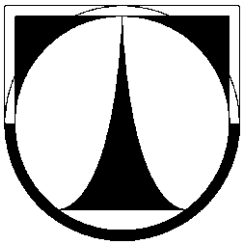
TECHNICAL UNIVERSITY OF LIBEREC
Faculty of Mechatronics, Informatics
and Interdisciplinary Studies

Assessing the impact of chlorinated ethenes remediation on indigenous microbial populations using molecular biology tools

Hodnocení vlivu sanace chlorovaných etylenů na původní mikrobiální společenstva pomocí nástrojů molekulární biologie.

Ph.D. Thesis
Author's summary



Study programme: P 3901 Applied Sciences in Engineering
Study branch: Applied Sciences in Engineering

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Liberec 2022

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ABSTRACT

This study assessed the effect of different remediation techniques on microbial community composition in the treated area and its changes over time. The research focused on sites polluted by chlorinated ethenes (CEs). The presence of organohalide-respiring bacteria (OHRB) was monitored along with the genes encoding enzymes of their degradation pathways. Isolation of deoxyribonucleic acid (DNA) from groundwater samples was challenging due to high concentration of CEs. As such, DNA extraction protocol had to be optimized and verified for each site studied. Furthermore, all primers for the detection of OHRB and specific genes had to be tested for their accuracy before standard use in real-time polymerase chain reaction (qPCR) and all qPCR reactions optimized due to the presence of inhibitors in some samples. Changes in the composition of the bacterial community and the shifts in selected populations were further analysed by the 16S rRNA amplicon sequencing, which was used to describe the composition of the whole autochthonous bacterial community in detail.

The application of reagents for oxidation (hydrogen peroxide to trigger the Fenton-like reaction) and reduction (nanoscale zero-valent iron in combination with electrokinetic treatment and zero-valent iron attached to activated carbon) of CEs caused a decrease in the levels of degrading bacterial strains. However, this effect was only temporary and, after a short time, OHRB populations recovered due to the inflow of untreated water and well-adjusted and favorable soil conditions. The application of biostimulation substrates sodium lactate and glycerol supported the growth of OHRB at contaminated sites. 16S rRNA sequencing also showed that glycerol-fermenting bacteria were the first to proliferate after glycerol application, followed by OHRB. Levels of degradation enzymes also increased. Oxidation-reduction potential of the groundwater was reduced after the application.

This study demonstrates the importance of autochthonous microbial community characterization of the polluted site before and during remediation for successful CEs decontamination. It was revealed that a mixed bacterial community providing sufficient syntrophic interactions is very important for CEs degradation. Monitoring of present degrading bacteria and their enzymes should be continued throughout the whole remediation action to support optimal biodegradation rates at the right time.

Three scientific publications have been published and one submitted on this topic.

ABSTRAKT

Tato práce se soustředí na vliv sanačního zásahu na složení mikrobiálního společenstva v zasažené oblasti a jeho změny v čase a sleduje přítomnost degradačních enzymů při dekontaminaci polutantů. Soustředí se na rozklad chlorovaných etylenů. Sleduje organohalide-respirující bakterie (OHRB), a také geny kódující degradační enzymy. Izolace deoxyribonukleové kyseliny (DNA) z podzemní vody byla komplikovaná pro vysoký obsah chlorovaných etylenů ve vzorcích, proto musel být extrakční protokol optimalizován a ověřen pro každou studovanou lokalitu. Pomocí metody polymerázové řetězové reakce v reálném čase (qPCR) byly detetekovány OHRB a specifické bakteriální geny v jednotlivých vzorcích. qPCR primery pro jejich detekci musely být otestovány pro co největší selektivitu a reakční podmínky optimalizovány pro každý vzorek zvlášť kvůli přítomnosti inhibitorů ve vzorcích. Změny ve složení a zastoupení přítomných bakteriálních populací byly dále sledovány metodou 16S rRNA amplikonové sekvenace, pomocí které lze detailně popsat složení celého autochtonního bakteriálního společenstva.

Bylo zjištěno, že aplikace činidel pro cílenou oxidaci (peroxid vodíku pro spuštění Fentonovy reakce) a redukci (nulmocné nanoželezo v kombinaci s aplikací elektrického napětí a nulmocné železo uchycené v aktivním uhlí) chlorovaných etylenů mohou způsobit pokles v hladinách sledovaných degradačních bakteriálních kmenů. Tento pokles je však pouze dočasný a po krátké době dochází k obnovení populací OHRB díky vhodně nastaveným půdním podmínkám a v některých případech i k podpoře jejich růstu. Aplikace biostimulačních substrátů laktátu sodného a glycerolu podpořila růst OHRB na kontaminované lokalitě. Pomocí sekvenace bylo zjištěno, že po aplikaci glycerolu došlo primárně k proliferaci glycerol-fermentujících bakterií, následovaných OHRB, současně se také zvýšily hladiny degradačních enzymů. Došlo i ke snížení oxidačně-redukčního potenciálu podzemní vody.

Tato práce ukázala, že pro úspěšnou dekontaminaci chlorovaných etylenů je nezbytné před sanačním zásahem co nejlépe charakterizovat vybranou lokalitu, a to jak z hydrochemického hlediska, tak za pomoci metod molekulární biologie. V monitoringu přítomných degradačních bakterií a jejich enzymů je třeba pokračovat v průběhu celého sanačního zásahu.

Na toto téma byly otištěny tři vědecké publikace a jedna odeslána.

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1 INTRODUCTION

Chlorinated ethenes (CEs), such as tetrachloroethene (PCE) and trichloroethene (TCE), are persistent groundwater contaminants. They are largely used in industry as cleaning and degreasing agents, solvents, etc. and are often released into the environment as a result of improper handling or storage. As degradation of these compounds under aerobic conditions is limited, they can easily soak into the groundwater and travel with the flow (Jugder et al., 2016). Degradation of CEs under anaerobic conditions is much more effective and is mostly achieved by the process of reductive dechlorination (Adrian and Löffler, 2016; Aulenta et al., 2006). PCE and TCE are dechlorinated abiotically or by microorganisms by sequential reductive dechlorination to *cis*-1,2-dichloroethene (*c*DCE), vinyl chloride (VC), and ethene as a final product. However, this process is sometimes incomplete, resulting in the accumulation of *c*DCE or VC in the aquifer. As VC is more toxic and carcinogenic than the parent compounds, stimulation of VC degradation and careful monitoring of the treated site are necessary (Tobiszewski and Namieśnik, 2012).

Characterization of remediation techniques using molecular biology tools was a new approach when I began to study in 2012 and its potential had not been fully exploited. Although the impact of selected remediation agents and substrates on bacteria had been described in laboratory studies, molecular biology monitoring of pilot or full-scale field applications were still rare. Even nowadays, the effect of remediation techniques on the bacterial community at a contaminated site is usually not monitored or evaluated, while it is precisely the bacteria capable of degrading the contaminants that are in charge of "cleaning up" the site after a physico-chemical remediation action. Characterization and monitoring of changes in bacterial community are also necessary during remediation using biostimulation through the application of different substrates to support the growth of indigenous bacteria capable of degradation of a selected contaminant. Molecular biology methods are defined as tools that target biomarkers (e.g., specific nucleic acid sequences) to provide information about organisms and processes relevant to the remediation of contaminants. They can contribute to a better knowledge of a contaminated site and help to optimize the bioremediation process (Stroo et al., 2006; Blázquez-Pallí et al., 2019).

2 OBJECTIVES

The overarching aim of this thesis was to evaluate the effect of different remediation techniques on autochthonous microbial community that is involved in CEs biodegradation and to describe fundamental principles and draw general conclusions.

As toxic environmental samples were analysed, the first objective was to optimize molecular biology methods (isolation of DNA and subsequent qPCR) to obtain robust and reproducible data.

The second objective was to evaluate changes in the composition of the bacterial community and the shifts in selected bacterial populations by sequencing specific regions of bacterial DNA.

Specific objective was to assess long-term *in situ* applications of the following remediation approaches and biostimulation substrates for induction of CE degradation:

- chemical oxidation: hydrogen peroxide applied to induce a Fenton-like reaction
- chemical reduction: nanoscale zero-valent iron (nZVI) supported by electrokinetic (EK) treatment

  nZVI embedded in activated carbon
- bioremediation: stimulation of indigenous bacterial populations capable of reductive dechlorination of CEs with sodium lactate or glycerol

4 THEORETICAL PART

4.1 Biodegradation of CEs

Biodegradation of CEs can be achieved through various bacterial metabolic processes. Anaerobic degradation is the most common, but degradation under aerobic conditions is also possible, either metabolically, when CEs are used for the cell growth, or by cometabolism, when bacteria gain neither energy nor organic carbon from pollutants transformation. In my thesis, I will focus mainly on anaerobic reductive biodegradation of CEs.

4.1.1 Anaerobic biodegradation

Microbial anaerobic biodegradation of CEs is achieved by the process of reductive dechlorination (also called dehalorespiration or organohalide-respiration) when CEs serve as final electron acceptors in the respiratory chain of OHRB. The exergonic dechlorination reaction generates energy for bacterial growth. Electrons and carbon for bacterial growth are obtained from molecular hydrogen and other organic substances (Molenda et al., 2020). During the degradation process, the CEs are gradually dechlorinated from PCE and TCE to *c*DCE, VC and subsequently to ethene as the final product, while the carbon in CEs changes from the oxidized (+2 for PCE) to reduced (-2 for ethene) state (Mattes et al., 2010) (Fig. 1). Lowering redox potential is required for each step. It means, that while PCE is dechlorinated relatively easily even under less reductive conditions, a strong reductive environment, such as sulfate-reducing and methanogenic, is required for the final dechlorination of VC to ethene (Kansas et al., 1998).
The biodegradation is not always complete, leading to the accumulation of the intermediates *c*DCE or VC in the environment. As VC is more toxic than its parent compounds and even carcinogenic, it is always necessary to carefully monitor the treated site and stimulate its degradation (Tobiszewski and Namieśnik, 2012).

Several anaerobic bacteria capable of PCE and TCE reduction to *c*DCE as a final product have been described, including the genera *Desulfitobacterium*, *Dehalobacter, Desulfovibrio*, *Comamonas, Sulfurospirillium,* or *Geobacter* (Dolinová et al., 2017), however, only *Dehalococcoides* spp. and the recently described *Dehalogenimonas* spp. (both Chloroflexi phyla) are capable of complete reduction of *c*DCE and VC to non-toxic ethene (Lee et al., 2013; Löffler et al., 2013; Yang et al., 2017) (Fig. 1). *Dehalococcoides* are strictly anaerobic and occur naturally in many localities contaminated with CEs (Hendrickson et al., 2002; Němeček et al.,

2016; Czinnerová et al., 2020b). Their presence at a contaminated site correlates with ethene production, while their absence leads to *c*DCE and VC accumulation (Kranzioch et al., 2013; Rossi et al., 2012).

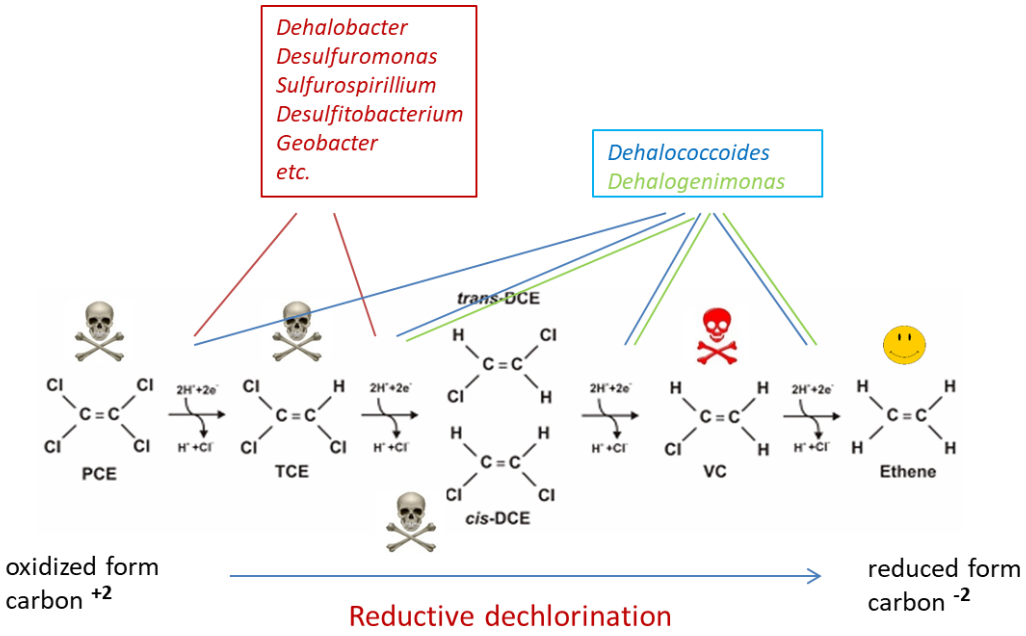

Fig. 1. Sequential reduction of CEs with key organohalide-respiring bacteria.

Successive dechlorination of CEs is catalyzed by different reductive dehalogenases (pceA, tceA, vcrA or bvcA), each cleaving specific carbon-chlorine bonds (Futamata et al., 2009; Pöritz et al., 2013; Dolinová et al., 2017). Detection of genes for one or more of these VC-reductases at a contaminated site by molecular biology methods suggests ongoing complete dechlorination of CEs and indicates the site potential for successful bioremediation (Dolinová et al., 2016; Saiyari et al., 2018).

4.1.2 Aerobic metabolic and cometabolic biodegradation

During metabolic degradation, the CEs are used as electron donors for cell growth (Mattes et al., 2010). In cometabolism, the degrading enzymes are actually produced for the degradation of bacterial primary growth substrates (named auxiliary substrates) like methane, ethene, ammonium, or aromatic pollutants which serve as electron donors (Tiehm and Schmidt, 2011). Cometabolic degradation has been described for all of the CEs, although only rarely for PCE (Ryoo et al., 2000) because opposite to anaerobic degradation, oxidation of CEs is more efficient with decreasing number of chlorine substituents (Dolinová et al., 2017).

Methanotrophs have been shown to cometabolically oxidize TCE, *c*DCE, and VC using enzyme methane monooxygenase (MMO) (Yoon et al., 2011; Liang et al., 2017). Ethene-oxidizing bacteria (ethenotrophs) can also cometabolize VC or *c*DCE in the presence of ethene as the primary growth substrate (Koziollek et al., 1999; Mattes et al., 2010).

A combination of sequential anaerobic/aerobic bioremediation can take advantage of both degradations processes and lead to complete mineralization of CEs. It means creating the conditions suitable for OHRB growth in the first step when PCE is dechlorinated to *c*DCE, and the subsequent *c*DCE degradation step being accomplished by the aerobic *Polaromonas* strain (Vogel et al., 2018; Czinnerová et al., 2020a).

4.2 Chemical methods of CE degradation

Chemical in situ remediation technologies are widely used to remove CEs from the environment, either alone or as a primary treatment prior to bioremediation. They use redox processes and can be divided into two groups, in situ chemical oxidation and chemical reduction. The main advantages of chemical treatment are rapid reduction of CE concentration, complete degradation of CEs to non-toxic products, and ease of use.

The main oxidizing agents used for remediation of CE-polluted sites are potassium permanganate, Fenton's reagent, hydrogen peroxide, ozone, and chlorine dioxide (Kao et al., 2008). Zero-valent metals in micro or nano-scale are increasingly used for CE reduction. An example of an application of nZVI for the treatment of CE-polluted site is schematically shown in Fig. 2. In addition to metals, organic substances, such as lactate, vegetable oils, molasses, ethanol, whey, or glycerol can be used to enhance microbial reductive dechlorination of CEs. Molecular hydrogen (H_2) is released into the environment through fermentation of the added substrate and serves as an electron source for OHRB (Aulenta et al., 2006; Černík and team of authors, 2010; Lacinová et al., 2013).

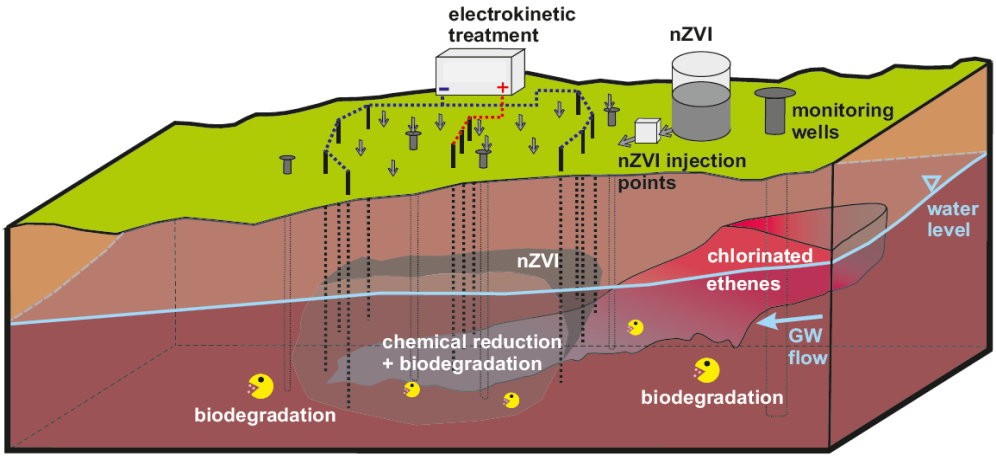

Fig. 2. The schema of remediation of CE-polluted site by application of nZVI combined with electrokinetic treatment, which also support the biodegradation (Czinnerová et al., 2020b).

This Ph.D. thesis is focused on the oxidation of CEs using Fenton's reagent, reduction using nZVI, and enhanced reductive dechlorination through addition of auxiliary substrates sodium lactate and glycerol.

4.2.1 Application of Fenton's reagent

Fenton's reagent (mixture of hydrogen peroxide [H_2O_2] and an iron catalyst [Fe^{2+}]) or a Fenton-like reaction (where there is a sufficient concentration of iron in the environment) has received a great deal of attention and is widely used for its strong oxidizing properties and low environmental impact. Groundwater remediation through Fenton's reagent application is a method based on the oxidation of CEs to CO_2. (Chapelle et al., 2005; Hrabák, 2012). In the Fenton's reagent reaction H_2O_2 and Fe^{2+} react together to form hydroxyl radicals which is then used to cleave the double bond in CEs (Černík and team of authors, 2010; Siegrist et al., 2011; Filip et al., 2019).

H_2O_2, one of the components of Fenton's reagent, is a very strong oxidizing agent toxic to microorganisms, it is even used in medicine to disinfect wounds. It is therefore likely that the application of large amounts of Fenton's reagent at the contaminated site could cause the elimination of autochthonous bacterial populations, together with OHRB, and thus stop the natural biodegradation of CEs in the treated site (Chapelle et al., 2005; Dolinová et al., 2016). Detailed monitoring of the impact of Fenton's reagent on the indigenous bacteria is therefore necessary.

4.2.2 Application of nZVI

In comparison with the microbial reduction of CEs, the abiotic reductive degradation is usually slower but complete and can be accelerated by addition of reductive agents, such as nZVI. The use of nZVI can be highly effective due to its high reactivity (large specific surface area) and its ability to completely dechlorinate CEs to non-toxic ethene (Elliott and Zhang, 2001; Zhang and Elliott, 2006; Lacinová et al., 2012; Czinnerová et al., 2020b). If oxygen is present in the environment, rapid oxidation of nZVI leads to oxygen depletion which helps form an anaerobic environment; at the same time, however, it decreases nZVI lifetime for reduction of the target contaminants (Stefaniuk et al., 2016). Under the anaerobic conditions typical for deeper zones of contaminated aquifers, nZVI reacts with water to form H_2 which serves as an electron donor for present bacteria (Bruton et al., 2015). Electrons from H_2 cause an immediate increase in the relative electron activity of groundwater, i.e. a decrease in the oxidation-reduction potential (Černík and Zeman, 2020). Both of these reactions lead to the creation of an environment suitable for the growth of OHRB.

During abiotic reduction of CEs by nZVI an electron is transferred from nZVI to the contaminant, and, concurrently, elemental iron Fe^0 is oxidized to Fe^{2+}. If the reduction is complete, non-toxic ethene or ethane are produced. The two main mechanisms responsible for reductive dechlorination of CEs by nZVI are sequential hydrogenolysis and reductive β-elimination (Lien and Zhang, 2001; Li et al., 2006).

The use of nZVI particles has several limitations, such as short-term reactivity and also rapid aggregation, that negatively affect their reactivity and mobility (Stefaniuk et al., 2016). To counteract this, different surface modifications of the nZVI particles may be used to accelerate their migration in groundwater and increase their contact with the contaminant (Lu et al., 2016).

Several studies have applied electrokinetic (EK) treatment to support nZVI and solve the above-mentioned problems (Moon et al., 2005; Gomes et al., 2016; Černík et al., 2019; Czinnerová et al., 2020b; Stejskal et al., 2020). The EK remediation process involves applying a low voltage direct current (DC) across a section of contaminated aquifer material (Acar and Alshawabkeh, 1993). Electrolysis reactions at the electrode produce hydronium ion (H_3O^+) at the anode, which lowers the pH in its proximity, and hydroxyls at the cathode, which increase the pH. Electrons released from the cathode, together with a higher pH, help to retain nZVI in a reduced state, thereby lowering reduction conditions over a longer period

(Černík et al., 2019). In addition to a positive effect of the DC field on nZVI longevity and migration, transport of nutrients to indigenous bacteria and pollutant availability is also significantly enhanced (Yeung and Gu, 2011).

4.2.3 Application of an auxiliary substrate to enhance reductive dechlorination

One of the promising approaches in bioremediation is so-called enhanced reductive dechlorination, which is achieved by the addition of an auxiliary substrate to the soil (Lacinová et al., 2012). As most OHRB require H_2 as an electron donor for CE reduction, selected carbon sources that can be fermented to H_2 are usually applied on polluted sites (Aulenta et al., 2006). Application of a readily degradable substrate results in the formation of an anaerobic reducing environment suitable for reductive dechlorination, both by depletion of oxygen, which is inhibitory to OHRB, and also due to released H_2. Electrons from H_2 cause an increase in the relative electron activity of groundwater, i.e. a decrease in the oxidation-reduction potential (Černík and Zeman, 2020).

The present OHRB use the H_2 released by fermentation of the added substrate as an electron source. The energy, generated by transporting an electron from a donor, i.e. H_2 dissolved in groundwater, to an acceptor, CE, is used for their growth (Fiedler and Keeley, 2000). Various types of organic easily fermentable substrates such as glucose, vegetable oil, yeast extract, whey, methanol, lactate, molasses, propionate, glycerol, and ethanol have been used as sources of H_2 for organohalide respiration. An important property of the applied substrate is its easy fermentability (ESTCP, 2004; Scheutz et al., 2010; Němeček et al., 2015; Dolinová et al., 2016; Atashgahi et al., 2017; Semerád et al., 2021).

4.3 Molecular biology methods

Molecular biology methods have received a great deal of attention in environmental sciences in recent years. They are increasingly being used for the monitoring of ongoing remediation processes. The molecular biology methods are especially beneficial in developing field rate constants for monitored natural attenuation, in optimization of a bioremediation process and characterization of poorly understood degradation pathways. When integrated with geochemical and physical data, the molecular biology methods can contribute to improved

design and management of remediation systems and could provide greater understanding and accurate predictions of natural and enhanced bioremediation processes (Stroo et al., 2006). Detection of bacteria capable of biodegradation and their enzymes through PCR, together with the characterization of the whole bacterial community by next-generation sequencing (NGS), are the methods most used today. Both methods work with bacterial DNA or RNA (ribonucleic acid) isolated from groundwater or soil samples. They allow for the detection and identification of selected bacterial taxa (or their enzymes) without the necessity of their cultivation.

4.3.1 Real-time PCR

Real-time PCR (quantitative PCR, qPCR) is now a well-established method for the detection, quantification, and typing of different bacteria and their genes in many fields, including remediations.

qPCR is a modification of the standard end-point PCR method when the final PCR product was visualized on an agarose gel. Instead, DNA amplification is detected in real time through the monitoring of fluorescence after each PCR cycle. The intensity of the fluorescent signal reflects the momentary amount of DNA amplicons in the sample at that specific time. The point at which the fluorescence intensity increases above the detectable level corresponds proportionally to the initial number of template DNA molecules in the sample. This point is called the threshold cycle (Ct; also named quantification cycle, Cq) and can be used to calculate the absolute quantity of the template DNA in the sample according to a calibration curve constructed of serially diluted standard samples with known concentrations or copy numbers (Kralik and Ricchi, 2017). qPCR can also provide semi-quantitative results when the measured Ct values are compared and expressed as higher or lower multiples. The results are evaluated as relative quantification, a fold change between two states, with the condition of the specific bacteria or gene at the beginning of the experiment (e.g. prior to the application of remediation agent) considered as the starting point.

4.3.2 Next-generation sequencing

Next-generation sequencing (NGS, or high-throughput sequencing) can be used in a wide range of analyses from the characterization of single bacterial species to the whole bacterial

community. In environmental microbiology, it can contribute to describtion of the function and changes in bacterial consortia during the remediation process, and, in the case of mRNA (messenger RNA) sequencing, also monitor the actively transcribed genes for degradation enzymes. The NGS method most used in environmental sciences is amplicon sequencing. It allows for sequencing of part of a genome, either selected functional genes or the 16S rRNA (ribosomal RNA) gene which can be used for phylogenetic bacterial analysis (16S rRNA amplicon sequencing). In this study, the NGS was performed on an Ion Torrent PGM (Thermo Fisher Scientific, USA).

5 EXPERIMENTAL PART, RESULTS AND DISCUSSION

This chapter is divided into four sections, each based on one article describing a selected remediation strategy for the removal of CEs from a real polluted site and evaluates its impact on indigenous bacterial populations using molecular biology tools.

Although the impact of selected remediation agents and substrates on bacteria can be evaluated in laboratory and batch studies, molecular biology monitoring of pilot or full-scale field applications is more important for describtion of the processes in the real environment. Following studies describe long-term in-situ applications of the following remediation approaches and biostimulation substrates for induction of CE degradation:

chemical oxidation: hydrogen peroxide applied to induce a Fenton-like reaction

chemical reduction: nZVI supported by EK treatment

nZVI embedded in activated carbon

bioremediation: stimulation of indigenous bacterial populations capable of reductive dechlorination of CEs with sodium lactate or glycerol

Three scientific publications have been published and one submitted on this topic.

5.1 Dynamics of organohalide-respiring bacteria and their genes following in-situ chemical oxidation of chlorinated ethenes and biostimulation

Application of Fenton's reagent and enhanced reductive dechlorination are currently the most common remediation strategies for the removal of CEs. The aim of this study was to examine the influence of a Fenton-like reaction and biostimulation through sodium lactate application on the dynamics of OHRB and their genes at a site contaminated with CEs. A wide spectrum of molecular genetic markers was used, including the 16S rRNA gene of the OHRB *Dehaloccocoides*, *Desulfitobacterium,* and *Dehalobacter*; reductive dehalogenase genes (*vcrA*, *bvcA*) responsible for dechlorination of VC and sulfate-reducing and denitrifying bacteria.

In situ application of H_2O_2 to induce a Fenton-like reaction caused an instantaneous decline in all markers below the detection limit (Fig. 3). Two weeks after application, the marker relative abundances increased to levels significantly higher than those prior to application. The increase depended mainly on the ORP of the groundwater, the dose of H_2O_2, and groundwater flow. No significant decrease in the concentration of CEs was observed due to the low H_2O_2 dose used. A clear increase in marker levels was also observed following in-situ application of sodium lactate, which resulted in a seven-fold increase in *Desulfitobacterium* and a three-fold increase in *Dehaloccocoides* after 70 days. An increase in the *vcrA* gene corresponded with an increase in *Dehaloccocoides*. After H_2O_2 application, most values returned to those prior to the application within one month. The increase in molecular genetic markers associated with OHRB lasted longer when dosing with sodium lactate. Application of sodium lactate led to the establishment of the reducing conditions necessary for the growth of anaerobic OHRB. Ongoing organohalide respiration was proven by an increase in markers along with an increase in ethene concentration.

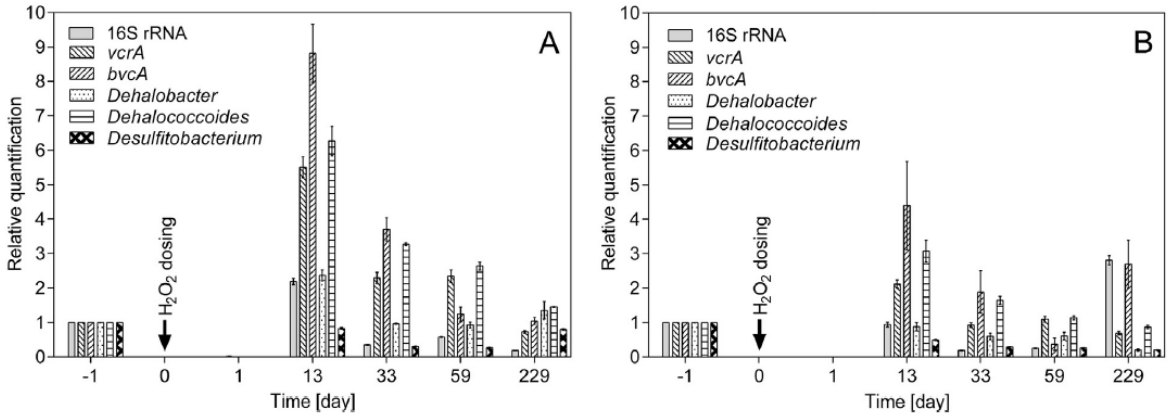

Fig. 3. Changes in the relative abundance of total bacteria (16S rRNA), OHRB (Dehalobacter, *Dehalococcoides*, and *Desulfitobacterium*), and VC reductase genes (*vcrA* and *bvcA*) following H_2O_2 application in well RW5-11: A) depth 8 m, B) depth 13 m. No molecular genetic markers were detected one day after dosing.

Analysis of selected markers clearly revealed a positive response of OHRB to biostimulation and unexpectedly fast recovery after the Fenton-like reaction.

A combination of a Fenton-like reaction followed by dosing with sodium lactate could prove efficient for CE degradation and subsequent biostimulation of OHRB during in-situ remediation of CEs at a highly contaminated site.

Citation:

Dolinová I., Czinnerová M., Dvořák L., Stejskal V., Ševců A., Černík M. (2016). Dynamics of organohalide-respiring bacteria and their genes following in-situ chemical oxidation of chlorinated ethenes and biostimulation.
Chemosphere **157**:276–285. https ://doi.org/10.1016/j.chemosphere.2016.05.030

5.2 Combining nanoscale zero-valent iron with electrokinetic treatment for remediation of chlorinated ethenes and promoting biodegradation: A long-term field study

This long-term field study explored nZVI-driven degradation of CEs supported by electrokinetic (EK) treatment, which can positively affect nZVI longevity and migration, and its impact on indigenous bacteria. In particular, the impact of combined nZVI-EK treatment on OHRB, ethenotrophs, and methanotrophs (all capable of CE degradation) was assessed using molecular genetic markers detecting *Dehalococcoides*, *Desulfitobacterium*, the reductive dehalogenase genes *vcrA* and *bvcA*, and ethenotroph and methanotroph functional genes. Changes in microbial abundance and groundwater properties were evaluated by combining physical-chemical parameters with molecular biology techniques.

This long-term field study demonstrates the great potential of the combined nZVI-EK bioremediation approach for cleaning up aquifers highly polluted by CEs. nZVI-EK treatment caused a rapid reduction in CEs in the treated area (Fig. 4) and, despite the constant inflow of contaminated water into the reactive zone, high *c*DCE degradation rates were observed throughout the experiment alongside increased production of the CE degradation products methane, ethene, and ethane. The long-term reactivity of nZVI was successfully supported by EK treatment, which additionally stimulated microbial degradation activity by elevating groundwater temperature.

The remediation treatment resulted in a rapid decrease of the major pollutant *c*DCE by 75% in the affected area, followed by an increase in CE degradation products methane, ethane, and ethene. OHRB continued CE reduction, even after partial nZVI exhaustion. The newly established geochemical conditions in the treated aquifer not only promoted the growth of OHRB but also allowed for the concurrent presence of VC- and *c*DCE-oxidizing methanotrophs and (especially) ethenotrophs, which proliferated preferentially in the vicinity of an anode where low levels of oxygen were produced. The nZVI treatment resulted in a temporary negative impact on indigenous bacteria in the application well close to the cathode; but even there, the microbiome was restored within 15 days.

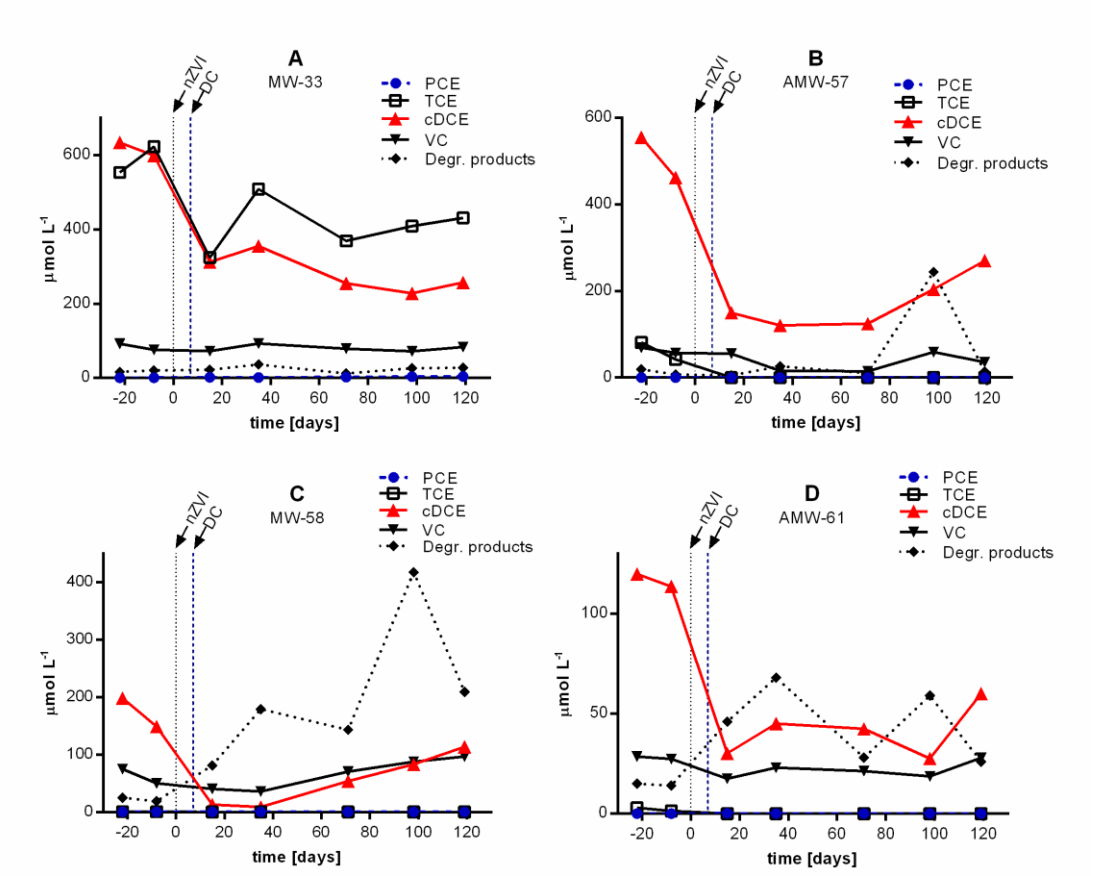

Fig. 4. Changes in the concentration of CEs and their final degradation products (the sum of methane, ethene, and ethane) following nZVI injection and direct current (DC) treatment. (A) reference well MW-33; (B) application well close to a cathode AMW-57; (C) monitoring well close to a cathode MW-58; (D) application well close to an anode AMW-61.

Our approach, combining nZVI as a strong CE reducing agent and EK for supporting nZVI reactivity and mobility proved highly effective in reducing CE contamination and creating a suitable environment for subsequent biodegradation by changing groundwater conditions, promoting transport of nutrients, and improving CE availability to soil and groundwater bacteria. As such, combined nZVI-EK treatment represents a suitable remediation strategy for cleaning sites highly polluted with CEs. To the best of our knowledge, this study presents the first field-scale, long-term exploration of native degrading bacterial population response to nZVI-EK treatment.

Citation:

Czinnerová M., Vološčuková O., Marková K., Ševců A., Černík M., Nosek J. (2020). Combining nanoscale zero-valent iron with electrokinetic treatment for remediation of chlorinated ethenes and promoting biodegradation: a long-term field study. *Water Res* **175**:115692. https://doi.org/10.1016/j.watres.2020.115692

5.3 In situ pilot application of nZVI embedded in activated carbon for remediation of chlorinated ethene-contaminated groundwater: effect on microbial communities

The nZVI is commonly used for remediation of groundwater contaminated by CEs, however, its long-term reactivity and subsurface transport are limited. A novel nZVI–AC material, consisting of colloidal activated carbon (AC) with embedded nZVI clusters, was developed with the aim of overcoming the limitations of nZVI alone. The purpose of this study was to examine the potential of novel nZVI–AC for cleaning a CE-polluted site and to elucidate abiotic and biotic processes triggered by nZVI–AC application and their impact on indigenous microorganisms. This study describes the effect of the application of the nZVI–AC composite on hydrochemical conditions and microbial community of an oxic aquifer. In doing so, we intended to elucidate the chemical and microbial processes involved in CE transformation.

Application of a limited amount of nZVI–AC to an oxic, nitrate-rich, highly permeable quaternary aquifer triggered a time-limited transformation of CEs, with noticeable involvement of reductive dechlorination. Reductive dechlorination of CEs was primarily abiotic as an increase in ethene and low concentrations of VC did not coincide with an increase in the abundance of reductive biomarkers indicating complete dechlorination of PCE (*Dehalococcoides*, *Dehalogenimonas*, VC reductase genes *vcrA* and *bvcA*).

Hydrochemical parameters (a temporal decrease in groundwater dissolved oxygen concentration and an insignificant, or temporary, decrease in nitrate and sulfate concentration) indicated a limited, short-term effect of nZVI–AC application, probably due to a high overall inflow of competing electron acceptors (CEs and oxidized inorganic compounds) and the low levels of $Fe^{(0)}$ applied to the treatment zone. This is in accordance with the changes observed in the bacterial community, where reducing effects only resulted in temporary and/or short-term proliferation of nitrate and iron reducers.

The generated reduced iron induced an increase in iron-oxidizing bacteria at a later stage (Fig. 5). Overall, we observed no significant inhibition effect of nZVI–AC on the bacterial community or its diversity. Oxic conditions in the aquifer prevented any significant growth of strictly anaerobic OHRB such as *Dehalococcoides* and their functional VC reductase genes *vcrA* and *bvcA* in the treatment zone; however, it did allow the survival of aerobic microorganisms of the genera *Pseudomonas*, *Polaromonas*, and *Rhodoferax*, known for their

ability to assimilate VC or *c*DCE. A potential for aerobic oxidative degradation of CE metabolites was also indicated through the detection of the ethenotroph functional gene *etnE*.

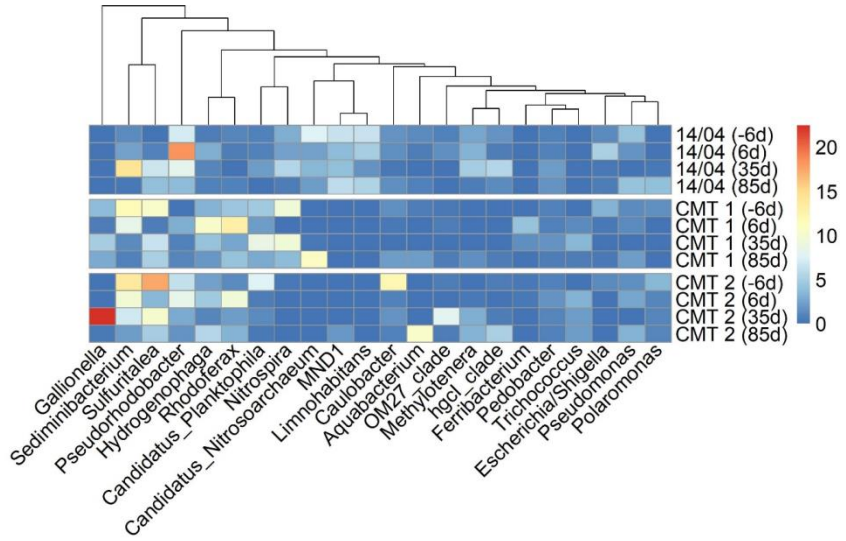

Fig. 5. Bacterial community composition in CMT 1, CMT 2 (application wells), and 14/04 (reference well) before (− 6 days) and after (6 days, 35 days, and 85 days) nZVI–AC injection. Only genera with a relative abundance > 1% are shown.

While this nZVI–AC application pilot study failed to produce a sustainable effect on CE contamination, it provided valuable insights into the hydrogeochemical and microbial processes induced, which could prove useful when designing full-scale applications.

Citation:

Czinnerová, M., Nguyen, N.H.A., Němeček, J., Mackenzie, K., Boothman, C., Lloyd, J., Laszlo, T., Špánek, R., Černík, M., Ševců, A. (2020).
In situ pilot application of nZVI embedded in activated carbon for remediation of chlorinated ethene-contaminated groundwater: effect on microbial communities.
Environ. Sci. Eur. **32**, 154. https://doi.org/10.1186/s12302-020-00434-2

5.4 Field application of glycerol to enhance reductive dechlorination of chlorinated ethenes and its impact on microbial community structure

This study summarizes the outcomes of a 7-month in-situ application of glycerol conducted to enhance reductive dechlorination of CEs on a highly contaminated site.

Glycerol injection resulted in an almost immediate increase in the abundance of fermentative Firmicutes (*Clostridium*, *Trichococcus*, and *Zymophilus;* Fig. 6) that produced essential sources of carbon (acetate) and electrons (H_2) for OHRB and changed groundwater conditions to more suitable for OHRB growth. A great increase in the abundance of OHRB *Dehalococcoides* and *Desulfitobacterium*, concurrently with VC-reductase gene levels (*bvcA* and *vcrA*), was revealed by the qPCR method (Fig. 7). Consistent with reduced redox potential sulfate and iron-reducing bacteria flourished at the end of the monitoring period (200 days) competing with OHRB for electron donors but at the same time producing acetate and essential corrinoid cofactors.

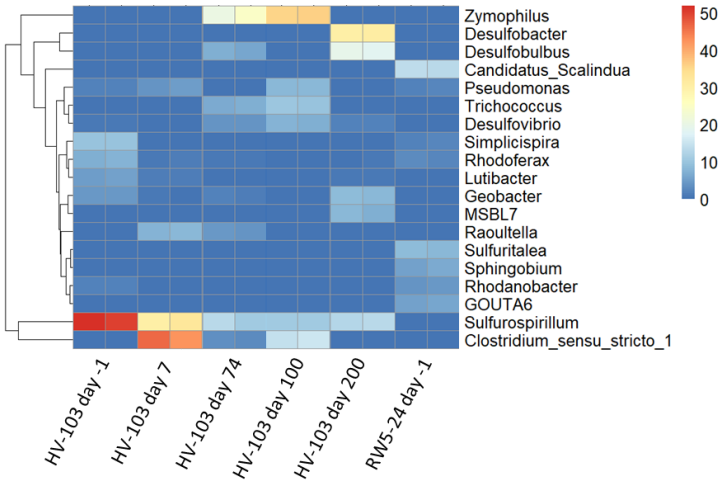

Fig. 6. Bacterial community composition in application well HV-103 and reference well RW5-24 before (− 1 day) and after (7 days, 74 days,100 days, and 200 days) glycerol injection. The scale expresses the percentage of each bacterial genus in a sample. Only genera with a relative abundance > 5% are shown.

Consistent with shifts in bacterial populations, concentrations of pollutants PCE and TCE decreased during the monitoring period, with rising levels of *c*DCE, VC, and, most importantly, final CE degradation products ethene and ethane (Fig. 8). 98.5% PCE and 99.4% TCE were reduced in the injection well HV-103 after glycerol applications with the increase of the degree of dechlorination from 21% to 62%.

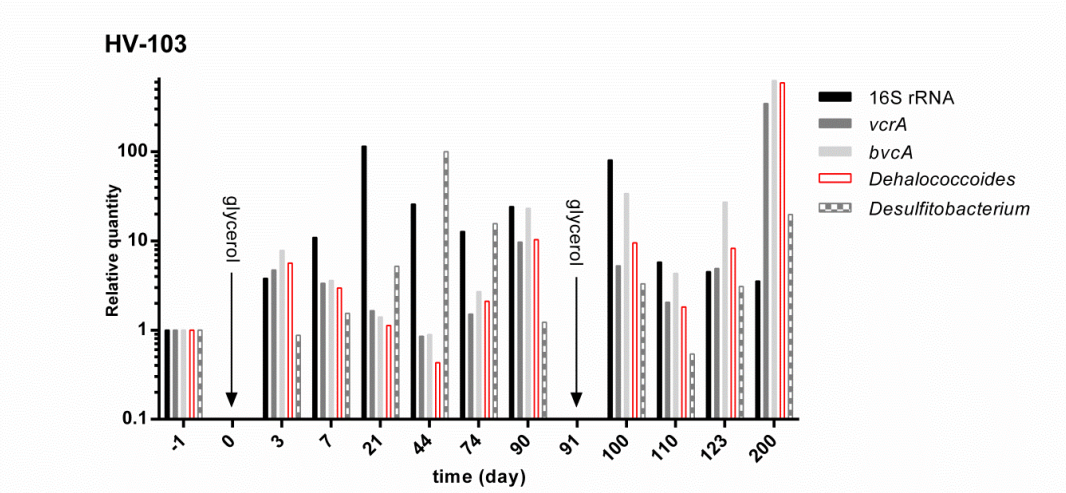

Fig. 7. Changes in the relative abundance of total bacteria (16S rRNA), OHRB (*Dehalococcoides* and *Desulfitobacterium*), and VC reductase genes (*vcrA* and *bvcA*) in the application well HV-103. All results are expressed as relative quantity to each marker abundance prior to glycerol application (-1 day). Note the logarithmic scale.

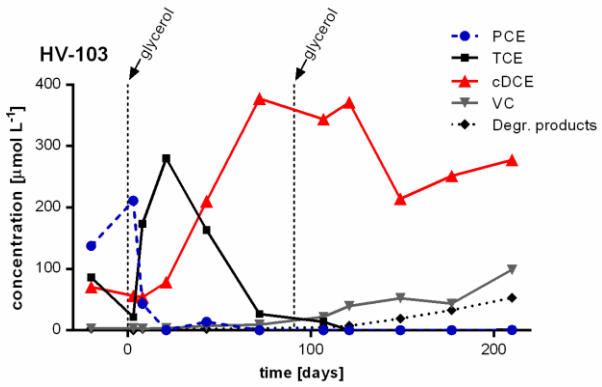

Fig. 8. Changes in the concentration of CEs and their final degradation products (the sum of ethene and ethane) following glycerol application in HV-103 well.

Our study implies the importance of syntrophic bacterial interactions for successful and complete CE degradation and evaluates glycerol as a low-cost substrate for enhancing reductive dechlorination and an effective source of electrons for OHRB.

Citation:

Czinnerová, M., Stejskal V., Nosek J., Špánek, R., Ševců A. (2022). Field application of glycerol to enhance reductive dechlorination of chlorinated ethenes and its impact on microbial community structure. Submitted to *Environmental Science: Processes & Impacts*.

6 CONCLUSIONS

The structure and function of the indigenous microbial community are still not commonly considered by technicians when planning the remediation treatments and molecular biology methods are still not commonly used, even though they offer an additional insight into the biodegradation processes at the treated site and can be very helpful in deciding the treatment strategy. Therefore, I focused my studies on establishing reliable and easy-to-use molecular biology methods for monitoring autochthonous bacteria populations and their enzymes at polluted sites. After optimizing the DNA isolation from the complicated environmental samples I concentrated mainly on establishing reliable qPCR protocol and reaction conditions for each genetic marker. The next step was designing field-scale applications of selected remediation agents in cooperation with remediation technicians and implementation of molecular biology methods in the diagnostics of ongoing remediation processes. I focused on describing changes in the microbial community during long-term *in situ* applications of selected remediation agents and biostimulation substrates for induction of CE degradation.

The main findings are as follows:

1. Detailed characterization of indigenous bacterial populations capable of CE degradation at the contaminated site together with physico-chemical analyses of groundwater and soil samples should be required for decision making which treatment strategy to use. Physico-chemical parameters alone do not provide sufficient information about ongoing microbial dechlorination.

2. Oxidative treatment of CE-contaminated site by a Fenton-like reaction had a temporary negative impact on indigenous bacterial populations followed by unexpectedly fast recovery due to the inflow of untreated colonized groundwater.

3. Negative impact of the reductive treatment by nZVI application proved to be time-limited as well with restoration of the microbiome within several days. nZVI injection led to the establishment of groundwater conditions suitable for OHRB growth.

4. Combined chemical treatment by application of oxidative or reductive agents followed by monitored biodegradation represents a suitable remediation strategy for cleaning sites highly polluted with CEs.

5. Application of auxiliary substrates sodium lactate and glycerol led to the enhanced reductive dechlorination of CEs by supporting OHRB growth. The variances in biodegradation at different contaminated sites were caused mainly by different rates of groundwater flow, physico-chemical parameters, and concentration of CEs.

6. Vertical stratification of biological and physico-chemical parameters must be considered when assessing the biodegradation potential of the polluted site. Reductive dechlorination of CEs was limited in an oxic environment, which did not allow for the proliferation of anaerobic OHRB even after nZVI treatment. On the other hand, low dissolved oxygen levels permitted the concurrent presence of OHRB and VC- and *c*DCE-oxidizing *Pseudomonas* and *Polaromonas.* The coupling of nZVI-induced support for microbial reduction of PCE with subsequent oxidation of cDCE and VC by oxidizing bacteria is a promising strategy for permeable, slightly anoxic aquifers.

7. Syntrophic bacterial interactions proved to be essential for successful and complete CE degradation, as in aquifers, bacterial populations influence the growth of each other by changing groundwater conditions, competing for sources, or producing nutrients essential for others. Complete biodegradation of CEs is possible only on sites with a high concentration of dissolved organic carbon and variegated bacterial populations (high relative abundance of OHRB together with fermenting and sulfate-reducing bacteria).

Future work should be aimed on the implementation of a multi-method assessment of the biodegradation potential of polluted aquifers to praxis. The assessment should combine hydro and geochemical data, metabolites concentrations, and the identification of selected biomarker genes through novel molecular biology methods like mRNA sequencing. The mRNA analysis will reveal actively transcribed genes for degradation enzymes and therefore will give more precise information about the ongoing metabolic processes involved in biodegradation.

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Kakosová E., Hrabák P., Černík M., Novotný V., Czinnerová M., Trögl J., Popelka J., Kuráň P., Zoubková L., Vrtoch L. (2017). Effect of various chemical oxidation agents on soil microbial communities. *Chemical Engineering Journal* **314**:257-265. https://doi.org/10.1016/j.cej.2016.12.065

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Czinnerová M., Sakmaryová I., Votrubová M., Ševců A. *Remediation of Chlorinated Ethenes Using Reactive Iron Barrier and Its Impact on Indigenous Bacteria*

ISBN : 978-1-927877-07-4; Paper No. 172

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