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MOLECULAR MONITORING OF DECHLORINATING MICRO-ORGANISMS

Final report

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Samenvatting
Een moleculaire detectiemethode, gebaseerd op de PCR-reactie, is ontworpen voor de specifieke detectie van *Desulfitobacteria*, een belangrijke groep van anaërobe VOCl dechlorerende bacteriën. De methode blijkt snel, zeer gevoelig en zeer specifiek in reinculturen en in beënte verontreinigde grond. In natuurlijke verontreinigde bodems treedt soms inhibitie op van de PCR-reactie, waarschijnlijk door verontreinigingen in het DNA-extract. Batchproeven blijken zeer geschikt voor het aantonen van het in situ biodegradatie-potentieel van VOCl's. De fysiologische MPN-methode voor dechlorerende bacteriën lijkt zeer ongevoelig en is tijdrovend. De MPN-PCR-methode kan een snel, gevoelig en specifiek instrument worden om de aan-wezigheid van anaërobe dechlorerende activiteit te monitoren. Betere DNA-extractiemethoden zijn daarvoor nodig.

Trefwoorden
Gecontroleerde termen:
anaerobic, dechlorination, detection, molecular, VOCs

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Abstract
A molecular detection method, based on the PCR reaction, was developed for the specific detection of *Desulfitobacteria*, an important class of anaerobic VOC dechlorinating bacteria. The method was shown to be highly specific and sensitive in pure cultures and inoculated contaminated soils. In natural contaminated soils, sometimes problems with PCR inhibition occurred, probably due to impurities in the DNA extract. Microcosms were successfully applied to demonstrate the anaerobic biodegradation potential for VOCs. The physiological MPN method for dechlorinating bacteria seemed to be highly insensitive and is labor intensive. The MPN-PCR method could form a quick, sensitive and specific tool to monitor the presence of in situ anaerobic dechlorination potential. Better DNA extraction methods are needed.

Keywords
Controlled terms:
anaerobic, dechlorination, detection, molecular, VOCs

Uncontrolled terms:

Project title
Molecular monitoring of dechlorating micro-organisms

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SAMENVATTING

Molecular monitoring of dechlorinating micro-organisms

Vluchtige organische gehalogeneerde verbindingen, zoals perchloorethyleen, kunnen onder anaërobe condities biologisch worden afgebroken, resulterend in de vorming van onschuldige verbindingen, zoals etheen en ethaan. Op dit moment zijn er geen geschikte instrumenten voor een snelle, specifieke en gevoelige detectie van deze bacteriën in grond. Het aantonen van het dechlorerende biodegradatiepotentieel is daarom afhankelijk van anaërobe batchproeven. Deze tests zijn algemeen geaccepteerd en gebruikt, maar hebben als belangrijkste nadeel dat ze weken tot maanden duren. Het doel van dit project is om een snelle, gevoelige en specifieke detectiemethode op te zetten voor de moleculaire detectie van anaërobe dechlorerende micro-organismen.

De ontwikkelde methode maakt gebruik van de Polymerase Chain Reaction (PCR) en is gebaseerd op verschillen in het DNA van *Desulfitobacteria*, een belangrijke groep van anaërobe dechlorerende micro-organismen. Een 'nested' PCR methode is ontwikkeld, met twee primerparen specifiek voor *Desulfitobacteria*.

De methode is uiterst specifiek gebleken voor *Desulfitobacteria*. De gevoeligheid van de methode in een verdunningsreeks (MPN) van een reincultuur was erg hoog: 6 DNA-kopieën per reactie, overeenkomend met ongeveer 100 cellen per monster. Indien deze bacteriën werden gemengd met verontreinigde grond, gevolgd door DNA-extractie en MPN-PCR bleef de gevoeligheid op een voldoende hoog niveau: 10^3 tot 10^4 cellen per gram droge grond (dg) konden worden gedetecteerd na toevoeging van 10^8 cellen/g dg grond. Deze gevoeligheid is waarschijnlijk voldoende om de aanwezigheid van anaërobe dechlorerende activiteit in de bodem te monitoren.

Anaërobe dechlorering in bodemmonsters van verontreinigde locaties, zoals bepaald in batchexperimenten, vertoonde een goede correlatie met het optreden van anaërobe dechlorering in situ. Batchexperimenten zijn daarom geschikt om het biodegradatiepotentieel op verontreinigde locaties aan te tonen. Het tellen van deze dechlorerende micro-organismen via een fysiologische MPN-methode bleek echter zeer ongevoelig. Een maximum van 10^2 cellen/g dg grond kon worden aangetoond, terwijl waarschijnlijk veel hogere (10^8 cellen/g dg) aanwezig waren.

Analyse van verontreinigde bodems met de moleculaire MPN-PCR-methode liet zien dat *Desulfitobacteria* aanwezig waren in deze bodems, waarmee is aangetoond dat de ontwikkelde methode toepasbaar is in 'natuurlijke' verontreinigde bodems. Tellen van bacteriën in deze monsters bleek mogelijk met eubacteriële primers in een MPN-PCR-methode. De gevoeligheid van de MPN-PCR-methode lijkt veel hoger (1000*) dan de fysiologische MPN-methode. De specifieke MPN-PCR-methode voor *Desulfitobacteria* echter kon niet worden gebruikt voor het accuraat tellen van dechlorerende bacteriën. De PCR-reactie werd verstoord, waarschijnlijk door onzuiverheden in het DNA-extract, waardoor vals negatieve reacties optraden in de verdunningsreeks. Andere DNA-extractie- en zuiveringsmethoden worden daarom getest om deze problemen te omzeilen. DGGE-analyse van reinculturen van *Desulfitobacteria* resulteerde niet in unieke bandpatronen, waarschijnlijk omdat er onvoldoende genetische verschillen zijn tussen de onderzochte stammen binnen het geamplificeerde DNA. Andere DGGE-PCR-primers moeten daarom worden ontworpen om deze vorm van monitoring mogelijk te maken.

SUMMARY

Molecular monitoring of dechlorinating micro-organisms

Volatile chlorinated hydrocarbons like perchloroethylene can be biodegraded under anaerobic conditions resulting in the formation of harmless components like ethene and ethane. At this moment there are no suitable instruments for a quick, specific and sensitive detection of these micro-organisms in soil. Demonstration of the biodegradation potential is therefore dependent on anaerobic microcosms studies. These tests are widely applied but have as main disadvantages that the tests take weeks to months to complete. The aim of this project was to set up a quick, sensitive and specific method for the molecular detection of anaerobic dechlorinating micro-organisms.

The developed method makes use of the Polymerase Chain Reaction (PCR) and is based on differences in the DNA of *Desulfitobacteria*, an important group of anaerobic dechlorinating micro-organisms. A nested PCR was designed, using two primer pairs specific for *Desulfitobacteria.*

The method proved to be highly specific for the detection of *Desulfitobacteria*. The sensitivity of the method in a dilution range (MPN) of a pure bacterial culture was very high: 6 DNA copies per reaction, approximately 100 cells per sample. When these bacteria were mixed with contaminated soil, followed by DNA extraction and MPN-PCR the sensitivity remained at a sufficiently high level: 10^3 to 10^4 cells per gram soil could be detected when 10^8 cells/g soil had been added. This sensitivity probably is high enough to monitor the presence of anaerobic dechlorination in soil.

Anaerobic dechlorination in soil samples from contaminated sites as determined in microcosm experiments correlated well with the presence of dechlorination products in situ. Microcosms are therefore well suited to demonstrate the biodegradation potential on contaminated sites. Counting of dechlorinating micro-organisms via a physiological MPN method however seemed to be very insensitive. A maximum of 10^2 cells/g soil could be detected while much higher cell numbers (10^8/g soil) were probably present.

Analysis of these soils with the molecular MPN-PCR method showed that *Desulfitobacteria* were present in these soils, indicating that the developed method is applicable in 'natural' contaminated soils. Counting of bacterial cells proved to be possible with eubacterial primers in a MPN-PCR approach. The sensitivity of the MPN-PCR method appears to be much higher (1000*) than the physiological MPN method. However, the specific MPN-PCR for *Desulfitobacteria* did not result in an accurate count of dechlorinating bacteria. Probably impurities in the DNA extracts interfere with the PCR reaction, causing false negative results. Other DNA extraction methods need therefore to be tested to overcome these problems.

DGGE analysis of pure cultures of *Desulfitobacteria* did not show unique band patterns probably because there are not sufficient genetic differences in the amplified DNA between the tested strains. Other PCR primers have to be developed to make this form of monitoring applicable.

NOTATIONS

VOC	volalite organic chlorinated compounds
PCE	perchloroethylene
TCE	trichloroethylene
c-DCE	cis-1,2-dichloroethylene
t-DCE	trans-1,2-dichloroethylene
1,1 DCE	1,1-dichloroethylene
VC	vinylchloride
PCR	polymerase chain reaction
GC	gas chromatography
BTEX	benzene, toluene, ethylbenzene, xylenes
RUG	Groningen State University

CHAPTER 1

INTRODUCTION

In situ dehalogenation of chlorinated contaminants is a bioremediation technique that gains increasing attention. The technique is applied at several sites in The Netherlands as a full scale remediation alternative. Within the NOBIS program several projects, e.g. 'Rademarkt, Combi-remediatie', 'Totaal concept Evenblij', use in situ anaerobic dechlorination of VOCl as part of the remediation strategy.

Monitoring of active biodegradation and intrinsic bioremediation is based on three independent links of evidence:

- removal of contaminants;
- geochemical data that indicate biodegradation;
- biological experiments, including batch tests.

In addition, detection and enumeration of specific dechlorinating micro-organisms could be useful for characterization of the biodegradation potential of contaminated sites and for monitoring of in situ dechlorination processes. This approach has been used for a range of biodegradation processes, e.g. mineral oil or BTEX biodegradation, to support evidence for in situ biodegradation.

At this moment conventional microbial counting techniques (Most Probable Number, MPN) can be used to detect the presence of specific anaerobic dechlorinating micro-organisms in soil.

The MPN method that can be applied for this purpose has however some serious drawbacks:

1. detection of growth requires GC analysis;
2. growth is only detectable after weeks to months of incubation;
3. in general only a minority (< 1 %) of environmental bacteria can grow in the laboratory.

These drawbacks make that biological analysis of anaerobic dechlorination is restricted to batch experiments and other types of laboratory experiments. Monitoring of active and intrinsic anaerobic biological remediation processes in the field is almost exclusively based on chemical analysis of the biodegradation of chlorinated solvents (PCE, TCE etc.) and biological analysis are not performed.

Molecular methods for detection of dechlorinating bacteria may be useful since:

- no culture step is needed;
- the analysis is quick (hours to days);
- the essential knowledge (DNA sequences) is available.

A molecular method for the specific detection and quantification of dechlorinating micro-organisms could therefore form a suitable instrument for a quick screening of the presence of dechlorinating potential situ and to monitor the effectiveness of bioremediating actions.

The main goal of this research project is to demonstrate the feasibility of molecular methods for the detection of dechlorinating micro-organisms in contaminated soil. In chapter 2 the origin of the tested soil samples and the used methods are presented. In chapter 3 the results are presented of the design of PCR primers, DNA extraction and quantitative detection of de-

chlorinating micro-organisms using a MPN-PCR assay. In chapter 4, the results of the 'conventional' MPN cell counts are presented. The results of the project are discussed in chapter 5, followed by the conclusions in chapter 6 and recommendations in chapter 7.

CHAPTER 2

METHODOLOGY

2.1 Samples

Soil samples were taken anaerobically from different polluted sites (see table 1). For some of these sites evidence of in situ dehalogenation was available. Some sites did not show indications of in situ dehalogenation and for the Haren site no information was available. Different soil samples taken from the same site were mixed anaerobically. This soil mixture was then used for further research. Soil samples were kept at low (4 ºC) temperatures precedeeding the experiments.

Table 1. Locations used for the batch experiments.

in situ dechlorination detected	in situ dechlorination not detected
Vaassen (Tauw b.v.)	Bunnik, DRAVO (Tauw b.v.)
Groningen, Rademarkt (Bioclear b.v.)	Emmen (Bioclear b.v.)
Leeuwarden (Bioclear b.v.)	Haren, Biologisch Centrum (RUG)

2.2 Soil slurries and MPN series

Soil slurries were incubated anaerobically at 30 ºC. Five 5 cm^3 soil and 5 ml medium were brought anaerobically in a 30 ml serum bottle, sealed with a butyl/viton septum. Batches were flushed three times with a N_2CO_2 atmosphere using a gas exchange system. The vacuum steps involved also removed any volatile contaminants that were present in the soil. Therefore the experiments were all supplemented with PCE to a final concentration of 100 µM in the water phase. Lactate (10 mM) was added to stimulate anaerobic dechlorination. For each type of soil a stock control was made to check for abiological losses of the added PCE. Furthermore a control which did not receive the lactate addition was analyzed to determine the intrinsic dechlorination potential of the soil. Soil samples were incubated in a range of 10 x dilutions to obtain a MPN range. The MPN range was incubated in duplicate. 'Stimulated' MPN ranges were diluted to a dilution of 10^6, controls to a dilution of 10^3. The formation dechlorination products was measured in time. The dechlorination rate was estimated on the basis of decrease or increase of VOC and products. Chloroethenes were measured by head space analyzing, with a capillary gaschromatograph as described by Gerritse et al. [1995].

2.3 Medium

The mineral medium used for batch experiments was prepared under N_2/CO_2 gas phase (80 %/20 %) and contained:

- yeast extract 0.10 g/l
- $NaK(NH_4)PO_4$ (0,5M) 50 ml/l
- trace elements A/B/C 1 ml/l (of each)
- lactate 10 mM

After autoclaving 10 ml/l KOH (10 % w/v/), 1 ml/vitamins [Heijthuijsen and Hansen, 1986], 10 ml/l Na_2S (2 % w/v), 30 ml/l $NaHCO_3$ (1M) and 100 µM PCE was added.

Composition of the trace elements (mg/l):

solution A: $FeSO_4.7H_2O$ (200), $MnCl_2.4H_2O$ (30), $CoCl_2.6H_2O$ (200), $NiCl_2.6H_2O$ (20);
solution B: $Na_2SeO_3.5H_2O$ (26.3), $Na_2WO_4.2H_2O$ (32.97);
solution C: $ZnSO_4.7H_2O$ (100), H_3BO_3 (300), $CoCl_2.6H_2O$ (200), $CuCl_2.2H_2O$ (10), $Na_2MoO_4.2H_2O$ (30).

The final pH of the medium was 6.8 ± 0.2. The batch slurries were incubated at 30 ºC.

2.4 **DNA extraction methods**

DNA was extracted from pure cultures of bacteria and from contaminated soil using a range of DNA extraction methods. The experimental protocols for DNA extraction are described in appendix A for the selected method. Other methods are described in the cited articles, listed in the reference list.

2.5 **PCR reactions**

The experimental protocols for PCR amplification of DNA are described in appendix B.

CHAPTER 3

RESULTS DESIGN MOLECULAR DETECTION METHOD DECHLORINATING MICRO-ORGANISMS

3.1 Micro-organisms

Chlorinated ethenes (perchloroethylene (PER), trichloroethylene (TCE), dichloroethylene (cis-DCE, trans-DCE) and vinylchloride (VC)) were chosen as the target contaminants for this project. A literature survey was performed to identify micro-organisms that can dechlorinate these components under anaerobic conditions and that have been isolated. Available 16S rRNA sequences for these organisms were retrieved from databases and pure cultures were collected if available (see table 2).

Table 2. Pure cultures of anaerobic dechlorinating micro-organisms.

dechlorinating micro-organism	author and year of publication	substrate	Genbank number	pure culture [1)]
Desulfitobacterium frappieri	Bouchard et al. [1996]	pentachlorophenol	U40078	no
Desulfitobacterium dehalogenans	Utkin et al. [1994]	2,4-dichlorphenol	L28946	yes
Desulfitobacterium sp. strain PCE1	Gerritse et al. [1996]	chlorophenols, perchloroethylene	X81032	yes
Desulfitobacterium sp. strain TCE1	Gerritse et al. [1996]	trichloroethylene	X95742 X95972	yes
Desulfitobacterium hafniense DCB2	Christiansen and Ahring [1996]	chlorophenols	X94975	yes
Desulfitobacterium chlororespirans	Sanford et al. [1996]	3-chloro-, 4-hydroxy-benzoate	U68528	yes
Desulfomonile tiedjei	El Fantroussi et al. [1997b]	3-chlorobenzoate and chloroethenes	M26635	yes
Dehalospirillum multivorans	Scholz-Muramatsu et al., [1995]	tetrachloroethylene	X82931	no
Dehalobacter restrictus	Wild et al. [1996]	tetrachloroethylene, trichloroethylene	Y10164	no

[1)] available to the project

3.2 Development of specific PCR primers for detection of *Desulfitobacterium* strains

3.2.1 *Design of PCR primers*

Many researchers have isolated *Desulfitobacteria* that can dechlorinate chloroethenes from contaminated soils. This group of micro-organisms seems therefore most promising as indicator organisms for anaerobic dehalogenation and were chosen as target organisms in this project.

The principle of PCR detection is in short as follows:
PCR primers (DNA sequences) are designed that bind on both sides of a highly specific region of the 16S rRNA of *Desulfitobacterium*. During the PCR reaction the specific DNA sequence between the primers is multiplied over many orders of magnitude.

At the end of the PCR reaction, the reaction mixture contains mainly the amplified specific 16S rRNA region. This PCR product is separated from remaining primers and original DNA on gel. If the PCR primers are not specific, DNA from many more organisms in a sample will produce a

PCR band giving a complex pattern. If no target DNA is available or the mixture contains toxic components, no product is formed.

16S rRNA sequences for *Desulfitobacteria* that were retrieved from databases were aligned (compared) to identity similarities (non-specific regions) and dissimilarities (specific regions) in the 16S rRNA sequences. Based on these alignments the primer sets A1F/A4R and N3F/N1R were designed for the specific detection of *Desulfitobacteria*.

The following criteria for selection of the primer set were used:

- identical annealing temperature for both primers;
- the 3' -end of the primers has to be a different base to improve the specificity of the primers;
- high overall specificity of the primers for *Desulfitobacteria.*

The specificity of the primers was checked with Genbank. Primer set A1F/A4R would yield a PCR-product of 568 basen pairs length. The positions of primer pairs on the 16S rRNA on the *Escherichia coli* 16S rRNA are shown in table 3.

Table 3. Used PCR primer sets.

name primer	length in base pairs	annealing in ºC	position primer in *Escherichia coli*	sequence primer 5' → 3'
A1F	20	51	114 - 133	TAACGCGTGGATAACCTACC
A4R	20	51	648 - 667	CCTCTCCTGTCCTCAAGATA
N3F	21	59	406 - 426	GTACGACGAAGGCCTTCGGGT
N1R	20	59	610 - 619	CCCAGGGTTGAGCCCTAGGT
U27F	20	55		AGAGTTTGATCMTGGCTCAG
U1492R	19	55		GGTTACCTTGTTACGACTT
EUB338(R)	18	55		GCTGCCTCCCGTAGGAGT

During optimization of the annealing temperature it was found that DNA from *Desulfotomaculum orientis* also gave a PCR product with the A1/A4 primer set. This means that the primer set A1/A4 is not specific enough for the detection of *Desulfitobacteria* only. It may be however that one of the deposited *Desulfotomaculum orientis* in the databases is a different organism and possibly a *Desulfitobacterium*. To avoid problems, it was decided to design a new primer set.

The new primer set, N3F en N1R, showed good specificity for *Desulfitobacteria* in Genbank. The PCR product of these primers is 225 base pairs long (see table 3). The primer set N3F/N1R was used to detect DNA, isolated from a series of pure cultures. It was shown that with the primer set N3F/N1R, only *Desulfitobacteria* were detected (see fig. 1 and table 4). This indicates that according to the database (Genbank) and according to a practical test the primer set N3F/N1R is in principal suitable for the specific detection of *Desulfitobacteria.*

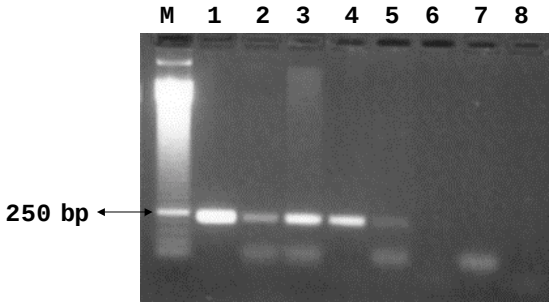


Fig. 1. PCR detection of *Desulfitobacteria* with primer set N3F/N1R.
Lanes: M, 50 bp-ladder; 1, *Desulfitobacterium* PCE1; 2, *Desulfitobacterium* TCE1; 3, *Desulfitobacterium dehalogenans*; 4, *Desulfitobacterium hafniense*; 5, *Desulfitobacterium chlororespirans*; 6, *Desulfotomaculum orientis*; 7, *Desulfovibrio* SULF1; 8, negative control (sterile demi water).

Table 4. PCR detection of *Desulfitobacteria* with primer set N3F/N1R.

lane	sample	PCR product expected	PCR product found
M	= 50 bp-ladder (the brightest band is 250 bp)		
1	= *Desulfitobacterium* PCE1	yes	yes
2	= *Desulfitobacterium* TCE1	yes	yes
3	= *Desulfitobacterium dehalogenans*	yes	yes
4	= *Desulfitobacterium hafniense*	yes	yes
5	= *Desulfitobacterium chlororespirans*	yes	yes
6	= *Desulfotomaculum orientis*	no	no
7	= *Desulfovibrio* SULF1	no	no
8	= sterile demi water	no	no

3.3 **DNA extraction**

When using a PCR detection for the quantitative detection of bacteria in soil, a good DNA extraction is essential. The target for the PCR is the extracted DNA and if the DNA extraction efficiency is poor, the actual numbers of target organisms in the soil will be underestimated. Several published methods for the extraction of DNA from soil were therefore tested.

The following methods were tested:

I. Modified method as described by Van Elsas and Smalla [1995], followed by a DNA clean-up step by sepharose CL-4B gel chromatography as described by Jackson et al. [1997].
II. Modified method as described by Van Elsas and Smalla [1995], followed by a DNA clean-up step by sepharose CL-4B gel chromatography as described by Jackson et al. [1997].
III. Method followed as described by El Fantroussi et al. [1997b].
IV. DNA extraction method as described by Porteous et al. [1997], followed by a DNA clean-up step by sepharose CL-4B gel chromatography as described by Jackson et al. [1997].
V. DNA extraction method as described by Porteous et al. [1997], followed by a commercial DNA clean-up step, Wizard ® DNA clean-up system (Promega).
VI. DNA extraction method as described by Lévesque et al. [1997], followed by a DNA clean-up step by sepharose CL-4B gel chromatography as described by Jackson et al. [1997] (see appendix A).
VII. DNA extraction method as described by Lévesque et al. [1997], followed by a commercial DNA clean-up step, Wizard ® DNA clean-up system (Promega).

An overview of the used DNA isolation methods is given in table 5.

Table 5. DNA isolation methods.

DNA extraction method	homogenization and lysis		concentration of crude DNA extract	DNA purification step	additional DNA purification step
	compounds	homogenizer			
I	lysozym SDS 125 mM phosphate buffer	bead beater	fenol/chloroform extraction	ethanol precipitation	sepharose CL-4B gel chromatography ethanol precipitation
II	lysozym SDS 62.5 mM phosphate buffer	bead beater	fenol/chloroform extraction	ethanol precipitation	sepharose CL-4B gel chromatography ethanol precipitation
III	lysozym SDS	ultrasoon waterbath	chloroform extraction isopropanol precipitation	sephadex G-50 gel chromatography ethanol precipitation	-
IV	guanidine isothiocyanate SDS	ultrasoon waterbath	potassium acetate PEG 8000 CTAB chloroform extraction isopropanol precipitation	ethanol precipitation	sepharose CL-4B gel chromatography ethanol precipitation
V	guanidine isothiocyanate SDS	ultrasoon waterbath	potassium acetate PEG 8000 CTAB chloroform extraction isopropanol precipitation	ethanol precipitation	Wizard® clean-up system (Promega)
VI	PVPP	bead beater	fenol/chloroform extraction	ethanol precipitation	sepharose CL-4B gel chromatography ethanol precipitation
VII	PVPP	bead beater	fenol/chloroform extraction	ethanol precipitation	Wizard® clean-up system (Promega)

Soil from the DRAVO site was used as test material, this soil is contaminated with chloroethenes.

The results were checked against the following criteria:

- amount of DNA retrieved as determined with the Genequant;
- extracted DNA visible on gel;
- PCR product formed with universal primers.

The results of the different methods are summarized in table 6.

Table 6. Results DNA extraction.

DNA extraction method	Genequant (µg DNA/g dw) DNA analysis	DNA visible on gel	PCR product formed [2)]
I	23.7	- [1)]	+
II	9 - 10	- [1)]	-
III	15 - 18	- [1)]	-
IV	3.3	-	-
V	2.8	-	+
VI	0.9	+	+
VII	2.2	-	+

[1)] the amount applied DNA was probably too low for visible detection
[2)] universal primers

The extracted DNA was in most cases still contaminated with organic materials and protein, which causes large differences in the Genequant assay. This method is designed for pure culture studies and is probably not suited for analysis of environmentally derived DNA.

The amounts of extracted DNA were probably too low to be directly visualized on gel in the applied volumes. Larger samples should be extracted and more sample should be applied to test this DNA detection method.

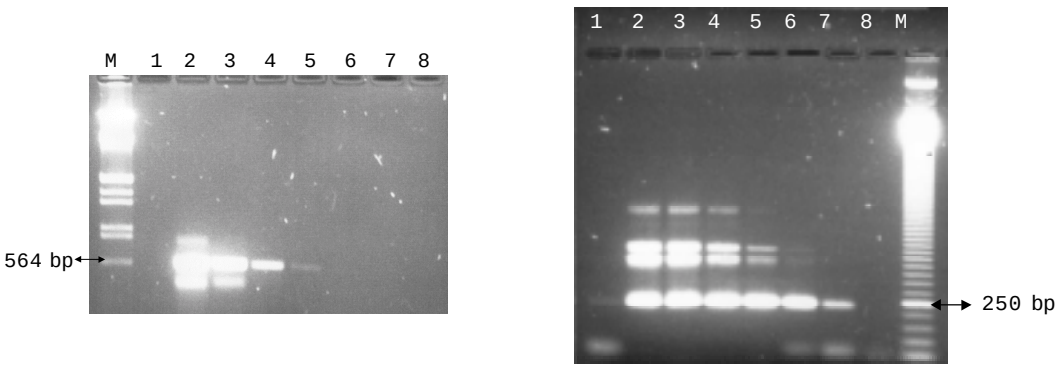
The PCR reaction that was applied made use of universal eubacterial primers that should react with any DNA from (eu)bacterial origin. It is therefore a positive control to the availability of extracted DNA and to the absence of components that may inhibit the enzymatic PCR reaction and that may be co-extracted with the DNA from contaminated soil.

It was shown that four out of seven methods gave positive PCR reactions in undiluted DNA samples.

Based on these results, it was decided to perform further DNA extractions with method VI (bead beating extraction, followed by sepharose clean-up) for extraction of DNA from contaminated soil. This method had shown both DNA on gel and resulted in the formation of products after PCR.

3.4 Detection limit MPN-PCR *D. hafniense* DCB2 in pure culture

The detection limit of the designed PCR method for specific detection of *Desulfitobacteria* was determined using a MPN approach. DNA was extracted from a *D. hafniense* pure culture according to the previously selected extraction procedure. The extracted DNA was diluted in a tenfold MPN dilution range and subjected to a nested PCR [Herman, 1995; El Fantroussi et al., 1997a] with PCR primer sets A1/A4 and N3/N1. The calculation from DNA to cell numbers was based on the assumption that the length of the *D. hafniense* chromosome is comparable to that of the *E. coli* chromosome, $4.7 \cdot 10^6$ base pair. The results after the nested MPN-PCR are shown in figure 2 and table 7.



a) b)

Fig. 2. PCR on diluted DNA of *D. hafniense* DCB2.

a) PCR with the primers A1F and A4R. Total volume of the PCR was 50 µl; 10 µl template DNA was used.
Lanes: M, Marker, λ·/EcoRI/HindIII; 1, $7.8 \cdot 10^6$ copies; 2, $7.8 \cdot 10^5$ copies; 3, $7.8 \cdot 10^4$ copies; 4, $7.8 \cdot 10^3$ copies; 5, $7.8 \cdot 10^2$ copies; 6, $7.8 \cdot 10^1$ copies; 7, $7.8 \cdot 10^0$ copies; 8, $7.8 \cdot 10^{-1}$ copies.

b) Nested PCR with the primers A1F/A4R and N3F/N1R. Total volume of the PCR was 50 µl; 3 µl PCR mix from the first PCR was used as template DNA.
Lanes: M, Marker, 50 bp-ladder; 1, $7.8 \cdot 10^6$ copies; 2, $7.8 \cdot 10^5$ copies; 3, $7.8 \cdot 10^4$ copies; 4, $7.8 \cdot 10^3$ copies; 5, $7.8 \cdot 10^2$ copies; 6, $8 \cdot 10^1$ copies; 7, $7.8 \cdot 10^0$ copies; 8, $7.8 \cdot 10^{-1}$ copies.

Table 7. Determination detection level MPN-PCR with *D. hafniense* DCB2.

lane	amount of DNA (g/PCR)	number of organisms per PCR	figure 2a	figure 2b
			single PCR with primer set A1/A4	nested PCR with primer sets A1/A4 and N1/N3
1	$4 \cdot 10^{-8}$	$7.8 \cdot 10^6$	- [1)]	+
2	$4 \cdot 10^{-9}$	$7.8 \cdot 10^5$	+++	+++
3	$4 \cdot 10^{-10}$	$7.8 \cdot 10^4$	+++	+++
4	$4 \cdot 10^{-11}$	$7.8 \cdot 10^3$	++	+++
5	$4 \cdot 10^{-12}$	$7.8 \cdot 10^2$	+	+++
6	$4 \cdot 10^{-13}$	$7.8 \cdot 10^1$	-	+++
7	$4 \cdot 10^{-14}$	$7.8 \cdot 10^0$	-	++
8	$4 \cdot 10^{-15}$	$7.8 \cdot 10^{-1}$	-	-
	0	0 (milli-Q)	-	-

[1)] - no PCR product detectable
+PCR product detectable
++ medium amounts of PCR product
+++ large amounts of PCR product

The intensity of the PCR products correlates with the amount of DNA template in the PCR mixture. The PCR with $7.8 \cdot 10^6$ DNA copies shows no PCR product with primers A1 and A4, but lower quantities of DNA are easily detected. This is probably caused by inhibition of the PCR reaction by high amounts of DNA or inhibiting compounds in the DNA extract. Further dilution relieved this inhibition.

The detection limit for the one step PCR with primer set A1F/A4R was $7.8 \cdot 10^2$ copies DNA in the PCR mixture. Using nested PCR with primer sets A1F/A4R and N3F/N1R, a positive result was obtained with as little as 8 DNA copies in the PCR mixture.

Both primer sets A1/A4 and primer sets N1/N3 showed more than one PCR product. This can be caused by the high amount of DNA in the mixture, resulting in non-specific hybridization and elongation. This seems indeed to happen as higher dilutions show fewer PCR products. Furthermore, minor amounts of A1/A4 primers will be present in the second PCR step, thus allowing the formation of hybrid PCR products. The following products may be formed in theory:

- PCR product A1(F) and A4(R); length 560 base pairs;
- PCR product A1(F) and N1(R); length 510 base pairs;
- PCR product N3(F) and A4(R); length 260 base pairs;
- PCR product N3(F) and N1(R); length 225 base pairs.

These PCR products are indeed visible on the agarose gels.

The detection limit could be further improved by raising the number of PCR cycli. The number of PCR cycli used was 30 in each PCR step.

3.5 Detection limit MPN-PCR *D. hafniense* DCB2 added to DRAVO soil

Following the determination of the detection limit of a pure culture, the detection limit of the nested PCR detection in soil was determined. For this purpose, a pure culture of *D. hafniense* was added to DRAVO soil. Based on biodegradation studies, it was assumed that DRAVO soil contains neglectable amounts of dechlorinating micro-organisms.

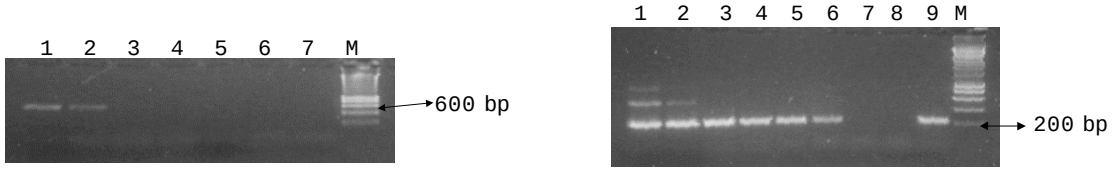
D. hafniense cells ($0.5 \cdot 10^8$) were added to Bunnik soil (0.5 g dw) and mixed. DNA was extracted according to the selected protocol and suspended in 80 µl sterile demi water. The DNA extract was diluted in a tenfold MPN dilution range and each dilution was subjected to nested PCR using the primer sets A1/A4 and N1/N3. Each PCR reaction used 10 µl DNA extract, PCR products were analyzed using gel electrophoresis.

In a single PCR with primer set A1/A4, $6 \cdot 10^4$ DNA copies of *D. hafniense* DCB2 could be detected (see fig. 3 and table 8). This is equivalent to a detection limit of $1 \cdot 10^6$ DNA copies per gram of soil. In a nested PCR approach the sensitivity of the detection improved to $6 \cdot 10^1$ - $6 \cdot 10^2$ DNA copies per PCR reaction. This equivalent to a detection limit of $1 \cdot 10^3$ - $1 \cdot 10^4$ *D. hafniense* DNA copies or cells per gram of soil.

These values compare well with published data for molecular detection of *Desulfofitobacteria* of 800 cells/g soil [Lévesque et al., 1997]. The detection limit can even be improved by decreasing the volume of the DNA extract or by using more DNA template in the second PCR reaction. This however may increase the chances of inhibition. The detection limit of the PCR reaction has increased from 8 to 60 - 600 DNA copies as compared to the pure culture.

This may have been caused by the following effects:

- the efficiency of DNA extraction from soil is lower;
- the cell lysis in soil is less efficient;
- the DNA is damaged or contaminated (sheared) resulting in poor templates for the PCR reaction;
- the soil extracts contains components that inhibit the PCR reaction. A wide range of soil compounds may interfere with the PCR reaction [Wilson, 1997].



a) b)

Fig. 3. MPN-PCR of *D. hafniense* in inoculated soil.
a) PCR with primers A1F, A4R (10 µl template).
Lanes: M, 200 bp-ladder; 1, undiluted; 2, 10 · diluted; 3, 10^2 · diluted; 4, 10^3 · diluted; 5, 10^4 · diluted; 6, 10^5 k · diluted; 7, negative control (milli-Q).
b) Nested PCR with primers A1F/A4R and N3F/N1R, 3 µl template.
Lanes: M, 200 bp-ladder; 1, undiluted; 2, 10 · diluted; 3, 10^2 · diluted; 4, 10^3 · diluted; 5, 10^4 · diluted; 6, 10^5 · diluted; 7, negative control (milli-Q) A1F/A4R and N3F/N1R; 8, negative control (milli-Q) N3F/N1R; 9, positive control N3F/N1R (*D. hafniense* DCB2).

Table 8. MPN-PCR of inoculated Bunnik soil (10^8 *D. hafniense* cells/g soil).

dilution factor	theoretical number of copies DNA per g soil	theoretical number of copies DNA per PCR	figure 3a		figure 3b	
			results single PCR with A1/A4 [2]		results nested PCR A1/A4 and N1/N3 [2]	
0 · diluted	$1{\cdot}10^8$	$6{\cdot}10^6$	+ [1]	+	++	++
10 · diluted	$1{\cdot}10^7$	$6{\cdot}10^5$	+	+	++	++
10^2 · diluted	$1{\cdot}10^6$	$6{\cdot}10^4$	+	+	++	++
10^3 · diluted	$1{\cdot}10^5$	$6{\cdot}10^3$	-	-	++	++
10^4 · diluted	$1{\cdot}10^4$	$6{\cdot}10^2$	-	-	++	++
10^5 · diluted	$1{\cdot}10^3$	$6{\cdot}10^1$	-	-	+	-
10^6 · diluted	$1{\cdot}10^2$	6	n.p. [3]	-	n.p.	-
10^7 · diluted	$1{\cdot}10^1$	$6{\cdot}10^{-1}$	n.p.	-	n.p.	-
negative control	-	-	-	-	-	-

[1] - no PCR product detectable
+PCR product detectable
++ medium amounts of PCR product
+++ large amounts of PCR product
[2] PCRs are done in duplo
[3] n.p.: not performed

3.6 PCR on soil DNA extracts with universal primers

3.6.1 *PCR on undiluted soil DNA extracts*

In a first approach to perform PCR on contaminated soil, DNA extracts from a range of contaminated soils were subjected to PCR using universal primers. The goal of this experiment was to verify that DNA could be extracted from these soils and that a PCR reaction could be performed with this DNA.

DNA extracts were obtained from DRAVO soil, DRAVO batch B1, DRAVO batch C1, Rademarkt soil, Rademarkt batch F0, Haren soil, Haren batch M0, Emmen soil, Emmen batch J0, Leeuwarden soil, Leeuwarden batch G0, Vaassen soil and Vaassen batch B0. The batch samples were taken from the batch experiments after completion of the biodegradation experi-

ment. The batches with codes x 0 contained non-diluted soil samples, the batches with codes x 1 contained 10 x diluted soil samples. The DNA was extracted from soil (0.5 g dw), suspended in demi water (80 µl) and used (5 µl) for nested PCR with the universal primer sets U27F/U1492R and U27F/EUB338R.

The samples from the Haren soil were high in humic substances as evidenced by the brown color of the extracts. The sepharose CL-4B clean-up step resulted in removal of the majority of these impurities, rendering the sepharose columns brown. The DNA pellets however were also still brown in color, indicating impurities.

DNA extract (30 µl) of each DNA extract was brought onto gel to detect DNA (see table 9). Only the Haren soil sample showed of DNA, visible as a smear.

Table 9. Nested PCR with universal/eubacterial primers in soil samples.

sample	MPN dechlorination [1)]	soil type	visibility DNA on gel (30 µl used)	single PCR (U27F/U1492R)	nested PCR (U27F/U1492R) and (U27F/EUB338R)
DRAVO soil		sand	-	-	+
DRAVO batch B1	positive	sand	+ 200 - 800 bp	-	+
DRAVO batch C1	negative	sand	+ 200 - 800 bp	-	+
Rademarkt soil		sand	-	-	+
Rademarkt batch F0	positive	sand	-	-	+
Haren soil		high organic content	+ 200 bp -20000 bp	-	+
Haren batch M0	positive	high organic content	+ < 100 bp	-	-
Emmen soil		clay	-	-	+
Emmen batch J0	negative	clay	-	-	+
Leeuwarden soil		clay	n.p. [2)]	-	+
Leeuwarden batch G0	positive	clay	n.p.	-	+
positive control				++	++
negative control (milli-Q)				-	+

[1)] see chapter 5
[2)] n.p.: not performed
- no PCR product detectable
+ PCR product detectable
++ medium amounts of PCR product
+++ large amounts of PCR product

A single PCR with the universal primers U27F and U1492R showed with none of the DNA extracts a visible PCR product (see table 9). This clearly indicated the presence of inhibiting factors in the DNA extract. The positive control, *E. coli* DNA, did show a PCR product of approximately 1500 base pairs. Nested PCR with primer sets U27F/U1492R and U27F/EUB338R resulted for all locations, except Haren batch, in PCR products.

The negative control also showed a positive signal. This result can possibly be caused by inaccuracy during the PCR work, or aerosol formation during the reaction. Another reason can be the presence of genomic *E. coli* DNA in the Taq-polymerase. Taq-polymerase is commercially produced with *E. coli* cells.

In following experiments, this potential source of contamination was eliminated by a 5 minute UV irradiation of the PCR mix (without template and primers) preceding the PCR reaction. No more positive signals in the negative controls were found after this.

3.6.2 *MPN-PCR with universal primers on diluted soil DNA extract from Rademarkt*

In a second approach to perform PCR on contaminated soil, soil extracts from Rademarkt soil and Rademarkt batch were analyzed in a nested MPN-PCR. DNA extracts were diluted and subjected to nested PCR with the primer sets U27F/U1492R and U27F/EUB338R.

After the first PCR with primer set A1/A4, none of the dilutions showed a PCR product (data not shown). The positive control with *E. coli* showed the expected product whereas the negative control (UV treated) did not show a product. This indicated again that in the first PCR inhibition of the PCR reaction occurs.

The second, nested, PCR did show PCR products for all dilutions, except for the negative control and the 10 times dilution of Rademarkt soil (see fig. 4 and table 10). The amount of PCR product does not correlate with the amount of template DNA in the first dilution. However, the exact amount of DNA in the PCR mixture at the start of the second (nested) PCR is unknown.

Assuming that a minimum number of 10 DNA copies is required for the generation of a PCR product, this result indicates that a minimum of $10 \cdot 10^6 = 10^7$ copies per 5 µl DNA extract was extracted. This means that from 0.5 g soil a minimum of $(80/5) \cdot 10^7 = 1.6 \cdot 10^8$ copies DNA or bacterial cells have been isolated.

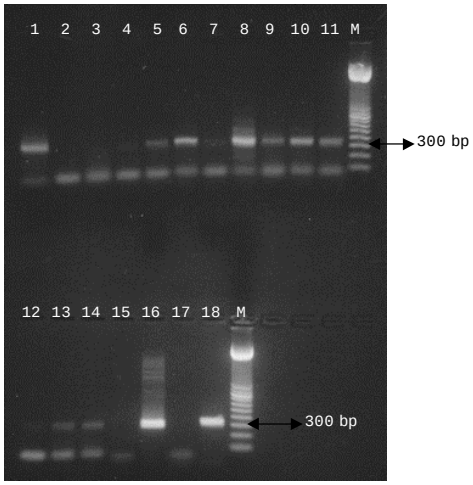


Fig. 4. Universal MPN-PCR in Rademarkt samples.
PCR with primer sets U27F/1492R (first, 5 µl template) and U27F/EUB338R (second PCR, 3 µl template).
Lanes: M, 1 kb-ladder; 1, Rademarkt soil undiluted; 2, Rademarkt soil 10 · diluted; 3, Rademarkt soil 10^2 · diluted; 4, Rademarkt soil 10^3 · diluted; 5, Rademarkt soil 10^4 · diluted; 6, Rademarkt soil 10^5 · diluted; 7, Rademarkt soil 10^6 · diluted 8, Rademarkt batch F0 undiluted; 9, Rademarkt batch F0 10 · diluted; 10, Rademarkt batch F0 10^2 · diluted; 11, Rademarkt batch F0 10^3 · diluted; 12, Rademarkt batch F0 10^4 · diluted; 13, Rademarkt batch F0 10^5 · diluted; 14, Rademarkt batch F0 10^6 · diluted; 15, negative control (milli-Q) U27F/1492R and U27F/EUB338R; 16, positive control (*E. coli*) U27F/1492R and U27F/EUB338R; 17, negative control (milli-Q) U27F/EUB338R; 18, positive control (*E. coli*) U27F/EUB338R.

Table 10. Results MPN-PCR on soil DNA extracts from Rademarkt with universal primer sets U27F/1492R and U27F/EUB338R.

sample	dilution factor						
	0	10^1	10^2	10^3	10^4	10^5	10^6
Rademarkt soil	++	-	+	+	++	++	+
Rademarkt batch F0	++	++	++	++	+	+	+

- no PCR product detectable
+ PCR product detectable
++ medium amounts of PCR product
+++ large amounts of PCR product

3.7. **Specific MPN-PCR or *Desulfitobacteria* in DNA extracts from soil**

The ultimate goal of the project was to detect and count numbers of dechlorinating micro-organisms in soils contaminated with VOC. As a sensitive detection of *D. hafniense* cells in soil had been demonstrated and nested PCR with universal primers in contaminated soils had been demonstrated, a specific nested PCR of *Desulfitobacteria* was attempted.

DNA was extracted from soils and batch experiments as described previously. The extracts were diluted and subjected to a nested PCR with the specific primer sets A1F/A4R and N3F/N1R.

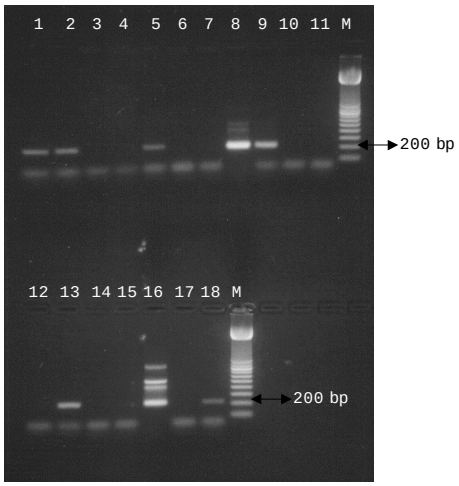


Fig. 5. Detection of *Desulfitobacteria* in soil samples using a specific nested MPN-PCR. PCR with primer sets U27F/1492R (first, 5 µl template) and U27F/EUB338R (second PCR, 3 µl template).
Lanes: M, 1 kb-ladder; 1, Rademarkt soil undiluted; 2, Rademarkt soil 10 · diluted; 3, Rademarkt soil 10^2 · diluted; 4, Rademarkt soil 10^3 · diluted; 5, Rademarkt soil 10^4 · diluted; 6, Rademarkt soil 10^5 · diluted; 7, Rademarkt soil 10^6 · diluted 8, Rademarkt batch F0 undiluted; 9, Rademarkt batch F0 10 · diluted; 10, Rademarkt batch F0 10^2 · diluted; 11, Rademarkt batch F0 10^3 · diluted; 12, Rademarkt batch F0 10^4 · diluted; 13, Rademarkt batch F0 10^5 · diluted; 14, Rademarkt batch F0 10^6 · diluted; 15, negative control (milli-Q) U27F/1492R and U27F/EUB338R; 16, positive control (*E. coli*) U27F/1492R and U27F/EUB338R; 17, negative control (milli-Q) U27F/EUB338R; 18, positive control (*E. coli*) U27F/EUB338R.

Table 11. MPN-PCR on soil DNA extracts with the specific primer sets A1F/A4R and N3F/N1R.

sample	dilution factor						
	0	10	10^2	10^3	10^4	10^5	10^6
Bunnik soil	+/-	-	-	-	-	-	-
Bunnik batch B1	-	-	-	-	-	-	-
Rademarkt soil	+	+	-	-	+	-	-
Rademarkt batch F0	++	+	-	-	-	+	-
Emmen soil	+	-	-	+	-	-	-
Emmen batch J0	+	-	-	-	-	-	-
Leeuwarden soil	-	-	-	-	+	-	-
Leeuwarden batch G0	+	-	-	-	-	-	-

[1)] - no PCR product detectable
\+ PCR product detectable
++ medium amounts of PCR product
+++ large amounts of PCR product

The highest number of positive results was found in the low (0*) dilution series of all samples (see fig. 5 and table 11). Soil samples from Rademarkt and Rademarkt batch showed the highest number of positive signals in the dilution series. These samples also showed positive results in the highest dilutions, indicating the presence of higher numbers of *Desulfitobacteria*.

At a dilution of 10^5, a positive signal was obtained, indicating a minimal cell concentration of $2.6 \cdot 10^7$ cells/g dw. However, negative results were present within the dilution series, which is not in line with the principle of MPN dilution. In each MPN series a negative control was applied and all negative controls were negative. This indicates that the positive results in the dilution series were not caused by contamination but were really due to the detection of autochtoneous DNA. The negative results of the intermittent dilutions must therefore be caused by inhibition of the PCR reaction. If it is assumed that the positive PCR reactions in the higher dilutions indeed indicate the presence of *Desulfitobacteria*, these data can be used to derive MPN-PCR numbers. This would result in a MPN-PCR of $2.6 \cdot 10^6$ cells/g in the Rademarkt soil sample. The physiological MPN for this sample was $2.5 \cdot 10^1$ cells/g dw (see chapter 4).

3.8 **Sequencing of PCR products**

The specificity of the detection was finally determined by sequencing of the obtained PCR products. The reasoning behind this action was that it needed to be proved that the positive signal that was obtained with a nested PCR was indeed caused by the amplification of DNA from the target organisms (*Desulfitobacteria*) and not by a DNA sequence that had sequence similairy in the primer region but was not a member of the *Desulfitobacteria* group.

One A1/A4 PCR product was sequenced: a PCR product from the 0·dilution from Rademarkt batch F0. The PCR fragments have been cloned and sequenced as described in appendix C. The sequence of the A1/A4 PCR product from Rademarkt batch F0 (duplo) is shown in appendix C1.

The sequence alignment of this product (C851 · A1F/A4R) with the database is shown below:

Table 12a. Sequence similarity of Rademarkt clone C851.

sequences producing significant alignments		
emb	|X95742|DSP16SRR1	*Desulfitobacterium sp.* 16S rRNA gene, clone 1
emb	|X95972|DSP16SRR2	*Desulfitobacterium sp.* 16S rRNA gene, clone 2
gb	|U40078|DFU40078	*Desulfitobacterium frappieri* 16S ribosomal R...
emb	|X94975|DH16SRRNA	*D. hafniense* 16S ribosomal RNA
gb	|U68528|DCU68528	*Desulfitobacterium chlororespirans* 16S ribos...
gb	|L28946|DTORRD	*Desulfitobacterium dehalogenans* 16S ribosomal...
emb	|X81032|DS16SRR	*Desulfitobacterium sp.* 16S rRNA gene

The retrieved sequences were compared with the 16S rRNA database in Genbank. The 7 sequences with the highest sequence similarity between the database and the Rademarkt sequence are listed in table 12a and 12b. The sequence similarity decreases from top to bottom.

The highest sequence similarity of the cloned PCR alignment is found with *Desulfitobacteria* in the databases. The sequence similarity of the cloned PCR product (C851 · A1F/A4R) was more than 99 % with *Desulfitobacteria sp.* 16S rRNA genbanknumber X95742. This result clearly shows that the obtained PCR product originates from a *Desulfitobacterium* that was present in the sample.

A second clone from the same PCR product (C852 · A1F/A4R) was sequenced with the following result:

Table 12b. Sequence similarity of Rademarkt clone C852.

sequences producing significant alignments		
emb	|X94975|DH16SRRNA	*D.hafniense* 16S ribosomal RNA
emb	|X95972|DSP16SRR2	*Desulfitobacterium sp.* 16S rRNA gene, clone 2
emb	|X95742|DSP16SRR1	*Desulfitobacterium sp.* 16S rRNA gene, clone 1
gb	|U40078|DFU40078	*Desulfitobacterium frappieri* 16S ribosomal R...
gb	|U68528|DCU68528	*Desulfitobacterium chlororespirans* 16S ribos...
gb	|L28946|DTORRD	*Desulfitobacterium dehalogenans* 16S ribosomal...
emb	|X81032|DS16SRR	*Desulfitobacterium sp.* 16S rRNA gene

The sequence similarity of the cloned PCR product (C852 · A1F/A4R) was more than 99 % with *Desulfitobacterium hafniense* 16S rRNA Genbank number X94975. Again, a *Desulfitobacterium* was identified. This clearly shows that *Desulfitobacteria* are present in the Rademarkt soil and even different types of *Desulfitobacterium* can be amplified in this PCR approach. The specificity of the selected primers is good.

3.9 **DGGE of *Desulfitobacteria***

In addition to the specific detection and enumeration of *Desulfitobacteria*, an attempt was made to distinguish dechlorinating microbial communities on the basis of community profiling. DGGE (denaturaling gradient gel electrophoresis) is a two-step approach to separate and identify DNA on the basis of base pair similarity [Muyzer et al., 1993]. First, a PCR step is applied. This PCR

step can be non-discriminating by e.g. amplifying the 16S rRNA of all bacteria in a sample. The DGGE can be made more specific by using specific PCR primers, resulting in PCR amplification of only a specified group of the microbiological community.

These PCR products are then separated on a DGGE gel. In this gel, the double stranded PCR products migrate through the gel until the DNA denaturates into single DNA strands that inhibit further migration and immobilize this PCR product on a distinctive spot in the gel. This denaturation is caused by chemicals in the gel and dependent on chemical concentration and DNA composition. By increasing the chemical concentration along the gel, each PCR product will immobilize on a different spot, depending on the DNA composition. A community profile can thus be obtained by creating DGGE profiles of known dechlorinating micro-organisms and comparing the separate DGGE profiles to identify unique bands.

To this end, the A1/A4 primer set was used to amplify total DNA from pure cultures of *Desulfitobacterium* PCE1, *Desulfitobacterium TCE1, Desulfotomaculum orientis* and *Desulfitobacterium chlororespirans*. Primer A4 contained a GC-clamp. After PCR, the products were subjected to DGGE analyses.

In a first attempt a DGGE gel with a ureum gradient of 35 % to 70 % was used. No clear differences in band pattern were visible between the different strains. The denaturating ureum % for the PCR products was approximately 55 %. Following this result, the A1/A4 PCR-products were separated on a 50 % - 60 % ureum DGGE.

This however did not improve the separation of bands. Probably the differences between the nucleotide sequences in the different PCR products is too small to result in visible retardation changes on the applied DGGE gels (see fig. 6).

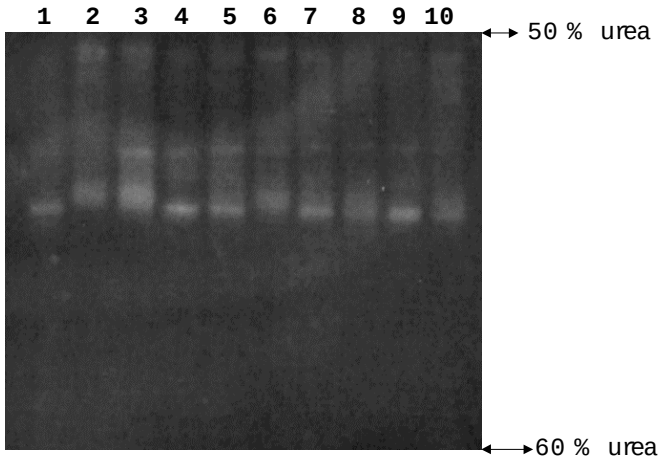


Fig. 6. DGGE of pure cultures of *Desulfitobacteria*.
Lanes: 1, *Desulfitobacterium chlororespirans*; 2, *Desulfotomaculum orientis*; 3, *Desulfitobacterium* TCE1; 4, *Desulfitobacterium* PCE1; 5, TCE1 and PCE1; 6, *D. orientis* and TCE1; 7, *D. orientis* and PCE1; 8, *D. chlororespirans* and TCE1; 9, , *D. chlororespirans* and PCE1; 10, *D. chlororespirans* and *D. orientis*.

The theoretical differences in nucleotide sequence in the amplified PCR product between *D. dehalogenans* and *Desulfitobacterium* PCE1 are shown in table 13.

Table 13. Base pair differences in A1 and A4 products between different subspecies from *Desulfitobacteria.*

strain	strain	base pair differences in A1/A4 PCR products
D. dehalogenans	PCE1	56
D. dehalogenans	TCE1	29
D. dehalogenans	*D. hafniense*	29
D. dehalogenans	*D. frappieri*	28
D. dehalogenans	*D. chlororespirans*	28

From table 13 it is clear that differences in nucleotide sequence are present in the different PCR products. The number of base pair difference that will allow separation on DGGE gel however is apparently is higher than the 28 - 56 that are present in this set of PCR products. Additionally the DGGE has to be optimized for separation of PCR products with low base pair differences.

CHAPTER 4

RESULTS BATCH EXPERIMENTS AND PHYSIOLOGICAL MPN DETECTION

4.1 Introduction

Bacterial numbers that can dechlorinate chloroethenes were determined using the MPN assay. In this assay soil is diluted in a step wise approach and diluted samples are incubated under anaerobic conditions that are favorable for anaerobic dechlorination. Dilutions that still contain sufficient numbers of dechlorinating micro-organisms will eventually start dechlorinating the chloroethenes in the incubation. Samples that were diluted beyond a minimum cell number will not dechlorinate, thus allowing the determination of cell numbers in the original sample in a statistical approach.

The purpose of this part of the study is to determine how many dechlorinating micro-organisms are present in chloroethene polluted soil and estimate the dechlorination rate of PCE and the dechlorinating products. These results can later be compared to the MPN-PCR analyses of the same soil.

The soil slurries were incubated with a carbon source and electron acceptor (lactate). Anaerobic dechlorination was followed in time through head space analysis of VOC and ethene.

4.2 Results

The degradation of PCE in the batches with non-diluted soil are shown in figures 7 to 10 and appendix D.

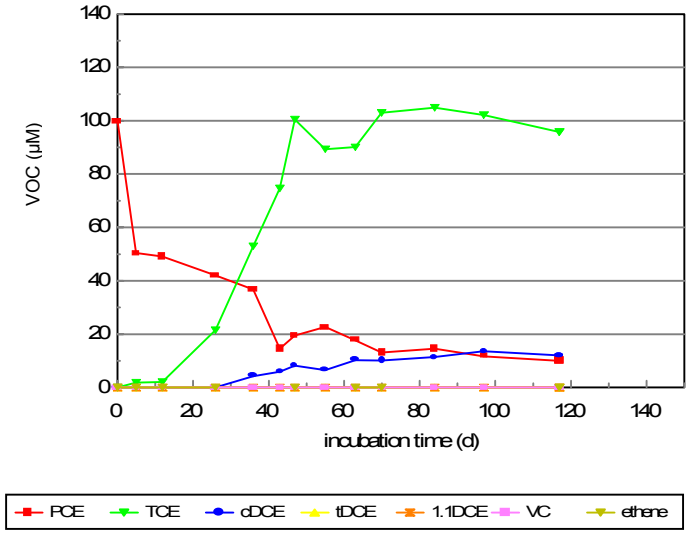


Fig 7. Dechlorination of PCE in a batch test with soil from the Groningen Rademarkt site.

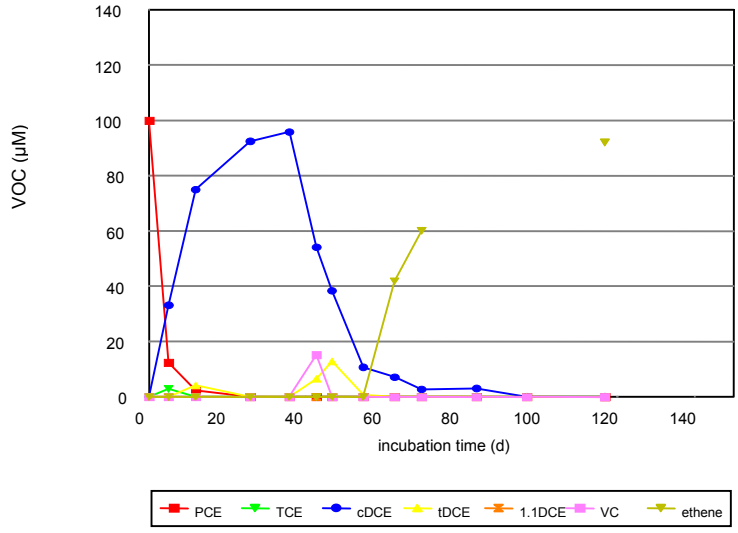


Fig. 8. Dechlorination of PCE in a batch test with soil from the Leeuwarden site.

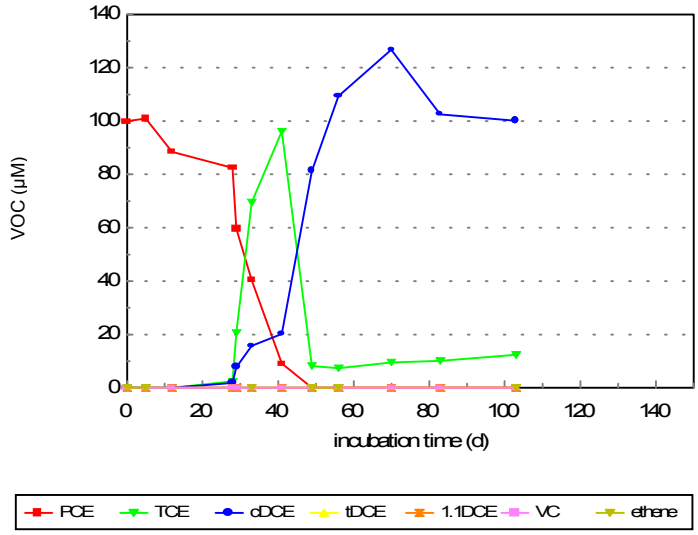


Fig. 9. Dechlorination of PCE in a batch test with soil from the Haren site.

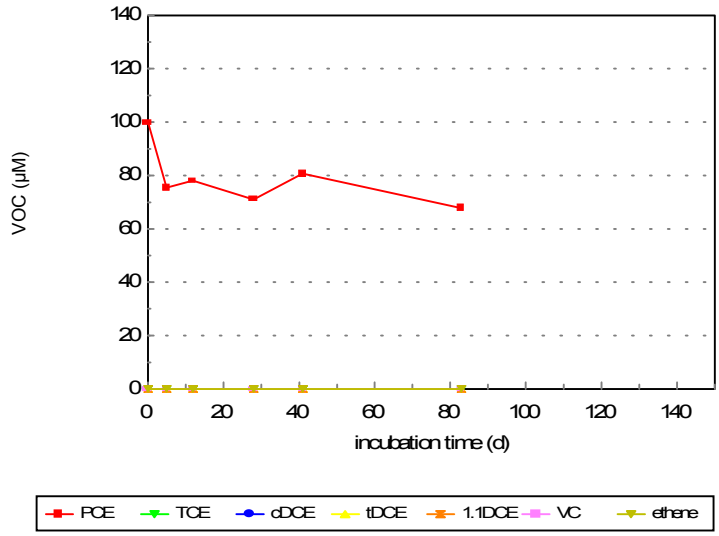


Fig. 10. Dechlorination of PCE in a batch test with soil from the Emmen site.

Addition of PER to soil samples from the Rademarkt site results in a relatively slow response from the microbial community, followed by the onset of dechlorination after a lag phase of 10 to 40 days (see fig. 7 and appendix D). Dechlorination is evidenced by the formation of TCE and some cis-DCE. In one incubation accumulation of TCE was found (see fig. 7). In two other incubations TCE was not detected and minor amounts of cis-DCE were formed that appeared to be recalcitrant during the course of the incubation (see appendix D). The mass balance on VOC was incomplete, indicating that some of the contaminant is lost from the incubation or bound to the soil (see appendix G). Indeed the sterile control shows a loss of PCE, which can only be explained by binding of the PCE to the soil, thus decreasing the PCR concentration in the head space analysis (see appendix E).

Soil from the Leeuwarden site immediately responds to PCE contamination and all PCE is converted to cis-DCE within 40 - 60 days of incubation (see fig. 8). All three incubations show the formation of the non-chlorinated product ethene. In two of the incubations no VOC is detectable after 60 - 100 days of incubation, indicating complete dechlorination of the added VOC (see appendix D).

Soil samples from the Haren site need an adaptation phase that lasts for 30 days after which in all incubations the PCE contamination is rapidly degraded (see fig. 9). This degradation results in a nearly stoichiometric conversion to cis-DCE, that is persistent in all incubations. TCE is seen as intermediate in all experiments (see appendix D).

Soil samples from the Emmen site are not capable of degrading the added PCE in the batch experiment (see fig. 10). No products are formed and the observed loss of PCE is comparable to that in the sterile control, indicating binding of PCE to the soil (see appendix E).

Samples from the Bunnik site also do not show any sign of dechlorination in the batch experiments (see appendix D). Binding of PCE tot the soil is higher (80 %) than in the Emmen incubations (see appendix E).

Batch incubations from the Vaassen site show formation of some dechlorination products (cis-DCE, VC), but the dechlorination is incomplete and no ethene is found (see appendix D).

Abiotic loss from PCE, presumably due to binding to the soil, occured in all batches (see appendix E). Only in batches from the Emmen site and the Bunnik site, this decrease was comparable to the decrease in the stimulated batches. This correlates well with the fact that in Emmen and Bunnik batches, no dechlorination products were found whereas all other batches showed the production of dechlorination products.

A second control was applied that simulated natural conditions and that was incubated with PCE but without lactate. In these incubations dechlorination needs to be fueled by the use of organic components that are already present in the soil and as such these incubations are comparable to the situation in the field. It is remarkable to see that none of these so-called 'intrinsic' batches show any evidence of biological dechlorination (see appendix F). The decrease in PCE is almost identical to that in the sterile controls and no dechlorination products are found.

The mass balance of ethenes in the batches is shown in appendix G. In most batches no complete mass balance can be obtained. The Vaassen batches show poor recovery of the added PCE, and the losses are only partly balanced by cis-DCE production. Abiotic losses of PCE due to binding to the soil is probably the cause of this incomplete mass balance. This is also true for Emmen soil, although the binding capacity for PCE seems to be less in this soil. In Groningen

soil a severe gap in the mass balance was observed for two batches, but a batch that showed complete dechlorination did show a good mass balance.

Possibly the PCE and higher chlorinated dechlorination products still bind to the soil in these incubations, but as soon as these components are further dechlorinated they are released from the soil matrix and become available for the head space analysis. The same appears to be true for the Leeuwarden and Haren incubations.

The increase and decrease in VOC concentrations in the incubations were used to calculate dechlorination rates in the experiments (see table 14).

Table 14. Dechlorination rates of chloroethenes as determined in batch experiments.

sample		dechlorination rate (nmol.day^{-1}.g^{-1}soil)				
		PCE	TCE	c-DCE	VC	ethene
Vaassen:	A	*		50		
	B	*		21		
Rademarkt:	D	15		17		
	E	4		18		
	F	500	714	23		
Leeuwarden:	G	36	400	421	160	
	H	564	100	1666		
	I	2333	800	522	462	408
Emmen:	J	0				
	K	0				
	L	0				
Haren:	M	489	800	786		
	N	555	1818	1000		
	O	500	29	526		
Bunnik:	P	0				
	Q	0				

* no PCE dechlorination measurable as compared to sterile control

The highest dechlorination rates were found in the batches with soil from the Leeuwarden site. This site has shown evidence of in situ dechlorination and contains a large fraction of organic material (peat) in the soil. These incubations are the only incubations that show complete dechlorination. Second best in dechlorination rate is the garden soil from Haren. This is a normal garden soil with no known history of contamination. Dechlorination however is incomplete and dechlorination stops at cis-DCE.

Diluted samples from the tested soils were also incubated in batch experiments in MPN assay and monitored for dechlorination of PCE. If dechlorination occurred, the dilution was regarded positive in the MPN range (see appendix H). Based on the results of these dilution ranges, a MPN for dechlorinating micro-organisms was determined (see table 15).

Table 15. Physiological MPN of dechlorinating micro-organisms in soil samples.

sample	MPN per gram soil	95 % confidence interval (MPN.g^{-1}soil)
Vaassen	10.2	2.5 - 42.0
Rademarkt	25.7	7.2 - 92.1
Leeuwarden	165.8	45.0 - 610.5
Emmen	< 0.1 [1)]	n.a. [2)]
Haren	226.2	5.8 - 978.1
Bunnik	< 0.1 [1)]	n.a. [2)]

[1)] no growth detected
[2)] not applicable

It is clear from these results that only low numbers of dechlorinating microorganisms can be counted with this MPN approach. Leeuwarden has the highest count of dechlorinating cells, which correlates well with the high activity observed in batch tests from this site. All samples that originate from contaminated sites that show evidence of in situ dehalogenation (Leeuwarden, Rademarkt, Vaassen) also show dehalogenation in the batch test. Sites that do not show any evidence of in situ dehalogenation on site (Emmen, Bunnik) also do not show dechlorination upon sample incubation in batch tests. As such, the results from the batch tests confirm the results obtained in the earlier groundwater characterization studies. The only exception is Haren soil. This garden soil has no known history of contamination with VOCs yet relatively high numbers of dechlorinating micro-organisms appear to be present.

A comparison was made between measured dechlorination rates in soil and known dechlorination rates from pure cultures to estimate the numbers of dechlorinating micro-organisms in the batches after incubation. Data from the pure culture *Desulfitobacterium sp.* strain PCE1 [Gerritse et al., 1996; Gerritse et al., 1997] were used to calculate the theoretical dechlorination rate per cell.

The dechlorination rate of PCE1 is 310 nmol.min^{-1}.mg^{-1} protein and the molar growth yield is 4.0 g cell carbon per mol pyruvate consumed in the presence of PCE. Assuming that the protein content of the dry weight is 50 % and the growth yield on pyruvate is the same on lactate, growth on 10 mM lactate produces 0.02 g protein.

The carbon content per cell is about 20 fg [Kemp et al., 1993], which equals about 10 fg protein. A dechlorination rate of 310 nmol.min^{-1}.mg^{-1} protein is comparable with $4 \cdot 10^{-6}$ nmol.day^{-1}.cell^{-1}. If the measured dechlorination rates in the batch experiments are divided by the dechlorinating activating per cell, the numbers of dechlorinating bacteria in the batch can be determined (see table 16).

From table 16 it becomes clear that high numbers of dechlorinating cells must be present in the batch experiments Rademarkt, Leeuwarden and Haren to account for the measured dechlorination rates. If the dechlorinating bacteria in the batch experiment have comparable biodegradation kinetics as the pure culture PCE 1, total dechlorinating cell numbers must range up to 10^8 cells/g dw in the active batches. These cell numbers however do not need to be present at the start of the batch experiments since growth will most likely have occurred during the course of the incubation. Therefore, the extremely low amount of dechlorinating cells as determined with the physiological MPN method may be correct. The dechlorination rate in the Leeuwarden soil is maximal at the start of the incubation, so growth of dechlorinating micro-organisms was not necessary to achieve this biodegradation rate. The fact that high cell numbers ($1.3 \cdot 10^8$/g dw) are

needed to achieve this production rate of cis-DCE whereas only low cell numbers ($1.6 \cdot 10^2$/g dw) were detected in this soil indicates that the MPN method largely underestimates the presence of dechlorating bacteria.

Table 16. Dechlorinating cell numbers in the batch experiments based on measured dechlorination rates.

site	batch	estimated cell numbers (MPN/g dw)				
		PCE	TCE	c-DCE	VC	ethene
Vaassen	A			1.3E+07		
	B			5.3E+06		
Rademarkt	D	3.8E+06		4.3E+06		
	E	1.0E+06		4.5E+06		
	F	1.3E+08	1.8E+08	5.8E+06		
Leeuwarden	G	9.0E+06	1.0E+08	1.1E+08	4.0E+07	
	H	1.4E+08	2.5E+07	4.2E+08		
	I	5.8E+08	2.0E+08	1.3E+08	1.2E+08	1.0E+08
Emmen	J					
	K					
	L					
Haren	M	1.2E+08	2.0E+08	2.0E+08		
	N	1.4E+08	4.5E+08	2.5E+08		
	O	1.3E+08	7.3E+06	1.3E+08		
Bunnik	P					
	Q					

CHAPTER 5

DISCUSSION

In this optimization study a selection was made for *Desulfitobacterium* as the model organism to detect dechlorinating activity. This selection seems to be suitable, since most micro-organisms that have been isolated and that can dechlorinate chloroethenes belong to this genus. It cannot be excluded however that other organisms are active in anaerobic dechlorination in situ as well (e.g. *Dehalobacter*, *Dehalococcus*, *Dehalospirillum*). *Desulfitobacteria* can however be a good indicator for anaerobic dechlorination.

There are many different methods to extract DNA from soil, and almost every laboratory seems to use several methods, depending on the application. Based on a preliminary review with soil contaminated with chloroethenes, the bead beating extraction method was selected. A clean-up step with sepharose column seems to be essential in order to remove co-extractants that inhibit the PCR reaction. Possibly, the DNA extraction method used needs to be selected and optimized for every type of soil that is tested. In this study, all DNA extractions were performed with the same method using bead beating and sepharose column clean-up.

Based on database sequence analysis, a specific PCR reaction was designed and tested for *Desulfitobacterium*. One PCR primer pair (A1/A4) was not completely specific for this genus, but this primer pair was shown to be useful as PCR primers for the first step in a nested PCR approach. This first step PCR resulted in amplification of all *Desulfitobacteria* and few non-related strains and served as a clean-up step for both non-specific DNA and potentially inhibiting compounds in the DNA extract. The PCR primer pair N3/N1 showed excellent specificity for *Desulfitobacterium* and was used for the specific amplification of *Desulfitobacterium* in the second step of the nested PCR.

When DNA was analyzed in a MPN-PCR approach using DNA extracts from a pure culture of *Desulfitobacteria*, the detection limit of the analysis was excellent: 8 DNA copies per reaction. This value compares well to published data for other MPN-PCR methods for detection of bacteria. When pure culture was added to soil, followed by DNA extraction and MPN-PCR analyses, this detection limit was somewhat raised to 60 - 600 DNA copies per reaction. This value equals a detection limit of 10^2 to 10^3 *Desulfitobacterium* cells per gram of soil. Assuming that a measurable physiological activity (dechlorination) in soil needs relatively high cell numbers (> 10^6/g dw), this detection limit (10^3 - 10^4/g dw) is sufficient to detect dechlorinating activity in soil. The method is therefore both specific and sensitive enough to be used for monitoring of *Desulfitobacteria* in soil.

DNA extraction from several different soils, most originating from sites that had a history of VOC contamination, did result in PCR products when universal PCR primers were used. However, the first PCR step did not show detectable PCR products, presumably caused by inhibition of the PCR reaction by components in the DNA extract. High cell numbers however were found with a nested PCR approach in soils from Rademarkt, a VOC contaminated site. Positive results were obtained in 10^5 diluted soil samples, indicating minimal cell numbers of $2.6 \cdot 10^7$ cells/g dw. Sensitive and quantitative PCR of *Desulfitobacteria* in contaminated soils using a nested MPN-PCR method is therefore possible.

Nested PCR with the A1/A4 and N1/N3 primer pairs in DNA extracts from these sites however did not always result in visible PCR product formation. The fact that within a dilution range positive and negative results could occur is not consistent with the theory of the MPN method. False negatives or false positives must have occurred. False positives cannot be excluded, but do not seem very likely since all negative controls were negative. False negatives may have been caused by the presence of inhibiting components.

However, there is no clear relation between the dilution of DNA extract and the presence of PCR products, so PCR does not seem to be related to the concentration of template DNA or inhibiting components. At this stage the reason for the encountered problems are unclear, but may in part be due to problems with the applied DNA extraction procedure. If these problems are solved, a highly sensitive and specific method for detecting and counting *Desulfitobacteria* will be available. Present research is therefore focussed on improving these extraction methods and improving the MPN-PCR analysis.

Using batch experiments, dechlorination has been demonstrated in three out of six tested soils. A good correlation was found between detection of dechlorination in situ and presence of dechlorination in batch incubations. This indicates that when dechlorination is detected in a batch experiment, dechlorination may well occur in situ as well. Batch experiments therefore form a good instrument to determine anaerobic biodegradation potential for VOCs.

Numbers of dechlorinating micro-organisms in soils that showed dechlorination as determined with the physiological MPN method were low (10^2/g dw). When the two methods for detection of dechlorinating micro-organisms are compared, it becomes clear that at this moment the only method that can unambiguously detect the presence of dechlorination in soil is the batch experiment. In these experiments, soils could be discriminated on the basis of the presence or absence of dechlorination of VOCs. Furthermore the potential for incomplete dechlorination to cis-DCE or complete dechlorination to ethene could be demonstrated. Dechlorination in batch tests correlated well with the presence of dechlorination on site, as evidenced by the presence of dechlorination products in the groundwater on site and production of these compounds in the batches. Quantification of rates of dechlorination was also possible, showing clear differences between the different soils tested. However, quantification of dechlorinating cells using a physiological MPN assay seemed very unsensitive. Even in the most active dechlorinating soils only 10^2 cells/g dw were counted.

For the Rademarkt soil sample, clear differences were found between the molecular and physiological cells counts. The molecular MPN-PCR, indicated *Desulfitobacteria* cell numbers of $2.6 \cdot 10^6$ cells/g dw., while the physiological MPN detected only $2.5 \cdot 10^1$ dechlorinating cells/g dw. This may indicate indeed that the molecular MPN-PCR method is more sensitive than the physiological MPN method. However, it is not certain yet that the detected *Desulfitobacteria* can indeed dechlorinate PCE and the MPN-PCR method was not consistent within the dilution range. This needs to be further investigated. In principle, the MPN-PCR method could be suitable as a tool for monitoring in situ anaerobic dechlorination. A second advantage of the molecular monitoring is the speed of the analysis. A MPN-PCR analysis, including DNA extraction, can be performed within two days. Batch experiments take much longer, ranging from 4 weeks to 3 months.

DGGE profiling of dechlorinating populations was attempted but found inconclusive with the applied PCR primers. This does not mean however that DGGE of dechlorinating populations is not possible. Different PCR primers may be applied to optimize the separation PCR products on DGGE and probably the DGGE has to be optimized for separation of PCR products with few base pair differences.

CHAPTER 6

CONCLUSIONS

The following conclusions can be drawn from this research:

- *Desulfitobacteria* have in the literature often been linked to anaerobic dechlorination of chloro-ethenes and may be good indicators for VOC biodegradation.
- A protocol for extraction of DNA from contaminated soil has been tested and selected.
- Two sets of PCR primers for specific nested PCR detection of *Desulfitobacteria* have been designed and tested.
- Specific detection of *Desulfitobacteria* has been demonstrated in pure cultures and inoculated soil using the PCR method.
- The detection limit of the MPN-PCR method for *Desulfitobacteria* in pure cultures is 8 copies of DNA.
- The detection limit of the MPN-PCR method for *Desulfitobacteria* in inoculated soil is 60 - 600 copies of DNA.
- The detection limit of the MPN-PCR method for *Desulfitobacteria* in inoculated soil is 10^3 - 10^4 cells/g dw.
- Dechlorinating activity has been demonstrated in anaerobic batch incubations of 3 out of 5 contaminated sites and 1 non-contaminated site.
- Batch experiments are highly suitable to determine the anaerobic biodegradation potential of contaminated soils.
- The physiological MPN method for anaerobic dechlorination is highly labor intensive and underestimates the numbers of dechlorinating micro-organisms present.
- Molecular MPN-PCR of *Desulfitobacteria* is potentially suitable for a specific and sensitive detection of dechlorinating micro-organisms.
- Molecular detection has the potential for a much faster (days instead of weeks), more sensitive (1000*) and more specific detection (discrimination between *Desulfitobacteria* and other dechlorinating micro-organisms) of dechlorinating bacteria than the physiological MPN.
- DGGE analysis of *Desulfitobacteria* with the A1/A4 primer set is not discriminative.
- More work is needed to render the MPN-PCR analysis quantitative in to contaminated soils.

CHAPTER 7

RECOMMENDATIONS

Based on the results of this work the following recommendations are made:

- Test and improve the procedure for extraction and clean-up of DNA from contaminated soil samples. Possibly the extraction procedure needs to be optimized for different types of soils and contaminations.
- Optimize the PCR conditions for the nested MPN-PCR to improve yield and purity of the PCR products.
- Design and test different sets of PCR primers and DGGE protocols to render the DGGE approach discriminative for *Desulfitobacteria.*

REFERENCES

Bouchard, B., R. Beaudet, R. Villemur, G. Mcsween, F. Lépine and J.G. Bisaillon, 1996.
Isolation and characterization of *Desulfitobacterium frappieri sp.* nov., an anaerobic bacterium which reductively dechlorinates pentachlorophenol to 3-chlorophenol.
Int. J. System. Bacteriol. 46: 1010-1015, 1996.

Christiansen, N. and B.K. Ahring, 1996.
Desulfitobacterium hafniense sp. nov., an anaerobic, reductively dechlorinating bacterium.
Int. J. System. Bacteriol. 46: 442-448, 1996.

El Fantroussi, S., J. Mahillon, H. Naveau and S.P. Agathos, 1997a.
Introduction of anaerobic dechlorinating bacteria into soil slurry microcosms and nested-PCR monitoring.
Applied and Environmental Microbiology 63: 806-811, 1997.

El Fantroussi, S., J. Mahillon, H. Naveau and S.P. Agathos, 1997b.
Introduction and PCR detection of *Desulfomonile tiedjei* in soil slurry microcosms.
Biodeg. 8: 125-133, 1997.

Elsas, J.D. van and K. Smalla, 1995.
Extraction of microbial community DNA from soils.
Molecular Microbial Ecology Manual 1.3.3: p 1-11, 1995.

Gerritse, J., V. Renard, J. Visser and J.C. Gottschal, 1995.
Complete degradation of tetrachloroethene by combining anaerobic dechlorinating and aerobic methanotrophic enrichment cultures.
Appl. Microbiol. Biotechnol. 43: 920-928, 1995.

Gerritse, J., V. Renard, T.M. Pedro Gomes, P.A. Lawson, M.D. Collins and J.C. Gottschal, 1996.
Desulfitobacterium sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or *ortho*-chlorinated phenols.
Arch. Microbiol. 165: 132-140, 1996.

Gerritse, J., R. Haanstra and J.C. Gottschal, 1997.
Monitoring van anaërobe bacteriën welke verantwoordelijke zijn voor *in situ* dechlorering van chloorethenen.
Eindrapportage NOVEM, projectnr. 351770/1710, Vakgroep Microbiologie, Rijksuniversiteit Groningen, 1997.

Heijthuijsen, J.H.F.G. and T.A. Hansen, 1986.
Interspecies hydrogen transfer in co-cultures of methanol-utilising acidogens and sulfate-reducing or methanogenic bacteria.
FEMS Microbiol. Ecol. 38: 57-64, 1986.

Herman, L.M.F, 1995.
Direct detection of *Listeria monocytogenes* in 25 milliliters of raw milk by a two-step PCR with nested primers.
Applied and Environmental Microbiology 61: 817-819, 1995.

Jackson, C.R., J.P. Harper, D. Willoughby, E.E. Roden and P.F. Churchill, 1997.
A simple, efficient method for the separation of humic substances and DNA from environmental samples.
Applied and Environmental Microbiology 63: 4993-4995, 1997.

Kemp, P.F., B.F. Sherr, E.B. Sherr and J.J. Cole, 1993.
Handbook of methods in aquatic microbial ecology.
Lewis Publishers, p 303-307,1993.

Lévesque, M.J., S. La Boissière, J.C. Thomas, R. Beaudet and R. Villemur, 1997.
Rapid method for detecting *Desulfitobacterium frappierri* strain PCP-1 in soil by the polymerize chain reaction.
Appl. Microbiol. Biotechnol. 47: 719-725, 1997.

Maniatis, T., E.F. Fritsch and J. Sambrook, 1989.
Molecular cloning: a laboratory manual (second edition).
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.

Muyzer, G., E.C. de Waal and A.G. Uitterlinden, 1993.
Profiling of complex microbial populations by denaturating gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA.
Applied and Environmental Microbiology 59: 695-700, 1993.

Porteous, L.A., R.J. Seidler and L.S. Watrud, 1997.
An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications.
Molecular Ecology 6: 787-791, 1997.

Sanford, R.A., J.R. Cole, F.E. Löffler and J.M. Tiedje, 1996.
Characterization of *Desulfitobacterium chlororespirans sp*. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate.
Applied and Environmental Microbiology 62: 3800-3808, 1996.

Scholz-Muramatsu, H., A. Neumann, M. Meßmer, E. Moore and G. Diekert, 1995.
Isolation and characterization of *Dehalospirillum multivorans gen.* nov., *sp*. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium.
Arch. Microbiol. 163: 48-56, 1995.

Utkin, I., C. Woese and J. Wiegel, 1994.
Isolation and characterization of *Desulfitobacterium dehalogenans gen.* nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds.
Int. J. System. Bacteriol. 44: 612-619, 1994.

Wild, A., R. Hermann and T. Leisinger, 1996.
Isolation of an anaerobic bacterium which reductively dechlorinates tetrachloroethene and trichloroethene.
Biodeg. 7: 507-511, 1996.

Wilson, I.G., 1997.
Inhibition and facilitation of nucleic acid amplification.
Applied and Environmental Microbiology 63: 3741-3751, 1997.

APPENDIX A

DNA EXTRACTION METHOD

Rapid DNA extraction for DNA extraction from soil
(modified protocol from [Lévesque et al., 1997])

- 500 mg of soil samples were mixed with 500 mg glass beads (diameter 0.25 - 0.5 mm) and 1 ml extraction buffer (10 mM Tris.HCl, pH 8.0; 1 mM EDTA; 1 % PVPP (polyvinylpolypyrrolidone)) in 2 ml tubes with screw-cap.
- The samples were homogenized in a homogenizer by giving 3 pulses lasting 40 seconds. Between the pulses the samples were kept on ice for 30 seconds. The homogenate was centrifuged then at 16,000 g for 10 minutes.
- The liquid phase (around 800 µl) was saved in a new 2 ml tube and extracted once with 0.5 volume phenol and 0.5 volume chloroform/isoamylalcohol (24:1). The mixture was centrifuged for 3 minutes at 16,000 g.
- Another extraction step was carried out with 1 volume of chloroform/isoamylalcohol. The mixture was centrifuged for 10 minutes at 16,000 g.
- The DNA in the supernatant was precipitated with 0.1 volume 3 M sodiumacetate (pH 5.2) and 2 volumes of ethanol (96 %). The minimal precipitation time was 2 hours at -20 ºC.
- DNA was pelleted by centrifugation (16,000 g for 25 minutes), the supernatant removed, the pellet washed once with 1 ml of 80 % ethanol and finally dried.
- The DNA pellet was dissolved in 100 µl TE buffer. The solution was heated at 65 ºC for 5 minutes to inactivate DNases.

DNA extraction for DNA extraction from soil
(modified protocol from [Porteous et al., 1997])

- 500 mg of soil sample was mixed with 925 µl SDS lysis buffer (0.25 M sodiumchloride, 0.1 M EDTA, 4 % SDS) and 75 µl guanidine isothiocyanate) in 2 ml centrifuge tubes.
- The sample was vortexed for 1 minute and was sonificated in a ultrasonic waterbath for 2 minutes.
- The sample was incubated at 68 ºC for 1 hour followed by centrifugation at 13,000 g for 15 minutes.
- 600 µl supernatant was saved in a clean 2 ml tube and mixed with 75 µl 5 M potassium acetate and 250 µl 40 % polyethylene glycol 8000. This mixture was incubated at -20 ºC for at least 1 hour followed by centrifugation at 13,000 g for 15 minutes.
- The supernatant was removed.
- The pellet was dissolved in 900 µl CTAB solution (2 % CTAB (hexadecyltrimethylammonium bromide), 1.4 M NaCl and 0.1 M EDTA) and was sonificated for 1 minute followed by a incubation at 68 ºC for 15 minutes.
- 900 µl chloroform was added and gently mixed. The mixture was centrifuged at 13,000 g for 10 minutes.
- The liquid phase was saved in a new 2 ml tube and 1 ml isopropanol was added for precipitating the DNA. This solution was incubated at -20 ºC for at least 1 hour followed by centrifugation at 13,000 g for 15 minutes.
- The supernatant was removed and the pellet was dissolved in 450 µl 2.5 M ammonium acetate.
- 1000 µl 96 % ethanol was added to the solution followed by a incubation at -20 ºC for at least 15 minutes. The DNA was pelleted by centrifugation at 13,000 g for at least 15 minutes.

- The supernatant was removed and the pellet was, after drying, resuspended in 450 µl TAE (0.04 M Tris-acetate, pH 8; 0.001 M EDTA).

Clean-up with sepharose CL-4B column
(modified protocol from [Jackson et al., 1997])

- 1 ml syringes were filled with 0.3 - 0.5 cm glasswool and filled with gelmatrix (sepharose CL-4B; Pharmacia, Uppsala, Sweden). The columns were centrifuged at 1,100 g for 5 minutes and repacked till the gel size in the columns were 4 cm.
- The columns were washed with TE buffer (10 mM Tris.HCl, pH 8.0; 1 mM EDTA) by adding approximately 500 µl TE buffer to the columns. The columns were centrifuged for 5 minutes at 1,100 g to remove TE buffer. The washing step was repeated another 2 times.
- The columns were packed in aluminium foil and autoclaved in a TE buffer saturated container. The columns could be storaged now for weeks at 4 ºC.
- Before using the columns another washing step with TE buffer was carried out.
- 100 µl of the crude DNA extract was applied to the column. The column was centrifuged at 4 ºC at 1,100 g for 5 minutes. The eluate was collected in a 2 ml eppendorf tube.
- 300 µl TE buffer was applied to the column. The column was centrifuged another time at 4 ºC at 1,100 g for 5 minutes. The eluate was collected in a 2 ml eppendorf tube.
- The DNA in the eluate was precipitated with 0.1 volume 3 M sodiumacetate (pH 5.2) and 2 volumes of ethanol (96 %). The minimal precipitation time was 2 hours at -20 ºC.
- DNA was pelleted by centrifugation (16,000 g for 25 minutes), the supernatant removed, the pellet washed once with 1 ml of 80 % ethanol and finally dried.
- The DNA pellet was finally dissolved in 80 µl TE buffer. The solution was heated at 65 ºC for 5 minutes to inactivate DNases.

APPENDIX B

PCR METHODS

PCR mixtures contained 10 mM Tris.HCl (pH 9); 50 mM KCl; 1.5 mM $MgCl_2$; 200 µM each of the four deoxynucleoside triphosphates, 0.4 µM of each primer, 1 unit Taq-polymerase (Pharmacia, Uppsala, Sweden) and 5 µl template DNA in a total reaction volume of 50 µl.

The positive control PCR contained target DNA of *D. hafniense* DCB2. Negative control PCRs did not contain DNA.

The following cycling conditions were used:

- PCR with primer set U27F/U1492R: 3 minutes at 94 ºC; 35 cycles with 1 cycle consisting of 45 seconds at 94 ºC, 1 minute at 55 ºC and 2 minutes at 72 ºC; and a final extinction-step of 5 minutes at 72 ºC.
- PCR with primer set A1F/A4R: 3 minutes at 94 ºC; 35 cycles with 1 cycle consisting of 30 seconds at 94 ºC, 30 seconds at 59 ºC and 50 seconds at 72 ºC; and a final extinction-step of 5 minutes at 72 ºC.
- PCR with primer set N3F/N1R: 3 minutes at 94 ºC; 35 cycles with 1 cycle consisting of 30 seconds at 94 ºC, 30 seconds at 63 ºC and 30 seconds at 72 ºC; and a final extinction-step of 5 minutes at 72 ºC.
- Deviations from this standard protocol are mentioned in the results of the different experiments.

Amplified PCR products were analyzed by agarose gel electrophoresis and stained by ethidium bromide. Analysis of large PCR products (500 to 1500 base pairs) was done through gels containing 1 % agarose and analysis of small PCR products (smaller than 500 base pairs) was done through gels containing 2 % agarose.

Agarose gels were prepared and runned in 1·TAE buffer [Maniatis et al., 1989].

APPENDIX C

DNA SEQUENCING METHODS

The PCR products were sequenced according to the following scheme:

1. Purification PCR product.
2. Ligation PCR product in pGEM vector (AMP-gen, lacZ (β-galactosidase transforms X-gal in a water insoluble blue product) multiple cloning site is located on the lacZ-gen.
3. Transformation in *E. coli* DH5α.
4. Cultivation under ampeciline pressure with X-gal (→ blue-white screening).
5. Plasmid DNA isolation + purification.
6. Sequencing reaction (Big Dye Terminator Cycle Sequencing). This is a PCR with only 1 primer, the PCR mix contains fluorescently labeled ddTTPs ('stopnucleotides'; ddATP, ddTTP, ddGTP and ddCTP have different fluorescent labels).
7. The obtained PCR mix is analyzed with an automatic sequencer. The obtained PCR fragments are separated on size with a capillary column. Short fragments migrate faster than long segments. Discrimination is possible at the 1 nucleotide level.
8. The final ddNTP from the fragment is analyzed with a laser. All four different fluorochromes have a specific wavelength.
9. The DNA sequence is derived from the fluorescent data.

The obtained DNA sequence is compared to sequences in the database to identify the DNA.

C1. Results DNA sequencing PCR products

Sequence from Rademarkt PCR product after nested PCR with primer set A1/A4 and N1/N3.

TAACGCGTGGATAACCTACCTGCTAGACCGGGACAACCCTTGGAAACGAGGGCTAATACC
GGATGAGCTTAATTAGTGGCATCACTGATTAAGGAAAGATGGCCTCTGAAAATGCTATCGTT
AGTAGATGGATCCGCGTCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGA
TCAGTAGCCGGCCTGAGAGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGTACGACGAAGGCCTTCGGGTTGTAAAGTACTGTCTTCAGGGACGAACGGTAAGT
ATGTAAATAATGTACTTACATGACGGTACCTGAGGAGGAAGCCCCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATCATTGgGCGTAAAGGGCGC
GTAGGCGGATaCTTAAGTCTGGTGTGAAACCTAgGGCTcaACCCTGGGACTGcATCGGAAAC
TGGGTATCTTGAGGACAG

C851 (Rademarkt x A1 x A4), 561 bases, 99 % identity with #1 in table 12a.

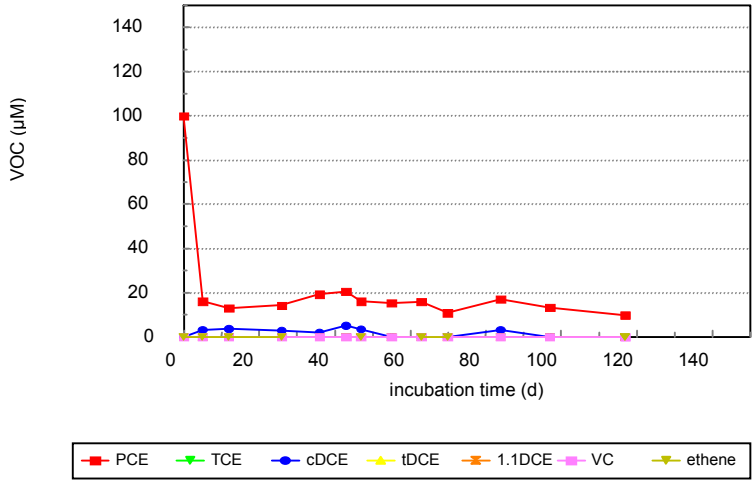
TAACGCGTGGATAACCTACCTGCTAGACCGGGACAACCCTTGGAAACGAGGGCTAATACC
GGATGAGCTTAATTAGTGGCATCACTGATTAAGGAAAGATGGCCTCTGAAGATGCTATCgTT
aGTAGATGGATCCGCGTCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGA
TCAGTAGCCGGCCTGAGAGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGTACGACGAAGGCCTTCGGGTTGTAAAGTACTGTCTTCAGGGACGAACGGTAAGT
ATGTAAAGAATGTACTTACATGACGGTACCTGAGGAGGAAGCCCCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATCATTGGGCGTAAAGGGcgC
GTAGGCGGATaCTTAAGTCTGGTGTGAAACCTAGGGCTcAACCCTgGGACTGcATCGGAAaC
TGGgTATcTTGAgGACAG

C852 (Rademarkt x A1 x A4), 561 bases, 99 % identity with #1 in table 12b.

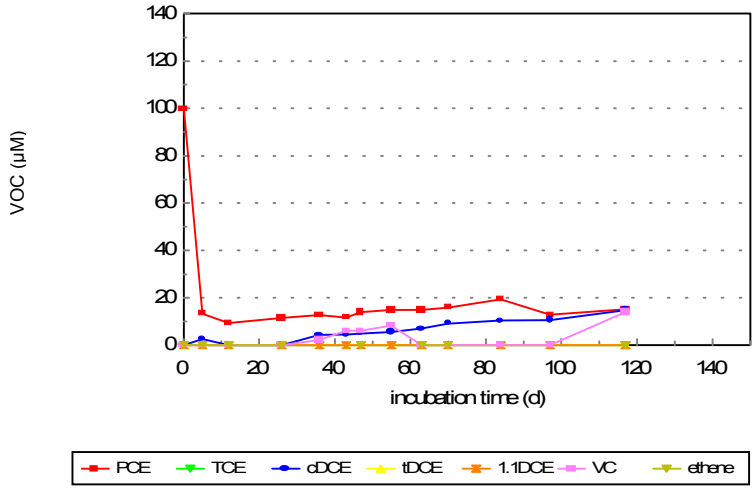
APPENDIX D

STIMULATED BATCH EXPERIMENTS WITH CONTAMINATED SOIL

1A

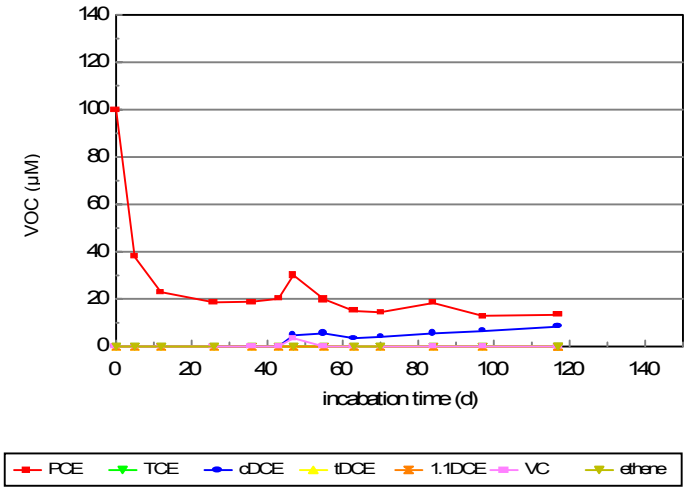


1B

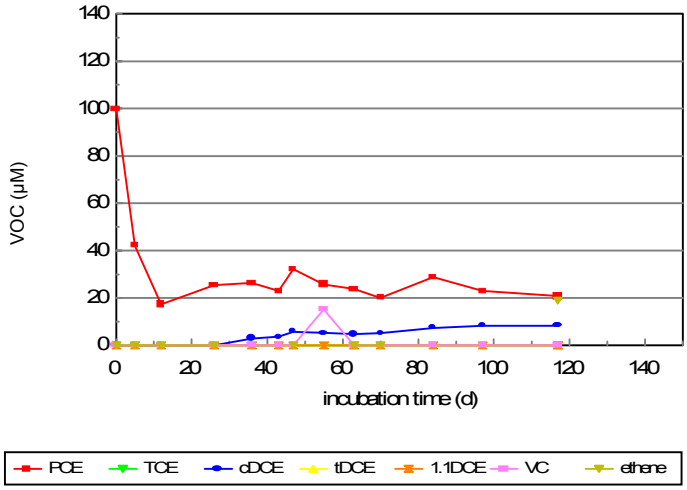


Stimulated batch experiments 1A, 1B with soil from the Vaassen site.

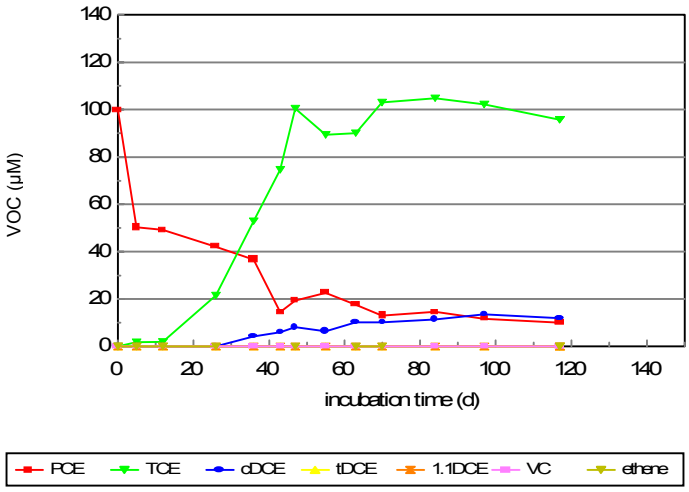
1D



1E

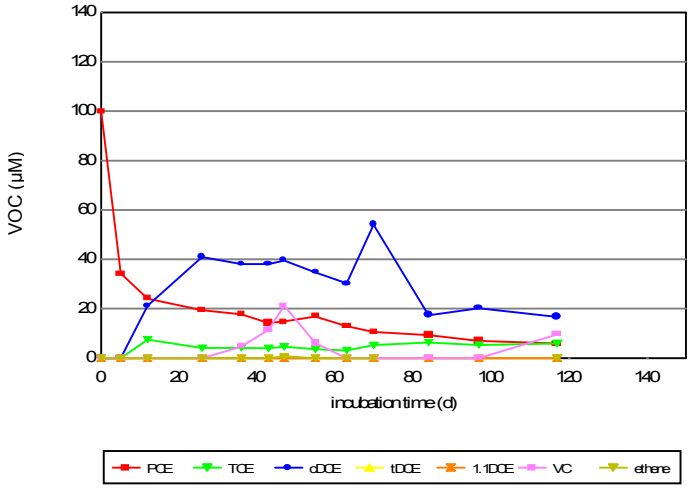


1F

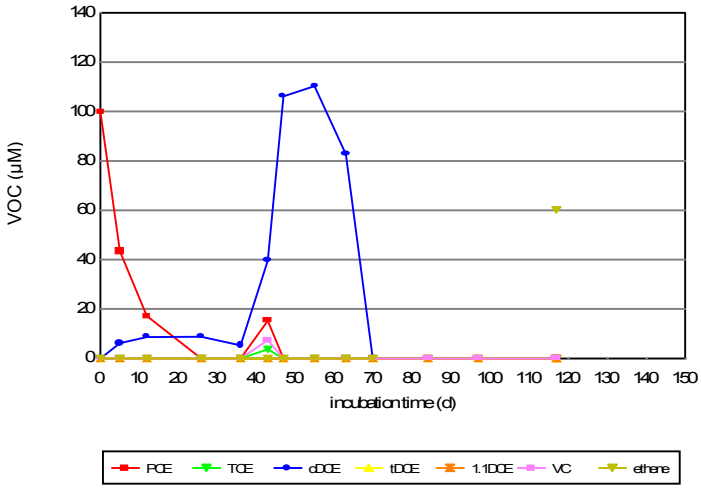


Stimulated batch experiments 1D, 1E and 1F with soil from the Rademarkt site.

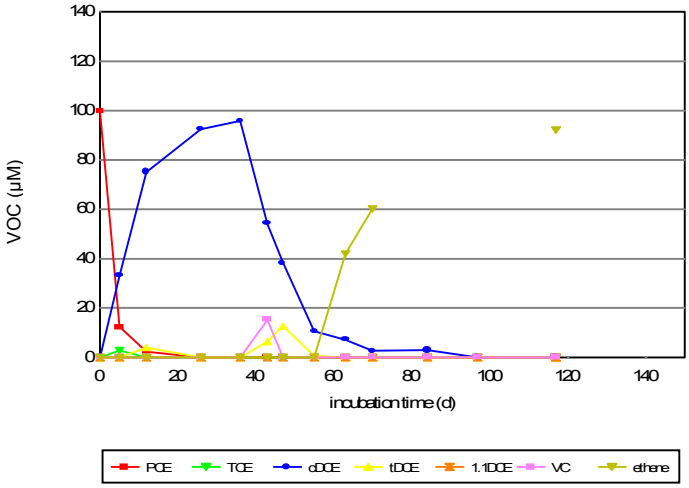
1G



1H

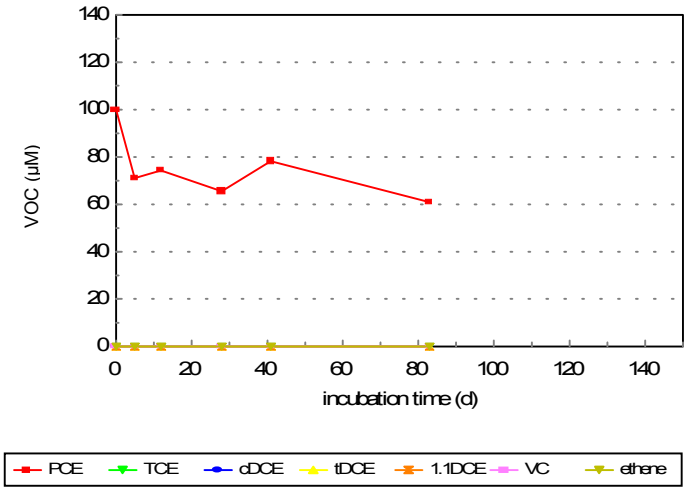


1I

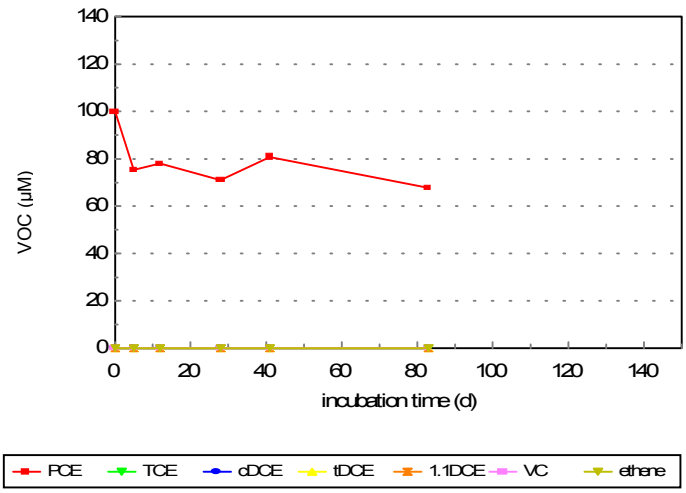


Stimulated batch experiments 1G, 1H,and 1I with soil from the Leeuwarden site.

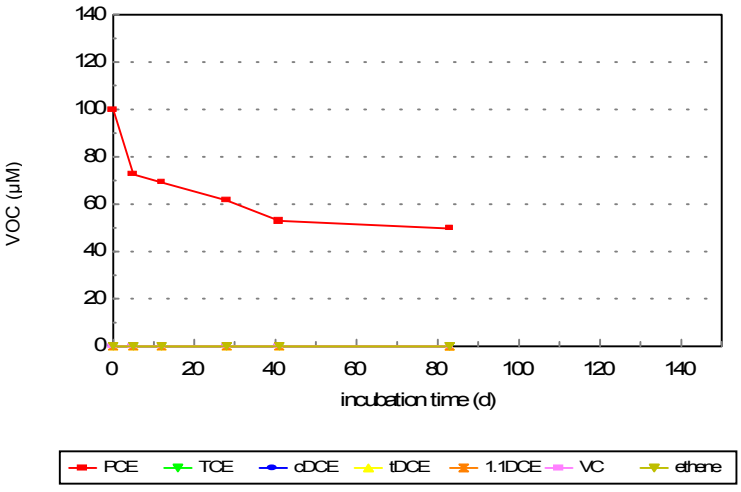
1J



1K

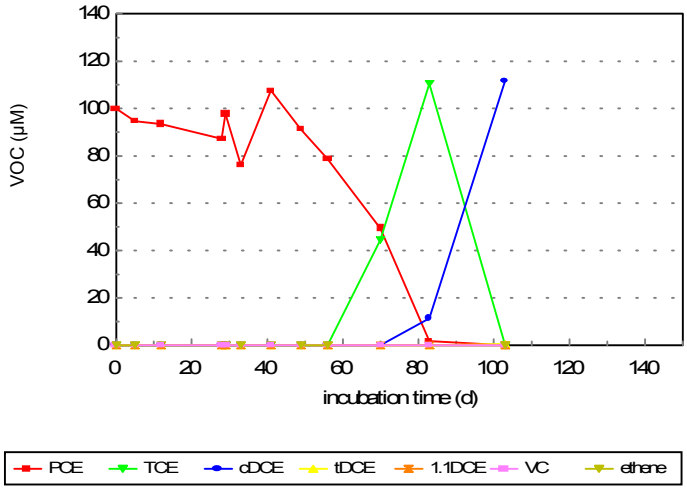


1L

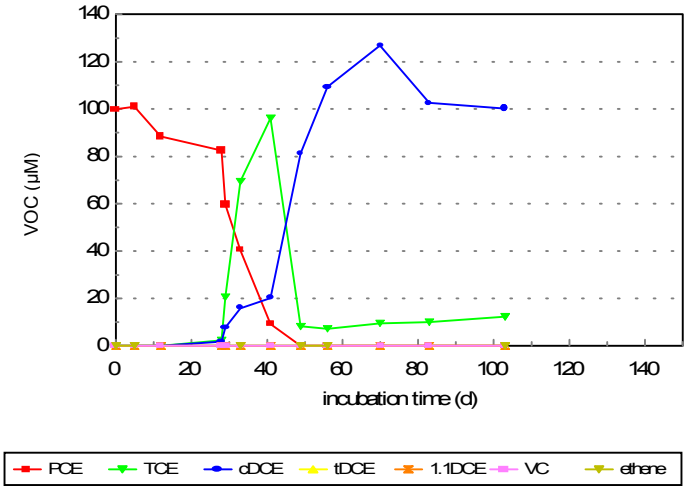


Stimulated batch experiments 1J, 1K and 1L from the Emmen site.

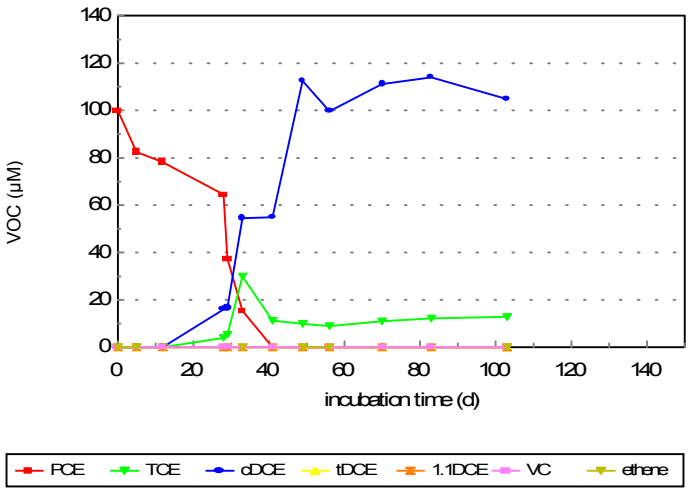
1M



1N

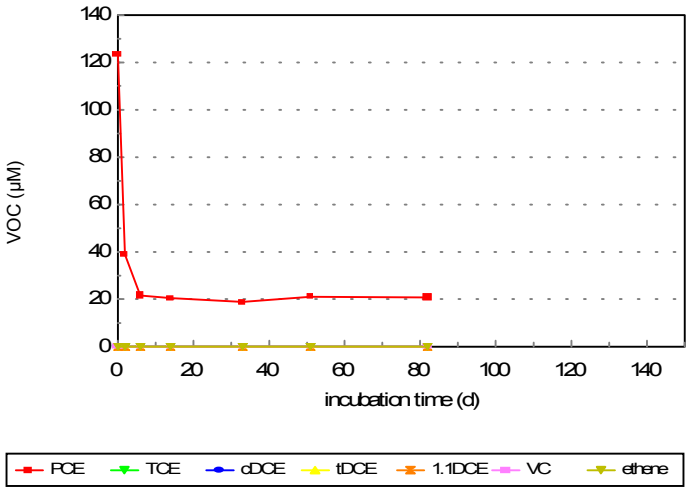


1O

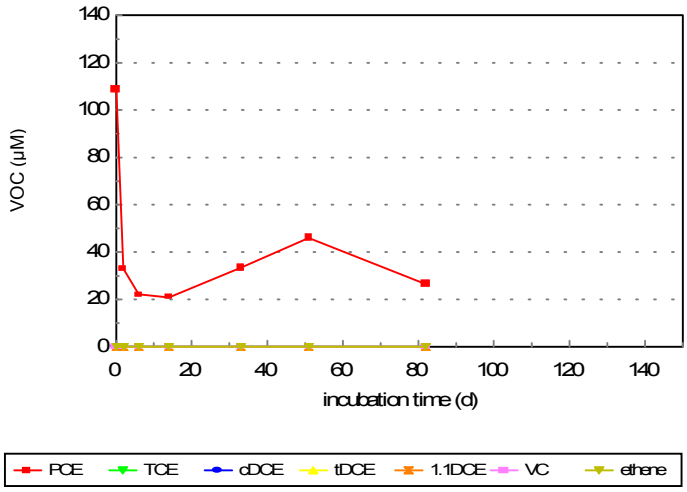


Stimulated batch experiments 1M, 1N and 1O with soil from the Haren site.

1P



1Q

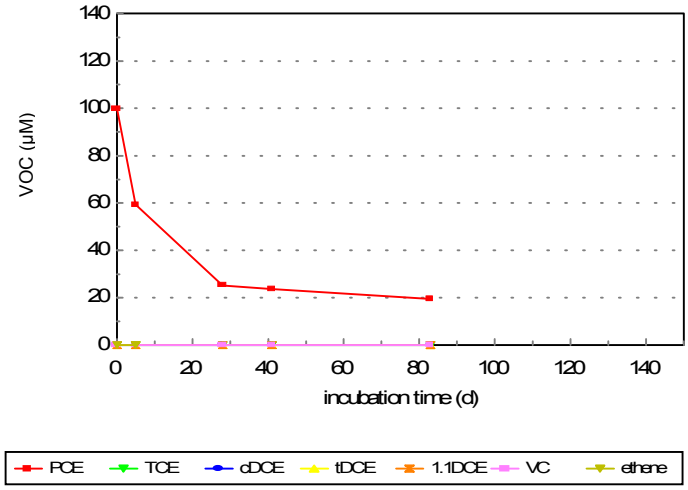


Stimulated batch experiments 1P and 1Q with soil from the Bunnik site.

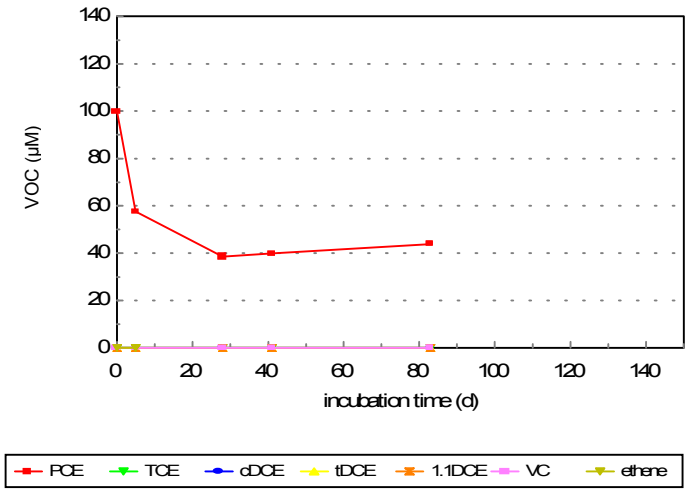
APPENDIX E

STERILE CONTROLS BATCH EXPERIMENTS

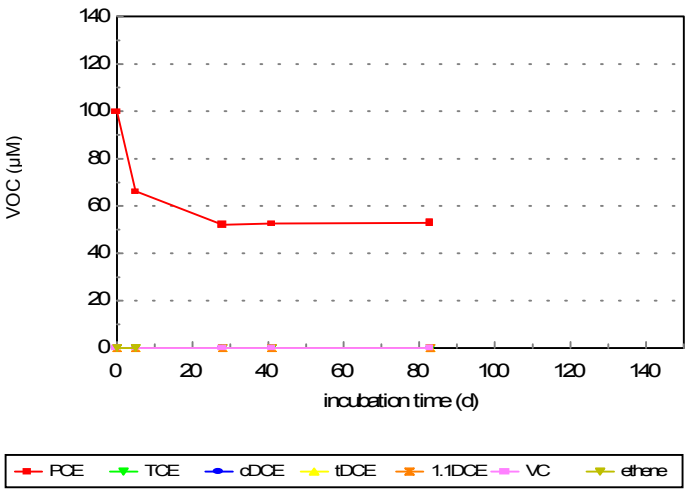
Vaassen



Rademarkt

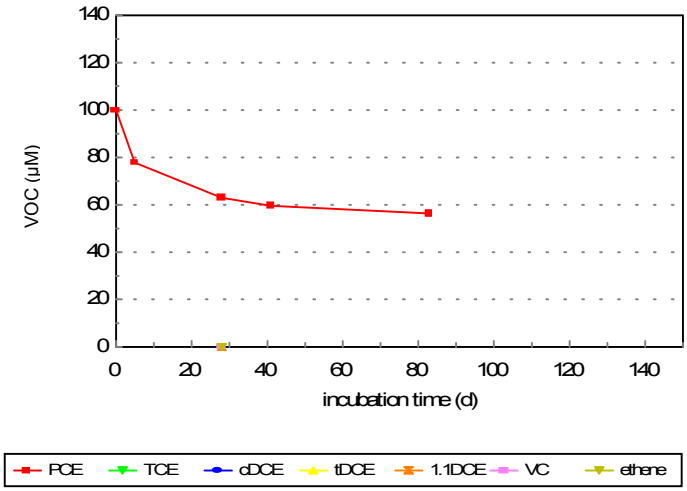


Leeuwarden

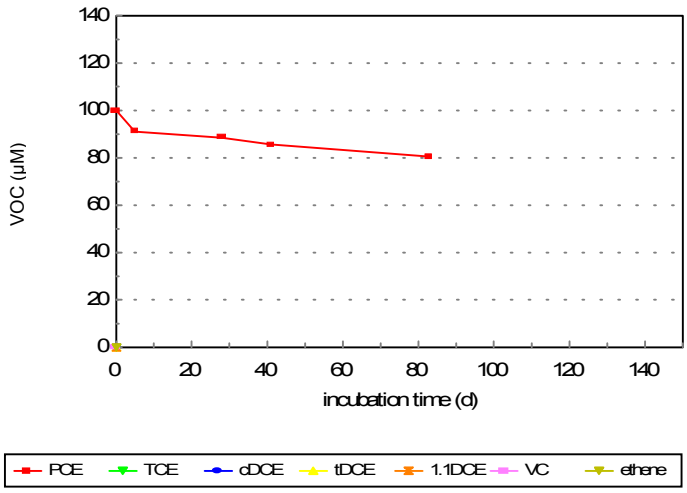


Sterile controls with soil from the Vaassen, Rademarkt and Leeuwarden sites.

Emmen



Haren

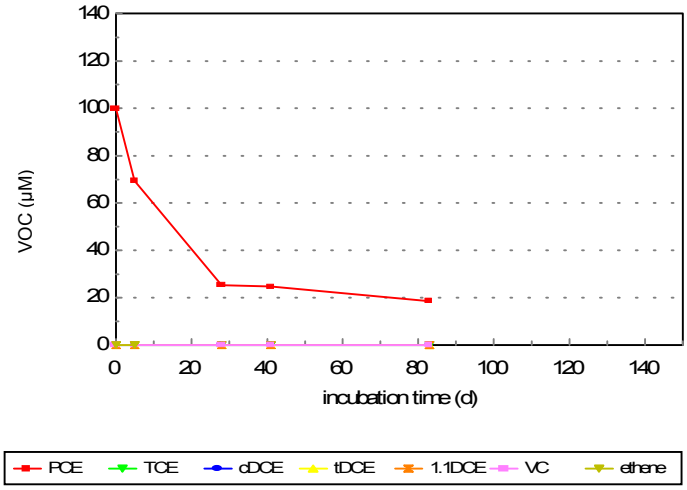


Sterile controls with soil from the Emmen and Haren sites.

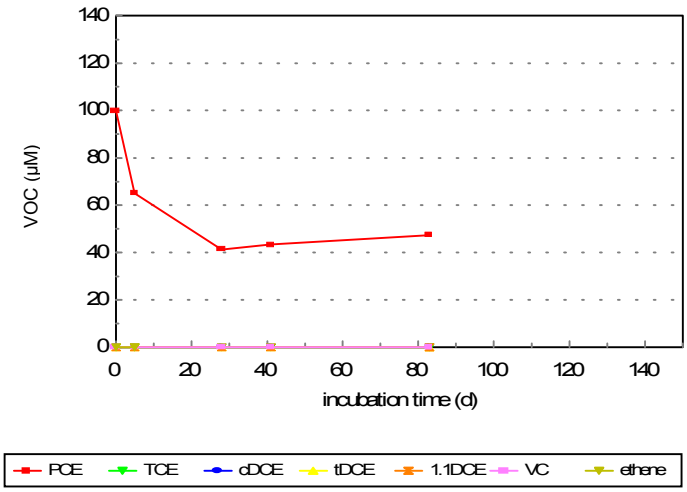
APPENDIX F

INTRINSIC BATCH EXPERIMENTS

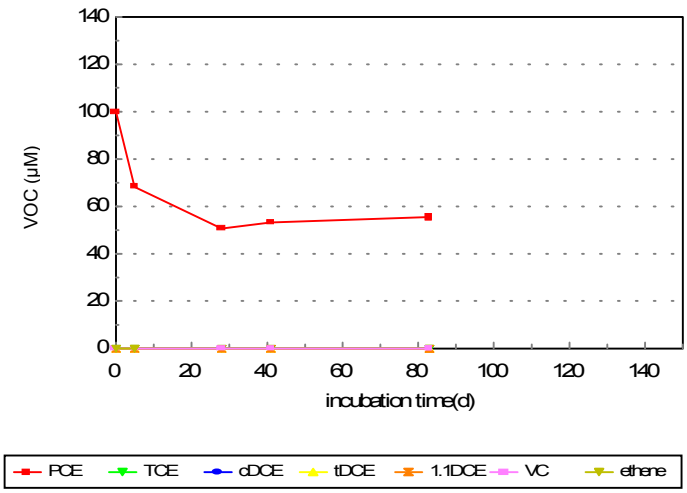
Vaassen



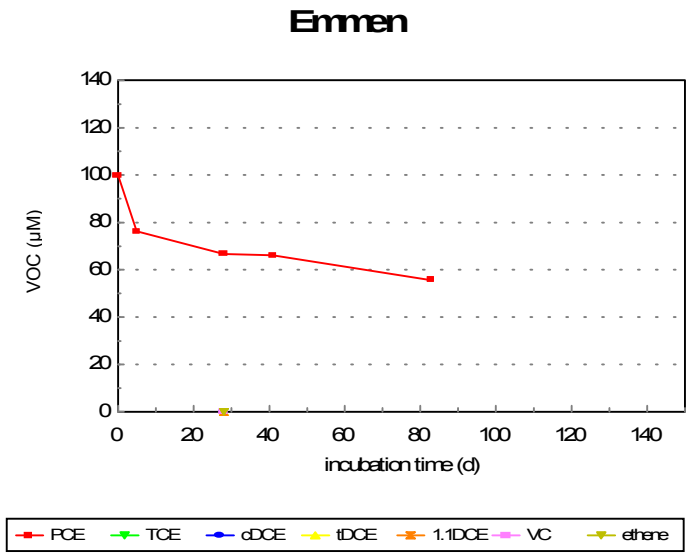
Rademarkt

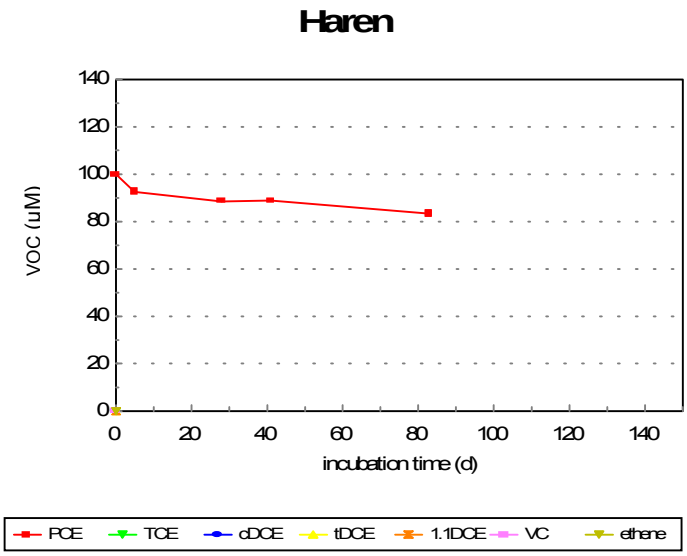


Leeuwarden



Intrinsic batches with soil from the Vaassen, Rademarkt and Leeuwarden sites.



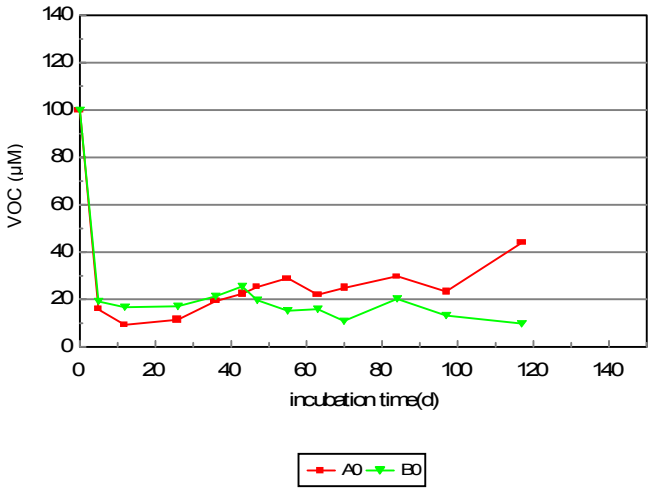


Intrinsic batches with soil from the Emmen and Haren sites.

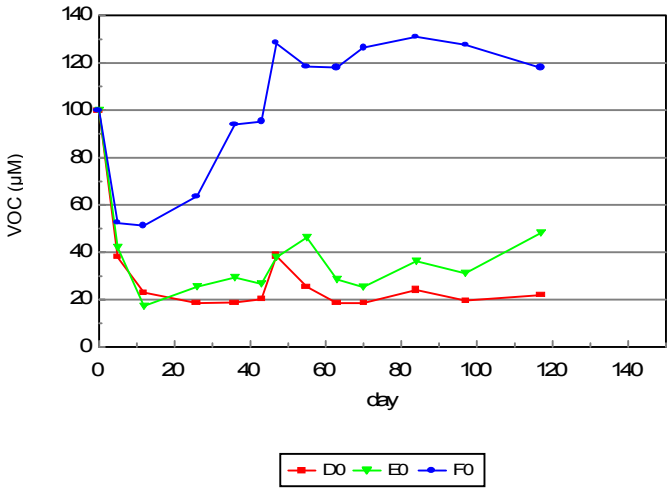
APPENDIX G

MASS BALANCE BATCH EXPERIMENTS

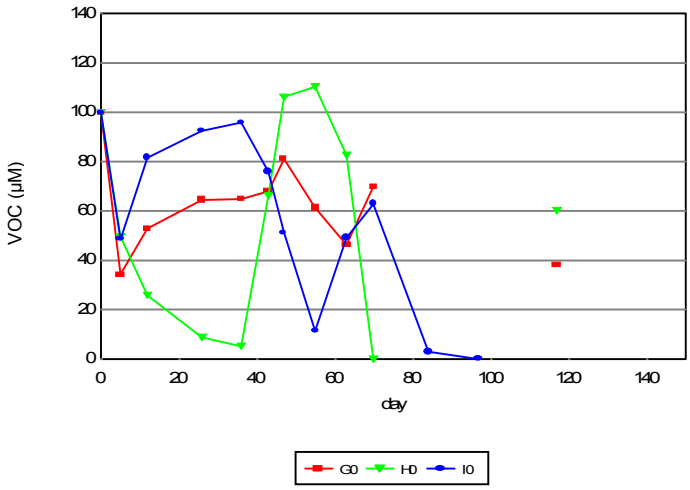
Vaassen



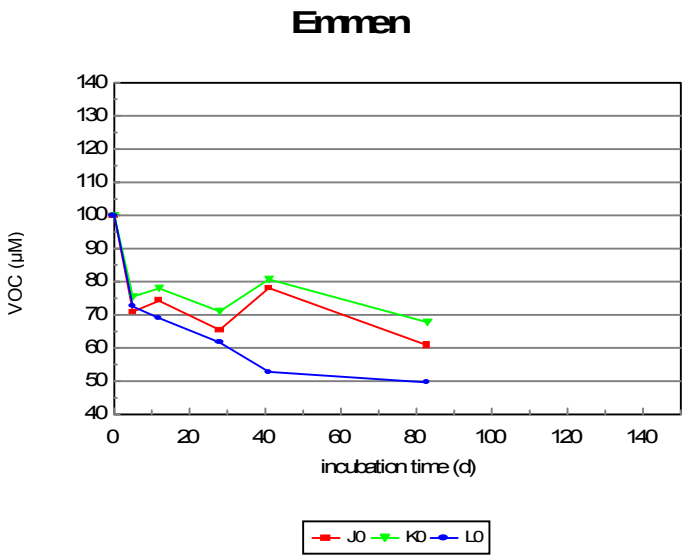
Rademarkt

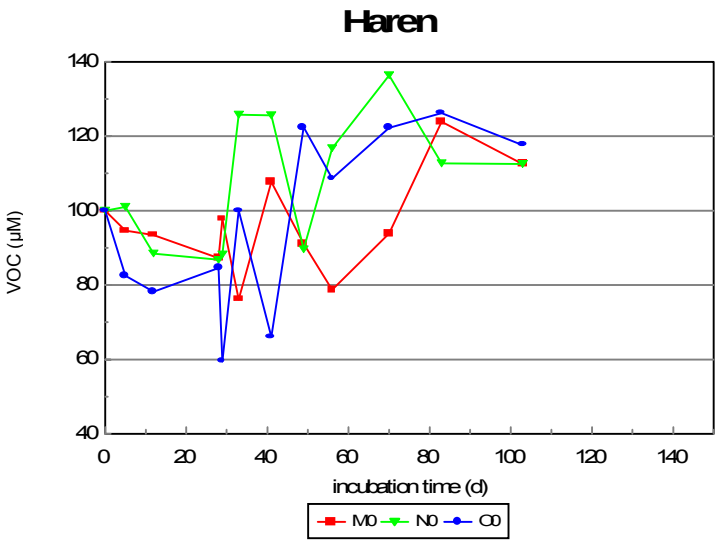


Leeuwarden



Mass balance over the stimulated batches with soil from the Vaassen, Rademarkt and Leeuwarden sites.





Mass balance over the stimulated batches with soil from the Emmen and Haren sites.

APPENDIX H

RESULTS FROM PHYSIOLOGICAL MPN DECHLORINATION

Table H1. Dechlorination products formed in batches with soil samples from sites with evidence of in situ dehalogenation.

location	dechlorination product	dilution						
		10^0	10^1	10^2	10^3	10^4	10^5	10^6
Vaassen	TCE	-	+	-	-	-	-	-
	c-DCE	+	+	-	-	-	-	-
	t-DCE	-	-	-	-	-	-	-
	1.1DCE	-	-	-	-	-	-	-
	VC	+	-	-	-	-	-	-
	ethene	-	-	-	-	-	-	-
Rademarkt	TCE	+	+	-	-	-	-	-
	c-DCE	+	+	-	-	-	-	-
	t-DCE	-	-	-	-	-	-	-
	1.1DCE	-	-	-	-	-	-	-
	VC	+	-	-	-	-	-	-
	ethene	-	-	-	-	-	-	-
Leeuwarden	TCE	+	+	+	-	-	-	-
	c-DCE	+	+	-	-	-	-	-
	t-DCE	-	-	-	-	-	-	-
	1.1DCE	-	-	-	-	-	-	-
	VC	+	-	-	-	-	-	-
	ethene	+	-	-	-	-	-	-

Table H2. Dechlorination products formed in batches with soil samples from sites with no evidence of in situ dehalogenation.

location	dechlorination product	dilution			
		10^0	10^1	10^2	10^3
Haren	TCE	+	+	+	-
	c-DCE	+	+	-	-
	t-DCE	-	-	-	-
	1.1DCE	-	-	-	-
	VC	-	-	-	-
	ethene	-	-	-	-
Emmen	TCE	-	-	-	-
	c-DCE	-	-	-	-
	t-DCE	-	-	-	-
	1.1DCE	-	-	-	-
	VC	-	-	-	-
	ethene	-	-	-	-
Bunnik	TCE	-	-	-	-
	c-DCE	-	-	-	-
	t-DCE	-	-	-	-
	1.1DCE	-	-	-	-
	VC	-	-	-	-
	ethene	-	-	-	-