

Geochemical and microbial community determinants of reductive dechlorination at a site biostimulated with glycerol

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Summary

Biostimulation is widely used to enhance reductive dechlorination of chlorinated ethenes in contaminated aquifers. However, the knowledge on corresponding biogeochemical responses is limited. In this study, glycerol was injected in an aquifer contaminated with *cis*-dichloroethene (cDCE), and geochemical and microbial shifts were followed for 265 days. Consistent with anoxic conditions and sulfate reduction after biostimulation, MiSeq 16S rRNA gene sequencing revealed temporarily increased relative abundance of Firmicutes, Bacteroidetes and sulfate reducing Deltaproteobacteria. In line with ¹³C

cDCE enrichment and increased *Dehalococcoides mccartyi* (*Dcm*) numbers, dechlorination was observed toward the end of the field experiment, albeit being incomplete with accumulation of vinyl chloride. This was concurrent with (i) decreased concentrations of dissolved organic carbon (DOC), reduced relative abundances of fermenting and sulfate reducing bacteria that have been suggested to promote *Dcm* growth by providing electron donor (H₂) and essential corrinoid cofactors, (ii) increased sulfate concentration and increased relative abundance of Epsilonproteobacteria and Deferribacteres as putative oxidizers of reduced sulfur compounds. Strong correlations of DOC, relative abundance of fermenters and sulfate reducers, and dechlorination imply the importance of syntrophic interactions to sustain robust dechlorination. Tracking microbial and environmental parameters that promote/preclude enhanced reductive dechlorination should aid development of sustainable bioremediation strategies.

Introduction

The widespread use of chlorinated ethenes (CEs) as solvents has resulted in severe groundwater contamination (Abelson, 1990). The incomplete transformation of CEs such as tetrachloroethene (PCE) and trichloroethene (TCE) in aquifers leads to the accumulation of *cis*-dichloroethene (cDCE) and vinyl chloride (VC) (Stroo *et al.*, 2012), the latter known as a human carcinogen (Kielhorn *et al.*, 2000). *In situ* bioremediation via enhanced reductive dechlorination (ERD) has become a widely applied remediation approach (Löffler and Edwards, 2006), and is achieved by biostimulation and in some cases bioaugmentation to activate microbial reductive dechlorination by organohalide-respiration (OHR) (Ellis *et al.*, 2000; Major *et al.*, 2002; Lendvay *et al.*, 2003; Scheutz *et al.*, 2010). Organohalide-respiring bacteria (OHRB) conserve energy by OHR (Leys *et al.*, 2013) and belong to distinct bacterial genera distributed among the phyla Chloroflexi, Firmicutes, and Proteobacteria (Atashgahi *et al.*, 2016). While many OHRB can only dechlorinate PCE and TCE to cDCE,

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Dehalococcoides mccartyi (*Dcm*) is capable of dechlorinating CEs to VC and nontoxic ethene. *Dcm* has been recognized as an essential member of the microbial community linked to biotransformation of a broad range of organohalides. This is due to its highly specialized metabolism restricted to OHR, rich repertoire of reductive dehalogenase genes (*rdhAB*) ranging from 10 to 36 copies in a single genome (Löffler *et al.*, 2013) and widespread distribution in pristine and contaminated sites (Hendrickson *et al.*, 2002). *Dcm* growth and dechlorination is more robust in mixed microbial communities as it depends on other organisms to provide hydrogen and acetate as electron donor and carbon source, respectively (Löffler *et al.*, 2013), to produce organic cofactors (Schipp *et al.*, 2013), to scavenge oxygen (Hug *et al.*, 2012) and to remove the carbon monoxide that it produces but cannot tolerate (Zhuang *et al.*, 2014).

Following addition of an electron donor in contaminated aquifers, OHRB will depend on composition and activity of the resident microbial community, the local geochemical and hydrological conditions and the interactions between these factors. Aquifers are biogeochemically and hydrologically highly heterogeneous/diverse and understanding biostimulation-induced subsurface feedback demands insight in microbial community dynamics and physiology and their interactions with geochemical and hydrological parameters across space and time. This knowledge is crucial to guide design and optimization of ERD efforts but remains limited. To date, ERD-related microbial monitoring efforts have mainly focused on quantitative PCR (qPCR) based tracking of key OHRB and their *rdhA* genes (Lendvay *et al.*, 2003; Lee *et al.*, 2008; Scheutz *et al.*, 2008; Ritalahti *et al.*, 2010; Scheutz *et al.*, 2010; Damgaard *et al.*, 2013; Révész *et al.*, 2014; Sutton *et al.*, 2015b) or occasionally of selected non-dechlorinating microbial guilds (Pérez-De-Mora *et al.*, 2014). While application of qPCR as a specific assay has highly refined diagnostic power and guided bioremediation efforts, it normally assays a limited number of targets and hence provides only fragmented information regarding microbial community composition, interactions between community members and community dynamics in response to ERD. Former studies on biogeochemical trajectories of ERD either applied low resolution microbial community analysis methods such as clone libraries and terminal restriction fragment length polymorphism of PCR-amplified 16S rRNA genes (Lendvay *et al.*, 2003; Macbeth *et al.*, 2004; Rahm *et al.*, 2006) or were not complemented with detailed analysis of geochemical parameters and CE concentration dynamics (Lee *et al.*, 2012). Knowledge of the interaction between geochemical parameters and the microbial communities involved in ERD will give insight into the “black box” of processes underlying this accepted bioremediation solution. Some studies have addressed

microbial community dynamics (Dojka *et al.*, 1998; Bowman *et al.*, 2006) and interactions with geochemical parameters (Hohnstock-Ashe *et al.*, 2001; Lowe *et al.*, 2002; Imfeld *et al.*, 2008; 2011; Rossi *et al.*, 2012) at CE contaminated sites undergoing natural attenuation without ERD. However, unlike the steady-state conditions under natural attenuation, biostimulation induces perturbations in the aquifer ecosystem that can reshape both living systems and the abiotic environment (Shade *et al.*, 2012).

In this study, an *in situ* glycerol injection experiment was conducted to stimulate reductive dechlorination of cDCE and VC as products of a former TCE contamination at a site in an industrial area in Vilvoorde, Belgium (Schneidewind *et al.*, 2014). Before and after injection groundwater geochemical parameters and bacterial community composition were analyzed over a period of 265 days. In parallel, the progress of ERD was followed by enumeration of *Dcm* and relevant *rdhA* genes using qPCR and monitoring of cDCE and VC isotopic signatures using compound specific isotope analysis (CSIA). This way, we aimed to gain an integrated geochemical and microbial insight that can be used to guide the design and optimization of ERD. Results showed how temporal succession of the predominant microbial guilds governed by their known/predicted physiology and aquifer geochemical feedback promoted and/or precluded ERD. Data presented here support the notion that microbial guilds known to sustain robust *Dcm* growth/dechlorination by syntrophic interactions seem pivotal for the success of ERD.

Results

Geochemical characterisation

Geochemical parameters were stable in the control well throughout the experiment. After biostimulation, DOC in the impacted filters was significantly ($P < 0.05$) higher than before biostimulation over the experiment duration (Supporting Information Fig. S1, Table S1). The opposite pattern was observed for dissolved oxygen (DO) and oxidation-reduction potential (ORP) that declined and were significantly ($P < 0.05$) lower throughout the experiment relative to pre-biostimulation values. ORP and DO slightly increased toward the end of the experiment. Nitrate was not detected. Fe(II) concentrations varied between days 0 and 72 in the down-gradient wells but showed a significant ($P < 0.05$) reduction by days 195 and 265 relative to pre-biostimulation concentrations. This was accompanied by appearance of black precipitate indicative of ferrous sulfide formation. In most samples throughout the experiment, the Fe(II)/Fe ratio was close to 1 indicating lack of reducible Fe(III). Starting from a background concentration of 463 ± 133 mg/l, sulfate steadily decreased to 212 ± 116 mg/l by day 195 in down-gradient wells and rebounded in most wells by day 265 reaching 267 ± 180 mg/l (Supporting Information Fig. S1, Table S1).

Reductive dechlorination of CEs, CSIA, and qPCR analysis

The pre-stimulation carbon isotope values of cDCE (average $\delta^{13}\text{C}$, $-19.3\text{‰} \pm 0.6$) and the sum of carbon isotope values of all CEs weighted by their molar fractions (average $\delta^{13}\text{C}_{\Sigma(\text{CE})}$, $-21.4\text{‰} \pm 0.8$) were highly consistent in the seven filters later impacted by biostimulation. By day 195, there was clear onset of cDCE dechlorination with concomitant increase in its $\delta^{13}\text{C}$ values and increased VC concentration in most filters. $\delta^{13}\text{C}$ of VC remained more negative compared with cDCE in shallow filters of 2A, 3A and 7A and VC was not further transformed to ethene. Further degradation of cDCE to VC was observed at day 265 with progressive enrichment of cDCE in ^{13}C . In addition, a significant increase (3‰) in the resultant $\delta^{13}\text{C}_{\Sigma(\text{CE})}$ relative to the control well provided evidence for incipient dechlorination of VC in filters 2A, 2B, and 7A between days 195 and 265 (Fig. 1, Supporting Information Table S2) (Hunkeler *et al.*, 2008). The evidence from CE

concentrations and isotopic signatures was further corroborated by over 1–2 orders of magnitude increased abundances of *Dcm* and *vcrA* and *bvcA* genes in the impacted wells relative to the control well. Among the *rdh* genes, *bvcA* became dominant in most filters, whereas *vcrA* was dominant in well 2 reaching above 10^7 copies/l by day 265. No ERD was noted in well 5 and the control well, in which cDCE concentration as well as isotopic and qPCR signatures were stable (Fig. 1, Supporting Information Table S2).

Impact on bacterial community alpha-diversity

Biostimulation significantly affected bacterial community alpha-diversity. The post-biostimulation samples (seven samples at each sampling time point, excluding control well samples) showed significantly higher ($P < 0.05$) predicted (Chao 1) and observed OTU richness, and phylogenetic diversity (PD) relative to the pre-biostimulation

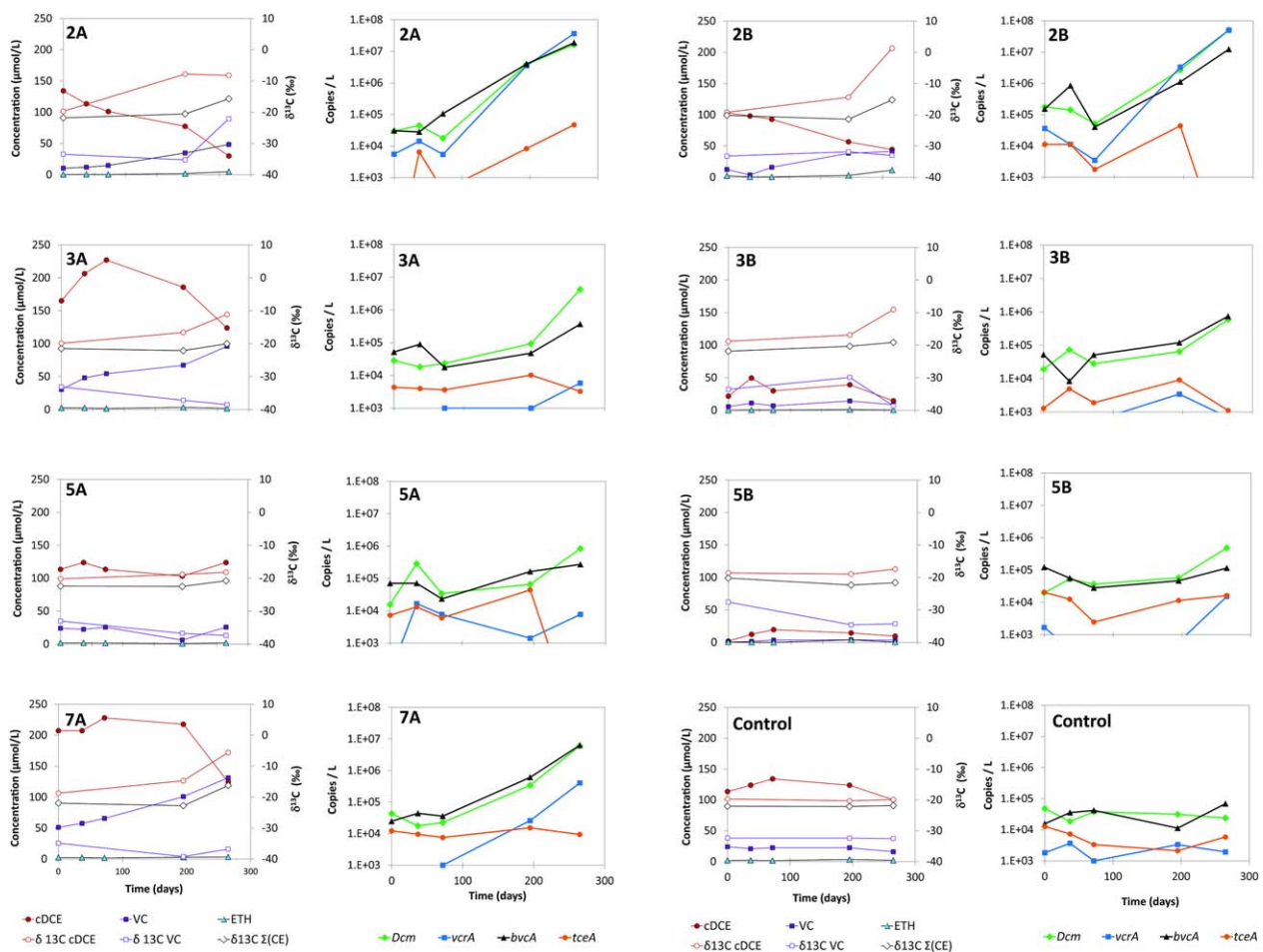


Fig. 1. Progress of reductive dechlorination over time. Left: concentrations of cDCE, VC, ethene, $\delta^{13}\text{C}$ of cDCE and VC and concentration-weighted average of CE $\delta^{13}\text{C}$ values ($\delta^{13}\text{C}_{\Sigma(\text{CE})}$). Right: qPCR quantification of 16S rRNA gene copy numbers of *Dcm* and reductive dehalogenase encoding *vcrA*, *bvcA*, and *tceA* genes. Each qPCR value represents the average of triplicate reactions. ETH, ethene.

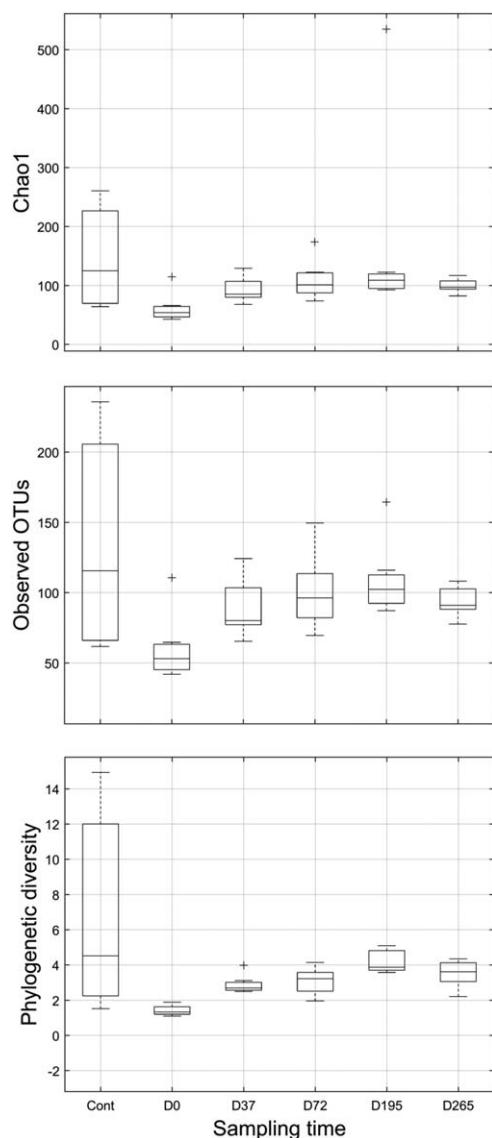


Fig. 2. Predicted (Chao 1) and observed OTU richness, and phylogenetic diversity in the control (five samples) and impacted filters at each sampling campaign (seven samples). D, day; Cont, control well.

samples (Fig. 2). No significant difference was found between the post-biostimulation sampling dates, except for the PD at day 195 that was significantly higher ($P < 0.05$) than at days 37 and 72 (Fig. 2). The control well showed progressively increasing alpha-diversity over time until day 195 (Supporting Information Fig. S2).

Bacterial community succession

The pre-biostimulation bacterial community composition showed high between-sample similarity including the control samples (Supporting Information Fig. S3), and was significantly different from all post-biostimulation communities

(Supporting Information Table S4). Remarkably, among the post-biostimulation communities, samples of day 265 were not significantly different from those taken at the control well (Supporting Information Fig. S3, Table S4). At day 0, the bacterial community was dominated by Campylobacteriales (Epsilonproteobacteria) followed by Flavobacteriales (Bacteroidetes), representing 75% and 18% of the average relative abundance (ARA) respectively (Fig. 3, Supporting Information Table S3). Recorded Campylobacteriales comprised members of *Sulfuricurvum*, *Sulfurospirillum*, and *Arcobacter* genera, while *Flavobacterium* was the main genus within Flavobacteriales (Supporting Information Fig. S4, Table S3). Following biostimulation, clear shifts in the bacterial community structure were noted. Except in the 2B and 7A filters that did not show DOC change at day 37 (Supporting Information Fig. S1, Table S1), Campylobacteriales and Flavobacteriales were greatly reduced in ARA relative to day 0 and replaced by fermentative members of the Firmicutes (Clostridia and Bacilli orders) and Bacteroidetes (Bacteroidia order) and by sulfate reducing Deltaproteobacteria (Desulfobacteriales order) (Fig. 3, Supporting Information Table S3). Within the Campylobacteriales members of the genus *Sulfurospirillum* became predominant whereas *Sulfuricurvum*, *Arcobacter*, and *Flavobacterium* showed drastic reduction in ARA. Members of the genus *Trichococcus* (Bacilli) within the Firmicutes increased substantially in relative abundance in 5A and 5B filters (Supporting Information Fig. S4, Table S3) that received the highest DOC input, whereas Clostridia genera of *Acetobacterium*, *Clostridium*, and *Pelosinus* were noted in most filters. By day 72, Campylobacteriales and Flavobacteriales dropped to below 20 and 1% ARA, respectively in impacted filters. In contrast, the sulfate reducing Desulfobacteriales dominated by *Desulfobulbus* genus flourished followed by Bacteroidia, Clostridia, and Bacilli. Desulfobacteriales continued to dominate communities observed in day 195 samples while Bacteroidia, Clostridia, and Bacilli were fading. Interestingly, at day 195 and day 265, the Campylobacteriales became predominant again in 5A and 5B filters, reaching 60% and 30% in relative abundance respectively. Instead, Desulfobacteriales were reduced in ARA at day 265. Dehalococcoidetes and *Geobacter* did not surpass 1% of the community at any time point, and other OHRB including *Dehalobacter*, *Desulfitobacterium*, *Desulfomonile*, *Desulfuromonas* were not detected. Furthermore, there was notable emergence of sequences associated with Deferribacteriales (Deferribacteres) and the candidate phylum OD1 by days 195 and 265, which were not observed in pre-biostimulation samples. The bacterial community showed a distinct succession pattern in the control well compared with stimulated wells. While Campylobacteriales was the most predominant taxon at days 0 and 37, their relative abundance dropped to 41% by day 72 and below 2% by days 195 and 265. The community in the control well became

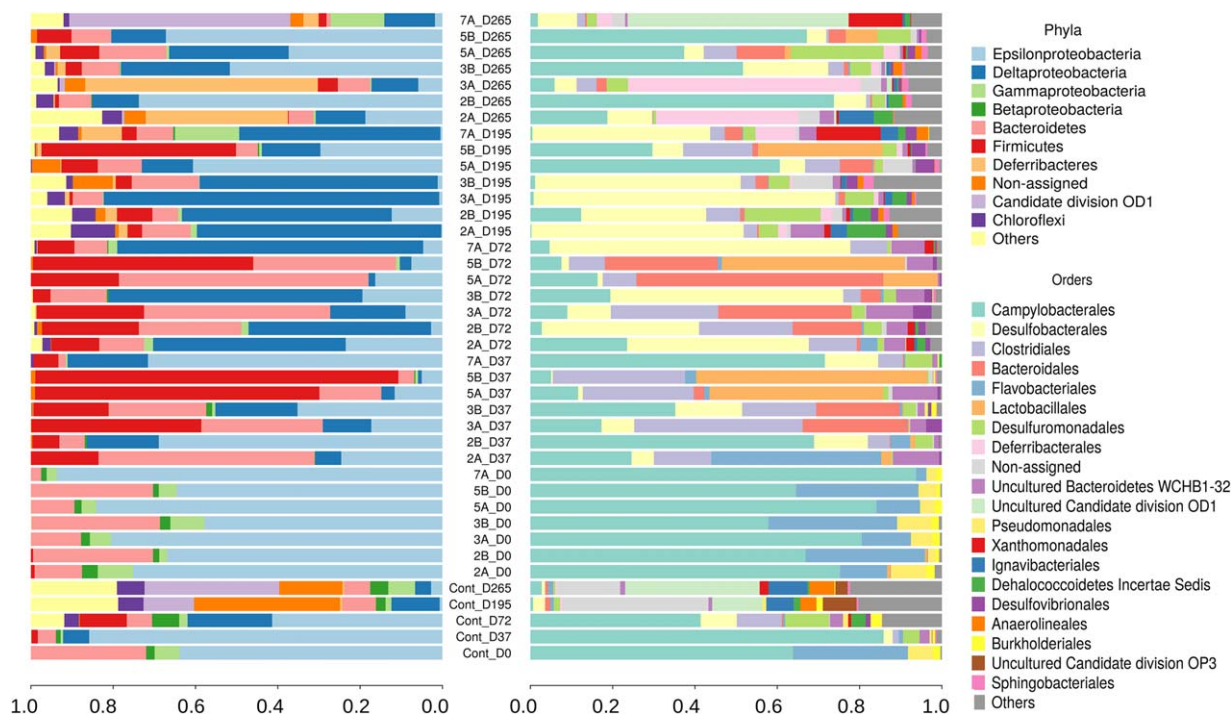


Fig. 3. Distribution of the most abundant phyla (>1% average relative abundance across all samples, left panel) and orders (>0.1% average relative abundance across all samples, right panel) in the control and impacted filters. D, day; Cont, control well.

more diverse over time (Fig. 2) with increased abundance of non-assigned reads and candidate phylum OD1 (Fig. 3, Supporting Information Table S3).

Geochemical and microbial interactions

The relative abundances of the orders Campylobacterales, Flavobacteriales, Burkholderiales and Pseudomonadales were positively correlated with DO, ORP, and sulfate (Fig. 4). Putative fermenters belonging to Clostridiales, Lactobacillales, Bacteroidales and uncultured Bacteroidetes WCHB1-32 showed a positive correlation with DOC whereas DO was inversely correlated with the relative abundance of Clostridiales and uncultured Bacteroidetes WCHB1-32. A negative correlation was noted between Fe(II)/Fe and Dehalococcoidetes.

Discussion

There is growing interest in using the capacities of indigenous microbial communities to remediate CE contaminated aquifers by ERD. However, the ERD bioremediation efforts are mostly applied as a “black box” without having fundamental insight into the underlying biogeochemical processes that govern the activity of key OHRB. To improve CE contaminated site bioremediation, the biogeochemical shifts induced by biostimulation must be understood, and the abundance and activity of key OHRB must be placed into the context of interlinked microbial networks,

including the supporting/competing electron donor/acceptor processes.

Geochemical and microbial dynamics: pre-biostimulation phase

The almost complete removal of TCE and the dominance of cDCE before biostimulation indicated naturally occurring reductive dechlorination and hence TCE depletion (Supporting Information Table S4) formerly reported to be present at this site (Schneidewind *et al.*, 2014). This could be an explanation for the presence of *Sulfurospirillum* as non-obligate dechlorinator of TCE to cDCE (Luijten *et al.*, 2003) in the pre-biostimulation samples that was also reported from other TCE contaminated aquifers (Macbeth *et al.*, 2004; Rahm *et al.*, 2006; Dugat-Bony *et al.*, 2012). By dechlorinating PCE and TCE to cDCE, *Sulfurospirillum* can facilitate CE dechlorination by *Dcm*. For example, synergistic interactions between *Sulfurospirillum* and *Dcm* members were reported for enrichment cultures where *Sulfurospirillum* dechlorinated PCE to cDCE and *Dcm* dechlorinated cDCE to ethene (Maillard *et al.*, 2011). These reports imply a potential role of *Sulfurospirillum* at CE contaminated sites. *Sulfurospirillum multivorans* has a distinct genomic region linked to OHR (Goris *et al.*, 2014) that can be used as a target for design of molecular monitoring tools. Some members of this genus are capable of chemolithoautotrophic growth by coupling nitrate and

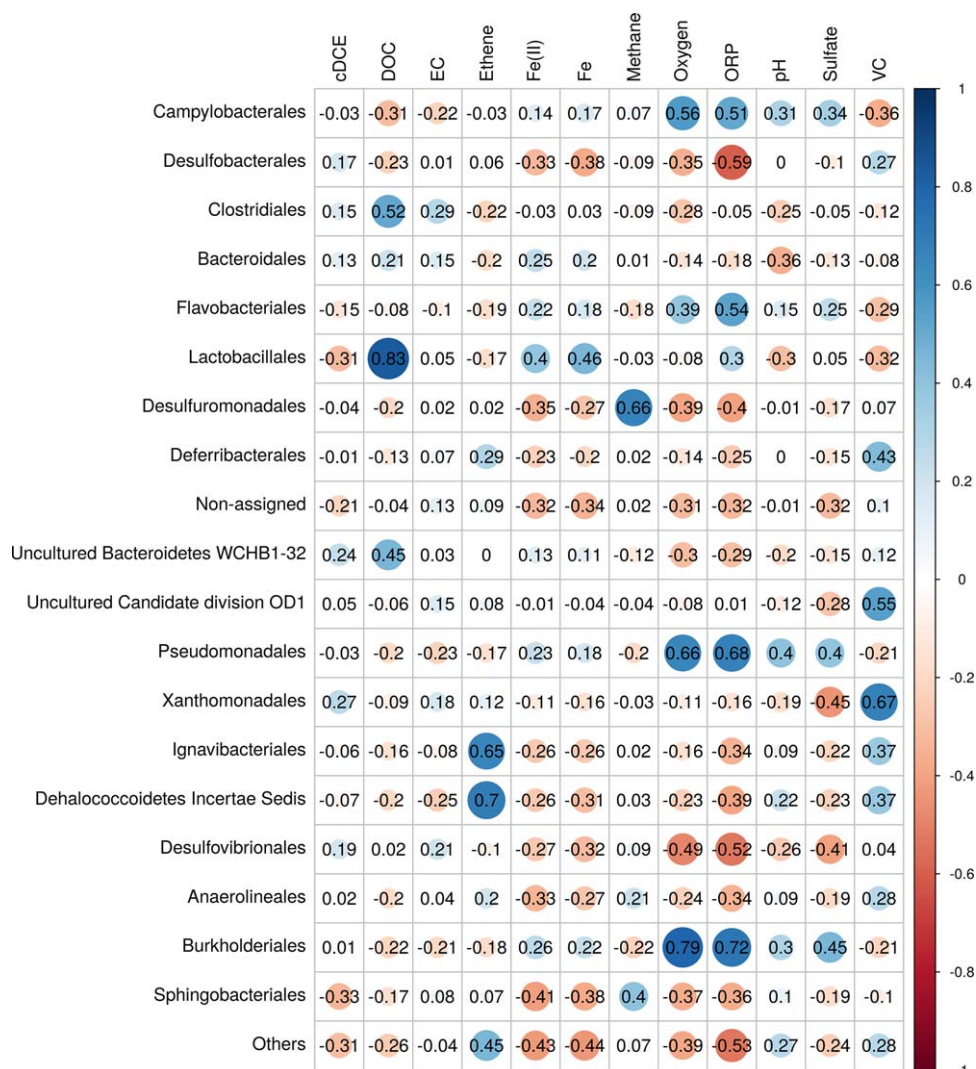


Fig. 4. Correlation between bacterial orders and geochemical parameters. A color gradient from dark blue to dark red depicting the strength and direction of the correlation is given.

oxygen reduction to the oxidation of sulfide, sulfur, and thiosulfate (Eisenmann *et al.*, 1995; Campbell *et al.*, 2006) and therefore, their growth using sulfur released into groundwater from soils, rocks, and minerals (Canfield, 2004) coupled to oxygen reduction cannot be excluded. *Sulfuricurvum* as another predominant genus currently contains only one characterized isolate, *Sulfuricurvum kujiense* strain YK-1, an obligate chemolithoautotroph (Kodama and Watanabe, 2004) that grows by oxidation of reduced sulfur compounds coupled to nitrate and oxygen (microaerophilic condition) respiration (Kodama and Watanabe, 2003). Although dechlorination or syntrophic interactions with OHRB by this strain are not reported, *Sulfuricurvum* was detected at TCE contaminated sites (Tsai *et al.*, 2014; Kao *et al.*, 2016) and in 1,2-dichloroethane dechlorinating enrichment cultures (Merlino *et al.*, 2015). Another predominant genus in pre-biostimulation samples was *Flavobacterium* whose members play an important role in decomposition of organic materials by hydrolysing

organic polymers such as proteins and polysaccharides produced from cell debris in oligotrophic environments (Bernardet and Grimont, 1989; Bernardet *et al.*, 1996).

Geochemical and microbial dynamics: post-biostimulation phase

Glycerol injection induced cDCE concentration reduction was accompanied by a shift of carbon isotope values. The most dynamic period was after day 72 during which the average $\delta^{13}\text{C}$ cDCE values increased by +3.9‰ at day 195 and by +5.7‰ at day 265 (Fig. 5). This development was reflected by a successional change in groundwater bacterial community composition. There was a sharp increase in Firmicutes (Clostridiales and Lactobacillales) ARA followed by Bacteroidetes (Bacteroidales). These taxa that also showed positive correlation to DOC (Fig. 4) had low initial ARA and decreased again toward the end of the field experiment. Other studies at CE contaminated

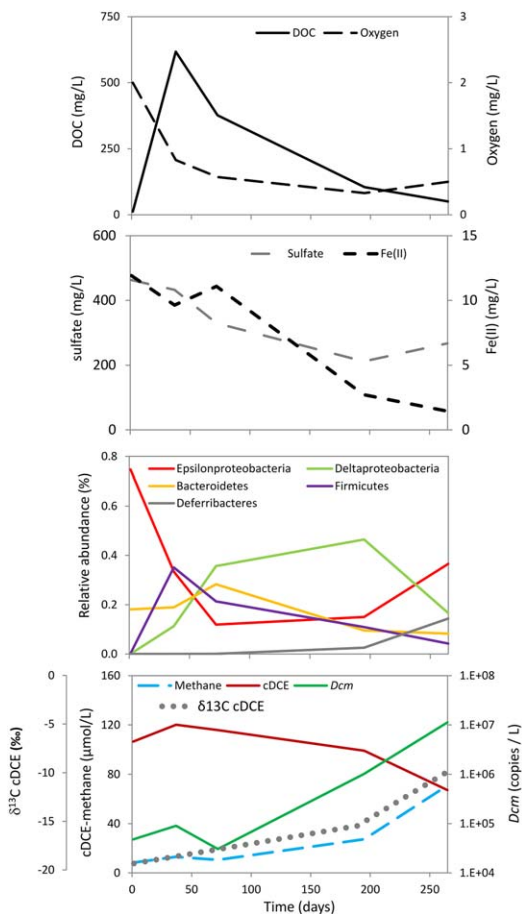


Fig. 5. Summary of major geochemical and microbial evolution before and after biostimulation (left), and a conceptual model of glycerol degradation based on the observed taxa from MiSeq analysis and their known/putative physiology (right). The proposed sulfur cycle is shown in red dotted square and the putative corrinoid supply to *Dcm* is shown by dashed gray arrows. All values are averages of the seven impacted filters. Error bars are not shown for clarity. PDO, 1,3-propanediol; SCFA, short chain fatty acids.

sites reported a similar transient peak of these microbes upon biostimulation (Macbeth *et al.*, 2004; Lee *et al.*, 2012; Pérez-De-Mora *et al.*, 2014). The emergence of members of the genera *Trichococcus*, *Clostridium*, and *Pelosinus* following biostimulation (Supporting Information Fig. S4, Table S3) is likely due to their glycerol fermentation capacity (Biebl, 2001; van Gelder *et al.*, 2012; Moe *et al.*, 2012; Wilkens *et al.*, 2012). Moreover, *Pelosinus* strains were shown to sustain growth of *Dcm* by providing the necessary corrinoids (Men *et al.*, 2014; 2015) and were found to co-occur with OHRB in CE contaminated aquifers (Puigserver *et al.*, 2016). The observed putative acetogenic bacteria belonging to *Acetobacterium*, *Clostridium*, and Spirochaetaceae (Supporting Information Fig. S4, Table S3) have been proposed to stimulate *Dcm* growth by production of acetate and corrinoid cofactors (Macbeth *et al.*, 2004; He *et al.*, 2007; Pérez-De-Mora *et al.*, 2014).

Sulfate reduction appeared to be the prevalent electron-accepting process at the site. Between days 37 and 195, Desulfobulbaceae flourished that can perform incomplete oxidation of propionate coupled to sulfate reduction (Rabus *et al.*, 2006) yielding acetate and sulfide that can stimulate (Löffler *et al.*, 2013) and inhibit (He *et al.*, 2005) *Dcm* growth respectively. On the other hand, both glycerol and

its degradation product 1,3-propanediol can be converted to acetate using sulfate as electron acceptor by members of the genus *Desulfovibrio* (Qatibi *et al.*, 1991; 1998) that were present during the same period. Furthermore, *Desulfovibrio* strains are known to sustain *Dcm* growth by providing hydrogen, acetate, and corrinoid cofactors (Men *et al.*, 2012). However, sulfate at >500 mg/l as observed in 3B and 7A filters (Supporting Information Fig. S1, Table S1) might be inhibitory to *Dcm* due to electron donor exhaustion (Hoelen and Reinhard, 2004), though this seems unlikely on the basis of the observed dechlorination at these filters (Fig. 1). Methanogenesis as a sink of hydrogen and acetate (Smatlak *et al.*, 1996) did not seem to be a major concern for OHR except at filters 5A and 5B that showed the highest DOC levels upon biostimulation and enhanced methanogenesis by day 265 (Supporting Information Fig. S1, Table S1) which can be an explanation for the lack of dechlorination at these filters.

Geochemical and microbial dynamics: the control well

Despite stable geochemical parameters in the up-gradient control well, the bacterial community observed at days 195 and 265 highly diverged from the original state. This could

be due to the high hydraulic conductivity of 1–3 m/day at this site (Hamonts *et al.*, 2009) facilitating a rapid dispersion and community succession (Zhou *et al.*, 2014). Accordingly, the pre-biostimulation bacterial community in the stimulated wells was significantly different from the control well (Supporting Information Table S4) whereas the geochemical parameters were similar between the pre-biostimulation and control well samples (Supporting Information Fig. S1, Table S1). On the other hand, the bacterial community in the stimulated wells at day 265 was significantly different from the pre-biostimulation community but not from the control well (Supporting Information Table S4). This indicates that the resident taxa in the impacted wells exploiting the stimulated conditions were also exposed to the arriving immigrant taxa brought by groundwater flow, in line with what has previously been suggested by Shade *et al.* (2012). However, it was only toward the end of the experiment (and hence fading stimulation) that the immigrant taxa became established in stimulated wells which can be described by the niche occupation concept outlined in Supporting Information Fig. S5. The observed geochemical stability but compositional instability of the control well over time indicates the necessity of previous knowledge of community behavior in relation to site geochemistry/hydrology as baseline prior to biostimulation to better guide intensity, duration, and location of post-biostimulation sampling.

Putative sulfur cycle following biostimulation

After the dominance of sulfate reducers between days 37 and 195 and concurrent sulfate depletion, sulfate concentrations increased again in most wells at day 265. This was accompanied by a substantial reduction of Desulfobulbaceae ARA, re-establishment of initially abundant *Sulfuricurvum* and *Sulfurospirillum*, and appearance of putative sulfur oxidizers, i.e. *Deferribacteres* and the candidate phylum OD1, which were barely detectable in pre-biostimulation samples (Fig. 3). The chemolithoautotrophic oxidation of the reduced sulfur compounds by *Sulfuricurvum* and *Sulfurospirillum* coupled to reduction of DO that was slightly increasing during the late phase may explain the increased sulfate levels. Accordingly, the epsilonproteobacterial Campylobacterales showed positive correlation with DO and ORP (Fig. 4). Enrichment of Epsilonproteobacteria as the successors of Deltaproteobacteria was previously reported during biostimulation of aquifers contaminated with uranium (Handley *et al.*, 2012; 2013) and CEs (Lee *et al.*, 2012). The resulting reduced sulfide levels can in turn support reductive dechlorination by decreasing the toxicity of sulfide to *Dcm* (Hoelen and Reinhard, 2004; He *et al.*, 2005).

Emergence of candidate phylum OD1

Members of the candidate phylum OD1 (also known as Parcubacteria) (Brown *et al.*, 2015) have mostly been connected with suboxic and anoxic pristine sulfur-rich environments (Elshahed *et al.*, 2005; Bri e *et al.*, 2007; Peura *et al.*, 2012). However, OD1 (Gihring *et al.*, 2011; Kocur *et al.*, 2016) and another candidate division, OP11 (Microgenomates), (Lee *et al.*, 2012) also appeared at a later stage during aquifer biostimulation for uranium and CE bioremediation (Elshahed *et al.*, 2005; Wrighton *et al.*, 2012). Interestingly, the study of Kocur *et al.* (2016) showed dominance of OD1 where the environment was not strongly reduced (–80 to –140 mV). This may explain their increased relative abundance at a later aquifer biostimulation stage when the redox potential increased and organic-rich condition faded. Owing to their fermentative lifestyle, the production of hydrogen and organic acids (e.g., acetate) from complex organic materials such as decaying biomass from microbial blooms by these candida phyla can fuel respiratory processes with nitrate, sulfate, and Fe(III) (Wrighton *et al.*, 2012; 2014) and perhaps CEs as terminal electron acceptors. Interestingly, OD1 has not been reported as member of CE dechlorinating communities in enrichment cultures (Richardson *et al.*, 2002; Dennis *et al.*, 2003; Duhamel *et al.*, 2004; Gu *et al.*, 2004; Freeborn *et al.*, 2005; Yang *et al.*, 2005; Duhamel and Edwards, 2006; Daprato *et al.*, 2007; Rowe *et al.*, 2008; Hug *et al.*, 2012; Sutton *et al.*, 2015a) that usually receive constant supply of easily accessible electron donors. As such, the OD1 is presumably outcompeted by fast-growing microbes and exterminated from the dechlorinating enrichment cultures, circumventing the services provided by this phylum. In line with this hypothesis, OD1 was recently shown as a persistent member of benzene-degrading methanogenic enrichment cultures where the slowly degradable benzene was used as the sole electron donor (Luo *et al.*, 2016).

Resilient and sensitive taxa

Epsilonproteobacterial *Sulfuricurvum* and *Sulfurospirillum* recovered in ARA toward the end of the experiment, though remarkably only in stimulated wells. Resilience of the members of *Sulfurospirillum* spp. was previously reported in permanganate treated PCE dechlorinating enrichment cultures (Sutton *et al.*, 2015a). This could be due to adaptation of some members like *S. multivorans* to oxic condition by hosting genes encoding key enzymes for detoxification of reactive oxygen species (Goris *et al.*, 2014) and protection of PCE dechlorinating enzyme (PceA) by a peroxidase-like protein (Goris *et al.*, 2015). In contrast, the initially predominant genera *Arcobacter* and *Flavobacterium* did not recover, which could be due to their

high sensitivity to biostimulation-induced perturbation. However, they were also barely detectable from the up-gradient control well toward the end of the experiment indicating a role of other factors such as hydrology in controlling community succession.

Conclusions and perspectives

Based on the observed geochemical, isotopic, and bacterial patterns, a conceptual model of the metabolic interactions within the microbial foodweb during biostimulation was proposed (Fig. 5). cDCE dechlorination was only noted by day 195 and it was not fully converted to ethene likely due to the fading organic-rich condition toward the end of the experiment. Remarkably, during the same period, the relative abundance of non-dechlorinating fermenters and sulfate reducers decreased that are considered to stimulate robust *Dcm* dechlorination by providing organic cofactors such as the key vitamin B12 (Hug *et al.*, 2012; Men *et al.*, 2012). The increased relative abundance of Deferribacteres and Epsilonproteobacteria by the end of the field experiment likely supported the *Dcm* population by reducing sulfide level/toxicity. However, to our knowledge, these taxa are not known to provide the more important organic cofactors such as vitamin B12 needed by *Dcm*, hence they are not likely “sufficient” as companions to sustain *Dcm* growth. In line with this, other studies showed the importance of particular syntrophic partners belonging to Firmicutes and Deltaproteobacteria in providing the “right” cobamide lower base of vitamin B12 that is vital for *Dcm* dechlorination (Men *et al.*, 2014; 2015) but cannot be provided even by closely related microbes (Yan *et al.*, 2012; 2013).

In case of incomplete dechlorination, biostimulation is performed together with bioaugmentation with dechlorinating enrichment cultures containing *Dcm* populations but also their non-dechlorinating partners (Rahm *et al.*, 2006; Pérez-De-Mora *et al.*, 2014). In fact, sustained presence of non-dechlorinating guilds that support *Dcm* growth might be the key to success of bioaugmentation as opposed to the initial biostimulation. Accordingly, a recent field biostimulation failed to induce complete dechlorination while dechlorination to ethene was achieved after bioaugmentation. This was concurrent with enrichment of Bacteroidetes while *Dcm* and *vcrA* concentrations were rather stable (Pérez-De-Mora *et al.*, 2014). Therefore, in addition to monitoring dechlorinating guilds, future ERD monitoring efforts must consider composition and successional patterns of supportive non-dechlorination community members as well as geochemical factors controlling them to ensure robust *Dcm* growth and activity. This knowledge is pivotal to establish and maintain the required syntrophic relationships for OHR under challenging field conditions.

Experimental procedures

Site description, treatment, and sampling procedures

At the contaminated site, a 1.4 km-wide groundwater plume mainly contaminated with cDCE flows toward the Zenne River in a northwesterly direction (Supporting Information Fig. S6). Four injection wells were used to inject a single pulse of a glycerol:water mixture (1: 3 v/v) by sonic drilling injection, at 20 l per vertical meter from 7 to 13 m below surface (mbs) at each injection point. Eight monitoring wells were used for groundwater sampling and monitoring. Seven wells were located downstream of the injection points (wells 1–7) and one upstream (well 0, designated as the control well). All monitoring wells contained a shallow filter at 7–9 mbs (A-filters) and a deep filter at 11–13 mbs (B-filters), except the control well that only contained a shallow filter. Groundwater was sampled from the control well and the wells impacted by glycerol injection (wells 2, 3, 5, 7A; Supporting Information Fig. S6) on day 0 (March 15th 2011) before glycerol injection, and 37, 72, 195, and 265 days after injection as outlined in supplementary information.

DNA extraction and qPCR

For DNA extraction, 2 l groundwater samples were vacuum-filtered over a 0.22 µm membrane filter (Millipore, USA), and filters were stored at –80°C. Filters were cut into small strips for DNA extraction by the FastDNA Spin Kit for Soil (MP Bio-medicals, Santa Ana, CA). qPCRs were performed in triplicate in 25-µl reactions in an iQ5 iCycler using the iQ SYBR Green Supermix kit (Bio-Rad, Veenendaal, the Netherlands). A list of target genes, primers, and thermal cycling conditions for qPCRs is shown in Supporting Information Table S5. Standard curves were obtained using serial dilutions of a known concentration of plasmid DNA containing a suitable fragment of the target genes.

Bacterial community analysis

A two-step PCR strategy was used to generate barcoded amplicons from the V1-V2 region of the 16S rRNA gene (primers and thermal cycling conditions shown in Supporting Information Table S6). The first PCR (50 µl) contained 10 µl 5× HF buffer (Thermo Scientific™, the Netherlands), 1 µl dNTP Mix (10 mM; Promega, Leiden, the Netherlands), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific™), 500 nM of 27F-DegS forward primer, 500 nM of 338R I and II reverse primers, and 1 µl template DNA (15–20 ng/µl). The forward and reverse primers were appended at the 5' end with 18 bp Universal Tag (Unitag) 1 and 2 respectively. The PCR product size was examined by gel electrophoresis. The second PCR (100 µl) contained 20 µl 5× HF buffer, 2 µl dNTP Mix, 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase, 500 nM of a forward and reverse primer equivalent to the Unitag1 and Unitag2 sequences, respectively, that were each appended with an 8 nt sample specific barcode (Ramiro-Garcia *et al.*, 2016) at the 5' end, and 5 µl PCR product of the first reaction. The PCR product size was examined by gel electrophoresis, purified with HighPrep™ (Magbio Genomics, Rockville, MD, USA) and quantified using a Qubit

2.0 Fluorometer (Life Technologies, Darmstadt, Germany) in combination with the dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA). Purified PCR products were pooled, underwent adaptor ligation and sequenced on a MiSeq platform (GATC-Biotech, Konstanz, Germany).

Analysis of the MiSeq data

NG-Tax, an in-house pipeline (Ramiro-Garcia *et al.*, 2016) was used for the analysis of the 16S rRNA gene sequencing data. In brief, paired-end libraries were filtered to contain only read pairs with perfectly matching barcodes, and those barcodes were used to separate reads by sample. Finally operational taxonomic units (OTUs) were assigned using an open reference approach and a customized SILVA 16S rRNA gene reference database (Quast *et al.*, 2013). Bacterial composition plots were generated using a workflow based on Quantitative Insights Into Microbial Ecology (QIIME) v1.2 (Caporaso *et al.*, 2010).

Chemical and carbon isotope analysis

Concentrations of CEs, ethene, ethane, and methane in groundwater samples were determined on a Varian GC-FID (CP-3800) as described previously (Atashgahi *et al.*, 2013). Sulfate concentrations were analyzed by ion chromatography using a Dionex DX-120 ion chromatograph equipped with a Dionex AS14A column (Dionex, Sunnyvale, CA). Concentrations of aqueous Fe(II), total Fe and NO₃-N were determined using HACH kits (HACH, USA) according to the manufacturer's instructions. Dissolved organic carbon (DOC) was determined from samples as the difference between total dissolved carbon and dissolved inorganic carbon, measured with a Shimadzu TOC-5000 analyzer equipped with an ASI-5000 auto-sampler. The stable carbon isotope composition of CEs in the groundwater samples were determined as described in supporting information. Concentration-weighted average values of the isotope signature for CEs ($\Sigma(\text{EC})$) was calculated by weighting compound-specific values $\delta^{13}\text{C}$ weighted by their molar fractions (x) (Aeppli *et al.*, 2010):

$$\delta^{13}\text{C}_{\Sigma(\text{CE})} = \delta^{13}\text{C}_{\text{DCE}}x_{\text{DCE}} + \delta^{13}\text{C}_{\text{VC}}x_{\text{VC}}$$

Statistical analysis

To compare microbial communities observed at different times and locations, different statistical approaches were used based on Bray–Curtis distances. PERMANOVA (Permutational Multivariate Analysis of Variance) (Anderson, 2001) and ANOSIM (Analysis of Similarity) (Clarke, 1993) were performed using the Fathom Toolbox for Matlab (Jones, 2015). We used 10⁴ permutations to assess significance of observed differences. MixOmics R package (Lê Cao *et al.*, 2009; González *et al.*, 2012) was used to integrate microbiota abundance data and geochemical parameters and to perform regression analysis. Sparse partial least squares was used for simultaneous variable selection and integration to avoid selection of spurious associations. Geochemical and microbial data were set as dependent and independent variables

respectively. Interchanging both sets did not significantly impact the results. An additional threshold on correlation values was imposed by retaining only the 20% most relevant associations in the final network.

Nucleotide sequences

Nucleotide sequence data reported are available at the European Bioinformatics Institute under accession number PRJEB13312.

Acknowledgements

This study was supported by a VITO/KU Leuven PhD scholarship (EU FP7 project SQUAREHAB, grant 226565) to S Atashgahi. Furthermore, S Atashgahi and H Smidt received support by a grant of BE-Basic-FES funds from the Dutch Ministry of Economic Affairs and D Springael by the Inter-University Attraction Pole (IUAP) “ μ -manager” of the Belgian Science Policy (BELSPO, P7/25). We thank Richard Lookman for his assistance in the field experiment and acknowledge the China Scholarship Council for the support to Y Lu and Y Zheng.

Conflict of Interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Groundwater geochemical parameters. Scales on Y-axes are: dissolved organic carbon (DOC) (mg/l), electrical conductivity (EC) ($\mu\text{S}/\text{cm}$), Fe (mg/l), Fe(II) (mg/l), methane ($\mu\text{g}/\text{l}$), oxidation-reduction potential (ORP) (mV), oxygen (mg/l), sulfate (mg/l). Cont: control well.

Fig. S2. Predicted (Chao 1) and observed OTU richness, and phylogenetic diversity at each filter over time (five samples per filter). D: day; Cont: control well.

Fig. S3. Ordination of community composition by nonmetric multidimensional scaling (NMDS) based on Bray–Curtis distances. D: day; Cont: control well.

Fig. S4. Distribution of the most abundant phyla (>1% average relative abundance across all samples, left panel) and genera (>0.7% average relative abundance across all samples, right panel) in the control (Cont) and impacted filters. D: day.

Fig. S5. Niche occupation concept. According to phylogenetic instability in the control well (well 0 in left figure), groundwater brings in immigrant microbes (the arrow from the control well toward the stimulated zone or active growth zone). The resident taxa in the stimulated wells exploit the stimulated conditions and occupy the available niches in the active growth zone. However, they were exposed to the newly arriving taxa brought by natural groundwater flow but it is only toward the end of the experiment (and hence fading stimulation) that the immigrants pass the threshold after day 195 (dashed line in right figure) and become established in the stimulated wells by day 265.

Fig. S6. Schematic presentation of the study site close to the Zenne River. Monitoring wells (black circles) and injection wells used for glycerol delivery (red stars) at the site are indicated. Well 0, located upstream of the injection wells, is taken as control. Samples were taken from the monitoring wells impacted by glycerol injection (shown in the dashed oval), i.e. well 2, 3, 5 and 7 (only shallow filter, 7A) as well as for the control well throughout the study.

Table S1. Groundwater geochemical parameters. Cont: control well.

Table S2. Concentrations of TCE, cDCE, VC, ethene and $\delta^{13}\text{C}$ of cDCE and VC and concentration-weighted average values of the isotope signature ($\delta^{13}\text{C}_{\Sigma\text{CCE}}$). Right: qPCR quantification of 16S rRNA gene copy numbers of *Dcm*, and *vcrA*, *bvcA* and *tceA* genes. Each qPCR value represents the average value obtained from triplicate reactions. NA: not analyzed; ETH: ethene.

Table S3. Distribution of the most abundant phyla, orders and genera in the control and impacted filters. D: day; Cont: control well.

Table S4. Significance tests of the differences of microbial communities at order level. The groups with no significant differences ($P > 0.05$) are shown in bold.

Table S5. Overview of primer sequences and thermal cycling conditions used for qPCR in this study.

Table S6. Overview of MiSeq primers and Unitags and thermal cycling conditions.