

Unveiling complete natural reductive dechlorination mechanisms of chlorinated ethenes in groundwater: Insights from functional gene analysis

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Abstract

Monitored natural attenuation (MNA) of chlorinated ethenes (CEs) has proven to be a cost-effective and environment-friendly approach for groundwater remediation. In this study, the complete dechlorination of CEs with formation of ethene under natural conditions, were observed at two CE-contaminated sites, including a pesticide manufacturing facility (PMF) and a fluorochemical plant (FCP), particularly in the deeply weathered bedrock aquifer at the FCP site. Additionally, a higher abundance of CE-degrading bacteria was identified with heightened dechlorination activities at the PMF site, compared to the FCP site. The reductive dehalogenase genes and *Dhc* 16S rRNA gene were prevalent at both sites, even in groundwater where no CE dechlorination was observed. *vcrA* and *bvcA* was responsible for the complete dechlorination at the PMF and FCP site, respectively, indicating the distinct contributions of functional microbial species at each site. The correlation analyses suggested that *Sediminibacterium* has the potential to achieve the complete dechlorination at the FCP site. Moreover, the profiles of CE-degrading bacteria suggested that dechlorination occurred under Fe³⁺/sulfate-reducing and nitrate-reducing conditions at the PMF and FCP site, respectively. Overall these findings provided multi-lines of evidence on the diverse mechanisms of CE-dechlorination under natural conditions, which can provide valuable guidance for MNA strategies implementation.

Keywords

Chlorinated ethenes; Natural reductive dechlorination; RDase genes; Groundwater

1. Introduction

Chlorinated ethenes (CEs), such as tetrachloroethene (PCE) and trichloroethene (TCE), have been widely used as solvents for dry cleaning and degreasing metal parts [1, 2]. Their widespread presence across various environmental media stems from inappropriate handling, storage, disposal practices, and accidental spills [3, 4]. CE in groundwater is a major concern due to their carcinogenicity, teratogenicity and mutagenicity [3, 5]. As dense non-aqueous phase liquids (DNAPL), CEs have the capacity to infiltrate impermeable formations such as silt and clay layers and migrate into the underlying bedrock. This characteristic enables them to persist as a prolonged source of groundwater contamination.

Monitored natural attenuation (MNA) has been applied as an environmentally friendly and cost-effective remediation approach for sites contaminated with chlorinated hydrocarbons [6-9]. Various biological, physical, and chemical processes contribute to the MNA of CEs. Among them, microbial reductive dechlorination is the primary process for achieving the complete detoxification of CEs [10]. Under anoxic environmental conditions, microorganisms can transform highly chlorinated parent CEs into less chlorinated derivatives such as *cis*-1,2-dichloroethene (*cis*-1,2-DCE), and vinyl chloride (VC) and ethene, through reductive dechlorination process [11]. Reductive dechlorination can be achieved by several bacteria either through organohalide respiration or co-metabolic processes. Organohalide-respiring bacteria (OHRB) such as *Dehalococcoides*, *Dehalobacter*, *Dehalogenimonas*, *Desulfitobacterium*, *Sulfurospirillum*, and *Geobacter*, among others, are able to couple reductive dehalogenation to energy conservation and microbial growth [12]. To date, *Dehalococcoides* and *Dehalogenimonas* are the only two OHRB reported to be able to dechlorinate VC to ethene via organohalide respiration. Those members of *Dehalococcoides* group, which can utilize CEs as electron acceptors with H₂ serving as the electron donor are defined as one species, *Dehalococcoides mccartyi* (*Dhc*) [13]. Additionally, *Dhc* are frequently found indigenous in many CE-contaminated sites [12, 14-18]. Hence, the presence of *Dhc* can serve as a useful indicator in assessing the likelihood of achieving complete dechlorination at CE-contaminated sites [18].

Nonetheless, the natural reductive dechlorination is not solely attributed to OHRB, but also involves others CE-degrading bacteria. For instance, it has been reported that *Sulfuricurvum kujiense* can dechlorinate TCE under sulfate-reducing conditions [19]. *Pseudomonas* can utilize DCE and VC as growth substrates through co-metabolism [20]. *Sediminibacterium*,

Aquabacterium and *Nocardioides* were found to be able to degrade VC [21]. Hence, investigating the profiles of degrading bacteria can provide insights into the natural reductive dechlorination of CEs taking place in the field amidst intricate site conditions. Furthermore, reductive dehalogenase (RDase) genes played an important role for the dechlorination of CEs. The enzyme encoded by the *tceA* gene facilitates the dechlorination of TCE, *cis*-1,2-DCE as well as VC [5, 22]. Similarly, the *vcrA* and *bvcA* genes enable *Dhc* to derive energy from the reduction of VC for growth [23, 24]. The abundance of *Dhc* 16S rRNA gene and RDase genes including *tceA*, *vcrA* and *bvcA*, typically indicative of CEs degradation and the detoxification VC to ethene, can also serve as biomarkers for monitoring the efficacy of CE reductive dechlorination [18, 25, 26]. Despite the insights gained so far, there remains a scarcity of studies examining CE dechlorination and its relationship with microbial community composition, diversity, and the prevalence of RDase genes in historically CE-contaminated groundwater.

This study aimed to address this gap by investigating the natural reductive dechlorination of CEs and the generation of ethene in two distinct CE-contaminated sites situated at varying depths within saturated aquifers. It involved identifying the microbial composition profiles and RDase gene abundance at these sites while assessing the relationship between degrading bacteria/RDase genes and the observed degradation of CEs in the field.

2. Materials and methods

2.1 Description of the study areas

In this study, two study areas were chosen from two sites: a pesticide manufacturing facility (PMF) and a fluorochemical plant (FCP). The PMF site was in active pesticide production for decades until 2006, and the FCP site has been used for fluorinated and chlorinated chemicals production for more than 20 years. Both sites were identified with contamination of CEs during previous site investigations. The PMF site was located in Suzhou city, Jiangsu province, China (coordinates: 120.64°E, 31.28°N) and the FCP site was in Zibo city, Shandong province, China (coordinates: 118.04°E, 36.98°N) (Fig 1a and 1b). The stratigraphy at the PMF consists of refill, silty clay, clayey silt and silty sand, with the groundwater level at about 1.5 m below ground surface (bgs) (Fig. 1d). Groundwater flows from northeast to southwest (Fig. 1c). At the FCP site, the stratigraphy is composed of refill, clay, silty clay, fine sand, silty clay, clay and partial sandstone

(Fig. 1f). Groundwater level was located at 4.5 m bgs and the groundwater flows from southeast to northwest (Fig. 1e).

Two sets of groundwater monitoring well clusters were installed at each site, namely PMF-1/2 and FCP-1/2. Each well cluster consists of three monitoring wells, in which S/M/D represent monitoring wells screened in shallow/middle/deep aquifers, respectively (Fig. 1d and 1f). At the PMF site, groundwater monitoring wells screens were located at 5-8 m bgs, 12-15 m bgs, 17-20 m bgs, respectively (Fig. 1c and 1d). At the FCP site, groundwater monitoring wells were screened at 21-24 m bgs, 39-42 m bgs, 57-60 m bgs, respectively (Fig. 1e and 1f).

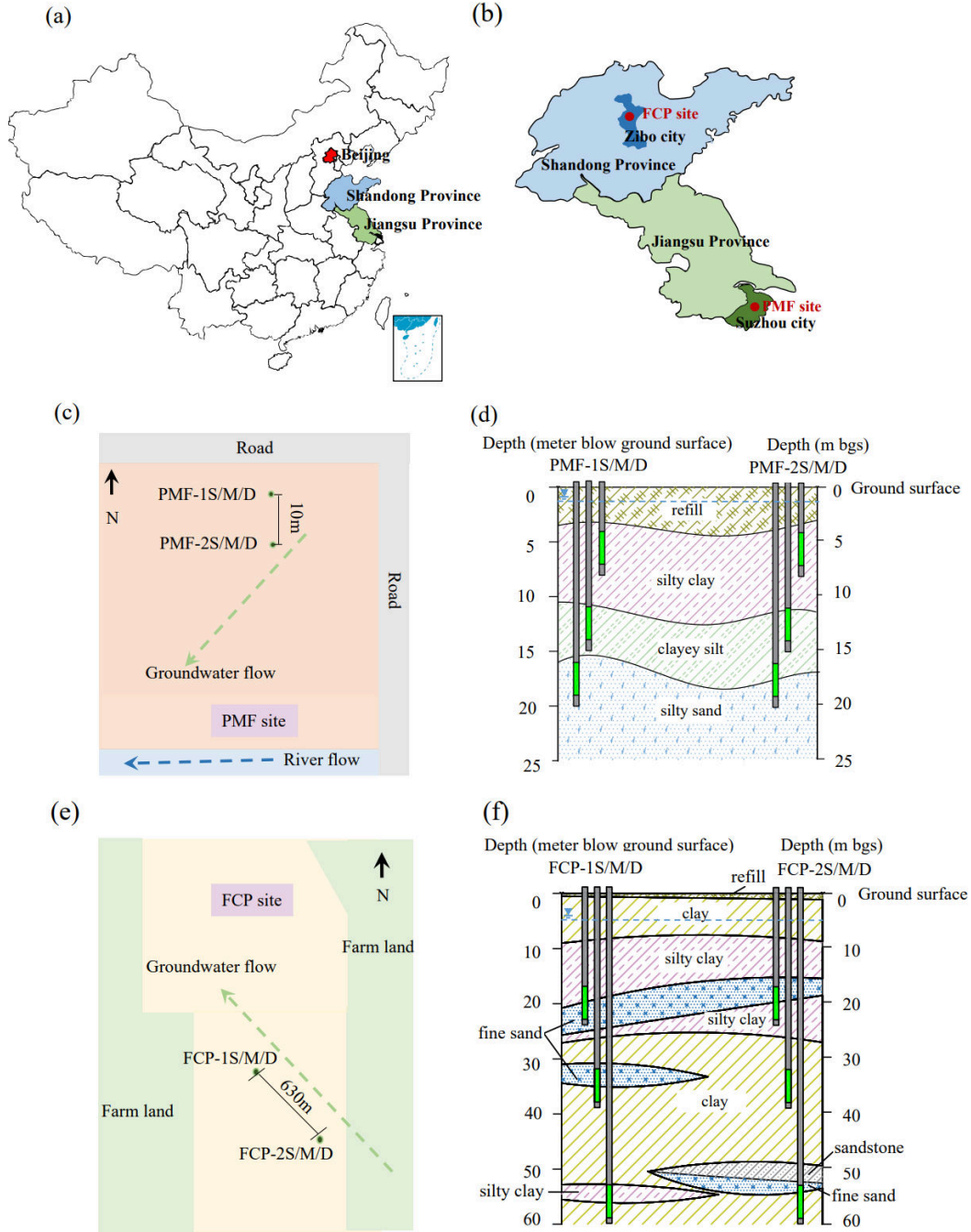


Fig. 1. Site locations (a, b), and layouts of groundwater monitoring wells and hydrogeological strata at the PMF (c, d) and FCP (e, f) sites.

2.2 Groundwater sampling and field measurements

Samples of groundwater were obtained from two clusters of groundwater wells located at each site in June 2022. Groundwater samples were collected from each monitoring well when the pumped

groundwater quality parameters such as pH, dissolved oxygen (DO), temperature, oxidation-reduction potential (ORP), electrical conductivity (EC) and dissolved hydrogen (DH) were stable. The groundwater quality parameters were analyzed using a hand-held water quality meter (HQ40d, HACH, USA) [27]. The samples for the CEs and ethene analyses were collected in 40 mL brown headspace bottles containing 2 drops of hydrochloric acid (1 mol/L). Groundwater samples for TOC analysis were collected into 40 mL brown glass bottles, followed by the TOC extraction through combustion oxidation nondispersive infrared absorption method. For the analyses of sulfate, nitrate, nitrite, Fe^{2+} and Fe^{3+} , groundwater samples were collected in 1 L plastic bottles. Samples were collected in 1 L brown glass bottles with 5 mL sodium hydroxide solution (1 mol/L) and 4 g ascorbic acid as protective agents for sulfide analysis. For total Fe and Mn analysis, 1 L glass bottles containing 1 mL HNO_3 were used to collect samples. All bottles were stored in dry-ice and transported to laboratory for analysis. Microbial samples were obtained by filtering 2 L groundwater through sterile 0.22 μm nylon filters with a diameter of 50 mm and the filtered biomass-containing membranes were kept in sterile bags and stored at -80 °C in the lab for further analysis.

2.3 Analytical methods

Concentrations of CEs, including PCE, TCE, *cis*-1,2-DCE, and VC, as well as the non-toxic terminal product ethene, were analyzed in groundwater samples collected at both the PMF and FCP sites. CE concentrations were determined using a purge-and-trap concentrator (Eclipse 4552&4660, OI Analytical, USA) coupled to a gas chromatography-mass spectrometer system (GC-MS 7890B/ 5977B, Agilent, USA), with separation on a capillary column (J&W Scientific DB-624 60 m $\times$ 0.25 mm $\times$ 1.4 μm , Agilent). Ethene was analyzed with a gas chromatography-flame ionization detector (GC-FID) equipped with capillary column (HP-PLOT/Q, 30 m $\times$ 0.32 mm). Sulfide, nitrate and nitrite were determined with UV-2600 (Shimadzu, Japan). Chloride and sulfate were analyzed with ion chromatography method with Dionex Aquion RFIC (Therom, USA). Total organic carbon (TOC) was detected with the combustion oxidation nondispersive infrared absorption method with TOC-V CPH 200V (Shimadzu, Japan). Total Fe, Mn, Fe^{2+} and Fe^{3+} were determined with ICP-OES (Shimadzu, Japan) and UV-2600 (Shimadzu, Japan), respectively.

2.4 DNA extraction, Illumina sequencing and quantitative real-time PCR (qPCR)

The total DNA from the triplicate samples was extracted using the FastDNA spin kits (MP Bio, USA) as recommended by the manufacturer. A set of universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') from 16S rRNA gene of bacteria were used to conduct a polymerase chain reaction (PCR) [28]. 4 μ L 5 $\times$ FastPfu Buffer, 2 μ L dNTPs (2.5 mM), 0.8 μ L forward primer (5 μ M), 0.8 μ L reverse primer (5 μ M), 0.4 μ L FastPfu polymerase, 0.2 μ L BSA, and 10 ng DNA were added in 20 μ L volumes to perform the PCR amplification reactions. The amplification for the 16S rRNA gene was performed using an automated thermal cycler (Eppendorf, Germany). The reaction started with an initial denaturation at 95°C for 3 minutes, followed by three series of 27 cycles in which each cycle consists of a denaturing step of 95°C for 30 s, an annealing 55°C for 30 s and an extension 72°C for 45 s, finally, with an extension at 72°C for 10 minutes. The DNA quality of PCR products was assessed by using NanoDrop instrument (ND-2000, Thermo Fisher Scientific, Wilmington, USA) [29].

The PCR products were detected by electrophoresis on 2% (w/v) agarose gel, purified with an Axy Prep DNA gel recovery kit (Axygen, California, USA), and eluted with Tris-HCl. The purified amplicon products were then sequenced using an Illumina MiSeq platform at Allwegene Biotechnology Co., Ltd. (Shanghai, Nanjing). After sequencing, image analysis, base calling and error estimation were performed using Illumina Analysis Pipeline Version 2.6.

Total bacterial 16S rRNA gene, *Dhc* 16S rRNA gene, and RDase genes (*tceA*, *vcrA*, *bvcA*) at the PMF and FCP sites were quantified via qPCR and the primers were shown in Table S1. The qPCR assay of standards, samples, and no-template control was performed in triplicate on an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, USA) with a 96-well format by Allwegene Technology (Allwegene Technology Co., Ltd. Beijing, China). Protocols to prepare qPCR calibration curves and quantify 5 genes followed procedures as shown in Text body in Supporting Information (SI).

2.5 Data analysis

All the samples were analyzed in triplicates and the alpha diversity indices of microbial community (Chao 1 and Shannon index) were calculated from Amplicon sequence variants (ASVs) using QIIME2. Beta diversity of microbial community was assessed by principal coordinates analysis

(PCoA) based on weighted UniFrac scores of ASVs level. Redundancy analysis (RDA) and canonical correspondence analysis (CCA) were used to evaluate the relationship between key environmental factors and specific microbial genera. Correlation heatmap was performed to evaluate the correlation between CE-degrading bacteria (abundance > 1%), genes and environmental parameters. These correlation analyses were performed using the Vegan package in R software. Moreover, analysis of variance (ANOVA) was performed to describe the significant differences among the different samples for the abundance of genes using one-way Complete Randomized Design (CRD) with ($p \leq 0.05$) through SPSS software.

3. Results and discussion

3.1 Dechlorination of CEs and formation of daughter products under natural conditions

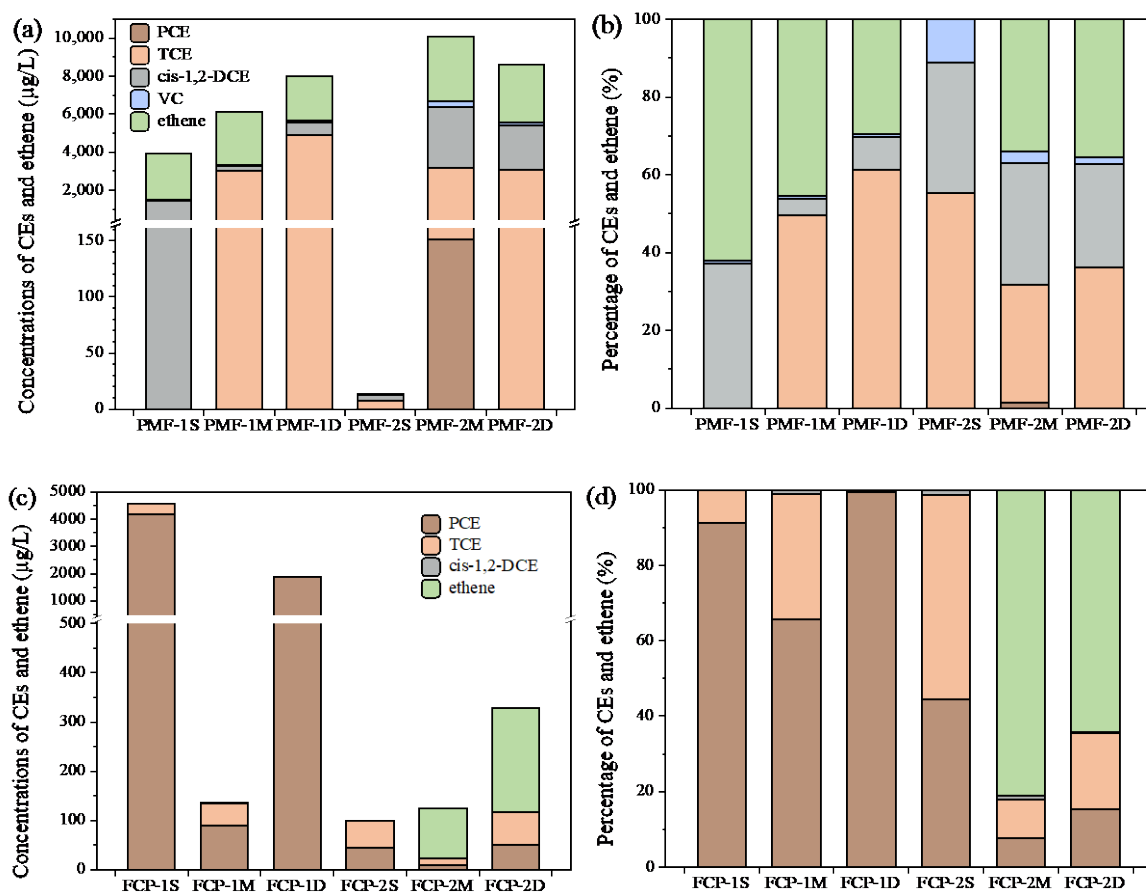


Fig. 2. Concentration and percentage of CEs and ethene in groundwater collected at the PMF (a, b) and FCP (c, d) sites.

The cumulative concentrations of PCE, TCE, *cis*-1,2-DCE, VC, along with the ethene concentrations are shown in Fig. 2a and 2c, while their relative abundances are shown Fig. 2b and 2d (Table S2). At the PMF site, TCE is the predominant parent CE, although PCE was detected at $151 \pm 2.42 \mu\text{g/L}$ at PMF-2M. Significant amounts of *cis*-1,2-DCE, VC, and ethene were detected at both well clusters, indicating the occurrence of the typical reductive dechlorination pathway from TCE to *cis*-1,2-DCE to VC and to ethene [7, 11, 30]. In addition, the widespread presence of ethene in all 6 monitoring wells at different depths, except for PMF-2M, suggested a complete reductive dechlorination under natural conditions [7, 30].

At the FCP site, the dominating parent CE was PCE, while TCE also detected in most of the monitoring wells. However, no significant byproducts of *cis*-1,2-DCE and VC were detected at the FCP site, though it is worth noting that these compounds were detected at selected wells in the site-wide investigation at the FCP site (data not shown). Furthermore, significant amounts of ethene, the final product of dechlorination, were found at FCP-2M and FCP-2D, with concentrations of 100.93 ± 1.91 and $210.97 \pm 2.45 \mu\text{g/L}$, respectively. Similar to the conclusions drawn above at the PMF site, the detection of ethene indicated complete dechlorination at FCP-2D, which was screened between 57-60 m bgs. This observation is significant and provides new insights for the treatment of CEs in deep bedrock aquifers, which was considered a persistent source, posing a great challenge for remediation. Moreover, the dissimilar relative abundances of CEs at the two sites suggest varying dechlorination mechanisms, despite following the same microbial reductive biodegradation pathway involving diverse degradation consortia, a concept that was further explored in the subsequent sections.

Furthermore, previous studies reported the generation of dechlorination product acetylene during the abiotic dechlorination of CEs in Fe/S system [31, 32], in which the ability of FeS in soil responsible for CEs abiotic degradation and acetylene generation followed the order of *trans*-1,2-DCE > *cis*-1,2-DCE > TCE > PCE [31]. There were detectable Fe^{2+} and sulfide in 5 out of the 6 monitoring wells (with the exception of PMF-2S) at the PMF site (Table S3), indicating the potential contribution of abiotic dechlorination of CEs. However, our analysis results showed there was no detectable acetylene in groundwater samples. It is worth noting that acetylene was detected in a microcosm study conducted for the microbial degradation of TCE with indigenous microbial community collected from soils at the PMF site (results not published yet), demonstrating the

occurrence of abiotic dechlorination in soils collected at the PMF site. The potential contributions of CE dechlorination by Fe/S compounds under natural conditions warrant further investigation.

3.2 Diversity of microbial community

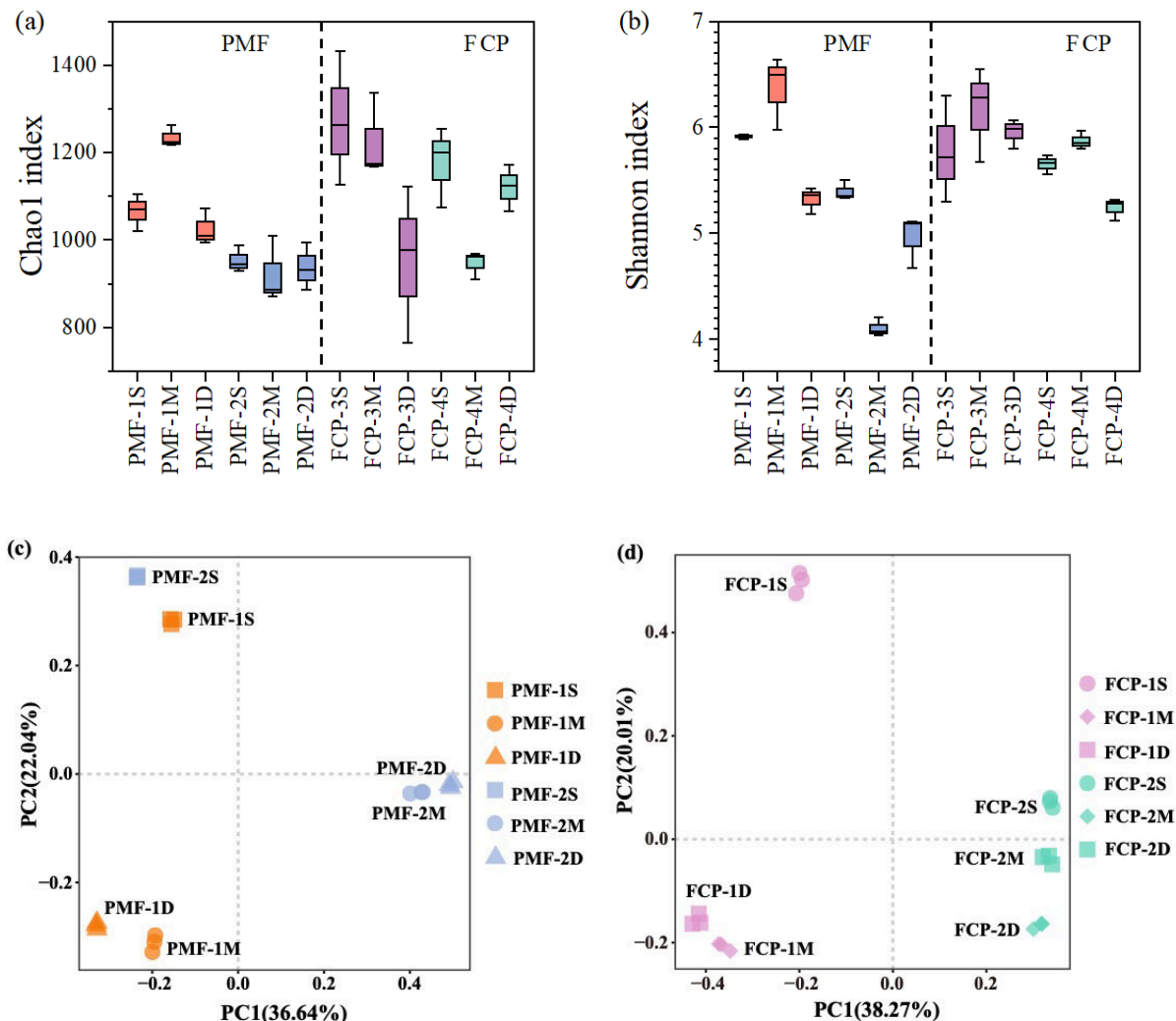


Fig. 3. Alpha diversity indices Chao1 index (a) and Shannon index (b) of the bacterial community and principal coordinates analysis based on weighted UniFrac scores on ASVs level of groundwater samples collected at sites PMF (c) and FCP (d).

Changes in microbial alpha diversity indices at the ASVs level within groundwater from both the PMF and FCP sites under varying depths are shown in Fig. 3 and Table S4. The Chao1 index varied at the PMF and FCP sites, indicating different species richness between the two sites. Studies have indicated that contaminant stress can modify microbial diversity and elevate the abundance of specific microbial species [33]. Notably, the presence of higher *cis*-1,2-DCE

concentrations coupled with VC at PMF-2M correlated with lower species richness due to their higher toxicity compared to the parent CEs [30]. Conversely, the FCP site had the highest Chao1 index, indicating a greater number of species compared to the PMF site [27]. However, the Shannon index showed similar diversity levels at both sites. Upon comparing the microbial diversity and richness at varying depths, both the Chao1 and Shannon index showed varying responses to depth variations at the PMF and FCP sites. At the PMF site, the highest microbial richness and diversity were observed in PMF-1M, potentially associated with lower CE concentrations exhibiting lower toxicity [33]. In contrast, lower TOC correlated with decreased species richness at FCP-2M suggested that insufficient TOC in groundwater influenced the indigenous microbial richness (Table S3) [34].

Comparison of the microbial community profiles in the middle and deep aquifers tended to cluster together, indicating similarities in microbial compositions between the two aquifers (Fig. 3). Conversely, the profiles in shallow wells were significantly different from those in the middle and deep aquifers. This observation suggested the potential hydraulic connections between the middle and deep aquifers, consistent with the hydrogeological strata and the distribution profiles of contaminant shown in Fig. 1 and Fig. 2, respectively. It is worth noting that the microbial communities in wells FCP-1S and FCP-2S, despite being sourced from the same depth of the aquifer, exhibited distinctive different community compositions. This dissimilarity can be attributed to significant variations in the geochemical parameters, particularly the higher EC, TOC, nitrate, and sulfate concentrations observed in FCP-1S as shown in Table S3. These factors may serve as key factors that influence the microbial community assembly in these wells, leading to their distinct microbial populations, as discussed further in Section 3.5.

3.3 Microbial community compositions and degraders of CEs

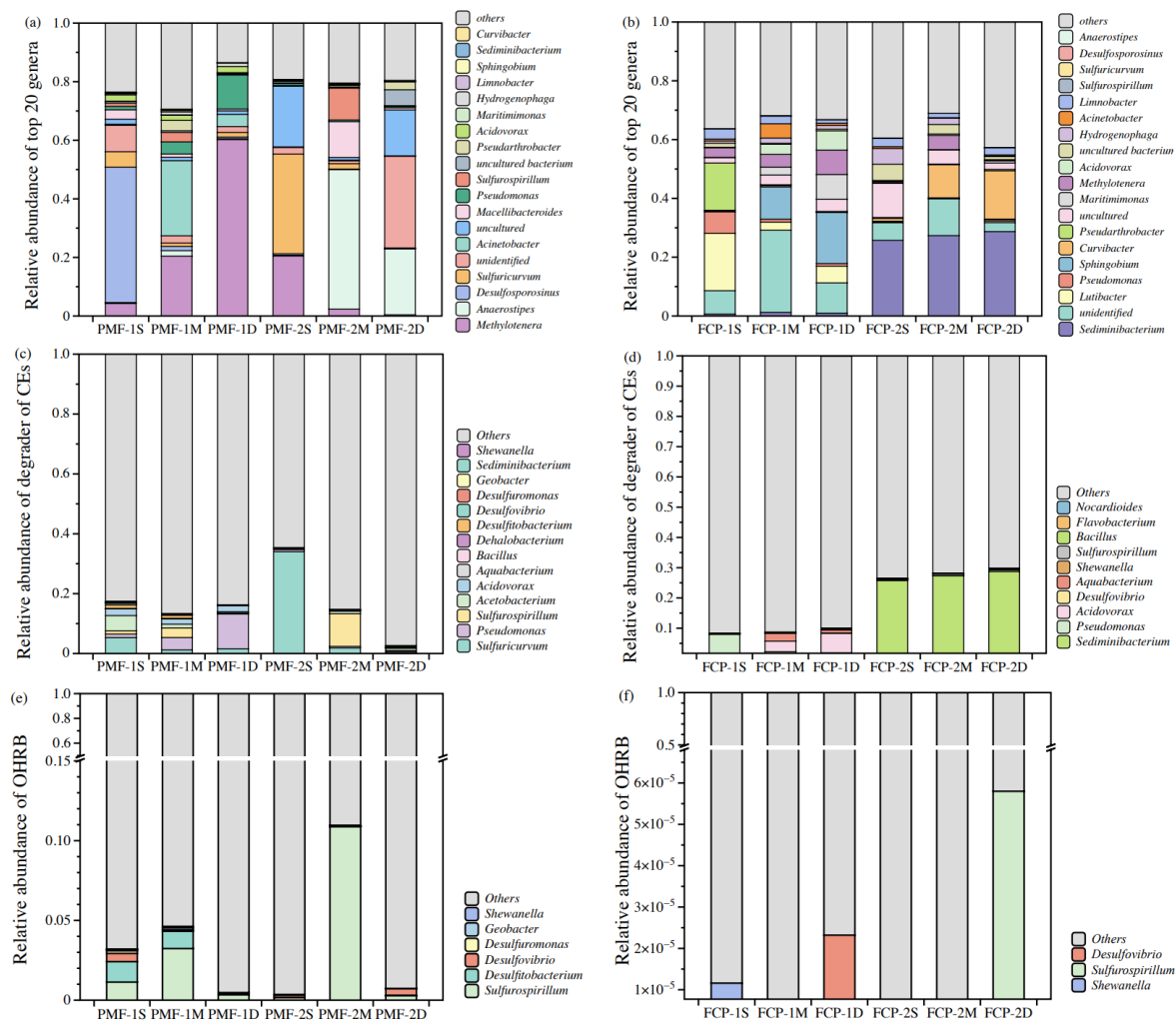


Fig. 4. Relative abundance of top 20 genera in the microbial consortium, degraders of CEs, and OHRB in groundwater at sites PMF (a, c, e) and FCP (b, d, f).

To further investigate the microbial community structure and composition at the PMF and FCP sites, the top 20 most abundant genera in contaminated groundwater at varying depths in different wells were analyzed (Fig. 4). The dominant genus varied significantly in each monitoring well at the PMF site. *Desulfosporosinus* and *Acinetobacter* were the dominant genera at PMF-1S and PMF-1M, representing 46% and 26% of the microbial abundance, respectively. In PMF-1D, *Methylothermus* was the dominant genus, with an abundance of 60%. Previous studies have reported the capability of *Desulfosporosinus* to enhance *Dehalococcoides* for dechlorination of CEs to ethene through syntrophic interactions and adjustment of redox potential [35, 36]. Additionally, it

is reported that *Acinetobacter* has ability to degrade chlorinated aromatic hydrocarbons and can degrade TCE and DCE with dimethyl sulfide (DMS) serving as the sole sulfur [37, 38], indicating that *Acinetobacter* may support the dechlorination of CEs at the PMF site. *Methylothermobacter* is known for its denitrification ability and methane production, indicating the presence of reductive environmental conditions conducive to CEs dechlorination [39, 40].

At PMF-2S, *Sulfuricurvum* was observed in high abundance of 34%, while *Anaerostipes* was 48% and 23% at PMF-2M and PMF-2D, respectively. *Sulfuricurvum* has been cultured during the reductive dechlorination of PCE and TCE under sulfate-reducing condition [19, 41]. The presence of *Anaerostipes*, a well-known anaerobic microbe for the reduction of sulfate/iron, is consistent with the detection of sulfate-sulfide and Fe^{3+} - Fe^{2+} redox couples at the PMF site (Table S3) [42, 43]. Moreover, the presence of *Anaerostipes* positively correlated with butyrate production, serving as an electron donor for the dechlorination process [44]. Similarly, *Dehalobacterium* played an essential role in the dechlorination of TCE [45, 46]. The correlation of dominant genera and reductions of nitrate, iron, sulfate as well as CEs and the fermentation for electron donors, indicated the importance of natural reductive conditions for CEs dechlorination at the PMF site. Moreover, genera, such as *Acinetobacter*, *Sulfuricurvum* and *Dehalobacterium*, known for their CEs degradation abilities, likely contributed to the natural reductive dechlorination of CEs at the PMF site, as discussed further below.

The abundance of the dominating genus in two well clusters (FCP-1 and FCP-2) were different with varying depths at the FCP site as shown in Fig. 4b. The dominant genera were *Lutibacter* and *Pseudarthrobacter* with the abundance of 19.5% and 16.1% at FCP-1S, *Sphingobium* with abundance of 10.9% and 17.4% at FCP-1M and FCP-1D, respectively. Moreover, at FCP-2 wells at the FCP, *Sediminibacterium* and *Curvibacter* ranked as the first and second dominant genera. A microbial community including *Sediminibacterium*, *Curvibacter*, *Pseudomonas*, *Acinetobacter*, *Acidovorax*, which were also present with high abundance in groundwater at the FCP site, was reported to convert chlorinated hydrocarbons when H_2/O_2 was applied [47, 48].

Degraders of CEs at two sites were selected and listed in Table S5 and S6, and their composition and profile are shown in Fig. 4c and 4d. It's worth noting that there were only VC and ethene detected indicating the active reductive dechlorination of CEs at PMF-1S, with relative lower abundance of degrader with much contribution from *Sulfuricurvum* and *Acetobacterium*. The

abundance of degraders at PMF-1M and PMF-1D of 38.96% and 20.34% with *Acinetobacter* and *Pseudomonas* dominating also led to the dechlorination of CEs to *cis*-1,2-DCE and ethene. The composition of microbial community at PMF-2S was distinct from that at PMF-2M as well as at PMF-2D (Fig. 3a) and the concentration of CEs was lowest at PMF-2S. Notably, *Sulfuricurvum* accounted for highest abundance at PMF-2S with the ability to reductively degrade TCE in sulfate-reducing condition [16, 19, 41], whereas, *Sulfurospirillum*, which can dechlorinate PCE and TCE to *cis*-1,2-DCE [49-51], was detected at PMF-2M with a high abundance, leading to the high concentration of *cis*-1,2-DCE at PMF-2M.

At the FCP site, *Sediminibacterium*, *Pseudomonas*, *Acidovorax*, *Acinetobacter* and *Aquabacterium* were the dominant degrading bacteria of CEs in FCP-1 wells. *Acidovorax* required sulfate and nitrate to initiate the dechlorination process [52-54] and the detectable nitrite concentrations at the FCP site (Table S3) suggested that *Acidovorax* was involved in the microbial degradation processes of CEs [48]. Unfortunately, sulfite concentrations were not analyzed in this study, yet the detections of high sulfate concentrations ranging from 334 mg/L to 700 mg/L could induce the enrichment of *Acidovorax* in the microbial consortium. *Sediminibacterium*, a facultative anaerobe, was the most dominant degrader at FCP-2 wells. It was reported that *Sediminibacterium* is associated with the degradation of VC and enriched with high abundance during anaerobic and aerobic microbial transformation of CEs [21, 48]. It is important to note that occurrence of ethene together with higher abundance of *Sediminibacterium* in FCP-2 wells, indicating the potential key role of *Sediminibacterium* in the transformation of PCE and TCE to ethene. Interestingly, there was no *cis*-1,2-DCE and VC accumulation noted in FCP-2 wells, specifying that *Sediminibacterium* may actively participate in quickly dechlorinating the toxic CE daughter products (such as *cis*-1,2-DCE and VC). Previous studies have revealed that *Sediminibacterium* has the potential to obtain carbon from VC [21, 55], but no report on the capability of *Sediminibacterium* for the biodegradation *cis*-1,2-DCE. It is worth noting that ethene was widely detected at both sites without the detections of *D. mccartyi*. It was reported that not all genera could be identified due to the limitations of 16S high-throughput sequencing [56], which could be overcome by the result of *Dhc* 16S rRNA gene, as discussed later in Section 3.4.

The distributions and diversity of OHRB among the degrading bacteria in groundwater samples at both the PMF and FCP site were shown in Fig. 4e and 4f. The relative abundance of OHRB

accounted a significant portion of the microbial community at the PMF site, with *Sulfurospirillum* dominating followed by *Desulfitobacteriu*. At the FCP site, extremely low relative abundance of OHRB was detected, only *Shewanella*, *Desulfovibrio*, and *Sulfurospirillum* identified at FCP-1S, FCP-1D and FCP-2D, respectively. *Sulfurospirillum* was the common OHRB that was detected at both sites.

3.4 Abundance of total bacterial 16S rRNA gene, *Dhc* 16S rRNA gene and RDase genes

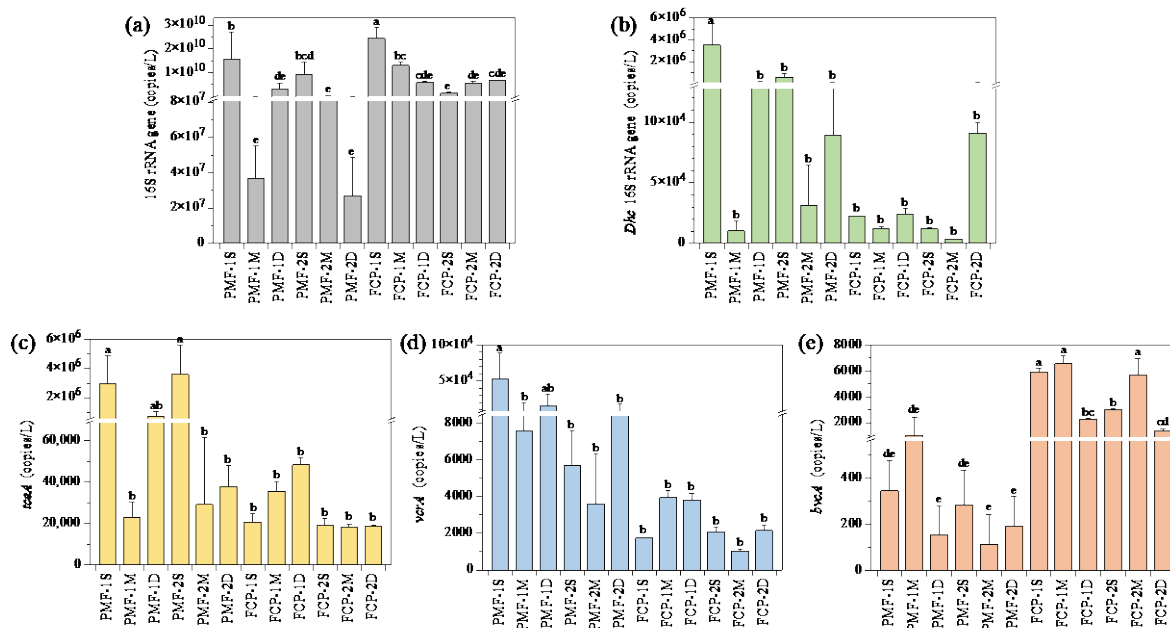


Fig. 5. Abundance of 16S rRNA gene (a), *Dhc* 16S rRNA gene (b), *tceA* (c), *vcrA* (d) and *bvca* (e) in groundwater collected at the PMF and FCP sites. Different letters showed significant differences ($p \leq 0.05$) among the samples.

The relative abundance of total bacteria (16S rRNA gene) and *Dhc* 16S rRNA gene, as well as the RDase genes, including *tceA*, *vcrA*, *bvca* genes at the PMF and FCP sites, are presented in Fig. 5 and Table S7. The abundance of 16S rRNA gene at the two sites were in the magnitude with 10^9 to 10^{10} copies/L, except for those at PMF-1M and PMF-2D with abundance of 10^7 copies/L (Fig. 5a). Nevertheless, the abundance of *Dhc* 16S rRNA gene at the two sites varied significantly, with those at the FCP site much lower than those at the PMF site (Fig. 5b). It may be due to the conducive reductive conditions, such as high TOC concentrations and relatively low ORP (Table S3), observed at the PMF site [57]. Moreover, the detections of sulfide and Fe^{2+} (Table S3) verified

that the more reductive conditions at the PMF site [7], which was essential for influencing the activities and abundance of *Dhc* [22, 58].

The RDase genes of *tceA*, *vcrA* and *bvcA* were detected in all the monitoring wells at both the PMF and FCP sites, with varying magnitudes of abundance. *tceA* was quantified with the highest copy number, followed by *vcrA* and *bvcA*. The abundance of *tceA* was in the range of 1.82×10^4 copies/L (at FCP-2M) to 3.61×10^6 copies/L (at PMF-2S). However, the abundance of *vcrA* was in the range of 1.02×10^3 copies/L (at FCP-2M) to 5.32×10^4 copies/L (at PMF-1S), which were lower than abundance of *tceA*, but higher than that of *bvcA*. It was noted that the abundance of *bvcA* were in the range of 1.13×10^2 copies/L (at PMF-2M) to 5.69×10^3 copies/L (at FCP-2M), indicating that *bvcA* played an important role for the formation of ethene at the FCP site.

It is worth mentioning that the abundance of *Dhc* 16S rRNA, *vcrA* and *tceA* were higher at the PMF site than those at the FCP site, indicating that different functional genes contributed to the biodegradation of CEs occurring at the two sites. Studies have reported that the abundance of *vcrA* and *bvcA* varied in sites depending on the redox condition, with higher abundance of *vcrA* occurring in more reducing conditions and more *bvcA* in less reducing conditions [59], which is consistent with the higher abundance of *vcrA* at the PMF site where lower ORP was observed. Notably, among all samples, both *Dhc* 16S rRNA gene and *vcrA* were detected with the highest abundance at PMF-1S while *tceA* was detected with the second highest abundance, consistent with the observation of the largest portion of ethene observed at PMF-1S (Fig. 1b), indicating that these functional genes accounted for the complete dechlorination of CEs at the PMF site. A field study was conducted and concluded that significantly higher ethene concentrations could be observed when *Dhc* 16S rRNA gene and RDase genes abundances exceeded 10^7 and 10^6 copies/L, respectively [18]. However, in this study, relatively high abundance of ethene were detected at FCP-2M and FCP-2D, even though the abundance of *bvcA* and *vcrA* were only at levels of 10^3 copies/L at the FCP site, suggesting that complete dechlorination can occur in the field with functional genes at much lower concentrations. On the other hand, the abundance of *Dhc* 16S rRNA, *vcrA* and *tceA* at FCP-1 well clusters were close to those at FCP-2, yet no ethene was detected, suggesting that the microbial dechlorination activities in FCP-1 wells were limited. The gene analysis data suggested that *bvcA* played a role at FCP site, however, it is worth noting that the formation of ethene occurred without *vcrA* and *bvcA* being detected under natural attenuation

at many sites in a previous study [18]. Moreover, it has been reported that high concentration of PCE and TCE can inhibit dechlorination of VC [60], which may contribute to the limited microbial dechlorination and the absence of ethene at FCP-1 wells (Fig. 2c).

Finally, it is interesting to observe that abundance of *tceA* is triple of *Dhc* abundance in FCP-2S. A great number of similar results were presented in previous studies where higher *tceA* (or *vcrA*) than *Dhc* in groundwater samples were observed, as presented by a summary prepared by Clark et al. [18]. The two- or three-fold differences (such as the phenomenon observed in the current study) in RDase to *Dhc* cell abundance could be the result of qPCR variations or due to gene duplication with a dominant *Dhc* strain. However, Clark et al. [18] also pointed out that the much higher fold-differences (up to 10,000-fold) between RDase and *Dhc* 16S rRNA gene copy numbers can occur when *Dhc* cells were present at low abundance of the range of 10^3 - 10^4 cells/L, in which the enumeration of *Dhc* 16S rRNA gene copy numbers at FCP fell. They speculated that the horizontal gene transfer between *Dhc* and *Dehalogenimonas* can occur, though no evidence to date. Hence, further exploration is warranted to shed lights on the observation.

3.5 Key environmental factors shaping the CE-degrading bacteria compositions and correlations among degrading bacteria, genes, CEs and ethene

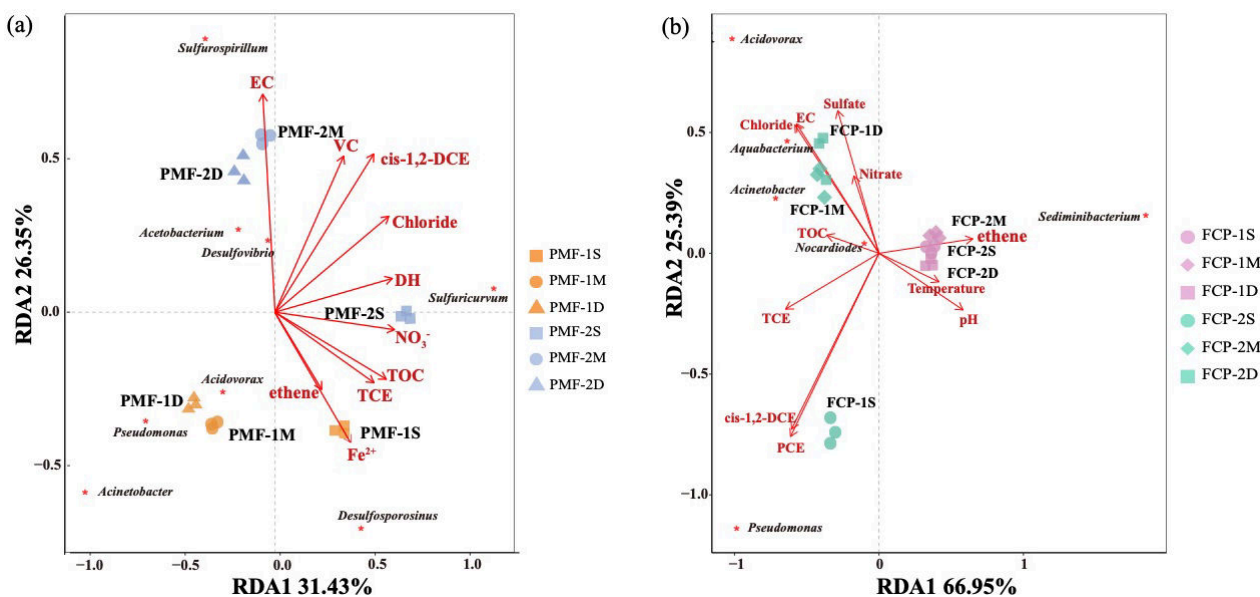


Fig. 6. Key environmental factors shaping the CE-degrading bacteria compositions in groundwater microbial community at the PMF (a) and FCP (b) site.

Geochemical parameters, including pH, temperature, DO, ORP, DH, EC, sulfate, nitrate, chloride, TOC, Fe^{2+} , Fe^{3+} , and the concentration of CEs with ethene were used for redundancy analysis (RDA) to explore the environmental factors shaping CE-degrading bacteria community at both the PMF and FCP sites and the results are shown in Fig. 6a and 6b. Environmental parameters showed different effects on the compositions of bacteria community at two sites. Fe^{2+} was the most influential parameter at PMF-1S, shaping the bacteria community with high abundance of *Desulfosporosinus*, which has ability for the reduction of Fe^{3+} and sulfate [46, 61], consistent with the detections of VC and ethene at this monitoring well. No key parameter was identified for the native communities in PMF-1M and PMF-1D wells, even though *cis*-1,2-DCE and VC were also detected in these wells, with TCE being the dominant CE. DH and nitrate affected the degrading bacteria compositions in PMF-2S, especially *Sulfurospirillum*, a nitrate-reducing bacterium, consistent with the low CEs detected. EC, primarily contributed by nitrate and chloride (Table S3), was the most dominant factor affecting the occurrence of *Sulfurospirillum*, *Acetobacterium* and *Desulfovibrio* at PMF-2M and PMF-2D. Furthermore, the high abundance of *Sulfurospirillum*, which was reported being able to reduce PCE and TCE to *cis*-1,2-DCE and VC [62], contributed to the accumulation of *cis*-1,2-DCE and VC observed at PMF-2M and PMF-2D (Fig. 2a).

PCE was the dominating CE at FCP-1S, with some TCE detected (Fig. 2c and 2d), indicating less dechlorination occurred in this well. The RDA results verified this and found that *Pseudomonas* was the only degrading bacteria identified at FCP-1S. At the well cluster of FCP-2S, 2M and 2D, ethene was recognized for a strong indicator of the degrading bacteria, suggesting the complete dechlorination occurred, especially at FCP-2M and FCP-2D. pH was identified as another factor influencing the microbial community structure of degrading bacteria at FCP-2M and FCP-2D. *Sediminibacterium* was recognized as the only dominant degrading bacteria at FCP-2 well cluster, indicating that *Sediminibacterium* played an important role in the formation of ethene. Similarly, the enrichment of *Sediminibacterium* was observed in the process of microbial reduction of PCE and TCE in a previous study [48]. In addition, *Sediminibacterium* can adapt to lower pH [63], consistent with the influence of pH observed in this study. Moreover, the detection of Fe^{2+} /sulfide at the PMF site and nitrite at the FCP site suggested the reductive dechlorination activities were coupled with different bioprocesses, consistent with those conclusions from previous studies [7, 64, 65].

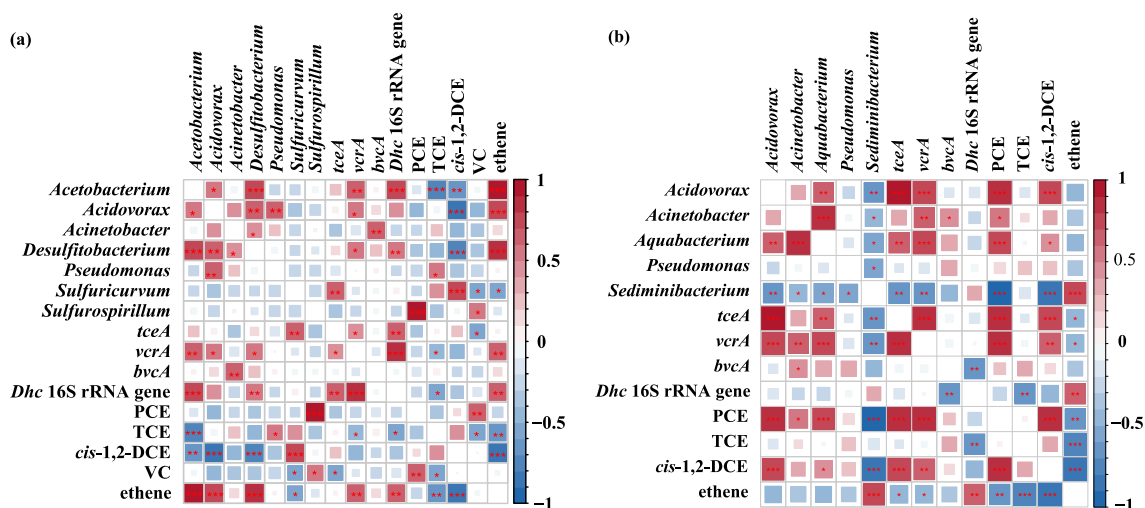


Fig. 7. Relationships between degrading bacteria, *Dhc* 16S rRNA, and RDase genes and CEs with ethene in groundwater at sites PMF (a) and FCP (b). *** $p \leq 0.001$; ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$.

To further investigate the relationship among CEs, ethene, *Dhc* 16S rRNA gene, RDase genes, and CEs degraders under natural conditions at two sites for guidance of degradation of CEs, the Spearman correlation coefficient was used to calculate the correlations among them and CEs degraders with relative abundances greater than 1% at both the PMF and FCP sites (Fig. 7). There are 7 degrading bacteria genera with abundance more than 1% at the PMF site, and among them *Acetobacterium* and *Desulfitobacterium* showed significantly positive correlations with each other. Furthermore, the genus of *Acetobacterium* was positively correlated to *Dhc* 16S rRNA gene, which could be due to the symbiotic and cooperative mechanisms with *Dehalococcoides*. It has been reported that *Acetobacterium* can facilitate the growth and dechlorination activities of *Dehalococcoides* by the fermentation to supply acetate and hydrogen and the biosynthesis of VB₁₂ to synthesize RDase [66, 67]. Cross-feeding among different degrading bacteria is an essential mechanism for biodegradation of CEs in natural condition [5, 68]. Furthermore, *Sulfuricurvum* and *Acinetobacter* were positively correlated to *tceA* and *bvcA*, respectively. *Acetobacterium*, *Acidovorax* and *Desulfitobacterium* showed negative correlation with concentration of TCE and *cis*-1,2-DCE, while a positive correlation with ethene was observed in this study, indicating that these genera potentially contributed to the biodegradation process of TCE and *cis*-1,2-TCE to ethene at the PMF site. However, an opposite trend was observed for *Sulfuricurvum*, which was positively correlated with *cis*-1,2-DCE, but negatively correlated with VC and ethene, consistent

with previous studies that *Sulfuricurvum* was able to degrade PCE and TCE to *cis*-1,2-DCE, leading the accumulation of *cis*-1,2-DCE [41]. Moreover, *Dhc* 16S rRNA had strong positive correlation with *tceA* and *vcrA* due to the contributions of *tceA* gene-*Dhc* and *vcrA* gene-*Dhc* among *Dehalococcoides* at the PMF site. The results also revealed that *vcrA* was positively correlated to ethene, indicating that the formation of ethene was contributed mainly by *vcrA* at the PMF site.

It has been reported that *Acidovorax* and *Aquabacterium* have the capacity to participate in the dechlorination process under the nitrate reducing condition [8, 48, 69-71], which was verified in this study that denitrification process was active with the detection of nitrite at the FCP site (Table S3). *Acidovorax*, *Acinetobacter* and *Aquabacterium* showed significantly positive correlation with *tceA* and *vcrA*. In addition, *vcrA* was positively related to *tceA*, indicating co-growth of *vcrA* gene-*Dhc* and *tceA* gene-*Dhc*. Interestingly, *Sediminibacterium* showed strongly negative correlation with *tceA*, *vcrA*, PCE as well as *cis*-1,2-DCE, with the only positive correlation observed with ethene. The positive correlations between *tceA* and *vcrA* with PCE and *cis*-1,2-DCE indicated that *tceA* and *vcrA* were the main *Dhc* genes responsible for the natural dechlorination of PCE to *cis*-1,2-DCE. However, further degradation to form ethene was contributed by *Sediminibacterium* and *Dehalococcoides* with other RDase genes, as indicated by the positive correlation between ethene and *Sediminibacterium*, as well as *Dhc* 16S rRNA.

4. Conclusions

The research presents key insights into natural reductive dechlorination of CEs within groundwater, shedding light on distinct microbial compositions and gene profiles at varied contamination sites. It notably identifies the potential for complete CE dechlorination at great depths in fully weathered bedrock aquifers. The potential of *Sediminibacterium* for complete dechlorination to ethene under natural conditions requires further investigation. Another potential contribution to the complete dechlorination of CEs under natural conditions is via the abiotic degradation by Fe/S compounds in groundwater and/or soil, and further investigation is also needed to quantify the corresponding contributions towards the complete dechlorination of CEs. Moreover, the study highlights the significance of molecular biological markers, particularly CE-degrading bacteria and their association with RDase genes, serving as effective indicators for evaluating MNA in groundwater remediation. The integration of these markers with essential biogeochemical factors enhances our understanding of the mechanisms driving reductive dechlorination in natural settings. Overall,

these findings offer a more comprehensive understanding of reductive dechlorination processes, potentially guiding the development of targeted remediation strategies for contaminated sites. The identification of *Sediminibacterium* opens avenues for exploring innovative approaches toward more effective and sustainable groundwater remediation techniques.

Author contributions

Zhuanxia Zhang: Conceptualization, Data curation, Methodology, Writing-original draft. **Mukhtiar Ali:** Writing – review & editing. **Zhiwen Tang:** Writing – review & editing. **Qi Sun:** Writing – review & editing. **Qing Wang:** Writing – review & editing. **Xin Liu:** Writing – review & editing. **Lipu Yin:** Resources. **Song Yan:** Resources. **Minmin Xu:** Resources. **Frederic Coulon:** Writing-review & editing. **Xin Song:** Conceptualization, Writing-original draft, Writing-review & editing, Supervision, Funding acquisition.

Declaration of competing interest

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

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