TECHNICAL UNIVERSITY OF LIBEREC
Faculty of Mechatronics, Informatics
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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

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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), one of the most common groundwater contaminants. The presence of organohalide-respiring bacteria (OHRB) capable of CE degradation 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 the 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 bacteria. 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 during remediation.

The application of biostimulation substrates sodium lactate and glycerol supported the growth of OHRB at the contaminated site. 16S rRNA sequencing also showed that glycerol-fermenting bacteria, which produce an essential source of carbon (acetate) and electrons (molecular hydrogen; H_2) for OHRB growth, 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 to levels suitable for the growth of sulfate and iron-reducing bacteria, which compete with OHRB for electron source but also produce enzyme cofactors essential for their growth.

This thesis demonstrates the importance of autochthonous microbial community characterization of the polluted site before and during remediation for successful CE decontamination. OHRB and other bacteria that act as primary or secondary "cleaners" of a

remediated site need not only certain soil conditions for successful growth, such as suitable oxidation-reduction potential, pH, dissolved oxygen, sulfate, or nitrate concentration, but also sufficient supply of nutrients and electron sources. Last but not least, it was revealed that besides OHRB, a whole bacterial community providing sufficient syntrophic interactions is very important for CE degradation. Monitoring of 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.

Keywords: remediation, chlorinated ethenes, organohalide-respiring bacteria, molecular biology methods, bacterial community, biodegradation

ABSTRAKT

Tato práce se soustředí na hodnocení vlivu sanačního zásahu na složení mikrobiálního společenstva a jeho změny v čase a sleduje přítomnost degradačních enzymů důležitých pro dekontaminaci polutantů. Soustředí se na rozklad chlorovaných etylenů, které představují jedny z nejrozšířenějších kontaminantů podzemních vod. Sleduje skupiny bakterií, které jsou schopny degradovat chlorované etyleny (tzv. organohalid-respirující bakterie; OHRB), a také geny kódující enzymy metabolických drah degradace těchto látek. 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 následně 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 bakterií. Tento pokles je však pouze dočasný a po krátké době dochází k obnovení populací OHRB díky vhodně nastaveným sanačním podmínkám a v některých případech i k podpoře jejich růstu.

Aplikace biostimulačních substrátů laktátu sodného a glycerolu podpořila růst OHRB na kontaminovaných lokalitách. Pomocí sekvenační analýzy bylo také zjištěno, že po aplikaci glycerolu došlo primárně k proliferaci glycerol-fermentujících bakterií, které produkují zdroj uhlíku (acetát) a elektronů (molekulární vodík; H_2) pro růst OHRB, současně se také zvýšily hladiny degradačních enzymů. Došlo i ke snížení oxidačně-redukčního potenciálu podzemní vody na hodnoty vhodné pro růst sulfát a železo-redukujících bakterií, které sice kompetují s OHRB o zdroj elektronů, ale současně produkují kofaktory enzymů důležité pro jejich růst.

Tato dizertační 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, protože OHRB i další bakterie, které fungují jako primární či sekundární „čističi" lokality, potřebují k úspěšnému růstu a práci nejen určité environmentální podmínky, jako je vhodný oxidačně-redukční potenciál, pH, koncentrace rozpuštěného kyslíku, sulfátů či nitrátů, ale také dostatek živin, zdroj elektronů, a v neposlední řadě se ukázalo, že i celé bakteriální společenstvo poskytující dostatek syntropních interakcí. V monitoringu přítomných degradačních bakterií a jejich enzymů je třeba pokračovat v průběhu celého sanačního zásahu, aby bylo možné ve správný čas znovu podpořit optimální biodegradaci.

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

Klíčová slova: remediace, chlorované etyleny, organohalid-respirující bakterie, metody molekulární biologie, bakteriální komunita, biodegradace

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ATTACHMENTS

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TABLE OF CONTENTS

1 ABBREVIATIONS

16S rRNA	16S ribosomal RNA
AC	activated carbon
ATP	adenosine triphosphate
bvcA	gene encoding vinyl chloride reductase
CCD	charge-coupled device
*c*DCE	*cis*-1,2-dichloroethene
CE	chlorinated ethene
Cq	quantification cycle
Ct	treshold cycle
DC	direct current
DNA	deoxyribonucleic acid
EK	electrokinetic
etnC	gene encoding alkene monooxygenase
etnE	gene encoding epoxyalkane: coenzyme M transferase
MMO	methane monooxygenase
mmoX	gene encoding soluble methane monooxygenase
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
NGS	next-generation sequencing
nZVI	nanoscale zero-valent iron
OHRB	organohalide-respiring bacteria
PCE	tetrachloroethene (perchloroethene)
pceA	gene encoding tetrachloroethene reductive dehalogenase
PCR	polymerase chain reaction
pmoA	gene encoding particulate methane monooxygenase
qPCR	real-time PCR
RNA	ribonucleic acid
TCE	trichloroethene
tceA	gene encoding trichloroethene reductive dehalogenase
VC	vinyl chloride
vcrA	gene encoding vinyl chloride reductase

2 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).

Different approaches can be applied to degrade CEs biotically. Monitored natural attenuation relies on indigenous bacterial populations and is based solely on monitoring of CE biotransformation and the presence of bacteria or specific enzymes capable of the degradation on site. This approach is usually quite slow and can be enhanced by biostimulation of degrading microorganisms by addition of nutrients and sources of carbon and electrons. On sites with insufficient native populations of degrading bacteria, bioaugmentation with specific degraders can be applied (Grandel and Dahmke, 2004; Pant and Pant, 2010; Li et al., 2021).

In comparison with the microbially-driven degradation of CEs, natural abiotic degradation is usually slower but complete and can be enhanced by chemical treatment through injection of oxidative (permanganate, Fenton's reagent, hydrogen peroxide) or reductive (often zero-valent metals) agents (Dolinová et al., 2016; Czinnerová et al., 2020b; Semerád et al., 2021). In addition to chemical treatment, physical processes such as venting, pump-and-treat, air sparging and thermal desorption can be applied (Černík et al., 2010; Aral et al., 2011; 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).

3 OBJECTIVES

The overarching aim of this thesis was to evaluate the effect of different remediation techniques on autochthonous microbial community that is involved in CE 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.

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). However, this process 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). The rate of reductive dechlorination is influenced by biogeochemical processes and competition for hydrogen between different bacterial groups, especially methanogenic bacteria and also nitrate, sulfate, and iron-reducing bacteria (Aulenta et al., 2007; Chambon et al., 2013).

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).

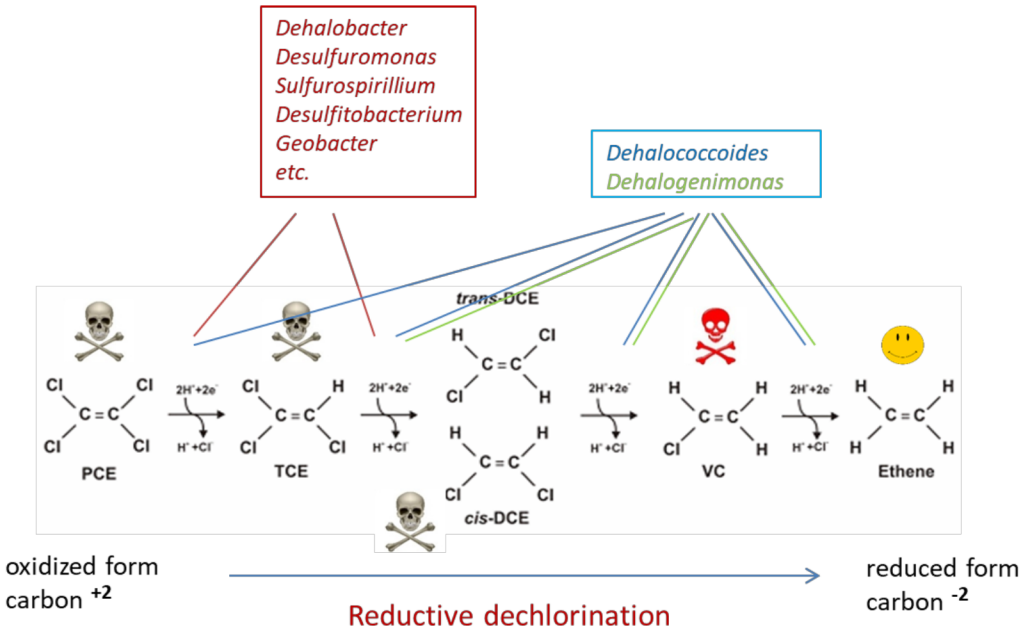

Fig. 1. Sequential reduction of CEs with key organohalide-respiring bacteria.

Several *Dehalococcoides* are usually required for complete dechlorination of CEs, as to date, no single species capable to obtain energy by dechlorination of all CEs has been described (Aulenta et al., 2006). For example, one strain of *Dehalococcoides* is able to metabolically dechlorinate PCE to VC, but dechlorination of VC to ethene occurs by cometabolism, while others use *c*DCE and VC transformation to obtain energy and degrade PCE and TCE only cometabolically. In summary, the "perfect" PCE degrader has not yet been described. Differing dechlorination abilities of individual species of *Dehalococcoides* occur due to the presence of genes for different types of reductive dehalogenases in their genome (Lee et al., 2013; Jugder et al., 2015).

Dehalococcoides have a remarkably small genome (approximately 1.2–1.4 Mb), yet it contains multiple distinct genes predicted to be corrinoid-dependent reductive dehalogenases (*rdhA* genes) accounting for a significant proportion of their genome (Molenda et al., 2020). As such, they lack the ability to synthesize several growth factors (Men et al., 2017). These nutrients must be provided exogenously in cultures or endogenously, from coexisting bacterial populations through a complex network of nutrient exchange (Chen and Wu, 2022). The

importance of syntrophic interaction between variegated bacterial populations has been implied, as *Dehalococcoides* growth and dechlorination rates are higher when grown in mixed cultures containing also fermentative bacteria and sulfate and iron-reducers (Atashgahi et al., 2017). Other species can provide some essential substances that members of *Dehalococcoides* are not able to synthesize, such as the corrinoid cofactors (Yan et al., 2012).

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). To date, three VC-reductases have been described, encoded by the *tceA*, *vcrA* and *bvcA* genes. They are directly involved in the reductive dechlorination of VC to ethene. TceA reductase, present in *Dehalococcoides mccartyi* strains 195 and FL2, mediates the sequential metabolic transformation of TCE to *c*DCE and VC and the subsequent cometabolic reduction of VC to ethene (Magnuson et al., 1998, 2000). *D. mccartyi* VS and GT strains also metabolize TCE but utilize vinyl chloride reductase vcrA (Muller et al., 2004), while strain BAV1 metabolically dechlorinates VC to ethene using the bvcA enzyme (Krajmalnik-Brown et al., 2004). 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

Anaerobic reductive dechlorination represents the most common degradation process for CEs, as they are mostly present in reductive environments. Consequently, only a minority of research studies have been focused on aerobic processes due to their lower relevance for *in situ* remediation. However, the aerobic oxidation of CEs is also possible, either metabolically, or by cometabolism (Schmidt et al., 2014).

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). Methanotrophs oxidize methane as a primary growth substrate, but the broader substrate range of MMOs allows for accidental CE degradation (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). VC-oxidizing methanotrophs and ethenotrophs have been found in anaerobic groundwater samples and can establish themselves at low levels of dissolved oxygen (Gossett, 2010; Richards et al., 2019). As such, the functional genes from ethenotrophs (*etnC* and *etnE*) and methanotrophs (*mmoX* and *pmoA*) are used as biomarkers of aerobic degradation of CEs in groundwater (Wymore et al., 2007; Jin and Mattes, 2010; Czinnerová et al., 2020b; Němeček et al., 2020).

The problem of cometabolic degradation is the possibility that the presence of CEs or toxic degradation products in the environment will lead to inhibition of auxiliary substrate metabolism, and thus to inhibition of the cell growth. Cometabolic degradation is also relatively slow compared to metabolic degradation (Luo et al., 2014).

During metabolic degradation, the CEs are used as electron donors for cell growth. Pure ethenotrophic strains of the genera *Mycobacterium* and *Nocardioides* have been described to degrade VC metabolically as their sole carbon and energy source (Mattes et al., 2010), while *Polaromonas* sp. strain JS666 and *Stenotrophomonas maltophilia* PM102 are the only microorganisms known so far that are capable of metabolically oxidizing *c*DCE and TCE, respectively (Mattes et al., 2008; Jennings et al., 2009; Mukherjee and Roy, 2012). Aerobic metabolic degradation of PCE has not yet been detected due to its high oxidized state.

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).

A comparison of aerobic metabolic and cometabolic degradation of CEs with anaerobic reduction is shown in Fig. 2.

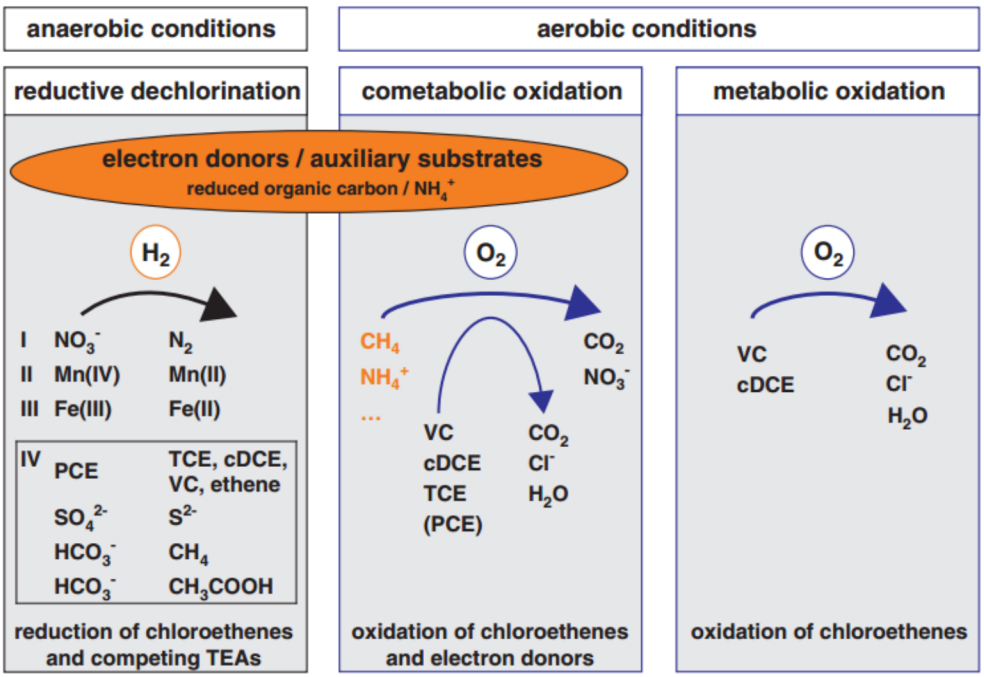

Fig. 2. Comparison of reductive dechlorination with cometabolic and metabolic oxidation in terms of the need for auxiliary substrates as electron donors and competing reactions. TEA: terminal electron acceptor. The numbers indicate the order of the different respiration processes (Tiehm and Schmidt, 2011).

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,

• in situ 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. Substances containing elements in a high oxidation state are used as oxidizing agents. They are unstable under normal conditions and have a high tendency to accept electrons, i.e. oxidize other substances in the environment. On the contrary, reducing agents easily donate electrons, i.e. reduce other substances (Filip et al., 2019).

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. 3. 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 et al., 2010; Lacinová et al., 2013).

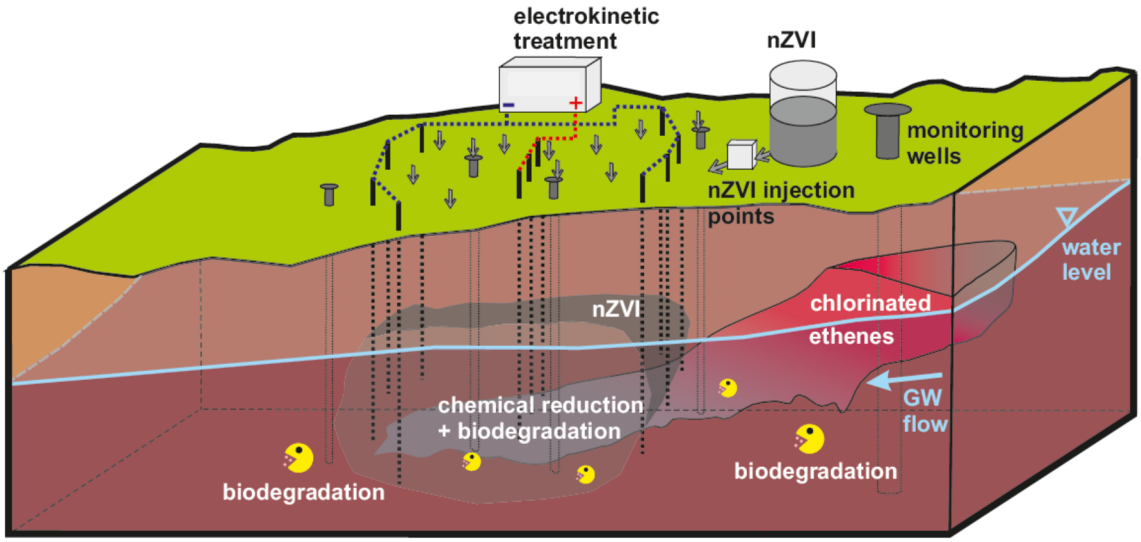

Fig. 3. The schema of remediation of CE-polluted site by application of nZVI combined with electrokinetic treatment, which also supported the biodegradation (Czinnerová et al., 2020b).

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. The use of Fenton's reagent has been tested for the removal of CE contamination in pilot and field-scale experiments. Some applications have been successful, while others observed rebounding of TCE concentrations several months after application (Chen et al., 2001; Chapelle et al., 2005; Hrabák, 2012).

In the Fenton's reagent reaction H_2O_2 and Fe^{2+} react together according to reaction 1 to form hydroxyl radicals (Černík et al., 2010; Siegrist et al., 2011; Filip et al., 2019).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (1)$$

Fe^{2+} is usually applied into the soil in the form of ferrous sulfate heptahydrate. In some localities, Fe^{2+} is naturally present in sufficient concentration in groundwater, and adjusting the pH by citric, hydrochloric, sulfuric or acetic acid is sufficient to trigger the Fenton-like reaction. The hydroxyl radical, which is produced by Fenton's reaction, is then used to cleave the double bond in CEs according to reaction 2.

$$C_2HCl_3 + 3OH^{\bullet} + 3OH^{-} \rightarrow 2CO_2 + 3H^{+} + 3Cl^{-} + 2H_2O \quad (2)$$

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 (reaction 3), 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).

$$2Fe^0 + 4H^+ + O_2 \rightarrow 2Fe^{2+} + 2H_2O \quad (3)$$

Under the anaerobic conditions typical for deeper zones of contaminated aquifers, nZVI reacts with water to form H_2 (reaction 4), 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).

$$2Fe^0 + 2H_2O \rightarrow 2Fe^{2+} + H_2 + 2OH^- \quad (4)$$

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).

During hydrogenolysis, the chlorine atoms in the CE are gradually replaced by hydrogen atoms. Thus, the more chlorinated ethenes PCE and TCE are sequentially dechlorinated to DCE, VC, and ethene or ethane as the final products (reaction 5, R represents a hydrocarbon residue and X represents halogen).

$$RX + H^+ + 2e^- \rightarrow RH + X^- \quad (5)$$

During β-elimination, two chlorine atoms are removed at the same time producing acetylene, which is rapidly converted to ethene or ethane (Černík et al., 2010; Bruton et al., 2015).

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). Three different mechanisms positively affect the transport of nZVI particles, electroosmosis, electromigration, and electrophoresis (Yeung, 1994; Hassan et al., 2018); however, in an environment where advective flow dominates, their contribution may be negligible (Černík et al., 2019). Moreover, 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). Electrokinetically-enhanced bioremediation has been extensively studied (Beretta et al., 2019) and applied in order to remove pollutants from soil and groundwater (She et al., 2006; Mousavi et al., 2011; Zhan et al., 2012; Zhang et al., 2013; Mena Ramírez et al., 2014; Zhang et al., 2014).

Although the use of nZVI for decontamination of CEs seems to be environmentally friendly, as it reacts very quickly to naturally occurring Fe^{2+} and even creates conditions suitable for microbial anaerobic dechlorination by OHRB, the impact of nZVI on naturally occurring bacterial populations needs to be described in detail and possible negative impact precluded (Coutris et al., 2020).

4.2.3 Application of an auxiliary substrate to enhance reductive dechlorination

As mentioned above, 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 stored in the form of ATP (adenosine triphosphate) or NAD (nicotinamide adenine dinucleotide) and 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 (ESTCP, 2004; Scheutz et al., 2010; Němeček et al., 2015; Dolinová et al., 2016; Atashgahi et al., 2017; Semerád et al., 2021). Even though H_2 is produced by fermentation of all of these substrates, the rate and extent of H_2 formation differ significantly and impact also the rate of dechlorination (ESTCP, 2004). OHRB compete for H_2 with methanogenic bacteria that also use H_2 as an electron donor, however, it has been shown that the minimum H_2 concentration for dechlorinating bacteria is ten times lower than for methanogens (Ballapragada et al., 1997). As such, the addition of slowly fermentable substrate such as butyrate or propionate, which release H_2 in low concentrations, should provide most of the produced H_2 for dechlorination reactions (Smatlak et al., 1996).

An important property of the applied substrate is its easy fermentability. Lactate fermentation produces acetate, carbon dioxide, and H_2 (reaction 6).

$$C_3H_5O_3^- + H_2O \rightarrow C_2H_3O_2^- + CO_2 + 2H_2 \quad (6)$$

H_2 releases the electron according to reaction 7.

$$H_2 \rightarrow 2H^+ + 2e^- \quad (7)$$

The electrons released from H_2 then chemically reduce CEs in groundwater (reaction 8) (Černík et al., 2010).

$$C_2Cl_4 + 2H^+ + 2e^- \rightarrow C_2HCl_3 + H^+ + Cl^- \quad (8)$$

As already mentioned, in the microbial reductive dechlorination process PCE is gradually dechlorinated via TCE, *c*DCE, and VC to ethene, with a lowering redox potential 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).

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. Fluorescent label (fluorescent probe or dye, for example, SYBR™ Green) marks the PCR product during thermal cycling and is detected by a CCD camera. The dye binds only to double-stranded DNA and emits fluorescence only when bound (Dorak, 2007).

The intensity of the fluorescent signal reflects the momentary amount of DNA amplicons in the sample at that specific time. In the beginning, the fluorescent signal is too weak to be distinguishable from the background. 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; Fig. 4) 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.

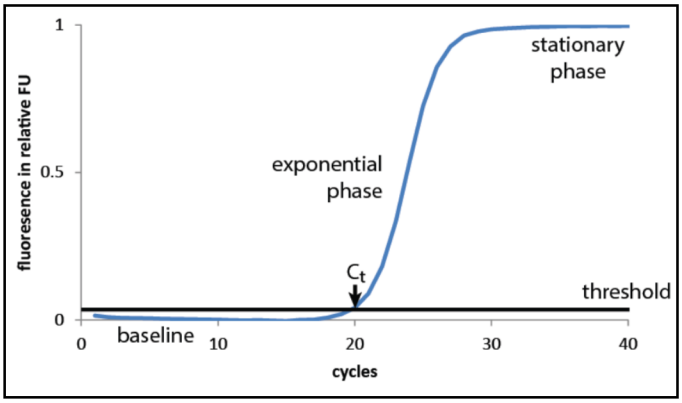

Fig. 4. Principle of qPCR. FU stands for fluorescence unit (PCR Compendium, 2016).

The PCR is an exponential process where the number of DNA molecules theoretically doubles after each cycle (if the efficiency of the reaction is 100%). Therefore, the lower the

Ct value, the more template was in the qPCR reaction, i.e. the higher the abundance of the selected marker gene in a sample.

For example, when performing a 1:10 dilution series of a template DNA, the following qPCR results would be expected:

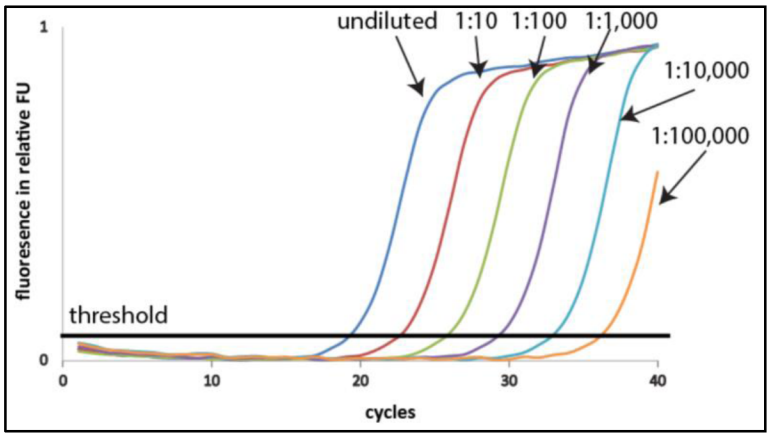

Fig. 5. qPCR graph of 1:10 dilution series of a template DNA. FU stands for fluorescence unit (PCR Compendium, 2016).

4.3.2 Next-generation sequencing

Next-generation sequencing (NGS, or high-throughput sequencing) is a rapidly developing molecular biology method, which replaced the more expensive and less effective Sanger sequencing. NGS offers the capability to produce a vast volume of data in a single run at a very low cost and in a short time. NGS 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.

In this study, the NGS was performed on an Ion Torrent PGM (Thermo Fisher Scientific, USA). The methodology for the whole genome sequencing is similar to other NGS technologies and involves library preparation (random fragmentation and ligation of adapters) followed by hybridization to the complementary sequences (primers) bounded with the beads followed by emulsion PCR. After clonal amplification, each bead is placed in the individual well on the semiconductor chip and is sequentially flowed by

nucleotides. A hydrogen proton (H^+) is released on the incorporation of each nucleotide by DNA polymerase, which results in a change of the pH, that is detected. This change in pH determines the base sequence (Gupta a Verma, 2019). The principle of the sequencing reaction is shown in Fig. 6.

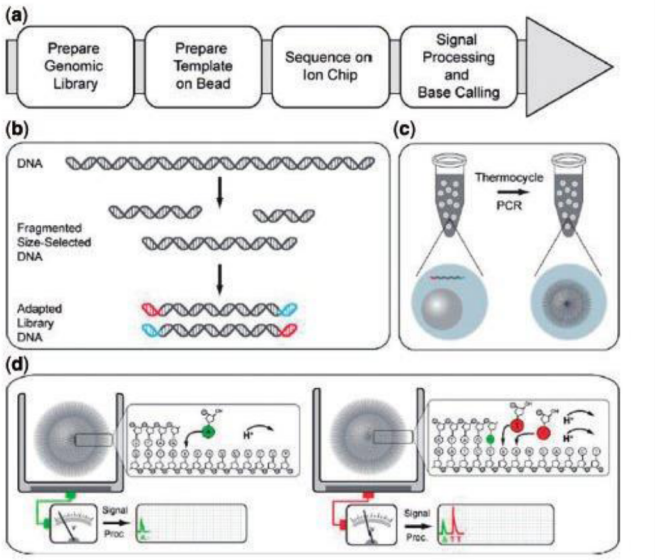

Fig. 6. Whole-genome sequencing workflow on Ion Torrent platform. (a) The overall workflow. (b) A genome library preparation. (c) Clonal amplification. (d) Sequencing on the chip. Modified from (Golan and Medvedev, 2013).

In addition to whole-genome sequencing, the other 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). The 16S rRNA is the RNA component of the 30S subunit of a prokaryotic ribosome. It contains nine hypervariable regions (V1–V9) that can be used for the identification of bacteria species (Yang et al., 2016).

NGS data can be displayed using different graphical outputs: heat maps, OTU graphs, Venn diagrams, Alpha or Beta diversity graphs, or bubble charts, an example of which is provided in Fig. 7.

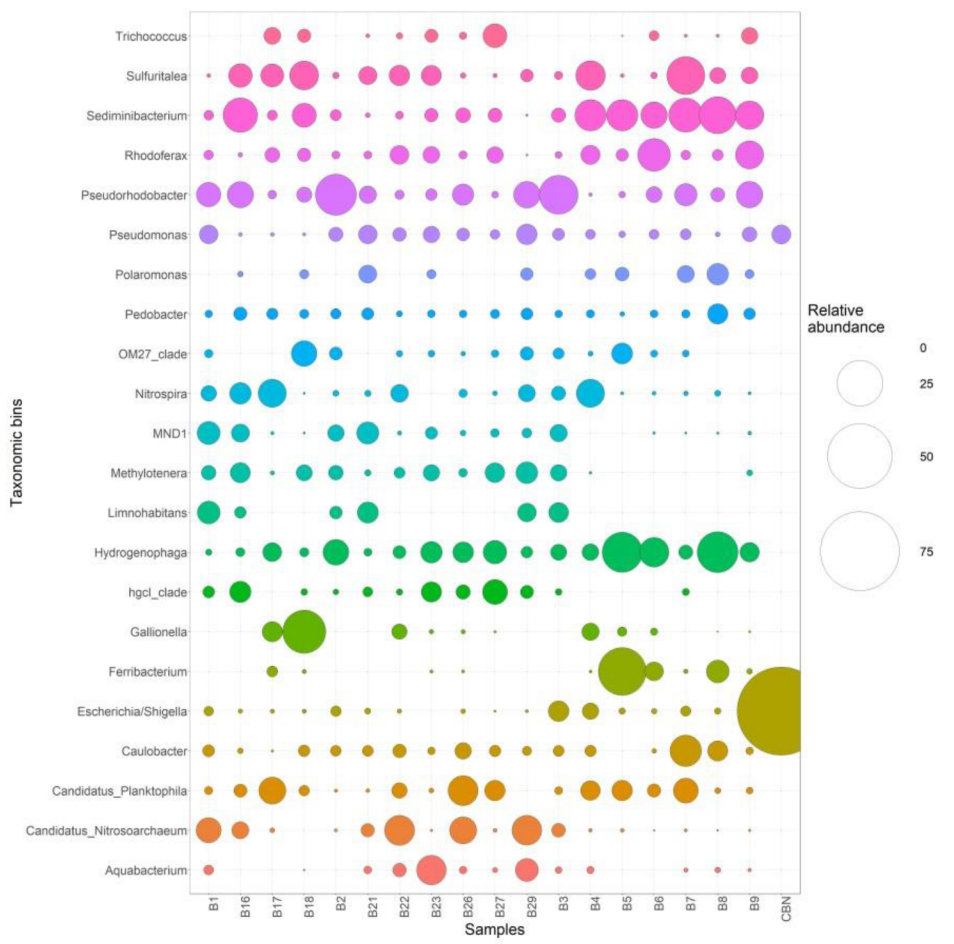

Fig. 7. Bubble chart showing individual microbial genera and their percentage representation in each sample (Czinnerová, unpublished data).

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. A combined approach based on molecular genetic assays along with the detection of chemical parameters was used in this study.

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. 8). 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.

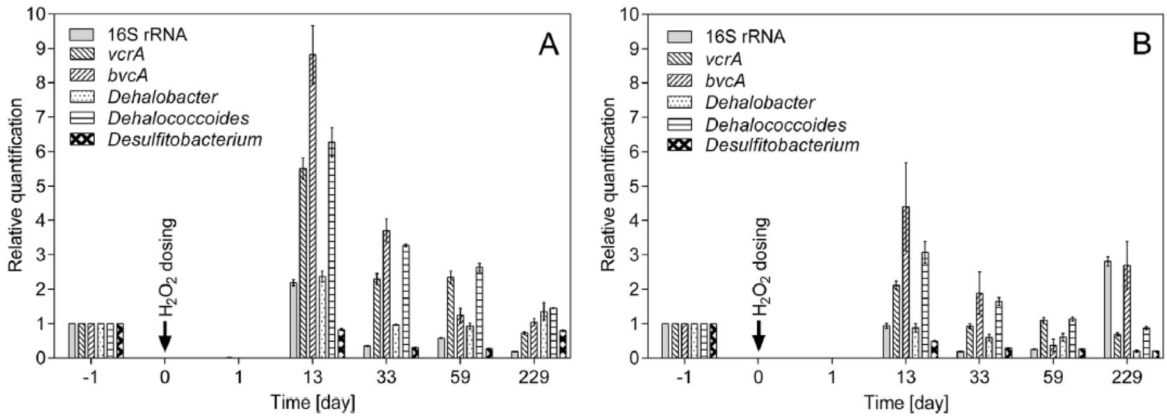

Fig. 8. Changes in the relative abundance of total bacteria (16S rRNA), OHRB (Dehalobacter, *Dehalococcoides,* and *Desulfitobacterium*), and VC reductase genes (*vcrA* and *bvcA*) following H_2O_2 application in well RW5-11: A) depth 8 m, B) depth 13 m. No molecular genetic markers were detected one 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* spp., *Desulfitobacterium* spp., 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. 9) 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 (Fig. 10). 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.

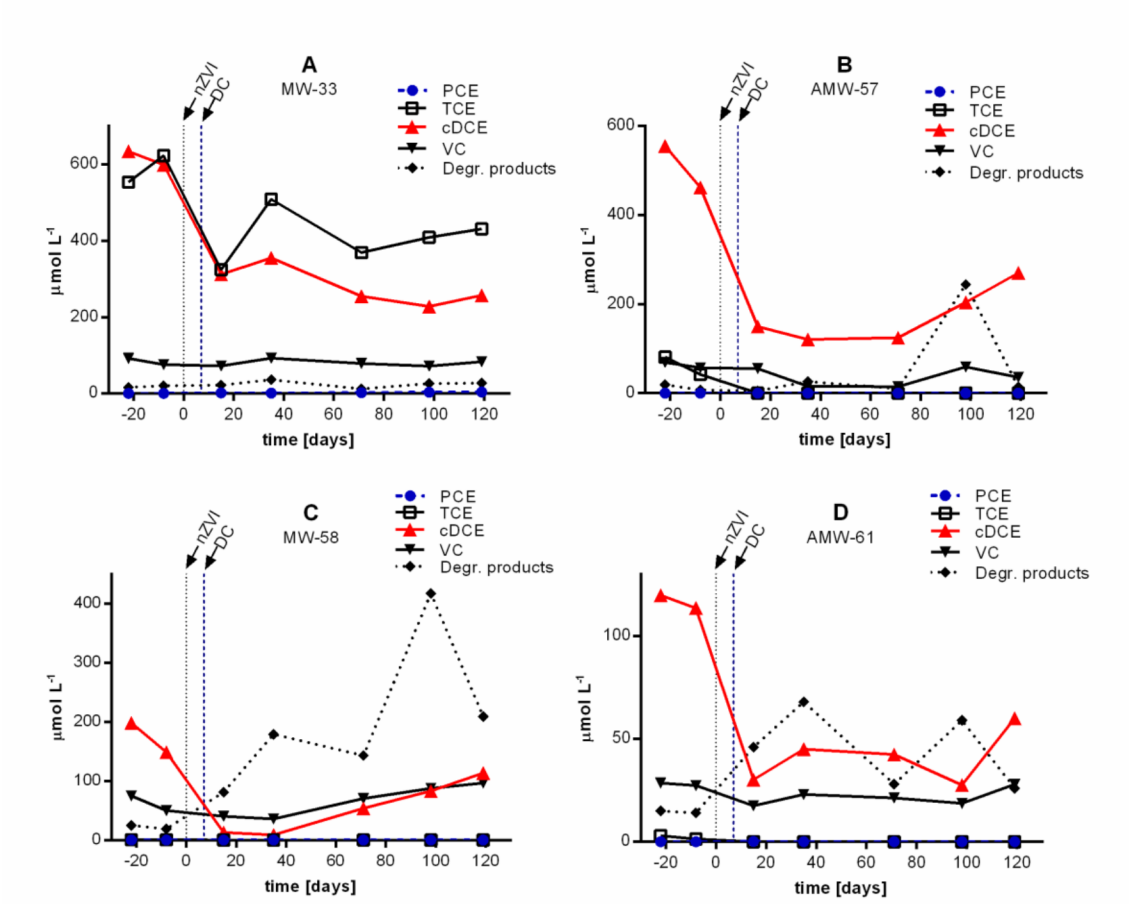

Fig. 9. Changes in the concentration of CEs and their final degradation products (the sum of methane, ethene, and ethane) following nZVI injection and direct current (DC) treatment. (A) reference well MW-33; (B) application well close to a cathode AMW-57; (C) monitoring well close to a cathode MW-58; (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.

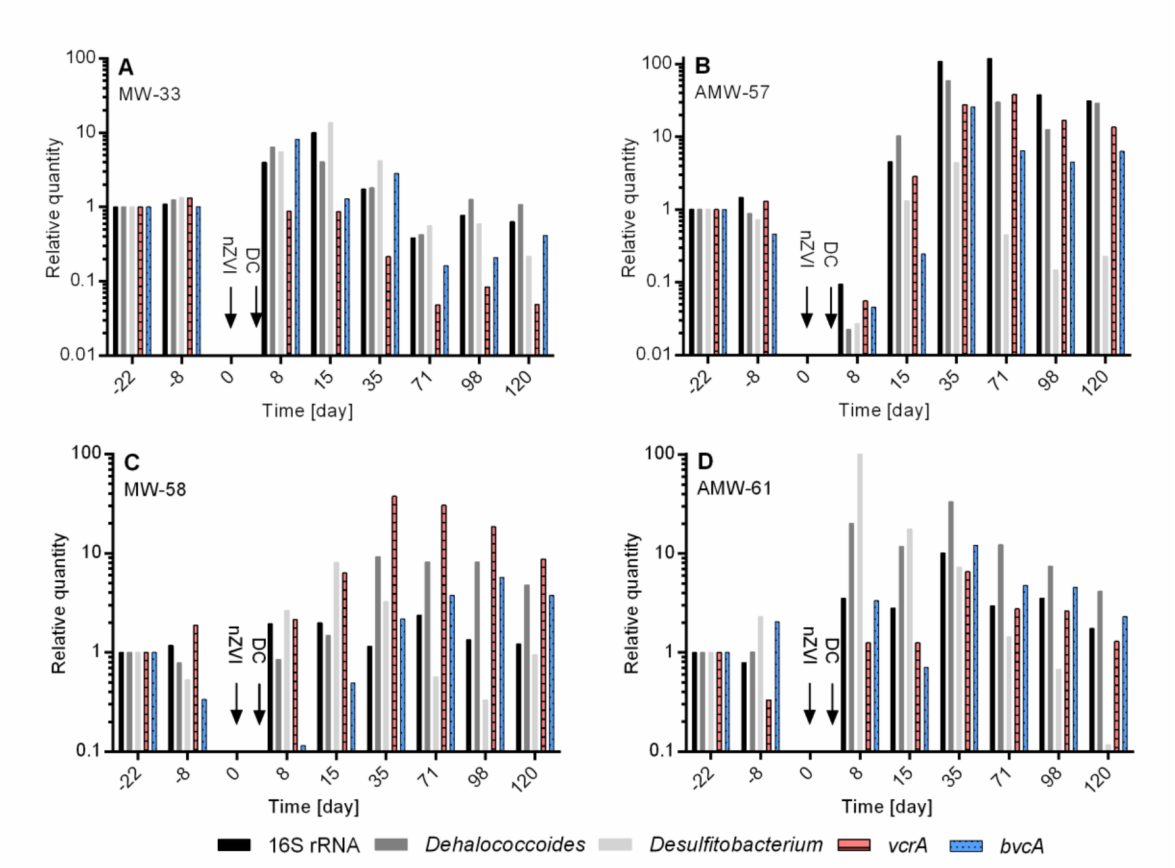

Fig. 10. Changes in the relative abundance of total bacteria (16S rRNA), OHRB (*Dehalococcoides* and *Desulfitobacterium*), and VC reductase genes (*vcrA* and *bvcA*) following nZVI injection and DC treatment. (A) reference well MW-33; (B) application well close to a cathode AMW-57; (C) monitoring well close to a cathode MW-58; (D) application well close to an anode AMW-61. All results are expressed as relative quantity to each marker abundance prior to application. Note the logarithmic scale.

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.

The main findings are as follows:

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. 11). 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*.

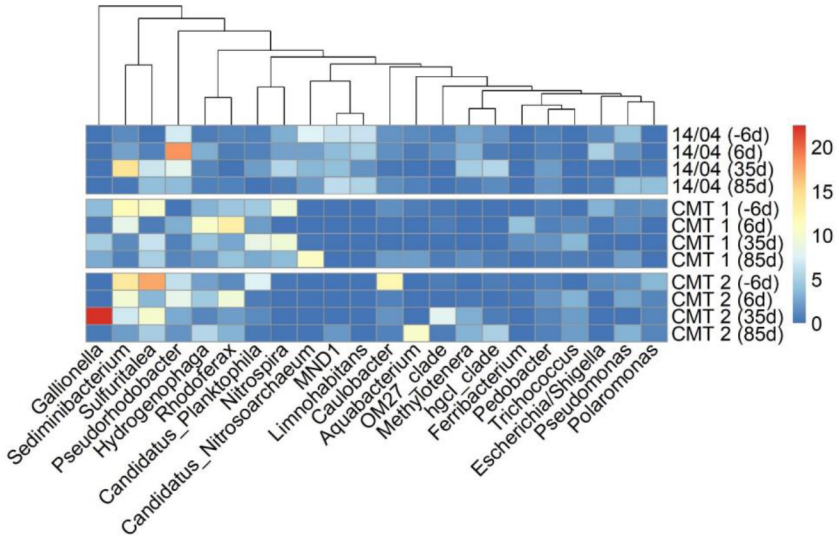

Fig. 11. Bacterial community composition in CMT 1, CMT 2 (application wells), and 14/04 (reference well) before (− 6 days) and after (6 days, 35 days, and 85 days) nZVI–AC injection. Only genera with a relative abundance > 1% are shown.

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 long-term in-situ application of glycerol conducted to enhance reductive dechlorination of CEs on a highly contaminated site. The physico-chemical parameters of groundwater together with changes in microbial community structure were analyzed prior to and after the glycerol injection over a period of seven months.

Glycerol injection resulted in an almost immediate increase in the abundance of fermentative Firmicutes (*Clostridium*, *Trichococcus,* and *Zymophilus;* Fig. 12) 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. 13).

Consistent with reduced redox potential sulfate-reducing genera *Desulfobacter*, *Desulfobulbus,* and *Desulfovibrio*, and iron-reducing *Geobacter* 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.

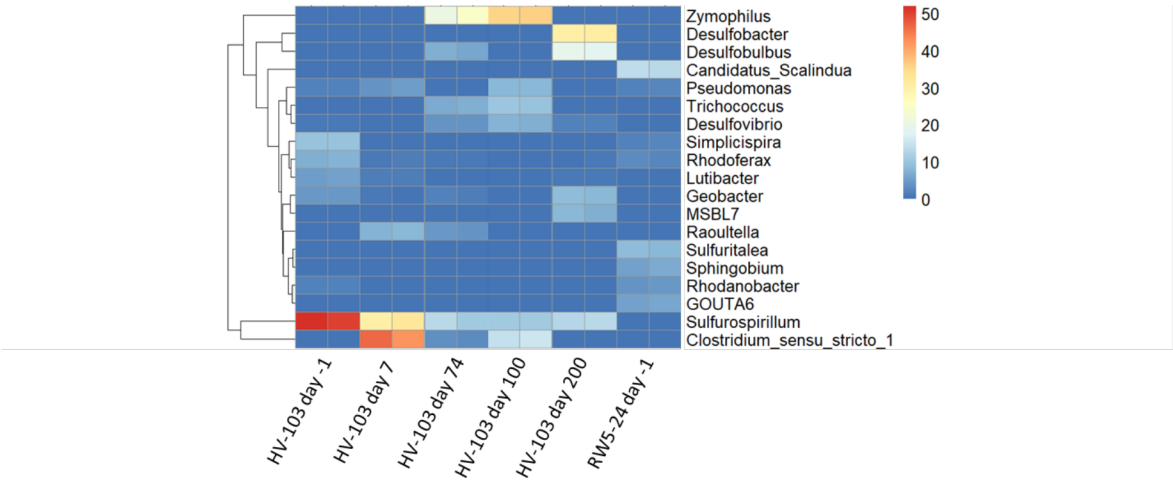

Fig. 12. Bacterial community composition in application well HV-103 and reference well RW5-24 before (−1 day) and after (7 days, 74 days,100 days, and 200 days) glycerol injection. The scale expresses the percentage of each bacterial genus in a sample. Only genera with a relative abundance > 5% are shown.

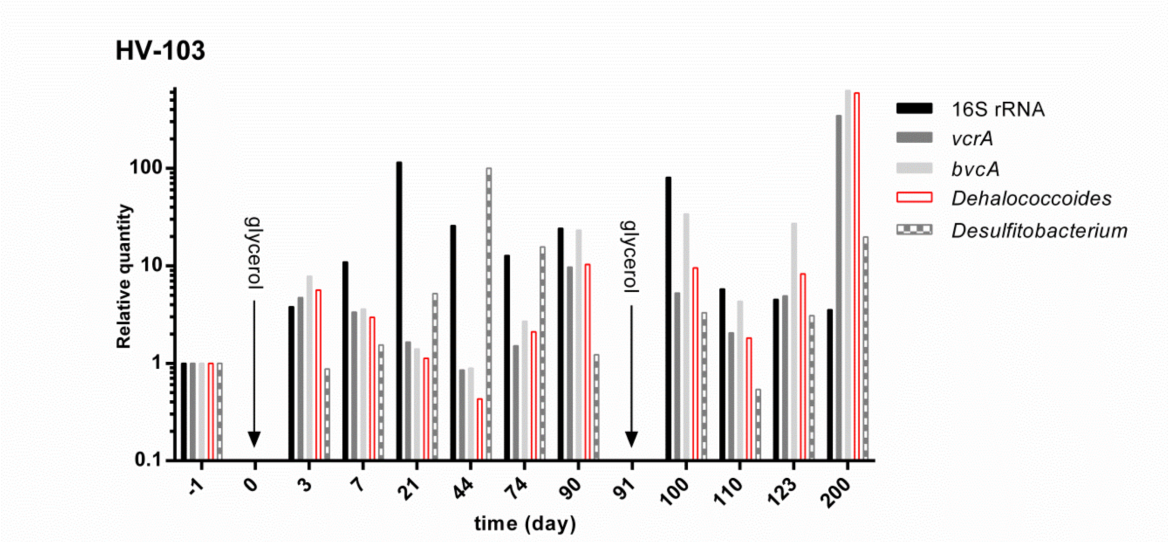

Fig. 13. Changes in the relative abundance of total bacteria (16S rRNA), OHRB (*Dehalococcoides* and *Desulfitobacterium*), and VC reductase genes (*vcrA* and *bvcA*) in the application well HV-103. All results are expressed as relative quantity to each marker abundance prior to glycerol application (-1 day). Note the logarithmic scale.

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. 14). 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%.

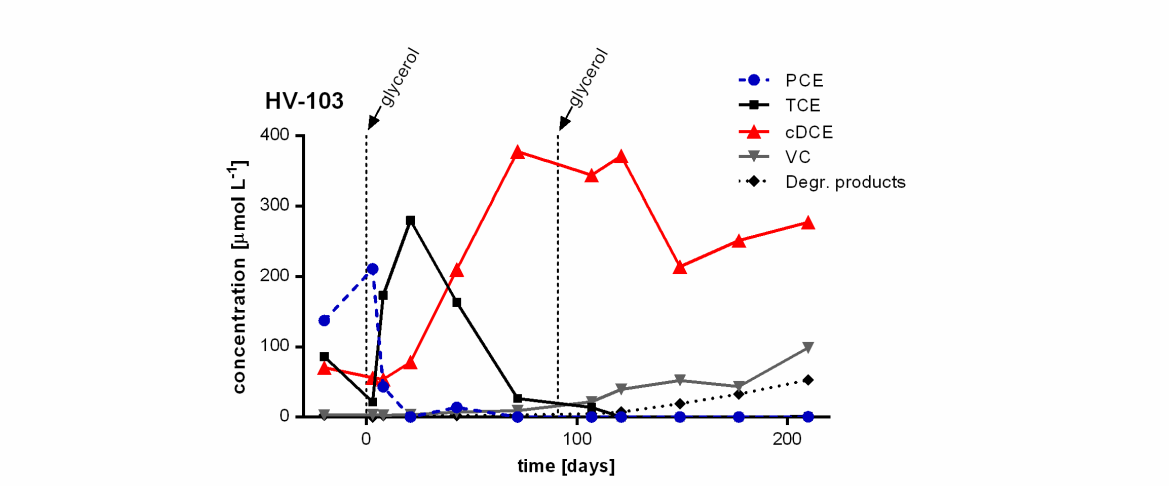

Fig. 14. Changes in the concentration of CEs and their final degradation products (the sum of ethene and ethane) following glycerol application in HV-103 well.

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.

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

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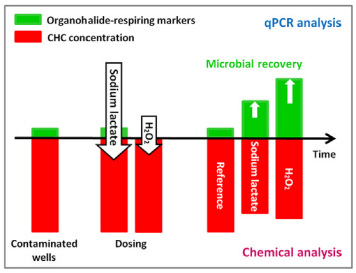
HIGHLIGHTS

- Effect of hydrogen peroxide (Fenton-like reaction) and lactate was evaluated.
- Changes in specific microflora after chemical oxidation/biostimulation documented.
- Stimulation of indigenous organohalide-respiring bacteria with lactate observed.
- Microbial recovery after the Fenton-like reaction was unexpectedly fast.
- Chemical analysis corresponded with the results of molecular genetic analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Application of Fenton's reagent and enhanced reductive dechlorination are currently the most common remediation strategies resulting in removal of chlorinated ethenes. In this study, the influence of such techniques on organohalide-respiring bacteria was assessed at a site contaminated by chlorinated ethenes using a wide spectrum of molecular genetic markers, including 16S rRNA gene of the organohalide-respiring bacteria *Dehaloccocoides* spp., *Desulfitobacterium* and *Dehalobacter*; reductive dehalogenase genes (*vcrA*, *bvcA*) responsible for dechlorination of vinyl chloride and sulphate-reducing and denitrifying bacteria.

In-situ application of hydrogen peroxide to induce a Fenton-like reaction caused an instantaneous decline in all markers below detection limit. Two weeks after application, the *bvcA* gene and *Desulfitobacterium* relative abundance increased to levels significantly higher than those prior to application. No significant decrease in the concentration of a range of chlorinated ethenes was observed due to the low hydrogen peroxide 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* spp. after 70 days. An increase in the *vcrA* gene corresponded with increase in *Dehaloccocoides* spp. Analysis of selected markers clearly revealed a positive response of

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organohalide-respiring bacteria to biostimulation and unexpectedly fast recovery after the Fenton-like reaction.

1. Introduction

Chlorinated ethenes are widely occurring contaminants that have been used in a range of industrial applications (Paul and Smolders, 2014). They represent one of the most environmentally persistent pollutants due to their strong carbon-chlorine bonds (Adetutu et al., 2015).

The most commonly used remedial activity for chlorinated ethenes in aquifers is monitored natural biodegradation; however, this has the disadvantage of slow reaction kinetics and possible accumulation of more hazardous by-products, such as *cis*-1,2-dichloroethene (cDCE) and vinyl chloride (VC) (Amaral et al., 2011; Nijenhuis et al., 2007). Nevertheless, the natural biological processes can be stimulated by addition of an organic substrate (carbon and electron source) and nutrients.

Microbial degradation of chlorinated ethenes takes place *via* anaerobic organohalide respiration. Chloroethene-contaminated aquifers may contain a range of coexisting bacteria with differing dechlorination activity, their occurrence and activity depending upon local biogeochemical conditions such as oxidation-reduction potential (ORP), pH and pollutant distribution (Imfeld et al., 2011; Paul and Smolders, 2014). Organohalide respiration is also influenced by competition for hydrogen by different bacteria, i.e. dechlorinating, fermentative, methanogenic and iron and sulphate-reducing bacteria (Aulenta et al., 2007; Chambon et al., 2013). Depending on the environmental conditions, organohalide respiration may lead to cDCE or VC accumulation (Conrad et al., 2010; Mattes et al., 2010; Taş et al., 2010). Accumulation of cDCE or VC occurs because the ORP needed for biodegradation of less-chlorinated ethenes is higher than required (Kansas et al., 1998). As both cDCE and VC are more toxic and carcinogenic than the original contaminants, treatment processes ensuring complete degradation of chlorinated ethenes are required.

While a number of bacteria are capable of incomplete degradation of tetrachloroethene (also known as perchloroethylene; PCE) and trichloroethene (TCE) (producing cDCE or VC as by-products), only *Dehalococcoides* spp. (e.g. *Dehalococcoides mccartyi*, phylum *Chloroflexi*) are known to fully degrade PCE to ethene (Chambon et al., 2013; Löffler et al., 2013; West et al., 2013). *Dehalococcoides* spp. are present at many chlorinated ethene contaminated sites (Kranzioch et al., 2013; Rossi et al., 2012) and there is a recognised correlation between *Dehalococcoides* spp. presence and formation of ethene (Rossi et al., 2012). Correspondingly, absence of *Dehalococcoides* spp. is frequently related to accumulation of cDCE and VC (Hendrickson et al., 2002; Kranzioch et al., 2013). Presence or absence of *Dehalococcoides* spp., therefore, may serve as an indicator of biodegradation potential as regards total removal of chlorinated ethenes under anaerobic conditions (Cupples, 2008; Kaster et al., 2014). The dechlorinating activities of *Dehalococcoides* spp. are known to be connected with the presence of reductive dehalogenases *rdhA*, which serves as a catalyst for cleaving the carbon-chlorine bond during organohalide respiration (Badin et al., 2014; West et al., 2013). While complete dechlorination of chlorinated ethenes is known to depend on the presence of one or more *rdhA* enzymes (*pceA, tceA, vcrA* or *bvcA*; Tang et al., 2013), the relationship between enzyme activity and expression is not as yet fully understood.

In addition to biological methods, a number of chemical techniques are also available for *in-situ* remediation, with chemical oxidation, e.g. using Fenton's reagent (a 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) being one of the most frequently applied methods for chlorinated ethenes. However, chemical oxidation can significantly restrict the activity of organohalide-respiring bacteria and cause changes in microbial population structure. Chemical oxidation can also oxidise organic matter in preference to chlorinated ethenes, resulting in a lack of carbon for autochthonous microorganisms (Chapelle et al., 2005). While changes to the microbial populations are a side-effect of chemical oxidation, application of a substrate (electron donor), such as lactate, directly improves conditions for biological organohalide respiration. This can be employed either alone or during the remediation of residual contamination, often present following the application of other techniques such as the Fenton reaction. Although biological methods exhibit slower reaction kinetics, they have the advantage of being less expensive than chemical methods and being more environmentally friendly. Despite this, many of the processes involved in biological methods are still not fully understood (Kang, 2014; Maphosa et al., 2010).

Previous studies involving Fenton-like reactions or application of sodium lactate ($NaC_3H_5O_3$) have either focused purely on chemical parameters or on total organohalide-respiring microbial colonisation (Chapelle et al., 2005; Mattes et al., 2010; Sutton et al., 2010). Though a number of studies have also conducted molecular genetics analysis, these have usually been undertaken at a laboratory scale only or following bioaugmentation with organohalide-respiring bacteria (Behrens et al., 2008; Damgaard et al., 2013a; Grostern and Edwards, 2006; Kranzioch et al., 2013; Scheutz et al., 2008).

The aim of this study was to examine the influence of a Fenton-like reaction and biostimulation through sodium lactate application on dynamics of organohalide-respiring bacteria and their genes at actual site contaminated with chlorinated ethenes. To obtain more information for the accurate diagnosis of ongoing organohalide respiration, new approach combining analysis of molecular genetic markers, together with physical and chemical parameters, was used.

2. Materials and methods

2.1. Study site

The Spolchemie Company a.s. (Ústí nad Labem, Czech Republic) has been one of the leading synthetic resins and freons manufacturers in Europe since the middle of the last century. Production of freons along with storage and distribution of raw materials (tetrachlormethane and tetrachlorethene), however, have led to extensive subsurface contamination with organic solvents, including chlorinated ethenes. The geological profile underlying the Spolchemie site consists of Mesozoic Cretaceous siltstones overlaid by a Quaternary terrace comprised mainly of fluvial sediments from the Rivers Bílina and Labe and the Klíšský stream. It is this subsurface Quaternary terrace that has been contaminated.

The Spolchemie site is currently undergoing remediation

treatment using a range of different approaches. In addition, newer methods such as application of nanoscale zero-valent iron and the Fenton reaction are also being tested at the site.

2.2. Application and reference wells

Samples were obtained from four wells at the Spolchemie site. Characteristics of sampling wells, including reagent and its dose applied, are displayed in Table 1.

Two wells located close to the source area of contamination (RW5-11 and RW5-12; Fig. 1A) were chosen to study the disposable effect of hydrogen peroxide application, used for the Fenton-like reaction, on organohalide-respiring bacteria. These wells are both situated along the outflow line from the former chlorinated hydrocarbons (CHC) production area and are predominantly contaminated with chlorinated ethenes and methanes, with methanes dominant at around 50–90%. No effect of *in-situ* chemical oxidation originating from neighbouring wells was observed on the microbial consortium prior to this study. No reference well was used for comparison with those dosed with hydrogen peroxide, the conditions prior to application being taken as a reference point.

Well RW5-49 was used to study biostimulation through the application of sodium lactate and well RW5-24 was chosen as a control (no reagents applied) as it had not been influenced by any previous remedial action conducted at either the source area or the area of plume treatment (Fig. 1A).

Both wells RW5-49 and RW5-24 are situated along the marginal edge of the contamination plume zone (Fig. 1A) and are predominantly contaminated with PCE. Furthermore, well RW5-24 is situated on the inlet side and data from previous monitoring (2 years prior to this study) confirmed its long-term stability.

As ferrous iron (Fe^{2+}) was present in the groundwater at a sufficient high concentration (reaching on average 51.7 mg L^{-1}) for a Fenton-like reaction at the contaminated wells (RW5-11 and RW5-12), no further iron was deemed necessary for the reaction to take place. Instead, reaction mixtures were prepared using hydrogen peroxide (3 wt%, Eurosarm, Czech Republic), together with citric acid ($C_6H_8O_7$; 3 g L^{-1}; Eurosarm, Czech Republic) for ferrous ion stabilisation. A total of 100 L of hydrogen peroxide solution was injected in one dose into well RW5-11, and 500 L of hydrogen peroxide in well RW5-12. Low volumes of hydrogen peroxide (common amounts at this site range from 3000 to 5000 L per well applied in several doses) do not result in any release of waste gases; therefore, no venting installation was needed.

A single 20 m^3 dose of sodium lactate (Galactic, Belgium), at a concentration of 12 g L^{-1}, was applied to well RW5-49 within 4 days. Application of sodium lactate was simple infiltration, it means without any overpressure applied during its dosing.

2.3. Physical and chemical analysis

All water samples were taken dynamically using a Gigant pump (Eijkelkamp, Netherlands). ORP was measured immediately after sampling using a WTW 3430 Multimeter (WTW, Germany) and recalculated against a standard H-electrode. The ORP depth profile was measured directly in the well using an Ekotechnika Ecoprobe 5 (Ekotechnika, Czech Republic).

Chlorinated hydrocarbons were determined according to EPA Method 8260 B. Concentration of ethene and ethane was analysed in accordance with the methodology described by Lewin et al. (1990). The degree of dechlorination (in %) was calculated according to the equation:

$$\textit{Degree of dechlorination} = \frac{[TCE] + 2\cdot[DCE] + 3\cdot[VC] + 4\cdot[ethene] + 4\cdot[ethane]}{4\cdot([PCE] + [TCE] + [DCE] + [VC] + [ethene] + [ethane])}\cdot 100 \ [\%]$$

where [*contaminant*] represents the concentration of the relevant contaminant in mmol L^{-1}.

Concentrations of nitrates, sulphates and ferrous iron were analysed according to Standard methods (APHA, 2012), while the chemical oxygen demand (COD) was measured using cuvette tests from Hach-Lange (Germany).

2.4. Molecular genetic analysis

All water samples used for molecular genetic analysis were immediately cooled and stored in the dark at 4 °C until further use. Before analysis, samples (0.2–0.5 L) were concentrated by filtration through a 0.22 μm membrane filter (Merck Millipore, Germany). DNA was extracted from the filter using a FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA) according to the manufacturer's protocol. The Bead Blaster 24 homogenisation unit (Benchmark Scientific, NJ, USA) was employed for cell lysis. Extracted DNA was quantified using a Qubit 2.0 fluorometer (Life Technologies, MA, USA).

Table 1
Characteristics of the sampling wells. Also shown are the reagents and doses applied and the concentration of chlorinated hydrocarbons (CHC) prior to the experiment; bgl = below ground level.

Well	Depth [m bgl]	Ground water level [m bgl]	Drilling diameter [mm]	Diameter [mm]	Volume of water [L]	Perforated casing [m bgl]	Backfill material	Reagent and dose	CHC prior dosing [mg L^{-1}]
RW5-11	15.0	3.3	220	180	280	4.0–14.0	4–8 mm gravel	3 wt% H_2O_2; 100 L	1.93[a]
RW5-12	15.0	3.9	220	180	280	4.0–14.0	4–8 mm gravel	3 wt% H_2O_2; 500 L	49.9[a]
RW5-49	15.5	3.3	220	180	220	4.0–14.5	4–8 mm gravel	sodium lactate (12 g L^{-1}); 20 m^3	0.15[b]
RW5-24	11.5	3.4	300	160	290	2.0–10.1	4–8 mm gravel	none (reference well)	0.62[b]

[a] 25 days prior to dosing.
[b] 35 days prior to infiltration.

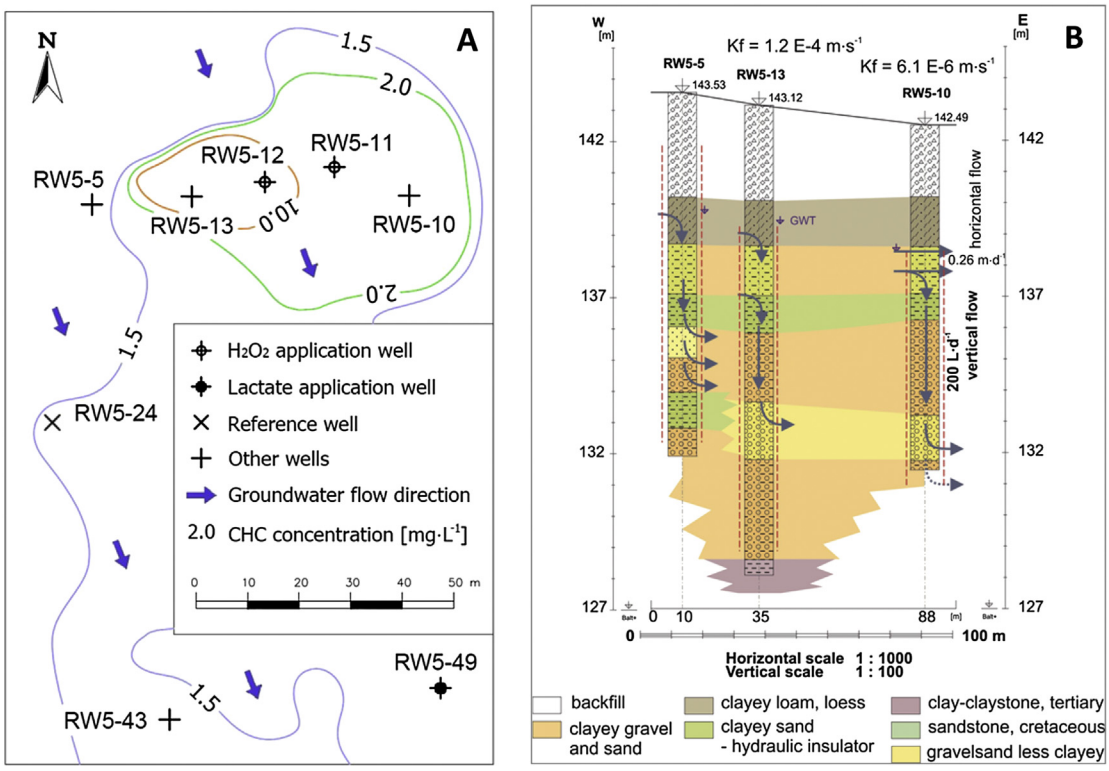

Fig. 1. A) Location of sampling and reference wells, including wells (RW5-5, RW5-10 and RW5-13) illustrating cross-section at the Spolchemie site (Ústí nad Labem, Czech Republic); and, B) geological cross-section of the site showing the open casings of the wells and direction of water flow (blue arrows).

Quantitative polymerase chain reactions (qPCR) were performed to assess the relative abundance of *Dehalobacter, Dehalococcoides* spp. and *Desulfitobacterium*, as well as the relative abundance of the VC reductase genes *vcrA* and *bvcA*. In addition, qPCR was also employed to monitor the presence of sulphate-reducing bacteria (by detecting levels of genes for the adenosine-5'-phosphosulphate reductase *apsA* and dissimilatory sulphate reductase *dsrA* enzymes) and denitrifying bacteria (by detecting levels of genes for the nitrite reductase enzymes *nirK* and *nirS*). Relative abundance of 16S rRNA gene (total bacteria marker) was determined as a control marker. All primers used for qPCR are listed in Table 2.

Reaction mixtures for qPCR were prepared as follows: per 10 μL reaction volume, 5 μL LightCycler® 480 SYBR Green I Master (Roche, Switzerland), 0.4 μL of 20 μM forward and reverse primer mixture (Generi Biotech, Czech Rep., IDT, US) with the addition of 3.6 μL ultra-pure water (Bioline, Great Britain). Each DNA template was diluted five-times with ultra-pure water. A 1 μL of this diluted DNA was then used for the qPCR reaction. Each sample was analysed in duplicate with ultra-pure water used as a template in a negative control.

All qPCR reactions were performed on a LightCycler® 480 instrument (Roche, Switzerland) with reaction conditions set as follows: 5 min at 95 °C initial denaturation, followed by 45 cycles of

Table 2
Specific primers used for qPCR.

Name	Sequence (5′ → 3′)	Product size (bp)	Target organism(s); gene(s)	Reference
U16SRT-F	ACTCCTACGGGAGGCAGCAGT	180	*Bacteria*; 16S rRNA genes	Clifford et al., 2012
U16SRT-R	TATTACCGCGGCTGCTGGC			
vcrA880F	CCCTCCAGATGCTCCCTTTA	139	*Dehalococcoides* sp. strain VS; *vcrA*	Behrens et al., 2008
vcrA1018R	ATCCCCTCTCCCGTGTAACC			
bvcA277F	TGGGGACCTGTACCTGAAAA	247	*Dehalococcoides* sp. strain BAV-1; *bvcA*	Behrens et al., 2008
bvcA523R	CAAGACGCATTGTGGACATC			
Dre441F	GTTAGGGAAGAACGGCATCTGT	205	*Dehalobacter* sp.; 16S rRNA genes	Smits et al., 2004
Dre645R	CCTCTCCTGTCCTCAAGCCATA			
DHC793f	GGGAGTATCGACCCTCTCTG	191	*Dehalococcoides* sp.;16S rRNA genes	Yoshida et al., 2005
DHC946r	CGTTYCCCTTTCRGTTCACT			
Dsb406F	GTACGACGAAGGCCTTCGGGT	213	*Desulfitobacterium* sp.; 16S rRNA genes	Smits et al., 2004
Dsb619R	CCCAGGGTTGAGCCCTAGGT			
RH1-dsr-F	GCCGTTACTGTGACCAGCC	164	*dsrA* (dissimilatory sulphate reductase)	Ben-Dov et al., 2007
RH3-dsr-R	GGTGGAGCCGTGCATGTT			
RH1-aps-F	CGCGAAGACCTKATCTTCGAC	191	*apsA* (adenosine-5'-phosphosulfate reductase)	Ben-Dov et al., 2007
RH2-aps-R	ATCATGATCTGCCAGCGGCCGGA			
Cd3aF	GTSAACGTSAAGGARACSGG	425	*nirS* (Nitrite reductase)	Michotey et al., 2000; Throbäck et al., 2004
R3cd	GASTTCGGRTGSGTCTTGA			
nirK876F	ATYGGCGGVCAYGGCGA	164	*nirK* (Nitrite reductase)	Henry et al., 2004
nirK1040R	GCCTCGATCAGRTTRTGGTT			

10 s at 95 °C, 15 s at 60 °C and 20 s at 72 °C. Finally, a melting curve ranging from 60 to 98 °C was assessed with a temperature gradient of 0.6 °C per 10 s. Purity of the amplified fragment was determined through observation of a single melting peak. Crossing point (Cp) values were obtained using the Second Derivative Maximum method available in LightCycler® 480 Software. The amplification efficiency of each primer set was determined by measuring standard curves using serial dilution of template DNA from five different environmental samples. Relative quantification of each parameter was expressed as a fold change between two states (a given sampling time and a sampling time of zero) using the delta Cp method. The results of qPCR were evaluated as relative quantification, with the condition of the organohalide-respiring bacteria prior to the application taken as the starting point.

3. Results and discussion

3.1. Application of hydrogen peroxide for the Fenton-like reaction

a) ORP measurement

Prior to the addition of hydrogen peroxide, ORP showed a relatively constant decrease with depth in well RW5-11 (Fig. 2). In well RW5-12, however, ORP only showed a slow decrease (ΔORP 20 mV over 7 m) down to a depth of 11 m below ground level (bgl), whereupon it decreased rapidly to −25 mV (ΔORP 250 mV over 3 m) at a depth of 14 m bgl (Fig. 2). The ORP profiles indicate the different geological structure in each well (Fig. 1B), with higher ORP in RW5-12 being due to faster groundwater flow (80–150 L d^{-1}) caused by the sandy gravel aquifer (down to 11 m bgl). Groundwater flow at the bottom of the well was much slower (10–20 L d^{-1}) due to the presence of loamy sands. Additionally, loamy sands could contain higher content of organic matter, along with slow groundwater flow resulting in a significantly lower ORP and thus ongoing biological reduction.

Application of hydrogen peroxide resulted in an increase in overall ORP from an initial level of approximately −30 mV at both depth strata in well RW5-11, and from 230 mV (8 m bgl) and 20 mV (13 m bgl) in well RW5-12 to more than 600 mV in both wells. Due to the small volume of hydrogen peroxide applied (Table 1), this increase in ORP only lasted five days, following which ORP returned to levels similar to those prior to application.

b) Influence on chlorinated ethenes

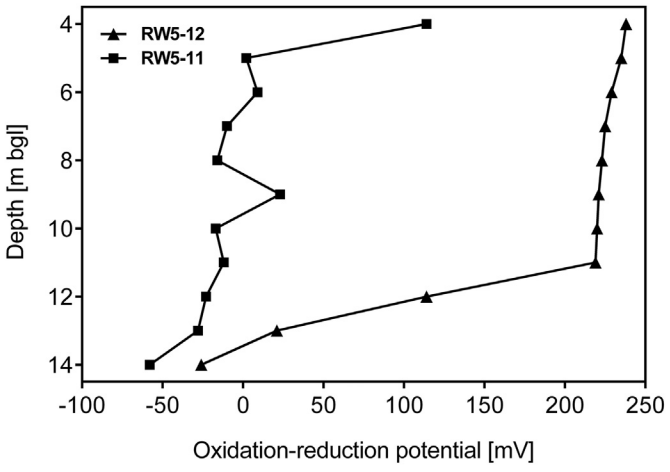

Fig. 2. Oxidation-reduction potential depth profile in wells RW5-11 and RW5-12 before dosing of hydrogen peroxide.

The small amount of hydrogen peroxide applied, i.e. 100 L in well RW5-11 and 500 L in well RW5-12 (amounts used at this site more commonly range from 3000 to 5000 L per well injected in several doses), did not result in a significant decrease in chlorinated ethenes concentration. Changes in the concentration of individual chlorinated ethenes were within the range of long-term fluctuations throughout the experiment (Table 3). Increase on Day 225 was caused by spreading of PCE contamination from source area, accompanied by higher concentration of degradation products. Prior to the addition of hydrogen peroxide to well RW5-11, the degree of dechlorination was around 13%, indicating partly ongoing organohalide respiration. After application, degree of dechlorination did not change suggesting both the removal of chlorinated ethenes and simultaneous inflow of contamination from the Fenton-unaffected environment nearby. At the end of the experiment, the degree of dechlorination had increased to 22%, indicating a gradual recovery of the organohalide-respiring bacterial populations (see below) and ongoing organohalide respiration. As almost no degradation products were detected in well RW5-12 (ca. 3%) and a low degree of dechlorination (<1.4%) was reached, it can be assumed that no organohalide respiration of chlorinated ethenes took place. Such a low ratio could be explained by the high prevalence of chlorinated methanes in this well, and especially trichloromethane and tetrachloromethane (Table S1). Moreover, Fenton-like reactions are known to be inefficient at removing chlorinated methanes (Teel and Watts, 2002).

c) Changes in the organohalide-respiring bacterial populations

In this study, the effect of small doses (disposable impact) of hydrogen peroxide on organohalide-respiring bacteria was evaluated in two contaminated wells and their recovery rate observed. In addition, the influence of depth was assessed by taking samples from 8 to 13 m in each well. The qPCR analysis prior to application confirmed the presence of organohalide-respiring bacteria (*Dehalococcoides* spp., *Dehalobacter*, *Desulfitobacterium*) in both wells, along with the VC reductase genes *vcrA* and *bvcA* (Figs. 3 and 4). All results are expressed as relative ratios, i.e. each marker in relation to its state prior to application (value #1).

One day after hydrogen peroxide application, all markers in both wells decreased rapidly to values below the detection limit (Figs. 3 and 4), independent of the volume of hydrogen peroxide applied. Such a rapid decrease cannot be explained by a flushing-out effect alone. Clearly, hydrogen peroxide affected both the organohalide-respiring bacteria and the levels of individual reductive dehalogenase genes, thereby confirming the toxic nature of hydrogen peroxide. Differences in local geological conditions and the ratio of chlorinated methanes to ethenes in each well resulted in differing changes in organohalide-respiring bacterial populations.

Well RW5-11 exhibited significant recovery of organohalide-respiring bacteria after 13 days, with *Dehalococcoides* spp. dominant. Furthermore, this increase corresponded with an increase in the level of both VC reductase genes (*vcrA* and *bvcA*). A similar rise was observed at both depth strata (i.e. 8 and 13 m) in well RW5-11, corresponding with the reducing conditions found at both levels. As only 100 L of hydrogen peroxide was dosed into this well, both groundwater flow system and surrounding well environment was not significantly affected. The recovery can be explained, therefore, by faster flushing of hydrogen peroxide being followed by re-inoculation from the microbial populations in new groundwater from areas unaffected by the Fenton-like reaction. Moreover, the newly inflowing microbial populations were able to establish themselves rapidly as the original populations had been removed by the Fenton-like reaction, simultaneously providing high nutrient and substrate content for growth. Chapelle et al. (2005) and Sutton

Table 3
Degree of dechlorination, including concentrations of individual chlorinated ethenes, ethene and ethane (in μmol L^{-1}) as end-products in application wells RW5-11 and RW5-12; LOQ - limit of quantification.

Well	Time [d]	VC	DCE total	TCE	PCE	Ethene	Ethane	Degree of dechlorination [%]
RW5-11	−25	0.07	1.21	1.30	4.48	<LOQ	<LOQ	13.9
	0[a]	0.05	1.45	1.08	5.21	<LOQ	<LOQ	13.3
	12	0.11	2.16	4.99	11.3	0.13	<LOQ	13.5
	32	0.78	1.31	0.60	9.77	<LOQ	<LOQ	11.2
	60	0.03	1.33	1.80	12.1	0.06	<LOQ	7.82
	225	1.48	13.6	5.19	21.9	0.26	<LOQ	22.3
RW5-12	−25	0.02	0.33	0.48	20.9	<LOQ	<LOQ	1.44
	0[a]	0.01	0.26	0.55	22.2	<LOQ	<LOQ	1.20
	12	0.01	0.25	0.66	25.7	<LOQ	<LOQ	1.05
	32	<LOQ	0.11	0.36	21.3	<LOQ	<LOQ	0.67
	60	<LOQ	0.18	0.51	26.1	<LOQ	<LOQ	0.81
	225	<LOQ	<LOQ	0.70	48.7	<LOQ	<LOQ	0.36

[a] Dosing time.

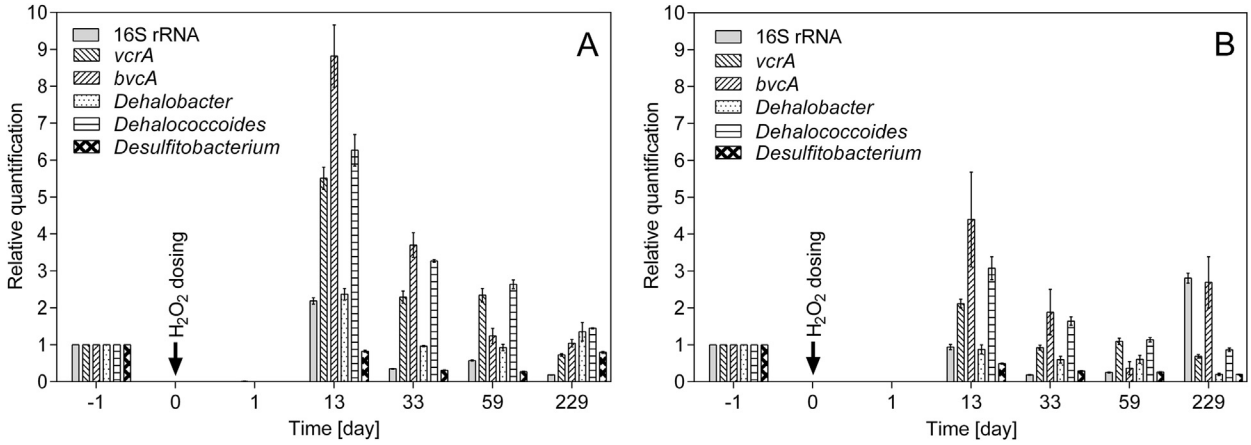

Fig. 3. Changes in the relative abundance of organohalide-respiring bacteria markers and 16S rRNA gene following application of hydrogen peroxide (100 L) in well RW5-11: A) depth 8 m, B) depth 13 m. No molecular genetic markers were detected one day after dosing.

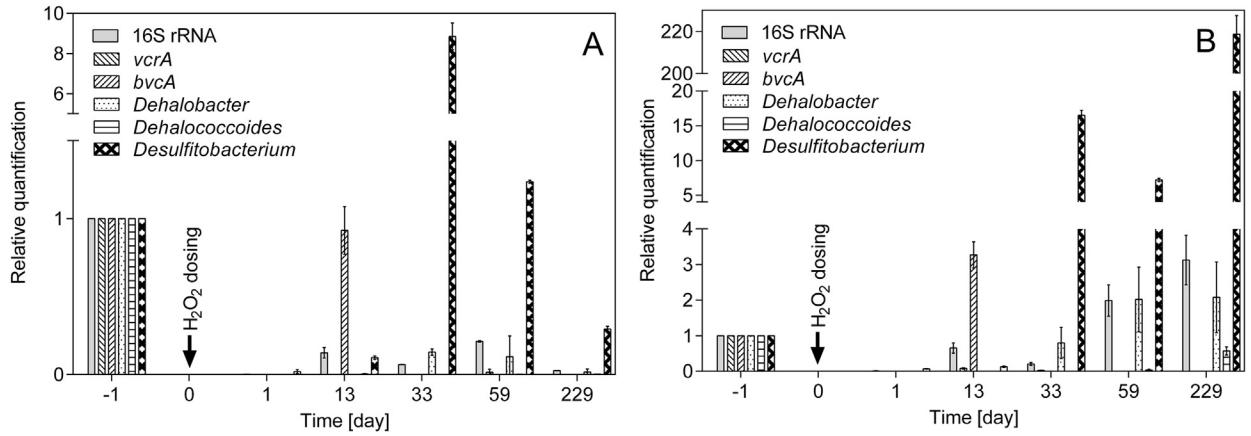

Fig. 4. Changes in the relative abundance of organohalide-respiring bacteria markers and 16S rRNA gene following application of hydrogen peroxide (500 L) in well RW5-12: A) depth 8 m, B) depth 13 m. Aside from the *Desulfitobacterium* (degrader of chloromethanes), no molecular genetic markers were identified one day after dosing. Note fragmentation of the y-axis.

et al. (2010) both reported that application of Fenton reagents led to the mobilisation of fermentable organic carbon, which can be utilised by new microbial populations to support growth. One month after application, the markers gradually decreased to levels comparable with those prior to application. Only *Dehalococcoides* spp. and both the *vcrA* and *bvcA* genes were still approximately three-times higher after one month, corresponding with the higher chlorinated ethene degradation product ratios observed due to ongoing organohalide respiration. At the end of the experiment (i.e. after 230 days), all markers were close to their original values, with the exception of the *bvcA* gene, again suggesting ongoing activity of organohalide-respiring bacteria.

The influence of the Fenton-like reaction on organohalide-respiring microbial populations was very similar at both depth strata 8 and 13 m (Fig. 3). In terms of microbial recovery, all markers had increased two-fold at 8 m, most likely due to more intensive groundwater flow in the well (approx. 80–150 L d^{-1} at 8 m bgl compared to 10–20 L d^{-1} in the underlying layer at 13 m bgl) and, therefore, faster re-inoculation by bacteria (Sutton et al., 2010).

As a higher volume of hydrogen peroxide was applied in well RW5-12, a wider surrounding environment was influenced. Zone of impact of hydrogen peroxide in aquifer was not, however, uniformly distributed over well depth. Whereas this zone was about 0.2 m in high-permeable layers (at 8 m bgl), at depth of 13 m bgl it ranged from 0.0 to 0.05 m due to the presence of low-permeable environment (Fig. 1B). Recovery of organohalide-respiring microbial populations in this well; therefore, differed to that in well RW5-11. Aside from *Desulfitobacterium*, no microbial recovery was observed and the levels of all markers remained at values only slightly above the detection limit observed at 8 m (Fig. 4). Faster groundwater flow at this depth, a more positive ORP (220 mV; Fig. 2) and decrease in pH caused by mixture of hydrogen peroxide together with citric acid (used for ferrous ion stabilisation) prevented recovery of anaerobic organohalide-respiring microbial populations. On the other hand, *Desulfitobacterium* appeared capable of growth also under such conditions, though only for a limited time as it needs reductive conditions (negative ORP) for stable and long-term growth (Kim et al., 2012). This would explain the decline in *Desulfitobacterium* relative abundance after 30 days (Miller et al., 1997). No further microbial recovery was observed at 8 m until the end of experiment (230 days), suggesting that reductive conditions are critical for recovery of organohalide-respiring bacteria. As relative quantification was used (not providing information on total amount), the presence of all markers was detected prior to the application of hydrogen peroxide (Fig. 4A) even though positive ORP (Fig. 2) at this depth of 8 m. It can be explained by occurrence of anoxic/anaerobic zones (Damgaard et al., 2013b), which disappeared after hydrogen peroxide application.

As at 8 m, organohalide-respiring bacteria disappeared completely at 13 m immediately after hydrogen peroxide application (Fig. 4B). In general, microbial recovery in well RW5-12 was affected by the different contaminants present, i.e. while chlorinated ethenes were the dominant contaminant in well RW5-11, trichloromethane and tetrachloromethane dominated in RW5-12. Organohalide respiration began again after 13-days, however, as indicated by the slow increase in marker levels and a two-fold increase in the levels of *bvcA* gene. Most likely due to the high concentration (~95%) of chlorinated methanes, *Desulfitobacterium* levels were up to 200-times higher at the end of the monitoring period compared to those prior to the experiment (Fig. 4B). These results agree with those of Odom and Singleton (2013), who reported that this bacterial strain is capable of utilising chlorinated methanes for growth. Organohalide-respiring bacterial activity could be influenced by the high *Desulfitobacterium* levels, with relative abundances comparable with those prior to the experiment. This corresponds well with the chemical analysis results (section 3.1b), which detected very low degradation product ratios throughout the experiment.

Chapelle et al. (2005) found comparable results, with a decrease in microbial colonisation in wells and microbial activity expressed as a rate of conversion of ^{14}C acetate to $^{14}CO_2$ two days after application of Fenton reagents. Hence, it would appear that the rate of microbial recovery depends on the groundwater flow, which influences the flushing out Fenton reagents as well as inflow of new microbial populations.

3.2. Application of sodium lactate

a) ORP measurement

The ORP in well RW5-49 decreased from 370 mV to 4 mV 30 days after application of sodium lactate (first measurement), with ORP remaining close to 0 mV until the end of the experiment after seven months, thereby indicating appropriate conditions for removal of chlorinated ethenes (Van der Zaan et al., 2009). In contrast, reference well RW5-24, which received no dosing, showed fluctuations of ORP around 250 mV throughout the experiment.

b) Influence on chlorinated ethenes

Concentrations of all contaminants in well RW5-49 decreased as a result of enhanced microbial degradation activity (Table 4; Fig. 5). The degree of dechlorination increased following sodium lactate addition from original value of 60.4–85.9% 30 days after infiltration, and 80.6% after 92 days (Table 4). In contrast, the degree of dechlorination in the reference well remained relatively stable over the same period, ranging from 18.3 to 26.0%. In addition to chlorinated ethenes, the concentrations of ethene and ethane, the end-product of degradation, were also analysed. Ethene concentrations were approximately two-times higher than those before application of sodium lactate. Higher ethene concentrations, along with high ratios for degradation products (maximum 92%), after 30 days clearly indicate an increased rate of chlorinated ethenes degradation. The presence of ethene prior to application can be explained by ongoing dechlorination in well RW5-49. Ethene concentrations remained below the limits of detection throughout the experiment in the reference well (RW5-24), indicating undetectable or incomplete degradation of chlorinated ethenes (also due to the higher ORP ~250 mV). No effect of sodium lactate dosing was observed on concentrations of chlorinated methanes in well RW5-49 (Table S2).

c) Changes in COD and redox sensitive parameters

As 20 m^3 of sodium lactate was infiltrated in well RW5-49, surrounding environment in diameter up to 10 m in high-permeable layers and about 0.1–0.3 m in the layers with low permeability was influenced. Due to the high-permeable layers, any significant increase in groundwater level and flow was not observed after sodium lactate infiltration. Only flow direction nearby to well was opposite during sodium lactate application. The amount of sodium lactate remaining in groundwater was determined by measuring the COD. COD increased by around 300% (from 82 to 250 mg L^{-1}) following application of sodium lactate in well RW5-49. Over the next 92 days, COD gradually decreased to 15 mg L^{-1}, whereupon they remained relatively stable until the end of experiment.

Nitrate in well RW5-49 decreased from about 1.5 mg L^{-1} to below the detection limit (0.20 mg L^{-1}) over the first 92 days after application, though they had returned to their original values after 200 days due to inflow of groundwater, thereby confirming exhaustion of lactate for biological reduction (ORP about 35 mV). Sulphate concentrations in well RW5-49 decreased from 460 mg L^{-1} to 160 mg L^{-1} 30 days after sodium lactate application, subsequently increasing to 290 mg L^{-1} after 90 days and remaining relatively constant at this level throughout the rest of the experiment. While the concentrations of nitrate fluctuated slightly (from 79 to 108 mg L^{-1}) in reference well RW5-24, the concentrations of sulphate remained relatively stable (1090–1280 mg L^{-1}) throughout the experiment.

Table 4
Degree of dechlorination, including concentrations of individual chlorinated ethenes, ethene and ethane (in μmol L^{-1}) as end-products in application well RW5-49 and reference well RW5-24; LOQ - limit of quantification.

Well	Time [d]	VC	DCE total	TCE	PCE	Ethene	Ethane	Degree of dechlorination [%]
RW5-49	−35	0.23	0.76	0.18	0.20	0.76	<LOQ	63.8
	0[a]	0.20	0.71	0.21	0.25	0.68	<LOQ	60.4
	30	0.09	0.42	0.13	0.03	1.92	<LOQ	85.9
	92	0.13	0.28	0.10	0.19	1.57	<LOQ	80.6
	153	0.13	0.18	0.13	0.20	0.95	<LOQ	74.0
	201	0.21	0.22	0.08	0.16	1.17	<LOQ	79.3
RW5-24	−35	0.20	0.68	1.64	1.93	<LOQ	<LOQ	20.2
	0	0.21	0.65	1.58	2.11	<LOQ	<LOQ	19.3
	30	0.36	0.66	1.50	2.80	<LOQ	<LOQ	18.3
	92	0.55	0.65	0.83	1.60	<LOQ	<LOQ	26.0
	153	0.25	0.64	1.17	1.86	<LOQ	<LOQ	20.4
	201	0.46	0.76	1.40	2.34	<LOQ	<LOQ	21.7

[a] Infiltration time for well RW5-49.

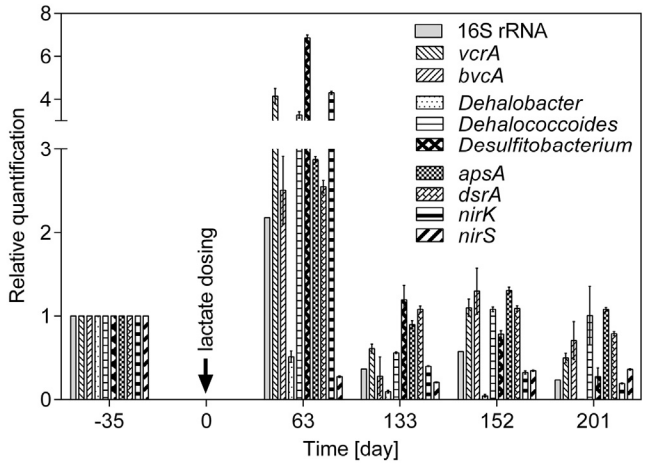

Fig. 5. Change in the relative abundance of markers in well RW5-49 following infiltration of sodium lactate. Note fragmentation of the y-axis.

d) Impact on bacterial populations

Before sodium lactate application, *Dehalococcoides* spp., *Dehalobacter*, *Desulfitobacterium*, VC reductase genes *vcrA* and *bvcA* and sulphate-reducing (identified through the *apsA* and *dsrA* genes) and denitrifying (identified through the *nirK* and *nirS* genes) bacteria were all detected in well RW5-49. Apart from the *bvcA* gene, the same markers were also identified in reference well RW5-24.

The clear differences in microbial response observed in well RW5-49 following sodium lactate dosing confirm a positive influence over a shorter time interval (Fig. 5). The relative abundance of those markers responsible for organohalide-respiration remained three- to seven-times higher than those prior to application two months after sodium lactate dosing. An increase in *Dehalococcoides* spp. and *vcrA* and *bvcA* genes was observed, with *Desulfitobacterium* showing the highest increase over all markers tested. The dominance of *Desulfitobacterium* (at seven-times their original level) is likely to have been due to its broad substrate specificity, enabling more rapid growth than other organohalide-respiring bacteria (Lupa and Wiegel, 2015). The increase in *Dehalococcoides* spp., along with levels of the *vcrA* and *bvcA* genes, is in accordance with the results of chemical analysis, which showed a clear transformation of VC to ethene (Table 4). On the other hand, sodium lactate application led to a decrease in the abundance of *Dehalobacter* (Fig. 5), primarily responsible for DCA degradation (Grostern and Edwards, 2006).

Approximately four months after sodium lactate application, levels of all markers had decreased, with final values similar to those before application. In well RW5-49, aside from the nitrite reductase gene *nirS* and *Dehalobacter*, all markers had returned to their original values within four months. Molecular genetic analysis and chemical analysis results both agreed, demonstrating a decrease in sulphates and nitrates together with a decrease in ORP. Increased relative abundance of denitrifying and sulphate-reducing bacteria corresponded with ongoing organohalide respiration, negative ORP being needed for both processes. This resulted in a clear decline in sulphate concentrations, accompanied by a simultaneous increase in the levels of *apsA* and *dsrA* genes responsible for sulphate reduction.

In the reference well (RW5-24), concentrations of chlorinated ethenes and marker relative abundance remained relatively stable throughout the experiment. 133 days after lactate application, however, marker levels had increased again, probably as a result of renewed contamination originating from inflow of untreated groundwater. Findings observed after sodium lactate application are in accordance with results by Damgaard et al. (2013) and Scheutz et al. (2008) who also reported an increase in molecular genetic markers for ongoing organohalide respiration after molasses and sodium lactate dosing, respectively.

If the remediation effect of the Fenton-like reaction is to be increased, dosing with an organic substrate would be needed to support organohalide-respiring bacterial growth. Based on this, the combination of a Fenton-like reaction followed by dosing with sodium lactate (or other organic substrate) would appear to be efficient approach for enhancing stimulation of organohalide-respiring bacteria during *in-situ* remediation of chlorinated ethenes.

4. Conclusions

Changes in anaerobic organohalide-respiring bacteria associated with *in-situ* dehalogenation of chlorinated ethenes following a Fenton-like reaction and infiltration of sodium lactate at a contaminated site was assessed. A combined approach based on molecular genetic assays along with detection of chemical parameters was used in this study.

While molecular genetic marker levels remained high two-months after sodium lactate application, most markers showed only a slight increase following the Fenton-like reaction. Although the Fenton-like reaction initially resulted in a decrease in all marker relative abundances, a subsequent increase was observed within 13 days. Most values returned to those prior to application within one month. Increase in molecular genetic markers associated with

organohalide-respiring bacteria lasted longer when dosing with sodium lactate. Sodium lactate led to establishment of the reducing conditions necessary for growth of anaerobic organohalide-respiring bacteria. Ongoing organohalide respiration was proven by an increase in markers along with an increase in ethene concentration.

Future work will be aimed at verifying whether a combination of a Fenton-like reaction followed by dosing with sodium lactate can be efficient approach for enhancing biostimulation of organohalide-respiring bacteria during *in-situ* remediation of chlorinated ethenes at contaminated site.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.05.030.

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

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ABSTRACT

Nanoscale zero-valent iron (nZVI) is recognized as a powerful tool for the remediation of groundwater contaminated by chlorinated ethenes (CEs). This long-term field study explored nZVI-driven degradation of CEs supported by electrokinetic (EK) treatment, which positively affects nZVI longevity and migration, and its impact on indigenous bacteria. In particular, the impact of combined nZVI-EK treatment on organohalide-respiring bacteria, ethenotrophs and methanotrophs (all capable of CE degradation) was assessed using molecular genetic markers detecting *Dehalococcoides* spp., *Desulfitobacterium* spp., the reductive dehalogenase genes *vcrA* and *bvcA* and ethenotroph and methanotroph functional genes. The remediation treatment resulted in a rapid decrease of the major pollutant *cis*-1,2-dichloroethene (cDCE) by 75% in the affected area, followed by an increase in CE degradation products methane, ethane and ethene. The newly established geochemical conditions in the treated aquifer not only promoted growth of organohalide-respiring bacteria but also allowed for the concurrent presence of vinyl chloride- and cDCE-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. The nZVI-EK treatment 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.

1. Introduction

Perchloroethylene (PCE) and trichloroethylene (TCE) are widely used in industry as dry cleaning fluids and degreasers and historical contamination has typically occurred due to their improper handling, storage or incidental releases. Degradation of PCE and TCE is very limited in aerobic environments; however, more efficient degradation is achieved under anaerobic conditions, primarily by the process of reductive dehalogenation (Vogel et al., 1987; Middeldorp et al., 1999; Aulenta et al., 2006). Despite this, it is very difficult to completely clean a site contaminated with chlorinated ethenes (CEs) due to the slow degradation kinetics of the less chlorinated by-products *cis*-1,2-dichloroethene (cDCE) and vinyl chloride (VC). As these compounds are more soluble and mobile in the environment than their parents, and VC also more toxic and even carcinogenic, their accumulation should be avoided and degradation processes stimulated and monitored (Tobiszewski and Namieśnik, 2012).

The most commonly used remediation process for CEs is enhanced anaerobic biodegradation, supported by the addition of organic substrates (as carbon and electron sources), and by changing groundwater conditions to make them more suitable for specific organohalide-respiring bacteria. Bioremediation is a cost-effective method compared to physical or chemical treatment (Chen et al., 2015), but is relatively problematic for sites with high initial pollutant concentrations, which usually inhibit bacterial

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growth (Haest et al., 2010; Xiu et al., 2010). As such, a combination of two or more remediation approaches (remedial train) usually proves a better strategy for efficient removal of these contaminants (Hassan et al., 2018).

The use of reducing agents, such as nanoscale zero-valent iron (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). 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 elemental hydrogen, which serves as an electron donor for present bacteria (Bruton et al., 2015). Both of these reactions lead to the creation of an environment suitable for the growth of organohalide-respiring bacteria. However, nZVI particles possess several limitations, such as short-term reactivity and also rapid aggregation, that negatively affects 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; 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). Three different mechanisms positively affect the transport of nZVI particles, electroosmosis, electromigration and electrophoresis (Yeung, 1994; Hassan et al., 2018); however, in an environment where advective flow dominates, their contribution may be negligible (Černík et al., 2019). Moreover, electrolysis reactions at the electrode produce hydronium ion (H_3O^+) at the anode, which lower 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). Electrokinetically-enhanced bioremediation has been extensively studied (Beretta et al., 2019) and applied in order to remove pollutants from soil and groundwater (She et al., 2006; Mena Ramírez et al., 2014; Mousavi et al., 2011; Zhan et al., 2012; Zhang et al., 2013, 2014).

Several anaerobic bacteria capable of reducing PCE and TCE to cDCE have been described, including the genera *Desulfitobacterium*, *Dehalobacter*, *Sulfurospirillium* and *Geobacter* (Dolinová et al., 2017); however, only *Dehalococcoides* spp. and the recently described *Dehalogenimonas* spp. (both *Chloroflexi* phyla) are capable of complete reduction of cDCE and VC to ethene (Lee et al., 2013; Löffler et al., 2013; Yang et al., 2017). Their presence at a contaminated site correlates with ethene production, while their absence leads to cDCE and VC accumulation. 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). The presence of genes for different reductases can indicate the contaminated site potential for successful bioremediation (Dolinová et al., 2016; Saiyari et al., 2018).

In addition to anaerobic degradation pathways, methanotrophs have been shown to cometabolicaly oxidize TCE, cDCE and VC using enzyme methane monooxygenases (MMO). Methanotrophs oxidize methane as a primary growth substrate, but the broader substrate range of MMO's allows for accidental CEs degradation (Yoon et al., 2011; Liang et al., 2017). Ethen-oxidizing bacteria (ethenotrophs) can also cometabolize VC or cDCE in the presence of ethene as the primary growth substrate (Koziollek et al., 1999; Mattes et al., 2010) and some ethenotrophs are even able to use VC as a sole carbon source (Verce et al., 2001). VC-oxidizing methanotrophs and ethenotrophs have been found in anaerobic groundwater samples and can establish themselves at low levels of dissolved oxygen (Gossett, 2010; Richards et al., 2019). As such, the functional genes from ethenotrophs (*etnC* and *etnE*) and methanotrophs (*mmoX* and *pmoA*) are used as biomarkers of aerobic degradation of CEs in groundwater (Wymore et al., 2007; Jin and Mattes, 2010; Němeček et al., 2020).

The aim of this long-term field study was to apply a strong CE-reducing agent (nZVI) supported by EK to enhance its performance and support complete biodegradation of CEs. In particular, we focused on the dynamics of organohalide-respiring bacteria, methanotrophs and ethenotrophs involved in both reducing and oxidizing processes. Changes in microbial abundance and groundwater properties were evaluated by combining physical-chemical parameters with molecular biology techniques.

2. Material and methods

2.1. Study site

The experiment took place on property owned by Spolchemie a. s. (Ústí nad Labem, Czech Republic), where poor TCE storage and handling practices have, over the years, led to significant contamination of soil and groundwater. Geologically, the Spolchemie site comprises Mesozoic Cretaceous siltstones overlaid by a Quaternary terrace, which is the layer containing most of the contamination. The Quaternary terrace consists of fluvial sediments located approximately 12.3 to 3 m below ground level (bgl) and its average thickness is 9 m. The base of the terrace consists of clay gravels, sandy gravel and alkaline boulders of the size 15–20 cm. The grain size of clastic sediments decreases towards the overburden. In the depth of 7.5–3 m bgl, there are parts of brown-rusty clay sands with smaller boulders of alkaline rocks or quartz. The terrace is covered by a 3 m thick layer of anthropogenic debris, originating from historical demolitions. The surface of the studied site is covered by a concrete layer with a thickness of 40–55 cm or with a coarse-grained gravel. The groundwater level is 3–4 m bgl, with the major horizontal flow south-east towards the River Bílina. As hydraulic permeability of the rock material decreases with depth, the highest groundwater flow rate is between 4.5 and 6.8 m bgl. The hydraulic conductivity coefficient at depths of 3.5–9 m bgl is in the range of 10^{-4} m/s to 10^{-6} m/s (Kvapil et al., 2011).

The field experiment took place in a contaminated area with a constant inflow of CEs from the source zone, situated north of the site. A central line of three anodes was surrounded by nine cathodes to create DC electric field (Fig. 1). All the electrodes were steel rods with a diameter of 20 mm installed directly into the aquifer to a depth of 9 m. Wells AMW-57, AW-60 and AMW-61 were used for nZVI application. Wells close to cathodes (AMW-57 and MW-58) and an anode (AMW-61) were monitored to assess the influence of nZVI and the electric field on CE degradation, physico-chemical parameters and abundance of indigenous bacteria. Well MW-33, situated on the inflow side of the study site, served as a control for monitoring the concentration of inflowing contaminants. Characterization of the groundwater from well MW-33 sampled 8 days before nZVI application is presented in Table S1.

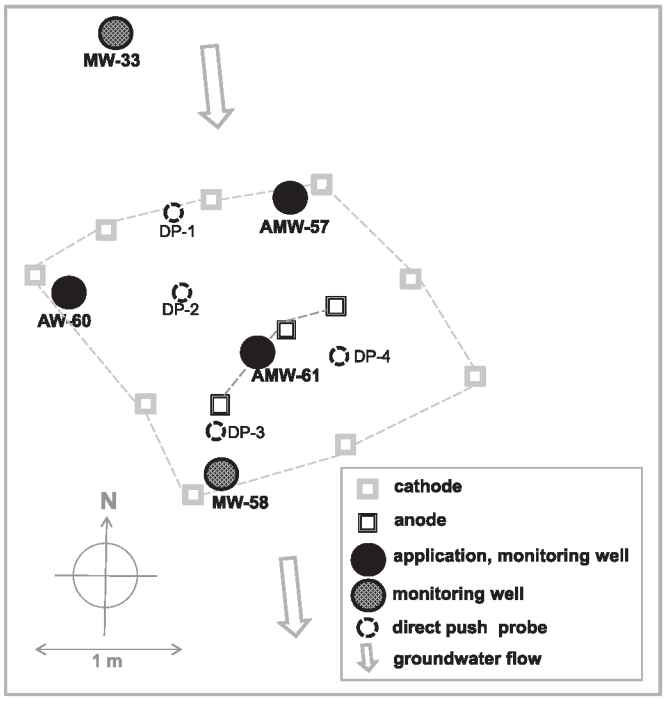

Fig. 1. Position of application and monitoring wells, direct push probes and DC electrodes at the study site.

2.2. Application of nZVI and EK treatment

A reaction mixture with a final nZVI concentration of 3 g/l was prepared fresh on-site by mixing 60 kg of nZVI (NANOFER STAR DC, Nanoiron Ltd., Czech Republic) and 1 kg of MSJ detergent (Schicht s. r.o., Czech Republic) in 20 m^3 of tap water. The suspension was injected into four direct-push probes (DP-1 − 4) and three narrow profile wells (AMW-57, AW-60, AMW-61) using a HP 10/80 pressure pump (Hapon s. r.o., Czech Republic) with a working pressure of 10–40 MPa (Table 1, Fig. 1). The injection was carried out for 12 h and continued for 8 h the next day.

A DC electric field, created by nine cathodes and three anodes (Fig. 1), was applied seven days after nZVI injection using a DC power supply (SP-750-24 24V-30A AC/DC convertor, Mean Well, Taiwan) with a voltage of 24 V and a maximum power of 750 W. When the system was switched on, the inverter output was approximately 550 W, but decreased slowly as a result of anode passivation and dissolving.

2.3. Sampling and measurement of chemical and physical parameters

Groundwater samples for molecular biology and physico-chemical analysis were obtained from wells AMW-57, MW-58, AMW-61 and MW-33 over a period of 140 days, with two samplings completed before nZVI application. The groundwater level was measured in all wells using a G30 electronic gauge (NPK Europe Mfg. s. r.o., Czech Republic). Subsequently, all water samples were taken from the depth of 8 m bgl using a Gigant pump (Eijkelkamp, Netherlands), after the physico-chemical parameters (pH, oxidation-reduction potential (ORP), water conductivity) of the continuously pumped water were stabilized. Water samples (0.3–0.5 L) for molecular genetic analysis were immediately cooled on ice, transported to the laboratory and stored in the dark at 4 °C until filtration the following day. Physico-chemical parameters

Table 1
Volume of nZVI suspension applied to individual wells (in m^3).

Horizon (m bgl)	Direct-push probes				Narrow profile wells		
	DP-1	DP-2	DP-3	DP-4	AMW-57	AW-60	AMW-61
5	2	1.7	1.5	1.5	3.3	3.3	2.3
6	0.8	0.8	0.6	0.6			
7	0.5	0.5	0.3	0.3			

(temperature, pH, ORP and water conductivity) were measured using a YSI Professional instrument (YSI, OH, USA) directly on site. ORP was recalculated to the standard hydrogen electrode.

Concentrations of CEs, methane, ethene and ethane were determined according to EPA Method 8260 B by gas chromatography-mass spectrometry (GC–MS; CP 3800, Saturn 2200, Varian, USA).

2.4. Molecular genetic analysis

The relative abundance of the genera *Dehalococcoides* and *Desulfitobacterium* and the genes 16S rRNA (representing total bacterial biomass), *vcrA* and *bvcA* (VC reductase genes capable of complete dehalogenation of cDCE to ethene), *mmoX* and *pmoA* (MMO genes of methanotrophs) and *etnC* and *etnE* (encoding the alkene monooxygenase and epoxyalkane: coenzyme M transferase of ethenotrophs) was determined by quantitative real-time PCR (qPCR).

Samples for molecular biology analysis were processed as described in Dolinová et al. (2016). Briefly, all water samples were concentrated by filtration through 0.22 μm pore size filters (Merck Millipore, Germany). DNA was extracted from the filter using the FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA) according to manufacturer's protocol. qPCR reactions were prepared and performed in a LightCycler® 480 instrument (Roche, Switzerland), with Cp values being obtained using the second derivative maximum method available in the LightCycler® 480 Software. Each sample was analysed in duplicate with ultra-pure water used as a template in a negative control. Relative quantification of each parameter was expressed as a fold change between two states (a given sampling time and sampling time zero) using the delta Cp method. The results of qPCR were evaluated as relative quantification, with the condition of the specific bacteria or gene before application taken as the starting point. Specific primer sets are listed in Table S2.

3. Results and discussion

3.1. Effect of nZVI-EK treatment on ORP, pH and temperature

When evaluating changes in physical and chemical parameters, it is necessary to take into account, that the nZVI-EK treatment formed a geochemical barrier with constant inflow of contaminated groundwater 1–3 m per day. Injection of nZVI resulted in a rapid decrease of ORP in the application wells (from approx. 100 mV to −200 mV in AMW-61 and to −300 in AMW-57). In well MW-58, which was not used for nZVI application, ORP decreased by 100 mV to −20 mV (Fig. 2A). Despite subsequent DC application, ORP values increased almost to their initial values within seven days due to relatively fast groundwater flow. Nevertheless, aside from well AMW-57, which was most affected by inflowing untreated water, ORP remained lower in the treated area than in inlet water (well MW-33) until the end of the experiment. Only short-term changes in pH were observed following nZVI injection (Fig. 2B). The aquifer environment was sufficiently buffered as described in other studies

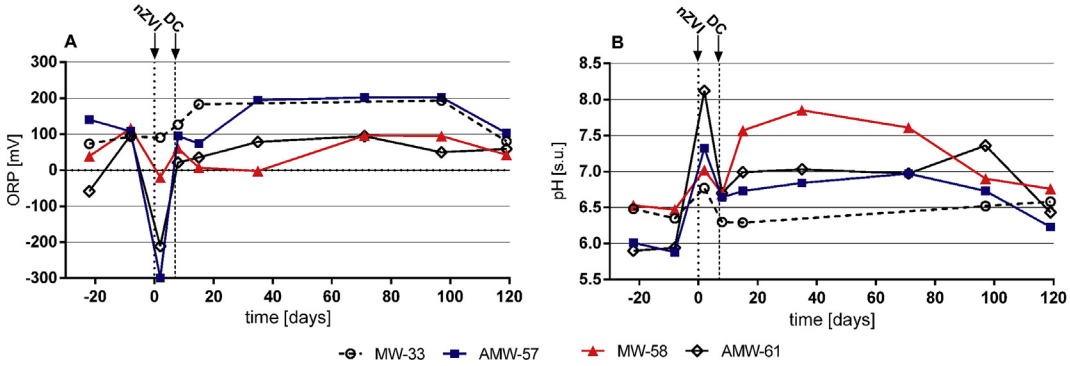

Fig. 2. Changes in (A) ORP and (B) Ph in each well following nZVI injection and DC application (time = 0 refers to nZVI injection).

(Gavaskar et al., 2005; Wei et al., 2010). In wells AMW-57 and AMW-61, pH increased directly after nZVI injection, from an initial level of around 6 standard units (s.u.) to more than 7 s. u. and 8 s. u., respectively. Compared with the starting point, pH values were still one standard pH unit higher after seven days, and showed a slight increase after DC commissioning. The downstream well MW-58, situated near the cathode and DP-3 probe, was most influenced as the inflowing water had been treated longest. The high volumes of nZVI suspension applied increased groundwater temperature within the treated area from approximately 15 °C to 18–21 °C. Application of DC current then maintained the temperature 5 °C higher in the treated area compared with the inlet water in well MW-33 (Fig. S1).

3.2. Degradation of CEs

The major contaminants detected in inflowing groundwater from well MW-33 were TCE (48% of total CEs) and cDCE (46% of total CEs). The proportion of cDCE increased in downstream groundwater due to ongoing, relatively rapid TCE degradation. As a result, initial contamination in the treated aquifer comprised mainly cDCE (70–80% of total CEs) and VC (10–15% of total CEs) (Fig. 3). The combination of nZVI and EK treatment resulted in a rapid decrease in TCE and especially cDCE. Inside the treated area, higher concentrations of TCE (42.4 μmol/L) were only recorded in upstream well AMW-57, and these were successfully reduced to 0.1 μmol/L after treatment. Initial levels of cDCE (120–633 μmol/L) rapidly decreased by approximately 75% in all wells except for the inlet well, where the reduction was nearer 50%. Concentrations of degradation products (the sum of methane, ethene and ethane) increased in all wells inside the treated zone following nZVI and DC application, indicating successful completion of CE dehalogenation. A significant rise in degradation products was observed from day 71 to day 98 of the experiment. Accordingly, high abundance of *Dehalococcoides* spp. together with both VC reductase genes (*vcrA* and *bvcA*) were detected (Fig. 4). In well AMW-57, situated on the edge of the affected area, cDCE slowly increased after an initial drop due to the constant inflow of contaminated groundwater that had not passed through the created geochemical barrier (Fig. 3B). Nevertheless, high concentrations of final degradation products, together with an increase in *Dehalococcoides* spp. and VC reductase genes, were observed (Fig. 4B). Highest concentrations of CE degradation products and VC were detected in well MW-58, situated downgradient of the anodes and close to a cathode (Fig. 3C).

In all wells, VC levels remained relatively constant (average 75 μmol/L in MW-33, MW-58 and AMW-57 and 23 μmol/L in AMW-61; Fig. 3) throughout the experiment, despite the presence of *Dehalococcoides* spp. and VC reductases. VC is probably produced from cDCE more rapidly than it is degraded. In inlet well MW-33, concentrations of TCE and cDCE decreased by approximately 50% after nZVI application (from 622 to 324 μmol/L and from 598 to 311 μmol/L, respectively; Fig. 3A). After 15 days, TCE levels increased slowly due to the continuous inflow of contaminated water. Levels of final degradation products remained low. Changes in CE concentration and pH observed in well MW-33 following nZVI application were most likely caused by the vast volume of water injected into nearby wells (only 2 m away), which could have diluted the local groundwater and affected conditions in well MW-33 over the medium-term.

Distinct changes in all physico-chemical parameters were observed in all wells after 100 days, with a drop in pH and ORP values (Fig. 2), an increase in water conductivity (Fig. S2), a 5 °C decrease in temperature (Fig. S1) and an increase in the concentration of CEs (Fig. 3). This was probably caused by a combination of two factors, i.e. a 0.5 m increase in groundwater level in the aquifer (Fig. S3), which could have resulted in washing of the unsaturated zone, and degradation and passivation of the anodes, resulting in a 50% decrease in the electric current (Fig. S4).

3.3. Impact of nZVI-EK treatment on native bacterial populations and degradation enzymes

Relative quantification, based on qPCR data, was used to evaluate the changes in bacterial populations and degradation enzymes following nZVI-EK treatment in individual wells over time (Fig. 4). Presence of organohalide-respiring bacteria of the genera *Desulfitobacterium* and *Dehalococcoides* and the functional VC reductase genes *vcrA* and *bvcA* was confirmed in inlet water from well MW-33 (Fig. 4A). However, the abundance of organohalide-respiring bacterial populations and degradation enzymes, as well as total bacterial biomass, was lower in the highly contaminated inlet water when compared with the aquifer and plume zone, as clearly shown on Ct values of each marker in different wells prior to nZVI-EK treatment (Fig. S5). Total CE concentration reached 1.3 mmol/L in well MW-33, while considerably lower levels were recorded in the other monitored wells (Fig. 3). Such high concentrations of CEs could be toxic to microorganisms and inhibit their growth (Duhamel et al., 2002; Haest et al., 2010). An exception was the TCE-dechlorinating genus *Desulfitobacterium*, which was more abundant in the inlet water from well MW-33 than in the wells situated downstream (Fig. S5). This can be attributed to high concentrations of TCE in MW-33, compared to low TCE levels in other monitored wells.

Interestingly, a different response to nZVI application was observed in two application wells close to a cathode and an anode. In well AMW-57, situated near the cathode, nZVI injection resulted

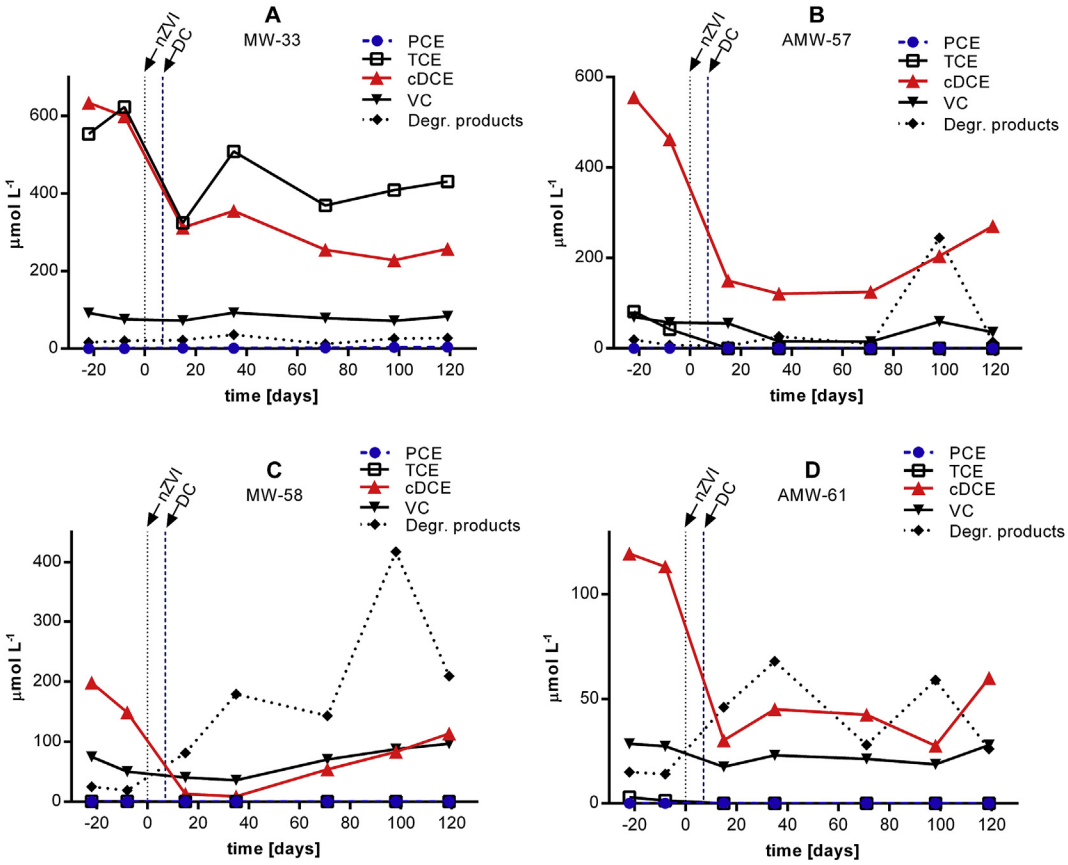

Fig. 3. Changes in concentration of CEs and their final degradation products (the sum of methane, ethene and ethane) following nZVI injection and DC application. (A) MW-33; (B) AMW-57; (C) ME-58; (D) AMW-61.

in a severe reduction of total bacterial biomass eight days after application (Fig. 4B). Such a rapid decrease can be explained by the nZVI suspension flushing-out a significant portion of the microorganisms present. Moreover, high doses of nZVI could be toxic to microorganisms in the short-term (Bruton et al., 2015; Stefaniuk et al., 2016). However, the initial decline in bacterial biomass and all monitored markers was followed by a rapid recovery, especially of *Dehalococcoides* spp. and both VC reductases, indicating enhanced biotic reduction of CEs. The groundwater microbiome was restored within 15 days after the nZVI application and, most notably, the growth of organohalide-respiring bacteria was enhanced. The increase in bacterial biomass corresponded to increasing concentrations of CE degradation products (Fig. 3B). On the other hand, no negative impact was observed on microorganisms eight days after nZVI injection in well AMW-61, situated close to the anode (Fig. 4D), with *Dehalococcoides* and *Desulfitobacterium* spp. even more abundant than before treatment. Electrolysis reactions constantly produce oxygen at the anode, as well as hydrogen ions, which can lower groundwater pH in the immediate vicinity(Hassan et al., 2018). Dissolved oxygen can react rapidly with nZVI, resulting in decreasing toxicity towards microorganisms (Černíková et al., 2020). This effect, together with new microorganisms carried into the well with groundwater flow, could have caused rapid restoration of the microbial community following nZVI injection.

The treatment's impact on the abundance of cDCE- and VC-oxidizing ethenotrophs and methanotrophs was assessed using markers for co-metabolic oxidation of CEs, targeting the genes *etnC*, *etnE*, *mmoX* and *pmoA* encoding degradation enzymes. Clear differences were observed in the abundance of ethenotrophs and methanotrophs near the cathode and anode (Fig. 5) in accordance with other monitored bacteria, with a decrease in abundance observed in well AMW-57 (located near the cathode) directly after application (Fig. 5A). Populations of methanotrophs and ethenotrophs re-established themselves within 35 days, but remained at pre-application levels due to a lack of dissolved oxygen. A slight decrease in ethenotrophs and methanotrophs was observed in well AW5-61 after nZVI and DC application (Fig. 5B), but higher levels of dissolved oxygen resulting from water oxidation on the anode allowed for a higher proliferation of VC-oxidizing bacteria. After 35 days, methanotrophs and ethenotrophs increased once again and ethenotroph levels especially remained up to 50-times higher compared to initial values until the end of the field study. Groundwater heterogeneity clearly supported divergent VC degradation processes. Anaerobic reductive degradation of VC produces ethene, which is oxidized by ethenotrophs that can also degrade VC co-metabolically (Atashgahi et al., 2017; Liang et al., 2017). Moreover, hydrogen produced at the cathodes served as an electron donor for organohalide-respiring bacteria, while oxygen produced at the anodes promoted growth of bacteria capable of aerobic co-metabolic VC oxidation, ethenotrophs and methanotrophs (Mattes et al., 2010; Liang et al., 2017), with the coupling of these two processes helping to completely clean the aquifer. The remediation treatment had no negative impact on indigenous microorganisms in well MW-58, even though it was situated near the cathode (Fig. 4C). The treated water flowing through this well allowed for better growth of *Dehalococcoides* spp., while the presence of both VC reductases indicated complete removal of CEs. The

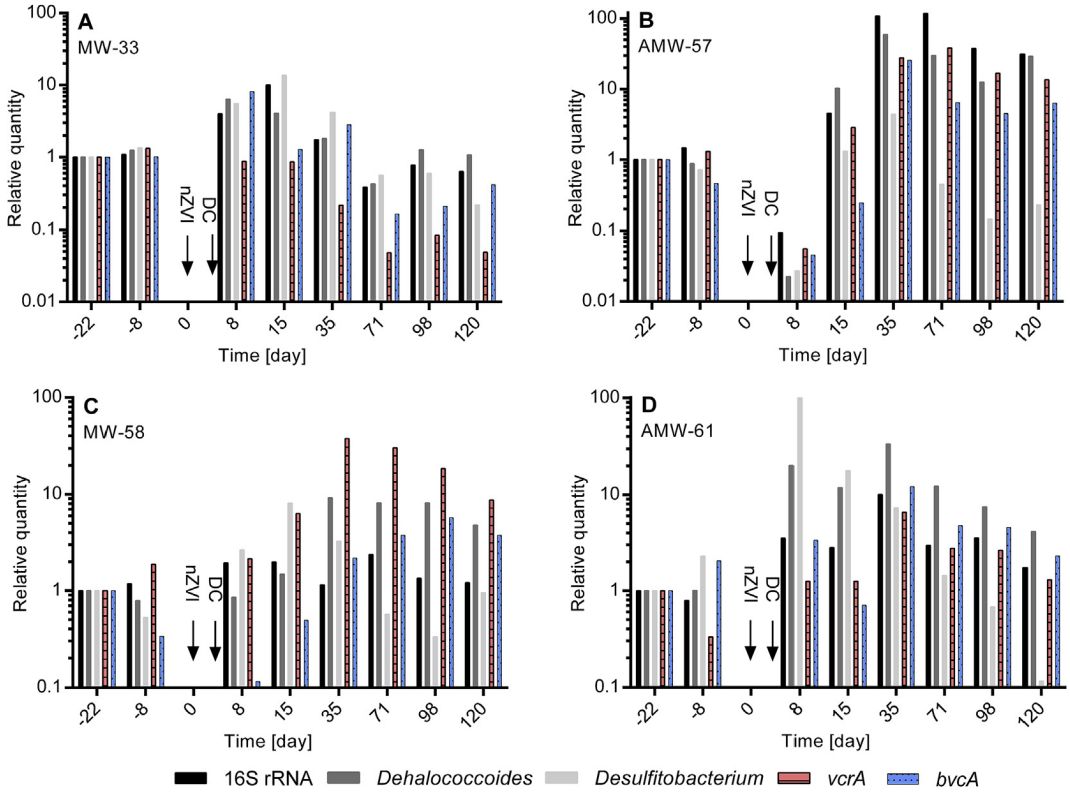

Fig. 4. Changes in the relative abundance of total bacteria (16S rRNA), organohalide-respiring bacteria (*Dehacoccoides* spp. and *Desulfitobacterium*) and VC reductase genes (*vcrA and bvcA*) following nZVI and EK (DC) treatment. (A) MW-33; (B) AMW-57; (C) MW-58; (D) AMW-61. All results are expressed as relative quantity to each marker abundance prior to application. Note the logarithmic scale.

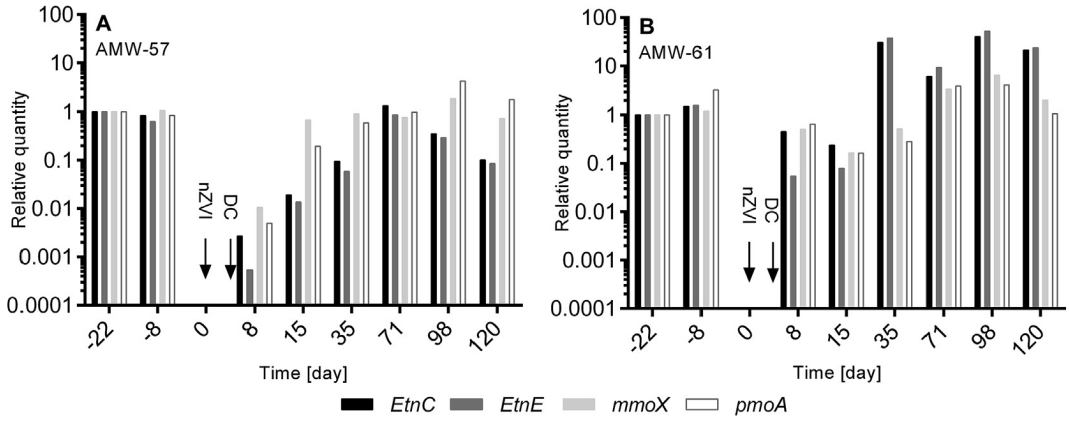

Fig. 5. Changes in the relative abundance of ethenotroph (*EtnC and Etn E*) **and** methanotroph (*mmoX and pmoA*) markers following nZVI and EK treatment. (A) AMW-57; (B) AMW-61. All results are expressed as relative quantity to each marker abundance prior to application. Note the logarithmic scale.

high abundance of these markers corresponded with the high concentration of CE degradation products (Fig. 3C). *Desulfitobacterium* sp. has been shown to be highly successful at colonizing newly formed niches (Villemur et al., 2006). After nZVI application, the relative quantity of *Desulfitobacterium* sp. increased rapidly in the affected wells, though they were soon outcompeted by more specialized organohalide-respiring bacteria favored by conditions in the treated groundwater. *Dehalococcoides* spp. and VC reductase genes were so abundant in the aquifer that they were able to buffer the diluting effect of rising groundwater levels 100 days after nZVI application, and remained relatively stable until the end of the study (Fig. 4).

nZVI-EK treatment elevated temperatures over the whole treated area by approximately 5 °C (Fig. S1), and this may also have accelerated CE biodegradation. Microbial activity is closely related to temperature and, as such, applying a DC current could represent a cost-effective method for both heating the soil and promoting enzymatic activity, especially in cold climates (Suni et al., 2007).

In this study, injection of nZVI induced depletion of indigenous microorganisms in application wells near the cathode. While the observed decrease in microbial biomass could have been caused by the high volume of injected nZVI solution diluting the groundwater, the groundwater microbiome was re-established within just eight and 15 days after nZVI application in wells AMW-61 and AMW-57,

respectively (Fig. 4D and B). Xiu et al. (2010) also observed stimulation in the growth of a culture containing *Dehalococcoides* spp. after a short lag period when exposed to nZVI. Several studies have documented that microorganisms can be negatively affected when exposed to different nZVI particles, however, the toxic effect of nZVI has usually only been observed under laboratory conditions and on pure bacterial cultures (Lee et al., 2008; Diao and Yao, 2009; El-Temsah et al., 2016; Ševců et al., 2017). In comparison, microcosm-scale tests on mixed bacterial cultures prove slightly more positive (Kirschling et al., 2010; Xiu et al., 2010), while nZVI shell oxidation and coating with natural organic matter can rapidly reduce its toxicity under natural environmental conditions (Li et al., 2010; Bruton et al., 2015). Data from field experiments are still scarce, but mostly show changes and shifts in the composition of bacterial communities. Generally speaking, nZVI effect is highly dose and species-dependent (Fajardo et al., 2012; Pawlett et al., 2013; Němeček et al., 2014, 2016; Lefevre et al., 2016; Nguyen et al., 2018), and rapid recovery, or even support, of organohalide-respiring bacteria has been reported in batch microcosms (Xiu et al., 2010) and after field injection of nZVI stabilized with carboxymethyl cellulose (Kocur et al., 2016). Accordingly, nZVI-EK treatment appears to have no long-term negative impacts on native bacterial populations, while promoting the growth of CE degrading bacteria, as clearly shown in this study.

4. Conclusions

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 and, despite the constant inflow of contaminated water into the reactive zone, high cDCE degradation rates were observed throughout the experiment alongside increased production of the CE degradation products methane, ethene and ethane. Long-term reactivity of nZVI was successfully supported by EK treatment, which additionally stimulated microbial degradation activity by elevating groundwater temperature. The negative impact of nZVI application on indigenous microorganisms observed proved to be temporary as the microbiome was restored within 15 days. Moreover, the established groundwater conditions promoted bioremediation. Organohalide-respiring bacteria continued CE reduction, even after partial nZVI exhaustion. cDCE and VC were also degraded by methanotrophs and ethenotrophs, which were favored by the substantial levels of oxygen produced at the anodes. Our approach, combining nZVI as a strong CE reducing agent and EK for supporting nZVI reactivity and mobility, also promoted biodegradation and, as such, 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.

Author contributions

All authors contributed to the writing of this manuscript. All authors have given approval to the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2020.115692.

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

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Abstract

Background: Nanoscale zero-valent iron (nZVI) is commonly used for remediation of groundwater contaminated by chlorinated ethenes (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.

Results: Application of a limited amount of nZVI–AC to an oxic, nitrate-rich, highly permeable quaternary aquifer triggered time-limited transformation of CEs, with noticeable involvement of reductive dechlorination. Reductive dechlorination of CEs was dominantly abiotic, as an increase in the concentration of vinyl chloride (VC) and ethene did not coincide with an increase in the abundance of reductive biomarkers for complete dechlorination of CEs (*Dehalococcoides*, *Dehalogenimonas*, VC reductase genes *vcrA* and *bvcA*). Application of nZVI–AC under unfavourable hydrochemical conditions resulted in no dramatic change in the microbial community, the reducing effect resulting in temporal proliferation of nitrate and iron reducers only. At a later stage, generation of reduced iron induced an increase in iron-oxidizing bacteria. High concentrations and a continuous mass influx of competing electron acceptors (nitrate and dissolved oxygen) created unfavourable conditions for sulphate-reducers and organohalide-respiring bacteria, though it allowed the survival of aerobic microorganisms of the genera *Pseudomonas*, *Polaromonas* and *Rhodoferax*, known for their ability to assimilate VC or *cis*-1,2-dichloroethene. A potential for aerobic oxidative degradation of CE metabolites was also indicated by detection of the ethenotroph functional gene *etnE*.

Conclusions: This pilot study, based on the application of nZVI–AC, failed to provide a sustainable effect on CE contamination; however, it provided valuable insights into induced hydrogeochemical and microbial processes that could help in designing full-scale applications.

Keywords: nZVI–AC, nZVI, Chlorinated ethenes, Reductive dechlorination, Organohalide-respiring bacteria, Microbial community, Next generation sequencing

Background

Chlorinated ethenes (CEs), which include tetrachloroethene (PCE), trichloroethene (TCE) and their toxic metabolites, constitute a large environmental problem due to their persistence in the environment [1]. In recent years, nanoscale zero-valent iron (nZVI) has

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proved effective in CE treatment when employing two major reaction mechanisms, reductive dechlorination (hydrogenolysis) and reductive β-elimination. During reductive dechlorination, C–Cl bonds in CEs are sequentially replaced by C–H bonds to form Cl^- and less chlorinated CEs. During reductive β-elimination of CEs, triple bonds are created between carbon atoms and halogens are simultaneously removed from molecules in the form of Cl^- [2]. However, the high but short-term reactivity of bare nZVI particles, together with scanty subsurface transport due to their fast aggregation and agglomeration, limit the use of nZVI in remediation processes [3, 4]. Recently, the use of novel particles consisting of colloidal activated carbon (AC) with embedded nZVI structures (nZVI–AC) has significantly improved the properties of nZVI for in situ groundwater remediation [5], and these have successfully been used in early pilot-scale field tests [6, 7].

The negative surface charge of the nZVI–AC particles prevents the particles becoming immobilized in the near-neutral aquifer environment, which normally has a negative charge. Moreover, the AC acts as a spacer between individual nZVI particles, reducing the tendency of nZVI–AC particles to aggregate, leading to higher sedimentation stability of nZVI–AC suspensions, even at higher particle concentrations. In addition, suspension stabilizers used during injection, such as carboxymethyl cellulose (CMC), improve the mobility of particles in the subsurface layers. Due to a relatively weak sorption of CMC onto the nZVI–AC surface, CMC is steadily desorbed in the contact with fresh groundwater after injection [8]. The solubility of CMC in water is also important in this context, the recommended maximum molecular weight is < 10 kDa and a substitution degree between 0.6 and 1.0 [8]. Previous field studies in anoxic aquifers have shown particle transport distances of several metres and a high rate of PCE decomposition with no vinyl chloride (VC) formation [5, 6, 8].

Enhanced anaerobic biodegradation by organohalide-respiring bacteria (OHRB) is a process commonly used for the remediation of CEs at polluted sites. This method relies on reductive dechlorination, where CEs serve as electron acceptors and molecular hydrogen and acetate, both released as by-products of organic substrate fermentation reactions, are used by the dechlorinating bacteria as electron donors and carbon sources, respectively [9]. nZVI application has been shown to improve groundwater conditions, making them more favourable for OHRB by lowering the oxidation–reduction potential (ORP), depleting oxygen and nitrate from the groundwater and producing molecular hydrogen during its corrosion process, which serves as an electron donor for chlorine removal during the organohalide respiration process [10, 11].

Although nZVI toxicity to microorganisms has been demonstrated in laboratory studies [12–14], field studies show only mild or short-term impacts on indigenous bacterial populations [15–17]. OHRB include a range of bacterial genera, including *Desulfitobacterium*, *Dehalobacter*, *Geobacter* and *Sulfurospirillum*, all of which utilize different reductases that can dechlorinate PCE to TCE and *cis*-1,2-dichloroethene (cDCE) [18]. However, only *Dehalococcoides* and *Dehalogenimonas* spp. are able to carry out the final and crucial step of CE degradation by dechlorinating cDCE via VC to non-toxic ethene [19, 20], using the specific VC reductases vcrA and bvcA [21].

In addition to anaerobic reductive dechlorination, degradation of CEs can also occur under aerobic conditions, either metabolically, where CEs are used as electron donors for cell growth, or by co-metabolism, where CEs are degraded fortuitously during the metabolism of other growth substrates, without gaining carbon or energy for bacterial growth [18]. Aerobic cometabolic degradation has been shown for all CEs, though only rarely described for PCE [22], and is related to certain aerobic bacteria, such as ethene oxidizers (ethenotrophs) and methane oxidizers (methanotrophs) [23–25]. Pure ethenotrophic strains of the genera *Mycobacterium* and *Nocardioides* can also degrade VC metabolically as their sole carbon and energy source [24], while *Polaromonas* sp. strain JS666 is the only microorganism known so far that is capable of oxidizing cDCE metabolically [26, 27]. A successful remediation treatment of a CE-polluted site in Lower Saxony (Germany) [7] showed how nZVI–AC injection could create conditions suitable for OHRB, with PCE subsequently dechlorinated to cDCE, though the subsequent cDCE degradation step was accomplished by an aerobic *Polaromonas* strain.

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.

Material and methods

Description of the study site

The pilot site, in the urban core of Balassagyarmat in Hungary, produced industrial electrical components between 1970 and 1994, but is mostly an industrial brownfield now. Geologically, the site comprises Quaternary alluvial sediments underlain by a sandstone bedrock weathered to silty fine-grained sand in its upper section. A two-layered aquifer system has developed in the Quaternary sediments, the upper layer consisting of Holocene fine-grained sand with a saturated thickness of

7 m and the lower layer consisting of Pleistocene coarse-grained sand and sandy gravel with a total thickness of 3 m (Fig. 1). The upper and lower layers are separated by a semipermeable layer of silt and silty sand 3 m thick. The upper layer is overlain by sandy-clay to clayey-sand of low permeability, approximately 2 m thick. The upper and lower layers have a hydraulic conductivity of $5.0 * 10^{-6}$ m/s and $5.0 * 10^{-3}$ m/s, respectively, based on pumping tests. Estimated groundwater flow velocities are approximately 0.01 m/day in the upper layer and 10 m/day in the lower layer.

The polluted groundwater plume, which is approximately 250 m wide (East–West) and 700 m long (North–South), is primarily contaminated with PCE, TCE and metabolites of reductive dechlorination—cDCE and VC present as minor co-contaminants. The volume of contaminated groundwater is estimated at about 190,000 m^3, and the plume is believed to contain around 15 kg of CEs (95 wt% PCE). Highest contaminant concentrations of between 85 and 120 µmol/L CEs (14–20 ppm PCE) were found in the coarse sandy–gravelly lower layer at 12 to 13 m below ground level (bgl). The groundwater of both layers was oxic (concentration of dissolved O_2 ranging from 5.2 to 6.2 mg/L) of the Ca-HCO_3^- type, with elevated mineralisation (total dissolved solids ranged from 900 to 1185 mg/L) and slightly alkaline (pH 7.3–7.4).

Continuous multichannel wells (CMT wells) were installed on site (Fig. 2), each with two tubes, separated by bentonite packers to ensure representative sampling, the depth and screening interval being different for each tube. The down-gradient CMT wells were monitored to examine the impact of nZVI–AC injection on groundwater conditions. Only in the closest down-gradient wells CMT 1 and CMT 2 the impact of nZVI–AC application was noticeable, therefore the suit of laboratory analyses of samples from these wells were extended with molecular biological tests and the whole dataset was assessed. Well CMT 1 was screened to a depth interval of 8.6–9.6 m bgl, representing the layer of fine-grained sand, whereas the well CMT 2 was screened at 13.4–14.4 m bgl, representing the lower course-grained sand and gravel layer. The up-gradient well, termed 14/04 (Fig. 2), was used to monitor the inflowing groundwater at a depth interval of 10.9–15.6 m bgl) and acted as a control.

nZVI–AC particles and injection parameters

nZVI–AC is an air-stable powder developed at the Helmholtz Centre for Environmental Research (UFZ; Germany), and is produced on an industrial scale by ScIDre GmbH (Germany). nZVI–AC consists of activated carbon colloids ($d_{50} \approx 1$ µm) with embedded nZVI structures with a mean Fe^0 content of 25 wt% [8]. Soluble CMC 75a was obtained from TiKEM (Hungary).

The nZVI–AC/CMC suspension used in this study was prepared by dispersion of the solid material in oxygen-free tap water under nitrogen atmosphere directly at the site. The suspension was injected by a Hydra-Cell G-10 hydraulic membrane pump (Wanner, UK) to a depth of 13 m bgl at three different injection points (Fig. 2) using TG63-150 penetrometer (Pagani,

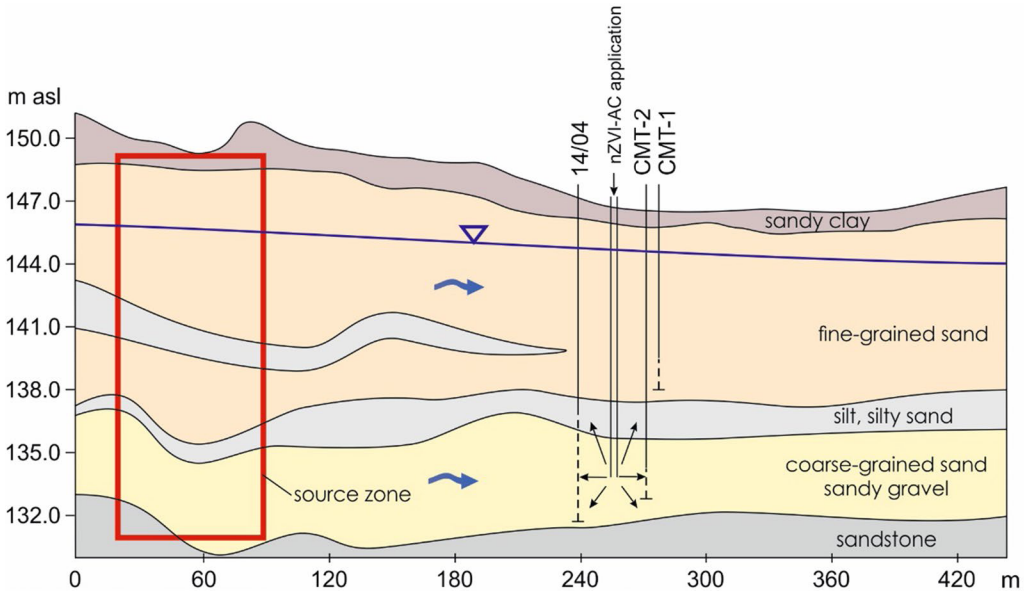

Fig. 1 Schematic geological cross section showing CEs source zone (red rectangle), up-gradient well 14/04, nZVI–AC application and down-gradient wells CMT 1 and CMT 2

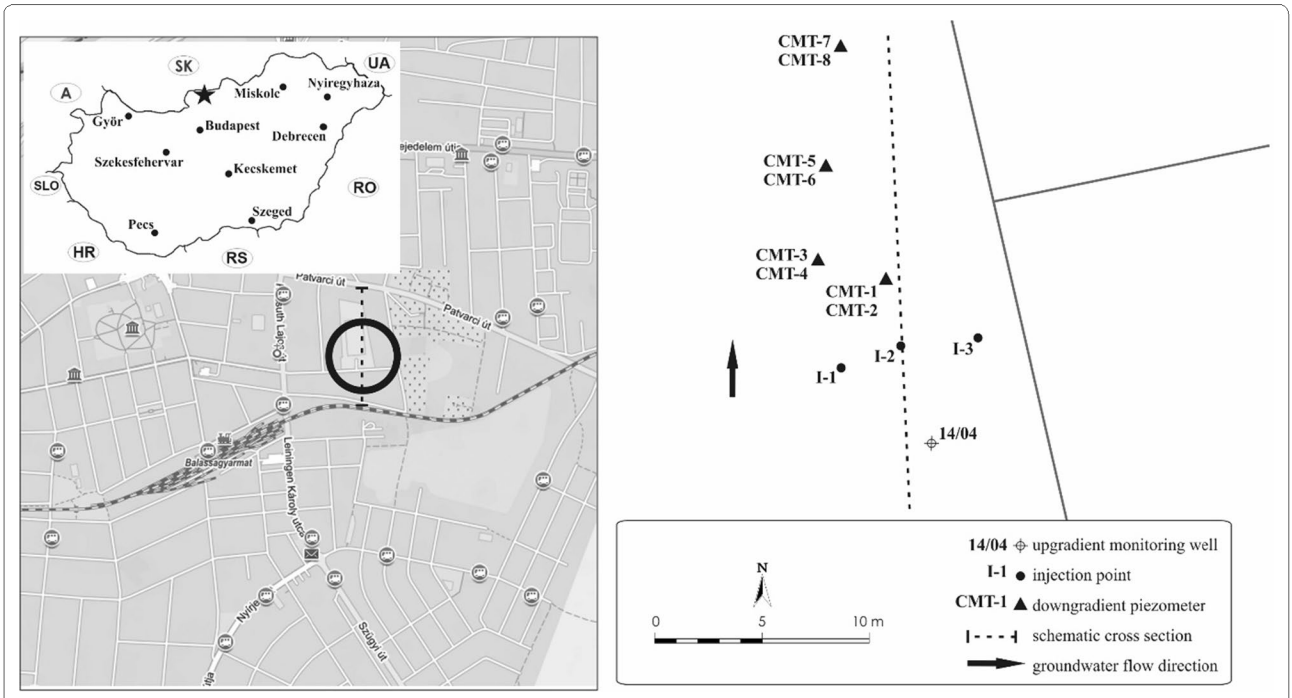

Fig. 2 Location and layout of the pilot test area in Balassagyarmat. CMT = continuous multichannel wells, I = nZVI–AC injection points, 14/04 = control monitoring well. Schematic cross section shown in Fig. 1 is indicated by dashed line

Table 1 Parameters of in situ nZVI–AC injection

Parameter	Dimension
nZVI–AC	176.8 kg
Fe^0 (25% in nZVI–AC)	44.2 kg
CMC	21.2 kg
Oxygen-free tap water	12.35 m^3
nZVI–AC concentration	~ 14.3 g/L
CMC concentration	~ 1.7 g/L
Injection pressure	0.5–4 bar
Injection flow rate	20–30 L/min
Injection depth at each injection point	13 m bgl
Soil porosity	30%

Italy) and Geoprobe pressure activated injection probe rods (Geoprobe Systems®, USA). The composition of nZVI–AC/CMC suspension and specific injection parameters are summarized in Table 1.

nZVI–AC injection took place in September 2015, with one groundwater sampling campaign undertaken prior to nZVI–AC application and three sampling campaigns taking place over an 85-day period after injection.

Measurement of chemical and physical parameters

Water samples were obtained using a low-flow technology Gigant pump (Eijkelkamp, Netherlands). Physico-chemical parameters were analysed directly in the field with groundwater level measured using a Heron interface meter (Heron Instruments, Canada), pH, dissolved oxygen, oxidation–reduction potential (ORP; recalculated to the standard hydrogen electrode (ORP_H)) and electrical conductivity measured with a Multi 350i multimeter (WTW, Germany). Concentration of nitrate and sulphate ions was analysed according to EPA method 9056A:2007 and concentration of total dissolved iron according to EPA method 6010C:2007. Concentration of PCE, TCE, cDCE and VC was assessed using an HP-7890 gas chromatography–mass spectrometer (GCMS; Agilent Technologies, Inc., USA) and concentration of ethene and ethane with an HP-5890 gas chromatograph with a flame-ionisation detector and thermal conductivity detectors (Agilent Technologies, Inc., USA). Reductive dechlorination of parent PCE by less chlorinated products to non-CE was expressed by the chlorine number (Cl no.), which was calculated as the weighted average number of Cl atoms per molecule of ethene [28].

DNA extraction and qPCR

Water samples for molecular genetic analysis were processed for DNA extraction immediately after sampling. The samples (0.5 l) were filtered through a 0.22 µm membrane (Merck Millipore, Germany) then DNA was extracted from the filter using the FastDNA Spin Kit for soil (MP Biomedicals, USA), according to the manufacturer's protocol.

The relative abundance of the 16S rRNA gene (representing total bacterial biomass), *Dehalobacter* spp., *Dehalococcoides* spp., *Dehalogenimonas* spp., *Geobacter* spp., *Desulfitobacterium* spp., *Gallionella*, *Geobacter*, the VC reductase genes *vcrA* and *bvcA* and functional genes encoding enzymes for ethenotroph-mediated aerobic biodegradation [i.e. alkene monooxygenase (*etnC*) and epoxyalkane:coenzyme M transferase (*etnE*)] were determined by qPCR, as described previously [29]. Briefly, qPCR reactions were prepared and performed in a LightCycler® 480 instrument (Roche, Switzerland). Crossing point (Cp) values were obtained using the Second Derivative Maximum method. The qPCR results were evaluated as relative quantification of the fold change between two states, with the condition of specific bacteria or genes prior to application taken as the starting point. Average Cp values (normalised to sample volume) of a given marker were summarised following calculation described in Nechanicka et al. [30]. The Cp values were first divided into two sets, one with values lower than 36 and the other with values equal to or higher than 36. The first set of values was divided into three intervals of the same size, with lowest values classified as 'high quantity', mean values classified as 'intermediate quantity'; values equal to or higher than 36 classified as 'low quantity', values between 36 and 39 classified as 'detection limit' and values equal to 40 classified as 'not detected'. All primers used for qPCR are listed in Additional file 1: Table S1.

16S rRNA sequencing

DNA for 16S rRNA sequencing (next generation sequencing, NGS) was extracted from a 30 mL sample using the MO BIO PowerWater DNA Isolation Kit (Carlsbad, California, USA). The 16S rDNA gene was amplified via PCR using the 8F (5′-AGAGTTTGATCC TGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTT ACGACTT-3′) primers [31]. Following PCR amplification, the DNA was stained and then placed in agarose gel, where it was subsequently separated using electrophoresis. The stained DNA was viewed under UV light and target ~ 1500 base pair products identified by comparison to a ladder of DNA fragments of varying lengths. Sequencing of the PCR amplicons for 16S rRNA was conducted using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) by targeting the V4 hyper-variable region (forward primer, 515F, 5′-GTGYCAGCMGCCGCGGTAA-3′; reverse primer, 806R, 5′-GGACTACHVGGGTWT CTAAT-3′) for 2 × 250-bp paired-end sequencing (Illumina) [31, 32]. PCR amplification was performed using the Roche FastStart High Fidelity PCR System (Roche Diagnostics Ltd, Burgess Hill, UK) on 50-µL reactions under the following conditions: initial denaturation at 95 °C for 2 min, followed by 36 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension step of 5 min at 72 °C. The PCR products were purified and normalised to ~ 20 ng each using the SequalPrep Normalization Kit (Fisher Scientific, Loughborough, UK). The PCR amplicons for all samples were then pooled in equimolar ratios. The run was performed using a 4 pM sample library spiked with 4 pM PhiX to a final concentration of 10%, following the method of Schloss and Kozich [33].

Sequencing data processing and analysis

Demultiplexed raw sequences were processed by DADA2 [34] following a standard DADA2 pipeline comprising (i) removal of low-quality and short reads, (ii) a search for and subsequent removal of chimeras (bimeras) and (iii) classification of reads against the SILVA reference database (version 13). Vegan [35] and phyloseq [36] R libraries were used for creation of the heatmap (only genera with a minimum relative abundance over 1% shown) and alpha-diversity comparison.

Results and discussion

Groundwater physical and chemical parameters

Injection of nZVI–AC resulted in a rapid decrease in groundwater ORP from both layers in the CMT well (Fig. 3a). ORP_H dropped from 192 to − 301 mV in the upper layer (CMT 1) and from 125 to − 347 mV in the lower layer (CMT 2). Six days after injection ORP_H increased to 102 mV at CMT 1 and up to − 45 mV at CMT 2, screened to the layer where nZVI–AC was injected and, therefore, more affected. The nZVI–AC suspension tended to sink slightly during and shortly after injection (13 m bgl, with the injection bulb having an estimated radius of 1.5 m in sediment porosity); hence, CMT 1 (8.6–9.6 m bgl) in the upper aquifer horizon was naturally less affected by the particles over time. ORP_H in both layers then increased further, returning to levels similar to pre-injection on day 35. A slight but temporary decrease in ORP_H was also observed in the upgradient well 14/04.

Groundwater pH increased immediately after injection from an initial value of around 7.3 to 7.6 in in well CMT 1 to 8.2 in well CMT 2 due to a reaction between nZVI and dissolved oxygen and water that generated

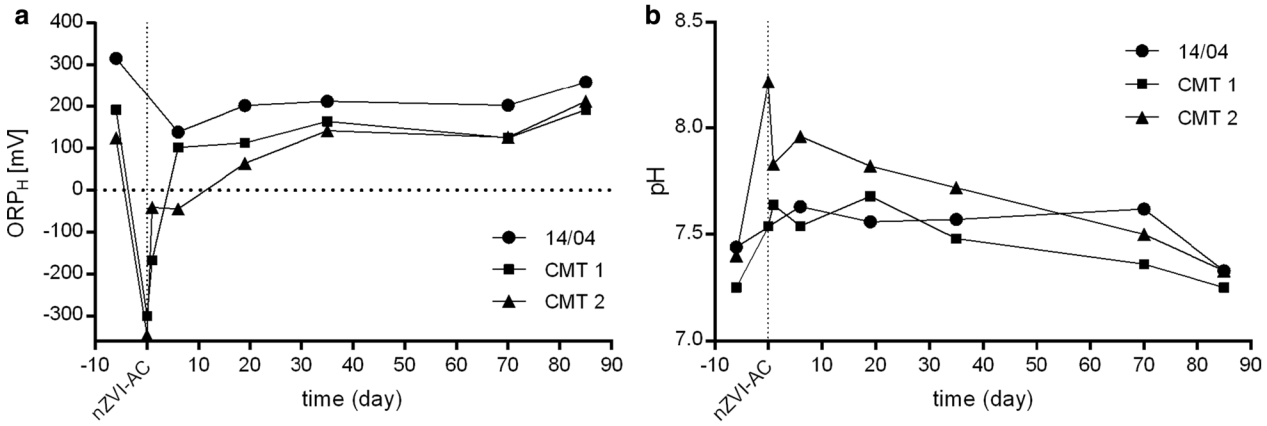

Fig. 3 Changes in ORP$_H$ (**a**) and pH (**b**) after nZVI–AC injection at day 0. CMT 1 and 2 = continuous multichannel wells, 14/04 = control monitoring well

OH^- [2]. The pH of both layers had dropped to the initial level of approximately 7.3 at the end of the study on day 85 (Fig. 3b). A similar pH trend to that in CMT 1 was also observed in groundwater from the upgradient well 14/04, indicating a temporal effect of nZVI–AC injection on groundwater in nearby upgradient areas (discussed further below).

An initially high dissolved oxygen concentrations of 5.2 to 6.2 mg/L indicated oxic conditions in both the upper and lower aquifer layers (Fig. 4a). While such conditions lead to a shorter lifetime for the reactive function of the material, and hence are outside the recommended application window for abiotic CE treatment with nZVI–AC, we decided to proceed with the injection. After nZVI–AC application, depleted dissolved oxygen was only

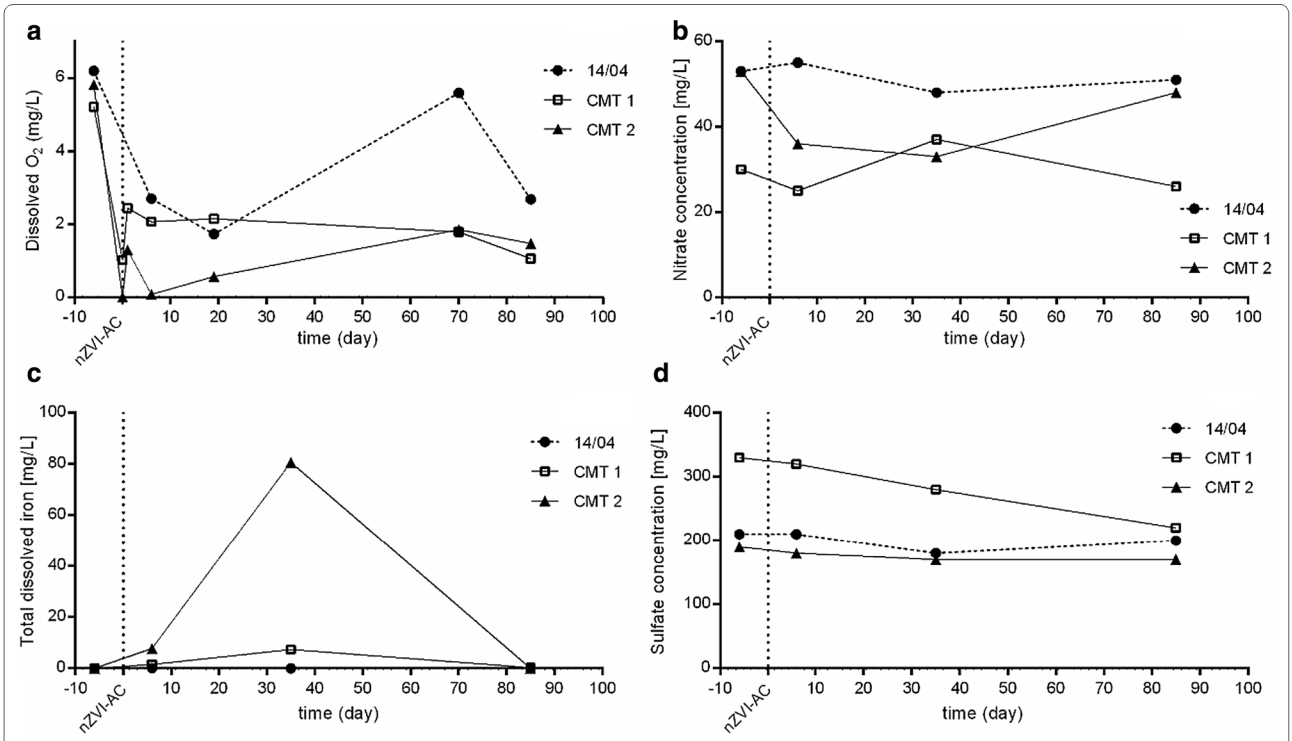

Fig. 4 Concentration of dissolved oxygen (**a**), nitrate (**b**), total dissolved iron (**c**) and sulphate (**d**) 6 days before and after nZVI–AC injection. CMT 1 and 2 = continuous multichannel wells, 14/04 = control monitoring well

observed in the lower layer of CMT 2 on day 0; however, on the following day dissolved oxygen increased to 1.3 mg/L, and similar levels were observed for the rest of the pilot study. This was due to the inflow of oxic groundwater being higher than the reducing capacity of the nZVI–AC. In comparison, the effect of nZVI–AC on groundwater dissolved oxygen concentration in the upper layer of CMT 1 and in the upgradient area (14/04) was much lower (Fig. 4a).

The dynamics of nitrate concentration in both layers (Fig. 4b) suggested an insignificant, or at most temporary, effect of nZVI–AC on overall redox conditions. Nitrate concentration showed a dramatic decrease of 32% in lower layer groundwater up to day 35; however, levels had been re-established by the end of the study due to the high inflow of nitrate-rich groundwater and the weakening effect of nZVI–AC. The original concentration of dissolved iron (Fe) was close to its detection limit (0.1 mg/L) at this site (Fig. 4c). Application of nZVI–AC led to a significant increase in the upper and lower layers (7.4 and 80.7 mg/L, respectively) on day 35. This 35-day increase in soluble Fe(II) probably occurred as a consequence of a complexing effect between CMC and microbial processes, where CMC fermentation products or H_2 (produced by reaction of nZVI) acted as an electron donor with Fe(III) as the electron acceptor. These data are in accordance with our previous studies [16, 37], where we suggested that Fe(II) is derived not only from naturally present iron, but is also recycled from the injected nZVI that was oxidized to Fe(III). The effect of nZVI–AC on sulphate concentration in lower layer groundwater (Fig. 4d) was even lower than that observed for nitrate (Fig. 4b) as conditions suitable for microbial sulphate reduction were not established. The linear decrease of sulphate concentration detected in the upper layer of CMT 1 does not appear to be related to abiotic reduction by nZVI–AC.

As the presence of electron acceptors in groundwater, mainly dissolved oxygen and nitrate has the negative effect on the CE degradation by nZVI–AC, consumption of the reducing power of nZVI by the reduction of dissolved oxygen and nitrate was estimated as follows.

Consumption of electrons by a reduction of oxygen and nitrate was calculated using the appropriate reduction reactions [38]:

$$\frac{1}{2}O_2 + 2H^+ + 2e^- = H_2O$$

$$NO_3^- + 6H^+ + 6e^- = \frac{1}{2}N_2 + 3H_2O$$

and a conservative assumption that the whole amount of applied nZVI–AC distributed in the lower layer with radius of influence of 1.5 m filled the void space of effective porosity of 20% in the area of approximately 26 m^2 (3 m diameter × 8.7 m depth). By taking the groundwater flow velocity of 10 m/day, the reducing power of nZVI (total number of electrons potentially donated from Fe^0 oxidation to Fe^{3+}) was theoretically exhausted in 9 days on the chemical reduction of dissolved oxygen and nitrate flowing into the treatment zone. Even though CMC also provided the reducing power in the process of its biological degradation and the fact that not all amount of nitrate was reduced, it was evident that the amount of nZVI–AC was not sufficient for substantial and long-term degradation of CE.

Monitoring of PCE and its degradation products

While CE concentrations in groundwater from the upgradient control well (14/04) remained more-or-less stable throughout the study (Fig. 5a), those in groundwater from the upper layer of CMT 1 and the lower layer of CMT 2 were lower (Fig. 5b, c), having been affected by nZVI–AC application.

Highest initial PCE concentrations were detected in groundwater from upgradient well 14/04 (121.8 µmol/L) and in groundwater from the lower layer of CMT 2 (84.4 µmol/L), with just 1.4 µmol/L detected in the upper layer of CMT 1. A rapid decrease in PCE concentration was observed in both downstream layers (90% decrease in CMT 1 and 99.8% in CMT 2) 6 days after nZVI–AC injection, followed by a slow increase to 72% (CMT 1) and 58% (CMT 2) of the initial concentrations by the end of the field experiment (day 85) due to continuous inflow of contaminated groundwater. The Cl no. ranged from 3.7 to 4 prior to injection, reflecting a very low dechlorination level of the parent contaminant PCE (Fig. 6). In comparison, the Cl no. remained high (4.0) throughout the study in groundwater from control well 14/04, indicating that reductive dechlorination did not play a significant role in the groundwater around this well. Cl no. dynamics in CMT 1 and CMT 2 indicated that reductive dechlorination contributed noticeably to a temporal decrease in PCE concentration and the subsequent formation of daughter products. This process could either have been abiotic or, after a lag phase, biotic, the process being mediated by OHRB where H_2, originating from the reaction between nZVI and water, serves as an electron donor. In CMT 1, the Cl no. decreased slightly from 3.7 to 3.0, 6 days after nZVI–AC injection, but had returned to its initial level (Cl no. 3.7) by day 35. Reductive dechlorination in CMT 1 appeared rather weak, most likely due to limited migration of nZVI–AC into the upper layer, as also observed by Mackenzie et al. [6] at a different site. The lower layer (CMT 2) exhibited the greatest decline in Cl no., falling from a baseline of 4.0 to 0.4 after 6 days.

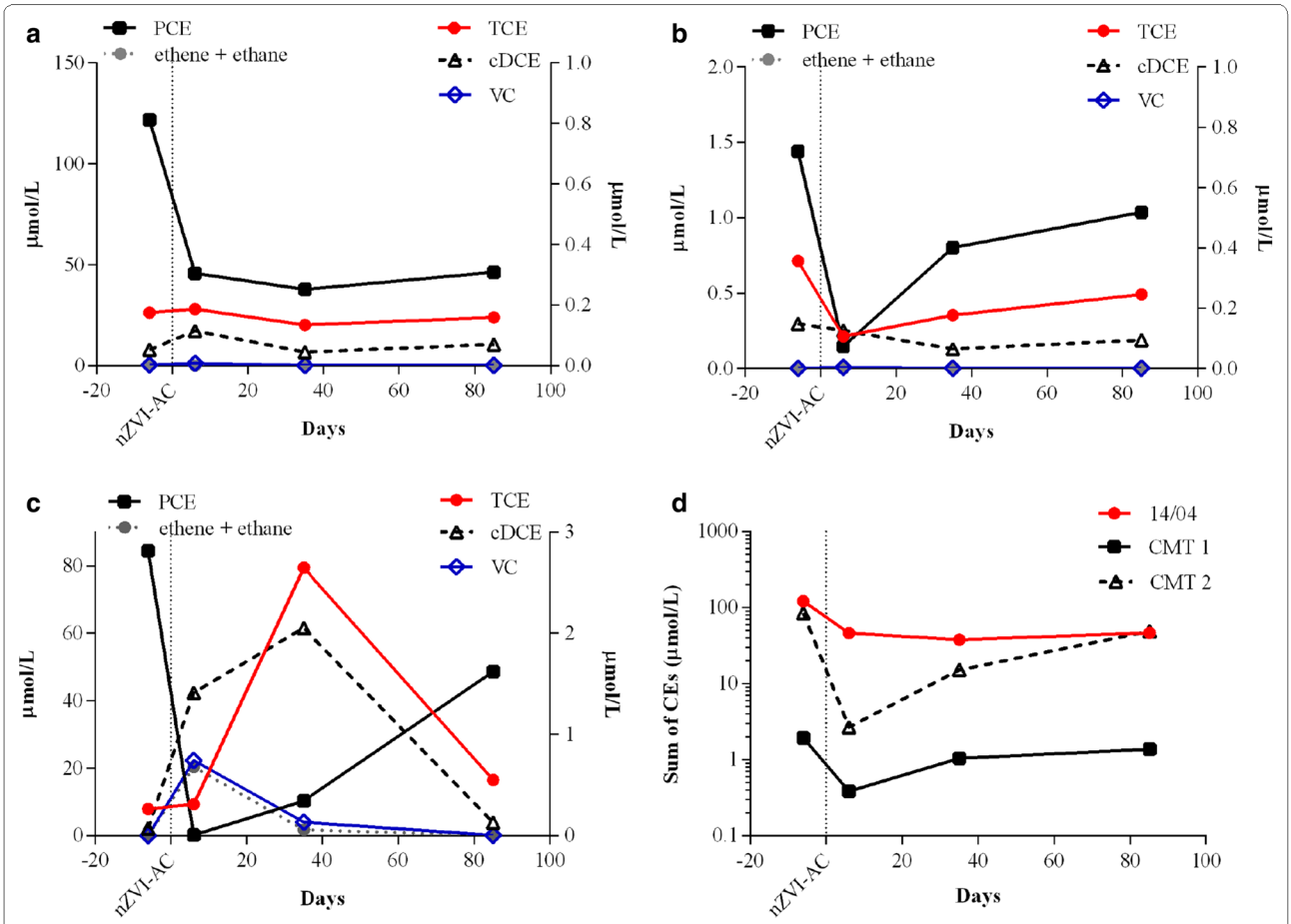

Fig. 5 Concentrations of CEs, ethene and ethane in groundwater from wells 14/04 (**a**), CMT 1 (**b**) and CMT 2 (**c**). Left axis represents PCE and the sum of ethene and ethane, while the right axis represents TCE, cDCE and VC. The sum of CE concentrations in a logarithmic scale (**d**)

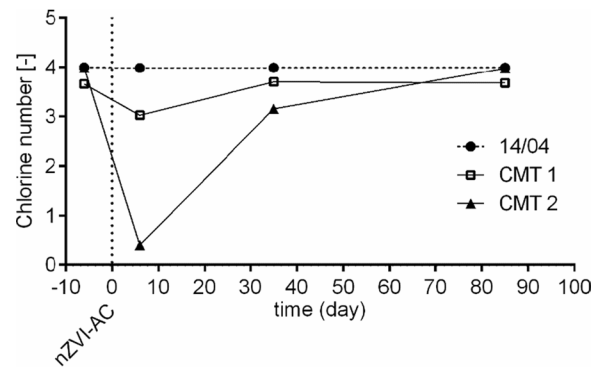

Fig. 6 Changes in Cl no. following nZVI–AC injection at day 0, calculated as the weighted average number of Cl atoms per molecule of ethene

An increase in pH from a baseline of 7.4 to 8.2 on day 0 documented corrosion of nZVI by water. The gradual restoration of both Cl no. and CE concentration (Figs. 5d, 6) to their initial values by the end of the test indicates that the effect of nZVI–AC injection was temporary. This was probably due to the material itself having a limited lifetime, due mainly to a strong inflow of other electron acceptors (such as dissolved oxygen and nitrate) into the treatment zone, together with PCE from upgradient areas.

Initial levels of less chlorinated metabolites (TCE, cDCE and VC) were low throughout the aquifer. Maximal initial concentrations were 0.357 µmol/L for TCE, 0.149 µmol/L for cDCE in well CMT 1 and 0.003 µmol/L for VC in well CMT 2. Less chlorinated metabolite concentrations differed between wells. On day 6, TCE and cDCE concentrations decreased temporarily in well CMT 1, while VC concentration increased slightly then dropped below the detection limit until the end of the study, both confirming low level of reductive dechlorination in CMT 1.

Responses differed in the lower layer at well CMT 2, where TCE, cDCE and VC concentrations increased

alongside nZVI–AC injection and PCE degradation (Fig. 5c). On day 35, TCE had increased by a factor of 10 (3.2% of initial PCE concentration transformed) and cDCE by a factor of 28 (2.5% of the initial PCE), while VC had increased by a factor of more than 900 by day 6 (0.8% of PCE before injection). Subsequently, there was a decline in metabolite concentrations, though by the end of the study levels were still higher than initial readings.

In the upgradient well 14/04, concentrations of the less chlorinated metabolites TCE, cDCE and VC showed only minor changes, all within typical deviation limits for field studies (Fig. 5a). By day 6, the cDCE concentration had increased by a factor of 2.2 and VC concentration by a factor 2.5, though levels subsequently dropped to (cDCE) or below (VC) their initial levels.

The initial levels of both ethene and ethane (non-chlorinated metabolites of CE anaerobic degradation) were below detection limits (< 0.001 µmol/L) in both monitoring wells and, aside from CMT 2, did not change significantly over course of the study. In CMT 2, application of nZVI–AC resulted in a significant increase in ethene and ethane to 10.4 µmol/L and 10.0 µmol/L, respectively, by day 6, followed by a rapid decrease toward the end of the study (Fig. 5c).

Overall, nZVI–AC injection resulted in mild and short-term reductive dechlorination of CEs in the upper layer (CMT 1), and noticeable, though time-limited, changes in the lower layer (CMT 2). Previous studies have shown that, while biological reductive dechlorination of CEs can occur under both nitrate-reducing and iron-reducing conditions, sulphate reducing and methanogenic conditions are most favourable for OHRB [39]. Unfortunately, such conditions were not established at our site.

Bacterial community

Bacterial community prior to nZVI–AC application

The microbial community in the aquifer was characterized by a high abundance of genera known for their involvement in nitrogen geochemical cycles, including nitrite-oxidizing *Nitrospira*, ammonia-oxidizing Candidatus *Nitrosoarchaeum*, nitrate-reducing *Rhodoferax* and *Methylotenera*, and iron: iron-oxidizing *Gallionella* and *Sediminibacterium*, iron-reducing *Rhodoferax*, *Ferribacterium* and *Geobacter*.

In contrast, the abundance of sulphate-reducing bacteria detected by NGS in our dataset was very low. The families Desulfobulbaceae and Desulfovibrionaceae were only detected in well CMT 1, with no assigned genera (data not shown). On the other hand, the sulphur-oxidizing genus *Sulfuritalea* was found in all samples and was recorded at highest abundance in the lower zone of well CMT 2. Finally, qPCR revealed a higher initial abundance of *Desulfitobacterium* in well 14/04.

Noticeable differences in the initial abundance of OHRB and VC reductase genes were detected by qPCR in the upper and lower layers of groundwater. In general, a higher abundance of OHRB (comprising the genera *Dehalobacter*, *Dehalococcoides* and *Dehalogenimonas*) were found in the upper groundwater layer in CMT 1 than the lower layer in CMT 2. Low numbers of OHRB in the lower layer most likely relate to its high permeability and the high mass fluxes of oxygen and nitrate that inhibit organohalide respiration [40]. The slightly higher degree of PCE reductive degradation observed in the upper groundwater layer (Cl no. 3.7 in the upper layer compared to 4.0 in the lower layer, Fig. 6) may reflect the stratification of OHRB.

NGS analysis did not reveal the presence of any OHRB other than the genus *Dehalogenimonas*, the only assigned member of the phylum Chloroflexi (below 1% of total genera detected), the genera *Desulfitobacterium*, *Dehalobacter* and *Dehalococcoides* not being detected. Nevertheless, qPCR confirmed the presence of these OHRB, together with VC reductase genes, in the aquifer. A similar discrepancy between NGS and qPCR data has been reported previously [41, 42], the previous authors explaining this by both a very low relative abundance of OHRB in the bacterial community and more efficient PCR amplification using the highly specific qPCR primers rather than the universal primers used for NGS.

On the other hand, NGS did reveal the presence of *Trichococcus* in the lower layer of CMT 2, a bacterial genus capable of dechlorinating PCE and TCE with the TceA enzyme [43]. Furthermore, the groundwater in both layers contained the genus *Aquabacterium*, capable of dechlorinating under anaerobic conditions [44]. Similarly, the proteobacterial group MND1, which was abundant in well 14/04, and *Hydrogenophaga*, detected in high numbers in both layers of the CMT wells, have both been recorded in relation to remediation of contaminated soils [44, 45]. In reference well 14/04, *Pseudorhodobacter* were the most abundant bacterial genus; however, while the genus has previously been detected on sites contaminated with CEs [41], there is no evidence of its association with CE degradation.

NGS also detected several genera known for their ability to degrade CEs aerobically, namely *Polaromonas* and *Pseudomonas*. Bacteria of the genus *Polaromonas* are commonly found in groundwater samples at CE-contaminated sites, even in deep wells where anoxic conditions predominate [7, 41, 43, 46]. Interestingly, *Polaromonas* sp. strain JS666 is the only microorganism known to oxidize cDCE by using it as a sole carbon and energy source [26]. The genus *Pseudomonas* comprises

strains capable of aerobic cometabolic PCE and TCE degradation and VC-assimilation [18, 24, 47].

Effect of nZVI–AC application

Soon after nZVI–AC injection, the bacterial genera *Rhodoferax* and *Ferribacterium,* both involved in iron reduction (*Rhodoferax* also respires with other electron acceptors, including nitrate), and *Methylotenera,* also involved in nitrate reduction, proliferated in both layers of in the CMT wells (Fig. 7). This increase is most likely related to the establishment of more favourable redox conditions and the availability of electron donors following application of the nZVI–AC/CMC suspension. The facultative anaerobe *Rhodoferax* has been shown to assimilate VC as a primary growth substrate [48]; further, it could regenerate iron for additional CE reduction by reducing oxidized Fe(III) to Fe(II).

Both iron-oxidizing *Gallionella* and iron-reducing *Geobacter* have previously been shown to dechlorinate PCE or TCE [49, 50]. In our study, *Gallionella*, which was initially found at a relatively high abundance in the upper layer of CMT 1, proliferated 1 day after nZVI–AC injection. However, a much greater increase was observed in the lower layer of CMT 2, 35 days after the injection (Table 2; Fig. 8). This corresponded with elevated levels of total dissolved iron (Fe(II)). *Gallionella* is commonly found in the groundwater of CE-contaminated sites [47, 51] and has been described as capable of dechlorinating TCE under sulphate-reducing conditions [49]. While iron-reducing *Geobacter* were already abundant in well 14/04 and in the upper layer of CMT 1, their abundance increased even further after nZVI–AC application (Table 2; Fig. 8).

On the other hand, relative abundance of the iron-oxidizing genus *Sediminibacterium* declined over the course of the study (Fig. 7). This may have been caused by an increasingly complex microbial community structure over time, leading to increased interspecific competition.

Application of nZVI–AC resulted in a decrease in the abundance of sulphur-oxidizing *Sulfuritalea* in the upper and lower layers of the CMT well by day 6, followed by partial recovery (Fig. 7). *Desulfitobacterium* was the only sulphate-reducing bacteria detected in the aquifer; thus, we can assume that groundwater conditions were insufficiently reduced by nZVI–AC to support sustained growth of sulphate-reducing bacteria. This is also supported by the changes in groundwater sulphate concentrations, with the most significant changes in CE concentration being observed on day 6 in the lower layer CMT 2 and an increase in the abundance of *Desulfitobacterium,* known to degrade PCE down to cDCE (Table 2; Fig. 8). However, other reductive biomarkers were also found in similar (*Dehalobacter*, *Dehalogenimonas,* VC reductase gene *bvcA*) or lower (*Dehalococcoides,* VC reductase gene *vcrA*) levels (Table 2; Fig. 8), suggesting that reductive dechlorination of CEs was dominantly

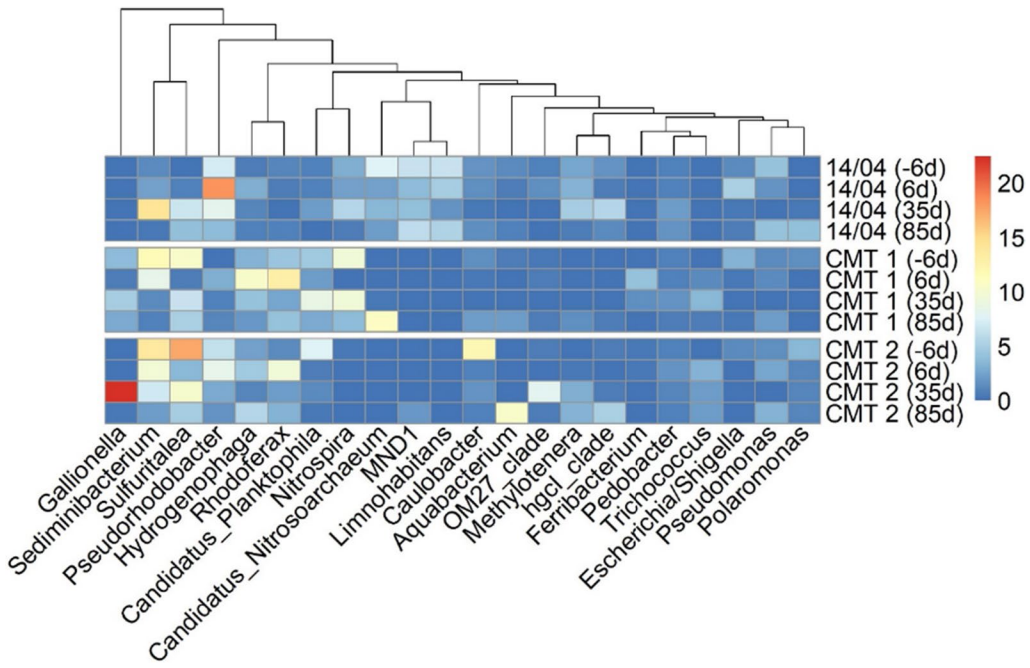

Fig. 7 Bacterial community composition in wells CMT 1, CMT 2 and 14/04 before (– 6 days) and after (6 days, 35 days and 85 days) nZVI–AC injection. Only genera with a relative abundance > 1% shown

Table 2 Summary of qPCR results for changes in the relative abundance of biomarkers following nZVI–AC injection (day 0)

Well	Time before and after nZVI–AC injection (days)				
14/04	**− 6**	**1**	**6**	**35**	**85**
16S rRNA	++	+++	+++	++	+
Desulfitobacterium	+++	+++	+++	++	+
Dehalobacter	++	+++	+++	++	+−
Dehalococcoides	ND	ND	ND	ND	+
vcrA	ND	ND	ND	ND	+
bvcA	ND	ND	ND	ND	+++
Dehalogenimonas	++	+	+−	+−	+−
Gallionella	+	+	+	+	++
Geobacter	++	++	+++	+++	+
etnC	+++	++	+++	+++	+
etnE	+++	++	+++	++	+−
CMT 1					
16S rRNA	+	++	++	++	++
Desulfitobacterium	+	++	+++	+++	+++
Dehalobacter	++	+++	++	++	+++
Dehalococcoides	+++	+++	++	+++	+++
vcrA	ND	++	ND	+++	ND
bvcA	ND	+++	ND	ND	++
Dehalogenimonas	+++	+++	++	++	+++
Gallionella	++	+++	++	++	+++
Geobacter	+	+++	++	+	+++
etnC	+	+	+	+−	+
etnE	+	+−	+	+−	+−
CMT 2					
16S rRNA	+	+	+	++	+
Desulfitobacterium	+	+	+++	+	+++
Dehalobacter	+	++	+	+	++
Dehalococcoides	+	+	+−	+	+
vcrA	++	ND	ND	+	++
bvcA	ND	ND	ND	ND	ND
Dehalogenimonas	+	++	+	+	+
Gallionella	+	++	++	+++	++
Geobacter	+	+	+	+	+
etnC	+−	+−	+−	+−	+−
etnE	+	+++	+	+v	++

Calculations were based on Cp values, with +++ = high abundance; ++ = medium abundance; + = low abundance; +− = at detection limit; ND = not detected

abiotic and can be attributed to nZVI–AC application. The abundance of *Dehalobacter, Desulfitobacterium* and the gene *vcrA* showed an increasing trend up to day 85, though not enough to result in significant reductive dechlorination activity as the Cl no. also increased at the same time (Fig. 6).

OHRB and VC reductase genes were more abundant in the upper layer of CMT 1 (compared to the lower layer) throughout the study; however, their abundance increased even further following nZVI–AC injection. Nevertheless, OHRB activity in this layer did not exhibit significant and sustainable reductive CE dechlorination.

Of the bacteria potentially participating in CE dechlorination, *Trichococcus* increased in both aquifer layers, culminating on day 6 in the lower layer and day 35 in the upper layer, after which it decreased again but not to its initial levels. Bacteria of the genus *Aquabacterium* declined in both layers following nZVI–AC application. By the end of the study (day 85), however, they had proliferated remarkably (Fig. 7).

Of the bacteria potentially degrading CEs aerobically, *Polaromonas* abundance decreased in both aquifer levels following nZVI–AC application (Fig. 7); however, while it declined to non-detectable levels in the upper layer (CMT 1) it remained detectable in the lower layer (CMT 2) throughout the study, indicating a potential for oxidative degradation of CEs. Similarly, the genus *Pseudomonas* exhibited had decreased in abundance by day 35 in both aquifer layers, but had staged a distinct recovery by the end of the study (day 85; Fig. 7). A slight increase in the abundance of the ethenotroph functional gene *etnE* was detected on day 35, indicating the potential for aerobic oxidative degradation of VC and cDCE (Table 2; Fig. 8).

Overall, NGS failed to show dramatic changes in the microbial community induced by nZVI–AC application. Bacterial community alpha-diversity showed high between-sample similarity prior to nZVI–AC injection, with no significant differences found after injection (Additional file 1: Fig. S1). The reducing effect of nZVI–AC resulted in short-term proliferation of nitrate and iron reducers only, with the reduced iron generated inducing an increase in iron-oxidizing bacteria at a later stage.

Owing to high initial concentrations and continuous mass influx of nitrate and dissolved oxygen, conditions for sulphate-reducing microorganisms and OHRB were unfavourable. As reported previously [39], presence of electron acceptors other than CEs has an inhibitory effect on organohalide respiration. Nevertheless, while optimal redox conditions required for anaerobic OHRB (i.e. sulphate-reducing or methanogenic [37]) were not achieved, these bacteria were detected in all wells prior to, and at slightly increased numbers after, nZVI–AC injection.

On the other hand, low dissolved oxygen levels permitted the concurrent presence of OHRB and CE oxidizing *Pseudomonas* and *Polaromonas* (both capable of assimilating cDCE), which is in accordance with the first field-scale study by Vogel et al. [7], who observed

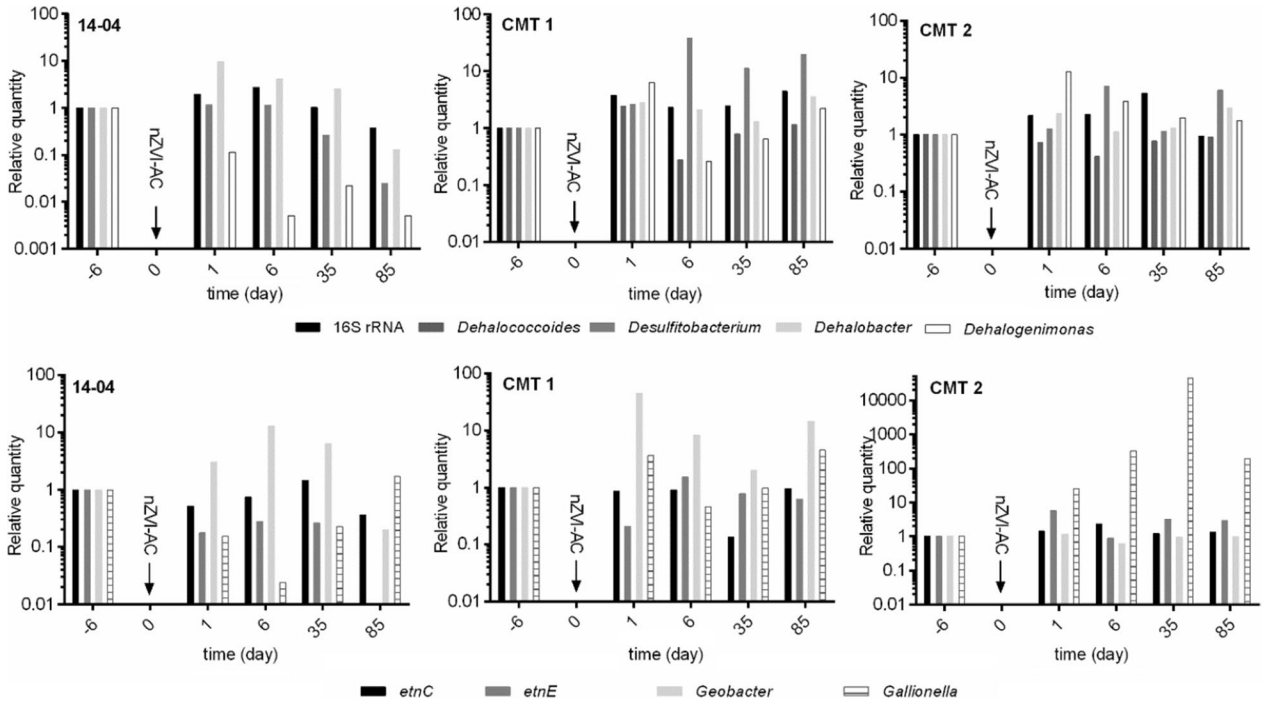

Fig. 8 Changes in the relative abundance of biomarkers following nZVI–AC injection. All results are expressed as relative quantity, each marker's abundance being compared to its state prior to injection. Note the logarithmic scale

nZVI–AC-supported organohalide respiration of PCE complemented by the oxidative degradation of cDCE by *Polaromonas*, even in deep anoxic wells 25 m bgl. Furthermore, detection of the ethenotroph functional gene *etnE* indicated the potential for ethenotroph-mediated aerobic biodegradation of CE metabolites. Co-occurrence of OHRB and VC assimilating bacteria has also been found in groundwater [17, 52, 53] and discrete aquifer soil samples [25] at other sites.

Conclusions

This study describes the effect of application of an 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.

The main findings are as follows:

Hydrochemical data indicated CE transformation with the 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 sulphate 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. 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 cDCE. A potential for aerobic oxidative degradation of CE metabolites was also indicated through the detection of the ethenotroph functional gene *etnE*.

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.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12302-020-00434-2.

Additional file 1: Table S1. Specific primer pairs used for qPCR method. **Figure S1.** Alpha-diversity measures: comparison of wells based on Chao 1 and Inv. Simpson alpha-diversity indexes.

Abbreviations

AC: Colloidal activated carbon; bgl: Below ground level; cDCE: *cis*-1,2-Dichloroethene; CE: Chlorinated ethene; Cl no.: Chlorine number; CMC: Carboxymethyl cellulose; CMT: Continuous multichannel well; DNA: Deoxyribonucleic acid; NGS: Next generation sequencing; nZVI: Nanoscale zero-valent iron; OHRB: Organohalide-respiring bacteria; ORP: Oxidation–reduction potential; PCE: Tetrachloroethene; qPCR: Quantitative polymerase chain reaction; TCE: Trichloroethene; VC: Vinyl chloride.

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Authors' contributions

LT, KM, JL, MC and AS designed the study. LT led in situ application of nZVI–AC and performed the chemical analysis. CB performed the 16S rRNA sequencing, NN and MC performed the qPCR analysis and RŠ analysed the data. MC, NN and JN wrote the draft manuscript, AS, KM and MC reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data necessary to interpret and replicate the findings reported herein are included in the published article and its additional files. Other data used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

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

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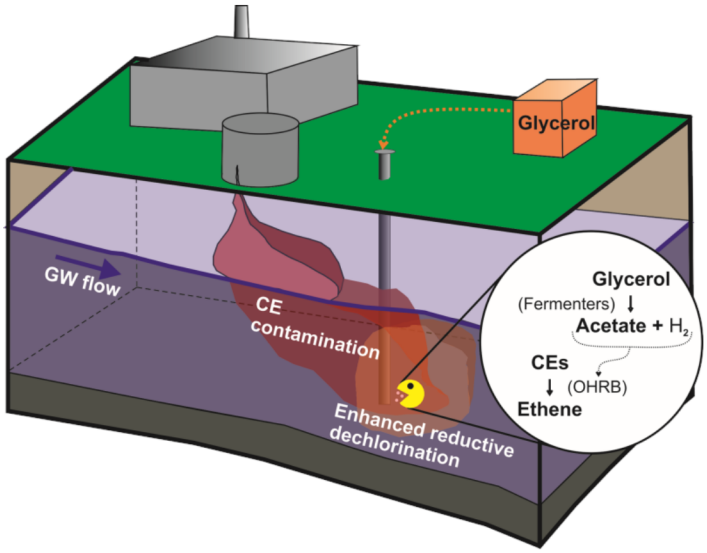
Glycerol-supported bacterial populations successfully degrade chlorinated ethenes (CEs). Detailed characterization of the microbial community is important for improving bioremediation strategies.



ABSTRACT

Chlorinated ethenes (CEs) are common and persistent contaminants of soil and groundwater. Their degradation is mostly driven by a process of bacterial reductive dechlorination (also called organohalide respiration) in anaerobic conditions. This study summarizes the outcomes of the long-term in-situ application of glycerol for the enhanced reductive dechlorination of CEs on a highly contaminated site. Glycerol injection resulted in an almost immediate increase in the abundance of fermentative Firmicutes, which produce essential sources of carbon (acetate) and electrons (H_2) for organohalide-respiring bacteria (OHRB) and change groundwater conditions to be suitable for OHRB growth. The decreased redox potential of groundwater promoted also the proliferation of sulfate and iron-reducing bacteria, which compete for electron donors with OHRB but at the same time support their growth by producing essential corrinoids and acetate. A considerable increase in the abundance of OHRB *Dehalococcoides*, concurrently with vinyl chloride (VC) reductase gene levels, was revealed by real time polymerase chain reaction (qPCR) method. Consistent with the shifts in bacterial populations, the concentrations of pollutants tetrachloroethylene and trichloroethylene decreased during the monitoring period, with rising levels of *cis*-1,2-dichloroethylene, VC, and most importantly, the final CE degradation products: ethene and ethane. Our study implies the importance of syntrophic bacterial interactions for successful and complete CE degradation and evaluates glycerol as a low-cost substrate to enhance reductive dechlorination and as an effective source of electrons for OHRB.

Keywords

Enhanced reductive dechlorination, Organohalide-respiring bacteria, Chlorinated ethenes, Glycerol, Bioremediation

INTRODUCTION

In-situ bioremediation via enhanced reductive dechlorination represents a widely applied remediation approach for the treatment of sites contaminated by chlorinated ethenes (CEs).[1] The incomplete degradation of the parent chlorinated compound tetrachloroethylene (PCE) and trichloroethylene (TCE) leads to the accumulation of less chlorinated but more soluble and mobile daughter products, namely, *cis*-1,2-dichloroethylene (*c*DCE) and vinyl chloride (VC), in the aquifer.[2] Biostimulation to enhance the reductive dechlorination of CEs by an indigenous microbial community capable of organohalide respiration is a cost-effective remediation method; however, careful monitoring of microbial community structure at the site together with CE transformation is necessary.[3–5] Organohalide-respiring bacteria (OHRB) comprise microorganisms that can use CEs as terminal electron acceptors in their respiration process to yield energy for growth.[6] Several anaerobic bacteria can carry out the incomplete degradation of PCE and TCE to *c*DCE (*Desulfitobacterium*, *Dehalobacter*, *Sulfurospirillium*, and *Geobacter*); however, only two members of phylum Chloroflexi, namely, *Dehalococcoides* and *Dehalogenimonas*, have been described thus far to be capable of complete dechlorination of *c*DCE to VC and ethene using VC reductases.[7] Their presence in contaminated sites correlates with ethene production, whereas their absence leads to *c*DCE and VC accumulation. Under field conditions, various environmental factors influence the proliferation and activity of OHRB, such as the availability of electron donors, competition of other naturally occurring electron acceptors (nitrate, sulfate, dissolved oxygen), or oxidation–reduction potential on site.[6,8]

Electron donor addition is usually necessary to enhance organohalide respiration on CE-contaminated sites. Given that most OHRB require molecular hydrogen (H_2) as an electron donor, selected carbon sources that can be fermented to H_2 are usually applied on polluted sites.[1] 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.[3,9–13] Although H_2 is produced by fermentation of all of these substrates, the rate and extent of H_2 formation differ significantly and affect the rate of dechlorination.[9] The application of a readily degradable substrate results in the formation of an anaerobic reducing environment suitable for reductive dechlorination by the depletion of oxygen, which is inhibitory to OHRB, and due to the released H_2. Electrons from H_2 cause an immediate increase in the relative electron activity of groundwater, i.e., a decrease in the oxidation–reduction potential.[14] The OHRB compete for H_2 with methanogenic bacteria, which also use H_2 as an electron donor; however, the minimum H_2 concentration for dechlorinating bacteria is ten times lower than that for methanogens.[15] Thus, the addition of slowly fermentable substrates, such as butyrate or propionate, which release H_2 in low concentrations, should provide most of the produced H_2 for dechlorination reactions.[16]

Among the above-mentioned substrates, glycerol is widely used in biological wastewater treatment plants to enhance biodegradation and seems as a suitable substrate for bioremediation purposes.[12] Bacteria can benefit from high emission of H_2 and high organic carbon content. Moreover, glycerol has a high chemical oxygen demand (COD, 950 g/L), helping to establish anaerobic conditions favorable for OHRB. Advantageous for field application is its low price and easy transportability in the form of a concentrate.

This study aimed to summarize and interpret the results of the long-term in-situ application of glycerol to stimulate the OHRB-driven dechlorination of a heavily CE-polluted aquifer. The physico-chemical parameters of groundwater together with changes in microbial community structure were analyzed prior to and after the glycerol injection over a period of seven months.

MATERIALS AND METHODS

Site description

The in-situ glycerol application and monitoring were held in the area of the Spolchemie Company a.s. (Ústí nad Labem, Czech Republic), which is historically one of the most important producers of synthetic resins and chlorinated hydrocarbons in the Czech Republic. The production and storage of chlorinated methane and ethenes caused extensive contamination of soil and groundwater on the site. The geological profile in the Spolchemie site consists of Mesozoic Cretaceous siltstones overlaid by a 15 m-thick CE-polluted Quaternary terrace comprised mainly of fluvial sediments from the Rivers Bílina and Labe and the Klíšský stream. The aquifer has moderate hydraulic conductivity (K 10^{-4} to 10^{-5} $m.s^{-1}$).[17]

Groundwater samples were obtained from glycerol application well HV-103 and reference wells RW5-24 and RW5-43 situated on the inlet side of the contamination plume zone.

Glycerol application and sampling

The application was performed via a pressure system with packers into sealed well HV-103. The injection of glycerol substrate was conducted in two campaigns. During the first and second application campaign, 3 (concentration 65 g/L) and 1 m^3 (concentration 195 g/L) glycerol solutions were applied at a 3-month interval, respectively.

Groundwater samples for molecular biology and physico-chemical analysis were obtained from application well HV-103 over a period of 210 days. However, groundwater in the reference well RW5-24 was accidentally affected by an alongside-running field experiment during the monitoring period, and the sampling had to be stopped after 43 days. Nevertheless, data from previous monitoring (1 year prior to this study) confirmed its long-term stability (Fig. S1). Well RW5-43 was sampled from day 72 of the experiment and served as a reference thenceforth (Fig. S2–S6).

All water samples were obtained dynamically after the stabilization of oxidation-reduction potential (ORP) and pH of the groundwater. The samples were obtained from the depth of 8 (HV-103), 10 (RW5-24), and 12.5 m below the ground level (RW5-43). Groundwater was pumped out of the wells by a submersible Gigant pump (Eijkelkamp, Netherlands).

Measurement of chemical and physical parameters

The ORP was measured directly at the site at the end of each sampling using a WTW 3430 Multimeter (WTW, Germany) and recalculated against a standard hydrogen electrode to Eh.

Chlorinated hydrocarbons were determined in accordance with the EPA Method 8260 B, and ethene was identified in accordance with the methodology described by [18] using gas chromatography-mass spectrometry (TRACE 1310 and TSQ 8000 Evo, Thermo Fisher Scientific, Inc., USA). COD was measured using cuvette tests (DR1900, LCK; Hach-Lange, Germany).

The degree of dechlorination (DoD) was calculated with the following equation:

$$DoD = \frac{[TCE] + 2 \cdot [DCE] + 3 \cdot [VC] + 4 \cdot [ethene] + 4 \cdot [ethane]}{4 \cdot ([PCE] + [TCE] + [DCE] + [VC] + [ethene] + [ethane])} \cdot 100\ [\%]$$

where *[contaminant]* represents the concentration of the relevant contaminant in mmol/L.

DNA extraction and real time polymerase chain reaction (qPCR)

Water samples for molecular genetic analysis were immediately cooled and stored in the dark at 4 °C. Before analysis, samples (0.03–0.5 L) were concentrated by filtration through a 0.22 mm membrane filter (Merck Millipore, Germany). DNA was extracted using a FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA) following the manufacturer's protocol. The extracted DNA was quantified using a Qubit 2.0 fluorometer (Life Technologies, MA, USA).

The relative abundance of 16S rRNA gene (representing the total bacterial biomass), selected OHRB (*Dehalobacter* spp., *Dehalococcoides mccartyi*, and *Desulfitobacterium* spp.), and VC reductase genes *vcrA* and *bvcA* were determined by qPCR as described previously.[3] Briefly, qPCR reactions were prepared and performed in LightCycler® 480 instrument (Roche, Switzerland). Each sample was analyzed in duplicate with ultra-pure water used as a template in negative control. Ct values were obtained using the second-derivative maximum method. The results of qPCR were evaluated as relative quantification, a fold change between two states, with the condition of the specific bacteria or gene prior to the application considered as the starting point. Table S1 lists all the primers used for qPCR.

16S rRNA sequencing, data processing, and analysis

Purified DNA samples were sequenced in duplicate. Two successive PCRs were performed per sample to amplify the DNA from the V4 region (using normal and barcode fusion primers). *In silico* analysis of primers was performed to cover as much diversity as possible while keeping the amplicon size below 400 bp. Amplification of the V4 region of the eubacterial 16S rRNA gene was performed with primers 515F (5′-TGCCAGCMGCNGCGG-3′ [19]) and barcode one 802R (5′-TACNVGGGTATCTAATCC-3′ [20]). A mock community (collection of 12 bacterial genomes) was subsequently sequenced to verify data evaluation. The PCR cycle conditions were as follows: first PCR: 95 °C for 3 min; 10 cycles at 98 °C for 20 s, 50 °C for 15 s, 72 °C for 45 s, and a final extension at 72 °C for 1 min; second PCR: 95 °C for 3 min; 35 cycles at 98 °C for 20 s, 50 °C for 15 s, and 72 °C for 45 s, with a final extension at 72 °C for 1 min. The concentration of purified PCR products was

measured with a Qubit 2.0 fluorometer (Life Technologies, USA). Barcode-tagged amplicons from different samples were then mixed in equimolar concentration. The sequencing of bacterial amplicons was performed on the Ion Torrent platform (Life Technologies, USA).

The raw data were processed and analyzed using Mothur software as described in a previous publication.[21] Only genera with a minimum relative abundance of over 5% were shown in a heatmap.

RESULTS AND DISCUSSION

Changes in Eh and COD

The first glycerol application resulted in a rapid decrease in Eh in the application well HV-103 (from approx. 203 mV to 50 mV). After the second glycerol injection (at 91 days), the Eh further decreased to values around −40 mV (Fig. 1). Such conditions were beneficial for the ongoing microbial reductive dechlorination of CEs by OHRB. By contrast, the Eh of the reference well RW5-24 remained relatively stable during the monitoring period, ranging between 229 and 258 mV, which indicated rather oxidative conditions, not optimal for OHRB growth.

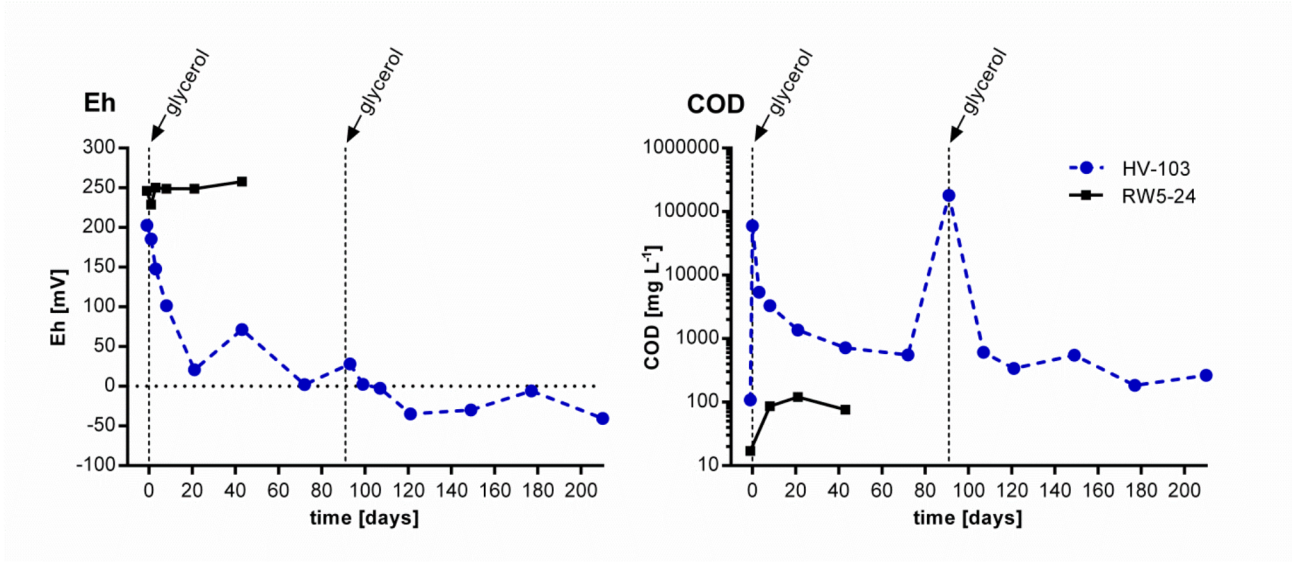


Fig. 1. Changes in Eh and COD in each well following glycerol injections. Note the logarithmic scale of the COD y axis.

COD concentrations are bound to Eh values because the application of a bioreductive substrate (in this case glycerol) and its subsequent microbial fermentation results in the formation of an anaerobic reducing environment beneficial for OHRB growth by the depletion of oxygen and due to the release of H_2.[14] The first glycerol application in well HV-103 caused an increase in the COD concentration by almost three orders of magnitude from the initial value of 108 mg/L (Fig. 1), and the rise was higher after the second application. After each application, the COD concentration gradually decreased but remained above the initial level until the end of the monitoring period. In general, the higher the COD concentration, the more substrate available for microbial processes in the environment. On the other hand, a decrease in COD concentration represents the organic substrate consumption. Our data show that most of the glycerol was fermented within approx. 30 days after the injection.

Degradation of CEs

A significant effect of glycerol application on CE degradation was observed in well HV-103 (Fig. 2). PCE concentration decreased from 137 µmol/L to 43 µmol/L 8 days after the first glycerol application,

followed by a further decrease to 0.03 µmol/L; the corresponding values in µg/l decreased from 22 800 µg/L to 5 µg/L. Concurrently, a temporal increase in the TCE from 86 µmol/L up to 280 µmol/L was detected due to intensive PCE degradation. However, before the second glycerol application, TCE concentration was reduced to 27 µmol/L and further to 0.23 µmol/L after the second injection. Concurrent with TCE degradation, a high production of *c*DCE was observed with the concentration increasing from 71 µmol/L to 377 µmol/L.

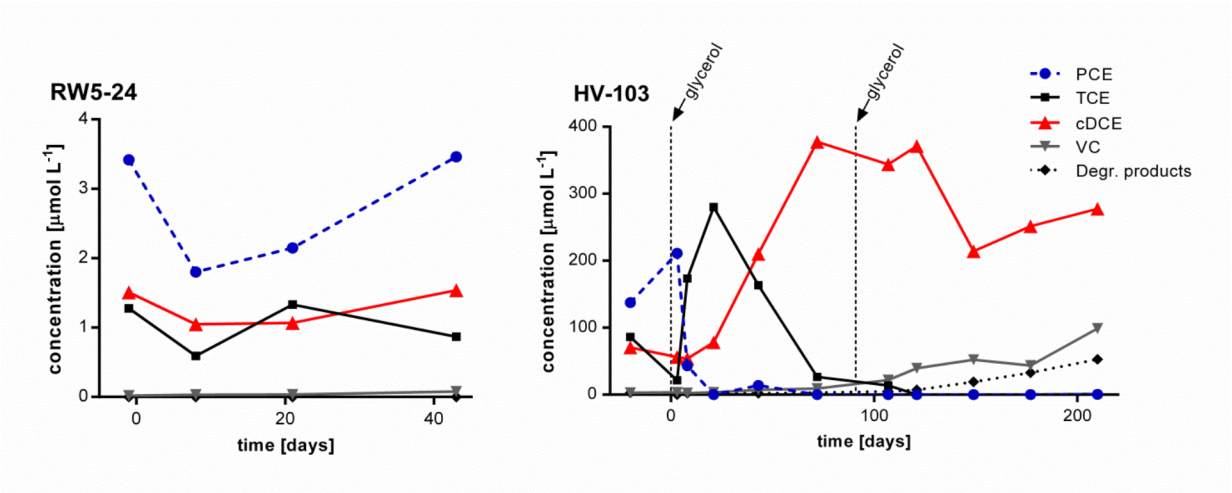


Fig. 2. Changes in the concentration of CEs and their final degradation products (the sum of ethene and ethane) following glycerol application.

Before the second glycerol injection, residual amounts of PCE and TCE were present in the groundwater. The second glycerol injection was therefore primarily aimed at *c*DCE reduction to VC or preferably, ethene. It successfully decreased the *c*DCE concentration down to 214 µmol/L, and non-toxic degradation products ethene and ethane were generated. The VC concentration increased from 3 µmol/L up to 99 µmol/L, and the sum of non-toxic degradation products (99% ethene) increased from 0 µmol/L to 53 µmol/L. Real production of VC or ethene may be higher than the monitored due to their fast volatilization. These results are in line with the increase in VC and decrease in dissolved organic carbon concentrations toward the end of a published field study.[12]

DoD represents an effective parameter for the comparison of results from different wells and is a perfect tool especially for the comparison of results of enhanced microbial dechlorination of CEs in the application and reference wells. DoD is the inverse chlorine number value that represents the amount of chlorine atoms in a mixture of chlorinated hydrocarbons. Thus, the fewer chlorine atoms in the mixture, the higher the DoD, the higher the PCE:TCE ratio, the lower the DoD. Several steps generally results in the higher DoD: decreased PCE:TCE ratio when the PCE and TCE are still present, decreased ratios of TCE:DCE, DCE:VC, and VC:ethene and the production of ethene and other non-toxic degradation products.

In accordance with CE degradation, the DoD of groundwater from HV-103 increased from 21% to 62% (Fig. 3) following the glycerol application, indicating the decreased PCE:TCE ratio and gradual production of less- or non-chlorinated hydrocarbons.

No significant changes in the monitored CE concentrations were observed in the groundwater of reference well RW5-24 during the monitoring period (Fig. 2). The concentration ratios between PCE, TCE, and *c*DCE also remained almost unchanged, as was very well represented by the DoD (Fig. 3), which remained stable during the monitored period, fluctuating from 16% to 19%.

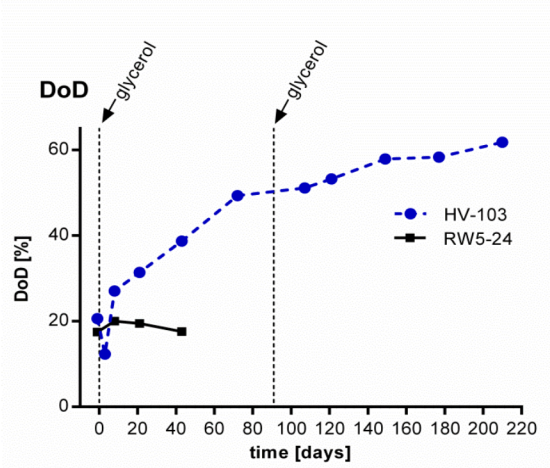


Fig. 3. Changes in DoD in each well following glycerol injections.

Changes in OHRB and VC reductase genes

The qPCR analysis of groundwater prior to glycerol injection confirmed the presence of OHRB genera *Desulfitobacterium* and *Dehalococcoides* and VC reductase genes *vcrA* and *bvcA* in the reference and application wells. The ongoing organohalide respiration in both wells was also confirmed by the presence of PCE and TCE degradation products, namely, *c*DCE, VC, and ethene. However, based on the Ct values (Fig. S5), the abundance of OHRB populations and degradation enzymes and total bacterial biomass was notably lower in the reference well RW5-24 than in the more contaminated HV-103.

While relatively stable levels of total bacterial biomass and all molecular markers of organohalide respiration were observed in reference well RW5-24 throughout the experiment, the injection of glycerol to well HV-103 caused rapid changes in the microbial abundance (Fig. 4). The total bacterial biomass increased by a factor of 4 three days after the first glycerol injection, increased until day 21 when it culminated with 100 times higher abundancy compared with the initial level, and increased again after the second injection. The abundance of *Dehalococcoides* in accordance with both VC reductase genes increased almost ten times immediately after the addition of glycerol, slowly decreased until day 44, reaching initial levels, and subsequently started to proliferate again. A similar trend was observed after the second glycerol injection with the abundance of *Dehalococcoides* almost 600 times higher at the end of the monitoring period (day 200) than the initial value, showing

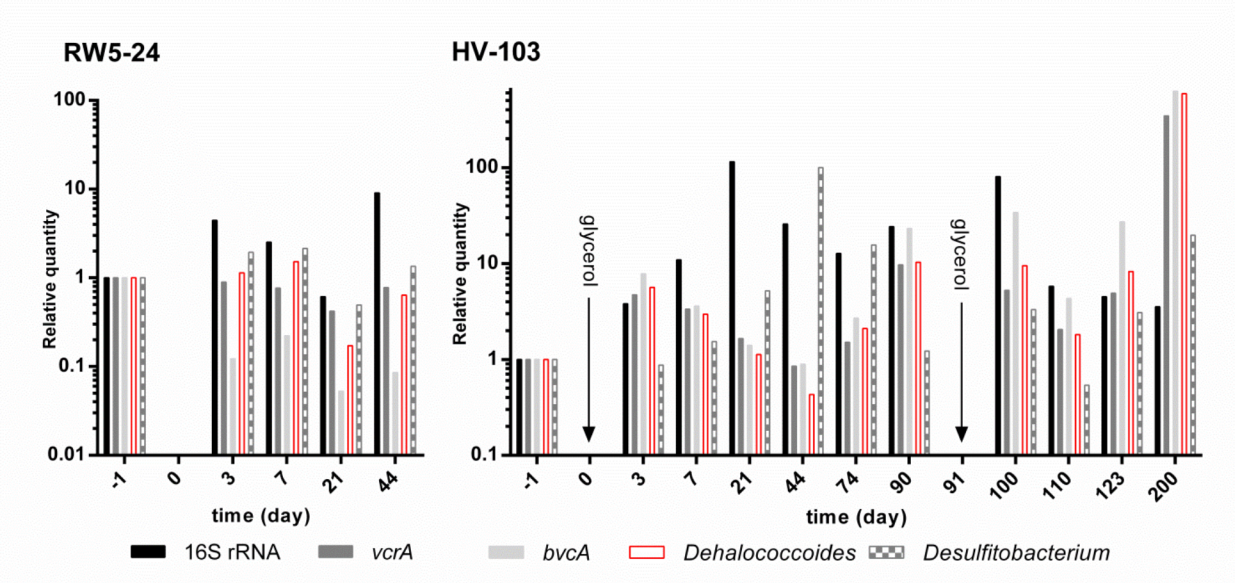


Fig. 4. Changes in the relative abundance of total bacteria (16S rRNA), organohalide-respiring bacteria (*Dehacoccoides* and *Desulfitobacterium*) and VC reductase genes (*vcrA* and *bvcA*). All results are expressed as relative quantity to each marker abundance prior to glycerol application (-1 day). Note the logarithmic scale.

comparable levels of VC reductase genes *vcrA* and *bvcA*. Correspondingly, steadily rising concentrations of CEs degradation products (ethene and ethane) were detected after the second injection until the end of the monitoring period (Fig. 2). The proliferation of *Desulfitobacterium*, on the other hand, was not readily stimulated by glycerol. Its population started to grow after 21 days, and its abundance correlated negatively with *Dehalococcoides*. The explanation is either the competition for the same electron donor or changed the CE composition in groundwater.

Changes in bacterial community composition

The structure of bacterial communities differed in the reference and application wells (Fig. 5).

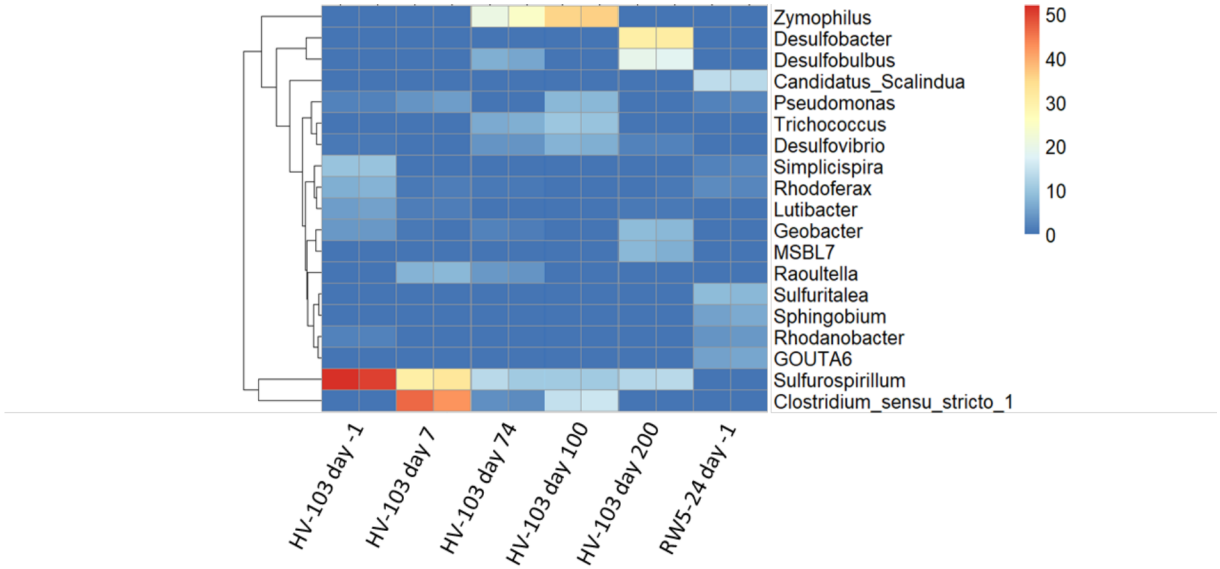


Fig. 5. Bacterial community composition in HV-103 and RW5-24 wells before (− 1 day) and after (7, 74, 100, and 200 days) glycerol injection. The scale expresses the percentage of each bacterial genus in a sample. Only genera with a relative abundance > 5% are shown.

Sulfurospirillum was the dominating bacterial genus in the application well HV-103 before glycerol addition, together with *Simplicispira* and *Rhodoferax*. *Sulfurospirillium* can dechlorinate PCE and TCE to *c*DCE, and its presence in pre-stimulated samples indicates naturally occurring reductive processes.[22] Importantly, *Sulfurospirillium* enables the complete dechlorination of CEs, leaving *c*DCE to *Dehalococcoides* for the final dechlorination to ethene. *Sulfurospirillum* can also oxidize reduced sulfur compounds to sulfates [12,23,24], detected in relatively high concentration on site. Sulfate reduction can inhibit OHRB growth because both processes compete for electron donors.[25]

After glycerol injection to the HV-103 well, the amount of dominating *Sulfurospirillum* decreased and was gradually replaced by glycerol-fermenting bacteria, namely, *Clostridium, Trichococcus*, and *Zymophilus* (all phylum Firmicutes). This result is in accordance with high COD concentration (i.e., high dissolved organic carbon) measured on site (Fig. 1). Fermentation of organic matter such as glycerol is necessary to supply electron donors (H_2) and carbon source (acetate) for the dechlorination of CEs by OHRB and to enhance favorable anaerobic conditions.[1] *Clostridium*, which was highly abundant 7 days after the first and second glycerol application, possesses a glycerol fermentation capacity with butanol, acetic acid, or propionate being the main fermentation products.[26,27] *Clostridium bifermentans* also dechlorinates PCE and TCE to *c*DCE cometabolically.[28] Another member of glycerol-fermenting bacteria, *Zymophilus*, predominated at 74 and 100 days after stimulation. *Zymophilus* belongs to Vellionellaceae family which includes obligate anaerobes that commonly

produce acetate and propionate.[29,30] Acetate is an important carbon source for *Dehalococcoides* and as such supports its growth.[31]

Glycerol also promoted sulfate reduction, with sulfate-reducing genera *Desulfovibrio*, *Desulfobulbus,* and *Desulfobacter* flourishing at the end of the monitoring period (200 days) by taking advantage of the decreasing redox conditions. They outcompeted glycerol-fermenting bacteria when most of the substrate was decomposed. In subsurface environments, the competition by nitrate or sulfate-reducing bacteria may have an inhibitory effect on organohalide respiration, as has been previously confirmed for nitrate and sulfate.[25,21] In general, sulfate reduction inhibits the growth of OHRB because both processes compete for electron donors;[32] however, *Desulfovibrio* strains can also promote the growth of *Dehalococcoides* by providing H_2, acetate, and corrinoid cofactors.[33] Desulfobulbaceae also sustains *Dehalococcoides* growth by producing acetate.[12,31] Acetate can be oxidized also by *Geobacter,*[34] which flourished together with acetate-producing bacteria 200 days after glycerol addition. *Dehalococcoides* uses corrinoid-dependent reductases for *c*DCE dechlorination to VC and ethene but lacks the ability for corrinoid synthesis. *Geobacter lovleyi* can dechlorinate PCE to *c*DCE and produces one type of corrinoid cofactor, that is, cobamide, to complement the nutritional requirements of *Dehalococcoides*, promoting its growth.[35]

Reference well RW5-24 was characterized by the presence of bacteria involved mainly in the geochemical cycles of nitrogen and sulfur: denitrifying *Rhodanobacter*, isolated also from polluted sites,[36] and *Simplicispira*, isolated from activated sludge,[37] ammonium oxidizing *Scalindua,*[38] and sulfur-oxidizing *Sulfuritalea.*[39] *Sphingobium*, which can degrade aromatic hydrocarbons was also abundant.[40]

Sequencing analysis did not detect any OHRB in the tested samples, nevertheless, *Desulfitobacterium* and *Dehalococcoides*, together with VC reductase genes *vcrA* and *bvcA*, were detected by qPCR in the reference and application wells (Fig. 4). This discrepancy between sequencing and qPCR data had been described before [21,41,42] and can be explained by the extremely low relative abundance of the selected OHRB in the bacterial community. Thus, qPCR is a better tool for detecting such rare but crucial bacteria due to more specific qPCR primers in comparison with the universal primers used for sequencing. When combining the results of both methods, *Dehalococcoides* population reacted positively to increase in the fermentative and sulfate-reducing bacteria in HV-103, which provided essential sources of carbon (acetate), electrons (H_2), and corrinoid cofactors. It also took advantage of decreasing redox potential. Concurrently with the growing *Dehalococcoides* population, we documented the steadily rising levels of CE degradation products (VC, ethane, and ethene) (Fig. 2).

As revealed in our study and described previously on laboratory-scale experiments [33,35] and one pilot field glycerol application,[12] the successful and complete dechlorination of CEs requires supporting the growth of OHRB together with variegated bacterial populations. The strong and complete dechlorination of CEs is possible only on sites with high concentration of dissolved organic carbon and sufficient syntrophic interactions (high relative abundance of fermenting and sulfate-reducing bacteria).[12] In aquifers, bacterial populations influence the growth of each other by changing groundwater conditions, competing for sources, or producing nutrients essential for others. Therefore, it is very important to study the targeted sites in detail to run the enhanced reductive dechlorination process successfully.

CONCLUSIONS

1. This long-term field experiment showed the great potential of glycerol application for enhanced reductive dechlorination on a site highly contaminated with CEs. It proved low-cost glycerol as an effective source of electrons and carbon for OHRB.

2. The concentrations of PCE and TCE decreased during the monitoring period with the increased levels of *c*DCE, VC, and non-toxic final CE degradation products (ethene and ethane) toward the end of the study.

3. A total of 98.5% PCE and 99.4% TCE were reduced in the injection well HV-103 after glycerol applications with the increase in DoD from 21% to 62%.

4. Glycerol injection started biological and geochemical processes that promoted the growth of OHRB *Dehalococcoides* and *Desulfitobacterium*.

5. Glycerol injection resulted in an almost immediate increase in the abundance of fermentative Firmicutes (*Clostridium*, *Trichococcus*, and *Zymophilus*), which produced essential sources of carbon (acetate) and electrons (H_2) for OHRB and changed groundwater conditions to be suitable for OHRB growth.

6. Consistent with the reduced redox potential, sulfate-reducing genera *Desulfobacter*, *Desulfobulbus* and *Desulfovibrio*, and iron-reducing *Geobacter* 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.

7. Syntrophic bacterial interactions proved to be very important for the complete dechlorination of CEs.

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