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Field test of a bioaugmentation agent for the bioremediation of chlorinated ethene contaminated sites

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Abstract
Chlorinated ethenes are toxic compounds that were widely used in the past, and their improper handling and storage caused notable pollutions worldwide. In situ bioremediation by reductive dechlorination of bacteria is a cost-effective and ecologically friendly way to eliminate these pollutions. During the present study, the efficiency of a previously developed bioaugmentation agent combined with biostimulation was tested under field conditions in contaminated soil. Furthermore, the preservation of dechlorinating ability was also investigated in a long-term experiment. Initially, aerobic conditions were present in the groundwater with possible presence of anaerobic micro-niches providing habitat for *Brocadia* related anammox bacteria. "*Candidatus* Omnitrophus" was also identified as a dominant member of community then. Significant changes were detected after the biostimulation, anaerobic conditions established and most of the dominant OTUs were related to fermentative taxa (e.g. *Clostridium*, *Trichococcus and Macillibacteroides*). Dominant presence of vinyl-chloride coupled with the lack of vinyl-chloride reductase gene was observed. The most notable change after the bioaugmentation was the significant decrease in the pollutant quantities and the parallel increase in the *vcrA* gene copy numbers. Similar to post-biostimulation state, fermentative bacteria dominated the community. Bacterial community composition transformed considerably with time after the treatment, dominance of fermentative—mainly Firmicutes related—taxa decreased and chemolithotrophic bacteria became abundant, but the dechlorinating potential of the community remained and could be induced by the reappearance of the pollutants even after 4 years.

Keywords Bioremediation · Bioaugmentation · Reductive dechlorination · *Dehalococcoides* · Trichloroethene

Introduction

Short-chain chlorinated aliphatic hydrocarbons (e.g.: perchloroethene—PCE, trichloroethene—TCE) were extensively used in the past few decades (Matteucci et al. 2018) as solvents, degreasing agents by the chemical industry and households as well (Aulenta et al 2005). Owing to the unregulated treatment obligations in the past in addition to the often improper storage, large amount of halogenated hydrocarbons penetrated into soils and groundwater causing contamination and their accumulation in the environment (Matteucci et al. 2015). Various technologies have been developed and applied to treat chlorinated ethene polluted sites, including physicochemical (e.g.: air sparging, pump and treat, chemical oxidation and thermal desorption) (Saiyari et al. 2018; Wang et al. 2018; Blázquez-Pallí et al. 2019) and biological treatments. Bioremediation techniques are cost-efficient and innovative methods to eliminate short-chain halogenated hydrocarbon contaminations (Saiyari et al. 2018). A wide range of chlorinated aliphatic hydrocarbons can be transformed by reductive dechlorination (Frascari et al. 2015; Puigserver et al. 2022) under anaerobic conditions using chlorinated ethenes as electron acceptor for respiration, which is one of the most effective, consequently the most significant microbiological process in the elimination of these pollutions. Diverse group of anaerobic microorganisms can reduce PCE and TCE to cis-dichloroethene (cis-DCE) to gain energy, such as some species of genus *Desulfitobacterium*, *Desulfomonile*, *Dehalobacter*,

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Desulfuromonas, *Sulfurospirillum* and *Geobacter* (Mohn and Tiedje 1991; Gerritse et al. 1996; Krumholz 1997; Holliger et al. 1998; Luijten et al. 2003; Sung et al. 2006). However, the most important microorganisms in reductive dechlorination are *Dehalococcoides* spp. within the phylum Chloroflexi. Certain strains of *Dehalococcoides mccartyi* are capable of the sequential reduction of chlorinated ethenes such as PCE and/or TCE to ethene via dichloroethene isomers (cis-DCE, trans-DCE, 1,1-DCE) and vinyl-chloride (VC) (Maymó-Gatell et al. 1997; McCarty 1997; Adrian et al. 2007; Löffler et al. 2013; Yan et al. 2021) to a harmless endproduct using hydrogen as sole electron source. A key enzyme in this complete type of dechlorination is the vinyl-chloride reductase, which only occurs in *Dehalococcoides* related bacteria and catalyzes the transformation of vinyl-chloride to non-toxic ethene. Currently, the microbial reductive dechlorination seems to be a common process in contaminated anoxic aquifers (Vogel et al. 2018), which can occur as natural attenuation. However, in many cases, microorganisms involved in dechlorination are underrepresented or absent at sites contaminated with chlorinated ethenes due to limited spreading, unfavorable environmental circumstances or competitive pressure (Löffler and Edwards 2006; Tas et al. 2010). On the other hand, despite the presence of diverse dechlorinating microbial community, including *Dehalococcoides* sp. natural dechlorination process could be incomplete (Perez-de-Mora et al. 2018; Nagymáté et al. 2020) resulting the accumulation of *cis*-DCE and the carcinogenic VC (Holmes et al. 2006). The efficiency of the dechlorination depends on the environmental conditions such as the subsurface heterogeneity (Perez-de-Mora et al. 2018; Yu et al. 2018) and on the presence and concentration of different types of halogenated compounds (Mészáros et al. 2013; Vogel et al. 2018; Nagymáté et al. 2020). However, the reductive dechlorination can be effective and successful even at high contaminant concentrations (e.g. 700–10,000 µg L^{-1}) (Kotik et al. 2013; Perez-de-Mora et al. 2014). Providing specific supplementary nutrients serving electron donors and/or as carbon source (e.g. ethanol, oils, Perez-de-Mora et al. 2018; glycerol, Atashgahi et al. 2017; acetate, Major et al. 2002; and lactate, Dugat-Bony et al. 2012) was successfully applied to enhance natural reductive dechlorination through the stimulation of autochthonous microorganisms involved in decomposition processes (*biostimulation*). In those cases when dechlorinating taxa are absent at the contaminated site, application of allochthonous microbial consortia (*bioaugmentation*) containing certain strains of *Dehalococcoides mccartyi* capable of complete reductive dechlorination provides the efficiency of the bioremediation. Furthermore, besides the dechlorinating taxa, other auto- or allochthonous microbes can also facilitate the dechlorination process indirectly by providing essential nutrients, vitamins (e.g.: Vitamin B_{12}, (Löffler, et al. 2013; Yan et al. 2013) for the growth of dehalorespiring bacteria and by releasing electron donors and carbon sources through the decomposition of organic matter.

Previously, Nagymáté et al. (2020) developed a bioaugmentation agent by enriching dechlorinating microbial communities inhabiting the groundwater of a contaminated site. As for the community composition of the agent, the major member was *Dehalococcoides* related (19.83–16.98%), partial dechlorinating bacteria were represented mainly by genera *Sulfurospirillum* and *Geobacter;* furthermore, fermentative (*Macellibacteroides*, *Trichococcus*, *Youngiibacter*) and amino-acid degrading bacteria (vadinBC_27_wastewater-sludge_group) were identified. Upscaling of the laboratory sized cultures (0.5–10 L) to industrial scale (100 L) was also carried out to provide enough volume for pilot tests. This large-scale dechlorinating microbial consortium thus developed was the predecessor of the later commercialized Ferm&Go 1 V inoculant (https://fermandgo.hu/en/fandgoproducts/fermgo-1v/). Firstly, the aim of the present study is to test the efficiency of the developed bioaugmentation agent in the elimination of TCE pollution under field conditions. To reveal the progress of dechlorination, the changes in the bacterial community composition, vinyl-chloride reductase gene (*vcr*A) copy numbers, physical–chemical properties of groundwater and pollutant concentrations were followed. Furthermore, survival of the applied key microbes and consequently the preservation of dechlorinating ability were also investigated in a long-term (4 years long) experiment.

Materials and methods

Pilot test of in situ bioremediation was carried out at a former industrial site located in Zalaegerszeg, Hungary. The size of the test site was 50 m^2, containing aprx. 105 m^3 contaminated groundwater. The area was characterized with high volatile chlorinated hydrocarbon (VOCl) contamination with main compounds of PCE, TCE and cis-DCE of which former leaked possibly from storage tanks. Previous investigations on the chlorinated pollutant concentrations of the test site revealed VOCl quantities of 11,300–85,800 µg L^{-1}. The released solvents polluted a shallow aquifer (1.9–3.6 m depth) endangering the deeper aquifer below 12 m. The two aquifers are separated by fine silt layer and a continuous clay layer providing good water retention capacity. Soils from the targeted bioaugmentation zone consisted of debris upload (0–1.8 m), yellow sand (1.8–3.5 m), light gray clay (3.5–3.7 m), yellow pebble sand (3.7–4.5 m), gray clay (4.5–5.1 m) and yellowish-gray sand (5.1–5.5 m). The flow rate of the groundwater in the targeted zone was estimated to 1.5×10^{-6} m s^{-1}. Biostimulation and bioaugmentation were carried out in a well having 12 m foundation depth

and a shallow filter at 1–5.5 m and deep filter 4.8–8.9 m. Prior to adding the dechlorinating agent, total volume of 500 L of anaerobic hydrogen releasing compound (HRC) (containing lactate and lactose; TOC: 24 g L^{-1}, pH 4.7) was injected into the well as biostimulation agent to create appropriate conditions for reductive dechlorination. Immediately after the biostimulation step, 10 L of dechlorinating consortium was injected anaerobically into the well monthly, for 3 months. Groundwater samples were collected using low-flow (5 L min^{-1}) technique in sterilized 2.0 L and 0.5 L bottles filled up without headspace and in 0.5 L volume bottles sealed and crimped with Teflon-coated butyl rubber septa (Wheaton Science Products, Millville, NJ, USA) for microbiological, chemical analysis and for gas chromatography, respectively. Water samples were kept at 10 °C during transportation to the laboratory and were processed within 24 h. *On site* measurement of dissolved oxygen concentration (DO), pH, temperature (T), specific electric conductivity (EC) and oxidation- reduction potential (ORP) was performed using a multimeter (Hach HQ40D portable multimeter; Hach, Loveland, CO, USA). Samplings were carried out at six occasions: before any treatment (ZDF1_IS), 1 month after the biostimulation (ZDF1_PBS), 1 month after the bioaugmentations (ZDF1_PBA), 6 months (ZDF1_06), 10 months (ZDF1_10) and 48 months (ZDF1_48) after the treatment.

Chemical parameters of the groundwater were determined by standard laboratory methods (Rice et al. 2012). Ammonium (ASTM 4500-NH3-F), nitrite (ASTM 4500-NO_2^--B), nitrate (ASTM 4500-NO_3^--B), sulfate (ASTM 4500-SO_4^{2-}-E), phosphate (ASTM 4500-P-E) and iron (3500-Fe-B) were measured photometrically. The quantity of chloride ion (4500-Cl^--B) was obtained by titrimetry. The concentration of total organic carbon content (TOC) was determined by persulfate oxidation method (ASTM 5310-C), and chemical oxygen demand (COD) was measured by colorimetry (ASTM 5220-D).

Biodegradation of the chlorinated hydrocarbons was followed by gas chromatography (HP 5890 instrument, Agilent Technologies Inc., Santa Clara, CA, USA) using flame ionization detection equipped with HP-PLOT Q type column (15 m × 0.53 mm) (Agilent Technologies Inc., Santa Clara, CA, USA). The carrier gas was 5.0 helium (Linde, Munich, Germany), and the injector was operated in split mode at a ratio 1:20. The following temperature protocol was used: 60 °C 2 min, 25 °C min^{-1} to 250 °C, the temperature of the detector and the injector was 250 °C. The method is applicable to separate methane, ethane, ethene, vinyl-chloride, *cis*- and *trans*-DCE, TCE and PCE.

The calibration standards were prepared as described by Nagymáté et al. (2020) and were stored and sampled in the same way as the groundwater samples. The injected volume of the headspace was 100 µl during manual injection using gas-tight Hamilton syringe. Prior to gas chromatography measurements, the samples were incubated for 24 h at 20 °C to reach the equilibrium of the volatile compounds among the phases.

For the extraction of the community DNA, 1 L of contaminated groundwater was filtered through 0.22-µm pore size cellulose nitrate filter (Millipore, Billerica, USA). DNA was extracted from the membrane filters using PowerSoil DNA Isolation Kit (MoBio Laboratories Inc. Carlsbad, USA) according to the manufacturer's instructions, with a modification at the physical cell disruption step: the samples were shaken in "Bead Solution tubes" (MoBio Laboratories) at 30 Hz for 2 min using mixer mill MM301 (Retsch, Haan, Germany).

The absolute quantification of the vinyl-chloride reductase gene (*vcr*A) was carried out using TaqMan-based quantitative PCR. Each 16 µL PCR reaction contained 8 µl of TaqMan gene expression master mix (Applied Biosystems, Foster City, CA), 300 nM of each of the primers (Vcr1022F and Vcr109R) and the probe (Vcr1042Probe), 6.19 µl DEPC (Diethyl pyrocarbonate)-treated water and 1 µL of template DNA (Mirza et al. 2016). For amplification, the following PCR conditions were applied: initial incubation at 50 °C for 2 min, an initial denaturation stage at 95 °C for 3 min, followed by 40 cycles of 10 s at 95 °C and 30 s at 58 °C for denaturation and annealing respectively. All reactions were made in triplicates and were run in ABI Step One plus (Applied Biosystems, Foster City, CA) device. DNA standard was obtained from the bioaugmentation agent used during the experiment containing *Dehalococcoides mccartyi* in high abundance. Data evaluation was carried out by StepOneTM software v2.3.

The bacterial community compositions of the groundwater samples were assessed by high throughput amplicon sequencing. PCR amplification was performed in triplicates in 20-µL final volume containing 1 × Phusion HF Buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.2 mM dNTPs (Fermentas Vilnius, Lithuania), 0.4 µg $µL^{-1}$ Bovine Serum Albumin (Fermentas), 0.3 µM of each primer and 0.4 U Phusion High-Fidelity DNA Polymerase (Thermo Fisher). The V3-V4 region of the 16S rRNA gene was amplified using CS1-TS-B341F (5′-CCT ACG GGN GGC WGC AG-3′) (Herlemann et al. 2011) and modified CS2-TS-806RM primers (5′-GGA CTA CHV GRG TWT CTA AT-3′) (Kozich et al. 2013) with the following thermal conditions: initial denaturation at 98 °C for 5 min, followed by 25 amplification cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, followed by final extension at 72 °C for 10 min. Library processing and amplicon sequencing were performed by Illumina Miseq platform using standard MiSeq v2 flow cell (Illumina) in a 2 × 250 bp paired end format with a v2, 500 cycle MiSeq reagent cartridge at Genomics Core Facility RTSF, Michigan State University,

USA (Szuróczki et al. 2020). For bioinformatic analyses of the resulting sequence reads, Mothur software (Schloss et al. 2009) was used and Miseq SOP pipeline was followed. Taxonomic affiliation was made by ARB-SILVA SSU NR reference database, release 138_1 (Quast et al. 2012). Operational taxonomic units (OTUs) were assigned at 97% similarity threshold levels (Tindall et al. 2010). Raw sequence reads were deposited in the NCBI SRA database and are accessible through BioProject ID PRJNA1124051.

For visualization of the data by multivariate statistical analysis, R software (https://www.R-project.org/) and vegan package (Oksanen et al. 2017) were used. To reveal the contribution of taxa to the observed dissimilarities, SIMPER test was carried out using PAST software (Hammer et al. 2001).

Results and discussion

Physical–chemical properties and bacterial community structure of the groundwater prior to treatment

The groundwater was characterized with 9.5 °C and neutral (7.13) pH at the pre-treatment phase (Table 1). Initially, high oxidation–reduction potential (228 mV), DO concentration (0.5 mg O_2 L^{-1}) and relatively low specific electric conductivity (665 µS cm^{-1}) was measured. Considering the water chemistry parameters, the well water was characterized with low total organic carbon content, inorganic nitrogen forms, phosphate and total iron concentration. Pollutant quantities were also relatively low with the dominance of TCE and cDCE.

As for bacterial community composition, Planctomycetota-, Verrucomicrobiota- and Patescibacteria-related sequences dominated the amplicon libraries on phylum level (Fig. 1). Considering the bacterial OTUs, the most abundant sequences were "*Candidatus* Brocadia" (Planktomycetota) and "*Candidatus* Omnitrophus" (Verrucomicrobiota) related, of which former was isolated during an investigation aiming the identification and quantification of anammox bacteria in nitrogen removal reactors (Hu et al. 2010), while latter was identified as a member of subsurface microbial community at Hanford site which is a representative of contaminated subsurface environment polluted by metals, radionuclides, nitrate, organic solvents and/or complexing agents (Lin et al. 2012) (Fig. 2). According to Shannon diversity calculated on OTU level, the highest diversity (4.98) was observed prior to any treatment. As for complete dechlorinating capacity of the bacterial community, *vcr*A cannot be detected by qPCR (Fig. 3).

Initially, aerobic conditions were present in the groundwater based on the ORP values, with possible presence of anaerobic micro-niches providing habitat for *Brocadia*-related anammox bacteria. Representatives of "*Candidatus* Omnitrophus"—giving the other dominant member of the community—are frequently identified as abundant taxon in groundwaters (Perez-Molphe-Montoya et al. 2022). Probably, neither of these can directly take part in the dechlorination process, and the absence of vinyl-chloride reductase encoding genes also supports the poor autochthonous bioremediation capacity of the site. The low pollutant

Table 1 Physical–chemical properties and pollutant concentrations of the groundwater samples taken from ZDF1 well before any treatment (ZDF1_IS), after the biostimulation (ZDF1_PBS), after the bioaugmentations (ZDF1_PBA), 6 months (ZDF1_06), 10 months (ZDF1_10) and 48 months (ZDF1_48) after the treatment

	ZDF1_IS	ZDF1_PBS	ZDF1_PBA	ZDF1_06	ZDF1_10	ZDF1_48
T (°C)	9.5	11.9	13.1	13.5	14.2	16.2
pH	7.13	6.5	6.38	6.41	6.93	7.24
DO (mg L^{-1})	0.5	0.23	0.6	0.31	0.2	0.8
ORP (mV)	228	−506.9	−87	−249.2	−660	−105.9
EC (µS cm^{-1})	665	1130	1239	1004	774	977
NH_4^+ (mg L^{-1})	0.35	0.01	0.03	18.2	21.9	1.5
NO_2^- (mg L^{-1})	0.02	0.01	0.01	0.01	0.01	0.23
NO_3^- (mg L^{-1})	43	34	48	8	28	9
Cl^- (mg L^{-1})	102	60	67	78	32	57
Fe (mg L^{-1})	0.01	0.01	2.2	2.6	0.01	0.4
SO_4^{2-} (mg L^{-1})	130	115	144	114	97	192
PO_4^{3-}(mg L^{-1})	1.5	2.6	6.1	10.1	71	1.94
TOC (mg L^{-1})	24	303	427	82	9	3
COD (O_2 mg L^{-1})	0	567	885	50	53	3
TCE (µM L^{-1})	1	13	0	50	53	8.7
cDCE (µM L^{-1})	1	2	0	0	0	12.7
VC (µM L^{-1})	2	472	0	0	0	3.1
VcrA (copy mL^{-1})	0.00E+00	0.00E+00	1.08E+03	2.94E+02	2.75E+01	1.67E+02

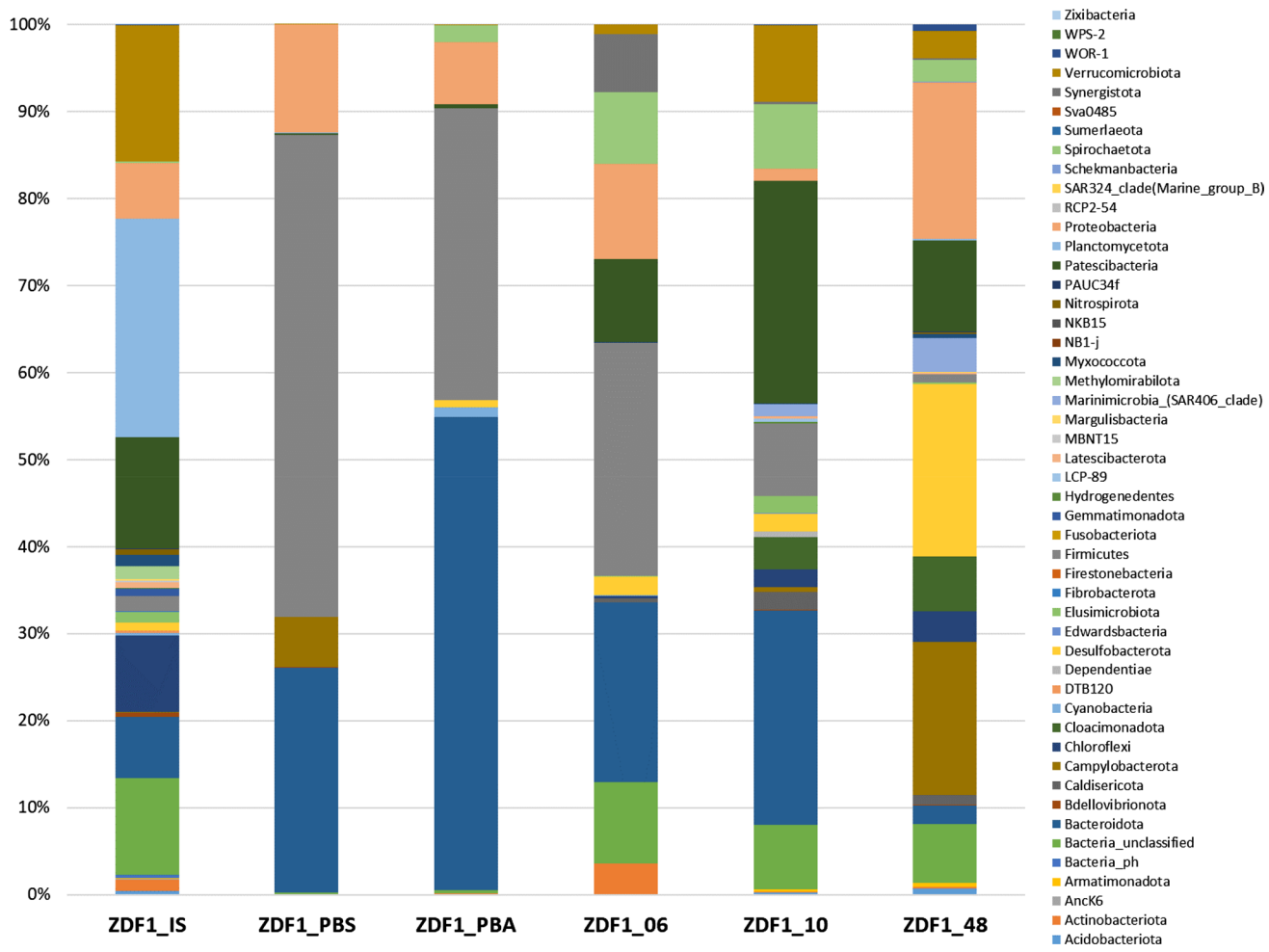

Fig. 1 Bacterial community compositions of the groundwater samples originating from.ZDF1 well before any treatment (ZDF1_IS), after the biostimulation (ZDF1_PBS), after the bioaugmentations (ZDF1_PBA), 6 months (ZDF1_06), 10 months (ZDF1_10) and 48 months (ZDF1_48) after the treatment on phylum level

concentrations at this time could be the result of the fluctuation caused by the combination of the location of the well (which was at the edge of a highly contaminated area) and the changes in direction and rate of groundwater flow.

Physical–chemical properties and bacterial communities of the groundwater after the biostimulation

After the determination of initial circumstances and prior to the bioaugmentation, biostimulation was carried out by adding 500 L of hydrogen release compound (HRC) solution into the well for a week. The treatment changed the water physical–chemical properties as well as the composition of the bacterial community considerably. Specific electric conductivity, TOC and COD values increased remarkably (Table 1, Fig. 2) indicating high quantities of organic matter. The DO concentration and the oxidation–reduction potential of the well water decreased to 0.23 1 mg L^{-1} and − 506.9 mV, respectively, providing highly reducing conditions. A notable pH drop was also observed to 6.5. Regarding the pollutant, notable quantities of vinyl-chloride (472 µM L^{-1}) were measured and cDCE (2 µM L^{-1}) and TCE (13 µM L^{-1}) were also presented. Considering the phylum-based bacterial community composition of the post-biostimulation sample, number of taxa significantly decreased compared to the non-treated sample. Firmicutes became the most dominant member comprising 55% of the community, but Bacteroidota (26%), Proteobacteria (12%) and Campilobacterota (6%) related sequences were also abundant (Fig. 1). On OTU level, members of *Clostridium* sensu stricto (Firmicutes), *Macellibacteroides* (Bacteroidota) and *Trichococcus* (Firmicutes) genera dominated the community and *Pseudarcobacter* (Campilobacterota), *Bacteroides* (Bacteroidota), Veillonellales-Selenomonadales (Firmicutes) and *Pseudomonas* (Proteobacteria)-related

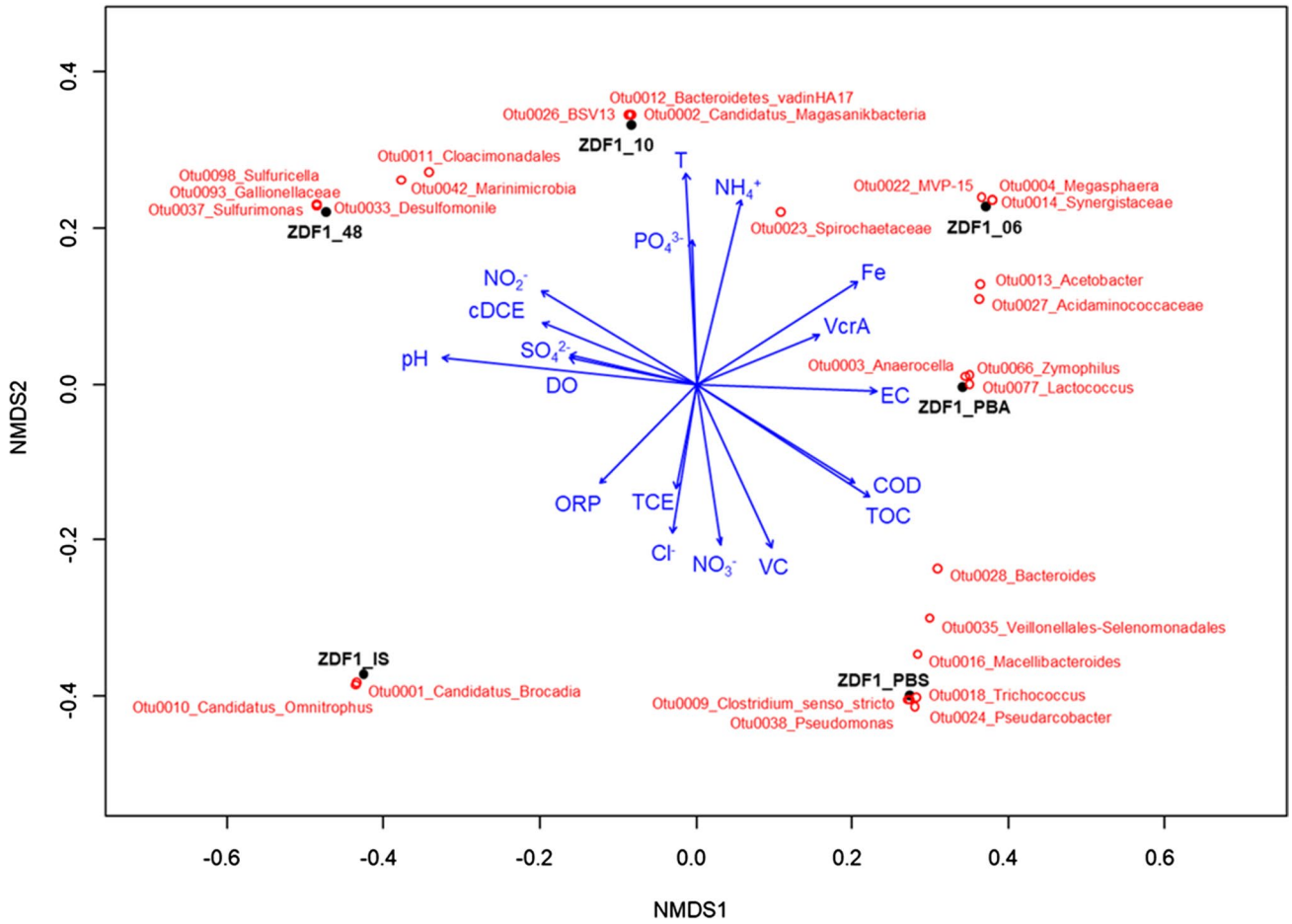

Fig. 2 Result of nonmetric multidimensional scaling on bacterial OTUs revealed from the groundwater samples before any treatment (ZDF1_IS), after the biostimulation (ZDF1_PBS), after the bioaugmentations (ZDF1_PBA), 6 months (ZDF1_06), 10 months (ZDF1_10) and 48 months (ZDF1_48) after the treatment. Environmental parameters were fitted as vectors onto the biplot by package vegan in R. Displayed taxa are limited to those OTUs whose cumulative contribution to group separation reached 50% by SIMPER (Stress = 0.0568)

sequences were also identified in large numbers (Fig. 2). According to the calculated Shannon index based on the OTU abundances, diversity decreased significantly (from 4.98 to 2.73) and dropped to the minimum considering all the samples. Similar to the pre-treatment state, vinyl-chloride reductase gene could not be detected (Fig. 3).

After the biostimulation, significant changes were observed in the abiotic parameters and in the bacterial communities of the groundwater as well. The most notable alteration regarding the physical–chemical parameters was the elevation of TOC, COD and EC which was caused by the high organic matter and ionic compound content of the biostimulation agent. The added HRC had two main facilitating effects on the dechlorination. Firstly, by providing large amount of organic matter, those bacteria that are using aerobic chemoorganotrophic metabolism can reduce the oxygen concentration in the ground water by oxidizing the added organic substrate and consequently creating anaerobic conditions suitable for the reductive dechlorination. Second, the created anaerobic conditions and the large quantity of lactate originating from the HRC solution favored the presence and activity of fermenting bacteria. On the other hand, addition of HRC considerably decreased the diversity which was also observed by Blázquez-Pallí et al. (2024) following lactate treatment. Most of the dominant OTUs were related to fermentative taxa (e.g. *Clostridium*, *Trichococcus* and *Macillibacteroides*) which could either be derived from the biostimulation agent or could be selected by the created circumstances from the autochthon community. Presence of these bacteria could enhance the efficiency of the bioremediation process by providing hydrogen as electron donor for the complete dechlorinating *Dehalococcoides*-related bacteria. Regarding the pollutant concentrations, TCE, cDCE and VC were all identified with the dominance of VC. Accumulation of vinyl-chloride could be the result of partial dechlorination, since many bacterial taxa could

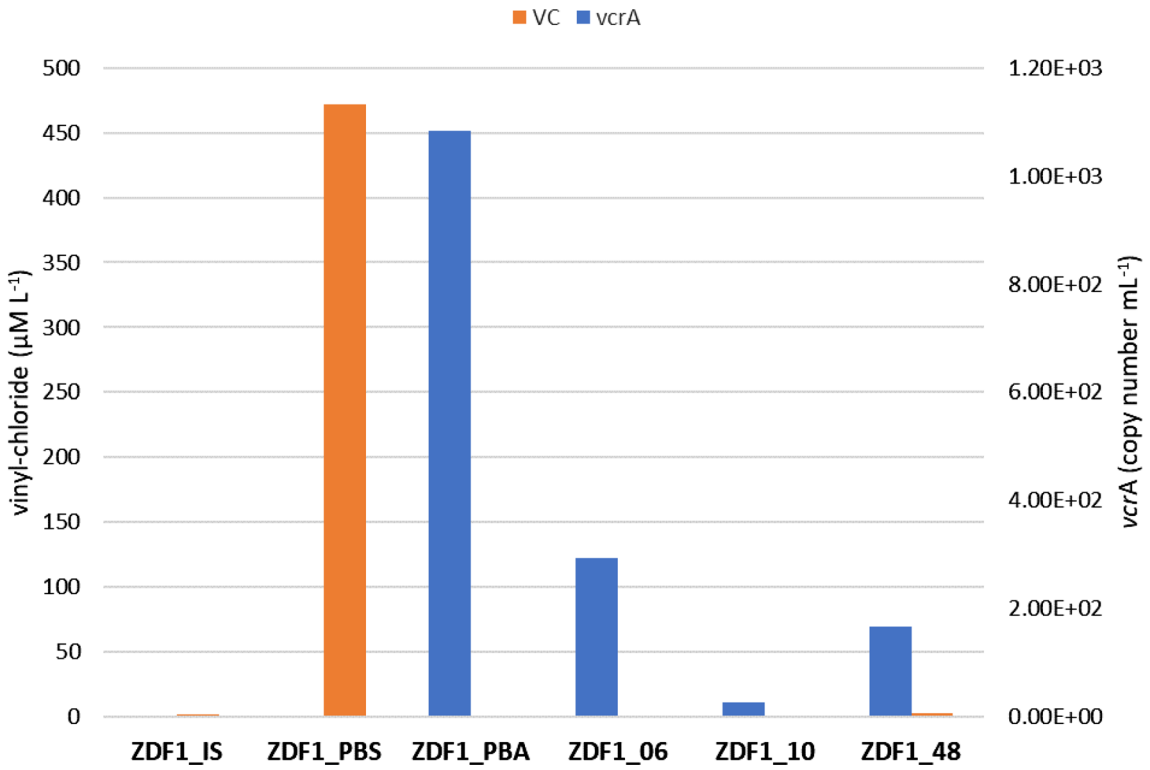

Fig. 3 Changes in the concentration of vinyl-chloride and in the copy number of vinyl-chloride reductase (*vcr*A) gene in the groundwater samples before any treatment (ZDF1_IS), after the biostimulation (ZDF1_PBS), after the bioaugmentations (ZDF1_PBA), 6 months (ZDF1_06), 10 months (ZDF1_10) and 48 months (ZDF1_48) after the treatment

have TCE reductase gene providing the ability for these to reduce TCE to VC; however, the relative abundance of the widely known organohalide-respiring microorganisms catalyzing partial dechlorination (*Sulfurospirillum* sp., *Geobacter* sp. and *Desulfitobacterium* sp.) was under 1% in the microbial community of groundwater. It suggests the role of other organohalide-respiring taxa in the partial dechlorinating process and results of some investigations imply that Firmicutes-related bacteria could have elevated TCE tolerance and could also take part in the dechlorination process (Koner et al. 2022; Tian et al. 2024). Since vinyl-chloride can only be reduced to ethene by the vinyl-chloride reductases of *Dehalococcoides*-related bacteria, in the absence of these, vinyl-chloride could accumulate, which is also supported by lack of *vcr*A gene.

Physical–chemical properties and composition of bacterial communities of the groundwater after the bioaugmentation

Anaerobic conditions established during the biostimulation was preserved after the bioaugmentation as well according to the negative ORP (Table 1). TOC and COD values increased further to 427 mg L^{-1} and 885 O_2 mg L^{-1}, respectively, while pH slightly decreased to 6.38. As for the pollutant concentrations, neither of the measured chlorinated ethene forms could be detected. Regarding the bacterial community composition of the groundwater on phylum level, number of taxa slightly decreased compared to the post-biostimulation state (Fig. 1). Dominance of Firmicutes-related bacteria reduced (to 34%) and Bacteroidota (54%) became the most abundant phylum. Members of Proteobacteria (7%) and Spirochaetota (2%) were also presented in high numbers. Considering the OTU composition, *Anaerocella*-related sequences were the most dominant (Fig. 2). Other significant OTUs were related to *Acetobacter*, Acidaminococcaceae, *Zymophilus*, *Lactococcus* and Spirochaetaceae. Considering the OTU abundance-based Shannon index, the diversity increased (2.97) compared with the post-biostimulation state. As for vinyl-chloride reductase gene copy numbers, 10^3 order of magnitude was determined in 1 ml of sample (Fig. 3).

Similar to post-biostimulation state, reductive conditions with elevated organic matter load were observed in the groundwater with the dominant presence of fermentative bacteria (Acidaminococcacea, *Zymophilus* and *Lactococcus*). The most abundant OTU showed the highest sequence similarity to an *Anaerocella*-related sequence previously obtained from lake water. The only described species of the mentioned genus (*Anaerocella delicata*) was isolated from

a methanogenic reactor and has a fermentative metabolism (Abe et al. 2012). Other than serving electron donor for the complete dechlorination, some taxa (e.g. *Acetobacter pasteurianus*) could also facilitate the complete dechlorination by producing the precursor of B12 vitamin, which is required for the proper growth of *Dehalococcoides*-related bacteria (Bernhardt et al. 2019). The most notable change in comparison with the post-biostimulation state was the significant decrease in the pollutant quantities and the parallel increase in the *vcr*A gene copy numbers. Significant presence of the vinyl-chloride reductase gene after the bioaugmentation indicates the success of the treatment. It is also proven by the absence of pollution in the groundwater which could be the result of the developed complete dechlorination preventing the accumulation of vinyl-chloride. Besides the complete dechlorinating *Dehalococcoides mccartyi* containing the *vcr*A gene, another significant OTU could also take part in the bioremediation process which was related to Spirochaetacea, since our sequences showed the highest similarity to a clone obtained from polychlorinated-dioxin dechlorinating microbial community (Yoshida et al. 2005).

Long-term effect of the treatment on physical–chemical parameters, bacterial community composition of the groundwater and preservation of dechlorinating capacity

To follow the long-term effect of the biostimulation and bioaugmentation, samples were taken 6 months, 10 months and 48 months after the treatment. As for the physical–chemical circumstances in the groundwater, anaerobic conditions maintained during the whole length of the experiment based on the measured ORP values which were − 249.2, − 660 and − 105.9, respectively. TOC and COD values gradually decreased (to 3 mg L^{-1} and 3 mg O_2 $L^{-1,}$ respectively) with time, while pH showed increasing tendency reaching 7.24 to the final sampling. Regarding the changes in sulfate quantities, the highest concentration (192 mg L^{-1}) was determined 48 months after the treatment. As for the measured nitrogen forms, nitrate concentration of the groundwater was typically higher before the treatment, after the biostimulation and bioaugmentation, while ammonium showed its highest concentration in the groundwater (21.9 mg L^{-1}) 10 month after the treatment. Pollutant concentrations were below the detection limit, except for last sampling when TCE, cDCE and VC were all in small concentrations (8.7, 12.7 and 3.1 µM L^{-1}, respectively) (Table 1). Considering the phylum level composition of bacterial communities, significant increase was observed in the number of taxa compared to post-bioaugmentation samples with the most identified phyla 10 months after the treatment (Fig. 1). Dominance of Bacteroidota-related sequences decreased (to 21%) 6 months after the treatment in comparison with the previous state, while ratio of Firmicutes (27%)—the other dominant member of the community—also slightly decreased. Other dominant taxa were Proteobacteria, Patescibacteria, Spirochaetota and Synergistota related. Besides Petscibacterota, Bacteroidota and Spirochaetota Verrucomicrobiota became the most abundant taxa after 10 months after the treatment. Dominance of Firmicutes-related sequences gradually reduced in line with passage of time after the treatment, showing the minimum abundance value (0.9%) at the last sampling. Then, members of Desulfobacterota, Campylobacterota and Proteobacteria were revealed in the highest proportion. As for bacterial OTU compositions, the most dominant taxa were related to an uncultured Synergistaceae (Synergistota), genus *Megasphaera* (Firmicutes) and genus MVP-15 within phylum Spirochaetota 6 months after the bioaugmentation. Members of Spirochaetaceae (Spirochaetota), *Acetobacter* (Proteobacteria) and *Acidaminococcus* (Firmicutes) were also found in large numbers then (Fig. 2). vadinHA17 within Bacteroidota, "*Candidatus* Magasinikbacteria" and Bacteroidota-related BSV13 comprised the largest OTUs 10 months after the treatment. At the final sampling occasion, Cloacimonadales, *Desulfomonile*, *Sulfuriomonas*, Marinimicrobia, Gallionellacea and *Sulfuricella* dominated the amplicon libraries. Copy numbers of the catalytic gene vinyl-chloride reductase showed decreasing tendency after the treatment except for the last sampling when almost one order of magnitude increase was observed (Fig. 3).

Bacterial community composition transformed considerably with time after the treatment. One of the most prominent part of this alteration is the gradual decrease of Firmicutes-related and other fermentative bacteria. Six months after the treatment, there was significant overlap with the bacterial community of post-bioaugmentation state yet by the dominant presence of *Acetobacter*- and Acidaminococcacea-related taxa. Besides Acidaminococcaceae, there were other possible fermentative taxa among the most dominant OTUs such as members of *Megasphaera* and an unclassified Synergistaceae. Our OTU belonging to the latter family showed the highest similarity to a sequence isolated from mesophilic anaerobic digester treating municipal wastewater sludge and could have major role in hydrogen production (Riviére et al. 2009) and consequently in the facilitation of dechlorination in our case. Ten months after the bioaugmentation, the most abundant community member on OTU level was a "*Candidatus* Magasanikbacteria" related, to which the most similar sequence (but with still only 90% identity) was revealed from an actively denitrifying community of a soil microcosm (Yoshida et al. 2012). Further two dominant OTUs showed the highest sequence similarity to vadinHA17 within phylum Bacteroidota isolated from a spring pit and to class Prolixibacteraceae-related BSV13 obtained from a polycyclic aromatic hydrocarbon contaminated soil. Another dominant member which was abundant

in the bacterial community of the last sampling time carried out 48 months after the treatment as well also gave high identity to a sequence previously isolated from an anaerobic, tar-oil contaminated aquifer sediment and belonged to phylum Marinimicrobia (Winderl et al. 2008). Due to the significantly reduced quantity of organic matter at the last sampling time indicated by the low TOC and COD values, major OTUs showed the highest similarity to chemolithotrophic bacteria taking part in the sulfur and iron cycling of groundwater. Members of genus *Sulfurimonas* are able to reduce nitrate and oxidize sulfur and hydrogen (Han and Perner 2015), similarly, *Sulfuricella*-related bacteria representing another dominant OTU 48 months after the treatment could also play role in the oxidation of sulfur compounds (Kojima and Fukui 2010). Sequences showing the highest similarity to genus *Gallionella* were also dominant, suggesting the presence of iron oxidization processes as well. However, the most abundant OTU of the bacterial community of the groundwater at that time was *Desulfomonile* related. Generally, members of the genus can reduce sulfur compounds, but *Desulfomonile tiedjei* could also play an important role in the dehalogenation process (Mohn and Tiedje 1991). Besides the abundance of the mentioned possible partial dechlorinating taxon the quantity of the vinyl-chloride reductase gene also increased, suggesting the elevated abundance of *Dehalococcoides*-related bacteria. Considering the low but detectable quantity of the pollution, the significant presence of the dechlorinating taxa could indicate ongoing bioremediation process 4 years after the last treatment and consequently the long-term preservation and the inducibility of the established bioremediation function.

Conclusion for future biology

Application of biostimulation in combination with a newly developed site specific bioaugmentation agent on a TCE contaminated site as a pilot test inflicted notable alteration both in the abiotic circumstances and in the bacterial community composition of the investigated groundwater. During the biostimulation, conditions favorable to reductive dechlorination were generated by adding HRC solution and by directly and indirectly creating a bacterial community facilitating the presence and activity of dehalorespiring taxa. The required catabolic potential for the complete dehalogenation was established by bioaugmentation, and as a result, copy number of catabolic gene encoding vinyl-chloride reductase notably increased parallel with the significant decrease in chlorinated pollutants. Regarding the data obtained from the long-term experiment, reductive conditions were maintained for the investigated 4 years, but in line with decreasing organic matter concentration, the bacterial community transformed. Dominance of fermentative – mainly Firmicutes related—taxa decreased and chemolithotrophic bacteria became abundant with time, but the dechlorinating potential of the community remained and could be induced by the reappearance of the pollutants even after 4 years.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare.

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