MULTIDISCIPLINARY EVALUATION OF THE REDUCTIVE DEHALOGENATION OF
CHLORINATED ETHENES

MULTIDISCIPLINÁRNÍ HODNOCENÍ PRŮBĚHU REDUKTIVNÍ DEHALOGENACE
CHLOROVANÝCH ETHYLÉNŮ

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Abstract:
This study presents results of a four-year collaborative Czech-Swiss research project, where two
universities, a research institute, a biotechnological company and four remediation companies are
involved. The project aims towards a deeper understanding of changes and interactions of the
indigenous groundwater microflora with the abiotic factors during stimulation of the reductive
dehalogenation (ERD) of chlorinated ethenes (CE) by application of organic substrate into the
geological environment. This research is a multidisciplinary approach using a coordinated monitoring
of groundwater (GW) samples from eight contaminated sites. The samples are analyzed by chemical,
microbiological and molecular genetic methods. All obtained data are continuously recorded in unified
database and statistically evaluated in order to identify key indicators of the biodegradation.
An example site is used to demonstrate the strategy for designing of an integrative technology for
assessment and enhancement of the complete removal of chloroethenes from the GW.

Keywords:
Chlorinated ethenes, enhanced reductive dehalogenation, abiotic factors, statistical
analysis, molecular genetic analysis, PCR, bvcA, verA, nanosamplers

Introduction
Wide range use of the cheap chlorinated solvents (machine, electro-technical, chemical and
pharmaceutical industry, dry cleaning, etc.) brought to a rapid growth of the contaminated sites all
over the world. Remedial companies have over 50 year’s practical experience in chlorinated ethenes
remediation. The competition among the remedial companies is enormous, as the main efforts are
focused to reducing of remedial price, cost efficient monitoring, reducing the time of remediation and
order of magnitude reduction of remedial limits. The traditional clean-up approaches are not enough
efficient at the final remediation stage. Nowadays, the limits for final remediation are much more
stringent, as frequently, the starting remediation point is comparable with the requirements for lowest
pollutant concentration several years ago. Dozens of methods and their combinations have been
developed and subsequently tested, and still, there is substantial room for improvement. A method
which works nicely at one site may fail completely elsewhere. That is why we still need to deepen our
understanding of chlorinated substances in geological environment, to better understand their
transformation by changes in oxidative-reductive conditions or by the microorganism activity, and to
improve the monitoring methods in order to lower the laboratory analyses costs. Thus, cooperation
between remedial companies and scientists is at present essential.
Methodology

Description of a model site – implementation by AECOM

The example site is contaminated primarily by chlorinated ethylenes (CE) – primarily tetrachlorethylene (PCE), and additional presence of oil hydrocarbons. The model site is located in an area of a former scrap yard (20x45m) within the premises of a machine production plant (since 1922), presently used as a temporary landfill. The foundation of the landfill consists of concrete panels grown over with vegetation. The communal and construction waste is stored in open piles. Further on, there is a roofed storage of paint, solvents and oils containers – Figure 1. In 1998 CE presence in the GW was determined in the adjacent gardens, at contaminations, similar to those in the scrap yard area (mainly for well C-279). The facility is situated in a shallow valley with a stream used in the past for drainage. The stream has been diverted into a piping system, not disrupting its drainage function (otherwise a wetland would have appeared in the gardens area). Thus, the sewer system forms a contamination border at the north-east direction – Figure 2. The GW circulation is bound to two aquifers – upper quaternary non-continuous (well NA-1), and lower eluvial continuous circulation (other wells labeled NA-X) (Havlíček, 2010). The interconnectivity of both aquifers has been verified in 2010 by a tracer test using fluorescein. The fluorescent dye was applied into the drain, first detected in well NA-1 and later detected in all other wells – (Heřmánek, 2010). The saturation of both collectors is influenced by series of layer half-insulators consisting of clayey sediments. The local saturation of the backfill also plays a significant role in limitation of the contamination expansion (formation of suspended aquifers).

![Shelter for storage of paints, solvents and oils, well HV-12 in the foreground.](image1)

![Stored construction and communal waste, well HV-14 in the background at left.](image2)

Figure 1: Documentation of state of the model site

By the summer of 2014, the bulk waste has been removed from the landfill. Traces of oil compounds were detected on the uncovered panels proving the usual lack of discipline during landfilling. However, based on the available information, it is obvious that the main source of contamination is located in the surroundings of wells NA-1 and NA-15, and mainly in the upper layers – Figure 2. Based on the precipitation intensity the contamination is periodically washed out and penetrates into the deeper aquifer. The GW level is located at 1,6-2,0m below the surface.
Contamination of saturated zone is depicted by violet color (based on AAR-2010), contamination of unsaturated zone based on atmochemical survey is depicted by red ellipse (based on information from AAR 2010 and AR 2008).

Figure 2: Occurrence of contamination by CE prior to the initiation of ERD (05/2010)

The prevailing contaminant at the example site is TCE which constituted 90% of the CE in 1998 (Pokorný, 1998). In 2010, 4 220 µg/l of CE were detected in well NA-1, comprised by 52% DCE (Havlíček 2010). Based on the above, it was judged about presence of microorganisms capable of transforming the CE at the site which used oil hydrocarbons as organic substrate. Table 1 shows an overview of the remedial activities on the site.

Table 1: Overview of the remedial activities on the example site

<table>
<thead>
<tr>
<th>Date</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/2008-2/2010</td>
<td>Remedial pumping combined with washing. Technology designed mainly for removal of the present oil hydrocarbons. The GW was pumped out from wells NA-1, NA-6, NA-12 and NA-14, cleaned in mobile remedial station and injected into 35m long drain. Gradually, the concentration of CE increased in the pumped water, the concentration of oil substances dropped below detection limit. Therefore new wells NA-15 and NA-16 have been installed, NA-15 to intensify pumping in the contamination source, NA-16 to intensify injection.</td>
</tr>
<tr>
<td>5/2010-11/2010</td>
<td>Pilot test of reductive dehalogenation, applied 2x6m³ of cheese whey, results – positive, method recommended for implementation during full scale remediation</td>
</tr>
<tr>
<td>10/2011-10/2012</td>
<td>Remediation by ERD, applied 3x6m³ of whey, remedial limits achieved in 10/2012</td>
</tr>
<tr>
<td>4/2013</td>
<td>Rebounding - identification</td>
</tr>
<tr>
<td>9/2013</td>
<td>New application of whey 1x7.7m³</td>
</tr>
<tr>
<td>2014</td>
<td>Monitoring</td>
</tr>
</tbody>
</table>

Based on AAR from 2010 (Havlíček 2010) ČIŽP (Czech Inspectorate of Environment) set the remedial limits on the example site as follows:

- 1,2-dichlorethlenes (∑ DCE) 200 µg/l
- Trichlorethlenes (TCE) 85 µg/l
- Tetrachlorethlenes (PCE) 350 µg/l

The model site is characterized using optimized methodology for characterization of sites contaminated by CE, which were elaborated based on discussions by the scientific as well as technical section of the project team. The usual standardized methodologies have been modified for the purpose of the research project. Newly, the presence of strontium in GW was monitored. A very close attention was paid to minimization of exposure of all samples to oxygen. All manipulations with samples for molecular genetic analyses are carried out using latex gloves to avoid their contamination. Groundwater samples were collected in dynamic state using Micro purging methodology (= pumping of small volume, 0.25-5 l/min) up to stabilization point of hydrochemical parameters measured in flow chamber (pH, redox-potential, conductivity, equipment by WTW company). In case that the stabilization was not reached for up to 30 minutes, the values at the 30th minute were recorded.
Collection of GW samples into dedicated sample containers for laboratory followed, as well as measurement of gas atmosphere in well 0,5m above the GW level using mobile gas analyzer Ecoprobe 5 (O2, CO2, CH4, PID-response, IR-response). The collected samples were analyzed by the accredited ALS laboratory for the following parameters: CE content including VC, organic substrate content using CHSκ(C), CH4, ethane, ethylene, H2+S2-, SO42-, PO43-, NO3-, NO2-, NH4+, Fe3+, Mn3+, Sr and K+.

Further on, samples of GW have been collected for molecular-genetic analyses carried out in MBÚ and EPFL and for microbiological analyses carried out in EPS.

Molecular-genetic analyses – by MBÚ
A main result of the project includes the optimization of environmental DNA extraction from the GW and soil samples using most advanced automated laboratory tool MaxwellTM 16 System (Promega, Madison, USA). The optimized methodology takes place in three steps and allows for processing of 16 samples at the same time. This accelerates the processing of dozens of samples for research purposes, as well as in the intended applied application. The first step includes filtration of 3-5 liters of GW through 0,2µm nylon membrane (Whatman, USA) at 50-500ml aliquots, based on the volume of colloid and solid particles in the GW sample. Filters with captured biological material are subsequently stored in sterile 50ml plastic test tubes (P-lab, ČR) at -20°C. Second step is the pre-extraction of DNA captured on four nylon membranes. The membranes are treated in sodium-phosphate pufr (pH=8) with SDS (Sodium dodecilsulphate) and Proteinase K (Machery-Nagel, Germany). The third step is the actual isolation of DNA in the automated device Maxwell.

The molecular-genetic methods were used for monitoring of five wells in the example site – four contaminated wells (NA-1, NA-6, NA-14 and NA-15) into which whey was applied, and one contamination free monitoring well (NA-16). The acquired volumes of DNA oscillated between 131-1 343 ng/100ml of filtrated GW. The detection of key bacterial species involved in the respiration process of CE, and genes coding two reductive dehalogenases (vcrA a bvcA) – i.e. enzymes crucial in the dehalorespiration pathways, transforming PCE → TCE → DCE → VC → ethylene, was carried out using polymerase chain reaction (PCR) method. The organisms and genes are detected using primer sets aiming at Dehalobacter sp. (Smits et al., 2004), Dehalococcoides sp. (Hendrickson et al., 2002, Adrian, 2007), Geobacter sp. (Duhamel et al., 2006), Sulfurospirillum sp. (Daprato et al., 2002), Desulfotobacterium sp. (Smits et al., 2004). In case of identification of presence of catabolic genes probes are used aiming at vinylchloride reductase (Behrens et al., 2008) from Dehalococcoides sp. which is known to be the main bacterial genus participating in the reductive dehalogenation of CE (see results – Table 2). Another bacterial group monitored by PCR is the sulphate-reducing bacteria (SRB) (Friedrich, 2002) which reflect the changes of in-situ oxidation-reductive conditions during remediation.

Nanosamplers – implementation of TUL
Currently, 3 to 5 liters of each GW sample must be processed in a laboratory in order to acquire sufficient DNA quantity for molecular-genetic analysis For the above reason an alternative procedure of acquiring DNA from GW is tested using stationary samplers containing nanofiber components submerged into a well. The development of nanosamplers and isolation and characterization of DNA nanocarriers is focus of the TUL activities. An optimal way of DNA/RNA stabilization during transport from site to laboratory had been examined (transport in common cooling box with frozen cooling inserts – RT, transport in common cooling box with dry ice – CO2, transport after rapid freezing in liquid nitrogen in a box of dry ice – N2, and immediate stabilization of sample by RNA later- RNA later) – see Figure 3.
Figure 3: Collection of nanofiber carriers (1. RT – room temperature, 2. - RNA later, 3. CO2 – dry ice, 4. N2 – liquid Nitrogen) – photo by Sakmaryová, 2013

For the purpose, the nanosamplers have been placed into the center of the water column and exposed for 4 and 6 months. DNA was isolated from the nanocarriers. The DNA yield (µg per gram of nanofiber) was determined, and the presence of bacteria and functional genes was examined as done by MBÚ. More about the nanosamplers - in Stavělová et al., 2013.

Aerobic cultivation – implementation of EPS

In the frame of the Techtool project, EPS focuses on technical solutions linking the precise analytical approaches from the field of microbiology with the demand for simple and cheap solutions applicable in real life laboratory operation. Currently, two main outputs can be highlighted: 1) Identification of life and death microorganisms by coloration (SITO 9 fluorescence dye), for determination and quantification of changes in the GW samples as a consequence of exposure to oxygen, and 2) development of robust technical device for quantification of metabolic gases in acc. with regulated experimental conditions.

Multiple Factor Analysis (MFA) - implementation of EPFL

The statistical evaluation of the data acquired from the model site was carried out using software R. MFA is a powerful tool for elaboration of large data files where the data are grouped and displayed in the form of 2D graphs (Borcard, D. 2011). This allows for determination of relations between variables and between variables and objects defined in these data files. To evaluate the data from example site four data matrixes have been elaborated using MFA containing crucial parameters of ERD (Shani, N., 2012): i) Terminal Electron Acceptors (parameters connected to bacterial respiratory processes – CE, ethylene, ethane, H2S+S2-, SO42-, NO3-, NH4+, Fe2+,Mn2+ concentration, percentage of particular CE), ii) Other environmental parameters (hydrochemical parameters e.g. pH, redox, CHSKCr, PO43-), iii) results with T-RFLP analysis (Rossi, P. et al., 2009), and iv) results from PCR analysis (present/not present). Each of the four matrixes includes data from seven monitoring campaigns for wells NA-1, NA-6, NA-14, NA-15 a NA-16.

Results and discussion

Characterization of the example site

The development of most important parameters from the shallow well in source point (NA-1) and a deep well further away from the source point (NA-14) is shown in Figure 4. The results demonstrate that from the very beginning, during the pilot test consisting of two whey applications, the primary contaminants PCE and TCE have been nearly completely transformed to 1,2-cis DCE. VC started accumulating, and transformation to nontoxic final products ethylene and ethane took place. The intensive and fast transformation of cis-DCE and VC continued during the remedial period, and within 12 months the final limits have been achieved in all wells. The ERD is still active, VC is intensively transformed, and the organic carbon content is not exhausted yet. The contaminant come-back (rebounding) was successfully treated by one dose of cheese whey application.
Nanosamplers

An optimal way of DNA stabilization during transportation from the site to the laboratory was tested in five wells on five different sites. Figure 5 shows the results from the model site well NA-14 for exposed nanocarriers for 4 months (grey columns) and for 6 months (blue columns). The way of conserving the samples for transportation had no significant impact on the yield and quality of DNA captured on the nanocarriers. The DNA seemed to be relatively stable even during transport in cooling box with frozen inserts (RT method). Using this simplest way of transportation showed the highest yield of DNA acquired.

Figure 4: Development of the most important parameters during ERD on the example site.
An important outcome is the trend of increase of biomass attached on the nanofibre carriers after two months. Based on PCR analysis of the DNA isolated from the nanocarriers, both the functional genes bvcA and vcrA, and presence of microorganisms from genus Dehalococcoides sp., as well as other monitored microorganisms similarly to the isolates from filtered GW has been identified. Thus, the DNA isolated from the nanosamplers represents rather the long term state of the wells, while DNA isolated during dynamic sampling represents the actual state of the GW.

Molecular genetic analyses
Based on the results included in Table 2, it is obvious that from the very beginning, after the pilot test in 2010, bacteria affiliated with Dehalococcoides sp. were present in the contamination cloud, including the functional genes bvcA and vcrA. Further on, it is evident that due to the stimulation by using repeated doses of whey the concentration of these crucial for the reductive CE dehalogenation organisms, as well as the concentration of other supporting organisms in the community gradually grows. Note: application of whey7/2010, 8/2010, 11/2011, 1/2012, 5/2012, 9/2013.

Table 2: Semi-quantitative PCR analysis of DNA isolated from GW samples for presence of microorganisms and functional genes during the process of reductive dehalogenation

<table>
<thead>
<tr>
<th>Year</th>
<th>Well</th>
<th>Sampling</th>
<th>16S-DHC</th>
<th>nested 16S-DHC</th>
<th>16S-GEO</th>
<th>16S-DRE</th>
<th>bvcA</th>
<th>vcrA</th>
<th>Sulfitoreducens</th>
<th>Desulfitobacterium</th>
<th>SRB</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>NA-1</td>
<td>10.11.2011</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>NA-6</td>
<td>10.11.2011</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>NA-14</td>
<td>10.11.2011</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>NA-15</td>
<td>10.11.2011</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>NA-16</td>
<td>10.11.2011</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>2011</td>
<td>NA-1</td>
<td>13.03.2012</td>
<td>+</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>+</td>
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<td>-</td>
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<tr>
<td>2012</td>
<td>NA-14</td>
<td>13.03.2012</td>
<td>+</td>
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<tr>
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<td>11.05.2012</td>
<td>+</td>
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<td>NA-1</td>
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</table>

Figure 5: Results from the DNA stabilization test (captured on nanocarrier during transport from site to laboratory: CO2 - transport in a common cooling box with dry ice, N2 - transport after rapid freezing in liquid nitrogen in a box with dry ice, RT - transport in a common cooling box with frozen cooling inserts, RNA later - immediate stabilization by RNA later solution)
Anaerobic cultivation

Figure 6 shows the results of fluorescence test with water sample collected from well NA-14. The test represents a state of microbial community stimulated by addition of lactic acid (1g/l) after five days of cultivation under anaerobic conditions (glove box) – figure A and aerobic conditions – figure B. The insignificant decrease of live bacteria indicates that many of the present microbial strains are surviving conditions where oxygen is present, and probably belong to facultative anaerobes. From practical point of view this proves that short term exposure of GW sample to oxygen during collection of the sample does not significantly influence the vitality of the present anaerobic microflora.

![Figure 6: NA-14 – consortium of fermentative and dehalogenating bacteria after 5 days of cultivation under anaerobic conditions (A) and under exposure to ambient air (B).](image)

Figure 7 reflects respiration activity of the anaerobic community in GW samples from the model site with support of modified device (U-tube) for quantification of the fermentative potential. The volume of produced gases under anaerobic conditions is compared. These results provide a useful insight into the microbial activity in each sample and can be used to optimize the management of the remedial action.
Figure 7: Respiratory activities of communities present in the wells of the model site

Multiple Factor Analysis (MFA)
The interpretation of MFA graphs provide a new model of interpretation of wide file of data from monitoring of remediated site. It aims at depicting the physical-chemical and biological parameters that play the main role during ERD of CE in GW. MFA reflects correlations between each of the physical, chemical and biological variables from four data matrixes – for specifics see methodology and Figure 8a. Each sample is characterized by specific redox conditions and state of bacterial population which show in MFA graphs. Each sample corresponds with particular date. The development in particular wells in time as well as changes in microbial population can be observed through varying redox conditions of the aquifer – see Figure 8b. It is possible to find out if the redox conditions are advantageous for transformation of chlorinated ethenes, which is important information for the management of an remedial action.

Each variable is depicted using one vector. The length of the vector corresponds to the influence/strength of the variable. The direction of the vector includes different set of information:
- Vectors pointing to the same direction (co-linear vectors) indicate that the variables develop in the same way, their relation corresponds to a direct proportion and they stimulate each other. The closer the vectors, the closer the mutual correlation.
- Vectors in opposite direction indicate anti-correlation, meaning that the parameters are in antagonistic relationship – indirect proportion. If two vectors are drawn with 90 degree angle there is no correlation between the variables and their values.

Figure 8a: MFA analysis of the mutual relationship of the parameters monitored during remediation
Each point corresponds to the center of gravity of four matrixes (Terminal Electron Acceptors, Other environmental parameters, Outcomes of T-RFLP and Outcomes of PCR.) Each point includes the name of a well (NA-X) and number corresponding to the monitoring campaign (1 to 7 for 10/2011, 01/2012, 03/2012, 05/2012, 9/2012, 4/2013, 7/2013). The red interrupted circles represent the redox conditions of the aquifer (in detail characterized by graph 8a). The state of the site at the beginning of remediation is represented by the red points, state during the active remediation by green ones. The blue points represent current state when solving rebounding. The black points refer to the background well NA-16 (no contamination).

Figure 8b: MFA analysis of the water quality change in particular well in time.

Summary
Based on the example of a particular site contaminated by chlorinated ethylenes and its remediation using ERD, the outcomes of the Techtool project were presented:

- Detailed description and characterization of remediated site
- Molecular-genetic analysis of the microflora contributing to the reductive dehalogenation, and the key functional genes including two vinylchloride reductases (vcrA and bvcA)
- Utilization of newly developed nanosamplers Presenting of modified methods of anaerobic cultivation optimized for operational laboratories
- Introduction of new model for interpretation of large data file from monitoring of remediated site using multivariable statistical analysis: Multiple Factor Analysis (MFA). This method shows a great interpretative potential which can become a powerful tool for the remedial companies.

Acknowledgement
This research is co-financed by the Technology Agency of the Czech Republic, project TECHTOOL (TA0202534), and the Swiss Federal Office for the Environment (FOEN). Data from the following project partners, not listed in this contribution, have been used: T. Lederer (AQUATEST, a.s.), R. Heřmánek (KHSanace s.r.o.), M. Pravečková, V. Reimannová (MBI) a P. Hlaváčová (VODNÍ ZDROJE, a.s.).

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