrients (not in microcosms that mimic 'natural attenuation').
- The pH of the microcosms has to 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. With an alkalinity lower than 50 meq/l, enough $NaHCO_3$ has to be 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 has to be added. In microcosms that mimic 'natural attenuation' pH adjustments are not necessary.
- Dependent on the presence of a phosphate or carbonate buffer the headspace has to contain N_2 or N_2/CO_2.
- Lactate can 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 can give additional information about the dechlorinating activity of the soil under study and about the feasibility to use these compounds in full-scale bioremediation.
- Based on the results of this research the ratio electron donor:chlorinated ethene has to be around 10:1 on a weight basis to support complete dechlorination. However, organic matter and co-contaminants that were also present in the soil could have contributed to the dechlorination process. These effects cannot be excluded since no control experiment was performed without additions of an electron donor. Further research is needed to obtain information about the minimum level of electron donor to stimulate anaerobic dechlorination. For now, it is advised to calculate the amount of electron donor needed for complete reduction of the amount of chlorinated ethenes and alternative electron acceptors (nitrate and sulphate), and to add a five-fold excess of the calculated amount of electron donor.
- The concentration of the chlorinated ethene applied in the microcosm study has to 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.
- Live microcosms have to be tested at least in duplicate, preferably in triplicate for each of the conditions applied. The dead controls can be carried out in singular tests. The dead controls have to be autoclaved two times for 1 h with at least 2 days in between. When $HgCl_2$ or NaN_3 are added to maintain the autoclaved control sterile it is sufficient to autoclave the sterile

controls once for 1 h. The autoclaved control (without $HgCl_2$ or NaN_3) can act as a control for sterile conditions during sampling.

Incubation conditions

- The microcosms have to be incubated for 6 months or until the formation of ethene is detected.
- Incubation has to be carried out in the dark, statically.
- Incubation temperature has to be between 10 - 30 ºC.

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 can be measured.

CHAPTER 7

CONCLUSIONS

Based on the results of the performed microcosm experiments the following conclusions can be drawn:

- Both the experimental protocols from Wageningen University and Bioclear are suitable for performing feasibility studies to demonstrate dechlorination potential in contaminated soil.
- No leakage of chlorinated ethenes occurred through the used septa during an incubation period of 12 weeks. In general, the performed microcosm experiments are highly reproducible. However, single sample experiments still have the risk of failure so triplicate (or at least duplicate) experiments from a single sample are preferred.
- The micro-organisms in the soil sample were capable of dechlorinating PCE or *cis*-DCE to ethene. No significant differences in the outcome of the microcosms spiked with PCE or *cis*-DCE were observed. The spiked concentration of PCE or *cis*-DCE (between 8,000 µg/l and 100,000 µg/l or 5,000 µg/l and 60,000 µg/l, respectively) does not have a significant effect on the degree and rate of dechlorination of PCE or *cis*-DCE. Anaerobic dechlorination experiments should therefore be performed with the dominant chlorinated ethene at the sampling site at the prevalent concentration.
- Molasses, a mixture of VFAs in compost percolate and lactate all support dechlorination of PCE to ethene. No difference was observed. Microcosms should therefore be spiked with an electron donor which can or will be used in a stimulated bioremediation of a contaminated site (accepted by the regulatory authorities, non-toxic, relatively cheap and available in large quantities) and sufficient nutrients (N and P). It is advised to calculate the amount of electron donor needed for complete reduction of the amount of chlorinated ethenes and alternative electron acceptors (nitrate and sulphate), and to add a five-fold excess of the calculated amount of electron donor.
- A sampling schedule of 0, 4, 8, 12, 16 and 24 weeks is sufficient for most soil samples when the level of dechlorination activity is unknown, but a (qualitative) analysis after 2 or 3 weeks of incubation is necessary to prevent loss of information with highly active soils.

Microcosm studies are a useful tool to provide evidence for the occurrence of dechlorination processes in the field. The guideline presented in chapter 6 gives the prerequisites for performing these microcosms studies, so the outcome of the microcosm experiments can be used to determine the possibilities for a (stimulated) bioremediation approach for the investigated contaminated site.

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CUR/NOBIS report 97-4-04, CUR/NOBIS, Gouda, The Netherlands.

APPENDIX A

RESULTS OF EXPERIMENT 1

Influence of the initial concentration of PCE on the dechlorination of chlorinated ethenes

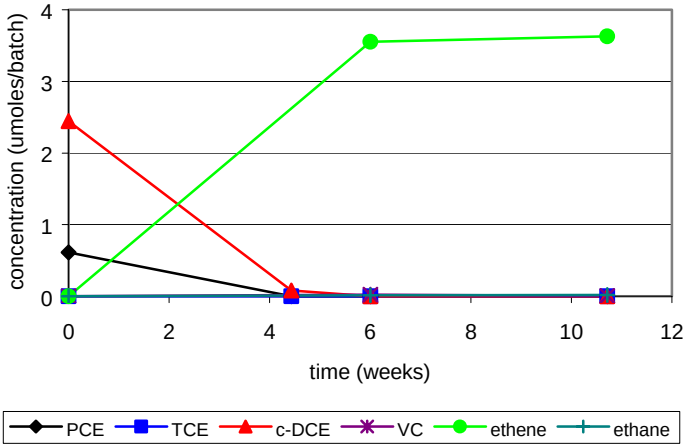

Fig. A1a. Wageningen University microcosm 1a, initial PCE concentration of 8,000 µg/l.

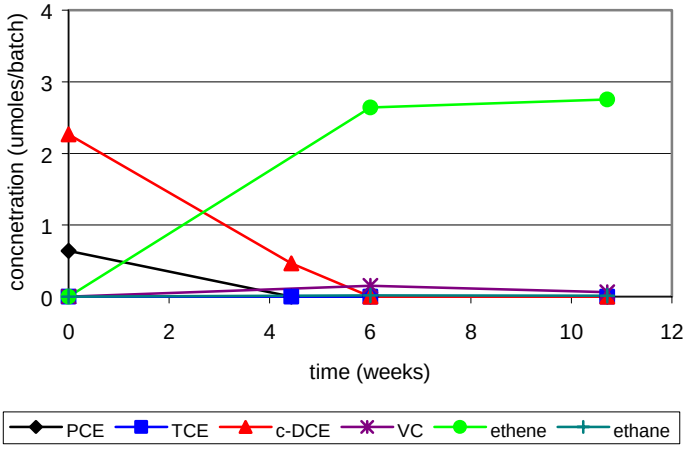

Fig. A1b. Wageningen University microcosm 1b, initial PCE concentration of 8,000 µg/l.

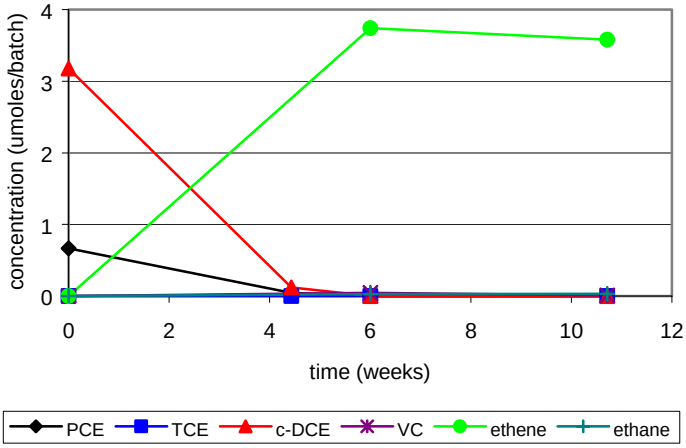

Fig. A1c. Wageningen University microcosm 1c, initial PCE concentration of 8,000 µg/l.

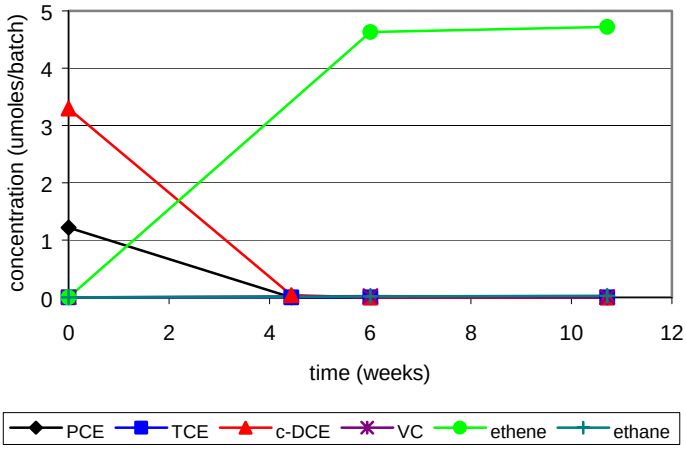

Fig. A2a. Wageningen University microcosm 2a, initial PCE concentration of 16,000 µg/l.

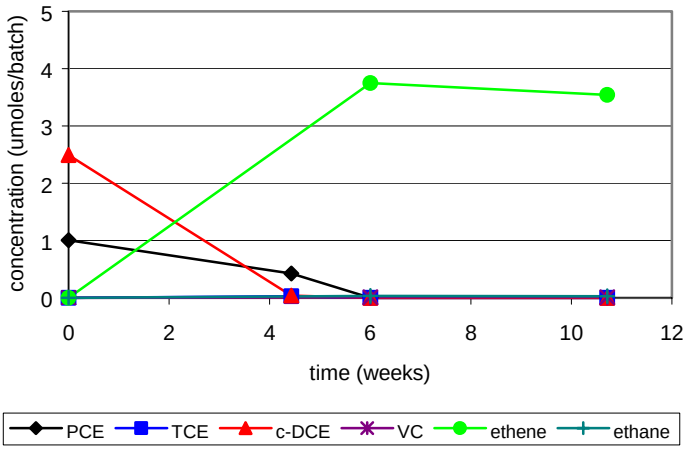

Fig. A2b. Wageningen University microcosm 2b, initial PCE concentration of 16,000 µg/l.

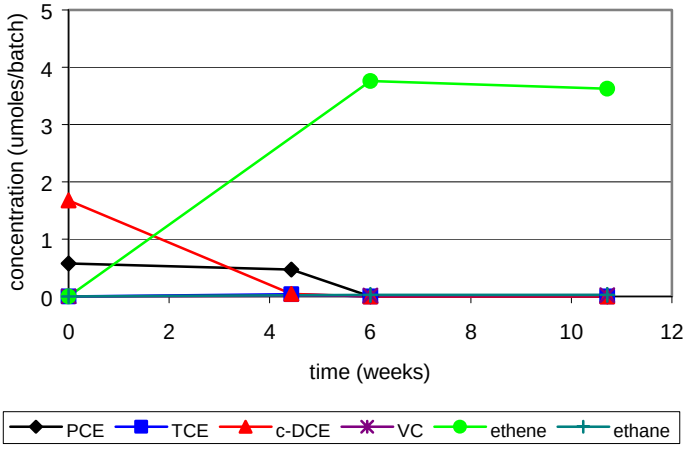

Fig. A2c. Wageningen University microcosm 2c, initial PCE concentration of 16,000 µg/l.

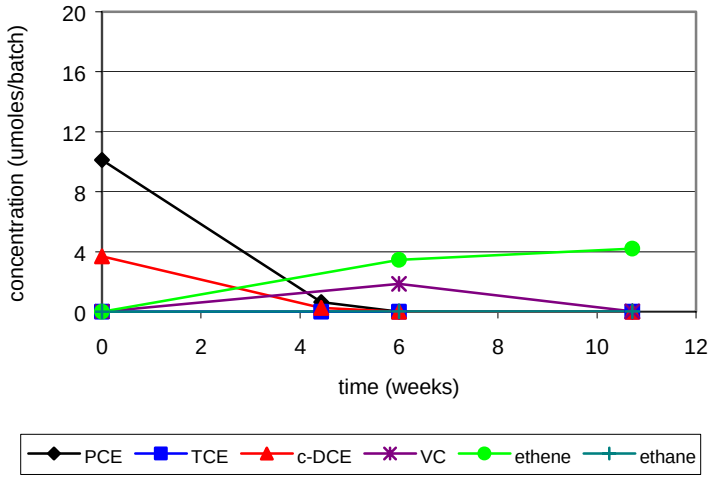

Fig. A3a. Wageningen University microcosm 3a, initial PCE concentration of 100,000 µg/l.

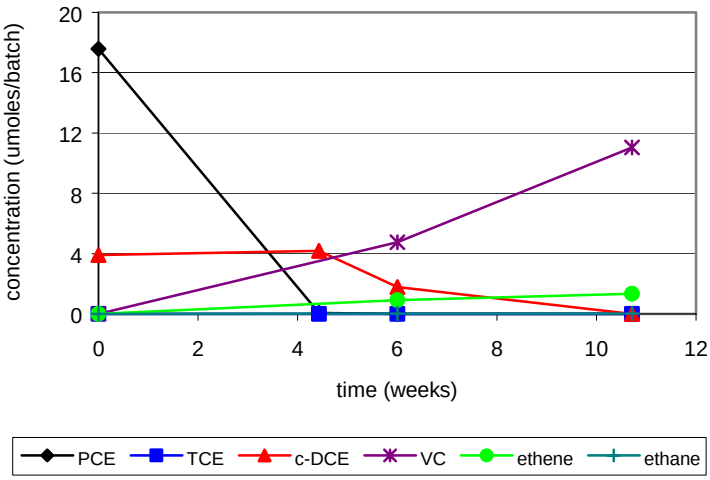

Fig. A3b. Wageningen University microcosm 3b, initial PCE concentration of 100,000 µg/l.

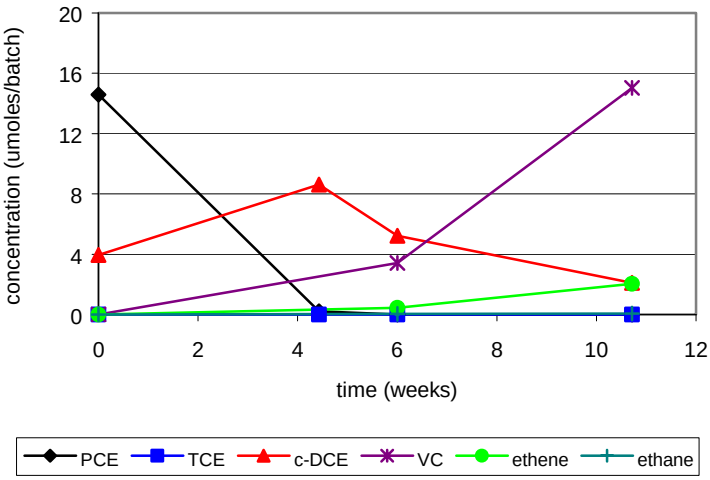

Fig. A3c. Wageningen University microcosm 3c, initial PCE concentration of 100,000 µg/l.

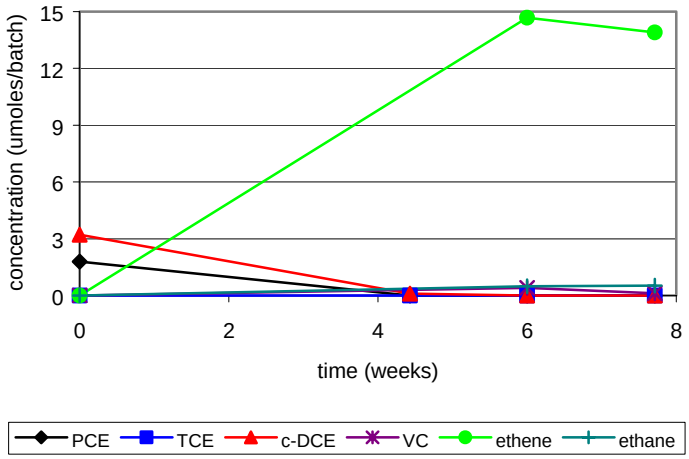

Fig. A4a. Bioclear microcosm 1a, initial PCE concentration 8,000 µg/l.

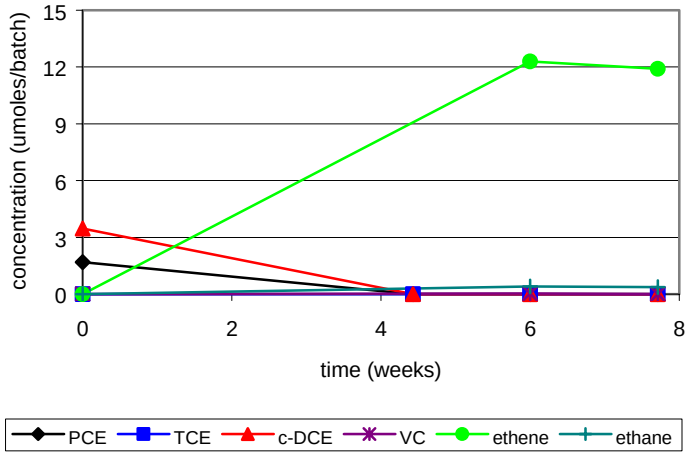

Fig. A4b. Bioclear microcosm 1b, initial PCE concentration 8,000 µg/l.

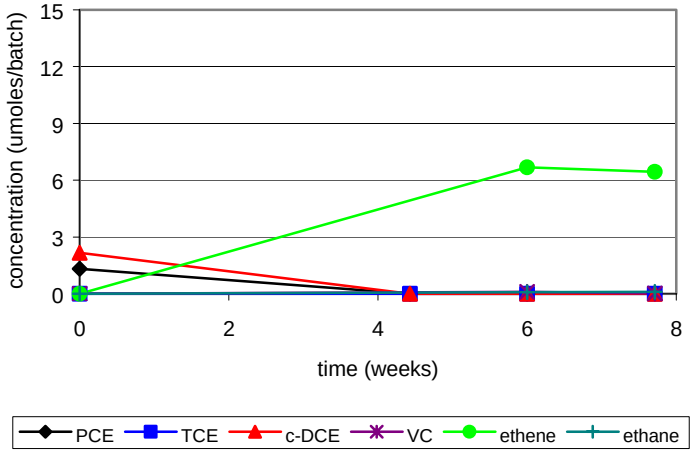

Fig. A4c. Bioclear microcosm 1c, initial PCE concentration 8,000 µg/l.

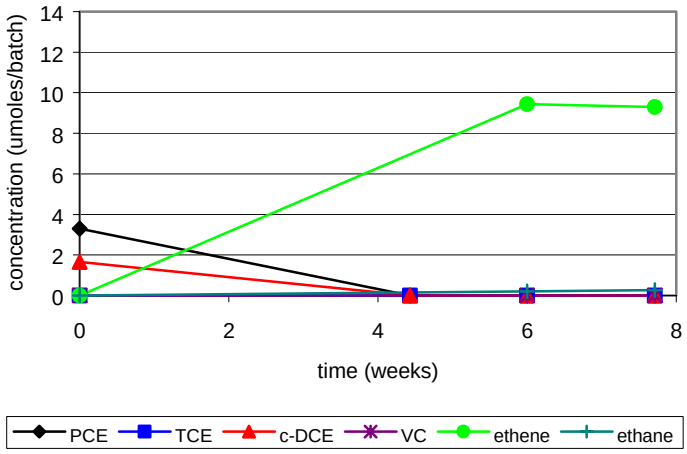

Fig. A5a. Bioclear microcosm 4a, initial PCE concentration 16,000 µg/l.

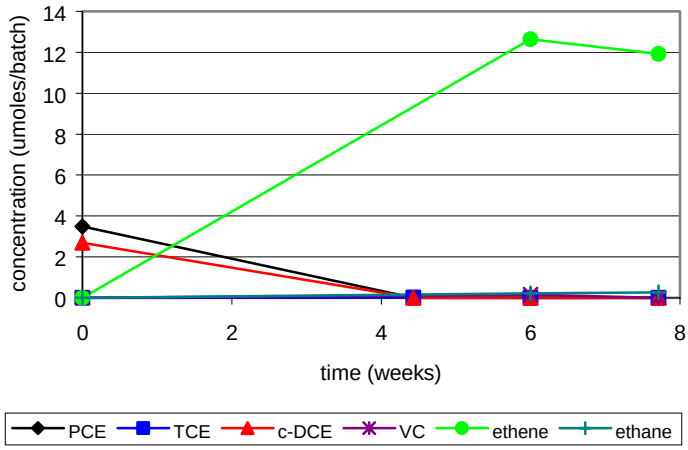

Fig. A5b. Bioclear microcosm 4b, initial PCE concentration 16,000 µg/l.

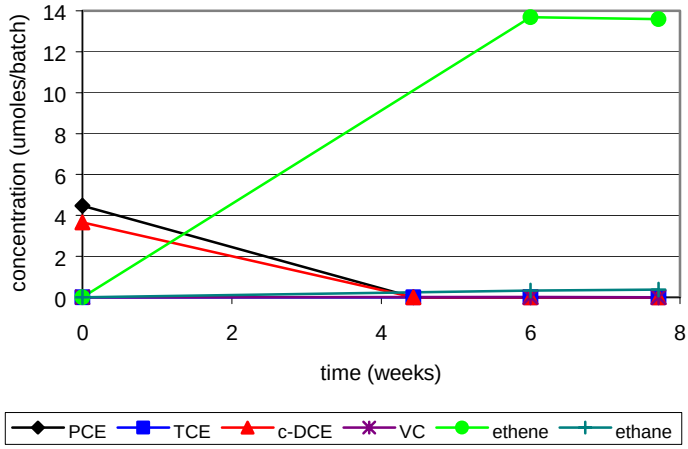

Fig. A5c. Bioclear microcosm 4c, initial PCE concentration 16,000 µg/l.

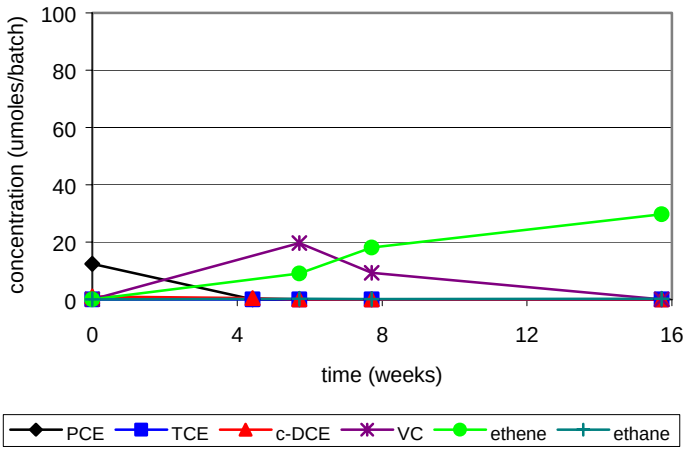

Fig. A6a. Bioclear microcosm 2a, initial PCE concentration 100,000 µg/l.

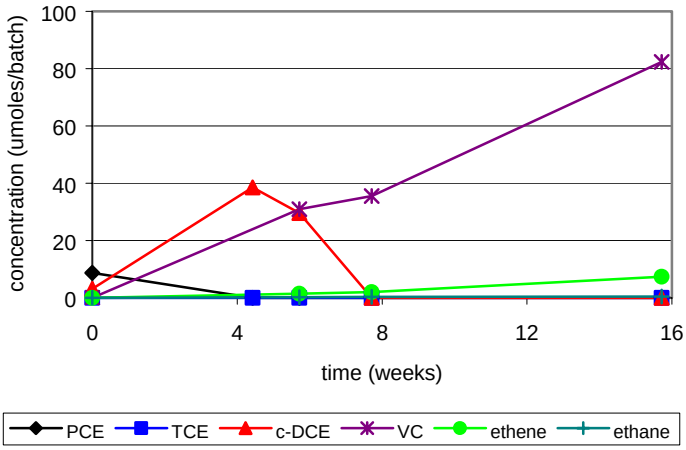

Fig. A6b. Bioclear microcosm 2b, initial PCE concentration 100,000 µg/l.

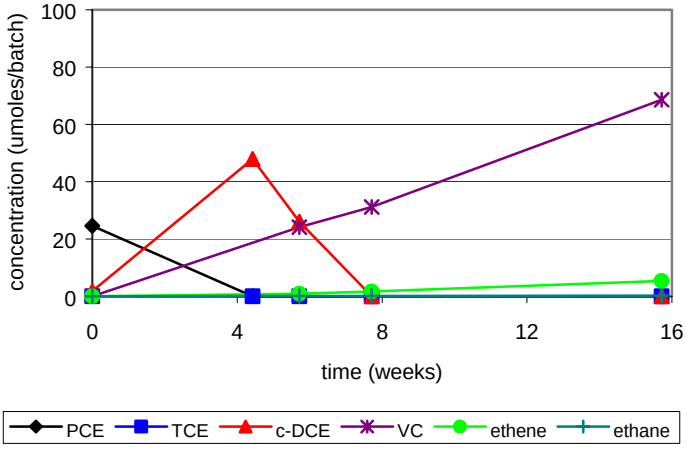

Fig. A6c. Bioclear microcosm 2c, initial PCE concentration 100,000 µg/l.

APPENDIX B

RESULTS OF EXPERIMENT 2

Influence of the nature of the spiked chlorinated ethene on the dechlorination of chlorinated ethenes

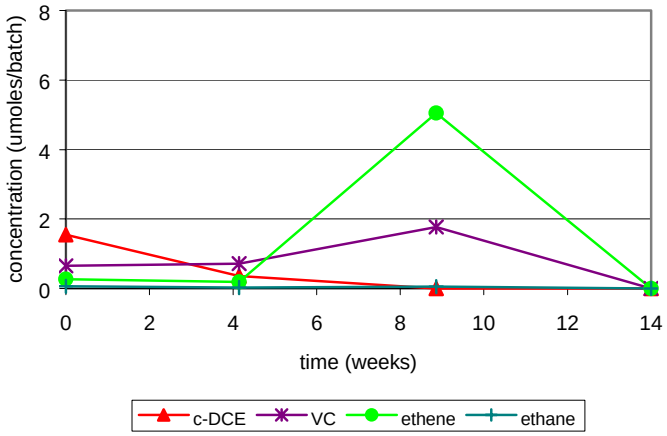

Fig. B1a. Wageningen University microcosm 6a, initial *cis*-DCE concentration of 5,000 µg/l.

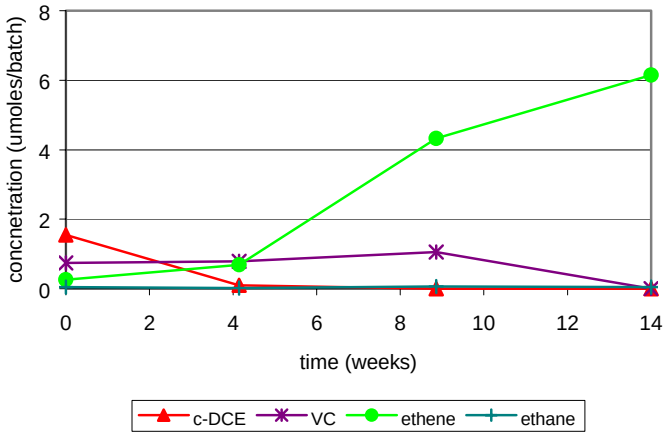

Fig. B1b. Wageningen University microcosm 6b, initial *cis*-DCE concentration of 5,000 µg/l.

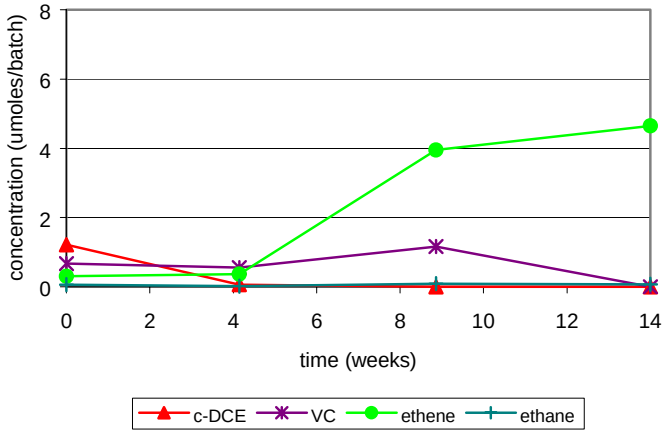

Fig. B1c. Wageningen University microcosm 6c, initial *cis*-DCE concentration of 5,000 µg/l.

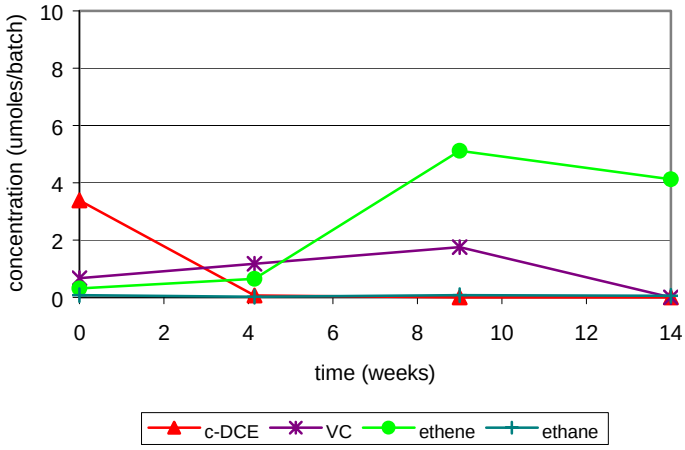

Fig. B2a. Wageningen University microcosm 7a, initial *cis*-DCE concentration of 10,000 µg/l.

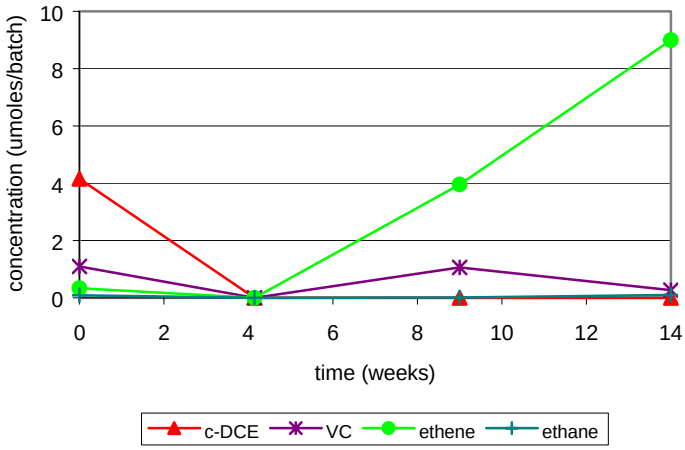

Fig. B2b. Wageningen University microcosm 7b, initial *cis*-DCE concentration of 10,000 µg/l.

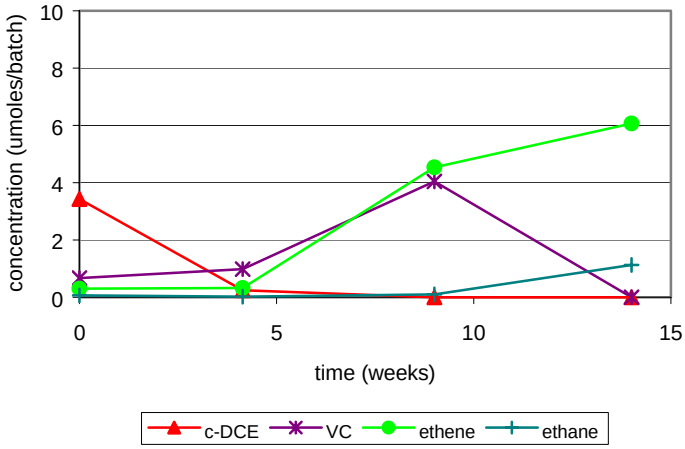

Fig. B2c. Wageningen University microcosm 7c, initial *cis*-DCE concentration of 10,000 µg/l.

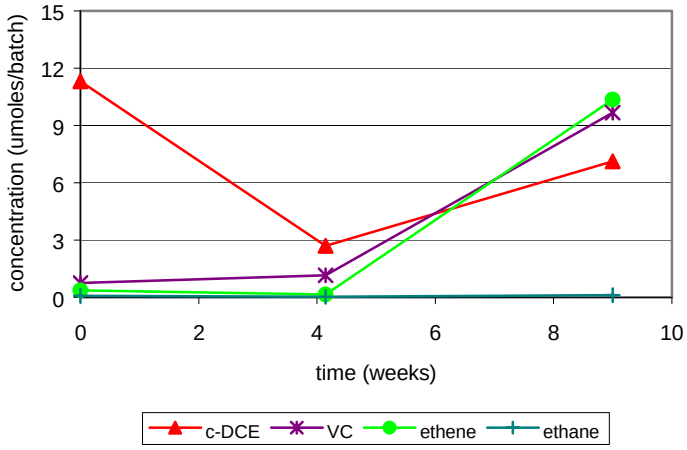

Fig. B3a. Wageningen University microcosm 8a, initial *cis*-DCE concentration of 60,000 µg/l.

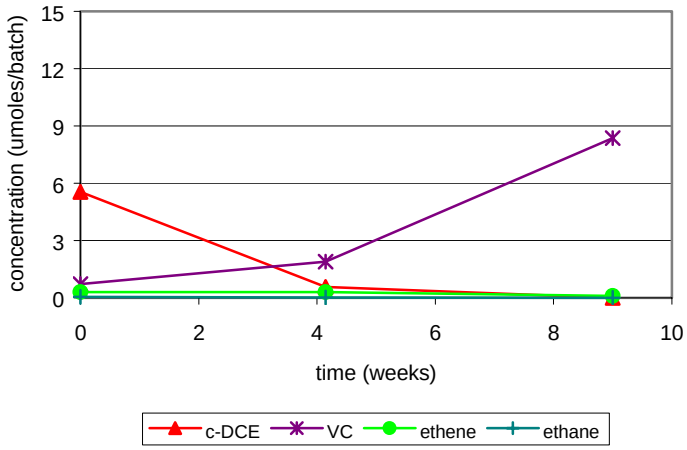

Fig. B3b. Wageningen University microcosm 8b, initial *cis*-DCE concentration of 60,000 µg/l.

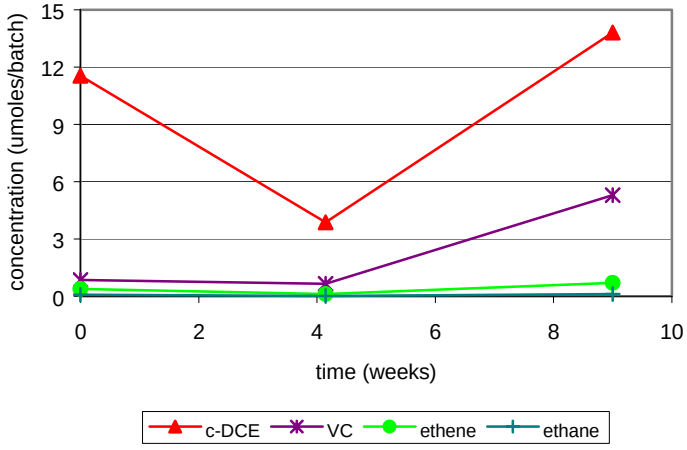

Fig. B3c. Wageningen University microcosm 8c, initial *cis*-DCE concentration of 60,000 µg/l.

APPENDIX C

RESULTS OF EXPERIMENT 3

Influence of the electron donor on the dechlorination of chlorinated ethenes

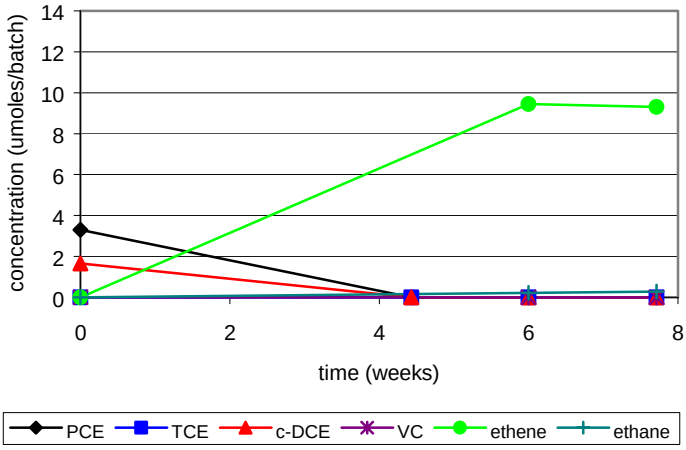

Fig. C1a. Bioclear microcosm 4a, molasses as electron donor.

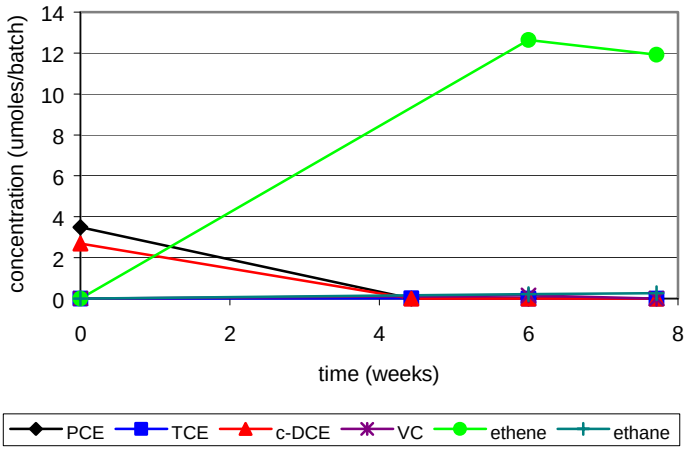

Fig. C1b. Bioclear microcosm 4b, molasses as electron donor.

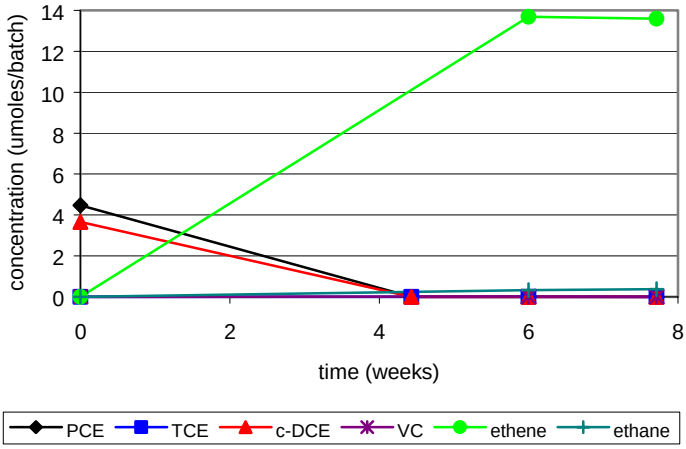

Fig. C1c. Bioclear microcosm 4c, molasses as electron donor.

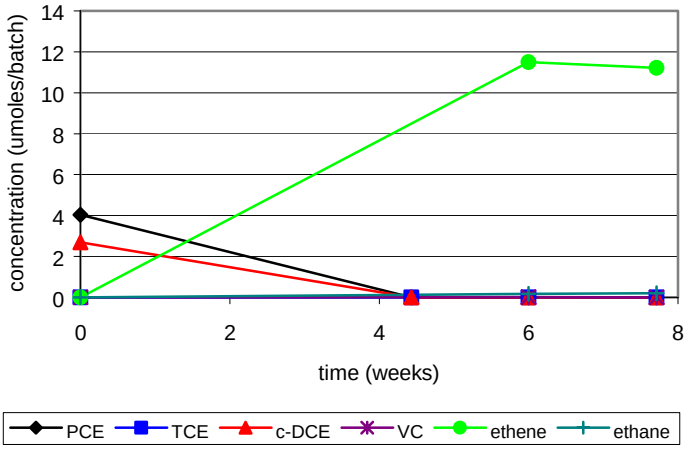

Fig. C2a. Bioclear microcosm 6a, VFAs in compost percolate as electron donor.

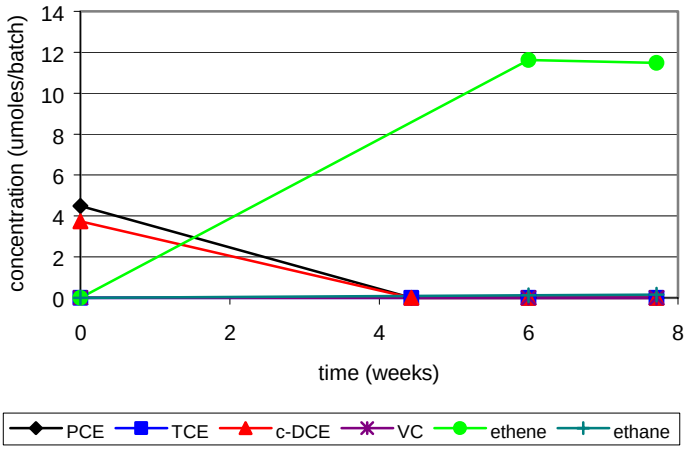

Fig. C2b. Bioclear microcosm 6b, VFAs in compost percolate as electron donor.

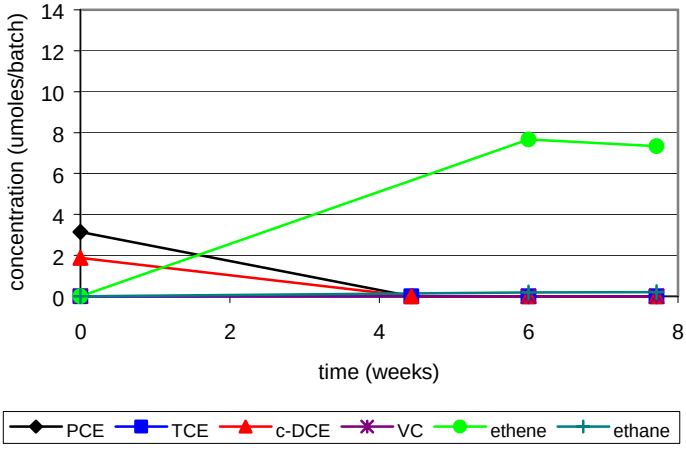

Fig. C2c. Bioclear microcosm 6c, VFAs in compost percolate as electron donor.

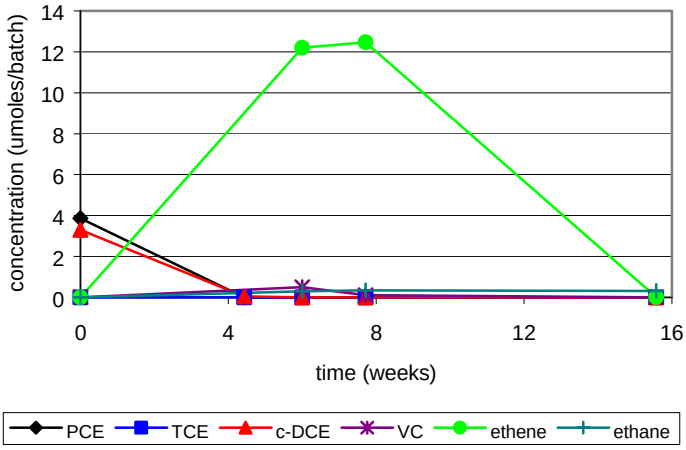

Fig. C3a. Bioclear microcosm 7a, VFAs in compost percolate as electron donor.

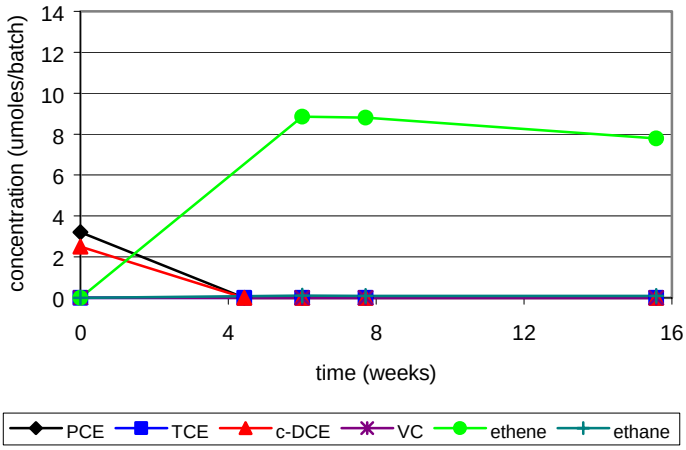

Fig. C3b. Bioclear microcosm 7b, VFAs in compost percolate as electron donor.

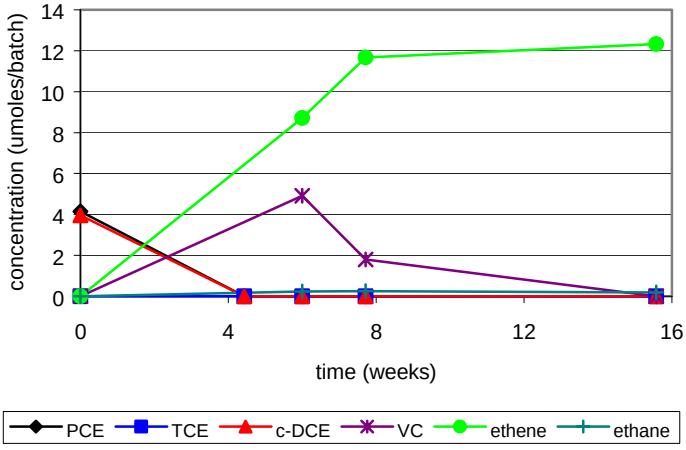

Fig. C3c. Bioclear microcosm 7c, VFAs in compost percolate as electron donor.

APPENDIX D

RESULTS OF EXPERIMENT 4

Influence of the concentration of molasses on the dechlorination of chlorinated ethenes

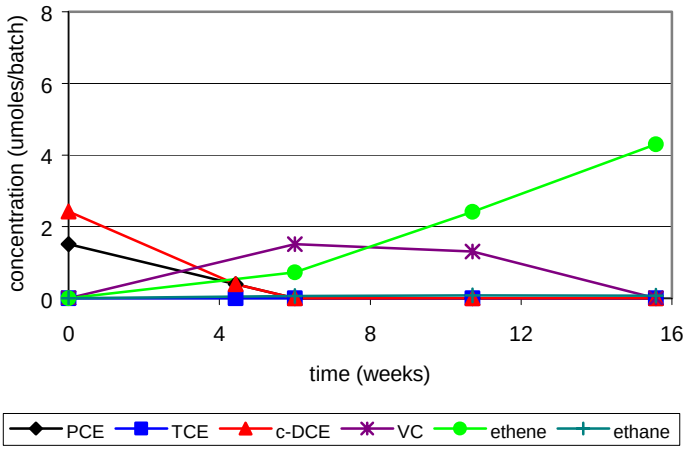

Fig. D1a. Wageningen University microcosm 4a, initial concentration of molasses of 80 mg/l.

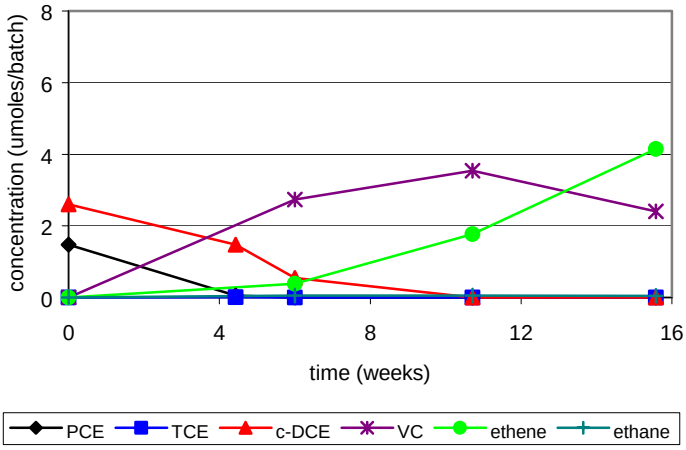

Fig. D1b. Wageningen University microcosm 4b, initial concentration of molasses of 80 mg/l.

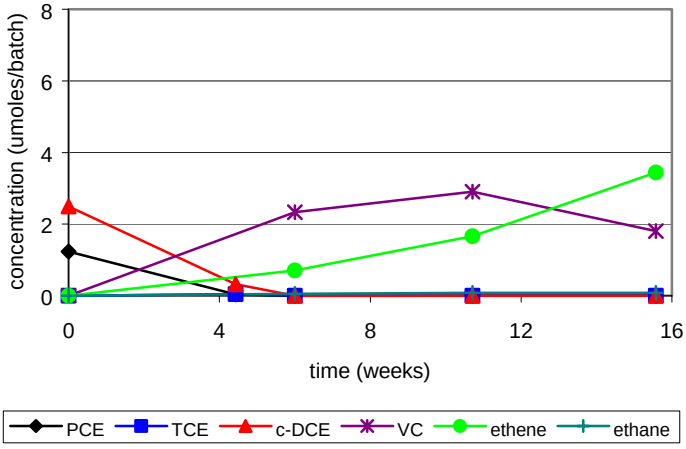

Fig. D1c. Wageningen University microcosm 4c, initial concentration of molasses of 80 mg/l.

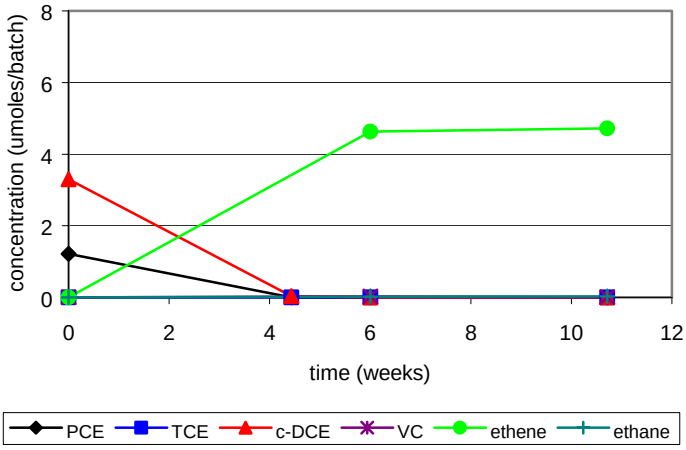

Fig. D2a. Wageningen University microcosm 2a, initial concentration of molasses of 160 mg/l.

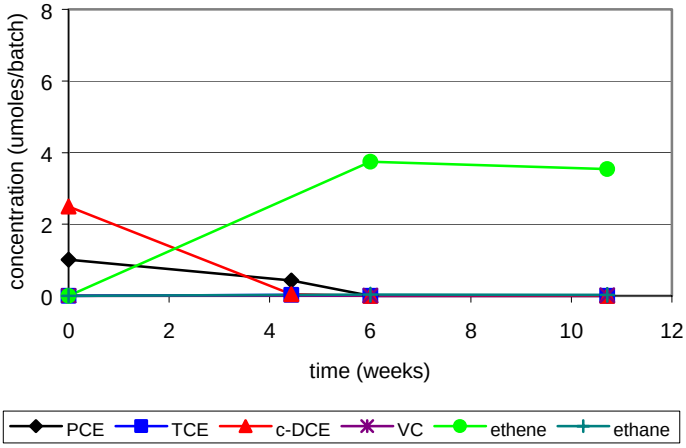

Fig. D2b. Wageningen University microcosm 2b, initial concentration of molasses of 160 mg/l.

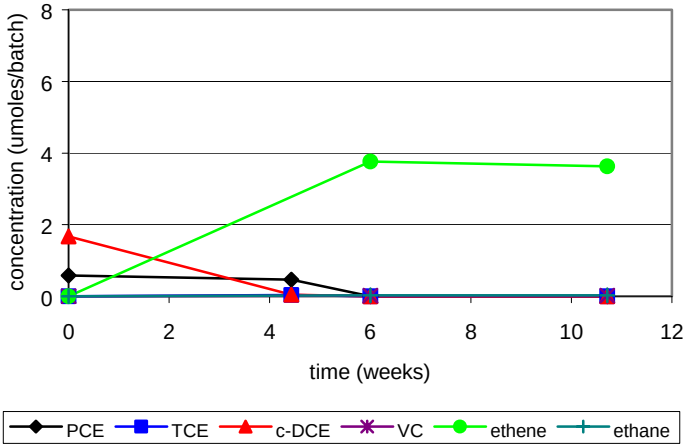

Fig. D2c. Wageningen University microcosm 2c, initial concentration of molasses of 160 mg/l.

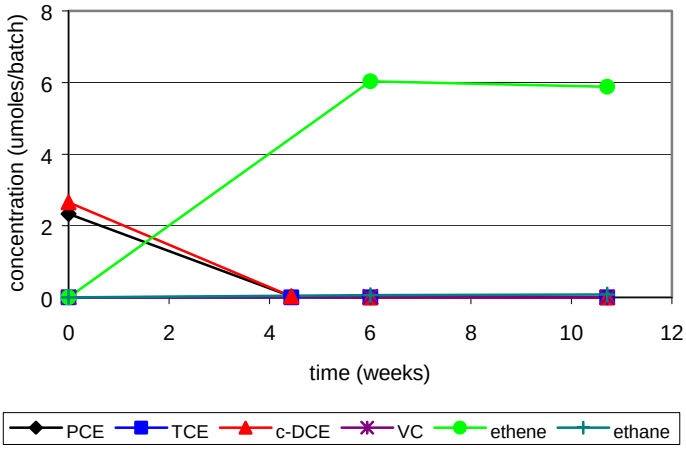

Fig. D3a. Wageningen University microcosm 5a, initial concentration of molasses of 1,600 mg/l.

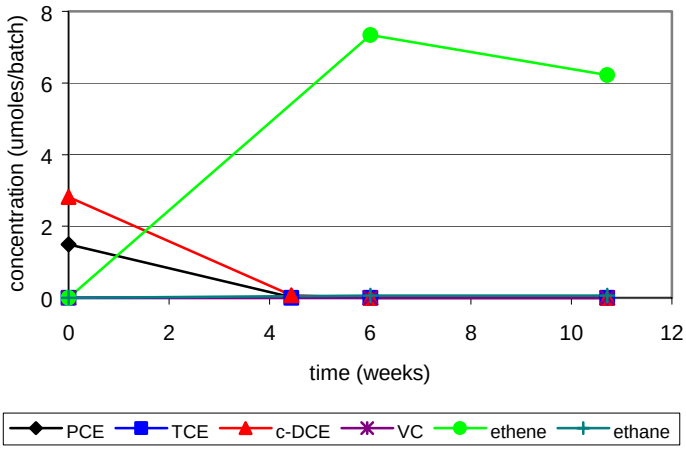

Fig. D3b. Wageningen University microcosm 5b, initial concentration of molasses of 1,600 mg/l.

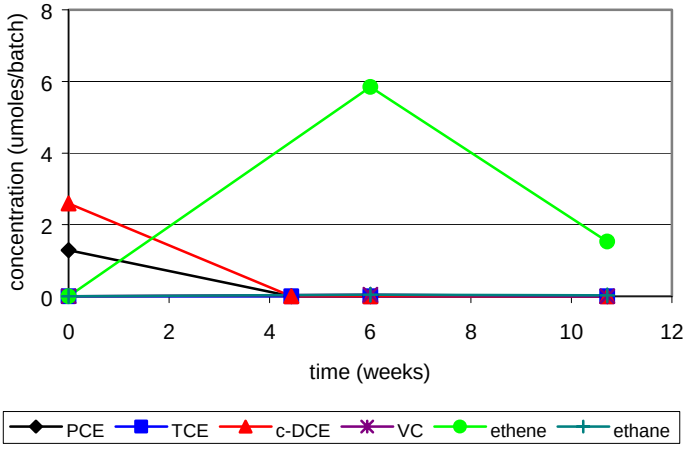

Fig. D3c. Wageningen University microcosm 5c, initial concentration of molasses of 1,600 mg/l.

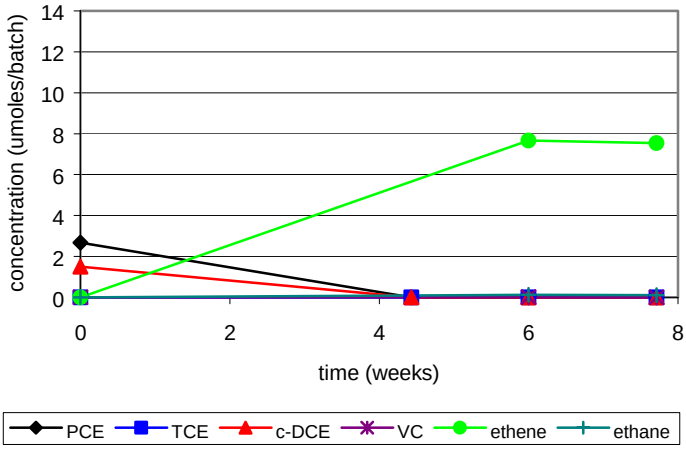

Fig. D4a. Bioclear microcosm 3a, initial concentration of molasses of 80 mg/l.

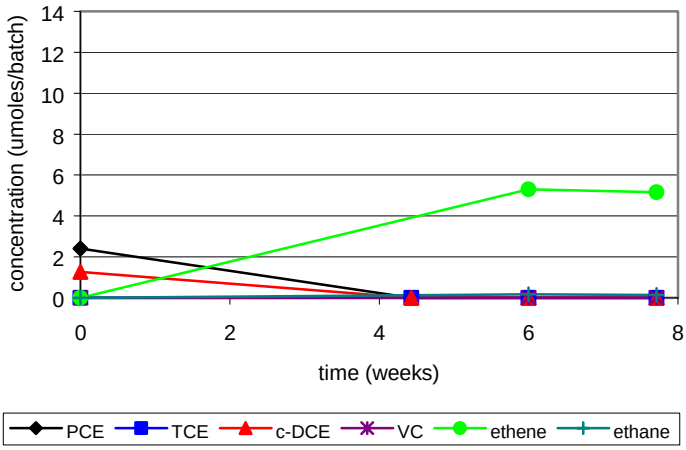

Fig. D4b. Bioclear microcosm 3b, initial concentration of molasses of 80 mg/l.

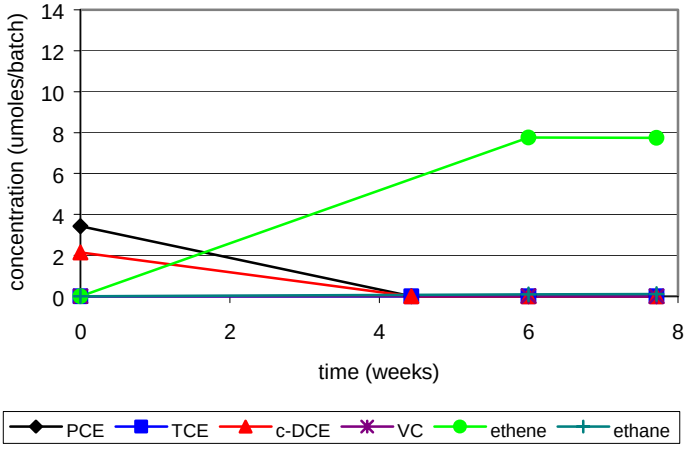

Fig. D4c. Bioclear microcosm 3c, initial concentration of molasses of 80 mg/l.

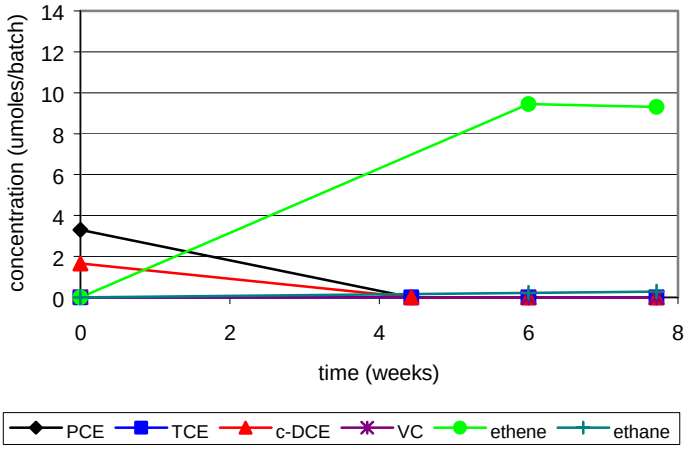

Fig. D5a. Bioclear microcosm 4a, initial concentration of molasses of 160 mg/l.

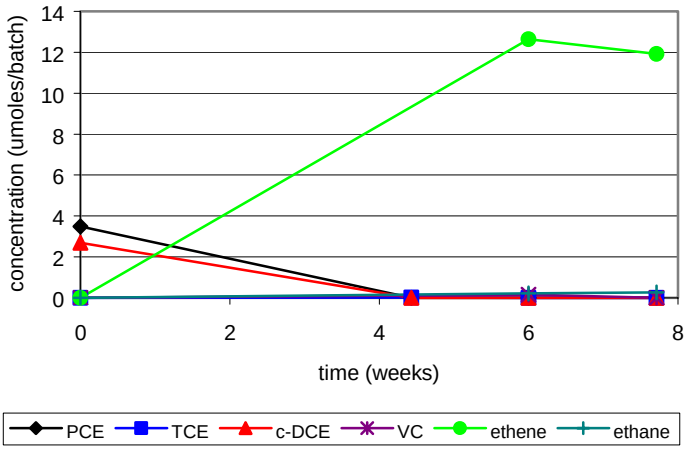

Fig. D5b. Bioclear microcosm 4b, initial concentration of molasses of 160 mg/l.

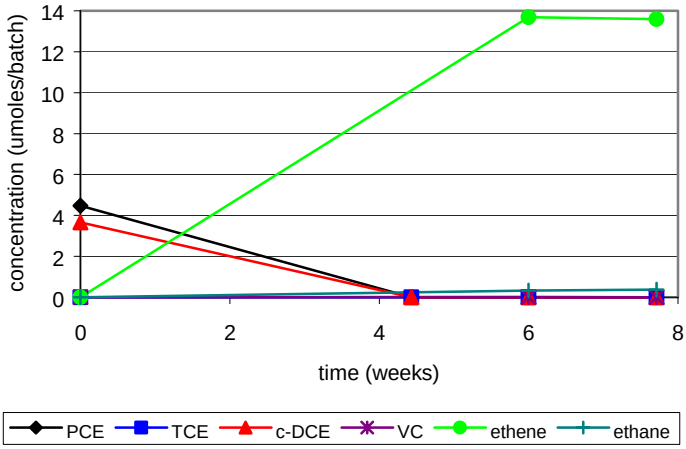

Fig. D5c. Bioclear microcosm 4c, initial concentration of molasses of 160 mg/l.

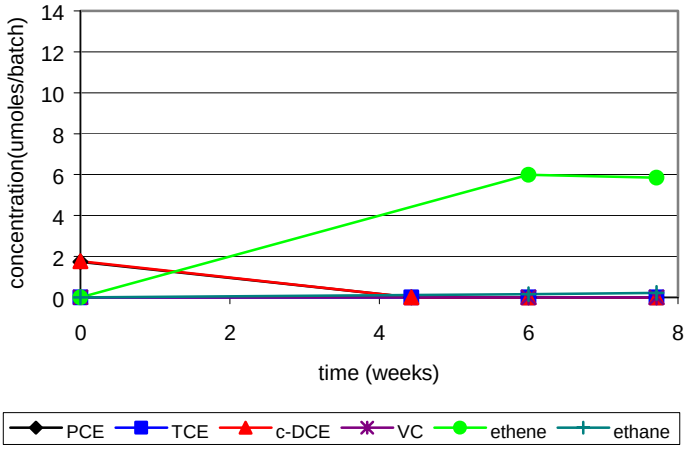

Fig. D6a. Bioclear microcosm 5a, initial concentration of molasses of 1,600 mg/l.

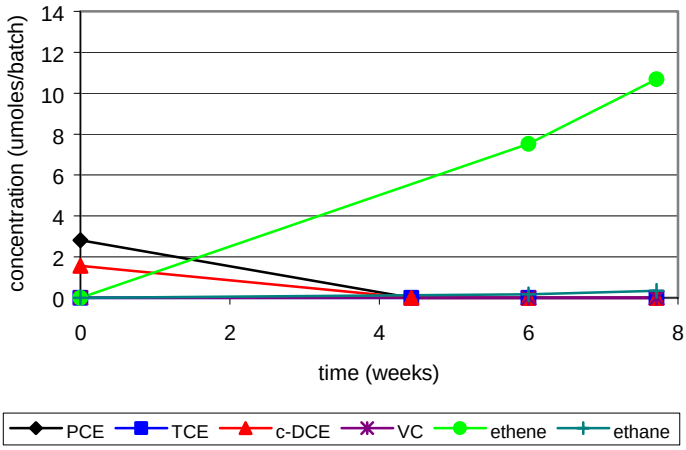

Fig D6b. Bioclear microcosm 5b, initial concentration of molasses of 1,600 mg/l.

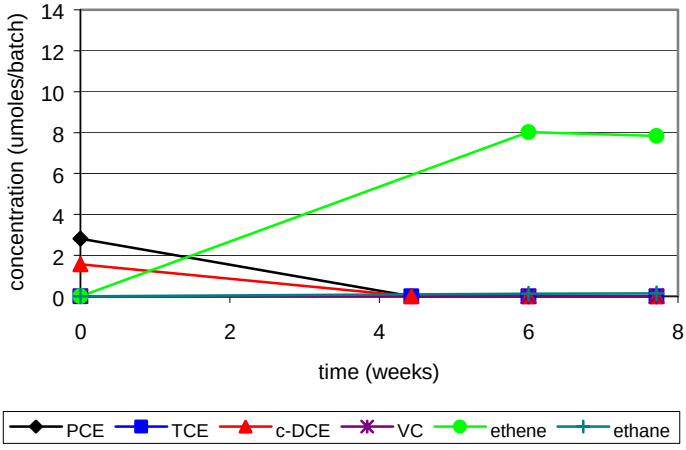

Fig. D6c. Bioclear microcosm 5c, initial concentration of molasses of 1,600 mg/l.