SYNERGISTIC COMBINATION OF BIOLOGICAL AND CHEMICAL DEGRADATION OF TETRACHLOROETHENE TO IMPROVE GROUNDWATER CLEAN-UP EFFICIENCY

Dissertation
Submitted to obtain the doctoral degree at
BOKU University of Natural Resources and Life Sciences, Vienna
In association with
AIT Austrian Institute of Technology GmbH

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Vienna, March 2020

ACKNOWLEDGEMENTS

I would like to thank my supervisor Univ.Prof. Dipl.-Ing. Dr. Dr.h.c.mult. Martin Gerzabek, Mag. Univ.Doz. Dr. Thomas Reichenauer and Priv.-Doz. Mag.rer.nat. Dr.rer.nat. Andrea Watzinger for their support and guidance. I want to especially thank Thomas not only for his professional supervision, but above all for his personal support and help.

Philipp Schöftner deserves a big “thank you” for all his professional support and input, as well as his moral help and friendship throughout my dissertation and beyond. I would also like to thank Christina Roschitz and Tamara Dinter for their support and great work during their internship. A special thank goes to all my colleagues at AIT in Tulln, who always put a smile on my face: It is hard to find a group of friends like you! To Anna Wawra: I am sorry I didn’t offer you any of my sweets on my first day!

Finally, I want to thank my mother for always encouraging me and her thorough spell checking. And special thanks and all my love go to Walter and my unborn, yet nameless son!

This study was funded in the scope of two projects:
MICROSYN funded by FFG – Austrian Research Promotion Agency (Proj. No.: 51762).
BIANO funded by the Austrian Federal Ministry of Sustainability and Tourism (BMNT) (former Ministry of Agriculture, Forestry, Environment and Watermanagement); Funding Management by Kommunalkredit Public Consulting (KPC) (Proj.No.: B420003).

CONTENTS

ABSTRACT

Due to its chemical characteristics the groundwater pollutant perchloroethene (PCE) can persist for decades when no proper remediation actions are taken. PCE can mainly be found in urban, build-on areas. This calls for innovative in-situ remediation technologies, like the application of zero-valent iron (ZVI) or dehalorespiring bacteria within the underground. ZVI reduces PCE to ethene, ehtyne and ethane but it also anaerobically reacts with water leading to particle consumption and H_2 production. The strictly anaerobic *Dehalococcoides spp.* completely dechlorinate PCE to ethene. For this process suitable electron donors, such as H_2, are needed. A lack thereof can lead to no or an incomplete dechlorination, resulting in an accumulation of hazardous metabolites. By combining the abiotic and biotic processes, ZVI can function as a H_2 source, increasing controllability and stability of the microbial dechlorination. Simultaneously, lower amounts of ZVI particles are needed reducing remediation costs drastically.

This dissertation investigated the influence of ZVI particles on dehalorespiring cultures in laboratory and lysimeter experiments:

Within study 1 microcosms experiments investigating the influence of different ZVI particles on two commercially available dechlorinating bacterial cultures were conducted. To do so nano-sized (nZVI) and micro-sized (mZVI) particles, with different reactivity and H_2 formation behaviors, were used. Bacterial cultures with different patterns resulting in different end products (ethene and *cis*-DCE respectively) were investigated. Furthermore, changes in methane production and PLFA-composition of the bacterial cultures were examined. The following findings were obtained:

- nZVI inhibits and mZVI stimulates anaerobic bacterial PCE dechlorination
- mZVI can start ethene production in an otherwise incomplete degrading culture
- H_2 produced by ZVI can be used by PCE degrading and methanogenic bacteria
- Slow H_2 formation at low levels by mZVI favors the dechlorinating metabolism

A lysimeter experiment with nZVI and the completely dechlorinating bacterial culture (both from study 1) was set-up during study 2. Under field-like, but still very controllable conditions an artificial aquifer was built and the influence of nZVI on the biotic dechlorination was observed for over 350 days. Additionally, the impact of the absence of an added electron source (molasses) was tested. The key-findings were:

- nZVI increased microbial PCE reduction and ethene formation
- nZVI reduced accumulation of hazardous metabolites by bacterial degradation
- Beneficial effects were pronounced in absence of an additional electron donor
- H_2 from anaerobic corrosion of nZVI was consumed by the bacteria present

The different results of the microcosm and lysimeter experiments with nZVI may be due to different spatial distances between the bacteria and the particles. While they exist in proximity within microcosm, the bacteria can establish themselves in lysimeters at a distance from the particles, avoid disruptive particle agglomeration on bacterial cells, while still benefiting from the H_2 produced.

ZUSAMMENFASSUNG

Aufgrund seiner chemischen Eigenschaften kann der gesundheitsschädliche Grundwasserschadstoff Perchloroethen (PCE) über mehrere Jahrzente bestehen, wenn keine geeigneten Sanierungsmaßnahmen durchgeführt werden. PCE kann hauptsächlich in städtischen, bebauten Gebieten gefunden werden. Daher müssen innovative in-situ Sanierungstechnologien, wie der Einsatz von nullwertigem Eisen (ZVI) oder dehalorespirierenden Bakterien im Untergrund, eingesetzt werden.

ZVI reduziert PCE zu Ethen, Ethin und Ethan. Jedoch korrodiert ZVI auch bei Kontakt mit Wasser unter anaeroben Bedingungen und es ensteht H_2 . Die strikt anaeroben *Dehalococcoides* spp. können PCE vollständig zu Ethen abbauen. Dafür benötigen sie Elektronendonoren (z.B.: H_2). Ein Mangel an H_2 kann zu keiner oder nur unvollständigen PCE-Dechlorierung führen und in Folge zu einer Akkumulierung von unerwünschten Metaboliten.

Durch die Kombination von abiotischer und biotischer PCE-Dechlorierung kann ZVI als H_2-Quelle dienen. Dadurch kann die Kontrollierbarkeit des mikrobiellen Abbaus erhöhrt werden. Gleichzeit können erheblich geringere Mengen an ZVI eingesetzt werden, wodurch eine drastische Reduktion der Sanierungkosten erzielt wird.

Diese Dissertation beschäftigte sich mit dem Einfluss von ZVI Partikeln auf dehalorespirierende Kulturen in Labor- und Lysimeterversuchen:

Im Rahmen der Studie 1 wurde in Mikrokosmosversuchen der Einfluss unterschiedlicher ZVI Partiklen auf zwei PCE dechlorierende Baktierenkulturen untersucht. Dazu wurden Nano- und Mikropartikeln, mit unterschiedlicher Reaktivität und H_2 Bildungseigenschaften, verwendet. Die ausgewählten Bakterienkulturen produzierten unterschiedliche Endprodukte. Zusätzlich wurden Unterschiede in der Methanproduktion und die PLFA-Zusammensetzung der Kulturen analysiert. Folgende Ergebnisse konnten gezeigt werden:

- nZVI inhibierte und mZVI stimulierte die bakterielle PCE-Dechlorierung
- mZVI induzierte die Ethenbildung in einer sonst unvollständig abbauenden Kultur
- ZVI produziertes H_2 konnte von PCE dechlorierenden und methanogenen Baktieren verwertet werden
- Die langsame H_2 Enstehung bei geringen Konzentrationen durch mZVI begünstigte den dehalorespirierenden Metabolismus

Während der Studie 2 wurde ein Lysimterversuch mit nZVI und der vollständig dechlorierenden Bakterienkultur durchgeführt. Unter feldnahen, jedoch kontrollierbaren Bedingungen wurde ein künstlicher Aquifer hergestellt und für über 350 Tage beobachtet. Zusätzlich wurde der Einfluss von der Abwesenheit einer zusätzlichen Elektronenquelle (Molasse) getestet. Folgende zentrale Ergebnisse konnten erzielt werden:

- nZVI erhöhte die baktierielle PCE Reduktion und Entstehung von dechlorierten Endprodukten
- nZVI reduzierte die Akkumulierung von gesundheitsschädlichen Metaboliten durch die bakterielle Dechlorierung
- Nach Beendigung der Zufuhr von Melasse waren die positiven Auswirkungen besonders ausgeprägt
- H_2, produziert durch die anaerobe Korrosion von nZVI, konnte von der mikrobiellen Gesellschaft verwertet werden

Die unterschiedlichen Ergebnisse der Mikrokosmen- und Lysimterversuche mit nZVI sind vermutlich auf die unterschiedlichen räumlichen Distanzen zurückzuführen. Während in den Mikrokosmen die Partikeln und Bakterien in unmittelbarer Nähe voneinander existieren müssen, konnten sich die Bakterien ohne physischen Kontakt zu den Partikeln im Lysimter etablieren. Dadurch wurde zellzerstörende Agglomeration der Partikeln an den Baktierenmembranen verhindert, während die dechlorierenden Baktieren dennoch vom chemisch entstandenen H_2 profitieren konnten.

INTRODUCTION

Perchloroethene in the underground

According to the Environment Agency Austria approximately 2000 severely contaminated sites in need of remediation are located in Austria. Chlorinated hydrocarbons (CHC) are very common contaminants found at those sites. On 98 % of these sites a contaminant transfer from the soil into the groundwater can occur, consequently leading to a negative influence on the groundwater quality. Groundwater is an essential part of the Austrian drinking water supply (99 %). Thus, measures, like remediation, are needed especially when groundwater used as drinking water is at risk (Granzin and Valtl, 2018).

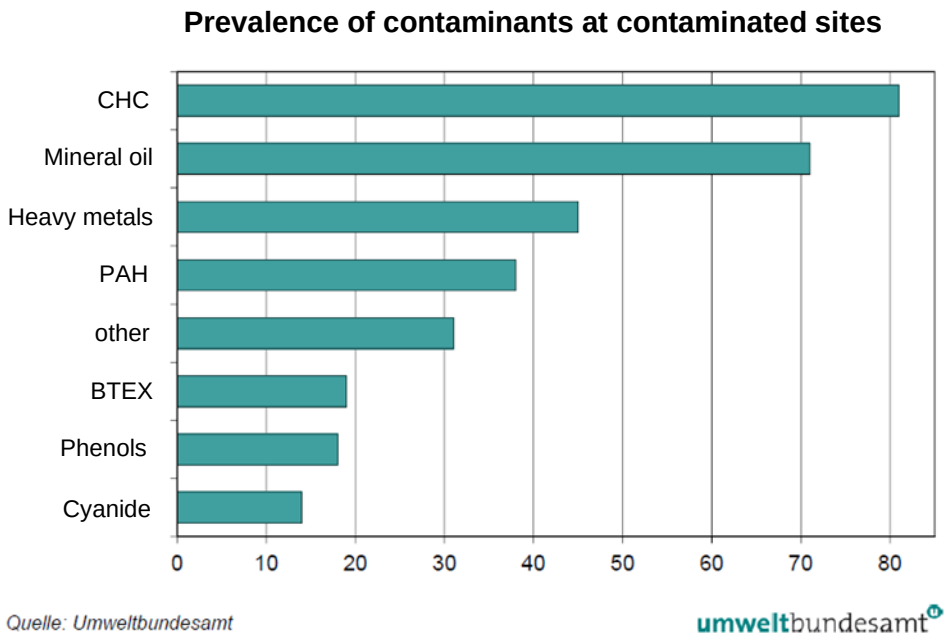

Figure 1: Prevalence of different contaminants on Austrian contaminated sites (Granzin and Valtl, 2018, translated)

Perchloroethene (PCE) is a four times chlorinated hydrocarbon and is highly hazardous for the human health. Exposure leads to damage to the central nervous system, kidneys, liver and reproductive processes and it is potentially cancerogenic (United States Environmental Protection Agency, 2017).
Due to its degreasing characteristics PCE was heavily used in the last century in dry-cleaners and the metal processing industry. Thus, it can mainly be found globally at contaminated sites in urban, build-up regions (Agency, 2017; Field and Sierra-Alvarez, 2004; Mueller et al., 2012; United States Environmental Protection Agency, 2017).
PCE is a dense Non-Aqueous Phase Liquid (DNAPL, density = 1.623 g/cm^3 at 20 °C) and thus trickles into the groundwater through permeable layers until it hits impermeable layers. There it forms pools, from which, detached droplets and dissolved PCE in the groundwater forms contamination plumes. Due to its low solubility in water (206 mg/L at 25 °C) these processes occur over several years, creating huge contaminated areas, which can exist for decades when no clean-up measures are taken (Grandel and Dahmke, 2008; ÖVA, 2012; Stroo et al., 2012).

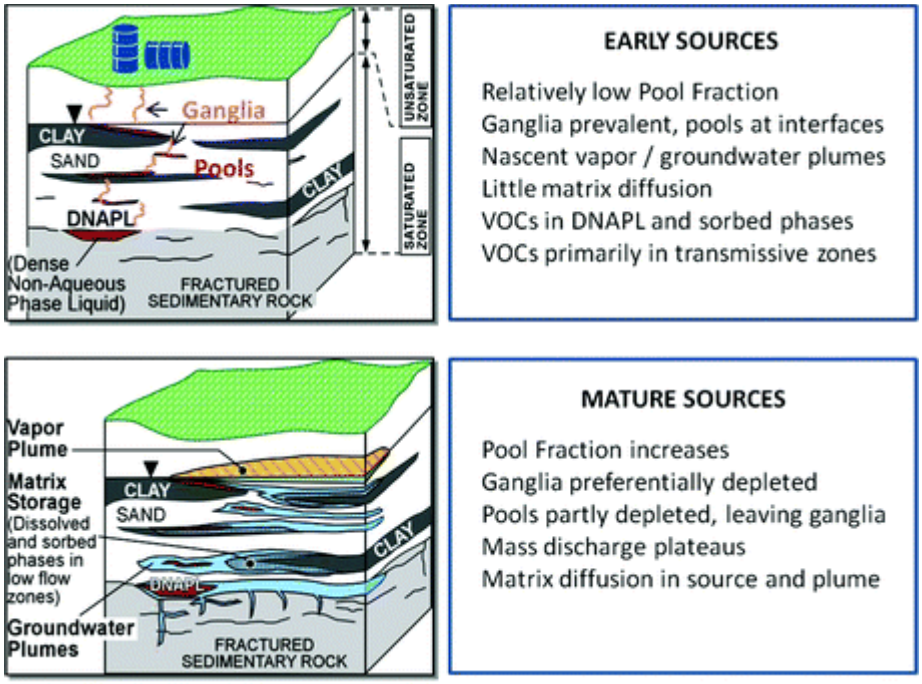

Figure 2: Behavior of DNAPL contamination in the underground (Stroo et al., 2012)

DNAPL remediation technologies

Using ex-situ clean-up technologies the contaminated subsurface material is brought to the surface, via excavation, pumping or suction and then treated in plants on or off-site (ÖVA, 2012). These techniques cannot be used at contaminated sites, with build-up surfaces (e.g. in cities or industrial sites). Thus, in-situ methods need to be established, where the contaminants are removed within the underground. Next to being applicable at urban sites in-situ remediation technologies are less disruptive, by keeping the underground structures intact. Figure 3 shows different in-situ methods for the remediation of DNAPL contaminated sites:

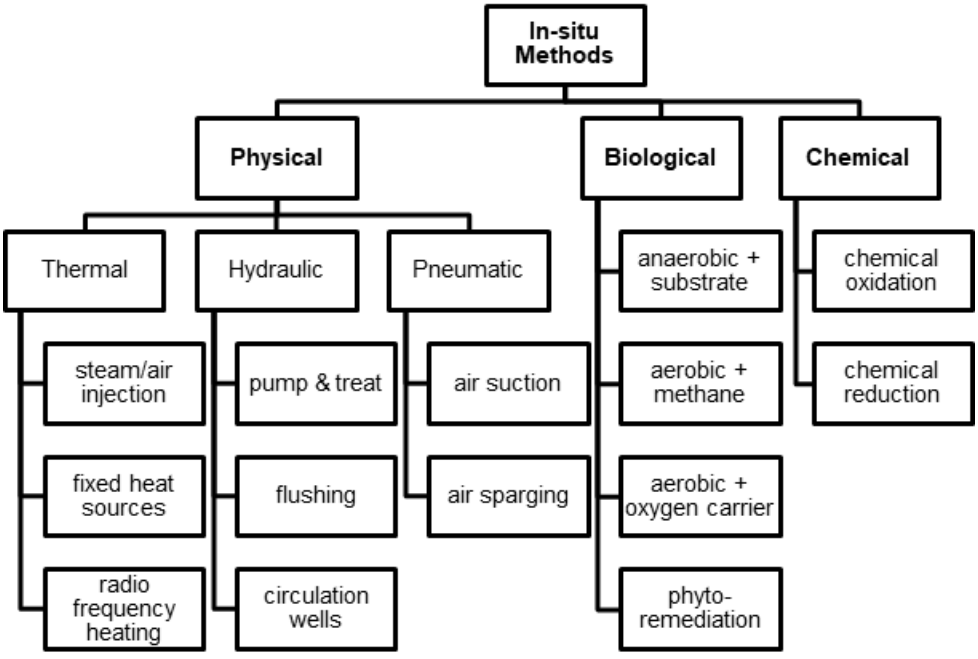

Figure 3: In-situ remediation methods for DNAPL contaminated sites (translated, ÖVA, 2012)

In- situ remediation methods can be divided into three main categories, physical, biological and chemical treatments (ÖVA, 2012, 2010):

Physical treatments

Thermal treatments

Steam-air injection

During the steam-air injection the saturated and unsaturated zones within the underground are heated. The injected steam condenses at the cold solid matrix and releases its energy onto it, heating it up and leading to the volatilization of CHC. The injected air acts as an inert carrier gas of the contaminants mobilizing them. This method cannot be used in subsurfaces with medium permeability and is limited by in-situ installation at the site.

Fixed heat sources

During this remediation technique electrical heating elements are used to conductively and continually heat up the unsaturated zone, vaporizing the soil water. This results in the mobilization of the contaminant into the gaseous phase. Fixed heat sources can be used in low permeable, sandy to loamy soils. The used materials need to withhold aggressive environments (low pH and high temperature) within the subsurface.

Radio frequency heating (RFH)

Organic contaminants are mobilized from the solid matrix into the gaseous phase by the heating of the saturated and unsaturated zone via low-frequency (3-50 MHz) radio frequency radiation. This technique is suitable for materials with low conductivity. Next to the volatilization of the CHC, RFH can be used to enforce hydrolyses, oxidation or pyrolysis, by heating the underground up to a few 100 °C. The subsurface can also be heated up to only 30-40 °C, creating an optimal temperature for microbial contaminant degradation.

The contaminant loaded air, produced during all three described thermal treatments, is removed from the subsurface by air suction and is sequentially treated on-site (e.g.: active carbon filters). The temperature increase leads to a raise in the solubility of the contaminants in the groundwater, calling for a safeguarding of the groundwater downstream.

Hydraulic treatments

Next to the conventional Pump & Treat methods, during which the contaminated groundwater is being pumped to the subsurface and then treated on-site, two other hydraulic treatment techniques exist:

Flushing

Contaminants, usually in the saturated zone, are mobilized using a flushing agent. Pure water and solubilizers (e.g.: surfactants, alcohols) can be used as flushing agents. The flushing agent can be percolated via drainage lines or flushing lances up-stream, shortly before to or directly into the contamination source. The groundwater-flushing agent-contaminant mixture is then pumped to the surface and treated. After the addition of the flushing agent the underground is flushed again with pure water to remove the agent residuals. For this technique the underground needs to be medium to highly permeable and it is not suitable for contaminants strongly

bound to the soil matrix or with low solubility. Furthermore, the groundwater flow characteristics need to be well known and not highly complex or changing.

Circulation wells

Air is blown into the saturated zone to create an upwards flow of the groundwater within the circulation well. This results in a cylindrical water movement, due to the escape of water at the top of the well causing an under pressure in the bottom area. This turbulent mixture of air and water leads to a transfer of the contaminants into the gaseous phase. The air loaded with CHC is removed by suction at the top of the well and treated on-site. This technique can be used in medium to high permeable unconsolidated rocks. However, it is not suitable for low permeable intermediate layers, strong secured aquifers or high groundwater gradients. Possible mobilization of the contaminant calls for protection wells downstream of the treatment zone.

Pneumatic treatments

Air suction

Highly volatile contaminants are being directly removed from the unsaturated zone using an air extraction system. Extraction wells or lances are ideally placed into the contamination source and a vacuum is applied. This leads to a removal of the contaminants from the soil air and consequentially to an imbalance of the contaminant concentration between the soil air and residual phases, adsorbed or dissolved contaminant. These are then mobilized by following air escapes from lower or uncontaminated zones. The contaminated air is treated in active carbon filters on-site. This technology can be used for medium to strongly permeable loose rocks and is easy to control. However, reaching satisfying remediation results can be time consuming.

Air sparging

Air sparging can be used for plume as well as source remediation. Highly volatile contaminants are removed from the saturated zone by blowing air directly into the groundwater using high pressure lances. The blown in air rises within in the groundwater and mobilizes contaminants into the gaseous phase, which carries them into the unsaturated zone. From there the contaminated air is removed and transferred into an exhaust air treatment system. Air sparging can be used in medium to highly permeable loose rocks. It is not suitable for contaminations at or close to the aquiclude. Possible mobilization of the contaminant calls for protection wells downstream of the treatment zone.

Biological treatments

Bacterial remediation

During biological treatment the microbiological degradation of organic contaminants is obtained by adjustment of the underground conditions to an optimum for the microbial degrading community. For CHC this usually means the input of electron donors to improve bacterial dechlorination. The following techniques are suitable for medium permeable, homogenic aquifers. Mixed contamination with cytotoxic contaminants (e.g. PAH, chromate) in high concentrations can limit this technique.

To push the anaerobic degradation of CHC usually organic carbon compounds are used. Highly soluble (e.g.: molasses, lactate or ethanol) as well as insoluble, slowly hydrolyzing additives can be used as degradable co-substrates. During the metabolic

breakdown of these co-substrates H_2 is produced, which is used as an electron donor under anaerobic conditions during a stepwise dechlorination of the CHC. The co-substrates are injected directly into the unsaturated zone through conventional wells as well as high pressure injection wells. ZVI can also function as an alternative H_2 source, this was investigated in this study (see chapters 0 and 0).

To force the aerobic C_2-CHC degradation methane-enriched air is brought into the underground during a conventional soil aeration. This selectively favors the growth of methanotrophic bacteria, which build methane-monooxygenases during methane degradation. The produced enzyme oxidates the CHC in a side reaction into an epoxide, which finally disintegrates into CO_2 and Cl^-. This technique is usually applied at the contaminant plume and the edge region of the source.

Oxygen carriers can be used to stimulate the aerobic, microbial dechlorination of lower chlorinated CHC, BTEX, MTBE, mineral oil HC and low molecular weight PAH in the saturated zone. Gaseous oxygen carriers (air, O_2 enriched air, pure O_2 and O_3) as well as liquid oxygen carriers (e.g.: H_2O_2) and solid oxygen carriers (e.g.: peroxides) can be applied.

Phytoremediation

Plants are used for the removal of inorganic and organic contaminants, either by degradation or immobilization. The following mechanisms can be used for phytoremediation:

- **Rhizodegradation**: degradation of organic contaminants by the root microbiome
- **Phytoextraction**: uptake of inorganic contaminants and enrichment in the aboveground biomass
- **Phytotransformation**: uptake and metabolization of organic contaminants within the plant (“green liver”)
- **Phytoimmobilization (-stabilisation)**: fixation of inorganic and organic contaminants by plant-produced root exudates
- **Rhizofiltration**: contaminant (organic and inorganic) filtration from surface water via adsorption or absorption by the roots of plants grown on rafts floating on the water
- **Phytovolatilization**: transfer of contaminants into the atmosphere via plant transpiration (often unwanted)
- **Hydraulic control**: limiting of contaminant horizontal and vertical (into the groundwater) spreading by the usage of deep rooting and water intensive plants

Phytoremediation is limited by high concentrations of phytotoxic substances and the root depth of the used plant.

Chemical Treatments

Chemical oxidation

Aggressive oxidative agents, like permanganate, ozone, peroxides and persulfates, are used to push a fast conversion of CHC. In the case of high contaminant concentrations exotherm reactions can cause the transition of volatile contaminants

into the soil air. Thus, additional subsurface air suction may be necessary. To achieve a successful remediation, result a homogenic and extensive oxidant distribution is crucial. High amounts of naturally occurring oxidable substances make an excess of oxidative agents is necessary, limiting this technique.

Chemical reduction

The chemical reduction of chlorinated HC is achieved by the injection of ZVI particles into the subsurface (see chapter 0). For a satisfying remediation result a homogenic and extensive distribution of the ZVI particles is necessary. This technique is suitable for medium to highly permeable soils.

To secure the protection and clean keeping of water bodies in Austria the input of substances, like solubizers, nutrients, oxidizing and reducing agents, into the underground is legally restricted. Injection of those substances is only granted case specifically, limiting the application of all technologies introducing such substances into the underground.

Different approaches may lead to a successful remediation depending on:

- contaminant type (e.g.: organic substances, heavy metals, mixed contamination)
- contaminant characteristics (e.g.: solubility, volatility)
- conditions in the subsurface (e.g.: pH, ORP, groundwater flow, microbiology, hydrochemistry, porosity)
- overall site conditions (e.g.: age, condition of surface, depth of contamination, underground characteristics, area of contamination)

Remediation set-ups can significantly differ from site to site and need to be carefully adapted to meet the needs for a successful clean-up. Thus, prior to remediation it is necessary to thoroughly investigate and evaluate the site and its conditions, by sampling and monitoring.

Chemical PCE degradation by zero-valent iron

Zero-valent iron (ZVI) is a strong reductive and sorptive agent and can be used in the remediation of sites contaminated with varying contaminants, like chlorinated hydrocarbons or heavy metals. ZVI is able to reductively dechlorinate PCE to ethene, ethyne and ethane (Cundy et al., 2008; Mueller et al., 2012; Taghavy et al., 2010).

Two possible pathways for the chemical PCE reduction exist (Arnold and Roberts, 2000; Gavaskar, 1999; Roberts et al., 1996):

During the hydrogenolysis PCE is reduced to TCE, following *cis*-1,2-Dichloroethen (*cis*-DCE) and finally vinylchloride (VC). VC is only slowly degraded to ethene and ethane by ZVI, leading to an accumulation of the cancerogenic metabolite. Only a small amount (around 5 %) of molecules undergo this degradation pathway. The vast majority of PCE molecules are dechlorinated via the β-elimination, where the contaminant is reduced to the wanted dechlorinated end products over short-lived intermediates (Figure 4).
The main products of the chemical dechlorination via ZVI are ethene and ethane and in small amounts *cis*-1,2-DCE, *trans*-1,2-DCE, 1,1-DCE and C_4-hydrocarbons. The

metabolites chloroethyne and ethyne are highly reactive and are therefore rapidly reduced to ethene and ethane.

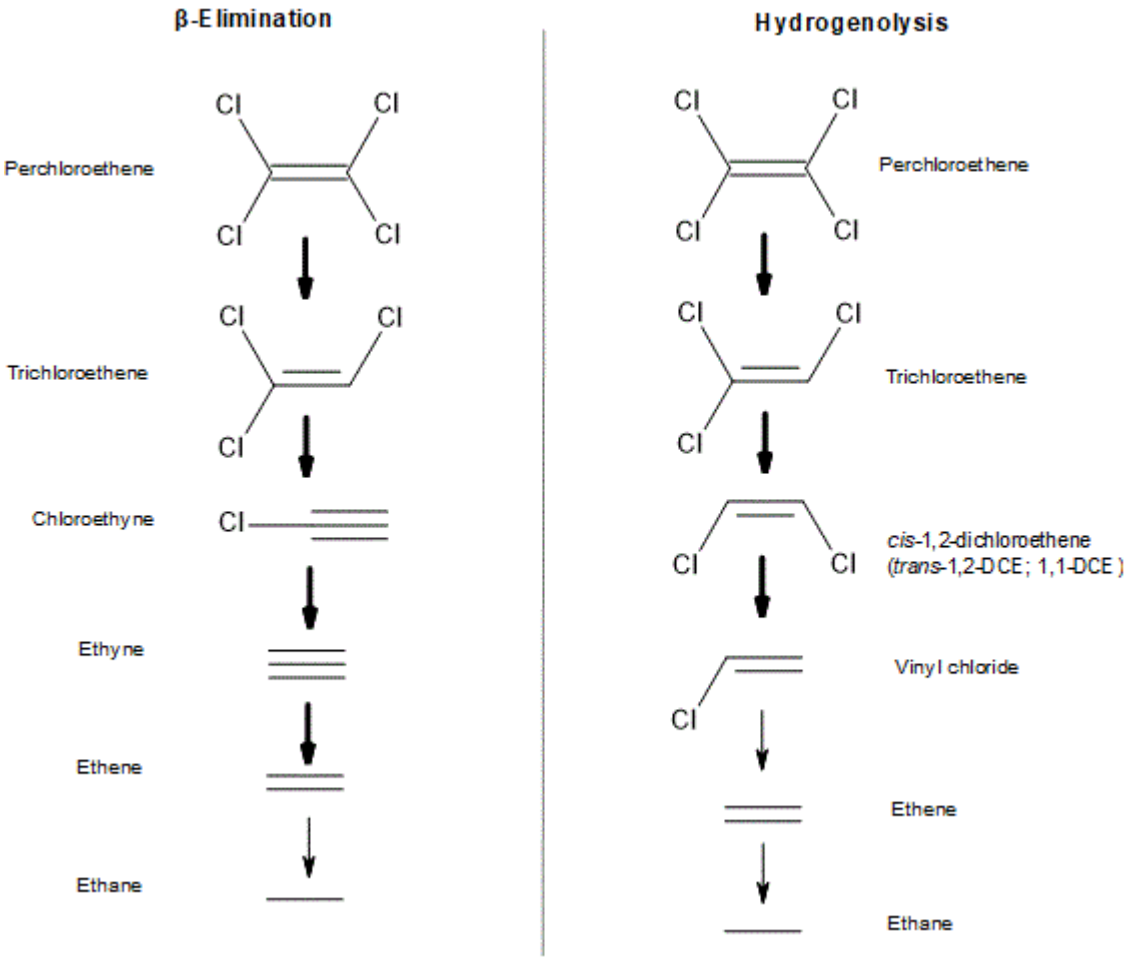

Figure 4: Main metabolites and products of β-elimination and hydrogenolysis during the abiotic PCE dechlorination by ZVI. Bolt arrows indicating fast reactions and thin arrows slower reactions.

Next to the wanted contaminant removal ZVI also reacts with water under anaerobic conditions. During the anaerobic ZVI corrosion H_2 is formed (*Equation 1*) (Filip et al., 2014). Most of the electrons available by ZVI can be used up during this side reaction, depending on the particle characteristics (Schöftner et al., 2015). Due to this unwanted consumption of ZVI during the anaerobic corrosion, an excess of ZVI particles is needed for a successful PCE clean-up. This need for a higher amount of particles can increase remediation costs extensively. Thus, strategies to reduce the amount of ZVI are necessary to accomplish a cost-effectively and economical usage.

$$2H_2O + Fe^0 \rightarrow Fe^{2+} + H_2 + 2OH^-$$ *Equation 1: Anaerobic corrosion of ZVI*

Different sized ZVI particles are used during PCE clean-up, depending on the technologies employed and the site conditions. The general rule is, the bigger the particle dimensions the more aggressive techniques are needed for ZVI delivery (Comba et al., 2011; Mueller et al., 2012). Several injection techniques are available to deliver ZVI to the contaminants underground (Comba et al., 2011; Kutzner, 1996):

Table 1: Relationship between injection techniques and particle dimension among case studies. There were 85 case studies considered for the analysis (Comba et al., 2011)

	Number of case studies			
Particle dimension	**Nano**	**Nano+Micro**	**Micro**	**Millimetric**
Soil mixing	0	0	1	8
Hydraulic fracturing	1	0	2	1
Pneumatic fracturing	1	0	8	0
Pneumatic injection	3	3	16	0
Pressure pulse technology	2	0	1	0
Pressurized injection	17	3	9	0
Gravity injection	8	1	0	0

Soil mixing technologies are used for the input of millimetric iron into the underground, while hydraulic fracturing, pneumatic injection, pressure pulse technology, pressurized injection and gravity injection are applied for nZVI and mZVI.

Soil mixing

Two soil mixing technologies can be used for the input of millimetric ZVI into the underground:

- **Mechanical soil mixing:** Soil is broken up and mixed with the grout using a mechanical auger
- **Jet grouting***:* break up of soil with a high-pressure jet and mixing of loosened soil with a stable grout, forming columns and panels

Both techniques require a sufficient overhead space for the mixing equipment and removal of buried obstructions. Soil mixing technologies are applicable at depths up to 100 ft bgs and are most effective at depths less than 40 ft bgs. Soil mixing should not be done, when a reduction of permeability is not wanted.

Hydraulic fracturing

This technology utilizes fluid pressure of water or a slurry of water, sand and a thick gel to create subsurface fractures. These can be filled with sand or other granular material.

Pneumatic injection and fracturing

During pneumatic injection, pressurized gas (e.g. N_2) is used as a carrier, introducing ZVI (dry or aerosol) into the porous medium. Pneumatic injection can be followed by pneumatic fracturing. During which existing fractures are extended and thus a secondary network of conductive subsurface fissures and channels are created, facilitating subsequent injections. This is done by the injection of high pressurized air into the soil, sediments or bedrock.

Pressure pulse technology (PPT)

Large-amplitude pulses of pressure are applied to the porous media at the water table or at variable depths. This opens pores and consequentially fluid level and flow increase. This facilitates the distribution of the ZVI slurry through the porous medium.

It also causes the detachment of DNAPL from porous medium, increasing its specific surface area.

Pressurized injection

The direct push technology is achieved by perforation using high pressure pump systems. The usage of a combined tool for drilling and injection increases the speed of the operation drastically.
To apply a tubes-a-manchette a sleeved pipe is installed inside a borehole. The ZVI fluid exits the pipe through small holes covered by rubber sleeves. Pressure is applied to the sleeves one at a time. The usage of tubes-a-manchette is an economically viable technique if injection needs to be repeated.

Gravity injection

During gravity injection a ZVI containing slurry is poured into a well. The slurry is then distributed within the underground by pressure differences between the injection slurry and the groundwater and natural groundwater movement.

The use of nanoparticles (nZVI) has risen in the last years. Due to their high surface to volume ratio they show a higher reactivity. This promises a faster contaminant clean-up. Next to their beneficial characteristics nZVI also show a series of disadvantages, like their tendency to agglomerate, low mobility and potential toxicity. mZVI particles show several advantages compared to nZVI particles: their lower price, higher selectivity and longevity compared to nZVI particles (Comba et al., 2011; Li et al., 2016; Ma et al., 2016; Nguyen et al., 2018; Zhao et al., 2016).

Table 2: Characteristics of the ZVI particles used in study 1 and study 2

ZVI Particles	**Scale**	**Spec. surface area [m^2/kg]**	**Abs. surface area [m^2]**	**Fe(0) content w/w %**
Höganäs AB	Micro	84	0.06	95
Nanofer Star	Nano	$17x10^3$	0.71	79

Biological PCE degradation by dehalorespiring bacteria

Different aerobic and anaerobic pathways exist for the degradation of PCE (Field and Sierra-Alvarez, 2004):

Aerobic degradation

- Microbial growth by usage of CHC as an electron donor and carbon source
- Co-metabolism of CHC by a simultaneous degradation of a different hydrocarbon

Anaerobic degradation

- Microbial growth by usage of CHC as an electron donor and carbon source
- Co-metabolism of CHC by a simultaneous degradation of a different hydrocarbon (e.g.: methanol)
- Halorespiration: microbial growth by usage of CHC as an electron acceptor, different electron donors (e.g.: H_2, lactate, formate, ethanol, pyruvate) and simple carbon source

Many aerobic and anaerobic bacteria have been identified as being able for CHC dechlorination resulting in different end products. Most of the bacteria capable of PCE degradation form hazardous metabolites, like cis-1,2-Dichloroethen (cis-DCE) and vinyl chloride (VC) (Field and Sierra-Alvarez, 2004; Men et al., 2013; Sung et al., 2006). Dehalococcoides spp. and recently Dehalogenimonas spp. are the only known bacteria species able to completely degrade PCE to ethene (Lee et al., 2004; Leitner et al., 2017; Maymó-Gatell et al., 1997; Yang et al., 2017a, 2017b). This degradation occurs stepwise with TCE, cis-DCE and VC as intermediates (Figure 5). The strictly anaerobic *Dehalococcoides* spp. enzymatically dechlorinate PCE and use this reductive dechlorination for cell growth. In this process they need an electron donor, such as H_2, which can be scarce in the underground (Löffler et al., 2013). Many different anaerobic bacteria (e.g.: methanogenic, sulfate-reducers) also need H_2 for their metabolisms and *Dehalococcoides* spp. need to compete with them, often unsuccessfully (Smatlak et al., 1996). A lack of *Dehalococcoides* spp. or H_2 in the underground can be the reason for no PCE dechlorination or the accumulation of even more hazardous intermediates like VC (Amos et al., 2008; Lu et al., 2006; Yang et al., 2017b, 2017a). During the competition for H_2 the level of H_2 concentration and its way of delivery is of high importance. While high H_2 concentrations and fast production of H_2 favors methanogenic bacteria, low levels of H_2 delivery are preferred by *Dehalococcoides* spp. (Löffler et al., 2013; Smatlak et al., 1996; Yang and McCarty, 1998). Other H_2 consuming bacteria species do not only pose a risk to the PCE dechlorination by *Dehalococcoides* spp. but are necessary for a naturally occurring biotic dechlorination (Wen et al., 2015).
The presence of *Dehalococcoides* spp. alone also does not guarantee a complete PCE dechlorination, the strain also needs to be equipped with the required dehalogenases. Two membrane bound enzymes are responsible for the complete degradation to ethene. The dehalogenase *bvcA* is responsible for the dechlorination of PCE to TCE, which is further dehalogenated to *cis*-DCE, VC and finally ethene by *vcrA* (Holmes et al., 2006).

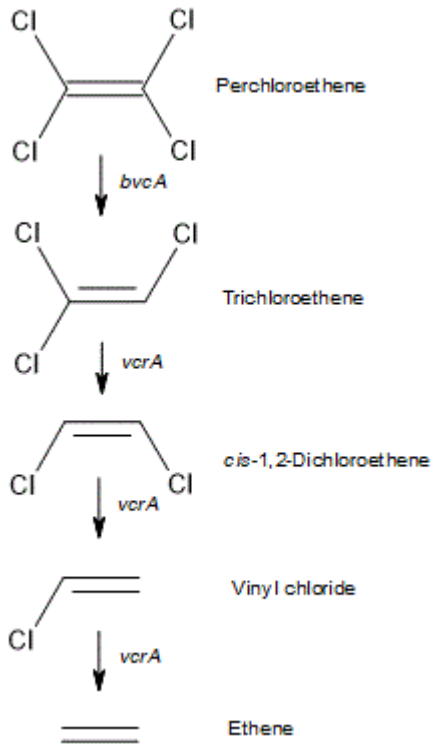

Figure 5: Pathway of the biotic PCE dechlorination by Dehalococcoides spp.

Combination of abiotic and biotic remediation

The combination of the abiotic PCE dechlorination by ZVI and the biotic dechlorination by *Dehalococcoides* spp. can lead to the removal of the downsides of both technologies used independently. Due to anerobic corrosion the ZVI can act as an H_2 delivery system for the *Dehalococcoides* spp. (Figure 6). Furthermore, it can create favourable conditions for the strictly anerobic bacteria (ORP, pH) and counteract an unwanted accumulation of hazardous intermediates, making the biotic remediation more controllable. Simultaneously, less ZVI particles are needed to successfully degrade the present amount of PCE, reducing remediation costs significantly.

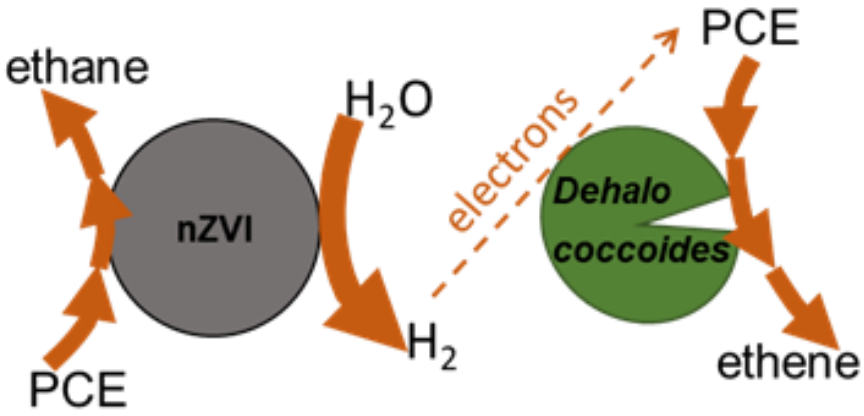

Figure 6: Schematic overview of the potential usage of ZVI produce H_2 by dehalorespiring Dehalococcoides spp.

Both beneficial and inhibitory effects of nZVI on the biotic PCE degradation have been observed (Barnes et al., 2010; Chen et al., 2011; Rónavári et al., 2016; Schiwy et al., 2016; Wang et al., 2016; Xie et al., 2017b). They tend to agglomerate to cell surfaces and sequentially disrupt the cells integrity (Auffan et al., 2008; Barnes et al., 2010; Chaithawiwat et al., 2016; Xiu et al., 2010). nZVI also forms ethyne, which is potentially toxic (Pon et al., 2003). Furthermore, the dechlorinating enzymes are membrane bound (Fung et al., 2007; Magnuson et al., 1998; Müller et al., 2004; Nijenhuis and Zinder, 2005). Thus, attachment of nZVI to cell membranes can lead to an inhibition of these enzymes. The gene down-regulation coding for dehalogenases has been observed in the presence of ZVI (Xiu et al., 2010), which could be caused by the fast elimination of the needed precursors *cis*-DCE and VC. The data of the effects of mZVI on the biotic dechlorination is scarce. But it was shown that it can have a beneficial effect on the dehalogenation decabromodiphenyl ether and 1,1,2-trichloroethane, suggesting a potential for the stimulation of the bacterial PCE dechlorination (Patterson et al., 2016; Shih et al., 2012; Xu et al., 2014).

RESEARCH QUESTIONS AND HYPOTHESIS

New approaches for the in-situ remediation of PCE are needed, tackling the downsides of the already established ones. Several studies have already been conducted regarding the combination of ZVI and dechlorinating bacteria for PCE dechlorination. Different nZVI particles and their influence on dechlorinating bacterial consortia in laboratory-scale and field-scale experiments have been investigated. Different results from stimulatory to inhibitory effects had been observed (Barnes et al., 2010; Kocur et al., 2016; Rónavári et al., 2016; Schiwy et al., 2016; Wang et al., 2016; Xie et al., 2017a; Xiu et al., 2010). On the other hand, only scarce information on the influence of mZVI on completely dechlorinating bacteria is available. Knowledge of the best possible combinations of ZVI and bacteria is a basic requirement for the successful application of this technology during a site remediation.

Hypothesis 1:

The combination of ZVI particles and *Dehalococcoides* spp. leads to a complete dechlorination of PCE. The H_2 produced during anaerobic corrosion of ZVI can be used by *Dehalococcoides* spp. for a complete PCE degradation. Thus, this technology is a promising option for in-situ remediation of contaminated sites.

Hypothesis 2:

The degradation performance is strongly dependent on the characteristics of the ZVI particles (size, reactivity, tendency to agglomerate).

Hypothesis 3:

Experimental parameters, like carbon source, supply of nutrients and vitamin B12, temperature and inoculum concentration influence the degradation performance drastically, but are adjustable to achieve a sufficient removal of PCE.

Hypothesis 4:
The prevalence of other bacterial species (e.g.: methanogenic) has a strong influence on the PCE degradation by *Dehalococcoides* spp..

In order to investigate the established hypothesis, the following experiments were conducted within the scope of the BIANO and the MICROSYN project:

Table 3: Overview of the conducted laboratory and lysimeter experiments during the study

Experiment	**Scale**
Screening of ZVI particles	Microcosms
Screening of bacterial cultures	Microcosms
Screening for suitable parameters for the bacterial dechlorination	Microcosms
Testing of different combinations	Microcosms
Lysimeter experiment	Lysimeter

Laboratory-scale experiments offer a fast, cheap and easy first step in investigating different ZVI and bacterial cultures under highly controlled conditions but are a weak model for the conditions present in the underground. Due to their spatial boundaries, direct contact of the bacterial consortia with the ZVI particles is inevitable. Negative effects of nZVI particles have mainly been accounted to aggregation onto the

bacterial cells (Auffan et al., 2008; Chaithawiwat et al., 2016; Li et al., 2010; Xiu et al., 2010). It was shown, that physical proximity is needed for the cytotoxic effect of nZVI particles (Li et al., 2010). In field conditions, bacteria would be able to establish themselves apart from the potentially inhibitory nZVI particles counteracting possible toxic effects, while still benefiting from the H_2 produced during anaerobic corrosion. The downside of field experiments is their low controllability, the difficulty in monitoring environmental parameters and high expenditure.
In this study the following experiments were conducted within the scope of the *BIANO* and the *MICROSYN* projects:

In first steps, different ZVI particles (nano, micro, flake-shaped, fresh and aged) were tested during microcosm experiments on their ability to dechlorinate PCE to ethyne, ethene and ethane as well as their production of H_2. Following different commercially available, *Dehalococcoides* spp. containing, dechlorinating bacterial cultures were tested on their dechlorinating behavior under different conditions (C-source, nutrient composition, temperature, inoculation concentrations).

So far two articles were published from the studies conducted. After these first screenings of suitable particles and cultures, two of each were chosen for further microcosm experiments to investigate the influence of their combinations (manuscript 1) mZVI and nZVI particles were chosen for the microcosm experiments, due to their different behaviors during dechlorination and anaerobic corrosion. The ZVI particles were combined with two differently chlorinating bacterial cultures:

- Complete PCE dechlorination to ethene
- Incomplete PCE dechlorination to *cis*-DCE

Following the laboratory scaled experiments, a lysimeter experiment was conducted, during which we were able to investigate the influence of nZVI particles on a completely dechlorinating culture over several months. Field-like conditions could be established, while keeping the controllability and possibility of environmental parameter monitoring high (manuscript 2).

PUBLICATIONS

Manuscript 1

Science of the Total Environment 722 (2020) 137802

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Inhibition and stimulation of two perchloroethene degrading bacterial cultures by nano- and micro-scaled zero-valent iron particles

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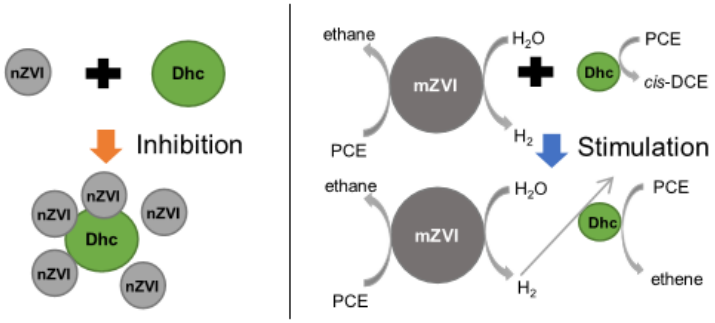
HIGHLIGHTS

- nZVI inhibits and mZVI stimulates anaerobic bacterial PCE dechlorination.
- Higher reactivity and heavy agglomeration nZVI impairs bacterial PCE degradation.
- mZVI can start ethene production in otherwise incomplete degrading culture.
- Slow H_2 production at low concentration by mZVI favours dehalorespiring metabolism.
- H_2 produced by ZVI can be used by PCE degrading and methanogenic bacteria.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 10 January 2020
Received in revised form 6 March 2020
Accepted 6 March 2020
Available online 07 March 2020

Editor: Frederic Coulon

Keywords:
Perchloroethene
Dehalococcoides
Nano-scaled zero valent iron
Micro-scaled zero valent iron
PLFA

ABSTRACT

The pollutant perchloroethene (PCE) can often be found at urban contaminated sites. Thus in-situ clean-up methods, like remediation using zero valent iron (ZVI) or bacterial dechlorination, are preferred. During the remediation with ZVI particles anaerobic corrosion occurs as an unwanted, particle consuming side reaction with water. However, in this reaction H_2 is formed, which is usually scarce during anaerobic microbial dechlorination. *Dehalococcoides* needs H_2 for cell growth using it as an electron donor to dechlorinate chlorinated hydrocarbons. Combining application of ZVI with bacterial dechlorination can turn ZVI in a H_2 donor leading to a more controllable bacterial dechlorination, a smaller amount of ZVI suspension and decreased remediation costs. In this study nano- and micro scaled ZVI particles (nZVI, mZVI) were combined in microcosms with two dechlorinating bacterial cultures. The two cultures showed different dechlorination behaviors with ethene and *cis*-DCE as final products. Phospholipid fatty acids (PLFA) associated with *Dehalococcoides* (18:1w7, 18:1w7c, 10:Me16:0) and *Geobacteriaceae* (16,1w7c; 15:0; 16:0) have been found in both bacterial cultures, slight differences in their abundance could explain the different dechlorinating behaviors. The combination of both bacterial cultures with mZVI led to a stimulated dechlorination process leading to about two times higher k_{obs} for PCE dechlorination (0.01–0.05 h^{-1}). In the otherwise *cis*-DCE accumulating culture complete dechlorination to ethene was achieved. While addition of nZVI inhibited both cultures. Combined with nZVI the completely dechlorinating culture produced lower amounts of dechlorinated products (3.2 μmol) as compared to the single biotic treatment (5.1 μmol). Combining the incompletely dechlorinating culture with nZVI significantly reduced the $k_{obs,PCE}$ (single: $8 \times 10^{-3} \pm 3 \times 10^{-4}$ h^{-1}; combination: $5 \times 10^{-3} \pm 2 \times 10^{-4}$ h^{-1}). H_2 produced by nZVI and mZVI was utilized by both bacterial cultures. The particle size, resulting specific surface areas, agglomeration tendencies and reactivity appears to be crucial for the effect on microbial cells.

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https://doi.org/10.1016/j.scitotenv.2020.137802
0048-9697/

1. Introduction

Perchloroethene is a common and hazardous groundwater pollutant found worldwide at contaminated sites. Due to its high usage in different industries (e.g.: dry-cleaning, metal processing) PCE contaminations are mainly situated in urban areas and thus, in situ methods are essential for their clean-up since dig&dump is mostly not applicable at such sites (Field and Sierra-Alvarez, 2004; Mueller et al., 2012; United States Enivironmental Protection Agency, 2017; Yang et al., 2017b).

Bacterial dechlorination of PCE has been observed on many contaminated sites (Kotik et al., 2013; Kranzioch et al., 2013; Men et al., 2013). Different anaerobic metabolic pathways capable of PCE dechlorination have been discovered. Most known PCE degraders (e.g. *Geobacter lovleyi*) form hazardous metabolites, like *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC) as their final product (Field and Sierra-Alvarez, 2004; Sung et al., 2006). *Dehalococcoides* and recently also *Dehalogenimonas* has been shown to be able to completely dechlorinate PCE and TCE to harmless ethene, by removing the chloride while keeping the carbon-backbone intact (Leitner et al., 2017; Maymó-Gatell et al., 1997; Yang et al., 2017a, 2017b). During dehalorespiration PCE gets reduced and is used as an energy source for the bacterial growth. *Dehalococcoides* are strictly anaerobic and need H_2 as an electron donor (Löffler et al., 2013). The absence of *Dehalococcoides* and/or H_2, as well as unfavorable environmental conditions, can results in no PCE dechlorination or the accumulation of unwanted, hazardous metabolites (Amos et al., 2008; Lu et al., 2006; Yang et al., 2017a, 2017b). Furthermore, *Dehalococcoides* are competing for the often scarce H_2 with other anaerobic bacteria (e.g. methanogenes) (Smatlak et al., 1996).

The strong reductive agent zero-valent iron (ZVI) was shown to degrade PCE to ethene, ethyne and ethane (Cundy et al., 2008; Mueller et al., 2012; Taghavy et al., 2010). For chemical clean-up different sized ZVI particles, nano (nZVI)- or micro (mZVI)-scaled, can be used in different installations (Comba et al., 2011; Mueller et al., 2012). Under anaerobic conditions ZVI however also reacts with water and forms H_2 during anaerobic corrosion (Filip et al., 2014). Depending on the particle used most of the available electrons can be used up by this unwanted side reaction (Schöftner et al., 2015). Thus, an excess of ZVI is needed to achieve a substantial PCE dechlorination leading to a drastic increase in remediation costs.

Combining the chemical and bacterial dechlorination could eliminate the down-sides of both single treatments. The H_2 produced by anaerobic corrosion of ZVI could be used by dehalogenating bacteria as an electron donor resulting in a controlled bacterial dechlorination. Furthermore, ZVI could establish suitable anaerobic conditions for *Dehalococcoides* and prevent accumulation of unwanted metabolites. nZVI particles have been shown to both stimulate and inhibit dehalogenating bacteria (Barnes et al., 2010; Rónavári et al., 2016; Schiwy et al., 2016; Wang et al., 2016; Xie et al., 2017; Xiu et al., 2010b) while information on the influence of mZVI on the bacterial PCE dechlorination is limited. However, mZVI has been found to have stimulatory effects on microbial dehalogenation of decabromodiphenyl ether and 1,1,2-trichloroethane and thus has potential to positively influence biotic PCE dechlorination (Patterson et al., 2016; Shih et al., 2012; Xu et al., 2014). The higher specific surface area of nZVI particles leads to a higher reactivity and tendency for agglomeration (Comba et al., 2011; Li et al., 2016; Ma et al., 2016; Zhao et al., 2016). Reduction in viable cell numbers, attachment to bacterial cells by bare nZVI particles and down-regulation of the expression of dehalogenase-genes have been observed in previous studies (Li et al., 2010; Xiu et al., 2010a). Lower reactivity of mZVI particles leads to a higher longevity, slower removal of needed precursors and slow release of H_2 favouring *Dehalococcoides* over their anaerobic competitors.

In this study the influence of ZVI particles on the PCE dechlorination by two commercially available bacterial consortia containing *Dehalococcoides* in microcosms was investigated. Different sized ZVI particles (nZVI and mZVI) were tested for their own PCE dechlorination potential as well as their effect on bacterial cultures. PCE dechlorination, metabolite production and formation of dechlorinated end products were monitored, as well as the H_2 development to investigate the potential of bacterial H_2 usage. CH_4 formation was measured to study the influence of the ZVI particles on another potentially competing anaerobic bacterial pathway.

2. Methods and materials

2.1. ZVI particles

Iron powder (zero valent iron; ZVI) in nano-scale (Nanofer Star; nZVI) purchased from NANO IRON, s.r.o. (Czech Republic) and in micro-scale (Höganäs AB; mZVI) purchased from Höganäs GmbH (Sweden) were used. A 20 times higher concentration of Fe(0) was applied using the mZVI particles compared to the nZVI particles to compensate at least partly for its lower specific surface area (Table 1). A complete compensation would have exceeded the volume of the microcosms and thus was not possible. The specific surface areas of the fresh ZVI particles were determined using BET (Brunauer, Emmett and Teller) measurements done by the *Palacký Unitversity of Olomouc, Faculty of Science, Regional Centre of Advanced Technologies and Materials*. Absolute surface areas of zerovalent iron particles were calculated by multiplying the specific surface area by the mass of particles per microcosm (0.70 g mZVI, 0.04 g nZVI) including the ZVI-content of the respective particles. The Fe(0) content of the particles was determined by measuring produced H_2 after treatment of the dry particles with hydrochloric acid. To counteract agglomeration of the nZVI during storage, they were dispersed directly prior to the experiments. A 240 g/L suspension was dispersed for 3 min (IKA ULTRA-TURRAX T 18 basic, IKA – Werke GmbH & Co.KG), subsequently transferred into an oxygen free 40 mL glass bottle closed with a mini-inert valve (Sigma-Aldrich Handels GmbH) and manually shaken until use. The procedure was done in a N_2 flushed glove tent to minimize oxygen input. Furthermore, the headspace of the bottle containing the nZVI suspension was also flushed with N_2 before use. For every experiment, a new nZVI suspension was freshly prepared. The fast sedimentation of the mZVI particles made it impossible to produce a homogenous particle suspension. Thus, mZVI particles were directly weighed into the microcosm bottles.

2.2. Bacterial cultures

Two commercially available, *Dehalococcoides* containing and PCE degrading mixed bacterial cultures were used (SiREM®, referred to as KB1, and Bioclear earth b.v.). Both cultures were stored at 10 °C upon receipt and were used within one month. All handling of the strictly anaerobic bacterial cultures was performed in an Ar-flushed glovebox with an O_2 concentration below 40 ppm. The bacterial cultures were thoroughly mixed by pipetting before putting them into the microcosm bottles, to ensure a homogenous and equal distribution within all microcosms.

2.3. Microcosm experiments

Table 2 shows the conducted microcosm experiments. Both ZVI particles were tested alone and in combination with both cultures regarding their PCE dechlorination potential and their production of

Table 1
Specific and absolute surface area of ZVI-particles in each microcosm and Fe(0) content (w/w%) of the used nano-scale and micro-scale ZVI particles.

ZVI particles	Scale	Spec. surface area [m^2/kg]	Abs. surface area [m^2]	Fe(0) content w/w %
Höganäs AB	Micro	84	0.06	95
Nanofer Star	Nano	17×10^3	0.71	79

metabolites (trichloroethene (TCE), *cis*-1,2-dichloroethene (DCE), *trans*-1,2-DCE, 1,1-DCE and vinyl chloride (VC)), fully dechlorinated end products (ethene, ethyne and ethane) and competition metabolites (CH_4). All microcosms (120 mL glass bottles, Th. Geyer GmbH & Co. KG, Germany) were prepared within an Ar-flushed glovebox. Quartz sand (84 g; 0.5/2 mm; Quarzwerke Melk, Austria) was used to simulate aquifer material. The mZVI containing microcosms were amended with 0.7 g of dry Höganäs particles. Subsequently the microcosms were filled with O_2 free water containing HEPES disodium salt (12 g/L; Sigma-Aldrich Handels GmbH, ≥99%) for pH stabilization during the experiment and resazurin sodium salt (1 mg/L; Alfa Aesar) as a redox indicator. Microcosms for bacterial investigations were amended with molasses (679 mg/L; AGRANA Beteiligungs-AG). Na_2S (0.5 g/L; Sigma-Aldrich Handels GmbH) was used to create a reducing environment. After the medium turned from pink to colorless, indicating suitable reducing conditions, 3.3 mL of bacteria cultures were added (to a final concentration of 100 mL/L). The microcosms were prepared to comprise 55 mL gaseous phase, 33 mL liquid phase and 32 mL solid phase (sand). pH and the ORP (oxidation-reduction potential, referred to standard hydrogen electrode E_h) were measured before and after adding the bacterial culture with a portable sensors (SenTix® 41; SenTix® ORP-T 900; Xylem Analytics Germany Sales GmbH & Co. KG, WTW) to check that the pH (6.5–8.0) and the ORP ($E_h < -120$ mV) were in a suitable range for bacterial dehalogenation (United States Environmental Protection Agency, 1998; Blume et al., 2010; Löffler et al., 2013; Yang et al., 2017c). The microcosm bottles were sealed with PTFE-layered septa and aluminum crimp caps (VWR international). PCE (Sigma-Aldrich Handels GmbH, ≥99,9%) was dissolved in acetone (3 mL/41 mL) and added to the microcosms. Then 170 μL of the nZVI suspension were added into the respective microcosms. The microcosms were weighed before and after addition of the nZVI suspension to control the amount of suspension added. To ensure gas tightness of the microcosms 100 μL of propane (60% in N_2; Linde AG) was used as an internal standard. Stable contents of propane have been measured over the course of the experiments indicating the tightness of the bottles. The microcosms were prepared in triplicates. The first measurements were done after 1 h of equilibration on the shaker. Microcosms were shaken vertically at 50 rpm/min (Universal Shaker SM 30C, Edmund Bühler GmbH) at room temperature and samples were taken in regular intervals.

Two types of control microcosms were prepared. Control 1 was filled with the liquid medium containing Na_2S and to control 2 additionally 84 g of quartz sand was added. Both controls were done in triplicates. The bottles were then autoclaved and closed. Control 1 was then spiked with 9 μL PCE-stock solution and 100 μL propane to prove air tightness of the system. Control 2 was spiked with 100 μL of propane, 90 μL PCE-metabolite solution and 80 μL VC solution (2000 μg/mL, Sigma Aldrich) to investigate sorption to quartz sand. The PCE-metabolite solution contained PCE, TCE, *cis*-1,2-DCE, *trans*-1,2-DCE and 1-1-DCE (200 μL each) dissolved in 41 mL acetone.

Table 2
Conducted microcosm experiments, their applied treatments, number of replicas and duration.

Experiment	Treatment	c_{aq} Fe(0) [g/L]		c_{aq} culture [%v/v]		No. of replicates	Duration [days]
		nZVI	mZVI	KB1	Bioclear		
ZVI	nZVI	1	–	–	–	3	36
	mZVI	–	20	–	–	3	36
KB1	KB1	–	–	10	–	3	14
	KB1 + nZVI	1	–	10	–	3	14
	KB1 + mZVI	–	20	10	–	3	14
Bioclear	Bioclear	–	–	–	10	3	37
	Bioclear + nZVI	1	–	–	10	3	37
	Bioclear + mZVI	–	20	–	10	3	37

Carbon balances from the microcosm experiments and both controls were calculated by adding up the moles of PCE, its metabolites and dechlorinated end products at the first sampling time compared to those measured over the course of the experiment.

2.4. PLFA extraction

Phospholipide fatty acids (PLFA) can be used as biomarkers for different bacterial groups and for analyzing the biomass content of a sample. To investigate the PLFAs present in both bacterial cultures separate bacterial microcosms (without ZVI) were prepared as described in Section 2.3. The microcosms were opened under anaerobic conditions after 12 days. 20 g of wet sand were taken from each replicate, frozen in liquid nitrogen and then freeze-dried. The freeze-dried samples were stored in a desiccator until extraction. The PLFA extraction was done according to Watzinger et al. (2014). About 2 g of each replicate sample were pooled together and lipids were extracted with a chloroform:methanol solution (1:2, v:v, pH 4). Using solid phase extraction (Isolute SI, 500 mg silica 3 mL, Biotage) the phospholipids were eluted and collected in methanol. Then they were transformed to fatty acid methyl esters (FAMEs) in a mild alkaline transmethylation (methanolic KOH). For quantification two internal standards were added. 19:0 PC 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, USA) was added to the samples before the extraction and 13:0 FAME fatty acid methyl ester (Supelco) was added during the methylation.

PLFAs were analyzed with a GC-FID (7890 A; Agilent Technologies) equipped with a HP-5 MS column (60 m × 0.25 mm; Agilent Technologies, Inc). More detailed information shown in supplement.

2.5. Chemical analysis

PCE, its metabolites (TCE, *cis*-1,2-DCE, *trans*-1,2-DCE, 1,1-DCE, VC) and end products (ethene, ethyne and ethane) as well as propane and methane were measured with a GC-FID (Fisons GC8000Top) equipped with a GS-Q column (30 m × 0.53 mm plot column; Agilent Technologies, Inc). For the measurement of H_2 a GC-TCD (Fisons GC8000Top) with a packed mole sieve-column (5 Å) was used. Detailed conditions are shown in supplement. For analysis 100 μL samples of the headspace were taken with a gas-tight syringe (100 μL, 50 mm, OD: 0.5 mm, Gauge: 25; SGE analytical science) and injected for each measurement. Generated overpressure was measured using an easy-going syringe (Fortuna Optima 5 mL; Poulten & Graf GmbH). To avoid oxygen input the sample taking syringe was flushed with N_2 beforehand. 100 μL of N_2 were added for each sample taken from the microcosms to avoid negative pressure causing a possible oxygen intrusion. According to Henry's law, the substance content in the aqueous phase (n_{aq} [mol]) was calculated:

$$\frac{n_{aq} = k_H \times V_{aq} \times m_g \times R \times T}{V_g \times M} \quad (1)$$

With k_H as Henry constant $[\frac{mol}{m^3 \times \frac{kg}{m \times s^2}}]$, V_{aq} as Volume of the aquatic phase [L], m_g as the substance content in the gas phase [g], R as ideal gas constant $[\frac{kg \times m^2}{s^2 \times mol \times K}]$, T as temperature [K], V_g as volume of the gas phase [L] and M as the molar mass of the analyte [g/mol]. Henry constant (k_H) can be described as a function of temperature (Eq. (2)):

$$k_H = k_H^{\theta} \times \exp\left(\frac{-\Delta_{soln}H}{R} \times \left(\frac{1}{T} - \frac{1}{T^{\theta}}\right)\right) \quad (2)$$

With k_H^θ being the Henry constant at a norm temperature, $\frac{-\Delta_{soln}H}{R}$ as the enthalpy of the solution and T^θ as the standard temperature (298.15 K). k_H^θ and $\frac{-\Delta_{soln}H}{R}$ for the different substances used were derived from published data (see supplement). Results for the total substance content within the microcosms were corrected with the losses during sample taking, overpressure release and additional losses of the internal standard propane. At the end of the experiment the pH was checked to verify a stable, neutral pH over the whole period of the experiment.

2.6. Calculation of reaction rates

Observed reaction rates (k_{obs}) of PCE dechlorination (all treatments), of H_2 formation (ZVI treatments) and CH_4 production (microbial and combined treatments) were calculated. PCE dechlorination followed pseudo-first-order kinetics. The reaction rates were derived using nonlinear regression (Eq. (3), Levenberg-Marquardt algorithm, SPSS Statistics 24.0).

$$n = A \times e^{B \times t} \tag{3}$$

With n as the amount of substance [μmol] and t as duration of the experiment [days].

The derived values for A and B were then used to calculate $k_{obs,PCE}$ (Eq. (4)).

$$k_{obs,PCE} = -\frac{\ln \frac{A \times e^{B \times t_0}}{A \times e^{B \times t_{end}}}}{\Delta t} \tag{4}$$

The H_2 production via ZVI and microbial CH_4 production followed a zero-order reaction kinetics. Thus, the corresponding k_{obs} [h^{-1}] were retrieved from the slope of the linear regression.

For the single ZVI treatments (without bacterial culture) particle surface area normalized reaction constants (k_{SA} [$L\,h^{-1}\,m^{-2}$]) were calculated. The retrieved k_{obs} were divided by the initial absolute surface area of the particles and the concentration of Fe(0) in the microcosms (Tables 1 and 2). In the combined microcosms (ZVI and bacterial cultures) two processes (chemical and bacterial) were responsible for the PCE dechlorination and H_2 was simultaneously formed and consumed. CH_4 was not produced by the ZVI particles. Thus, values for k_{obs} and/or k_{SA} were not calculated for these experiments.

3. Results

3.1. Dechlorination of PCE, formation of metabolites (TCE, cis-DCE and VC) and dechlorinated end-products (ethene, ethane and ethyne)

The highest observed reaction constants ($k_{obs,PCE}$) for the PCE dechlorination were achieved with all three KB1 treatments (Fig. 1). Combining KB1 with mZVI resulted in a more than two-fold increase in $k_{obs,PCE}$ compared to the single KB1 treatment, while the combination with nZVI did not result in a significant difference. The combination of Bioclear + nZVI significantly decreased $k_{obs,PCE}$ and Bioclear + mZVI did not result in a significant change in $k_{obs,PCE}$. The similar $k_{obs,PCE}$ of the two ZVI treatments, the 20 times higher Fe(0) content of the mZVI microcosms and the higher specific surface area of the nZVI particles resulted in the same surface area normalized reaction constants ($k_{SA,PCE}$).

PCE was fully removed in all treatments containing bacterial cultures apart from the Bioclear + nZVI microcosms (Fig. 2). *cis*-DCE was the main metabolite formed by both bacterial cultures. While it was degraded in the KB1 microcosms, it accumulated in the Bioclear microcosms without further dechlorination. mZVI accelerated the dechlorination of *cis*-DCE in KB1 and induced dechlorination of *cis*-DCE by Bioclear. The combination of KB1 with nZVI led to lower VC levels compared to the single treatments, while the combination with mZVI increased VC formation and dechlorination velocities. The mZVI particles induced the formation and dechlorination of VC by the Bioclear culture.

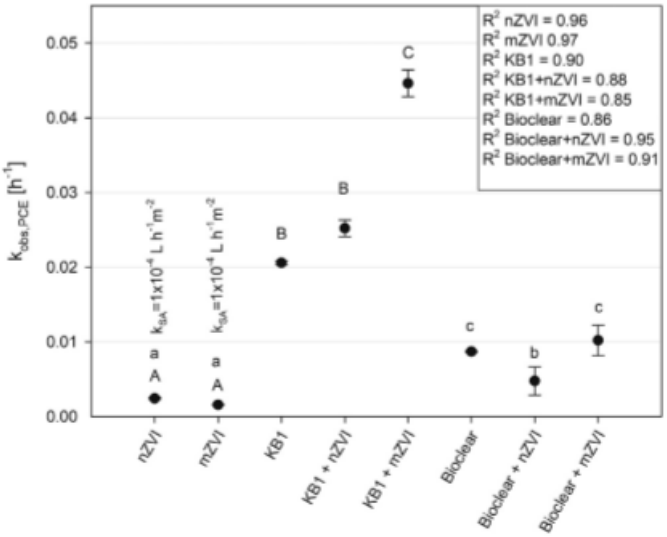

Fig. 1. Observed PCE degradation rates (k_{obs}) of all treatments (KB1, KB1 + nZVI, KB1 + mZVI, Bioclear, Bioclear + nZVI, Bioclear + mZVI, nZVI and mZVI). Surface normalized PCE degradation rates (k_{SA}) for the single ZVI treatments are shown above the respective symbols. Error bars represent standard deviations of three replicates. Different letters (A, B, C, D and a,b,c) above the symbols indicate statistically significant differences between the respective values ($\alpha = 0.05$ Duncan's post-hoc test).

The ZVI microcosms showed a similar behavior regarding the formation of the dechlorinated end-products (CH) ethene and ethane (Fig. 2). Except for ethyne which was detected only in low levels in those with nZVI (not shown). All KB1 microcosms led to higher CH contents than the single ZVI treatments, with highest levels detected in KB1 + mZVI and lowest in KB1 + nZVI. No ethane production could be observed in the single KB1 treatment. The combination of the KB1 culture with both ZVI particles led to earlier detectable CH levels than the single KB1 treatment. While the combination KB1 + nZVI produced ethene and ethane steadily from the beginning, the KB1 + mZVI treatments showed a drastic increase in ethene levels after eight days. High standard deviations for ethene showed differences between the replicates from day four onwards. While two replicates showed a drastic increase in ethene after eight days, this increase appeared one data point later for the third replicate. In the single Bioclear treatment no notable formation of ethene or ethane was detected. Combining Bioclear with nZVI resulted in a slightly higher amount of detected CH than combining it with mZVI. The ethene production in Bioclear + mZVI ceased after 14 days until it restarted on day 25. No ethyne could be detected in the microcosms containing the KB1 or Bioclear cultures.

3.2. Hydrogen development and CH_4 formation

The H_2 evolution in the nZVI microcosms was about three times higher than in the mZVI microcosms (Fig. 3). The H_2 content in both ZVI treatments increased gradually until the end of the experiment. In comparison in all combined treatments a distinct reduction in the H_2 concentration could be observed after an initial increase. The H_2 consumption started 2–4 days earlier when both cultures were combined with mZVI rather than nZVI. Significant differences in $k_{obs,H2}$ and $k_{SA,H2}$ between the two ZVI particles were detected. The mZVI particles achieved a lower $k_{obs,H2}$ ($0.2 \pm 0.04\ h^{-1}$; $R^2 = 0.96$) than the nZVI particles ($1.1 \pm 0.2\ h^{-1}$; $R^2 = 0.98$). In comparison the $k_{SA,H2}$ of the mZVI particles ($3.8 \pm 0.6\ h^{-1}\,m^{-2}$) was more than two times higher compared to that of the mZVI ($1.5 \pm 0.2\ h^{-1}\,m^{-2}$).

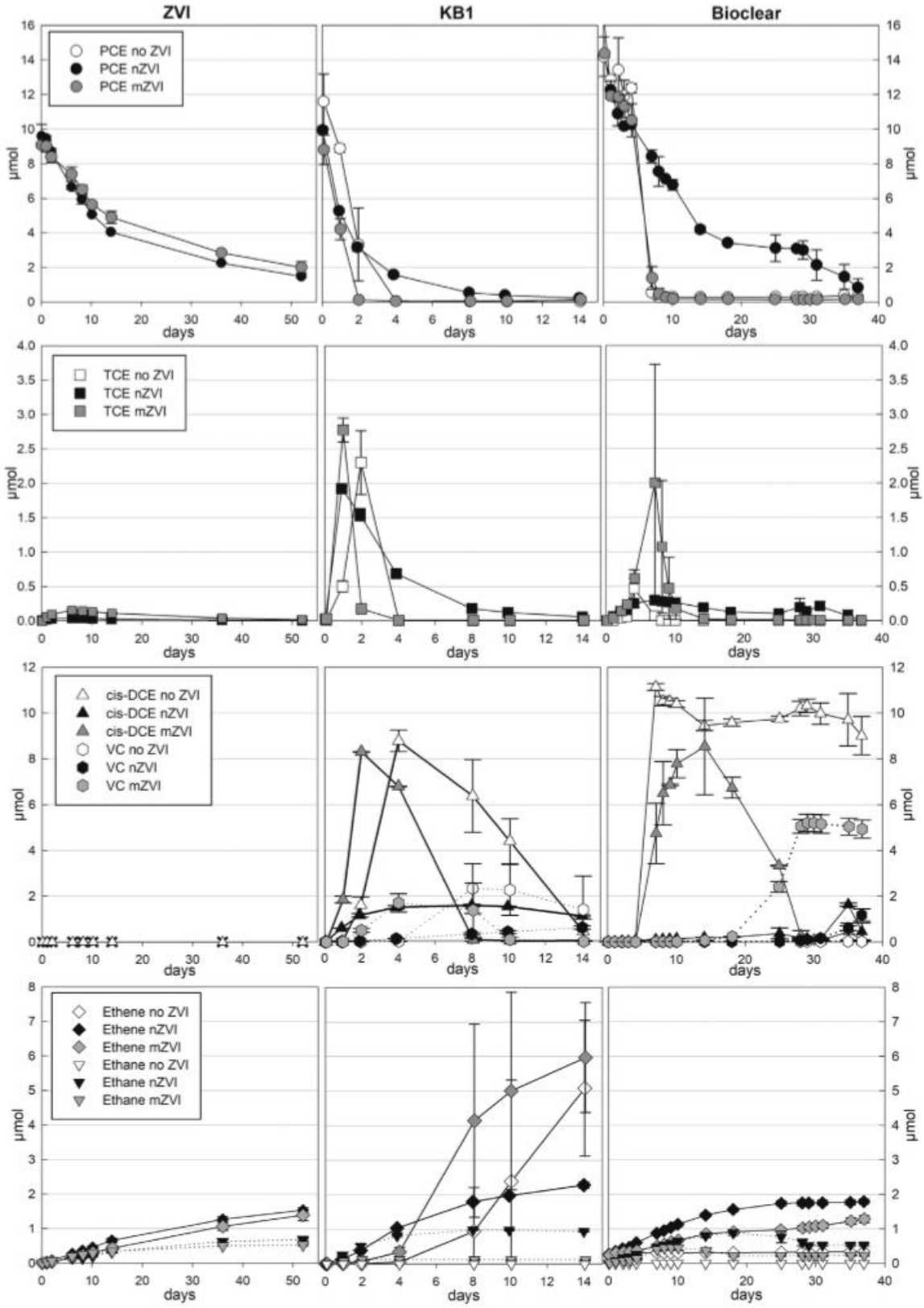

Fig. 2. Temporal changes in concentration of PCE (●), TCE (■), *cis*-DCE (▲), VC (●), ethene (♦) and ethane (▼) in microcosm experiments (µmol of aqueous and gaseous phase combined). Column ZVI: nZVI (black symbols) and mZVI (grey symbols); column KB1: KB1 (white symbols), KB1 + nZVI (black symbols), KB1 + mZVI (grey symbols); column Bioclear: Bioclear (white symbols), Bioclear + nZVI (black symbols), Bioclear + mZVI (grey symbols). Values were corrected with internal propane-standard. Error bars represent standard deviations of three replicates.

In all Bioclear treatments an elevated CH_4 content was detected at the start of the experiment, which was caused by CH_4 present in the Bioclear inoculum. For better comparability and calculation of formed CH_4 the measured starting CH_4 contents were subtracted from the measured CH_4 content in the microcosms. The combination of both bacterial cultures with mZVI led to 8–14 µmol higher maximum CH_4 levels

(Fig. 4). In the combinations with nZVI CH_4 concentrations increased by 4–7 μmol compared to their single bacterial treatment counterparts. In all four combined treatments a significant increase in the $k_{obs,CH4}$ with higher values in the combinations with mZVI was achieved (Fig. 5). Highest $k_{obs,CH4}$ values were observed in the combinations with mZVI (1.2–1.5 h^{-1}). A much more distinct increase in $k_{obs,CH4}$ with 39–678 times higher values occurred in the combined Bioclear microcosms compared to the combined KB1 treatments (1–2 times). The CH_4 production continued in all six treatments until the end of the experiments but amounts surpassed the upper measurement range limit of 23.5 μmol CH_4 in the headspace.

3.3. PLFA extraction

Both bacterial cultures showed similar PLFA patterns with only slight differences (Fig. 6). In both cultures the fatty acids 16:0 (KB1: 0.23 ng/g, Bioclear: 0.27 ng/g), 16:1ω7c + 6c (KB1: 0.26 ng/g, Bioclear: 0.34 ng/g) and 18:1ω7c (KB1: 0.10 ng/g, Bioclear: 0.13 ng/g) were predominant, with an overall higher abundance in the Bioclear culture. All other detected PLFAs were found at concentrations at or below 0.05 ng/g. The fatty acids 10Me16:0 + i17:1ω8 were found with higher abundance in the KB1 culture (KB1: 0.04 ng/g, Bioclear: 0.01 ng/g). Similar total PLFA amounts (KB1: 72 ng; Bioclear: 84 ng) were detected in both cultures.

3.4. Carbon balances and unidentified peaks

From the start over 20% less carbon could be recovered from batches containing medium and sand (control 2) compared to batches containing only medium (control 1). After 50 days the difference in the carbon balance increased to over 30% (see supplement). All experimental microcosms had a carbon recovery of ≤83% after 14 days. Compared within the experimental counterparts the lowest carbon recovery was found in the microcosms containing nZVI (32–56%) at day 14, 37 and 52, compared to those containing only bacterial culture (46–70%), only mZVI (43–64%) or combinations with bacterial cultures and mZVI (48–83%). At the end of the experiments, the percentage of the total peak areas of unidentified substances related to the total peak area was calculated (see supplement). In the microcosms containing only ZVI this percentage was 5–24 times higher than in the bacterial single treatment ones and 2–12 times higher than in the combined microcosms.

4. Discussion

4.1. Influence of nZVI and mZVI on bacterial dechlorination

Although the absolute surface area of the nZVI particles in the batches was over 10 times higher the batches containing nZVI showed no significant difference in the $k_{obs,PCE}$ or $k_{SA,PCE}$ during the course of the experiment (day 1, day 14 and day 38, *t*-test $\alpha = 0.95$) compared to those containing mZVI. $k_{SA,TCE}$ of 0.12 L h^{-1} m^{-2} have been reported using Nanofer Star particles in similar concentrations (Schöftner et al., 2015). In our microcosm experiment the shaking velocity during the experiment was five times lower than in the other study. This low shaking velocity (simulating a moderate groundwater flow) during the experiment could have favored agglomeration of the nZVI particles, leading to a decreased surface area and consequently the reduced reactivity of the nZVI particles.

Both bacterial cultures were able to degrade PCE and remove it from the system completely. However, KB1 showed an over two times faster PCE dechlorination rate compared to Bioclear. The KB1 culture was able to degrade *cis*-DCE further to VC and finally to ethene. In contrast in the Bioclear microcosms dechlorination stopped at *cis*-DCE and no VC or a fully dechlorinated final product were produced. Faster PCE dechlorination resulting in higher amounts of toxic intermediates when using biotic compared to abiotic dechlorination has already been observed (Rosenthal et al., 2004). The similar total PLFA content of both bacterial cultures suggests that a different biomass abundance cannot be accounted for the different dechlorination behaviors. Various metabolic pathways for PCE dehalogenation exist and not all result in a complete dechlorination of PCE to ethene (Field and Sierra-Alvarez, 2004). Presence of bacteria that are able to degrade PCE to *cis*-DCE (e.g.: *Geobacter lovleyi*) combined with an absence or low abundance of those capable of degrading lower chlorinated ethenes can cause an incomplete dechlorination. In both bacterial cultures PLFA biomarkers for degrading bacteria were detected. In pure *Dehalococcoides cultures* the mono unsaturated fatty acid 18:1w7 and the mid-chain branched saturated fatty acids (18:1w7c, 10:Me16:0) have been found besides ubiquitous straight chain fatty acids (14:0, 16:0 and 18:0) which are abundant in other *Chloroflexi* as well (Löffler et al., 2013; Wen et al., 2015). *Geobacter lovleyi* are represented in the family of *Geobacteriaceae* (Sung et al., 2006). 16:1w7c; 15:0; 16:0 are fatty acids reported to be associated with *Geobacteriaceae* (Anderson et al., 2003; Lovley et al., 1993). Overall the PFLA patterns of both cultures were similar only showing minimal differences. Fatty acids only associated with *Geobacteriaceae* (15:0 and

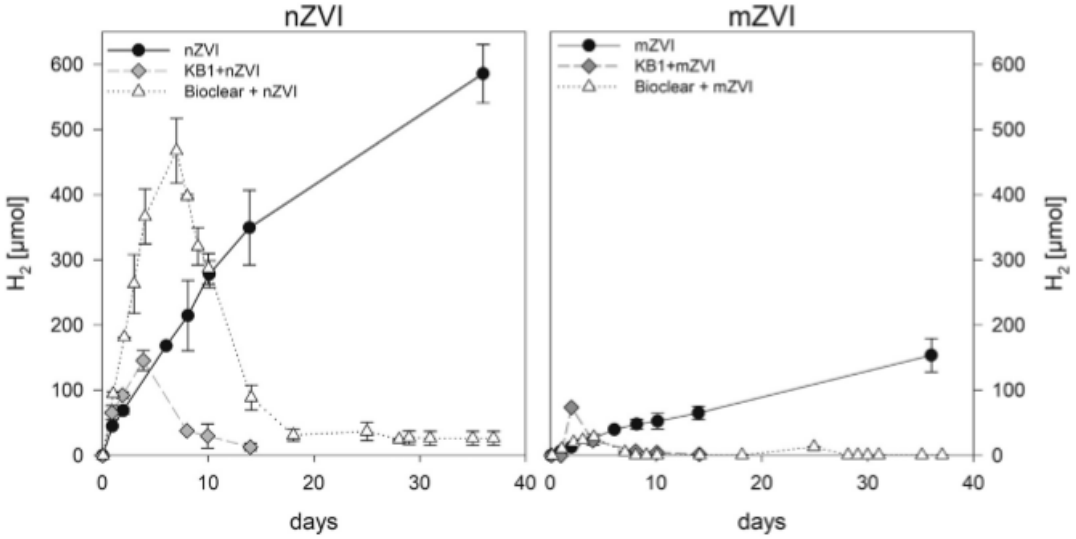

Fig. 3. H_2 development in the treatments containing ZVI particles (nZVI, KB1 + nZVI, Bioclear + nZVI, mZVI, KB1 + mZVI, Bioclear + mZVI). Error bars represent standard deviations of three replicates.

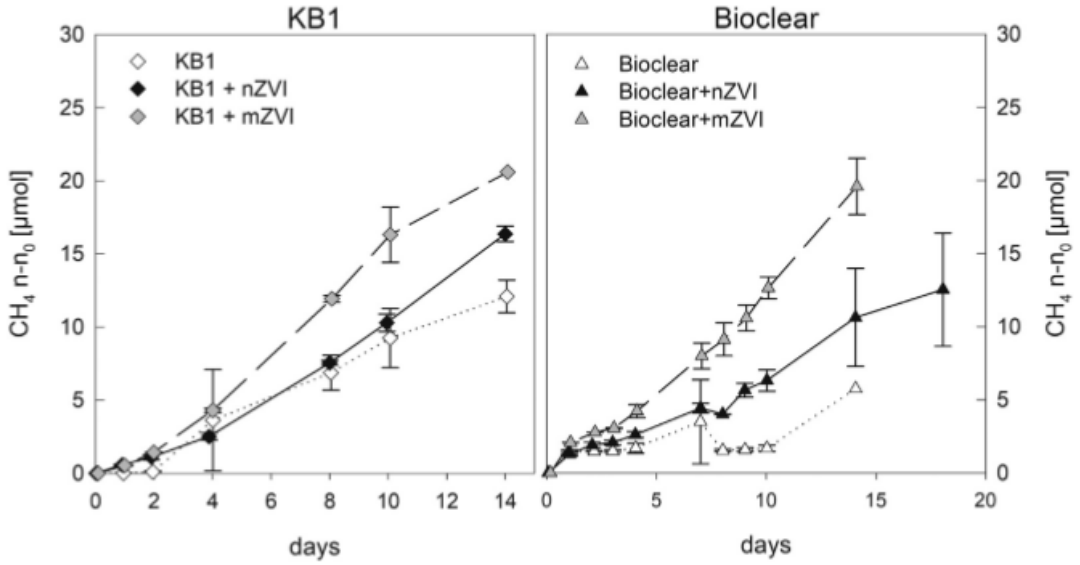

Fig. 4. Development of CH_4 contents in all bacterial microcosms (KB1, KB1 + nZVI, KB1 + mZVI, Bioclear, Bioclear + nZVI, Bioclear + mZVI). The CH_4 contents are those of the aqueous and gaseous phase combined. Values shown were derived by subtracting the measured CH_4 amount at day 0 (n_0) from the measured amount at the respective timepoint (n) and were corrected with internal propane-standard. Error bars represent standard deviations of three replicates.

16:1w7c) had a slightly higher abundance in the Bioclear culture. Furthermore, higher contents of the fatty acid markers 18:0 and 10Me16:0 related to *Dehalococcoides* in the KB1 culture. This suggests a higher prevalence of incomplete degraders in the Bioclear culture compared to culture KB1. The different degrading behaviors could have been caused by the lack of enzymes crucial for complete dechlorination. Different reductive dehalogenases have been found to be responsible for the dechlorination of higher and lower chlorinated ethenes in *Dehalococcoides* (Magnuson et al., 1998; Müller et al., 2004; Niño de Guzmán et al., 2018). Therefore, the presence of *Dehalococcides* does not guarantee a satisfying dechlorination of lower chlorinated ethenes. The absence of strains containing the reductive dehalogenase necessary for the dechlorination of lower chlorinated ethenes would result in accumulation of intermediate products. Additionally, a slower adjustment of degraders to the microcosm environment could have been the reason for the under-performance of Bioclear. This could have slowed down the growth of degrading bacteria and thus would have led to a slower and/or incomplete dechlorination under the used experimental conditions.

The combination of both bacterial cultures with mZVI led to a stimulation of the microbial dechlorination, however in slightly different ways. When combining KB1 with mZVI the $k_{obs,PCE}$ increased significantly. The $k_{obs;\ PCE}$ of mZVI was much lower (0.002), thus the faster dechlorination of PCE in the combination was not due to the dechlorinating activity of mZVI. The metabolites *cis*-DCE and VC, as well as ethene were both formed earlier than in the single treatment KB1 microcosms. Also, the measured concentrations of fully dechlorinated end products indicate the predominance of the microbial dechlorination in the combined treatment with mZVI. The percentage of ethane and ethyne of the total amount of dechlorinated end products

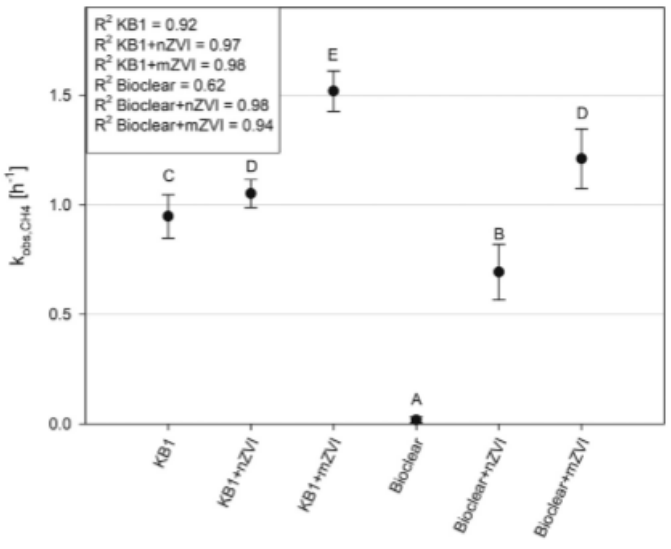

Fig. 5. Observed reaction constants (k_{obs}) for the microbial formation of CH_4 in all bacterial microcosms (KB1, KB1 + nZVI, KB1 + mZVI, Bioclear, Bioclear + nZVI and Bioclear + mZVI). Error bars represent standard deviations of three replicates. Different letters above the symbols indicate statistically significant differences between the respective values ($\alpha = 0.05$ Duncan's post-hoc test).

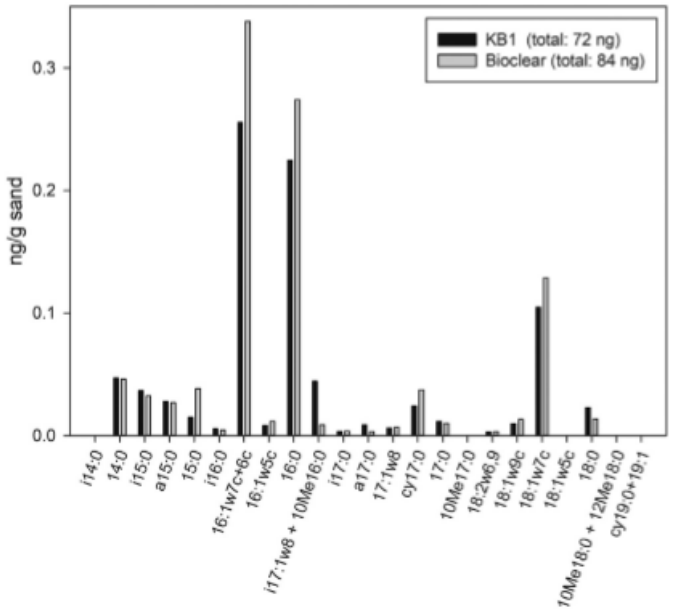

Fig. 6. Fatty acids detected from PLFA extraction in KB1 and Bioclear PLFA microcosms after 12 days.

can be an indicator for the prevalent degrading process (chemical or bacterial). Low amounts of dechlorinated end products in the ZVI microcosms were mainly caused by chemical side reactions resulting in unidentified final products, sorption of ethyne onto oxidized ZVI surfaces and sorption to the sand (see Section 4.2.). Ethane and ethyne are only produced by chemical dechlorination and not by microbial organohalide respiration. Anaerobic ethane oxidation by bacteria has been reported (Adams et al., 2013; Redmond et al., 2010). But under the conditions present in our microcosms (temperature and high abundance of favorable carbon sources) ethane dechlorination was unlikely. Thus, looking at the percentage of ethane and ethyne of the total amount of CH formed can give an insight into the on-going dechlorination processes. At the beginning of the experiment the percentage of ethane + ethyne in the KB1 + mZVI treatment was close to that of the single mZVI treatment, showing that the dechlorination process was mainly driven by mZVI (see supplement). At day eight the ethene production in the combined treatment increased drastically. Consequently, the ethane and ethyne percentage dropped significantly beneath that of the single mZVI treatment, indicating that the dechlorination was taken over by bacterial processes. The bacterial dehalorespiration is a step-wise process. The higher chlorinated ethenes are needed as a precursor for the formation of the lesser/not chlorinated ethene. Thus, VC is needed for the bacterial production of ethene. The co-occurrence of the significant increase of ethene with the decrease of VC further indicates that the production of ethene in this phase can mainly be accounted to bacterial processes. The combination of Bioclear with mZVI did not show a significant difference in the dechlorination of PCE compared to that of the single Bioclear treatment. But the dechlorination of *cis*-DCE and the formation of VC showed that the bacterial culture was stimulated by mZVI to generate and degrade lower chlorinated ethenes. Because of the stepwise dechlorination of the bacterial process, the immediate formation of ethene indicates a prevalence of chemical processes at the beginning of the experiment for the treatment Bioclear + mZVI. But the slight increase in ethene production combined with the presence of VC from day 25 indicated that bacterial processes contributed to the production of ethene from that phase. A change in the predominance of the main dechlorination process has also been described by Patterson et al. (2016) for 1,1,2-Trichloroethane. The mZVI reduced high contaminant concentrations to a suitable level for the *Dehalococcoides* containing culture, which then could consequently start its biotic dechlorination process.

The combination of both bacterial cultures with nZVI led to an inhibition of the bacterial dechlorination compared to the single treatments with bacterial cultures. This could be seen by formation of ethene from the beginning, lower amounts of metabolites (*cis*-DCE and VC) and insignificant differences in (KB1 + nZVI) or even lower (Bioclear + nZVI) $k_{obs,PCE}$. Even though no significant differences between $k_{obs,PCE}$ values of the KB1 and the KB1 + nZVI treatment were found, inhibition by nZVI was indicated by lower amounts of produced ethene in the combined treatment. As well as by the slowed down dechlorination of TCE compared to KB1 alone. However, this deceleration occurred only after four days, whereas at the beginning the speed of PCE and TCE dechlorination were similar between KB1 and KB1 + nZVI.

The low amounts of metabolites could either be accounted to a lower bacterial activity or their dechlorination by nZVI. The immediate ethene formation from the start indicated a prevalence of the chemical ethene production during that phase. This could also be seen in the similar percentages of ethane and ethyne in the total amount of dechlorinated products at the beginning.

ZVI reacts anaerobically with water and forms H_2, which is needed for bacterial dehalorespiration (Löffler et al., 2013; Magnuson et al., 1998). *Dehalococcoides* have been shown to be able to grow in the presence of ZVI (Niño de Guzmán et al., 2018; Rosenthal et al., 2004). Thus, ZVI can potentially support bacterial dechlorination by generation of H_2. This can explain the stimulation of the microbial consortia by mZVI particles in our experiments. Consumption of mZVI produced H_2 by *Dehalococcoides* and beneficial reduction of toxic contaminant concentrations by mZVI have also been observed in previous studies (Patterson et al., 2016; Xu et al., 2014).

The inhibition by nZVI, even though H_2 was produced, could have been caused by their higher reactivity and tendency to stick to the surface of cells, the elimination of needed metabolites (*cis*-DCE and VC) as precursors and/or their formation of ethyne which is toxic for cells (Kocur et al., 2016; Pon et al., 2003). Both, stimulation and inhibition of bacterial dechlorination has been observed in batch experiments (Barnes et al., 2010; Lee et al., 2001; Rónavári et al., 2016; Xie et al., 2017; Xiu et al., 2010b; Xu et al., 2014). The nZVI particles were shown to attach to the cell walls of gram-negative bacteria (Auffan et al., 2008; Chaithawiwat et al., 2016; Li et al., 2010; Xiu et al., 2010a). This can cause negative effects of the cell's membrane functions. Furthermore reductive dehalogenases are membrane bound (Fung et al., 2007; Magnuson et al., 1998; Müller et al., 2004; Nijenhuis and Zinder, 2005). Thus, attachment to the cell walls can interfere directly with the degrading enzymes and can inhibit them. It was shown, that physical proximity is needed for nZVI cytotoxicity (Li et al., 2010). Thus, in microcosms, where bacteria are in close distance the cytotoxic effects of nZVI are more pronounced than in the field. Under field conditions the bacteria can establish themselves at a distance to the particles but could still benefit from released H_2 (Summer et al., 2020). Down regulation of the genes coding for dehalogenases was observed in the presence of ZVI (Xiu et al., 2010a). This could be caused by the dechlorination of PCE and/or metabolites by the nZVI particles, since the lower chlorinated metabolites are needed to further stimulate the bacterial dehalogenation. Thus, reducing the metabolites shortly after their formation can down-regulate the expression of dehalogenase genes, thus reducing the bacterial formation of ethene.

The measured maximum H_2 amount in the nZVI microcosms was 586 ± 45 μmol which is only slightly below the theoretical maximum of H_2 that can be formed by the amount of nZVI used (595 μmol) showing that around 98.5 ± 7.6% of electrons available from the nZVI particles were used up by anaerobic corrosion rather than for dehalogenation. In comparison only 1.5 ± 0.4% of the electron equivalents provided by the mZVI particles were used by anaerobic corrosion. This could explain the similar PCE dechlorination behavior of both ZVI particles. The H_2 produced by the ZVI particles could be utilized by both bacterial cultures, utilization of H_2 produced by ZVI was also observed by Xu et al. (2014). Depending on the level and velocity with which H_2 is formed, different bacteria can benefit from it. Methanogenic and acetogenic usually benefit from a fast H_2 production at higher concentrations. Thus, the slower H_2 formation at lower concentrations by the mZVI could be the reason for their stimulatory effects on the bacterial dehalogenation, since halorespiration by *Dehalococcoides* requires low H_2 concentrations (Löffler et al., 2013; Smatlak et al., 1996; Yang and McCarty, 1998). Under field conditions lower H_2 concentrations, due to present O_2, dilution and transport by groundwater flow can be expected. Thus, the results from our batch-experiments cannot be simply transferred to field-conditions (Kocur et al., 2016).

The CH_4 detected in all microcosms with bacterial cultures indicates the presence of methanogenic bacteria. Furthermore, the methanogenic population was stimulated by the addition of either ZVI particles. The stimulated CH_4 production when combining both cultures with ZVI suggests that the chemically formed H_2 was used to produce CH_4. The more distinct stimulation of CH_4 formation with the mZVI particles furthermore shows the beneficial characteristics of the bigger particles on anaerobic microorganisms. The higher stimulation was probably caused by the lower reactivity and thus lower cell toxicity of the mZVI particles compared to the nZVI ones. High H_2 formation rates usually benefit methanogenic bacteria (Löffler et al., 2013; Smatlak et al., 1996; Yang and McCarty, 1998). This contradicts our findings with the nZVI microcosms having faster H_2 formation at higher concentrations. Showing that the H_2 formation rates and concentrations of the ZVI particles did not influence the stimulation. Both stimulation and inhibition of methanogenic

bacteria by nZVI particles have been observed. In contrast, we could not find studies on the influence of mZVI on methanogenic bacteria. Stimulation by nZVI was accounted to more favorable conditions (pH and ORP) (Carpenter et al., 2015; Rónavári et al., 2016; Yang et al., 2013). Different pathways for the bacterial production of CH_4 exist. The hydrogenotrophic pathway converts CO_2 to CH_4 using H_2 as an electron donor. During the acetoclastic methanogenesis and the dismutation of methanol or methylamines no H_2 is used up. Thus, CH_4 production does not necessarily have to be in competition to dehalogenation. It was even shown that methanogenic bacteria can have a beneficial influence on dechlorinating bacteria (Wen et al., 2015). The electron equivalents needed to form the measured maximum amount of CH_4 in the microcosms (46–157 eq^-) exceed the theoretical maximum input of electron equivalents by molasses and the ZVI particles (~5 eq^-). This indicates, that not only competing, hydrogenotrophic methanogens were present in the microcosms. Other possibilities for H_2 competing metabolisms (e.g.: sulfate reduction) have not been investigated. These could have been influencing the performance of the single and combined bacterial cultures even further.

The different effects of the two particle types on the bacterial cultures can mainly be accounted to their 1000 times difference in size, leading to a significant higher specific surface area of the nZVI compared to mZVI (Table 1). The small particle size results in an increased attachment to bacterial cells which can together with the high reactivity and the tendency for agglomeration explain the higher toxicity for bacterial cells. Comba et al. (2011) showed that nZVI has a higher reactivity compared to mZVI and millimetric ZVI, leading to a lower efficiency, selectivity and longevity. The impact on planktonic microorganisms was stronger with nZVI than mZVI, possibly caused by the higher reactivity. The rapid decline in O_2, ORP and bioavailable phosphorous led to a decrease in the number of algae and aerobic bacteria. mZVI treatment resulted in an increase in total species richness and number of less common bacteria under aerobic conditions (Nguyen et al., 2018). Comparing nZVI and mZVI for the potential in contaminant oxidation under aerobic conditions showed a lower transformation of mZVI than nZVI. nZVI rapidly degraded the contaminant but wasteful consumption and rapid reactions with O_2 and H_2O, makes the long-term release of oxidants by mZVI preferable (Ma et al., 2016). Another study on the impact of nZVI and mZVI on the enzymatic activities of an microbial community in an uncontaminated site could not observe negative effects by application of either sized particles (Cullen et al., 2011). Thus, it appears that toxic effects of ZVI are strongly dependent on environmental conditions and the affected organisms.

4.2. Carbon balances

The control 1 microcosms showed that the carbon losses were not caused by the experimental set up. The small amount of lost carbon can partly be accounted to diffusion into the septum. The septum is only layered with PTFE towards the inside of the microcosms. During sampling, a hole in the septum is created where the metabolites can diffuse into. Small amounts of PCE, metabolites and dechlorinated endproducts were measured, when heating up the used septa to 40 °C for 10 min in a crimp closed headspace vial. The lower carbon balance with the control 2 microcosms containing sand shows that some sorption of PCE and its metabolites occurred when quartz sand was present. Lower carbon balances in the experimental microcosms can additionally be explained by the formation of unidentified products. A higher percentage of unidentified peaks in the chromatograms of microcosms containing only ZVI particles could explain their lower carbon balances. This shows the occurrence of additional unwanted chemical reactions with PCE. The resulting unidentified substances could have been partially degraded by the present bacterial consortia (Xu et al., 2014). As well as the formation of ethyne which can sorb to oxidized iron particles and thus cannot be detected in headspace measurements. Carbon balances of 36%-92% after 4.5 h to 20 days were reported in other studies with ZVI particles, explained by the formation of unidentified peaks, other reaction pathways and sorption of ethyne polymerization products (Han and Yan, 2016; Liu et al., 2005; Schöftner et al., 2015).

5. Conclusion

This study shows that the combination of dehalogenating bacterial cultures with ZVI has a potential to either stimulate and inhibit bacterial PCE dechlorination, depending on the characteristics of the particles used. The combination with nZVI inhibited bacterial dechlorination, probably by dechlorination of needed precursors, disruption of cell integrity, and/or inhibition of dehalogenases in the cell membrane. In case of the completely dechlorinating bacterial culture the combination with nZVI led to a lower amount of fully dechlorinated end products and the $k_{obs,PCE}$ of the incomplete dechlorinating bacterial culture was significantly decreased.

The combination with mZVI could stimulate the formation of VC and ethene production of an otherwise *cis*-DCE producing bacterial culture and increased dechlorination efficiency in a completely degrading culture. Resulting in higher concentrations of fully dechlorinated end products and a faster *cis*-DCE dehalogenation.

Furthermore, H_2 formed during anaerobic corrosion was used by both bacterial cultures, showing that both particles can be used as a pool for needed H_2. A significantly lower $k_{obs,H2}$ of the mZVI particles could have played a role in their stimulatory effects. Significant stimulation of methanogenic bacteria has been observed with both particle types, with 1.1–1.6 (KB1) and 39–67 (Bioclear) times higher $k_{obs,CH4}$ than with the single treatments. Due to different methanogenic pathways this does not necessarily mean a competition for the dehalogenating species. It has to be noted, that the negative effects of the nZVI particles shown in this lab-scale study need not necessarily occur under field-like conditions, where bacteria can establish themselves at a spatial distance from iron particles.

Credit authorship contribution statement

Dorothea Summer: Investigation, Formal analysis, Visualization, Writing - original draft. **Philipp Schöftner:** Methodology, Investigation. **Andrea Watzinger:** Resources, Supervision. **Thomas G. Reichenauer:** Conceptualization, Funding acquisition, Project administration, Supervision, Resources, Writing - review & editing.

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.

Acknowledgement

This work was conducted within the scope of the projects BIANO and MICROSYN. The project BIANO was funded by the Federal Ministry for Sustainability and Tourism (BMNT); Management by Kommunalkredit Public Consulting (KPC) (Proj. No.: B420003). The project MICROSYN was funded by FFG – Austrian Research Promotion Agency (Proj. No.: 851762).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137802.

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Manuscript 2 (in press)

Synergistic effects of microbial anaerobic dechlorination of perchloroethene and nano zero-valent iron (nZVI) – a lysimeter experiment

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Abstract

Perchloroethene (PCE) is a hazardous and persistent groundwater pollutant. Both treatment with nanoscaled zero-valent iron (nZVI) and biological degradation by bacteria have downsides. Distribution of nZVI underground is difficult and a high percentage of injected nZVI is consumed by anaerobic corrosion forming H_2 rather than being available for PCE dechlorination. On the other hand, microbial PCE degradation can suffer from the absence of H_2. This can cause the accumulation of the hazardous metabolites *cis*-1,2-dichloroethene (DCE) or vinylchloride (VC). The combination of chemical and biological PCE degradation is a promising approach to overcome the disadvantages of each method alone. In this lysimeter study, artificial aquifers were created to test the influence of nZVI on anaerobic microbial PCE dechlorination by a commercially available culture containing *Dehalococcoides* spp under field-like conditions. The effect of the combined treatment was investigated with molasses as an additional electron source and after cessation of molasses addition. The combination of nZVI and the *Dehalococcoides* spp. containing culture led to a PCE discharge in the lysimeter outflow that was 4.7 times smaller than that with nZVI and 1.6 times smaller than with bacterial treatment. Moreover, fully dechlorinated end-products showed an 11-fold increase compared to nZVI and a 4.2-fold increase compared to the microbial culture. The addition of nZVI to the microbial culture also decreased the accumulation of hazardous metabolites by 1.7 (*cis*-DCE) and 1.2 fold (VC). The stimulatory effect of nZVI on microbial degradation was most obvious after the addition of molasses was stopped.

Keywords

Perchloroethene, *Dehalococcoides* spp., nano-scaled zero valent iron (nZVI), lysimeter

List of abbreviations

PCE – perchloroethene

nZVI – nanoscaled zero-valent iron

cis-DCE – *cis*-1,2-Dichloroethene

VC – vinylchloride

ORP – oxidation reduction potential

PTFE – Polytetrafluoroethylene

Dhc – *Dehalococcoides*

Introduction

Chlorinated hydrocarbons such as perchloroethene (PCE) are hazardous environmental pollutants which can enter the groundwater. PCE is found globally at urban contaminated sites due to its heavy use in the dry-cleaning and the metal processing industry [1,2]. Nanoscaled zero-valent iron particles (nZVI) are able to degrade PCE by reductive dechlorination to ethene and ethane [3–5]. In addition to the desired degradation reaction with PCE, nZVI also reacts with water. Under anaerobic conditions Fe^0 is oxidized and H_2 is formed [6]. Depending on the type of nZVI applied, a high percentage of available electron equivalents (e^- eq) is dissipated by this anaerobic corrosion [7]. Moreover nZVI is not very mobile in the underground due to its high adsorption to the aquifer matrix and sedimentation enhanced by aggregation [8,9]. Thus, an excessive amount of nZVI is necessary to achieve a satisfying remediation outcome resulting in increased remediation costs.

Bacterial biodegradation of chlorinated ethenes has been observed at several contaminated sites [10–12]. Different aerobic and anaerobic pathways have been described for the degradation of higher and lower chlorinated ethenes. *Dehalococcoides* spp. is known for its ability to dechlorinate PCE completely to ethene. In the metabolic pathway termed dehalorespiration, H_2 is used as an electron donor and PCE as an electron acceptor for growth [13,14]. In this process PCE is dechlorinated stepwise to trichloroethene (TCE), *cis*-dichloroethene (*cis*-DCE), vinyl chloride (VC) and ethene as the fully dechlorinated end-product. The lack of H_2 and/or *Dehalococcoides* spp. at contaminated sites can lead to a lengthy persistence of PCE or the accumulation of the hazardous metabolites *cis*-DCE or VC. Other anaerobic microbial processes, such as hydrogenotrophic methanogenesis, also use H_2 as an electron donor;thus *Dehalococcoides* spp. must compete for the H_2 present [15]. Furthermore, unsuitable environmental parameters, such as a low pH, dissolved O_2 or high redox values can negatively affect *Dehalococcoides* spp. [16–19].

The combination of chemical degradation via nZVI and biological degradation via *Dehalococcoides* spp. has the potential to overcome the weaknesses of either method alone [20]. nZVI could act as a possible H_2 donor for anaerobic bacterial PCE dechlorination and could create favorable conditions for *Dehalococcoides* spp., such as a low redox-potential or the depletion of O_2, and degrade hazardous metabolites. However, laboratory studies investigating the effects of nZVI on microorganisms are inconsistent, since both stimulation and inhibition of *Dehaloccocoides* spp. by nZVI have been shown in several studies [20–25]. Exposure to nZVI can lead to a significant down-regulation of genes encoding reductive dehalogenases. Uncoated nZVI has been shown to attach to bacterial cells [26]. However, the proximity of nZVI and *Dehalococcoides* spp. in batch experiments may not be representative of larger spatial distances under field conditions. A lysimeter experiment can thus help to gain a better insight into the interplay between nZVI and *Dehalococcoides* spp. under field-like, but nevertheless controlled conditions.

The aim of this lysimeter study was therefore to investigate if nZVI can stimulate PCE degradation by a PCE-degrading bacterial consortium under controlled field-like conditions. Apart from controlling the mass and position of the PCE source the lysimeter study facilitated calculation of a mass balance for PCE and its metabolites. In the lysimeter chambers an oxygen-free aquifer was established followed by a controlled injection of PCE. One of the chambers served as an untreated control, while in the other three treatments with nZVI, a dechlorinating culture and molasses, as well as a combination of bacteria and nZVI, were investigated. Following the injection, concentrations of PCE its metabolites and relevant environmental parameters were monitored for over 350 days.

Methods and Materials

Materials and chemicals

Nanofer Star (NANO IRON s.r.o., Topolová 933, Židlochovice 66701, Czech Republic) was used for the chemical PCE degradation. Prior to use, the nZVI was stored in an argon-flushed glovebox to minimize O_2 contact. nZVI solutions were produced by dispersing 50 g in 200 ml O_2-free water for 3 min using an Ultra-Turrax (ULTRA-TURRAX T 18 basic, IKA®-Werke GmbH & Co. KG), idle speed: 15600 rpm, immediately before use. nZVI solutions were produced in an argon-flushed glove tent. The KB-1 culture (SiREM, 130 Stone Road West, Guelph, Ontario, Canada) was stored at 10 °C in 1 L glass bottles sealed with Polytetrafluoroethylene (PTFE) to prevent O_2 exposure until use. The KB-1-culture delivered contained $3.6x10^{11}$ gene copies *Dehalococcoides (*Dhc*)*/L. The final KB-1 content in each chamber was $4.92x10^7$ Dhc/L. PCE (Sigma-Aldrich, ≥99,9%) and molasses (AGRANA Beteiligungs-AG, Tulln, Austria) were stored at 4 °C until use. Tap water (carbonated-earth alkaline; NO_3^-=2-4 mg/l; SO_4^{2-}= 70 mg/l) from Seibersdorf (Austria) was used within the lysimeters.

Establishing an oxygen-free operation

To ensure an anaerobic environment inside each lysimeter chamber, dissolved O_2 was removed from the in-flowing water by flushing with N_2 to reach an O_2 content of <0.05 mg/l. To counteract a resulting pH-change, CO_2 was used to adjust the pH to 6.5-6.7.

Filling and conditioning of the lysimeters

The lysimeter facility consisted of a construction made of reinforced concrete with a cellar level 1.2 m beneath ground level. It comprised four chambers, each equipped with lateral holes at different depth levels for the installation of sensors and sample-taking ports. Each chamber had a base area of 4 m^2 and a height of 2.9 m. Temperature loss during winter was counteracted by heating the chambers to 15-16 °C.

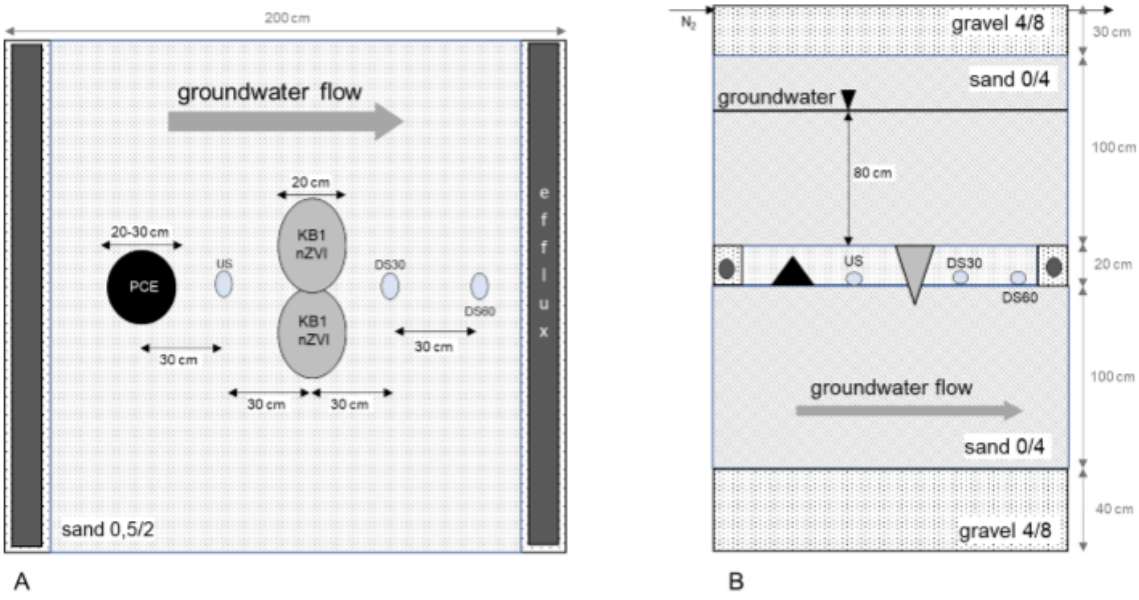

Fig. 1: (A) Horizontal and (B) Vertical layout of the simulated aquifer (depth 130-150 cm) in the lysimeter chambers and positioning of the sampling and injection tubes in the aquifer zone. Elements can vary according to the treatments of each chamber (PCE = PCE phase; treatment zone: Injection of nZVI and KB1-culture; US, DS30 and DS60 = sampling ports upstream, 30 cm and 60 cm downstream of the treatment zone).

To induce an artificial aquifer, layers with different hydraulic conductivities were built in each chamber. The artificial aquifer (layer with main water flow) consisted of a 20 cm layer of quartz sand (Quarzwerke Österreich GmbH, Wachbergstraße 1, 3390 Melk, Austria) constituting the layer where most water was flowing (Figure 1 B). The zone below and above the artificial aquifer consisted of 100 cm compacted fine sand followed by drainage gravel. The permeability difference between the quartz sand layer (aquifer zone) and the compacted sand layers beneath and above exceeded three orders of magnitude. This secured a horizontal flow and subsequently a horizontal contaminant transport within the quartz sand layer. Quartz sand was used due to its well-defined quality standard and inert features, thus reducing unwanted interactions with water components or PCE. The top of each chamber was sealed with a gas-tight PVC sheet, to hinder O_2 diffusion from the atmosphere. In addition,

the top gravel layer was continuously flushed with N_2. Perforated pipes at both sides of the aquifer zone allowed water influx and efflux (Figure 1). The water flow was kept at a constant rate of 12-14.6 ml/min, resulting in distance velocities of 0.11-0.13 m/d within the artificial aquifer, which is typical for many Austrian sites and also common in Europe.

The dissolved O_2 content of the entering water was measured hourly by an oxygen probe (Mettler Toledo InPro6860i). Samples for pH and oxidation reduction potential (ORP) analysis were automatically taken daily from positions US and DS30 (Figure 1) and measured in a throughflow probe equipped with a pH/ORP electrode (Mettler Toledo InPro®4800 SG).

Experimental design

After reaching constant flow conditions and an O_2-free environment in the aquifer zone of each chamber, the following treatments were established:

- Chamber NZVI: Infiltration of nZVI in treatment zone
- Chamber CONT: no infiltration (negative control) + flushing with molasses for 91 d
- Chamber KB1: Infiltration of KB-1culture into treatment zone + flushing with molasses

Chamber COMB: Infiltration of nZVI and KB-1 culture into treatment zone + flushing with molasses

The treatments during the experiment are shown in Table 1.

Table 1: Treatments of the four chambers throughout the experiment

Day	**Procedure**	**Chamber**			
		NZVI	KB1	COMB	CONT
0	PCE injection (pure phase)	100 ml	100 ml	100 ml	100 ml
44	nZVI injection	304 g	-	336 g	-
63-188	Molasses input 1	-	0.6 g/l	0.6 g/l	-
72	KB1 injection	-	$4.92x10^7$ CN *Dhc*/l	$4.92x10^7$ CN *Dhc*/l	-
212-303	Molasses input 2	-	-	-	0.7 g/l
303-343	Molasses input 3	-	0.5 g/l	0.5 g/l	-

Injection of PCE, nZVI, carbon source and KB1-culture

Prior to the lysimeter experiment, laboratory scale experiments were conducted to estimate the distribution behavior of nZVI and PCE (see Supplementary Material).

100 mL of PCE were injected resulting in a PCE pool with an estimated diameter of 30 cm, 2-3 cm above the compacted lower fine sand layer (Figure 1B). The estimation of the geometry of the PCE source was based on previously conducted laboratory scale injection experiments, where it was shown that no infiltration of PCE into the compacted sand layer occurred (results shown in Supplementary Material). Prior to the injection of the microbial culture the input of molasses was started in

chambers KB1 and COMB. A starting concentration of molasses of 0.6 g/l was adjusted in the aquifer zone. The same ports were used for injection of nZVI and the microbial culture. For the transport between laboratory and lysimeter, the microbial culture was filled into gas-sampling tubes within an argon-flushed glove box. At the lysimeter site the gas-sampling tubes were attached to the N_2 flushed injection tubes and the culture was injected with a hydrostatic pressure of 10 kPa. After four months molasses input into chamber KB1 and COMB was stopped while simultaneously being started in chamber CONT. Approximately one month before the end of the experiment, molasses addition was restarted in chambers KB1 and COMB and stopped for chamber CONT.

Sampling and analysis

All samples were transported in a cooling box from the lysimeter to the laboratory and stored at 4 °C prior to the analysis.

CHC, CH_4 and H_2 samples

5 ml of water were collected in 12 ml glass bottles, sealed with a PTFE-septum from sampling ports in one to a two-week interval. Samples were stored at 4°C and were brought to room temperature prior to the analysis. To measure the organic compounds, 100 µl of the headspace were manually injected into a gas chromatograph (Fisons GC8000Top GC-FID, CE Instruments, The Old Barn, Swan Lane, Hindley Green, Wigan WN2 4HF, UK). H_2 was measured by manual injection of 1ml of the headspace using a GC-TCD (Fisons GC8000Top). See Supplementary Material for more details.

Dehalococcoides spp. real-time PCR analysis

To verify the presence of *Dehalococcoides* spp. in the chambers, a real-time PCR assay was established and performed. Sampling for the analysis of *Dehalococcoides* spp. was performed after 89 and 145 d (three and nine weeks after KB1 injection). Samples were taken from points US and DS30 of all four chambers. 2 mL of water were collected from the sampling points into sterile 2 mL tubes and centrifuged at 29,000 RCF for 12 minutes (Eppendorf centrifuge 5415R). After discharging the supernatant, the pellet was frozen in liquid nitrogen and stored at -80 °C until further processing.

DNA extraction was performed using a DNA Isolation Kit (MoBio; now DNeasy UltraClean Microbial Kit, Qiagen, 19300 Germantown road, Germantown, USA) following a modified protocol including an additional phenol/chloroform/isoamyl alcohol (PCI) step. To do so, the pellets were re-suspended in 300 µl MicroBead Solution; 50 µl Solution D1, 350 µl PCI solution (25:24:1, Sigma-Aldrich Handels GmbH, Marchettigasse 7/2, 1060 Wien, Austria) and beads from the MicroBead tube were added and the samples were homogenized using a ball mill (Retsch Mühle MM400, Retsch GmbH, Retsch-Allee 1-5, 42781 Haan, Germany) at 15 s^{-1} setting for five minutes. The steps following were performed according to the manufacturer's protocol. DNA was eluted in 50 µl water. To establish that the obtained DNA was of sufficient quality for the real-time PCR analysis an inhibition test was performed, following a previously published protocol [27].

For the detection of *Dehalococcoides* spp. a new real-time PCR assay was established. First DNA was extracted directly from the KB-1 culture as described above. Subsequently, the 16S rRNA gene fragment was amplified using the *Dehalococcoides* spp. specific primers Dhc1f and Dhc264r [28]. Then the amplicons were cloned using the StrataClone PCR Cloning Kit (Agilent Technologies, 5301 Stevens Creek Blvd., Santa Clara, CA 95051, USA, Cat. No.: 240205) and sequenced following to standard procedures [29]. Sequences were edited and assembled using Sequencher v5.3 (Gene Codes Corporation, 525 Avis Drive, MI 48108, USA). Out of 24 analyzed sequences 15 were identical, in nine only single nucleotide differences were observed. The consensus sequence was used as a template for the real-time PCR primer and probe design that was performed using PrimerQuest tool (Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, Iowa 52241, USA, https://eu.idtdna.com/). The primers and probe developed and used for the subsequent *Dehalococcoides* spp. analysis are shown in the Supplementary Material.

Results

Dehalococcoides spp. in KB1 and COMB

Significant amounts of *Dehalococcoides* spp. could be detected in the samples taken downstream of the treatment zone (DS30 and DS60) of chamber COMB and KB1 (results shown in Supplementary Material).

Total cumulative efflux-loads of chloroethenes, ethene and ethane

Chamber CONT had the highest cumulative efflux-load of PCE (223 µmol) followed by NZVI (135 mmol), KB1 (47 mmol) and COMB (29 mmol) (Figure 2). For CONT, the amount discharged during the experimental period was 23 % of the injected PCE, indicating that the mass of PCE in each lysimeter chamber was sufficient for the period of investigation. The PCE loads were inversely correlated with cumulative loads of the fully dechlorinated end-products, ethene and ethane, in the efflux of the different treatments. The highest values were measured in COMB and no fully dechlorinated end-products were found in the untreated control.

The intermediate degradation products TCE and *cis*-DCE were found in all treatments, but in CONT they were produced mainly during the period when molasses was added to this chamber. VC was detected in higher amounts only in the two treatments were the microbial culture was added (Figure 2; see below for more details).

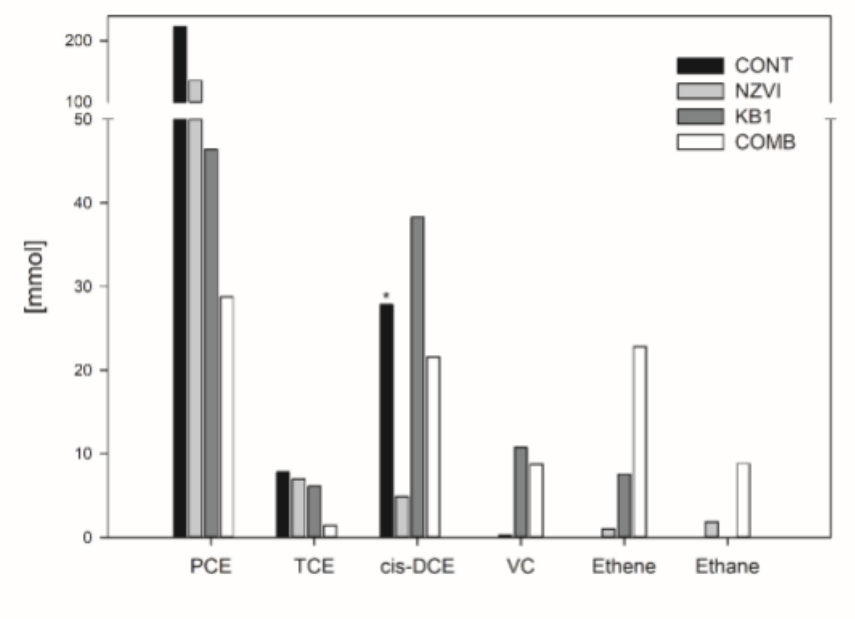

Fig. 2: Cumulative loads of PCE, metabolites and fully dechlorinated end products calculated from the volumes and concentrations of the efflux from each chamber. * marks the metabolites formed only during molasses input in chamber CONT

Effect of stop of molasses addition to the PCE load in the efflux

The lower load of PCE and the higher load of fully dechlorinated end products in COMB compared to KB1 were induced by stopping the input of molasses in these two treatments. During that time the cumulative load started to increase in KB1, but not in COMB, indicating a stimulation of microbial dechlorination by nZVI when the addition of the organic substrate was stopped (Figure 3).

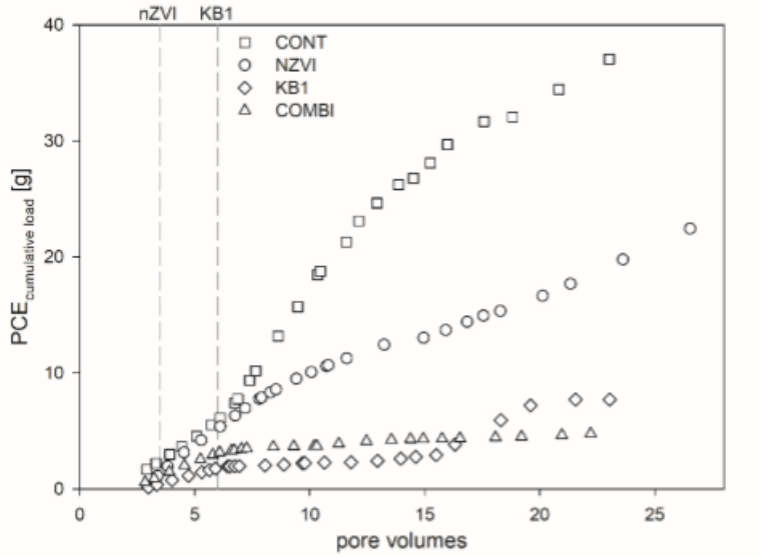

Fig. 3: Time course of PCE loads in the efflux of the chambers during the whole experiment. The vertical dashed lines indicate times of injection of nZVI and KB1. The hatched areas indicate the period of molasses addition into chambers KB1 und COMB. The dotted area indicates the period of molasses addition into chamber CONT.

Time course of concentrations of PCE, ethene and ethane

The divergent performances of COMB and KB1 after the stop of molasses addition that were clearly visible when comparing the PCE-loads were not seen by simply following the time course of PCE-concentrations at the sampling points upstream (US) and downstream of the treatment zone (DS30 and DS60) (Figure 4).

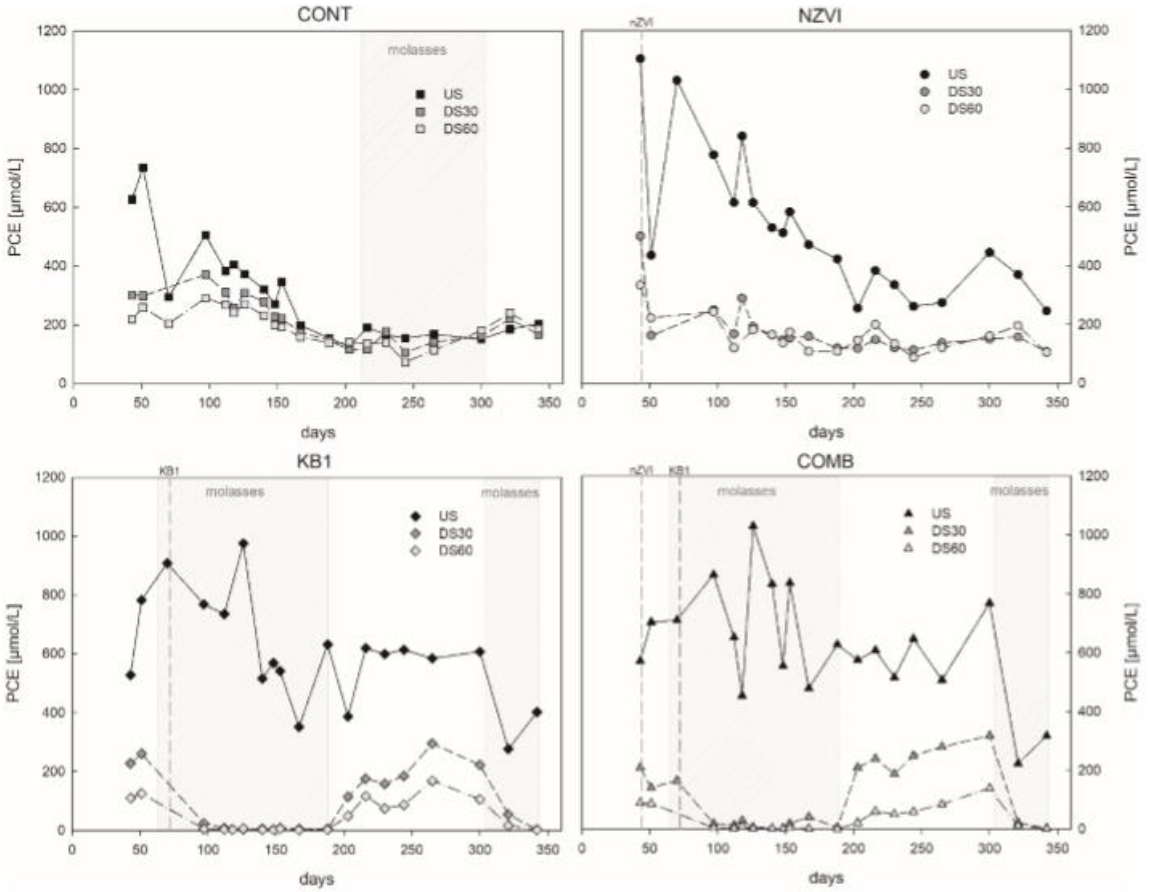

Fig. 4: PCE concentrations at the sampling points upstream (US) and downstream (DS30 = 30 cm, DS60 = 60cm) of the treatment zone. The vertical dashed lines indicate injection times of nZVI and KB1. Hatched areas indicate the periods of molasses addition.

The decrease in PCE downstream of the treatment zone expressed as a percentage of the upstream concentration showed no significant difference between COMB and KB1 (Table 2A). However, there was a significant difference between COMB and KB1 in the efflux-concentration of ethene+ethane during the period when no molasses was added (Table 2B), indicating a more effective dechlorination in COMB.

Table 2: Mean values during the period when no molasses was added to chambers KB1 and COMB (day 203 to 300) of (A) PCE concentration in sampling points DS30, DS60 and the efflux expressed as percentage of the upstream concentration (US) and (B) concentration of ethene+ethane at DS30, DS60 and the efflux. Different letters (a, b, c) above the values indicate statistically significant differences between values in each row (α=0.05 Duncan's post-hoc test).

(A) PCE as percentage of upstream concentrations

	CONT	**NZVI**	**KB1**	**COMB**
ΔUS-DS30	13.75±19.22^{B}	58.56±6.24^{A}	66,47±8.96^{A}	58.83±7.07^{A}
ΔUS-DS60	16.07±27.79^{B}	56.22±9.28^{C}	82.71±6.20^{A}	88.79±5.31^{A}
ΔUS-efflux	80.27±10.72^{B}	95.68±1.08^{A}	98.09±1.70^{A}	99.85±0.01^{A}

(B) concentrations of ethene+ethane

	CONT	**NZVI**	**KB1**	**COMB**
DS30	0.00±0.00^{A}	2.13±0.70^{A}	1.15±2.33^{A}	13.29±4.51^{B}
DS60	0.00±0.00^{A}	2.03±1.33^{A}	2.58±3.99^{A}	15.01±7.28^{B}
efflux	0.01±0.01^{A}	0.24±0.11^{A}	1.40±1.48^{A}	5.93±4.38^{B}

Ethene and ethane concentrations with molasses

Ethene was detected in chambers NZVI, COMB and KB1 downstream of their treatment zones, whereas ethane was only detected in chambers containing nZVI (NZVI and COMB). The highest total concentrations of fully dechlorinated end-products were detected in COMB, and were higher than the sum of the concentrations in NZVI and KB1 (Figure 5).

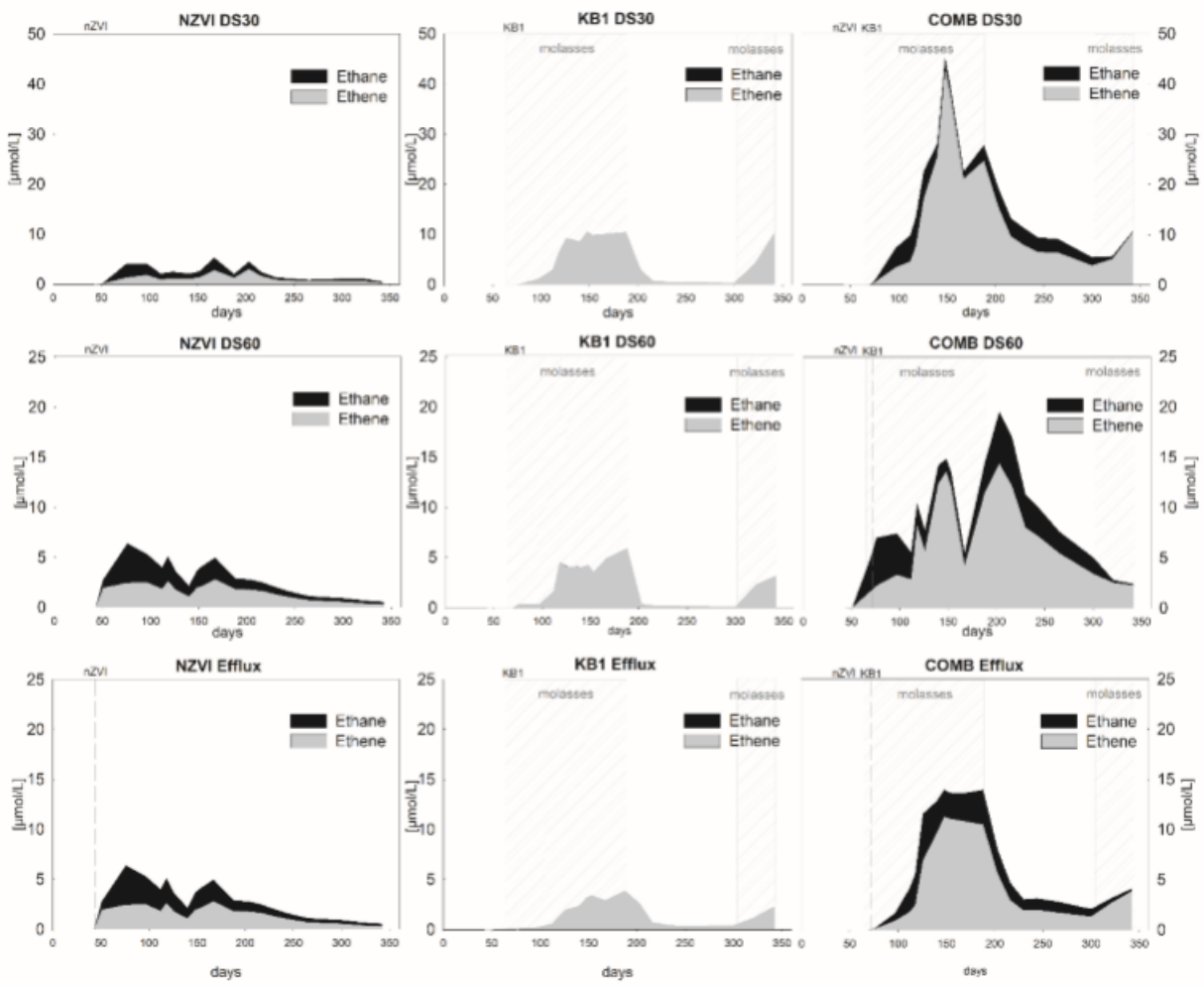

Fig. 5: Ethene and ethane concentrations at the sampling point DS30, DS60 and efflux of chambers NZVI, KB1 and COMB. The vertical dashed lines indicate injection times of nZVI and KB1. Hatched areas indicate the periods of molasses addition. The dotted lines shown in the three graphs for COMB indicate the sum of ethene+ethane concentrations measured in the chambers NZVI and KB1.

Metabolites (TCE, *cis*-DCE, VC)

Upstream of the treatment zone in chambers COMB and KB1, molasses addition induced an increase in concentrations of TCE, up to about 50 µmol/l, and of *cis*-DCE up to 342 µmol/l and 842 µmol/l respectively, but with no formation of VC (Figure 6). TCE was degraded downstream of the treatment zone to *cis*-DCE with decreasing concentrations in DS30, DS60 and the efflux, and further degradation to VC. When the addition of molasses was stopped, the TCE concentration upstream of the treatment zone (US) started to decline, while there was a steep increase in the downstream sampling ports DS30 and DS60 also followed by a decline (Figure 6). The concentrations of *cis*-DCE and VC both decreased after stopping the addition of molasses. In CONT, *cis*-DCE could only be detected in the efflux during molasses input, while in NZVI *cis*-DCE was only occasionally found in very low concentrations (mainly beneath the limit of detection = 0.3 µmol/L). VC was not detected in either CONT or NZVI (see Supplementary Material for graphs).

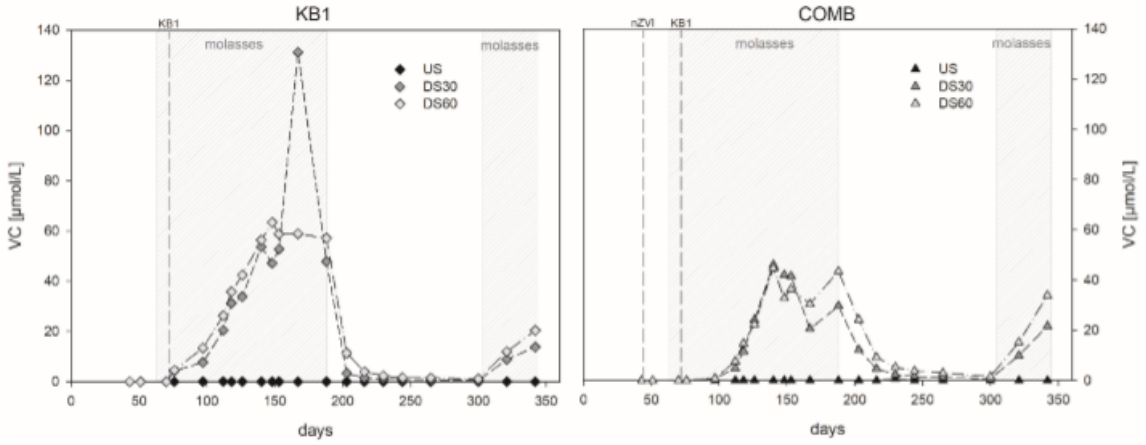

Fig. *6*: Metabolite concentrations (TCE, cis-DCE and VC) at the sampling points upstream (US) and downstream (DS30 = 30 cm, DS60 = 60cm) of the treatment zone. The vertical dashed lines indicate injection times of nZVI and KB1. Hatched areas indicate the periods of molasses addition.

Hydrogen

H_2 was only detected in treatments containing nZVI (NZVI and COMB). Between 10-20 days after nZVI injection, H_2 could be measured in the sampling points DS30 and DS60 (Figure 7). Approximately one month after iron injection, maximum H_2 concentrations at DS30 were reached and were 1.6 times higher in NZVI than in COMB. Thereafter, H_2-concentrations declined, indicating ageing of the nZVI particles. After about 150 d in NZVI and about 100 d in COMB, no further H_2 was detectable.

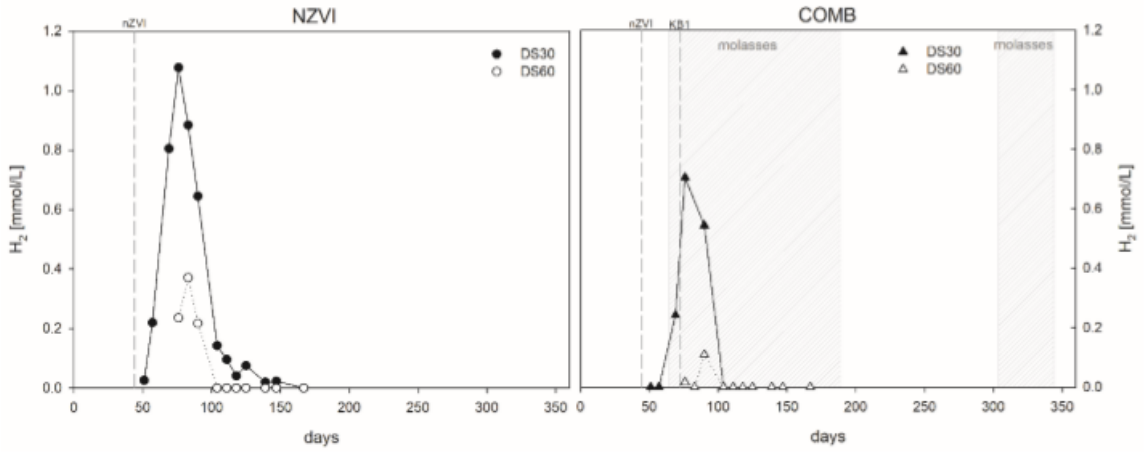

Fig. 7: H_2 concentrations at sampling points downstream (DS30 = 30 cm, DS60 = 60cm) of the treatment zone. The vertical dashed lines indicate injection times of nZVI and KB1. Hatched areas indicate the periods of molasses addition.

Methane

Increasing concentrations of CH_4 started to be detectable only about 1.5 months after the start of the molasses addition in COMB and KB1 (Figure 8). Stopping the addition of molasses caused the CH_4 concentrations to decrease immediately upstream of the treatment zone (US), followed by delayed decrease with increasing distance downstream of the treatment zone (DS30 and DS60) in both chambers. However, in CONT no increase in CH_4 concentration could be detected within a period of 91 d after molasses addition.

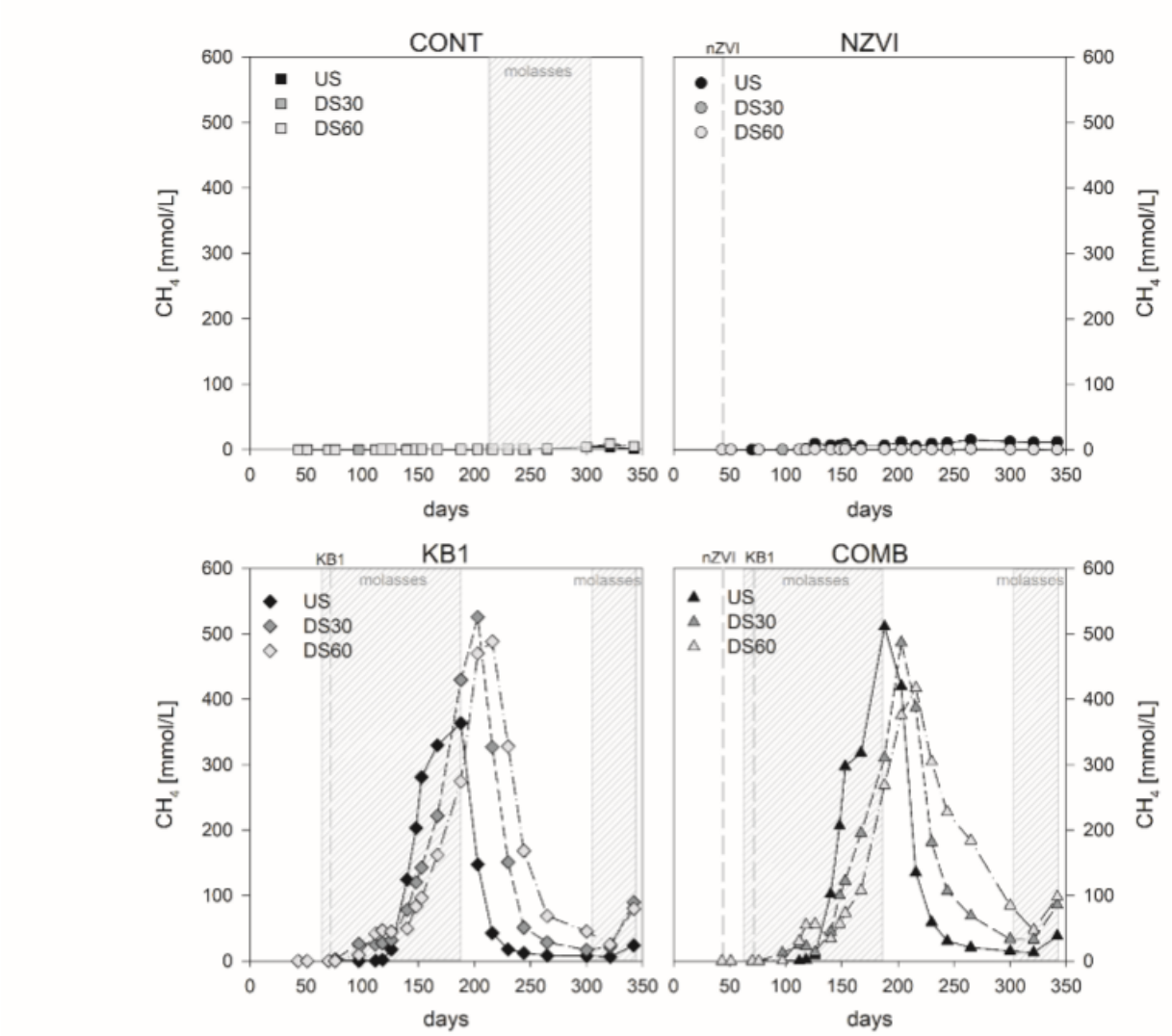

Fig. 8: CH_4 concentrations at sampling point upstream (US) and downstream (DS30 = 30 cm, DS60 = 60cm) of the treatment zone and in the efflux of each lysimeter chamber. The vertical dashed lines indicate injection times of nZVI and KB1. Hatched areas indicate the periods of molasses addition.

Discussion

Conditions

The conditions in the chambers enabled microbial PCE dechlorination to occur by the injected bacterial culture. The pH, temperature and ORP could be adjusted to a suitable range for dehalogenating bacteria (see Supplementary Material for detailed data). Different dehalogenating bacteria have been reported to dechlorinate in a pH range of 6.5-8.0 and at 10-35 °C [14,16,30–32]. Microbial reductive dehalogenation is most effective in the ORP range of SO_4^{2-} reduction (-50 to -180 mV) and methanogenesis (< -120 mV) [33]. Thus, the lysimeters represented field-like conditions for reductive dechlorination. Compared to a real field site, a lysimeter has the advantage that an exact value for the effluent load of each substance can be calculated by multiplying the measured concentration with the volume of the effluent water. By this means it was possible to identify a clear difference in PCE load between KB1 and COMB during the period when no molasses was added. This

difference was not visible by simply comparing concentrations of samples taken from sampling ports downstream of the treatment zone.

Stimulation of reductive dechlorination by nZVI

While both stimulatory and inhibitory effects of nZVI on the anaerobic microbial dehalogenation of chlorinated ethenes have been described [20–25] , the results of this lysimeter experiment indicate a stimulatory effect of nZVI on anaerobic dechlorination by a *Dehalococcoides* consortium under field-like conditions. The combined treatment showed lower total loads of PCE and of intermediate products (TCE, *cis*-DCE and VC) in the efflux together with the highest load and concentrations of fully dechlorinated end products. The positive effects of nZVI on the microbial community was particularly pronounced when addition of molasses was stopped after a period of molasses addition.

The fully dechlorinated end-products ethane and ethene can be used to differentiate between dechlorination by nZVI and *Dehalococcoides*, since ethane was only detected in the presence of zerovalent iron. Thus, it appears that part of the dechlorination found in the combined treatment was due to a direct reaction with nZVI. However, the concentrations of ethane and ethene, as well as their total loads in the efflux in COMB, were higher than the sum of NZVI+KB1, indicating a real stimulation of dechlorination and not just an additive effect. This effect could be due to production of H_2 by nZVI, which is needed by *Dehalococcoides* spp. for dechlorination as an electron donor [6,7,13,14]. However, during the period when molasses addition was stopped, the iron particles were already aged as indicated by the cessation of H_2 production. Nevertheless, since slow and stable H_2 release at low levels were found to favor dehalogenation [15,34], the possibility cannot be excluded that there was still some generation of H_2 below the limit of detection, but enough to stimulate dechlorination. If the H_2 was immediately consumed, it would not be detectable. Another possible mechanism could be a reactivation of the aged iron particles by a bio-reduction of (hydr)oxides in the particle shells by dissimilatory iron reducing bacteria [25].

Since molasses provides a lot of H_2 [35], this was not a limiting factor during the period of molasses addition. About 50 % of the applied molasses consisted of sucrose, which can be enzymatically split into glucose and fructose. Fructose shows a high similarity of metabolic transformation to glucose, with similar H_2 production. Approximately 40 moles of H_2 are produced during the fermentation of one mole of glucose [35]. One mole of H_2 provides 2 e^- eq. leading to a total e^- eq added via molasses of 18 for chamber CONT and 37 for chamber COMB and KB1 each. In comparison to the amount of e^- eq from molasses in the chambers KB1 and COMB, the 8-9 e^- eq from nZVI were low, making it unlikely that H_2-generation by nZVI further enhanced microbial dechlorination during the addition of molasses, especially since *Dehalococcoides* favors low concentrations of H_2 for its activity [15,34].

Zones with different populations of dechlorinating microorganisms

The lysimeter experiment also revealed a differentiation between the sections upstream and downstream of the treatment zone. Upstream, the intermediate dechlorination products TCE and *cis*-DCE, but not VC, were formed, if molasses was

added, indicating the activity of bacteria that are able to perform partial reductive dechlorination of PCE [36]. Downstream, all intermediate dechlorination products, including VC, and fully dechlorinated end-products ethene and ethane, could be detected. Also, in the lysimeter without addition of nZVI and KB1-culture (CONT) the intermediate degradation products TCE and *cis*-DCE, but not VC or fully dechlorinated end-products, were formed during the period of molasses addition. These findings correspond well with the detection of *Dehalococcoides* spp. by qPCR only downstream (DS30) of the treatment zone in KB1 and COMB, but not upstream (US), nor in CONT.

Influence of nZVI on methanogenic bacteria and potential competition with Dehalococcoides

Increasing CH_4 production was detected in KB1 and COMB during the period of molasses addition and when the addition was stopped, the decrease of CH_4 concentration appeared to be slower in COMB. However, it cannot be claimed from just one chamber that this difference is significant. In batch experiments both stimulation and inhibition of methanogenesis by nZVI have been shown [23,37,38]. While stimulation was mainly attributed to favorable conditions, inhibition was attributed to disruption of cell integrity. Compared to batch microcosms, the microorganisms in the lysimeter were able to establish themselves at a spatial distance from the particles. Thus, a negative effect of nZVI on cell integrity by a direct contact was strongly diminished under the lysimeter conditions. In CONT, no significant CH_4 concentrations were detected even during the period of molasses addition. This might be due to the toxicity of PCE, which was shown to have inhibitory effects on anaerobic and especially methanogenic bacteria at concentrations as low as 60 µmol/L, up to complete inhibition at 600 µmol/L [39,40]. A closer examination of the time course of CH_4 concentrations shows that in KB1 and COMB, CH_4 first appeared downstream of the treatment zone and only after a lag-period did its concentration begin to rise upstream. Thus, the CH_4 detected upstream was probably produced downstream in the zone where PCE was mostly degraded and reached the upstream sampling point via diffusion.

Conclusion

Under field-like and controlled conditions it was shown that the combination of nZVI and a microbial culture containing *Dehalococcoides* spp. stimulated the degradation of PCE and production of fully dechlorinated end-products (ethene and ethane). In particular, when the addition of the H_2 source (molasses) was stopped, the dechlorination process was maintained if nZVI was present. A potential stimulation of CH_4 production by aged nZVI remains to be further investigated. Overall, this study shows that, under field-like but controlled conditions, the combination of chemical and biotic PCE degradation is a promising approach for complete and long-term PCE degradation.

Acknowledgements

The project BIANO was funded by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water (BMLFUW); Management by Kommunalkredit Public Consulting (KPC) (Proj.No.: B420003).

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EVALUATION OF HYPOTHESES

Evaluation of hypothesis 1:

The results of this work show that the combination of ZVI with dechlorinating bacteria is a promising technology to increase PCE remediation efficiency. The H_2 produced by ZVI could be used by the present bacterial community. In laboratory experiments mZVI could stimulate an otherwise *cis*-DCE accumulating culture to completely degrade PCE to ethene and improve the dechlorination behavior in a completely degrading culture. nZVI on the other hand showed different effects in microcosms compared to those observed in a field-like lysimeter. While inhibition by nZVI occurred in the microcosms a stimulation, especially after the input of molasses was stopped, was observed in the lysimeter experiment. This discrepancy can be explained by the spatial distance of the dechlorinating bacteria to the nZVI in the lysimeter, thus omitting their possible toxic effects while still being able to benefit from the H_2 formed.

Evaluation of hypothesis 2:

The microcosm experiments showed, that the influence of ZVI on the biological PCE degradation is highly dependent on the characteristics of the particles used. While the bigger, less reactive mZVI particles led to a stimulation of the degrading bacterial community, the combination with nZVI resulted in an inhibition. The negative effects of nZVI particles can be accounted to their higher surface area, leading to a higher reactivity and tendency for agglomeration. This causes toxicity by attachment to bacterial membranes, which can lead to cell disruption and inhibition of membrane-bound dehalorespiring enzymes (Li et al., 2010; Xiu et al., 2010). Furthermore, nZVI rapidly removed metabolites (*cis*-DCE and VC) needed as precursors for the complete bacterial dechlorination to ethene, possibly leading to a down-regulation of dehalogenases gene expression. Additionally they formed ethyne, which is toxic to bacterial cells (Kocur et al., 2016; Pon et al., 2003).

During the lysimeter experiment, these detrimental effects of nZVI could not be observed. In contrary, a stimulation of the *Dehalococcoides* spp. containing culture was observed. Showing that close proximity is needed for the toxicity of nZVI to be effective. In field-like conditions the degrading bacteria could establish themselves without physical contact to nZVI particles, evading their toxic effects and benefiting from the produced H_2. Showing that not only particle characteristics but also their placement in the field is crucial for the success of the combined treatment.

Evaluation of hypothesis 3:

While some experimental parameters (carbon source, temperature and inoculum concentration) showed a severe influence on the bacterial degradation, others (H_2, vitamin and trace element addition) did not influence dehalorespiring behavior. All influential parameters could be adjusted, according to field-conditions, time and cost-efficiency, during a remediation process to achieve a sufficient and complete PCE removal.

Different carbon sources (molasses, Na-acetate, ethanol, Na-lactate, ethyl lactate and ethyl butyrate) led to different dechlorination and methane formation behaviors in microcosms. Ethene formation could be observed with all carbon sources except

ethyl butyrate. Even though Na-lactate showed the fastest ethene formation, molasses was chosen for all following experiments, due to its satisfying degradation results, coupled with its low costs and bulk-availability. Addition or omission of external H_2, vitamins (e.g. B12), yeast extract and trace elements did not have an effect on the bacterial dechlorination behavior.

A decrease in temperature (from 21 °C to 10 °C) resulted in an about five times slower PCE-degradation and consequentially slower metabolite formation, but ethene production could still be achieved. While temperatures in the field are highly dependent on seasonal changes and hard to control, there are techniques to increase them (see chapter 0) if a faster biological degradation is desired.

The lower the inoculum concentration (1%, 5% and 10%) the slower was the PCE degradation. But ethene formation could be observed in all experiments with about 30 days difference. Showing that also a low abundance of degrading bacteria can lead to a satisfying remediation result, but it takes more time. Thus, costs of bacterial cultures versus those of a longer remediation time must be balanced out during a remediation.

Evaluation of hypothesis 4:

The presence of methanogenic bacteria does not necessarily mean a competition for H_2 and negative effects on the dechlorinating bacteria. The present methanogenic bacteria were stimulated by ZVI particles in all conducted experiments. Although, higher H_2 formation by nZVI did not favor methanogenic bacteria compared to the slower H_2 production by mZVI. Furthermore, it was shown that not only competing methanogens (hydrogenotrophic, using H_2), but also bacteria depending on the acetoclastic methanogenic pathway were present. Demonstrating that the presence of methanogenic bacteria does not necessarily mean a competition for H_2 and can even be beneficial by providing the bacterial culture with needed electron donors.

CONCLUSIONS AND OUTLOOK

In order to use the combination of ZVI and *Dehalococcoides spp*. as a successful remediation technology on contaminated sites the interactions between the two components must be thoroughly considered and investigated.

This study shows that anaerobic bacteria are potentially capable of using the H_2 formed during the anaerobic corrosion of ZVI particles. This can enhance the bacterial dechlorination drastically, especially when the addition of a suitable carbon and electron source is stopped.

The positive effects of mZVI have been thoroughly proven during laboratory-scaled microcosm experiments, by increasing $k_{obs;\ PCE}$ and stimulation of ethene production in an otherwise *cis*-DCE producing bacterial culture. The effects of nZVI differed drastically between laboratory-scale experiments and the field-like lysimeter experiment. While an inhibition in PCE dechlorination in two different bacterial cultures could be observed in microcosm experiments, positive effects on bacterial dechlorination by nZVI could be observed in the lysimeter.

The different effects of mZVI and nZVI particles in the microcosms can be explained by their different sizes, leading to different reactivities and tendencies for agglomeration. Toxicity of nZVI is mainly caused by their attachment to cell surfaces, leading to a break-down of cell integrity and possible inhibition of cell-membrane bound dechlorinating enzymes (Li et al., 2010; Xiu et al., 2010). Furthermore, the high reactivity of nZVI particles also leads to a removal of metabolites (*cis*-DCE, VC), which are necessary precursors for complete bacterial dechlorination (Kocur et al., 2016; Pon et al., 2003). The lower reactivity of the mZVI also resulted in a slower H_2 release at lower concentrations compared to that of the nZVI.

With the results of the lysimeter experiments, where a stimulation of the bacterial dechlorination was observed, spatial distance of the dechlorinating bacteria to the highly reactive nZVI particles seems to be necessary for the bacterial consortia benefitting from the chemically produced H_2 without suffering from the toxic effects of nZVI. The positive effects of nZVI on *Dehalococcoides spp.* were especially pronounced when the addition of the carbon and hydrogen source (molasses) was halted.

Bacterial methane production was stimulated in all experiments (microcosms and lysimeter) using both ZVI particles and bacterial cultures. This shows a potential for H_2 competition between the different bacterial metabolisms, the outcome is highly dependent on the way H_2 is delivered by the ZVI particles. The slower H_2 production at lower concentration by mZVI, compared to nZVI, favors the dehalorespiring metabolism to the methane producing one. However, methanogenic bacteria can also be beneficial to the bacterial dechlorination by *Dehalococcoides spp*. (Wen et al., 2015).

The results of this study show, that bacterial dechlorinating behavior can potentially benefit from the combination with ZVI particles. But both bacterial cultures and ZVI particles must be carefully selected, based on dechlorination behavior and particle characteristics. Especially the stimulation of ethene production in an otherwise incompletely dechlorinating bacterial culture shows how promising this technology is. The differences between the laboratory-scaled and field-like experiments demonstrate, that the remediation set-up (spatial distance) is crucial for a successful remediation and needs to be thoroughly considered and planned. Therefore, further investigation of the influence of nZVI and mZVI particles of the dechlorination

metabolism is needed. Changes in bacterial culture composition, attachment to bacterial cells and influence of potentially beneficial particles modifications are of high interest for a better understanding and application of this remediation technology.

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SUPPLEMENTARY MATERIAL

Supplementary material to manuscript 1

Supplementary information

Inhibition and stimulation of two perchloroethene degrading bacterial cultures by nano- and micro-scaled zero-valent iron particles

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S.1. Used values for the calculation of the Henry constant

The following table shows the used values of the Henry constant at norm temperature and the enthalpy of the solution for the calcutlation of the Henry constant:

Table S.1: Used k_H^θ and $\frac{-\Delta_{soln}H}{R}$ values for PCE, TCE, *cis*-DCE, VC, ethene, ethane, ethyne, methane and H_2

Substance	k_H^θ [M/atm]	$\frac{-\Delta_{soln}H}{R}$ [K]	References
PCE	5.7×10^{-4}	4900	(Gossett, 1987)
TCE	1.1×10^{-3}	4800	(Gossett, 1987)
cis-DCE	2.7×10^{-3}	4200	(Gossett, 1987)
VC	3.8×10^{-4}	3300	(Gossett, 1987)

ethene	$4.7x10^{-5}$	1800	(Wilhelm et al., 1977)
ethane	$1.9x10^{-5}$	2300	(Abraham and Matteoli, 1988)
ethyne	$4.1x10^{-4}$	1800	(Wilhelm et al., 1977)
methane	$1.4x10^{-5}$	1600	(Burkholder et al., 2015)
H_2	$7.8x10^{-6}$	500	(Young, 1981)

S.2. Parameter chemical analysis

The conditions of the GC analysis of PLFA, PCE, its daughter products, methane and hydrogen are listed in the tables below:

Table S.2: Conditions of GC-FID analysis of PLFA (carrier flow, split-injection, injector and detector temperature, make-up flow and temperature program)

He-carrier flow [mL/min]	**Split-injection**	**Injector [°C]**	**Detector [°C]**	**N_2 make-up flow [mL/min]**	**Temperature program**
1.5	5:1	280	300	60 mL/min	40°C: 3 min 180 C (30°C/min): 4.8 min 200°C (80°C/min): 5 min

Table S.3: Conditions of GC-FID analysis of PCE, TE, *cis*-DCE, *trans*-1,2-DCE, VC, ethene, ethane, ethyne and methane (carrier flow, injector temperature and temperature program)

He-carrier flow [mL/min]	**Injector [°C]**	**Temperature program**
2.5	110	40°C: 3 min 180 C (30°C/min): 4.8 min 200°C (80°C/min): 5 min

Table S.4: Conditions of GC-TCD analysis of H_2 (carrier and reference flow, injector and detector block temperature, filament temperture and temperature program)

Ar-carrier flow [mL/min]	Ar-reference flow [mL/min]	Injector [°C]	Detector [°C]	Filament [°C]	Temperature program
16	17	110	50	190	40°C: 3 min 180 C (30°C/min): 0.5

S.3. Percentages of ethane+ethyne of the total CH content

To get an insight of the dominant degradation mechanism (chemical or microbial) the percentages of ethane+ethyne of the total dechlorinated end-products have been observed (Figure S.*1*).

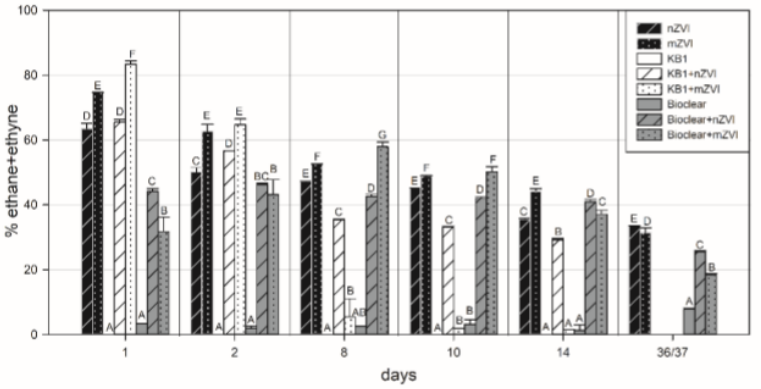

Figure S.1: Percentage of ethane and ethyne of the total amount of dechlorinated end products produced by all treatments (nZVI, mZVI, KB1, KB1+nZVI, KB1+mZVI, Bioclear, Bioclear+nZVI and Bioclear+mZVI). Error bars represent standard deviations of the three replicates. Different letters (A, B, C, ...) above the bars indicate statistically significant differences among treatments within one timepoint (α= 0.05, Duncan post hoc test).

S.4. Carbon balances

The carbon balances of all treatments and the two control microcosms over the course of the experiment are shown in Figure S.*2*.

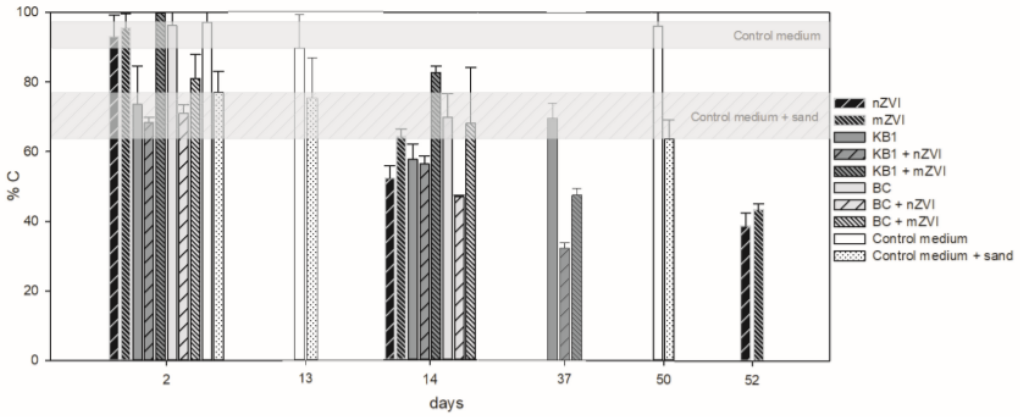

Figure S.2: Carbon balances of all treatments and the two control experiments at different time points (day 2, 13, 14, 37 and 50). The plain grey area and the hatched grey area show he lowest and the highest mean carbon recoveries of control 1 and control 2 respectively over the course of the experiment. Error bars represent standard deviations of the three replicates.

Percentages of unidentified peak areas of the total peak areas have been calculated at the end of all microcosm experiments (Figure S.3).

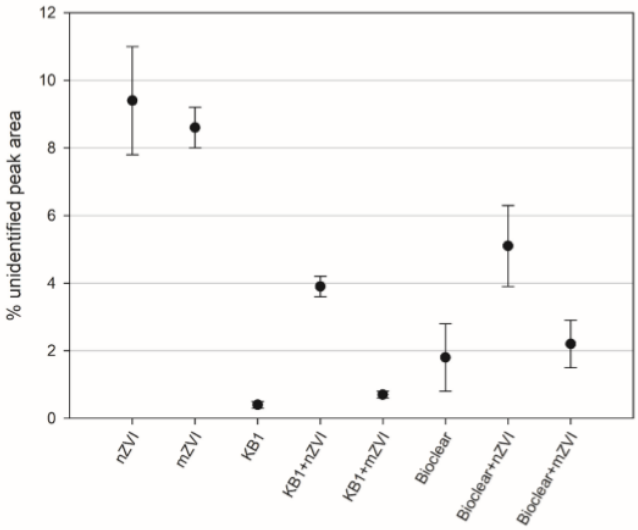

Figure S.3: Percentages of unidentified peak areas of the total peak area at the end of the experiments

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Supplementary material to manuscript 2

Supplement

S.1. Additional information on the lysimeter set up, PCE and nZVI injection

Chamber set-up

The four lysimeter chambers were separated by an accessible cross-way, from which the sample ports could be reached. At the bottom of each chamber a perforated tube was installed to enable the flushing of the whole chamber with N_2 bottom up. To reduce the leaching of fine material the perforated pipes were embedded into the drainage gravel. The input of molasses led to a build-up of exo-polymeric substances inside the tubes, which needed regular cleaning-treatment prevented clogging. The water levels in the chambers was kept 50 cm beneath the chamber surface and 80 cm above the top edge of the aquifer zone. A magnet valve system was used to automatically take pH and ORP samples.

Table S.1: Properties of the different chamber layers

Layer	Material	Grain size	Special treatment
Bottom/top layer	Drainage gravel	4/8 mm	-
Compacted layer	Quartz sand	0/4 mm	Compacted with a rammer (Bomag BT60/4, resulting in a hydraulic conductivity of $1x10^{-6}$ ms^{-1})
Aquifer layer	Quartz sand	0.5/2 mm	-

PCE injection

One liter of water was sucked from the chambers into a glass container, which was permanently flushed with N_2. PCE (100 ml per chamber) was injected into the lower zone of the container through a septum. After opening the injection valve, the PCE entered the chamber due to a pressure difference of +1 m of water.

nZVI injection

Perforated injection tubes (20 cm injection length) were embedded vertically within the aquifer layer. Between each set of perforations, the tubes were equipped with circular impermeable barriers (PTFE discs) to ensure an extended and even particle distribution during injection. The first barrier had an inner diameter of 20 cm followed by seven smaller barriers (di = 4.5 cm). O_2 input was prevented by flushing the storage container with N_2. The amount of nZVI particles injected was chosen to be considerably smaller than the stoichiometric demand for the degradation of the injected PCE. For the dechlorination of one mole PCE to ethene and ethane 8 and 10 electron equivalents (e^- eq) are needed respectively. 0.6 mol PCE was injected into each chamber resulting in 4.9 e^- eq needed for its complete dechlorination to ethene. About 8.6 e^- eq were introduced into the chambers by nZVI. With a particle electron efficiency of about 3 %, only 0.3 e^- eq are available for PCE dechlorination [6].

S.2. Analytical parameters of the GC and IC measurements

GC-FID measurements

The GC-FID was equipped with a GS-Q column (30m*0.53 mm plot column) using the following conditions/parameters: He-carrier: 2.5 ml/min, Injector temp.: 110°C; temperature program: 40°C for 3 min; 180 C (30°C/min) for 4.8 min, 200°C (80°C/min) for 5 minutes.

GC-TCD was equipped with a packed mole sieve-column (5 Å) using the following conditions/parameters: Ar-carrier: 16 ml/min; Ar-reference: 17 ml/min; injector temp.: 110°C; detector block temp.: 50°C; filament temp.: 190°C; temperature program: 40°C for 3 min, 180°C (30°C/min) for 0.5 min.

The IC was equipped with a guard column (DIONEX Ion Pac AG14, 4x50 mm) and an ion-exchange column (DIONEX Ion pac AS14, 4x250 mm). The system was equipped with an AS50 Autosampler (DIONEX).

S.3. Additional information on the real-time PCR analysis and results

Specificity of the primers was not tested extensively because the analysis was performed in a closed system. Instead we tested for inclusivity, i.e. amplification of *Dehalococcoides* spp. contained in the KB-1 culture.

Table S.2: *Dehalococcoides* spp. primers and probes used in this study

	Sequence (5' – 3')	**Length**	**Tm [°C]**	**GC %**
forward	CATGCAAGTCGAACGGTCTTA	21	63	47.6
reverse	CTTCAGTTTCCCGAAGCTATCC	24	63	50.0
probe*	ATAGTGGCGAACGGGTGAGTAACG	22	68	54.0

* probe was ordered with 5' 6-FAM/ZEN/3' IBFQ labelling

Real-time PCR reactions were performed in 20 µl total volume containing 2 µl DNA, 0.4 µM of each forward and reverse primer, 0.2 µM probe and 1x SsoFast™ Probes Supermix (BioRad Laboratories). For the standard serial dilution (10^7-10^1 copies/rxn) of the cloned Dhc1f-Dhc264r the KB-1 amplicon was used (re-amplified with M13 primers). CFX Connect™ Real-Time PCR Detection System (BioRad Laboratories) was used for the real-time PCR amplification with the following settings: initial denaturation for 2 min at 95 °C followed by 40 cycles of 15 sec at 95 °C and 30 sec at 60 °C. Fluorescence was captured at the end of each cycle. Each sample was tested in triplicate.

Table S.3: Results of the *Dehalococcoides* spp. real-time PCR (n.d. – not detected)

Chamber	Sampling point	Replicate	89 days	145 days
CONT	DS30	1	n.d.	n.d.
CONT	DS30	2	n.d.	n.d.
CONT	DS30	3	n.d.	n.d.
CONT	US	1	n.d.	n.d.
CONT	US	2	n.d.	n.d.
CONT	US	3	n.d.	n.d.
NZVI	DS30	1	n.d.	n.d.
NZVI	DS30	2	n.d.	n.d.
NZVI	DS30	3	n.d.	n.d.
NZVI	US	1	n.d.	n.d.
NZVI	US	2	n.d.	n.d.
NZVI	US	3	n.d.	n.d.
KB1	DS30	1	positive	positive
KB1	DS30	2	positive	positive
KB1	DS30	3	positive	positive
KB1	US	1	n.d.	n.d.
KB1	US	2	positive	n.d.
KB1	US	3	n.d.	n.d.
COMB	DS30	1	positive	positive
COMB	DS30	2	positive	positive
COMB	DS30	3	positive	positive
COMB	US	1	n.d.	n.d.
COMB	US	2	n.d.	n.d.
COMB	US	3	n.d.	n.d.

At the sampling point US of chamber KB1 *Dehalococcoides* spp. were detectable only in one replicate (day 89) at low levels close to the limit of detection.

S.4. Spatial arrangement of nZVI, PCE and KB1

Setup and results of the pre-experiments to estimate the nZVI distribution

In order to estimate the distribution and needed amount of nZVI and PCE within the lysimeter chambers lab-scaled pre-experiments were conducted. Therefore, a box was filled with sand, resulting in a compacted sand layer and an aquifer sand layer (equal to those in the lysimeter chambers). Both zones were saturated with water and an injection tube (for the tube preparation see supplementary information on nZVI injection) was installed. Prior to the injection the particles were dispersed and immediately afterwards dissolved in water. 10 L of a 0.5 g/L suspension was injected with a pressure of 0.2 bar. nZVI suspension was injected vertically into the aquifer layer. To determine the distribution of the particles in the injection zone two duplicate sand samples were taken (at 1-3 cm and 9-12 cm depth to the injection tube) directly next to the injection tube and at the edge of the injection zone. The samples were

dried and then measured for their iron content via Flame-AAS (Perkin Elmer, AAnalyst 400).

The iron zone was visually evaluated and was conically distributed along the injection length. Showing that the particles entered the sand layer preferred through the first injection holes. The total volume of the distributed particles was 2.1 L (a prior conducted experiment with 900 mL suspension led to a similar distribution zone). The iron content showed a regular distribution within the injection zone (19-25 g/L in the center, 10-16 g/L at the edge).

Setup and results of the pre-experiment for the behavior of the PCE phase within in aquifer layer

In order to predict the behavior of the injected PCE phase within the aquifer zone a lab-scale pre-experiment was conducted. Therefore, a see-through glass-box was used for visual evaluation. Fine-grained sand was used as an impermeable bottom-layer. On top the aquifer zone was created with coarse-grained quartz sand (materials see table S.1). Using a peristaltic pump both sand layers were saturated with water from the bottom. A vertical flow with a distance velocity of 30 cm/d was applied. Subsequently two times 2 mL PCE (dyed with 0.13 % Sudan III) were injected respectively. The first injection was done exactly onto the impermeable layer. The second one was implemented into the middle of the aquifer zone (approximately 6 cm above the impermeable layer). Neither of the two PCE phases entered the impermeable layer and both showed only little lateral spreading. Following 25 mL of dyed PCE were injected 2 cm above the impermeable layer. No infiltration of the impermeable layer was observed with the 12.5 higher volume as well. Lateral distribution mainly occurred at the depth of injection and after 2 hours no further spreading of the phase could observed.

Spatial distribution of PCE within the lysimeter

To monitor the spatial distribution of PCE five additional sampling ports have been installed:

- DS30-L: 25 cm left to DS30 (horizontally)
- DS30-R: 25 cm right to DS30 (horizontally)
- DS60-L: 25 cm left to DS60 (horizontally)
- DS60-R: 25 cm right to DS60 (horizontally)
- DS60-T: 10 cm above DS60 (vertically)

At the first sampling undissolved PCE was found at DS30-R of chamber CONT and KB1, thus no further samples could be taken. As well as no samples could be retrieved from DS60-L due to clogging.

Differences in the spatial PCE distribution could be observed and were caused by unavoidable differences during the chamber installations. Similar PCE concentrations to that of US could be found at DS30-L in chamber NZVI, indicating that the nZVI was not well distributed horizontally (as suspected after the pre-experiments). Which can also be seen in the slightly elevated concentrations at DS30-L in chamber COMB, where the lower concentrations are probably due to bacterial degradation. The horizontal PCE distribution at DS60 to the left and right was insignificant. Except in

chamber CONT where increased PCE concentrations at DS60-R have been observed. 10 cm above DS60 only low PCE concentrations could be measured. Due to the high density of PCE it sank unto and stayed on top of the impermeable layer.

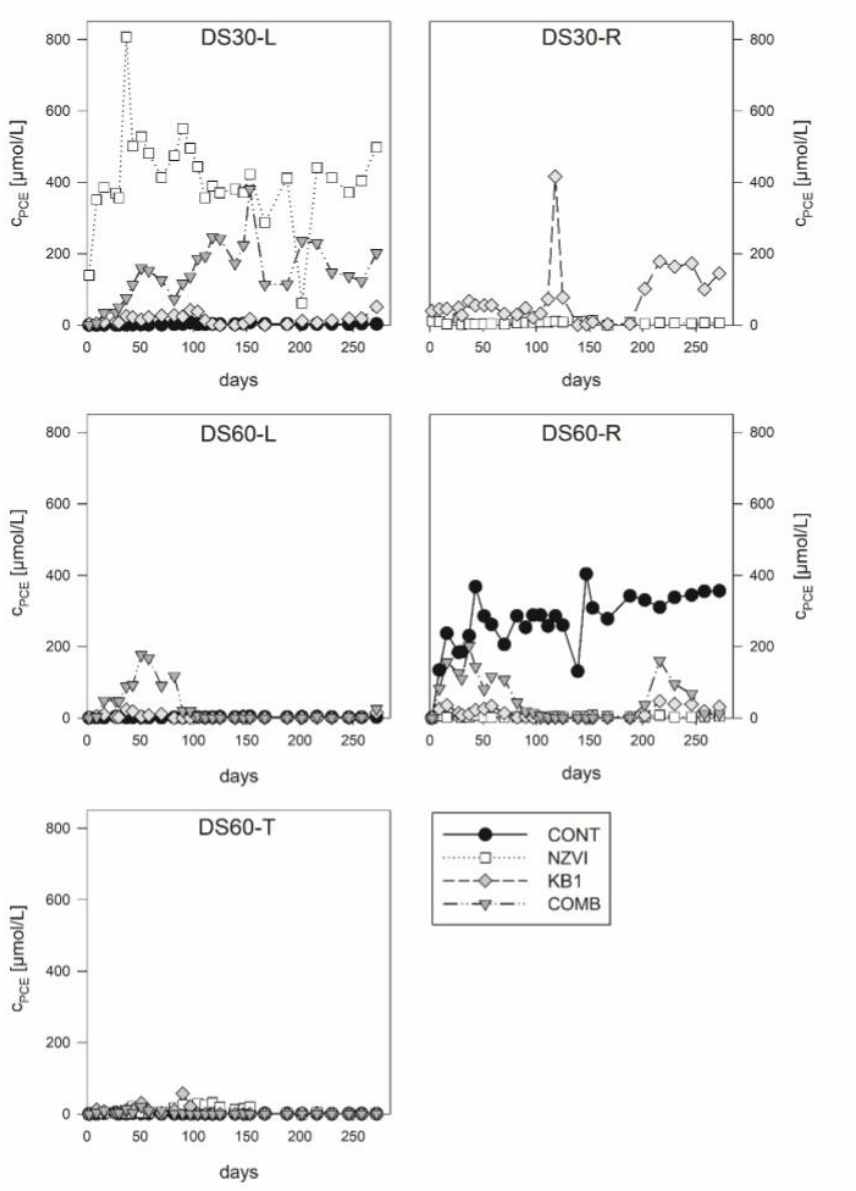

Fig. S.1: PCE concentrations at DS30-L, DS30-R, DS60-L, DS60-R and DS60-T of chamber CONT, NZVI, KB1 and COMB

5

Spatial distribution of KB1-culture

Samples for the qPCR analysis were only taken from the middle transect of the chambers. But *Dehalococcoides* spp. were still detected in the efflux of the chambers and thus about 1 m downgradient to their injection point.

S.5. Environmental parameters

The temperature in the aquifer zones of the four chambers could be kept at similar levels (Fig. S.). However, there were annual variations between 9 °C (winter) and 21 °C (summer).

The pH could be regulated between 6.1-7.1 from day 43 until the end of the experiment (**Error! Reference source not found.**). The addition of molasses caused the pH to drop to 6.1-6.2 in the chambers COMB and KB1. Whereas the injection of nZVI led to a pH increase in chamber nZVI (6.9) and COMB (7.0) downstream of the treatment zone. This pH increase is consistent with the formation of OH^- during anaerobic corrosion.

The oxidation-redox-potential (ORP, E_h compared to standard hydrogen electrode) started at 300-400 mV in all chambers (

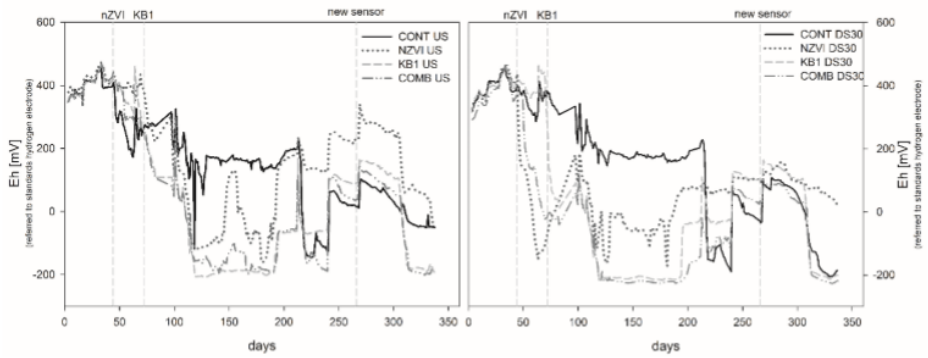

Fig. S.). The injection of nZVI decreased the ORP downstream of the nZVI zone in the chambers nZVI (up to -148 mV) and chamber COMB (up to -24 mV). The addition of molasses led to a decrease of the ORP (157 to -227 mV) in all the chambers where it was applied. After stopping the molasses input the ORP increased again to 22-164 mV.

The measured NO_3^- concentrations in the aquifer zones corresponded with the measured ORP in all chambers. In chamber CONT at all sampling points and in chamber NZVI at sampling point US hardly any NO_3^- was reduced. Either because the ORP was not low enough, the residence times were too low or not enough organic substances were present for the oxidation reaction. The nZVI injection into chamber NZVI led to a reduction of NO_3^- over 4-5 months from 2.2 mg/l to 0 mg/l. The molasses addition in chambers COMB and KB1 and the corresponding drop in the ORP led to a drastic reduction of NO_3^-. The stop of molasses addition caused a slight increase of the NO_3^- concentrations (0.5 mg/l) until the anew molasses input caused again a NO_3^- reduction. The NO_3^- concentrations in the chambers CONT

(0.4 mg/l) and KB1 (0.5 mg/l) were lower from the start compared to the other chambers.

The SO_4^{2-} concentrations in the aquifer zones of all chambers corresponded with the measured ORP. The nZVI injection increased the SO_4^{2-} concentrations at the DS30 sampling points of chamber NZVI (from 52.2 to 77.5 mg/l) and COMB (from 77.5 to 86.0 mg/l). The molasses addition to chambers COMB and KB1 caused the SO_4^{2-} concentrations to increase shortly after, probably due to a high SO_4^{2-} content of the molasses, followed by a decrease to 0-10 mg/l. After stopping the input of molasses in chamber CONT the SO_4^{2-} concentration increased from 19.2 mg/l to 71.0 mg/l, which was not in correspondence with the decreasing ORP (-200 mV).

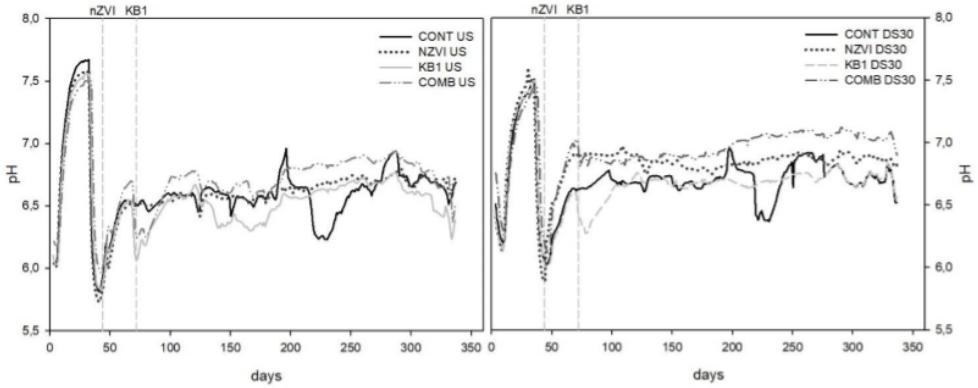

Fig. S.2: pH values at sampling points US and DS30 of chambers CONT, NZVI, KB1 and COMB (vertical dash lines indicating nZVI and KB1 injection)

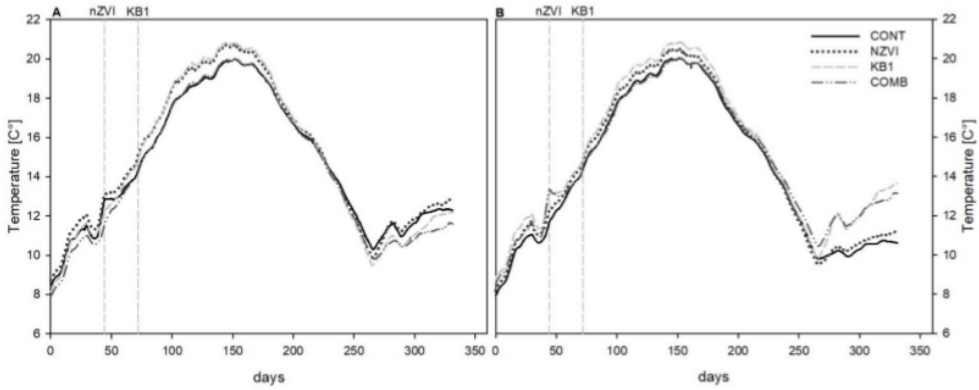

Fig. S.3: Temperature development before (A) and after (B) the treatment zones in chambers CONT, NZVI, KB1 and COMB (vertical dash lines indicating nZVI and KB1 injection)

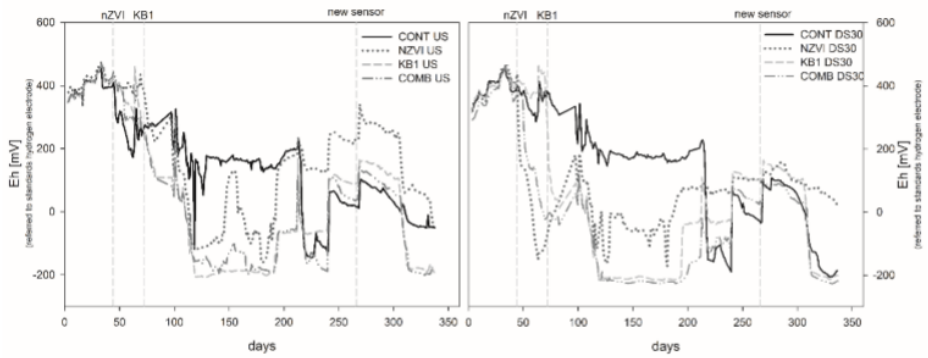

Fig. S.4: Eh (referred to standard hydrogen electrode) development at the sampling points US and DS30 of chambers CONT, NZVI, KB1 and COMB (vertical dash lines indicating nZVI and KB1 injection and installation of a new ORP sensor)

S.6. Cumulative CH_4 loads

Total cumulative methane loads were highest with similar values in chambers KB1 and COMB followed by chambers NZVI and CONT (Table 3). In NZVI methane was only detected in the efflux. The origin of this methane remains unclear. We assume that this was an artefact.

Table S.4: Cumulative CH_4 loads in the efflux of the chambers CONT, NZVI, KB1 and COMB at the end of the experiment.

Chamber	[mmol]
CONT	35
NZVI	264
KB1	596
COMB	584

ABBREVIATIONS

BTEX	benzene, toluene, ethylbenzene and xylene
CHC	Chlorinated hydrocarbons
DCE	Dichlorothene
DNAPL	dense non-aqueous liquid
HC	Hydrocarbon
mZVI	micro-scaled zero-valent iron
nZVI	nano-scaled zero-valent iron
ORP	oxidation-reduction potential
PAH	Polycyclic aromatic hydrocarbons
PCE	Perchloroethene
PLFA	Phospholipid fatty acids
TCE	Trichloroethene
VC	Vinyl chloride
ZVI	Zero-valent iron

FIGURES

TABLES

EQUATIONS

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Scientific work experience

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2015	**Austrian Institute of Technology, Tulln, AUT** Fem-Tech Internship at the groundwater remediation group, lab work and data evaluation

Scientific contributions & experiences

Aug. 2018	Summer D., Schöftner P., Wimmer B., Reichenauer G. T.: **Inhibition and Stimulation of Different Bacterial Cultures Using Iron Particles** (Poster: 8th International Conference on Environmental Pollution and Remediation, Madrid, ESP, Poster award winner)
Jan. 2017	Summer D., Schöftner P., Wimmer B., Reichenauer G. T.: **Synergistic degradation of chlorinated hydrocarbons with microorganisms and zero valent iron** (Oral presentation: 6th AIT PhD Seminar, Hirschegg, AUT, 3rd place winner)
Oct. 2016	Summer D., Schöftner P., Wimmer B., Reichenauer G. T.: **Comparison of micro- and nano-scaled zero-valent iron particles regarding their PCE dehalogenation and H2 production potential** (Poster: Internano Conference 2016 Koblenz, DEU)

Oct. 2016	Summer D., Schöftner P., Watzinger A., Wimmer B., Kostic T., Sessitsch A., Reichenauer G. T.: **Synergistic combination of abiotic and biotic PCE-dechlorination** (Poster: Docday 2016, UFT Tulln, Austria)
Nov. 2015	Summer D., Schöftner P., Wimmer B., Watzinger A., Reichenauer G. T.: **Synergistic degradation of chlorinated hydrocarbons with microorganisms and zero valent iron** (Poster: presentation, 14th Stable Isotope network Austria SINA, Tulln, AUT)
Oct. 2015	Summer D., Schöftner P., Wimmer B., Reichenauer G. T.: **Remediation of TCE, arsenic and nickel contaminated groundwater using permeable barriers - Column experiments with zero-valent iron** (Oral presentation: 5th International Conference “Applied Natural Science 2015”, Jasna, SK)
Jul. 2015	Summer D.: **Dehalo-Man vs. Evil PCE** (Oral presentation: AIT PhD Science Slam, Tulln, AUT, 1st place winner)