ENHANCED REDUCTIVE DEHALOGENATION OF CHLOROETENES BY APPLICATION OF CHEESE WHEY IN SITU: A CASE STUDY WITH FIELD, CHEMICAL AND MOLECULAR BIOLOGY ANALYSIS

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Abstract:
This study presents results within a four-year collaborative research project TECHTOOL, where two universities, a research institute, a biotechnological company and four remediation companies were involved. The project aims towards deeper understanding of changes and interactions of indigenous groundwater microflora with abiotic factors during stimulation of the reductive dehalogenation (ERD) of chlorinated ethenes (CE) by application of organic substrate (in our case cheese whey) into the geological environment. Groundwater (GW) samples from eight contaminated sites were monitored and analyzed by multidisciplinary approach using chemical, microbiological and molecular genetic methods. All data was continuously recorded in unified database and evaluated in order to identify key indicators of the biodegradation. Alternatively, GW sampling using newly designed nanofiber carriers submerged into the well was tested. Interpretation of the field and chemical data together with the molecular biological analysis for present dechlorinating bacteria and reductive dehalogenase genes was used for a better controlling of the remediation procedure. An example site was used to demonstrate the strategy for designing of complex technology for assessment and enhancement of the complete removal of chlorinated ethenes from the groundwater.

Keywords:
Chlorinated ethenes, enhanced reductive dehalogenation, abiotic factors, field measurement, PCR, *bvc*A, *vcr*A, nanofiber carrier

1. Introduction

Wide range application of the cheap chlorinated solvents (machine, electro-technical, chemical and pharmaceutical industry, dry cleaning, etc.) brought to the rapid growth of contaminated sites all over the world. Remedial companies have over 50 years of practical experience in the chlorinated ethenes decontamination. Their main efforts are focused on reducing of the remedial price, with time- and cost efficient monitoring and minimizing of the remedial limits. The traditional clean-up approaches (pump & treat, venting) are not enough efficient at the final remediation stage. Nowadays, the limits for final remediation are more stringent as frequently the starting remediation point is comparable with the requirements for lowest pollutant concentration several years ago. While plenty of methods and their combinations have been developed and subsequently tested, still, there is a substantial room for their improvement. A method which works nicely at one site may completely fail elsewhere. Thus, the cooperation between remedial companies and scientists is essential for better understanding of the transformation of chlorinated substances in a geological environment and for improving of monitoring and analyses costs.

2. Material and methods

Organic substrate selection and characterization

We have selected cheese whey (CH-W) as an organic substrate for ERD stimulation. It is a rich organic carbon source with appropriate macronutrients content (N, P, K) and low cost (1 m^3 of CH-W = 180 CZK ~ 6,5 EUR + transport = 50 CZK/km ~ 2 EUR/km). In addition, it has an ecological aspect positively appreciated by the public in a sense of waste recycling. The CH-W is a waste from the production of casein, cheese and cottage cheese. It is a rich source of lactic acid bacteria and its dry matter consists of ~ 85 % lactose, 10 % mineral salts from milk (Cl^-, PO_4^{3-}, Na, Ca,…), 3 % lactic acid, and 2 % fat.

ERD stimulation = *in situ* anaerobic reactor management

In advance, a baseline of the contaminated site was determined for the complete range of field measurements, laboratory and molecular genetic assays. CH-W was then repeatedly injected into wells containing chlorinated ethenes at intervals of several months. The CH-W amount applied to each well was dependent on the progress of contaminant transformation. Primary contaminants PCE and TCE were nearly completely transformed to 1,2-cis DCE followed by VC accumulation and VC transformation to the final nontoxic products ethylene and ethane.

GW field measurement and sampling for chemical analyses

Groundwater (GW) samples were collected in a dynamic state using Micro purging methodology up to the stabilization point of the hydrochemical parameters (pH, redox-potential, conductivity, groundwater level). The collected samples were transported within one or two days in cooling boxes to the accredited laboratory for chemical analysis:

- CE content including VC, ethane, ethylene, CH_4,
- organic substrate content using COD,
- NO_3^-, NO_2^-, NH_4^+, Fe^{2+}, Mn^{2+}, SO_4^{2-}, H_2S+S^{2-}, PO_4^{3-}, Sr and K

Attention was paid for minimal oxygen exposure of all samples.

Molecular genetic analyses – Institute of Microbiology (IMB)

The main goal of the project required the optimization of environmental DNA extraction from the GW by using the advanced automated Maxwell™ 16 System (Promega, Madison, USA). The optimized methodology allowed for processing of 16 samples at the same time (before: 8-10 samp./2 days, now: 16 sampl./2 hours). The first step included filtration of 3-5 liters of GW through 0,2 µm nylon membrane (Whatman, USA) at 50-500 ml aliquots based on the volume of the colloid and solid particles in the GW sample. Filters with captured biological material were subsequently stored in sterile 50ml plastic tubes at -20°C. The second step was the pre-extraction of DNA captured on four nylon membranes. The membranes were treated in sodium-phosphate pufr (pH=8) with SDS (sodium dodecilsulfate) and proteinase K (Machery-Nagel, Germany). The third step was the isolation of DNA in the automated device Maxwell™. Detection of key bacterial species involved in the respiration of CE, and genes coding two reductive dehalogenases (*vcr*A a *bvc*A) – i.e. enzymes crucial in the dehalorespiration pathways, transforming PCE →TCE→ DCE→VC→ ethylene, was carried out using polymerase chain reaction (PCR). The organisms and genes were 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), *Desulfitobacterium* sp. (Smits et al., 2004). For identification of catabolic genes probes were used aiming at vinylchloride reductase (Behrens et al., 2008) from *Dehalococcoides* which are known to be the main bacteria participating in the reductive dehalogenation of CE. Additionally, the group of sulfate-reducing bacteria (SRB) was monitored (Friedrich, 2002). The latter is reflecting changes of the in-situ oxidation-reductive conditions during the remediation.

Nanosamplers – Technical University of Liberec (TUL)

Currently, 3 to 5 liters of each GW sample must be processed in order to acquire sufficient DNA quantity for molecular genetic analysis. For the above reason, an alternative procedure acquiring DNA from GW is tested using stationary samplers containing nanofiber components submerged into a well. The inspiration for samplers with nanofiber carriers comes from their successful using in WWTP. The type of nanofiber and design of nanocarriers is important for the further yield quantification. Development of the nanosamplers, isolation and characterization of DNA from the nanocarriers are focus of the TUL activities. An optimal way of DNA/RNA stabilization during transportation from the site to the laboratory was examined (in cooling box with frozen cooling inserts – RT, in cooling box with dry ice – CO2, after rapid freezing in liquid nitrogen in a box of dry ice – N2 , and immediate stabilization of the sample by RNA*later*) – see Figure 1.

Figure 1: Collection of nanofiber carriers (1. RT – ice, 2. - RNA *later,* 3. CO2 – dry ice, 4. N2 – liquid Nitrogen) – *photo by Dolinova*

The nanosamplers were placed into the center of the water column at specified level, and exposed for at least 3 months. DNA was isolated from the exposed nanofiber, the DNA yield (µg per gram of nanofiber) was determined, and the presence of bacteria and functional genes was examined by the same way as in IMB.

3. Results and discussion

Field measurement standardization

a) Redox-potential measurement on-site

The redox-potential is a useful parameter for evaluation the changes in in-situ bioreactor environment in time. However, the measured redox values using argentochloride reference electrode on site are usually higher than the theoretically calculated Eh, which is due to chemical and/or electrochemical disequilibrium (Wilson N, 1995). For example, the theoretical redox potential calculated for hydrogen electrode (Eh) for monitoring of the sulfate-reducing conditions is -220 mV. From comparing of the results during groundwater sampling in a dynamic state (Table 1) it is clear that sulfate reduction does not take place only in well HV-60. However, recalculated Eh values are higher than -220 mV. In our case, the E (AgCl) measured in GW is very close to the expected theoretical Eh values.

Table 1: Redox measurement example for sulfate-reducing conditions

WEL:	**Unit**	**HV-2**	**HV-4**	**HV-17**	**HV-37**	**HV-38**	**HV-59**	**HV-60**	**HV-61**	**HV-62**	**HV-58**
sampling date	**Unit**	*4.MAR 2015*	*4.MAR 2015*	*4.MAR 2015*	*4.MAR 2015*	*4.MAR 2015*	*4.MAR 2015*	*4.MAR 2015*	*4.MAR 2015*	*4.MAR 2015*	*4.MAR 2015*
Redox meassured: E(AgCl)	mV	*-127*	*-230*	*-270*	*-260*	*-239*	*-300*	*-9*	*-314*	*-294*	*-224*
Redox recalculated: Eh	**mV**	***89***	***-14***	***-53***	***-44***	***-22***	***-84***	***207***	***-98***	***-78***	***-8***
SO_4^{2-}	mg/L	<5	106,00	<5	<5	<5	41,60	105,00	57,00	9,39	88,80
$H_2S + S^{2-}$	mg/L	0,576	<0,05	4,220	2,750	0,367	2,580	<0,05	5,770	1,880	<0,05
CH_4	µg/l	39000	14000	13000	15000	30000	8800	23	34000	21000	3000

b) Dissolved oxygen measurements

Oxielectrodes, exactly a permeable oximembranes, are very sensitive to mechanical damage or to suspended solids/clayey particals in GW. Thus, the site-manager supervision during a field measurement is important for the purpose of identifying of insignificant data. Fast maintenance on site, including comparing data from two or three oximeters in one well ensures the accuracy of the measured DO values.

c) Fe^{2+} and Mn^{2+} sampling, two methods testing (by Hlavacova, VZ)

We have tested two methods. The first one - immediate on-site sample filtration (0,45 µm) and acidification by 2M HNO_3, AAS measurement in laboratory. The second one – immediate sampling to lab-prepared vessels with 1,10-phenanthroline + acetate buffer, red colour solution appears in case that Fe^{2+} is present, termination by UV/VIS analysis at 510 nm. Mn^{2+} measurement correlation for the both methods was very good – see Table 2; Fe^{2+} measurement correlation was acceptable for

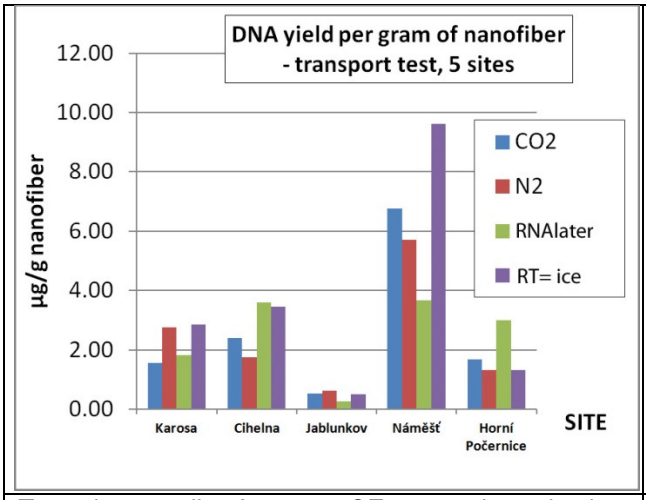
concentrations up to 10 mg/L of total Fe, higher concentration of the total Fe needs more detailed site-manager supervision and detailed monitoring of Fe^{2+}.

Table 2: Fe^{2+} and Mn^{2+} sampling and chemical analysis, an example

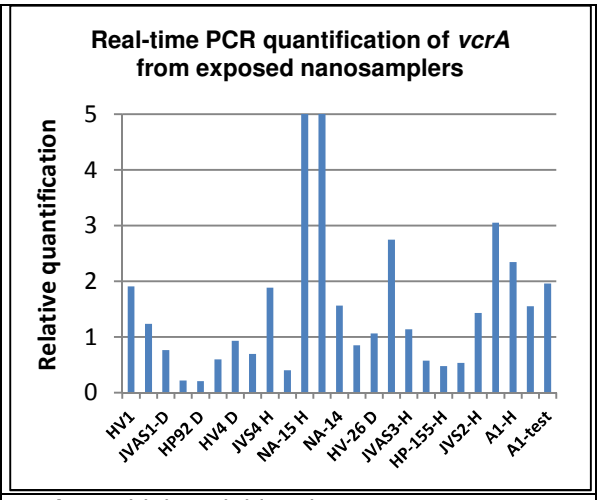
Sampling SEPT.2013	Method	HP-83	S-889	HP-92	HP-155
Fe2+	1,10-phenantroline	2,00	7,70	2,70	7,80
Fe2+	filtration 0,45 µm + acidificatin on site	1,90	33,20	2,20	8,40
Fe total	AAS	6,30	36,50	3,80	9,10
Mn total	AAS	1,40	0,09	0,05	0,15
Mn2+	filtration 0,45 µm + acidificatin on site	1,30	0,09	0,05	0,17

Nanosamplers

The stability of DNA extracted from the GW-submerged nanosamplers after transportation from the site was followed in detail from 2013 to 2014. Comparison of the yields of DNA isolated from colonized nanofiber (Fig.2) proved that the transportation in cooling box with ice is enough efficient and it is not necessary to exploit other more financially and mechanically demanding methods (N_2, CO_2, RNA*later*). PCR could target the dehalorespiring markers - *vcr*A and *bvc*A genes, in both the nanosamples and the GW.



Tested 5 wells from 5 CE-contaminated sites. Stabilization of 6 months exposed nanosamplers by solid CO2, liquid N2, RNAlater solution and in cooling box with ice (=RT).

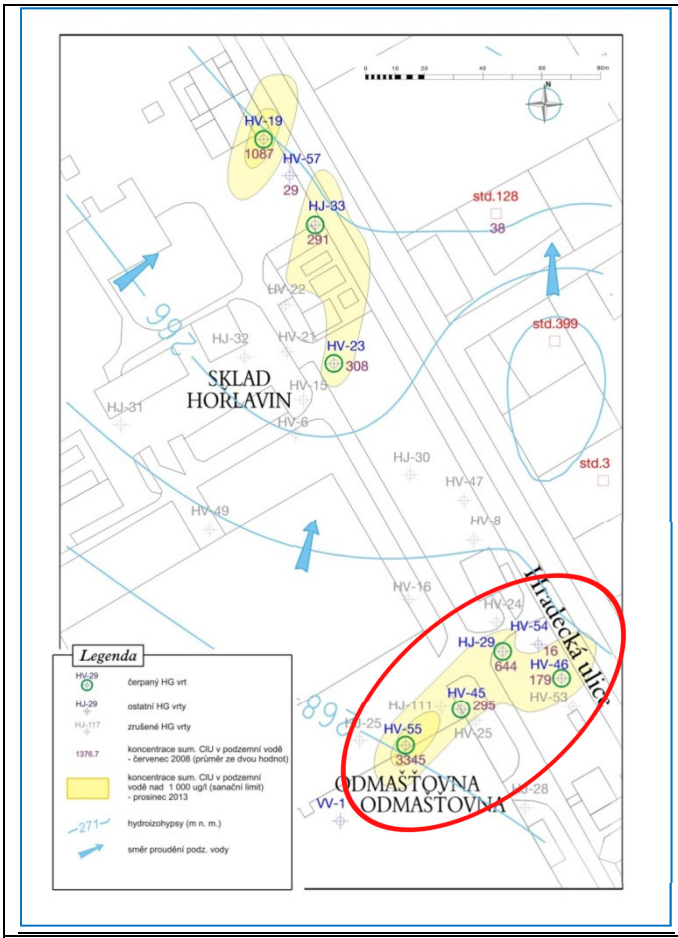


vcrA >1= higher yield on ice transport
vcrA <1= higher yield RNAsolution transport
14 samples from 25 (56%) had a higher yield from nanosampler transportation on ice

Figure 2: Transport tests of exposed nanosamplers (by Dolinova)

4. Case studies

CASE STUDY I . – Site A: Early ERD process, early stage



Date	Site A - activities
1997 - 2000	Venting
9/2003 -10/2005	Venting
1997 - 2/2014	P&T
6/2014 - ?	ERD, cheese-whey

Site A: Cheese-whey application			
	Date (M_YY)	**volume (m3)**	**Inj. wells**
1.	6-14	4,5	HV-55, HV-45, HJ-29, HV-46
2.	11-14	3	HJ-29, HV-46,HV-54
3.	4-15	7	HV-55, HJ-29, HV-46, HV-54
Totaly Applied		**14,5**	**m3**

Site A treated area:
75 x 35 m

Site A – closure limits:
SUM PCE,TCE,DCE: 200 µg/l except HV-55 which is 600 µg/l

Figure 3: Site A characterization

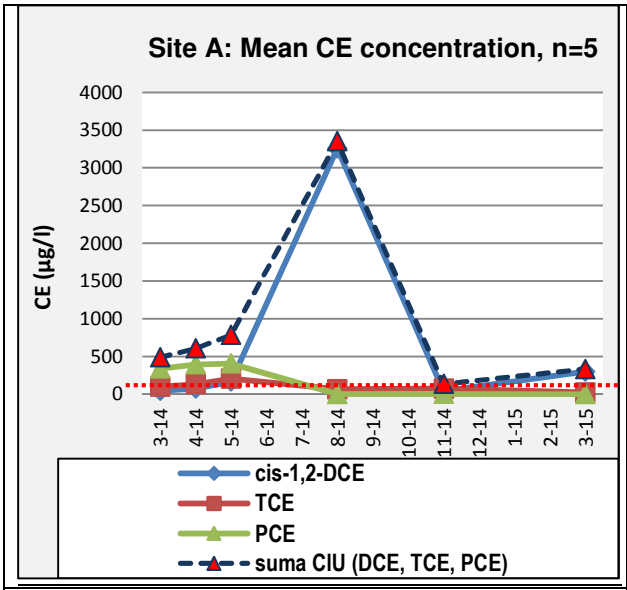


Figure 4: Site A - chloroethenes development (mean)

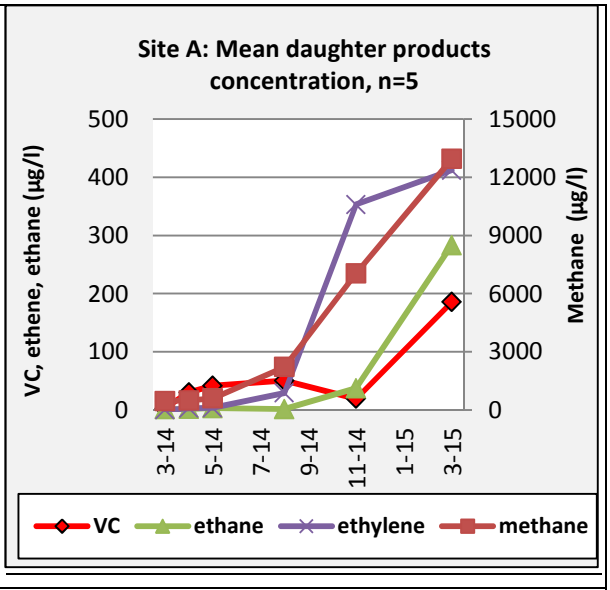


Figure 5: Site A – daughter products development (mean)

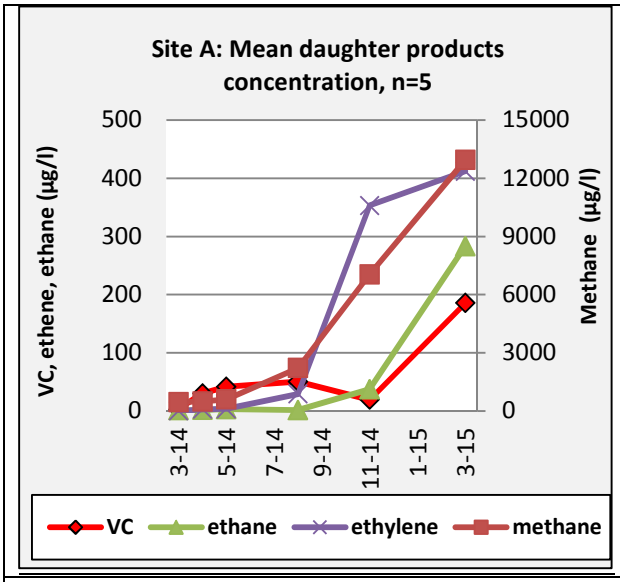


Figure 6: Site A – redox and sulphides development (mean)

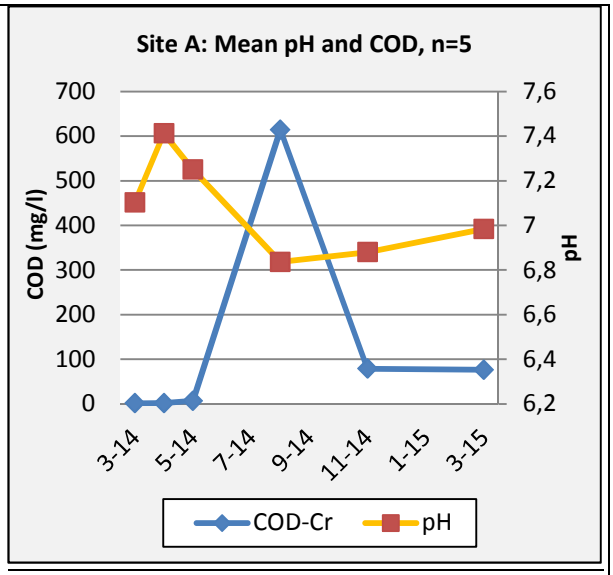


Figure 7: Site A – pH and organic substrate concentration development (mean)

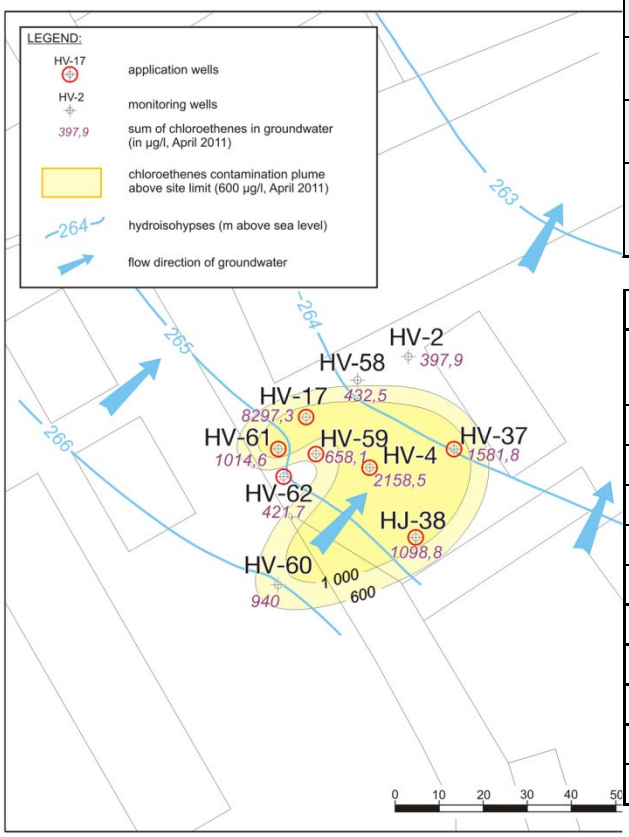
Table 3: Molecular-genetic analyses – PCR detection of key bacterial species involved in the CE organohalide respiration, and genes coding two reductive dehalogenases (vcrA a bvcA), an example – well HV-55, hot spot. Grey highlighted cells are results after CH-W application

Well	sampling	16S - DHC	nested 16S-DHC	16S - GEO	16S - DRE	*bvcA*	*vcrA*	*Sulfurospirillum*	*Desulfitobacterium*	SRB
HV-55	12.3.2014	-	++	-	+	+/-	-	+	+	+
HV-55	24.4.2014	-	+++	+	-	-	-	-	++	+++
HV-55	27.5.2014	-	+	+	-	-	-	+++	+	+/-
HV-55	7.8.2014	+++	+++	+	-	+	+++	+	+++	++
HV-55	5.11.2014	+	++	++	++	++	+++	+++	-?	+++

Conclusions for site A

1. Site A was in the early ERD stage. The primary contaminants (PCE, TCE) were completely transformed into cis-DCE within 6 months after the 2nd CH-W injection (7,5 m^3 totally)
2. pH 6,73 – 7,53 within the plume area
3. VC production was proved 2 months after 1st CH-W injection in the hot spot (HV-55, HV-45), max. 148 µg/l
4. Massive ethene production was detected 4 months after 1st CH-W injection in the hot spot (HV-55, HV-45), max. 1700 µg/l
5. Spring 2015 – organic substrate almost depleted, ethene production in stagnation (still the same speed of production), VC and ethane production accelerated – next organic substrate injection necessary.

CASE STUDY II . – Site B: ERD in progress



Date	Site A - activities
1991 - 04/2011	P&T
5/2011 - 12/2013	ERD full scale, cheese-whey
2014 - 2015	Rebounding solution, Occasional low volume CW injection

Site B: Cheese-whey application		
	Date (M_YY)	volume (m3)
1	5-11	8,0
2	7-11	7,5
3	10-11	8,0
4	12-11	2,0
5	4-12	8,0
6	11-12	7,0
7	5-13	2,0
8	11-13	5,8
9	4-15	2
Totaly Applied	**m3**	**50,3**

Site B treated area:
70 x 60 m

Site B – closure limits:
SUM PCE,TCE,DCE: 600 µg/l

Figure 8: Site B characterization

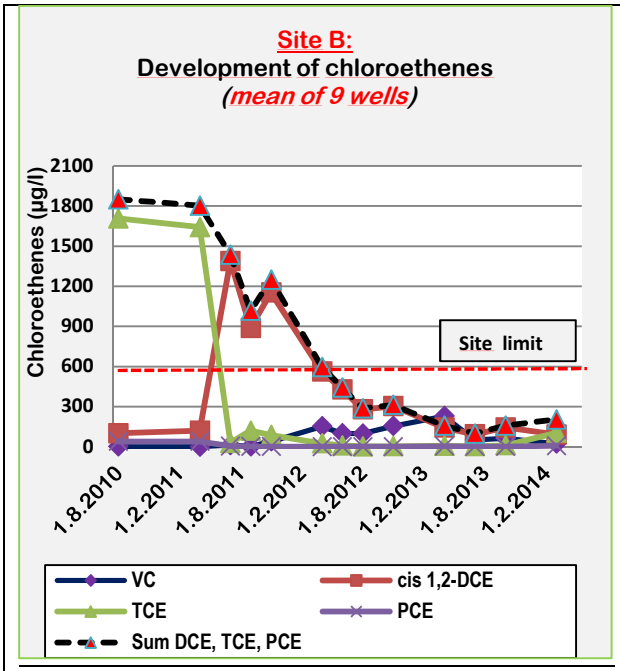


Figure 9: Site B - chloroethenes development (mean)

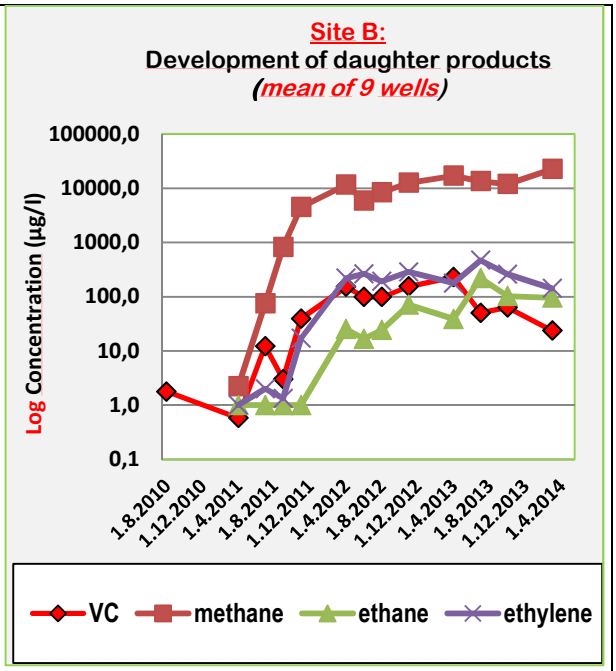


Figure 10: Site B – daughter products development (mean)

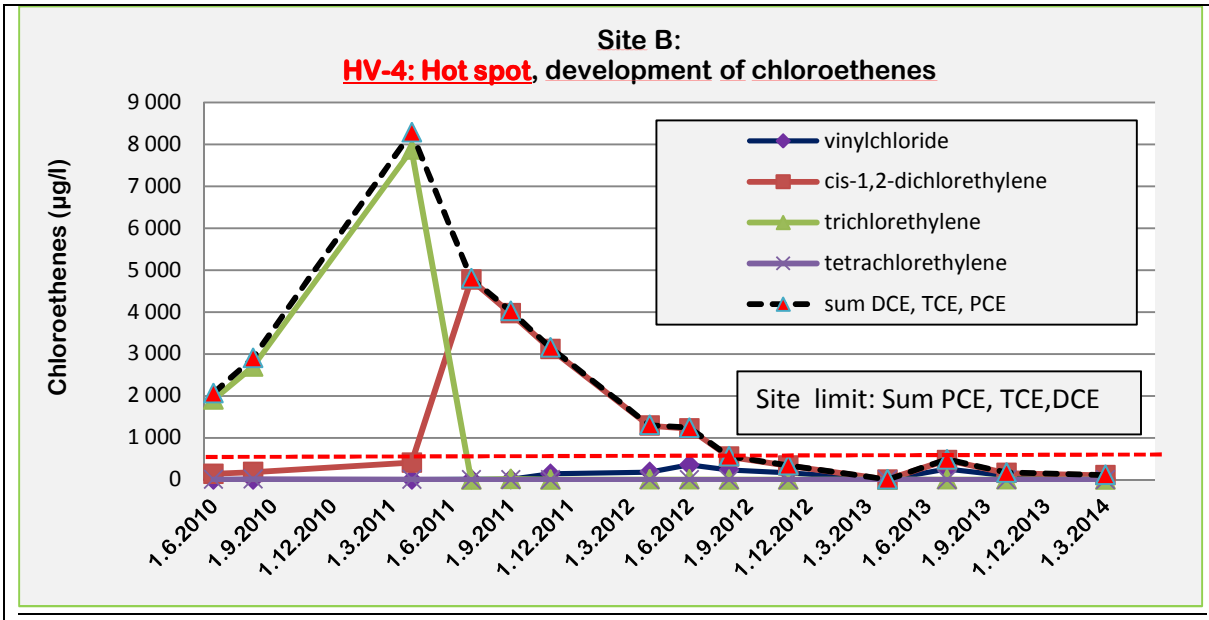


Figure 11: Site B - chloroethenes development in hot spot, well HV-4

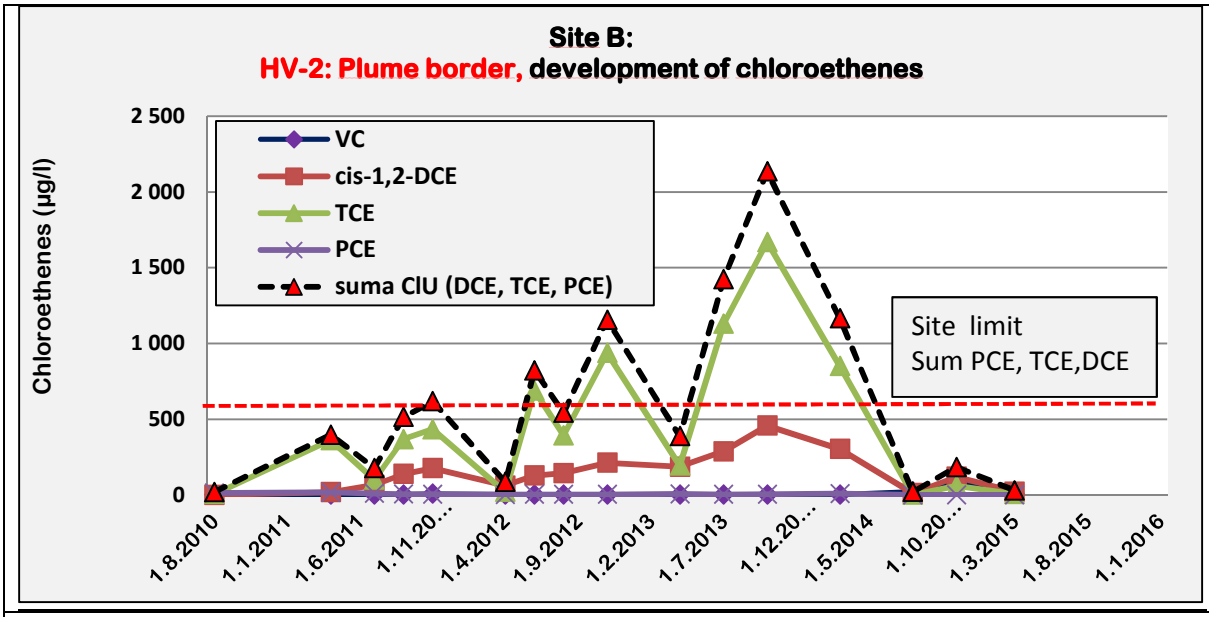


Figure 12: Site B - chloroethenes development in plume border, well HV-2

Table 4: Molecular-genetic analyses – PCR detection of key bacterial species involved in the CE organohalide-respiration, and two reductive dehalogenases genes (vcrA a bvcA), an example – well HV-4, hot spot and HV-2, plume border. Grey highlited cells are results after CW application

Well	sampling	16S - DHC	nested 16S-DHC	16S - GEO	16S - DRE	*bvcA*	*vcrA*	*Sulfuro spirillum*	*Desulfito bacterium*	SRB
HV-2	3.4.2012	+	+	-	-	-	-			
HV-4	3.4.2012	+	+	-	-	+ +	+			
HV-2	4.6.2012	+	-	-	-					
HV-4	4.6.2012	+	+	-	-					
HV-2	14.8.2012	+/-	-	-	-					

Table 4: Molecular-genetic analyses – cont.

Well	sampling	16S - DHC	nested 16S-DHC	16S - GEO	16S - DRE	*bvcA*	*vcrA*	*Sulfuro spirillum*	*Desulfito bacteriu m*	SRB
HV-4	14.8.2012	+	+	-	-					
HV-2	6.11.2012	+/-	+	+	-	-	-			
HV-4	6.11.2012	+/-	+	+	-	++	+ +			
HV-2	16.04.2013	-	+	+/-	-	-	-	+/-	-	++
HV-4	16.04.2013	+	+	-	+	++	+	+++	+	+++
HV-2	30.7.2013	-	+	+	-	-	+/-	+	-	+++
HV-4	30.7.2013	+	+	+	+	++	++	+++	+	+++
HV-2	30.10.2013	+/-	+	+	-	-	+/-	++	+/-	++
HV-4	30.10.2013	+	+	+	++	++	++	++	+/-	+++
HV-2	13.03.2014	-	+++	-	+/-	+/-	-	+	-	-
HV-4	13.03.2014	+	+++	-	+/-	+++	+	+++	+	+
HV-2	27.5.2014	+++	+++	+	++	-	+++	-	++	++
HV-2	07.08.2014	+++	+++	-	-	-	++	+	-	+++
HV-4	07.08.2014	+	+++	-	+	++	++	+++	-	++
HV-2	4.11.2014	-	++	+	-	++	++	+++	+	++
HV-4	4.11.2014	+	++	+	-	+	++	++	+/-	+++

Conclusions from site B

1. ERD on site B proceeds according to the expectation. The site remediation limit was reached within 2 years.
2. The first CE rebounding in the hot spot appeard 10 months after reaching the site limit.
3. Some VC is still remaining, thus, CH-W was injected only into one well (HV-59, in 4/2015) Border plume was temporary involved in increase of CE concentration, see Figure 12. Later on the complete CE transformation to ethene was proved.

5. Conclusions

- ERD using CH-W application is a successful and cheap remediation choice; substrate price: $1m^3$ (=1000L) per 6,5 EUR + transport; the cost of PCR analysis/GW sample with identification of ERD community and *vcrA* and *bvcA* = cca. 100 EUR (in Institute of Microbiology); complete ERD Chemical analysis/GW sample = app. 95 EUR (in accredited ALS laboratory)
- ERD is a slow anaerobic process. Site-specific limits may be reached within 2 years. However, it is necessary to count with additional time due to the rebounding of the pollutant (2-3 years).
- The rebound is a postremediation pollutant reversal due to residual contamination and new physical-chemical equilibrium after the treatment. Thus, a short term successful remediation without rebounding cannot be anticipated.
- For each in-situ injection, regardless of the selected type of injected solution, a “washing effect” appears demonstrated by subsequent higher contaminant concentration in GW, contaminant mobilization and contaminant migration. Therefore, an interpolation of all data for the whole plume area is recommendable.
- All field measurements require a diligent and continual on-site control for accuracy, correctness and compatibility of the measured values due to their further utilization for site management and interpretation.
- The nanosamplers are an appropriate tool for long term microbial community monitoring. Transferring of the exposed nanosamplers on ice from site to lab guarantees the DNA stability.
- Statistical data-mining of all Techtool results is currently in progress. We have investigated in detail eight CE-contaminated sites within four years (aprox. 700 complete well analysis and 480 MBT analyses). Our aim is i) minimizing the monitoring data volume, and ii) answering the question whether there are common signs for ERD management on the majority of CE sites or if a site specific management model is the only possible remediation choice.

Acknowlegement
This work is funded by the Technology Agency of the Czech Republic (project TA02020534). Many thanks for the excellent cooperation on the project TECHTOOL belong to Ch. Holliger, P. Rosi and S. Tarnawski (École polytechnique fédérale de Lausanne), J. Mikes (EPS s.r.o.), T. Lederer (Aquatest a.s.), R. Hermanek (KHsanace s.r.o.)

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