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RESEARCH ARTICLE

Effects of ferrous iron supplementation on reductive dechlorination of tetrachloroethene and on methanogenic microbial community

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One sentence summary: The mechanisms associated with the enhancement of reductive dechlorination of chloroethenes upon ferrous iron supplementation have been examined through a series of laboratory biodegradation tests.
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ABSTRACT

Chloroethenes are common soil and groundwater pollutants. Their dechlorination is impacted by environmental factors, such as the presence of metal ions. We here investigated the effect of ferrous iron on bacterial reductive dechlorination of chloroethenes and on methanogen community. Reductive dechlorination of tetrachloroethene was assayed with a groundwater sample originally containing 6.3×10^3 copies mL^{-1} of *Dehalococcoides* 16S rRNA gene and 2 $mg\,L^{-1}$ of iron. Supplementation with 28 $mg\,L^{-1}$ of ferrous iron enhanced the reductive dechlorination of *cis*-dichloroethene (*cis*-DCE) and vinyl chloride in the presence of methanogens. The supplementation shortened the time required for complete dechlorination of 1 $mg\,L^{-1}$ of tetrachloroethene to ethene and ethane from 84 to 49 d. Methanogens, such as *Candidatus* 'Methanogranum', *Methanomethylovorans* and *Methanocorpusculum*, were significantly more abundant in iron-supplemented cultures than in non-supplemented cultures ($P < 0.01$). Upon methanogen growth inhibition by 2-bromoethanesulfonate and in the absence of iron supplementation, *cis*-DCE was not dechlorinated. Further, iron supplementation induced 71.3% dechlorination of *cis*-DCE accompanied by an increase in *Dehalococcoides* 16S rRNA and dehalogenase *vcrA* gene copies but not dehalogenase *tceA* gene copies. These observations highlight the cooperative effect of iron and methanogens on the reductive dechlorination of chloroethenes by *Dehalococcoides* spp.

Keywords: ferrous iron supplementation; *Dehalococcoides*; methanogen; chloroethenes; dehalogenase gene; 2-bromoethanesulfonate

INTRODUCTION

Chloroethenes, represented by tetrachloroethene (PCE) and trichloroethene (TCE), are common soil and groundwater pollutants (United States Environmental Protection Agency 2013; Ministry of the Environment, Japan 2019). PCE and TCE, and intermediate products of their microbial reductive dechlorination, *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC), are toxic and carcinogenic, especially VC is more toxic than PCE and TCE (United States Environmental Protection Agency 2010; International Agency for Research on Cancer 2019).

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Some microorganisms, such as *Dehalobacter* (Holliger *et al.* 1993), *Desulfitobacterium* (Gerritse *et al.* 1999), *Desulfuromonas* (Krumholz, Sharp and Fishbain 1996), *Geobacter* (Sung *et al.* 2006), *Sulfurospirillum* (Scholz-Muramatsu *et al.* 1995) and *Dehalococcoides* (Maymó-Gatell *et al.* 1997; Löffler *et al.* 2013), reductively dechlorinate chloroethenes. Growth experiments indicated that *Dehalogenimonas* dechlorinates VC (Yang *et al.* 2017b). Only *Dehalococcoides* spp. are thus far described to be able to dechlorinate both PCE and TCE, and their intermediate products *cis*-DCE and VC, to ethene (Yoshikawa, Zhang and Toyota 2017a); hence, *Dehalococcoides* plays an important role in the complete dechlorination of chloroethenes. Bacteria from this genus harbor various reductive dehalogenase genes associated with the dechlorination of chloroethenes (Seshadri *et al.* 2005; Ismaeil, Yoshida and Katayama 2017; Türkowsky *et al.* 2018). For example, *Dehalococcoides mccartyi* 195 harbors dehalogenase genes *pceA* and *tceA*, which encode proteins that primarily dechlorinate PCE and TCE, and TCE and *cis*-DCE, respectively (Maymó-Gatell *et al.* 1997; Magnuson *et al.* 1998; Magnuson *et al.* 2000). *Dehalococcoides mccartyi* BTF08 and *Dehalococcoides* sp. UCH007 harbor *vcrA* genes, which encode for a DCE and VC reductive dehalogenase (Pöritz *et al.* 2013; Uchino *et al.* 2015). Another well-known dehalogenase dechlorinating *cis*-DCE and VC is encoded by *bvcA*, and is harbored by *D. mccartyi* BAV1 (Krajmalnik-Brown *et al.* 2004; Tang *et al.* 2013). The existence of *Dehalococcoides* strains harboring *vcrA* and/or *bvcA*, or the existence of *Dehalogenimonas* has been considered as a necessary condition for complete dichlorination of chloroethenes. (Krajmalnik-Brown *et al.* 2004; Müller *et al.* 2004; Yang *et al.* 2017b).

Bioremediation technology that depends on the microbial ability to detoxify pollutants is currently receiving increasing attention because of its low cost and high environmental efficiency (Juwarkar, Singh and Mudhoo 2010; Zhang and Yoshikawa 2016). Bioremediation approaches require scientifically integrated design and engineering. For instance, when designing a rapid anaerobic bioremediation by *Dehalococcoides* spp., one should also consider various abiotic and biotic environmental factors (Juwarkar, Singh and Mudhoo 2010). One such abiotic factor is pH. For instance, *Dehalococcoides* spp. fail to completely dechlorinate chloroethenes at pH 5.5 (Yang *et al.* 2017a; Puentes Jácome *et al.* 2019). According to Puentes Jácome *et al.* (2019), *Dehalococcoides* spp. was activated when the pH was increased to be circumneutral, although this has not been observed in the other study (Yang *et al.* 2017a). Further, excessive oxygen exposure (Yoshikawa, Zhang and Toyota 2017b) and a high concentration of *cis*-DCE (Ise, Suto and Inoue 2011) inhibit the dechlorination of chloroethenes. Considering biotic factors, the effect of methanogens on dechlorination by *Dehalococcoides* has been investigated, but the findings are inconsistent. Enhancement (Löffler, Ritalahti and Tiedje 1997; Heimann, Batstone and Jakobsen 2006; Futagami *et al.* 2011), interference (Smatlak, Gossett and Zinder 1996), and no effect of such interactions (Heimann, Batstone and Jakobsen 2006; Men *et al.* 2012) have been reported. The studies with microbial cultures including *Methanosarcina* or *Methanomicrobiales* showed enhancement of the dechlorination (Heimann, Batstone and Jakobsen 2006; Futagami *et al.* 2011). The observed interference could be explained by competition for electron donors (Yang and McCarty 1998).

Ferrous iron is the prevalent form of iron in groundwater at polluted sites also harboring *Dehalococcoides* (Imfeld *et al.* 2008; van der Zaan *et al.* 2010). Hence, iron might be an important abiotic factor that affects the reductive dechlorination of chloroethenes by *Dehalococcoides*, but the underlying mechanisms are unclear. According to the Pourbaix diagram of iron (Pourbaix 1974), iron exists in the ferrous state under specific redox (approximately −220 mV to −240 mV) (Parsons 2004) and pH conditions (approximately 6.0 to 8.3) (Yang *et al.* 2017a) that can support the dechlorination reaction catalyzed by *Dehalococcoides* spp. At the optimal redox potential, ferric compounds are reduced to soluble ferrous iron (−50 mV), which is followed by sulfate reduction (−220 mV) and methanogenesis (−240 mV) (Parsons 2004). Furthermore, soluble ferrous iron can transform to insoluble ferrous minerals, including pyrite and magnetite (Parsons 2004; Koenig, Lee and Manefield 2015). The effects of iron compounds on methanogenesis under anaerobic environments can be positive or negative, depending on their physicochemical properties and environmental conditions (Baek, Kim and Lee 2019). Vlyssides, Barampouti and Mai (2006) reported that the addition of ferrous iron stimulated generation of methane in an anaerobic sludge blanket reactor, while Hu *et al.* (2020) reported that ferrous iron addition decreased methane emissions induced by rice straw in flooded paddy soils.

Here, our preliminary analysis of groundwater at a polluted site revealed notable dechlorination of chloroethenes when high concentration of total iron was present. This was accompanied by high abundance of methanogens. Accordingly, we then investigated in detail the effects of ferrous iron on the reductive dechlorination of chloroethenes by *Dehalococcoides* spp. and on the methanogenic community, using iron supplementation (within the range observed at polluted sites) and methanogen inhibition experiments.

MATERIALS AND METHODS

Assessment of groundwater at polluted site

In the current study, groundwater from four monitoring wells in Japan (F-2, F-2-2, D-5-5 and E-5-4) at a study site polluted with chloroethenes (Fig. S1, Supporting Information) was analyzed to select a sampling well for a reductive dechlorination experiment. Groundwater taken 14 m below the ground level, i.e. the center of the second aquifer, was evaluated. The concentration of chloroethenes in the groundwater sampled from the monitoring well F-2-2 was the lowest among those in the four monitoring wells (Table S1, Supporting Information). F-2-2 groundwater sample contained 0.033 mg L^{-1} of *cis*-DCE, while F-2 groundwater sample, from a well located 1 m away from well F-2–2, was markedly contaminated (1.063 mg L^{-1} of *cis*-DCE). Non-chlorinated products were detected in F-2-2 groundwater sample (ethene and ethane, 0.140 and 1.051 mg L^{-1}, respectively). The temperature and pH values at the four wells were similar, i.e. 17.3–18.4°C and 6.6–7.1, respectively; the oxidation–reduction potential values of F-2 and F-2-2 groundwater were lower than those of D-5-5 and E-5-4 groundwater (Table S2, Supporting Information). Groundwater from well F-2-2 contained the highest concentration of total iron (insoluble and soluble) among the groundwater sampled from the four wells (Table 1).

For microbiota analysis, 50 mL of groundwater were collected from each well and DNA was extracted. *Dehalococcoides* 16S rRNA gene and dehalogenase (*tceA*, *vcrA* and *bvcA*) genes were detected in all four groundwater samples (Fig. 1A–D). Based on next-generation sequencing analysis, *Dehalococcoides* accounted for 0.1% of the microbial community in the F-2-2 groundwater sample, while *Dehalogenimonas* was not detected. Further, *Euryarchaeota*, including methanogens, accounted for 39.4% of the microbial community in the F-2-2 groundwater sample, although other groundwater samples contained low amounts of

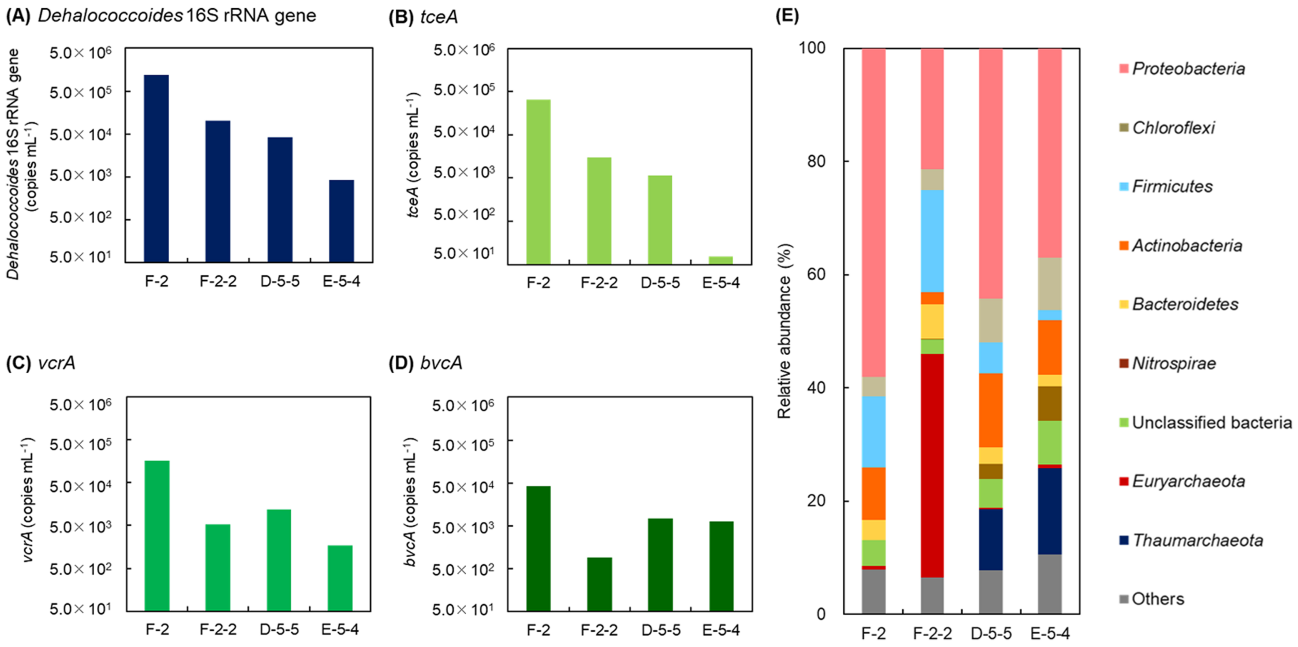

Figure 1. Microbial characterization of groundwater sampled from the second aquifer at the study site. Copy numbers of the *Dehalococcoides* 16S rRNA gene **(A)**, and the *tceA* **(B)**, *vcrA* **(C)** and *bvcA* genes **(D)**, and the relative abundance of microbial phyla based on 16S rRNA amplicon sequencing **(E)** are shown.

Table 1. Iron concentrations in groundwater sampled from the second aquifer at the study site.

	Iron concentration (mg L^{-1})		
Well no.	Insoluble iron	Soluble iron	Ferrous iron ion
F-2	1.3	4.3	4.1
F-2–2	10.6	0.4	0.2
D-5–5	1.1	5.3	<0.1
E-5–4	3.7	5.5	<0.1

these archaea (Fig. 1E). These observations indicate that groundwater from well F-2-2 was suitable for a reductive dechlorination experiment with *Dehalococcoides*, particularly for the assessment of reductive dechlorination of chloroethenes over a short period of time, and for the elucidation of the effects of ferrous iron on the dechlorination reaction and on the methanogen community.

Reductive dechlorination experiment for chloroethenes

An experiment was performed to investigate the effect of iron on the reductive dechlorination of chloroethenes. A sample was collected from the F-2-2 well 14 m below the ground level. The sample was immediately sealed in a glass bottle to prevent oxygenation, and stored below 4°C during subsequent transportation and storage. Before the experiment, nitrogen gas was bubbled through the sample for 30 min to remove background chloroethenes. Then, 47 mL of the sample were dispensed into a sterile vial (nominal volume of 60 mL). Because the previous assessment revealed a lower copy number of *Dehalococcoides* 16S rRNA gene than that reported in other studies conducted with enriched cultures (Ise, Suto and Inoue 2011; Yang *et al.* 2017a; Puentes Jácome *et al.* 2019), the groundwater sample was used directly in the assay, without diluting. Sodium lactate solution and yeast extract were added to the final concentrations of 122 and 120 mg L^{-1}, respectively, as carbon sources and/or electron donors for microorganisms. Such level of organic compounds has been commonly used in laboratory studies, but are much higher than that in natural groundwater conditions. The headspace of the vial was filled with N_2/CO_2 (v:v = 80:20) gas mixture. PCE was added to the vial to an initial concentration of 1 mg L^{-1}, as this amount is sufficient for an effective microbial reductive dechlorination (Yoshikawa and Zhang 2020). The volume of the solution in the vial was adjusted to 50 mL with anaerobic sterile water. All vials were placed in the dark at 20°C, which was approximately the *in situ* temperature of groundwater at the study site (Table S2, Supporting Information). Reductive dechlorination assays were performed in triplicate for each condition.

To investigate the effect of iron on the dechlorination of chloroethenes, the iron levels were increased to 15-fold those in the original groundwater sample, i.e. 30 mg L^{-1} (supplemented) vs. 2 mg L^{-1} (original) (equivalent to 0.04 and 0.54 mmol L^{-1} of iron, respectively). Henceforth, (+) denotes supplementation and (−) denotes no supplementation. Iron at 30 mg L^{-1} is within the concentration range at sites of reductive dechlorination (van der Zaan *et al.* 2010). In the experiments, iron levels were adjusted using $FeCl_2{\cdot}4H_2O$. To determine whether the effect of iron would be noticeable upon the inhibition of methanogen growth, methanogen growth was inhibited by the addition of 844 mg L^{-1} of sodium 2-bromoethanesulfonate (BES) (equivalent to 4.0 mM). The concentration was two-fold that used in a study of Löffler, Ritalahti and Tiedje (1997) but does not inhibit the dechlorination of chloroethenes itself (Lu *et al.* 2017). Overall, the reductive dechlorination experiment was performed under four conditions: Fe(−)BES(−), Fe(+)BES(−), Fe(−)BES(+) and Fe(+)BES(+).

Concentrations of chloroethenes, ethene, ethane and methane during reductive dechlorination

The concentrations of chloroethenes, end products (such as ethene and ethane) and methane were analyzed over time.

Chloroethene concentrations in the headspace gas in the test vials were analyzed by gas chromatography-mass spectrometry (GC-MS) (GCMS-QP2010, Shimadzu, Kyoto, Japan) with an RTX-624 column (0.25 mm diameter, 60 m length; Restek, Bellefonte, PA, USA). The concentrations of ethene, ethane and methane were determined by gas chromatography-flame ionization detection (GC-FID) (GC-2014, Shimadzu) with a GS-Q column (0.53 mm diameter, 30 m length; J&W Scientific, Agilent Technologies, Santa Clara, CA, USA). The column oven programs for GC-MS and GC-FID were as described previously (Yoshikawa and Zhang 2020). The concentrations in culture solutions were calculated using Henry's law constant (Mackay and Shiu 1981).

DNA extraction

DNA was extracted from each culture solution to analyze the changes in microbial copy numbers and microbial community diversity over time. For the experiment, 0.5 mL of culture solution, periodically collected during the dechlorination of chloroethenes, were used. DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). As described previously by Takada-Hoshino and Matsumoto (2004), 20 mg of skim milk was added to increase the efficiency of DNA extraction (Volossiouk, Robb and Nazar 1995; Garcia-Pedrajas *et al.* 1999). The extracts were further purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA) to prevent inhibition of polymerase chain reaction (PCR). The purified DNA was then used for downstream analyses, as described below.

Quantification of microbial 16S rRNA genes and functional genes

The copy numbers of bacterial 16S rRNA gene, *Dehalococcoides* 16S rRNA gene and *Dehalococcoides* dehalogenase genes that have been extensively studied [i.e. *tceA*, *vcrA* and *bvcA* (e.g. Magnuson *et al.* 2000; Krajmalnik-Brown *et al.* 2004; Müller *et al.* 2004; Tang *et al.* 2013)] were determined using StepOnePlus™ real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Each reaction mixture contained TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific), and specific primers and probes (Table S3, Supplementary data). The reactions were performed as recommended by the manufacturer, following the cycling protocol: 50°C for 2 min, 95°C for 20 s and 40 cycles of 95°C for 1 s and 60°C for 20 s. The copy numbers of gene for the α-subunit of methyl coenzyme M reductase (*mcrA*) harbored by all methanogenic archaea (Friedrich 2005) were quantified using the same real-time PCR system. The reaction mixture contained PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) and specific primers (Table S3, Supporting Information). The PCR was performed according to the manufacturer's protocol: 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 1 min. To ensure the quality and reliability of the data, negative controls with nucleotide-free water instead of the DNA template were analyzed with each reaction set. The gene copy numbers were determined using a calibration curve generated using 10-fold dilutions of specific templates, i.e. *Escherichia coli* 16S rRNA gene, cloned genes of *Dehalococcoides* sp. 16S rRNA and dehalogenase, and *Methanothermobacter thermautotrophicus mcrA* genes. Details of the quantitative PCR assays are presented in Table S4 (Supporting Information).

Characterization of microbial communities

Microbial communities in culture solutions were analyzed by next-generation sequencing targeting the V4 region of the bacterial and archaeal 16S rRNA genes. The region of interest was amplified from the extracted DNA using T100™ Thermal Cycler (BioRad, Hercules, CA, USA), universal primers 515F and 806R with adapter sequences for the Illumina sequencer (Caporaso *et al.* 2012), and PrimeSTAR® GXL DNA polymerase (Takara Bio, Kusatsu, Japan). The initial PCR products were purified using the AMPure XP beads (Beckman Coulter) and then used in subsequent PCR to incorporate index sequences. Purified products of the second PCR step were then sequenced as 300-bp paired-end reads using the Illumina Miseq platform (Illumina, San Diego, CA, USA). The obtained sequences were deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA) under the accession number DRA010066.

UCHIME algorithm (Edgar *et al.* 2011) was used to obtain non-chimeric sequences. The sequences were grouped at an identity cut-off value of 97% using mothur (version 1.44.3) (Schloss *et al.* 2009). Low-abundance operational taxonomic units (OTUs) (<0.05%) were removed using R (version 4.0.2) (R Core Team 2019) in R package vegan 2.5-6 (Oksanen *et al.* 2019), and a normalized data set containing 26 699 sequences was generated for each sample. Taxonomic affiliations were assigned using the EzBioCloud 16S database (Yoon *et al.* 2017) as the reference taxonomy. High-abundance OTUs (>5%) were visualized as a heatmap using heatmap3 package (Zhao *et al.* 2019) in R. The metastats command (White, Nagarajan and Pop 2009) in mothur, a *t*-test, was applied to the normalized OTU data set to identify significant differences ($P < 0.01$) in the relative OTU abundances between the test conditions. Here, traditional OTU-based approach was used rather than the relatively new method of exact sequence variants (Callahan, McMurdie and Holmes 2017) for sequence grouping. This is because the diversity increment of genetic mapping using exact sequence variants (e.g. 2.1 times that of OTU richness) (Glassman and Martiny 2018) would make it difficult to identify changes in microbial abundance in culture solutions originated from environmental groundwater.

Analysis of iron speciation and concentrations

After the reductive dechlorination experiment, iron levels in culture solutions were analyzed using 1,10-phenanthroline method (The Japan Society for Analytical Chemistry 2005). Concentrations of total iron, soluble iron and ferrous iron ion were first determined; the concentration of insoluble iron was determined as the difference between the concentrations of total iron and soluble iron. To analyze the total iron concentration, culture solutions were digested with HCl before the 1,10-phenanthroline reaction. For soluble iron determinations, the culture solutions were filtered (Millex-GP filter unit, 0.22 μm; Merck Millipore, Burlington, MA, USA) and digested with HCl before the 1,10-phenanthroline reaction. For ferrous iron ion determinations, the culture solutions were filtered as above and analyzed without HCl digestion. The absorbance of the phenanthroline iron (II) complex in solution was measured at 510 nm using Evolution 220 spectrophotometer (Thermo Fisher Scientific). The iron concentrations were calculated based on a calibration curve prepared using Fe $(NH_4)_2(SO_4)_2{\cdot}6H_2O$ solutions. The detection limit of iron was 0.2 mg/L.

RESULTS

Changes in chloroethene and non-chlorinated product levels during the reductive dechlorination experiment

The dechlorination rates of chloroethenes under different conditions differed during the 84-d analysis period (Fig. 2). Chloroethenes were completely dechlorinated to ethene and converted to ethane in the absence of BES [Fe(−)BES(−) and Fe(+)BES(−), Fig. 2A, B]. Upon iron supplementation and without BES [Fe(+)BES(−)], chloroethenes were completely dechlorinated to nonhazardous ethene and ethane within 49 d, while their dechlorination took 84 d without iron supplementation and BES [Fe(−)BES(−)]. The first-order dechlorination rate constants (Chambon *et al.* 2013) were 0.03 and 0.08 d^{-1} for *cis*-DCE, and 0.06 and 0.09 d^{-1} for VC in Fe(−)BES(−) and Fe(+)BES(−) experiments, respectively. Methanogen inhibition by BES and without additional iron [Fe(−)BES(+)] led to the inhibition of the sequential dechlorination of *cis*-DCE to VC [Fig. 2C]. Iron and BES supplementation [Fe(+)BES(+)] stimulated the dechlorination of *cis*-DCE to VC and ethene [Fig. 2D]. No chloroethene dechlorination was observed in the uninoculated negative control (data not shown).

Changes in methane levels during the reductive dechlorination experiment

Methane was produced in test vials in the absence of BES [Fe(−)BES(−) and Fe(+)BES(−), Fig. 3A and B]. Its levels increased exponentially from day 9, and reached 346.0 and 368.4 μmol per vial on day 28 in Fe(−)BES(−) and Fe(+)BES(−) experiments, respectively. In the experiments upon BES supplementation [Fe(−)BES(+) and Fe(+)BES(+)], the concentrations of methane only reached 0.20 and 0.17 μmol per vial, respectively [Fig. 3C and D].

Changes in the copy number of genes related to reductive dechlorination and methanogenesis during the reductive dechlorination experiment

The initial abundance of bacterial 16S rRNA genes was 1.4×10^7 copies mL^{-1}, and increased 6- to 9-fold under all conditions tested during the analysis period (Fig. S2, Supporting Information). The copy numbers of specific microbial genes (*Dehalococcoides* 16S rRNA, *tceA* and *vcrA*) are shown in Fig. 4. The *Dehalococcoides* 16S rRNA gene copy number increased from 6.3×10^3 to 3.8×10^5, 1.3×10^5 and 2.9×10^5 copies mL^{-1} in Fe(−)BES(−), Fe(+)BES(−) and Fe(+)BES(+) experiments, respectively, during the experimental period. In the Fe(−)BES(+) experiment upon BES supplementation, the *Dehalococcoides* 16S rRNA gene copy number was at most 2.8×10^3 copies mL^{-1}. The *tceA* gene was detected from days 28 and 14 in Fe(−)BES(−) and Fe(+)BES(−) experiments, respectively, and its abundance increased in the absence of BES. However, it was not detected upon BES supplementation [Fe(−)BES(+) and Fe(+)BES(+)]. The *vcrA* gene was detected under all culture conditions tested, from days 42, 28, 56 and 28 in Fe(−)BES(−), Fe(+)BES(−), Fe(-)BES(+) and Fe(+)BES(+) experiments, respectively. Its copy number was below 1.7×10^3 copies mL^{-1} in Fe(−)BES(+), and increased to 3.3×10^5, 4.3×10^4 and 9.7×10^5 copies mL^{-1} in Fe(−)BES(−), Fe(+)BES(−) and Fe(+)BES(+) experiments, respectively. The dehalogenase gene *bvcA* was not detected under any conditions during the study period. Further, the initial 3.2×10^5 copies mL^{-1} of *mcrA* gene increased to 3.1×10^6 and 6.4×10^6 copies mL^{-1} by day 28 in Fe(−)BES(−) and Fe(+)BES(−) experiments, respectively [Fig. 4D]. Then, the number decreased to 4.4×10^5 copies mL^{-1} on day 42 in Fe(−)BES(−) experiment, while it remained stable until the end of experiment in Fe(+)BES(-). Upon BES supplementation, the maximum copy number of *mcrA* was 3.8×10^4 and 3.5×10^4 copies mL^{-1} without and with iron supplementation, respectively.

Microbial community dynamics during the dechlorination of chloroethenes

We then analyzed microbial phylum diversity in samples, with 26 699 sequences obtained for each growth condition (Fig. S3, Supporting Information). *Proteobacteria*, *Firmicutes* and *Bacteroidetes* were the major and common phyla under all conditions tested. *Euryarchaeota* was the most abundant phylum in Fe(+)BES(−), accounting for 35.2%–41.4% of all microbes on days 28–84 and for 6.3%–18.8% in Fe(−)BES(−) during the same period. Upon BES supplementation [Fe(−)BES(+) and Fe(+)BES(+)], *Euryarchaeota* accounted for up to 0.3% and 0.9%, respectively, of all microbes. *Firmicutes* was the most frequently observed phylum in Fe(−)BES(+) and Fe(+)BES(+) on days 28–84. On the genus level, *Desulfitobacterium* was detected on days 7–14 under all conditions tested, and *Sulfurospirillum* was detected upon iron supplementation (OTU156 and OTU189; Table S5, Supplementary data). Neither *Dehalococcoides* nor *Dehalogenimonas* were detected by amplicon sequencing during the dechlorination experiment.

Under the conditions tested, microbial composition among test conditions varied from day 28 but was relatively stable on days 28–84 (Fig. S3, Supporting Information), as revealed by non-metric multidimensional scaling (NMDS) analysis (Fig. S4, Supporting Information). On days 7 and 14, microbial community composition under all conditions tested was similar; however, the community compositions from day 28 to 56 clustered by test conditions. Based on these observations, we next focused on the changes in the relative OTU abundance on days 28–84.

The relative abundances of major OTUs, accounting for >5% of microbial community in each culture solution from day 28 to 84, and on day 0, are shown in Fig. 6. The data including less abundant OTUs that account for >0.1% of each microbial community are shown in Table S5 (Supporting Information). The abundance of unclassified *Dehalobacterium* spp. significantly increased upon BES supplementation ($P < 0.01$; namely, it accounted for 3.2%–13.0%, 0.4%–16.1%, 24.6%–46.3% and 38.8%–48.8% of all microbes in Fe(−)BES(−), Fe(+)BES(−), Fe(−)BES(+) and Fe(+)BES(+), respectively. All *Euryarchaeota* OTUs were more abundant in the absence of BES than upon BES supplementation ($P < 0.01$). The abundance of *Candidatus* (*Ca.*) 'Methanogranum', *Methanomethylovorans* sp. and *Methanocorpusculum* (*M.*) *sinense* was significantly higher in Fe(+)BES(−) than that in Fe(−)BES(−) ($P < 0.01$). The three OTUs accounted for up to 0.7% in the initial microbial community. The abundance of *Methanosarcina* sp. peaked at 14.3% in Fe(−)BES(−) on day 28.

Speciation and concentration of iron at the end of the reductive dechlorination experiment

At the end of the experiment, soluble and insoluble iron levels were higher with iron supplementation [Fe(+)BES(−) and Fe(+)BES(+)] than without iron supplementation ([Fe(−)BES(−) and Fe(−)BES(+)], Table 2). Overall, 86%–88% of iron remained soluble upon ferrous iron supplementation. The solution of Fe(+)BES(−) contained 11.5 $mg\,L^{-1}$ of ferrous iron ion, whereas

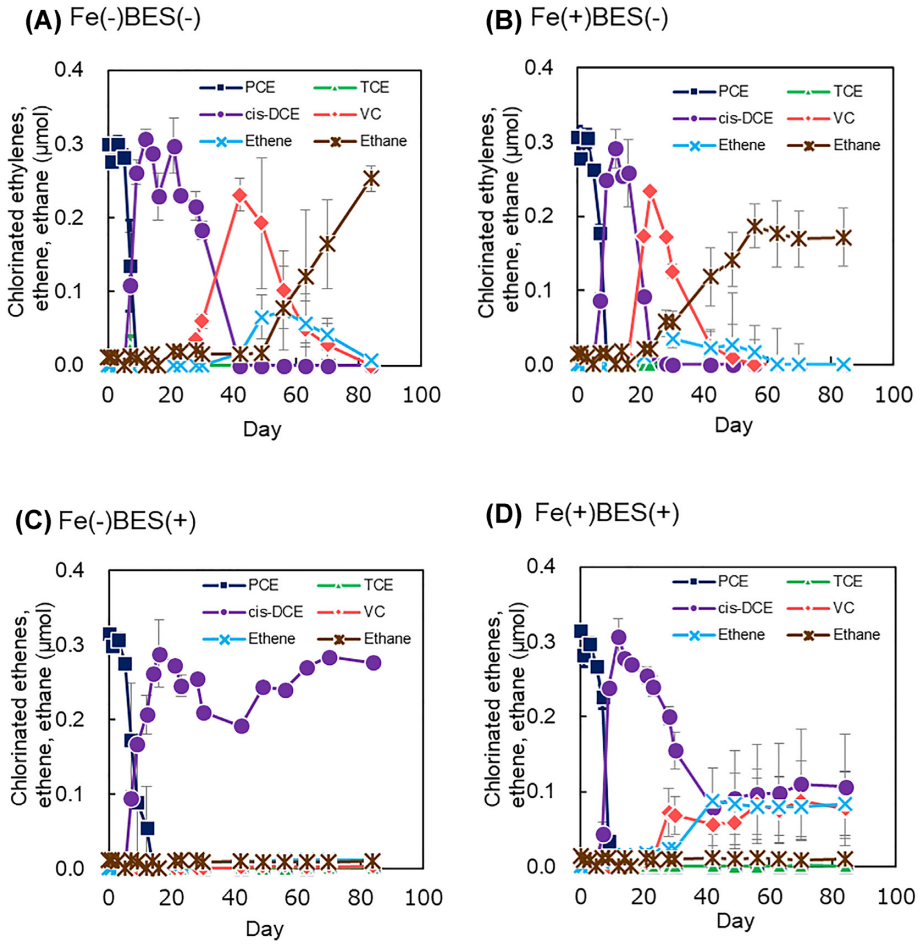

Figure 2. Dechlorination of tetrachloroethene under the indicated conditions of ferrous chloride (Fe) and 2-bromoethanesulfonate (BES) supplementation: (+), supplemented; (−), not supplemented. PCE, TCE, *cis*-DCE and VC denote tetrachloroethene, trichloroethene, *cis*-dichloroethene and vinyl chloride, respectively. Average values and standard errors from three experiments are shown.

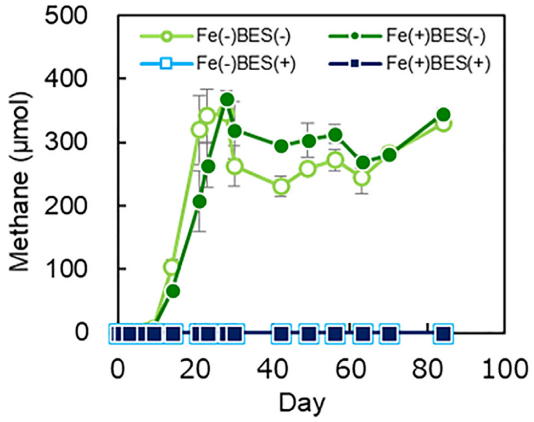

Figure 3. Methane production under the indicated conditions of ferrous chloride (Fe) and 2-bromoethanesulfonate (BES) supplementation: (+), supplemented; (−), not supplemented. Average values and standard errors from three experiments are shown. The values of methane concentration in Fe(−)BES(+) and Fe(+)BES(+) are close to 0, and the maximum value in Fe(−)BES(+) is 0.20 μmol per vial on day 84 while that in Fe(+)BES(+) is 0.17 μmol per vial on day 84.

that of Fe(+)BES(+) contained 27.9 mg L^{-1} of ferrous iron ion. In the solution of Fe(+)BES(−), other speciation of soluble iron, i.e. the difference between soluble iron and ferrous iron ion, was observed after the 84-d analysis period, and its concentration was 15.3 mg L^{-1}.

Table 2. Speciation and concentration of iron under different culture conditions at the end of the reductive dechlorination experiment.

	Concentration (mg L^{-1})		
	Insoluble iron	Soluble iron	Ferrous iron ion
Fe(-)BES(−)	1.8	0.5	0.4
Fe(+)BES(−)	3.5	26.8	11.5
Fe(−)BES(+)	1.5	0.6	0.6
Fe(+)BES(+)	4.5	27.9	27.9

Culture conditions: ferrous chloride (Fe) and 2-bromoethanesulfonate (BES) supplementation is indicated by (+); no supplementation is indicated by (−). Average values from three experiments are shown.

DISCUSSION

Enhancement of the dechlorination of chloroethenes by ferrous iron supplementation in the presence of methanogens

To examine the effects of ferrous iron supplementation on the dechlorination of chloroethenes in the presence of methanogens, we here compared the results of concentrations of chloroethenes and their intermediate products, microbial copy numbers and microbial community diversity between Fe(−)BES(−) and Fe(+)BES(−). As shown in Fig. 2A and B, dechlorination of *cis*-DCE and VC by the microbial communities were

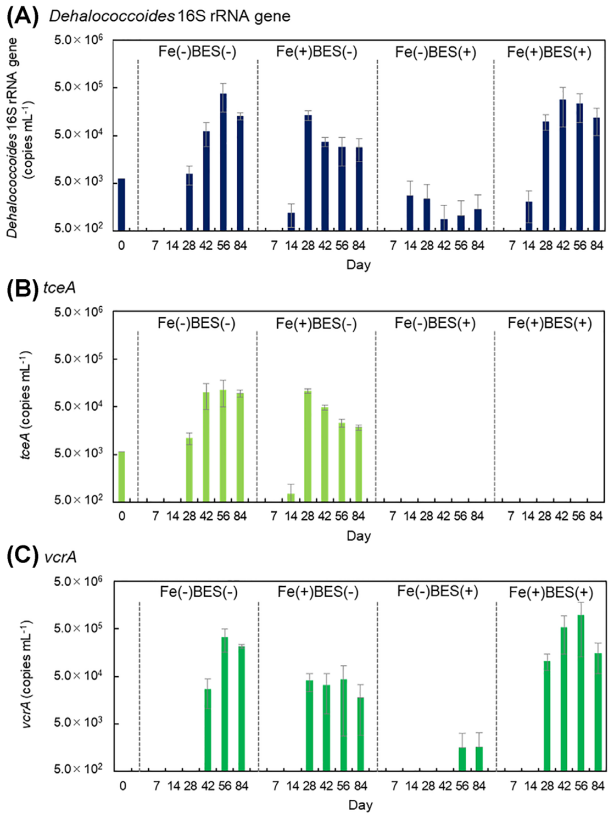

Figure 4. Time-course changes in the copy numbers of **(A)** *Dehalococcoides* 16S rRNA gene, **(B)** *tceA* gene and **(C)** *vcrA* gene. Ferrous chloride (Fe) and 2-bromoethanesulfonate (BES) supplementation conditions tested: (+), supplemented; (–), not supplemented. Average values and standard errors from three experiments are shown.

more rapid upon iron supplementation than without supplementation. Further, ferrous iron supplementation induced earlier growth of *Dehalococcoides*, as the *Dehalococcoides* 16S rRNA gene, and the *tceA* and *vcrA* genes were detected in Fe(+)BES(–) 2 weeks earlier than in Fe(–)BES(–) [Fig. 4B and C]. The increments in copy numbers of *tceA* and *vcrA* genes corresponded to the dechlorination of *cis*-DCE and VC, respectively, suggesting that *Dehalococcoides* dechlorinates the intermediate products. These observations are in agreement with previous studies indicating that the reductive dechlorination of chlorinated ethenes is generally associated with *Dehalococcoides* growth (Maymo-Gatell *et al.* 1997), and that the rate of reductive dechlorination is related to the density of *Dehalococcoides* (Chambon *et al.* 2013).

In terms of using iron in wastewater treatment, nanoscale zerovalent iron is often used for abiotic rather than biotic dechlorination of chloroethenes (Zhang 2003). The results of a published study on industrially produced nanoscale zerovalent iron (Xiu *et al.* 2010) contrast with the findings of the current study as copy numbers of *tceA* and *vcrA* increased. The nanoscale zerovalent iron may inhibit the expression of *tceA* and *vcrA* genes, e.g. by producing strong reducing conditions at the *Dehalococcoides* membrane (Xiu *et al.* 2010). Our results demonstrated that supplementation of ferrous iron could stimulate biotic dechlorination of chloroethenes (Fe(+)BES(–)).

We observed that ethene was further reduced to ethane in Fe(–)BES(–) and Fe(+)BES(–) [Fig. 2A and B]. Methanogens are thought to be involved in the reduction of ethene to ethane (De Bruin *et al.* 1992; Koene-Cottaar and Schraa 1998; Elsgaard

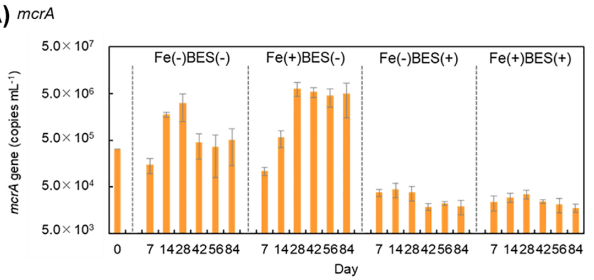

Figure 5. Time-course changes in the copy numbers of *mcrA* gene under different ferrous chloride (Fe) and 2-bromoethanesulfonate (BES) supplementation conditions: (+) supplemented; (–), not supplemented. Average values and standard errors from three experiments are shown.

2013), although pure methanogenic cultures were not shown to reduce ethene (Koene-Cottaar and Schraa 1998). In the current study, any microorganisms' increases were not strictly consistent with ethene reduction (Figs 2 and 6), and further research is needed to clarify the microorganisms associated with reduction of ethene to ethane. Other biotic and/or abiotic reactions of ethene transformation might also occur upon ferrous iron supplementation [Fe(+)BES(–)], as reported by Berns *et al.* (2019), because ethane was not stoichiometrically produced from the depleted chlorinated ethenes [55.9% recovery of PCE, Fig. 2B]. Besides the ethane, transformation to other substances, such as butane, propane, propylene and propyne could also be possible (Berns *et al.* 2019).

Improvement of the dechlorination of chloroethenes upon ferrous iron supplementation after methanogen inhibition

We also investigated whether the enhancement of chloroethene dechlorination upon ferrous iron supplementation occurs even in the presence of BES. BES critically inhibited *Methanosarcina* originally present in the groundwater sample with a relatively high abundance up to 3.2% (Figs 5 and 6; Table S5, Supporting Information) and impacted the dechlorination of *cis*-DCE in the absence of iron supplementation [Fig. 2C], as has been also reported by Futagami *et al.* (2011). Similarly, Heimann, Batstone and Jakobsen (2006) reported inhibition of VC dechlorination upon BES supplementation in a microbial culture containing *Dehalococcoides* spp. and *Methanosarcina* spp. *Methanosarcina* spp. may potentially produce growth factors for *Dehalococcoides*.

However, even upon BES supplementation in microbial cultures, ferrous iron supplementation improved *cis*-DCE dechlorination to VC or ethene (as 71.3% of *cis*-DCE was dechlorinated) and the growth of *Dehalococcoides* strains harboring *vcrA*. One potential explanation for this improvement is that soluble ferrous iron supports the growth of *Dehalococcoides* strains harboring the *vcrA* gene, but not those with the *tceA* gene. Ferrous iron participates in the abiotic dechlorination of chloroethenes, with the specific intermediate product acetylene (Berns *et al.* 2019). In the current study, acetylene was not detected by GC-MS analysis in cultures during the experiment (data not shown). Hence, it is reasonable to conclude that, in the current study, the dechlorination of chloroethene was a result of biotic reactions.

In the microbial communities that contain *Dehalococcoides* strains harboring both *tceA* and *vcrA*, VC was completely dechlorinated with increase in *vcrA* (Holmes *et al.* 2006; Mayer-Blackwell, Azizian and Green 2017). However, in the current study, *Dehalococcoides* strains harboring *tceA* are potentially

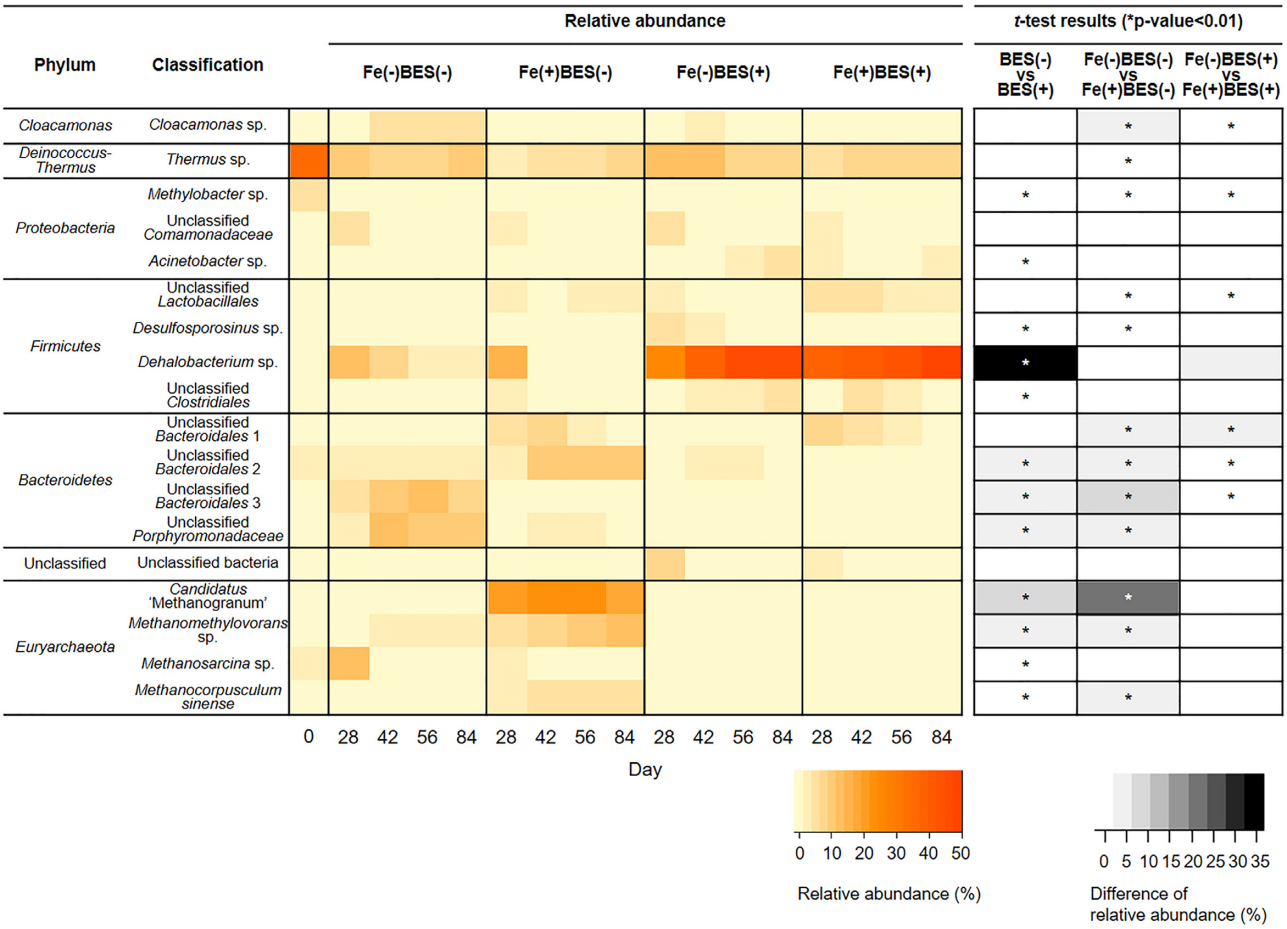

Figure 6. Heatmap of the major operational taxonomic units (>5%) in the microbial communities on day 0 (left), and from day 28 to day 84, with *t*-test analysis (right). The asterisks indicate $P<0.01$. Ferrous chloride (Fe) and 2-bromoethanesulfonate (BES) supplementation conditions tested: (+), supplemented; (−), not supplemented.

required for the dechlorination of *cis*-DCE and VC upon BES and ferrous iron supplementation, because the intermediate products were completely dechlorinated upon BES non-supplementation, with increment of *tceA* gene copies [Figs 2A, B and 4B]. Dehalogenase TceA dechlorinates *cis*-DCE at a rate of 12.1 μmol min^{-1} mg^{-1} (Magnuson *et al.* 2000), which is more rapid than the reaction catalyzed by VcrA (0.4 μmol min^{-1} mg^{-1}) (Müller *et al.* 2004), indicating that *Dehalococcoides* strains harboring *tceA* dechlorinate *cis*-DCE faster than strains harboring *vcrA*. Rapid dechlorination of *cis*-DCE by TceA might induce the dechlorination of VC because *cis*-DCE accumulation inhibits the subsequent dechlorination of VC (Yu and Semprini 2004). Although the underlying mechanisms remain unclear, interactions with methanogens appear to be more beneficial for *Dehalococcoides* strains harboring *tceA* than for those harboring *vcrA*. Further, while Futagami *et al.* (2011) showed that *Dehalococcoides* does not grow in the presence of BES, the current study suggests that the growth of *Dehalococcoides* under these conditions is strain-specific and depends on iron concentration. Furthermore, using the appropriate electron donor for hydrogen production, at an optimal concentration, is a useful approach for achieving a complete dechlorination reaction (Yang and McCarty 1998; Daprato, Löffler and Hughes 2007). In the current study, the most abundant microorganism was *Dehalobacterium* in the presence of BES, which have been shown to dechlorinate dichloromethane (Mägli, Wendt and Leisinger 1996). *Dehalobacterium* could be a competing bacterium associated with dechlorination of chloroethenes by *Dehalococcoides*, but the mechanisms remain unknown. Further reductive dechlorination experiments designed to clarify this point are necessary.

Effects of ferrous iron supplementation on the dechlorinating culture

Ferrous iron is an essential metal, with roles in DNA biosynthesis, regulation of gene expression, and other biological processes that depend on iron, e.g. in the form of iron–sulfur clusters, incorporated into proteins (Andrews, Robinson and Rodríguez-Quiñones 2003). The supplied ferrous iron remained in the ferrous form at the end of the experimental period (Table 2), potentially enhancing the biological processes of dechlorination. Iron supplementation in the absence of BES affected the speciation of soluble iron, e.g. iron may have formed a bicarbonate complex (Jensen *et al.* 1998) because some methanogens, including *Methanomethylovorans*, produce CO_2 during methanogenesis (Lomans *et al.* 1999). Detection of such bicarbonate complex can be practically difficult because anaerobic condition should be sustained during the analysis.

In the current study, supplementation with ferrous iron enhanced the growth of specific microorganisms, such as *Dehalococcoides* and some methanogenes, but not that of all bacteria. From the possible dechlorinators of PCE and TCE, the abundance of *Sulfurospirillum* (OTU189; Table S5, Supporting Information), similar to the PCE- and TCE-dechlorinating bacterium

Sulfurospirillum multivorans DSM 12446 (Scholz-Muramatsu *et al.* 1995) (99.2% similarity), increased upon iron supplementation. However, no such abundance increase was apparent for *Desulfitobacterium* (OTU 156), a bacterium that shares 99.2% similarity with *Desulfitobacterium hafniense* PCE-S (Gerritse *et al.* 1996).

The primary cellular transport system for ferrous iron is the Feo system (Lau, Krewulak and Vogel 2016), and the genomes of all (33) *Dehalococcoides* spp. strains deposited in the EzBioCloud (https://www.ezbiocloud.net/, last accessed 23/1/2021) contain genes encoding FeoB homolog. Incorporated ferrous iron can be used by dehalogenase proteins that contain the iron–sulfur cluster (Fincker and Spormann 2017). As for methanogens, iron is used as a constituent of important proteins, e.g. iron–sulfur proteins, or in electron shuttles for methanogenesis (Baek, Kim and Lee 2019). Ferrous iron supplementation in the presence of methanogens may assist *Dehalococcoides* both directly and indirectly (*vide infra*).

The reported concentrations of ferrous iron for the cultivation of *Dehalococcoides* for chloroethene dechlorination vary widely. For example, enrichment medium for the cultivation of *Dehalococcoides* contains 0.4 mgL^{-1} (Löffler, Sanford and Ritalahti 2005), 1.4 mgL^{-1} (Yang and McCarty 1998) or 61.4 mgL^{-1} (Duhamel *et al.* 2002) of ferrous iron, but almost no ferrous iron in a minimal medium was used for the isolation of *Dehalococcoides* (Maymó-Gatell *et al.* 1997; He *et al.* 2005). In the current study, 2 mgL^{-1} of iron was sufficient for a complete dechlorination of 1 mgL^{-1} of PCE by the *Dehalococcoides* microbial community, while 30 mgL^{-1} of iron enhanced the dechlorination reaction. Similarly, ferrous iron supplementation can accelerate dechlorination rates in the presence of less than 30 mgL^{-1} ferrous iron in *Dehalococcoides* cultures, possibly induced by the activity of *Dehalococcoides* iron–sulfur proteins, such as dehalogenase (Fincker and Spormann 2017). The dehalogenase VcrA was purified from the culture containing ferrous iron at similar concentration (~28 mgL^{-1}) and the activity was confirmed (Müller *et al.* 2004).

Effect of ferrous iron supplementation on the methanogenic community

Methanogenic microbial community markedly changed upon ferrous iron supplementation [Fe(+)BES(−)] (Fig. 6). Iron supplementation appears to have significantly enhanced the growth of specific methanogens, *Ca.* 'Methanogranum', *Methanomethylovorans* sp. and *M. sinense*. The representative sequence of the obtained *Methanomethylovorans* sp. OTU is similar to that of *Methanomethylovorans* strains EK1 (Cha *et al.* 2013) and DMS1 (Lomans *et al.* 1999), sharing 99.6 and 99.2% similarity with these strains, respectively. Consistent with the current study, $FeCl_2$ supplementation stimulated the growth of these *Methanomethylovorans* strains (Lomans *et al.* 1999; Cha *et al.* 2013). Different methanogens require different iron concentrations (Baek, Kim and Lee 2019) and, accordingly, in the current study, ferrous iron supplementation appeared to create preferable conditions for the growth of three specific methanogens. Enhancing methanogen growth is probably one factor stimulating the dechlorination of *cis*-DCE and VC by *Dehalococcoides*. In the current study, in Fe(+)BES(−) samples, drastic increment of *mcrA* gene copies from day 14 to 28 accompanied accelerated dechlorination of *cis*-DCE and VC (Figs 2 and 5). No such observations with the three methanogens have been reported to date. Heimann, Batstone and Jakobsen (2006) showed that the dechlorination rate of VC of a culture dominated by *Methanosarcina* was higher than that after methanogen inhibition. In the current study, *Methanosarcina* abundance did not increase during dechlorination in Fe(+)BES(−) (Fig. 6). Any direct relationships between dechlorination and the three dominant methanogens remain to be further explored in the future.

Some methanogens compete for hydrogen and acetate with *Dehalococcoides*. The latter use hydrogen as an electron donor and acetate as a carbon source (Löffler *et al.* 2013). Accordingly, some methanogens utilize hydrogen and acetate for methanogenesis (Garcia, Patel and Ollivier 2000). *Ca.* 'Methanogranum' and *Methanomethylovorans* utilize methyl compounds, including methanol and dimethyl sulfide, for methanogenesis (Cha *et al.* 2013; Iino *et al.* 2013). Indeed, we have detected dimethyl sulfide in cultures using GC-MS (data not shown). *Ca.* 'Methanogranum' is suggested to require external hydrogen for methyl-reducing methanogenesis (Iino *et al.* 2013), and thus, may compete with *Dehalococcoides*. The abundance of both species observed in this study suggests the occurrence of other interactions between these species, such as inter-species amino acid transfer (Embee *et al.* 2015). Collectively, these observations may explain the successful dechlorination of chloroethenes by *Dehalococcoides* and a concurrent methanogenesis with methyl compounds upon ferrous iron supplementation, even under the conditions of high methanogen abundance.

Presence of methanogens, including *Methanomethylovorans* and *Methanocorpusculum*, has been reported in microbial communities that dechlorinate chloroethenes (Duhamel and Edwards 2006; Kittelmann and Friedrich 2008). This implies that methanogens play some important roles in the dechlorination of chloroethenes, although the conditions for dechlorination remained unclear. In the current study, we showed that ferrous iron supplementation stimulates the growth of *Methanomethylovorans* and *Methanocorpusculum*. *Ca.* 'Methanogranum' has not been isolated to date (Iino *et al.* 2013) and has not been reported as a member of microbial communities that dechlorinate chloroethenes. However, interestingly, the *Ca.* 'Methanogranum' OTU dominated the microbial community in Fe(+)BES(−) during days 28–84, suggesting that growth of *Ca.* 'Methanogranum' was beneficial for chloroethene dechlorination. Further studies are required to verify this finding.

Practical implications of this study

Accumulation of *cis*-DCE and VC has often been a problem associated with anaerobic dechlorination of chloroethenes in polluted sites (Bradley 2000). Both *tceA* and *vcrA* were detected in the culture solution of Fe(+)BES(−) that resulted in rapid dechlorination of *cis*-DCE and VC, and consequently complete dechlorination of chloroethenes (Fig. 4). If *Dehalococcoides* strains that harbor *tceA* and *vcrA*, and methanogens detected in the current study exist simultaneously in a polluted site, complete dechlorination of chloroethenes can be accelerated upon introduction of ferrous iron to the site. If the abundances of these microorganisms are not enough in a polluted site, introduction of the culture solution containing *Dehalococcoides* strains that harbor *tceA* and *vcrA* and the methanogens, as that of Fe(+)BES(-) in the current study, can be effectively used for bioaugmentation.

CONCLUSIONS

We here showed that in the culture supplemented with 28 mgL^{-1} of ferrous iron the reductive dechlorination of chloroethenes was enhanced when methanogen growth was

not inhibited. The iron supplementation altered the structure of the methanogen community, increasing the abundance of *Ca*. 'Methanogranum', *Methanomethylovorans* and *Methanocorpusculum*, and allowing for more rapid dechloriation. Furthermore, iron supplementation improved the reductive dechlorination of *cis*-DCE and growth of *Dehalococcoides* strains harboring the *vcrA* gene even when methanogen growth was inhibited. Ferrous iron influenced the growth of specific strains of methanogens and *Dehalococcoides*, and the changed microbial communities accelerated the dechlorination reaction. The findings obtained from this study may provide practical considerations for designing biostimulation with ferrous iron and/or bioaugmentation with *Dehalococcoides* and the methanogens associated with accelerated, complete dechlorination of chloroethenes *in situ*.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflicts of interest. None declared.

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