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**Analysis of anaerobic microbial community capable of degrading
tetra- and trichloroethene groundwater pollutants**

PhD thesis



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I. Introduction

From the beginning of the last decade due to extended industrial, agricultural and military activity enormous environmental pollution occurred worldwide. Several anthropogenic compounds entered the environment, causing a major challenge for it, since natural ecosystems are not adapted to the rapid degradation of this type of compounds.

Halogenated hydrocarbons, such as chlorinated aliphatic compounds (for example tetrachloroethene [PCE], trichloroethene [TCE], dichloroethene [DCE], vinyl chloride [VC], carbontetrachloride, chloroform, dichloromethane and chloromethane) are among the most abundant aquifer contaminants in Hungary (there are thousands of contaminated sites). These compounds have been widely used in industry, agriculture and households as degreasing agents, biocides and solvents. Halogenated hydrocarbons are well known because of their acute and chronic toxicity, thus their accumulation and persistence in the ecosystems adversely affect both the environment and us.

Because of their physicochemical properties they have the tendency to accumulate in anoxic ecosystems, such as: sediment, sludge, soil and groundwater. Groundwater dissolves the contaminants continuously from the sediment and soil in low concentration causing long-term pollution. As groundwater flows through contaminated sites, pollutants can spread far from their source, endanger drinking-water sources.

Since in Hungary 95% of drinking water originates from subsurface environments, protection and/or bioremediation of groundwater systems from widespread and highly toxic chlorinated ethenes are in major concern.

II. Objectives

In this study, four different approaches were performed in order to reveal dechlorinating microbial communities.

The aim of the first experiment was to assess the Archaeal community changes with fingerprinting methods in several different types of contaminated groundwater. Samples were collected from sites with different chemical and geological characteristic, and from different depths. We developed monitoring methods, which are effective, rapid, suited to analyze large sets of samples and feasible to detect

differences in community diversity to correlate them to chemical parameters. On the basis of chemical results we aimed to evaluate the presence of microorganisms other than Archaea. In addition applied *in situ* bioremediation techniques were analyzed.

In the second experiment, diversity of *Dehalococcoides* sp. and its reductive dehalogenase (RDase - *tceA*, *bvcA* and *vcrA*) was analyzed in different dechlorinating systems (groundwater, constructed wetland, microcosm and enrichment cultures). These systems have the same source groundwater; however they differ in terms of the prevailing environmental conditions, system heterogeneity and complexity, as well as the stage of dechlorination activities. In this study, we addressed the following main questions: 1) is the *Dehalococcoides* sp. enriched in laboratory cultures representative of the *Dehalococcoides* diversity in the complete investigated field site; 2) how do environmental conditions, including enrichment conditions, affect the diversity of *Dehalococcoides* and its key dehalogenase genes; and 3) are the *vcrA* and *bvcA* genes both associated with the complete dehalogenation of chlorinated ethenes?

The third assay aimed to develop a new molecular method „Single-Nucleotide Primer Extension” (SNuPE). With this new technique we intended to detect and identify specific sequences present in PCR products (in this special case, different reductive dehalogenase genes).

In the fourth experiment, three-phase microcosms were set up by addition of soil, substrates and trace elements, because previous investigations showed only partial dechlorination of TCE in a two-phase system, possibly due to the absence of adequate surface and biofilm formation capability. We applied complex chemical and molecular biological approaches to follow the changes in the bacterial community with molecular fingerprinting techniques and to determine the presence and activity of halo-respiring bacteria under different microcosm conditions. The goal of this study was to stimulate complete degradation of TCE and to determine the most auspicious electron donor for application during *in situ* bioremediation.

III. Applied methods

Sampling. To analyze the diversity of Archaea community and to set up three-phase microcosms samples from a Hungarian industrial contaminated site were collected. For the diversity analysis of *Dehalococcoides* and for the development of the SNuPE method samples from groundwater, constructed wetland and microcosms were derived from a plume of chlorinated ethenes, located in the industrial Bitterfeld/Wolfen contaminated mega-site in Germany.

Experimental set up. Anaerobic microcosms were set up for the development SNuPE method. Acetate served as carbon source and hydrogen as electron donor. We added VC, c-DCE, TCE and PCE as electron acceptors. Microcosms were inoculated with 1 ml of *BTF08* enrichment culture.

For the three-phase microcosm experiments, 2 l water samples were concentrated for the microcosm experiments by filtering through a sterile membrane with pore size of 0.2 μm under anaerobic conditions. During filtration, the collected groundwater was purged with N_2 through one PTFE tube in order to produce overpressure in the bottle, and forced the groundwater through another tube into the vacuum filtration funnel unit which contained a sterile 0.2 μm pore size filter. The upper part of the vacuum filtration unit was kept under CO_2 overflow to keep out any O_2 . Each membrane containing concentrated autochthonous bacteria were stored in anaerobic medium. Soil served as third phase. We added acetate as carbon source, hydrogen served as electron donor, electron acceptor was TCE.

Chemical analysis. The following chemical parameters were measured: water chemical parameters, ethane, ethene, methane, halogenated hydrocarbons. The dynamics of biodegradation was monitored by using gas chromatography and flame ionization detection.

Nucleic acid extractions. Archaeal DNS extraction was performed from the membranes with UltraClean™ Water DNA Isolation Kit. During *Dehalococcoides* diversity experiment, DNA was extracted from the membranes and from the microcosms. From the three-phase microcosms 1.5 ml sample was collected for community DNA extraction, which was performed with MoBio Soil Kit alkalmazásával.

In the course of SNuPE method development, total RNA was isolated from the different microcosms. The total RNA isolation was performed with RNeasy mini kit, cells were mechanically revealed, using FastPrep machine. In the three-phase microcosm experiment we followed an alternative protocol to extract total RNA.

Polimerase chain reaction (PCR). 16S rRNS gene specific PCR was performed with Bacteria-, and Arhcaea specific universal primers. Taxon-specific 16S rRNS-based PCR amplification was applied to detect dechlorinating microorganisms, such as, *Dehalococcoides* sp., *Desulfitobacterium dehalogenans*, *Dehalobacter restrictus*, *Desulfomonile tiedjei*, *Desulfuromonas chloroethenica*. Three RDase genes - *vcrA*, *bvcA* and *tceA* - were amplified using PCR.

Calibration processes for the analysis of *Dehalococcoides* diversity. In the course of assessing the diversity of *Dehalococcoides*, Cornell and Pinellas subgroups were detected by direct sequencing in the different samples.

New method - SNuPE. We developed a new molecular probe to detect expression of different dehalogenase groups. The method was based on SNuPE technique. We identified seven different RDase groups based on clone sequences and five group-

specific SNUPE probe were designed. These probes aimed the five main groups of dehalogenases.

Reverse transcription. For RT reaction RevertAid™ First Strand cDNA Synthesis Kit was applied and random hexamer primer.

Analysis of Archaea community. PCR products were separated by capillary electrophoresis. Electropherograms were analyzed with GeneMapper v3.7 program and Microsatellite method.

Analysis of Bacteria community. To examine the bacterial community changes over time we applied the T-RFLP molecular fingerprinting method. Analysis was performed as it was described at the analysis of Archaea diversity. Data were analyzed with T-REX (T-RFLP Analysis Expedited) program.

Clone library. We established clone libraries based on blue-white selection method. We grouped our clones using ARDRA method and the representatives of the different groups were sequenced.

Phylogenetic analysis. Sequences of related taxa derived from databases RDP and NCBI. Obtained sequences were aligned with ARB-SILVA. Phylogenetic trees were created.

Statistical analysis. During Archaea diversity approach, we applied multivariate statistical analysis to examine the correlation between the environmental variables and the T-RFLP results, using ordination method. To analyze the result of three-phase microcosm T-RFLP data, matrix created by T-REX based on peak areas was used for two dimensional Principal Component Analyses (PCA) using PAST.

IV. Results and discussion

I. In the first study, we examined the Archaeal community changes in an *in situ* biostimulation process. Molecular fingerprinting methods were applied.

1. We developed a **rapid monitoring method**. With this new method we could **detect diversity changes** correlated to chemical parameters.

2. From the analysis of chemical results we determined, that **effective dehalogenation** processes are **influenced by the concentration of sulphate and TOC, the properties of the added substrate and the geological parameters of the contaminated site**.

3. Results of **multivariate statistical approach** showed **which chemical parameters have an effect on the separation of the samples**, thus indirectly on its diversity of Archaea community. It was noted, that particular T-RFs (or the

operational taxonomic unit) were abundant in every samples, while others proved to be site specific.

II. In the second experiment, we investigated the ubiquity and diversity of *Dehalococcoides* sp. and its three RDase genes in different systems supplied with groundwater originating from the same source in Bitterfeld/Wolfen.

1. Our study indicates that the **enriched *Dehalococcoides* sp.** from the field in Bitterfeld **is relevant for the field site** and is present in all investigated systems. Further, both key genes for vinyl chloride reductive dehalogenation to ethene, *vcrA* and *bvcA*, were found in parallel suggesting **that both genes are important for the dehalogenation of chlorinated ethenes to ethene.**

2. Additionally, **16S rRNA gene diversity of *Dehalococcoides* was not affected by the different environmental or enrichment conditions.** However, RDase gene diversity was, confirming that **for monitoring purposes, key metabolic gene detection is of greater importance** compared to the detection of the ribosomal genes only for analysis of the metabolic potential. In the case of *tceA*, absence of a positive PCR amplification may not be relevant, as the same or many other organisms with similar genes can take over the metabolic process of TCE dehalogenation. Nevertheless, *tceA* was not found in our studied systems. However, this does not mean that TCE was not dechlorinated, since this process was observed in the microcosms; thus it also confirms **that *Dehalococcoides* spp. with uniform 16S sequences have different functionality.**

3. Our investigations suggest that **one species, even one ribotype can be present, but behind this ribotype there must be several genetically different inventories of RDase genes and depending on the environmental conditions one or the other population is getting predominant.**

4. In practice, when the potential for chlorinated ethene dehalogenation to ethene is assessed, evaluating both the presence and diversity of specific dehalogenase genes is essential. We determined, that **a regular monitoring of the geochemical parameters together with the presence/absence of the taxon-specific and functional genes indicative of dechlorination activities might be necessary for a more comprehensive understanding of microbial processes occurring in the various chlorinated ethene contaminated systems.**

III. In the case of halogenated hydrocarbon pollution it is important to know the diversity of functional gene pool of the present microbes.

1. Therefore, **a new molecular approach was designed to detect the expression of different groups of dehalogenase genes in contaminated sites.** Moreover we

determined the substrate range of putative dehalogenases and we examined their expression under different conditions.

2. We assessed that with its limitation/error the SNuPE **method is applicable to analyze functional gene expressions** (in this particular case, **expression of RDase genes**). We could detect two different RDase groups based on mRNA assays. One is presumably a **novel trichloroethene reductase gene**.

IV. We composed microcosms able to degrade TCE with the use of concentrated groundwater collected from a contaminated site.

1. **The most efficient TCE dechlorination was achieved by the indigenous microbes in the absence of an added electron donor**, where VC was produced in amounts higher than in other microcosms. The dehalogenation process went one step further in TCE metabolism and the **appearance of ethene** was detected.

2. **The addition of an electron donor and hydrogen resulted in rapid but incomplete TCE degradation.** These observations **contradict previous reports** presenting complete dehalogenation of TCE to ethene with the addition of molecular hydrogen and acetate as electron donor and carbon source, respectively.

3. In the case of the biotic control, we assume that **due to the previous sour whey amendment performed at the site where the groundwater was derived from, the inoculant native microbial community might well have been capable of dehalogenation merely by nutrient addition.** Dechlorinating bacteria, already present in the initial sample were enriched by concentrating the autochthonous microorganisms and by adding nutrients and vitamin B₁₂.

4. The analysis of chemical, taxon- and function-specific detections indicated that **there was a potential for the complete degradation of TCE to ethene in all types of microcosms.** In particular, **acetate amendment moderated the community metabolism**, which was further confirmed by T-RFLP molecular fingerprinting results. **We detected a remarkable decline of bacterial diversity in all microcosms, and an indication of selection of dechlorinating bacteria.** In the biotic microcosms in which biodegradation of TCE to ethene took place, the main T-RF peak using *AluI* digestion was at 130 bp, which corresponds to *Dehalococcoides* sp. In the acetate amended microcosms, the main T-RF peak was at 113 bp, which corresponds to *Sulfurospirillum halorespirans*. In addition, with reference to the relative T-RF peak areas at the RNA level, **in the initial sample the ratio between *Sulfurospirillum halorespirans* and *Dehalococcoides* sp. was 15.8:4.3%. In the case of the acetate amended microcosm, the ratio was 32.5:0.3%, while in the biotic control it was 2.4:29.7%.** These results confirmed that the difference observed in bacterial community structure correlated with the completely different dehalogenation potential.

5. The results obtained with the laboratory microcosm study provided valuable information for planning *in situ* bioremediation applications, and justified the need for a three-phase microcosms to be able to follow more authentically the microbial processes that take place *in situ*.

V. Publications in connection with the thesis

Mészáros, É., Sipos, R., Pál, R., Romsics, Cs., Márialigeti, K. 2013. Stimulation of trichloroethene biodegradation in anaerobic three-phase microcosms. *International Biodeterioration and Biodegradation* 84: 126-133.

Mészáros, É., Imfeld, G., Nikolausz, M., Nijenhuis, I. 2013. Occurrence of *Dehalococcoides* and reductive dehalogenase genes in microcosms, a constructed wetland and groundwater from a chlorinated ethene contaminated field site as indicators for *in situ* reductive dehalogenation. *Water, Air, & Soil Pollution*, 224: 1768.

VI. Publications in connection with the subject of the thesis

Révész, S., Sipos, R., Kende, A., Rikker, T., Romsics, C., **Mészáros, É.,** Mohr, A., Táncsics, A., Márialigeti, K. 2006. Bacterial community changes in TCE biodegradation detected in microcosm experiments. *International Biodeterioration and Biodegradation* 58, 239-247.

Imfeld, G., Aragones, C.E., Fetzer, I., **Mészáros, É.,** Zeiger, S., Nijenhuis, I., Nikolausz, M., Delerce S., Richnow H.H. 2010. Characterization of microbial communities in the aqueous phase of a constructed model wetland treating 1,2-dichloroethene-contaminated groundwater. *FEMS Microbiology Ecology* 72, 74-88.

VII. Other publications

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