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Coexistence of two distinct *Sulfurospirillum* populations respiring tetrachloroethene – genomic and kinetic considerations

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Abstract

Two anaerobic bacterial consortia, each harboring a distinct *Sulfurospirillum* population, were derived from a ten year old consortium, SL2, previously characterized for the stepwise dechlorination of tetrachloroethene (PCE) to *cis*-dichloroethene (*cis*-DCE) via accumulation of trichloroethene (TCE). Population SL2-1 dechlorinated PCE to TCE exclusively, while SL2-2 produced *cis*-DCE from PCE without substantial TCE accumulation. The reasons explaining the long-term coexistence of the populations were investigated. Genome sequencing revealed a novel *Sulfurospirillum* species, designated '*Candidatus* Sulfurospirillum diekertiae', whose genome differed significantly from other *Sulfurospirillum* spp. (78-83% ANI). Genome-wise, SL2-1 and SL2-2 populations are almost identical, but differences in their tetrachloroethene reductive dehalogenase sequences explain the distinct dechlorination patterns. An extended series of batch cultures were performed at PCE concentrations of 2-200 μM. A model was developed to determine their dechlorination kinetic parameters. The affinity constant and maximal growth rate differ between the populations: the affinity is 6- to 8-fold higher and the growth rate 5-fold lower for SL2-2 than SL2-1. Mixed cultivation of the enriched populations at 6 and 30 μM PCE showed that a low PCE concentration could be the driving force for both functional diversity of reductive dehalogenases and niche specialization of organohalide-respiring bacteria with overlapping substrate ranges.

Keywords

Organohalide respiration, *Sulfurospirillum*, reductive dehalogenase, tetrachloroethene, kinetics, numerical modeling

Introduction

Organohalide respiration (OHR) is a key bacterial metabolic pathway for the biodegradation of a wide range of halogenated compounds such as tetrachloroethene (PCE), a major groundwater pollutant (Adrian & Löffler, 2016). Organohalide-respiring bacteria (OHRB) are spread among many phylogenetic groups and share the capability to produce reductive dehalogenases, the catalytic enzymes directly involved in the dehalogenation reaction (Atashgahi*, et al.*, 2016). The major known PCE-respiring bacteria belong to the genera *Dehalococcoides*, *Dehalobacter*, *Desulfitobacterium* and

Sulfurospirillum, of which the two latter are facultative OHRB. The range of organohalide substrates converted by these genera varies extensively within *Dehalococcoides* (Zinder, 2016) and *Dehalobacter* (Maillard & Holliger, 2016), exhibiting a wider range than for *Desulfitobacterium* (Futagami & Furukawa, 2016) and *Sulfurospirillum* (Goris & Diekert, 2016). This broader organohalide substrate range is also reflected by the number and diversity of reductive dehalogenase homologous genes (*rdhA*) present in the available genomes. Although the sequences of identified PCE reductive dehalogenases (PceA) vary significantly, all of them except one are able to dechlorinate PCE to *cis*-dichloroethene (*cis*-DCE) or further to vinyl chloride. Two notable exceptions are PceA of *Desulfitobacterium* sp. PCE1 (van de Pas*, et al.*, 2001), and PteA of *D. mccartyi* 11a5 (Zhao*, et al.*, 2016), both of which dechlorinate PCE to TCE. *Sulfurospirillum multivorans*, one of the most studied OHRB, is able to dechlorinate PCE to *cis*-DCE (Goris & Diekert, 2016). PceA of *S. multivorans* is extensively characterized and its crystal structure was solved (Bommer*, et al.*, 2014). The kinetic parameters associated with OHRB can be ascertained by models of various levels of complexity. The state-of-the-art, as described in (Chambon*, et al.*, 2013), are numerical models that use Monod kinetics to describe the multiple guilds present in OHR ecosystems, where importance must be placed on the bacterial function rather than the individual species (Wade*, et al.*, 2016). Furthermore, numerical models can account for physical processes that may affect the bacterial activity and degradation of chlorinated ethenes (Aeppli*, et al.*, 2009, Kouznetsova*, et al.*, 2010, Buchner*, et al.*, 2017).

An anaerobic bacterial consortium, named SL2-PCEb, has been maintained for more than a decade in our laboratory. This consortium, which primarily consists of two different but coexisting *Sulfurospirillum* populations, was obtained from a fixed-bed bioreactor treating PCE contaminated groundwater (Rouzeau-Szynalski*, et al.*, 2011). This consortium is characterized by the ability to perform stepwise dechlorination of PCE to trichloroethene (TCE) and *cis*-DCE (Maillard*, et al.*, 2011). Two consortia were enriched from SL2-PCEb, each harboring a unique *Sulfurospirillum* population and each exhibiting distinct dechlorination potential. Consortium SL2-PCEc, harboring population SL2-1, dechlorinates PCE to TCE only. Consortium SL2-TCE, which was selected on TCE and harbors population SL2-2, has kept the potential to dechlorinate both PCE and TCE to *cis*-DCE. The reductive dehalogenase gene of both populations were identified as $pceA_{TCE}$ and $pceA_{DCE}$. Their respective gene products were shown to use PCE only in the case of $PceA_{TCE}$ from population SL2-1, or PCE and TCE ($PceA_{DCE}$ from SL2-2) (Buttet*, et al.*, 2013). However, we think that the ability of population SL2-2 to dechlorinate TCE, in contrast to population SL2-1, cannot fully explain the long-term coexistence of both SL2 populations in the parental consortium.

PCE dechlorination occurs via the successive elimination of chlorine atoms. PceA of *S. multivorans* catalyzes the reduction of PCE to *cis*-DCE without substantial accumulation of TCE (Neumann*, et al.*, 1994), which suggests that PCE to TCE dechlorination is the rate-limiting step during successive dechlorination. Bacterial consortia containing several OHRB that compete for PCE, such as the two SL2 populations in this study, constitute an ideal case study by which more complex ecological systems can be better understood. Therefore, the first aim of the present work was to identify the factors that enable the long-term coexistence of the *Sulfurospirillum* populations SL2-1 and SL2-2 in the consortium SL2-PCEb. The second aim was to investigate the population-specific properties that may explain the populations' successive appearance in the parent consortium (please note that in this study, successive appearance is distinct from the ecological concept of succession). The genomes of the SL2 populations were sequenced in order to identify genetic features possibly responsible for

the difference in their dechlorination potential, and to compare the predicted metabolic pathways of the new *Sulfurospirillum* populations with those of *S. multivorans.* Next, the dechlorination parameters of both populations were monitored in batch cultures, and then the growth kinetics were determined with a numerical model that was specifically developed for the culture conditions involving two liquid phases and one gas phase. Lastly, additional culture experiments were performed by mixing both *Sulfurospirillum* populations to highlight their sequence in dechlorination depending on the initial PCE concentration.

Material and Methods

All chemicals were analytical grade and used without purification. The gases (N_2, CO_2 and H_2) were purchased from SLGas (Sauerstoffwerk Lenzburg, Switzerland).

Bacterial strains and growth conditions

The bacterial consortia SL2-PCEc and SL2-TCE were cultivated anaerobically in 125-mL serum bottles sealed with rubber-stoppers in 50 mL of medium as described previously (Buttet*, et al.*, 2013), if not stated differently. Formate was added as electron donor to a final concentration of 20 mM. PCE stock solutions (10 mM to 1 M) were prepared in hexadecane (Holliger*, et al.*, 1998) and were used in a system with two liquid phases. The hexadecane phase allows the addition of substantial amounts of chlorinated compounds to a batch system without affecting growth. The expected initial PCE concentration in the aqueous phase ([PCE_{aq}]) was calculated with the partition coefficient between hexadecane and water (log K_{HW}) of 3.7 (~5000 part in hexadecane for 1 part in the aqueous phase) (Holliger*, et al.*, 1993). The total available amount of PCE in all culture flasks was kept at 500 μmol by varying both the volume of added organic phase and the PCE concentration in hexadecane [PCE_{HD}]. SL2 consortia routinely cultivated at 20 μM [PCE_{aq}] (supplied from 100 mM [PCE_{HD}]) were systematically used as inocula. The cultures were incubated in the dark at 30°C and set on a rotary shaker at 100 rpm after an initial static incubation of 1 h. In order to compare the growth yields of the two populations, the SL2-TCE consortium harboring the SL2-2 population was also cultivated with TCE instead of PCE as electron acceptor at a starting concentration of 100 mM in hexadecane [TCE_{HD}] (corresponding to 212 μM [TCE_{aq}]).

Escherichia coli DH5α was used as a host for molecular cloning (**Table 1**). *E. coli* competent cells were prepared using the standard $CaCl_2$ method (Sambrook*, et al.*, 1989). *E. coli* was cultivated at 37°C in Luria-Bertani (LB) liquid medium or agar plates containing 100 μg mL^{-1} of ampicillin when needed.

Chemical analyses

The aqueous chloride concentration was monitored in all batch cultures. One mL of the aqueous phase was collected at defined time intervals, filtered (0.2 μm), and stored at –20°C. Chloride concentration was analyzed with ion chromatography (ICS-90, IonPac AS14A-5 μm/3 × 150 mm column, blower; ACRS 500 2 mm, Dionex) with a mix of 8 mmol L^{-1} Na_2CO_3 and 1 mmol L^{-1} $NaHCO_3$ as the eluent and 50 mmol L^{-1} H_2SO_4 as the regenerating solution.

Chloroethenes were analyzed by gas chromatography with the Agilent Technologies 7890B system equipped with an Optima 624 LB (30m by 0.32 mm; M&N GmbH & Co. KG) coupled to a flame ionization detector. The carrier gas was helium, utilized at a flow rate of 1.8 mL min^{-1}. The column was kept at the initial temperature of 30°C for 5 min, then raised to 60°C at a rate of 10°C min^{-1}, then raised to 200°C at a rate a 40°C min^{-1}, and lastly the column was kept at the final temperature of

200°C for 5 min. Each culture was analyzed by collecting 1.5 mL of the gas phase from the culture flask in sterile conditions. Percentile proportions of PCE, TCE and *cis*-DCE were calculated from the area of corresponding peaks obtained in each chromatogram that were divided by a response factor relative to the area of PCE (5.8 for TCE, and 21.8 for *cis*-DCE, as determined from analyzing known concentrations of individual chloroethenes in the same conditions).

Quantitative PCR (qPCR)

For qPCR, plasmids containing fragments of the target genes (*rpoB*, $pceA_{TCE}$ and $pceA_{DCE}$) were used as standards (**Table 1**). Primers to clone the *rpoB* gene, encoding the B subunit of the RNA polymerase and used here as a reference gene to follow *Sulfurospirillum* population size in the SL2 consortia, were designed based on the genomes of *S. multivorans* (Genbank CP007201.1), *S. deleyianum* (CP001816.1) and *S. halorespirans* (CP017111.1) (**Table 2**). The cloning procedure and preparation of qPCR standards were completed as previously described (Maillard*, et al.*, 2003, Buttet*, et al.*, 2013). Reaction mixtures were prepared as previously described (Buttet*, et al.*, 2013) and run in a MIC apparatus (Bio Molecular Systems). The thermocycling program for qPCR was as follows: an initial denaturation of 2 min at 95°C; then 45 cycles of 10 s at 95°C, 30 s at 55°C (*rpoB*) or 60°C ($pceA_{TCE}$ and $pceA_{DCE}$), and 20 s at 72°C, after which the acquisition of fluorescence took place using the SYBR detection channel. A melting curve ranging from 72°C to 95°C was performed at 0.1°C s^{-1}. For each run, triplicates of samples and standards were run concurrently and the concentration of samples was calculated from the derived standard curve. The MicPCR v1.6.0 software was used for data analysis. Average run performances are given in **Table S1**. Replicates with standard deviation of less than 15% of the average value were kept for analysis.

Genomic DNA extraction and library preparation

For genome DNA extraction, two 200 mL batch cultures of the SL2-PCEc and SL2-TCE consortia were cultivated. The biomass was collected by centrifugation at 3,300 × *g* for 10 min at 4°C. Biomass pellets were washed in 1 mL of 50 mM Tris-HCl buffer (pH 8.0) and collected by centrifugation at 8,800 × *g* for 5 min. Biomass pellets (18.6 mg and 19.7 mg for SL2-PCEc and SL2-TCE, respectively) were flash-frozen in liquid N_2 and stored at −80°C. DNA extraction was performed with the DNeasy Blood & Tissue kit following the recommendations of the manufacturer (Qiagen). Preparation of the genomic libraries was carried out according to (Diaby*, et al.*, 2015). DNA samples were purified with magnetic beads (AxyPrep Mag PCR Clean-Up, Axygen) and fragmented using the enzyme mix provided in the Ion Xpress Plus Fragment Library Kit according to the manufacturer instructions (Life Technologies). Size selection (max. 370 bp) was carried out on commercial agarose gels (E-Gel System, Life Technologies). Quantification and size analysis of the selected fragments was carried out using the BioAnalyzer 2100 and the High Sensitivity DNA kit (Agilent Technologies).

Semiconductor sequencing with Ion Torrent PGM

Emulsion PCR was prepared using the Ion XPress Template Kit (Life Technologies) as described in the user guide provided by the manufacturer. Sequencing was carried out on the Ion Torrent Personal Genome Machine (PGM) using the Ion Sequencing 300 bp kit (Life Technologies) equipped with a 316 chip and following the corresponding protocol. This work was done in collaboration with P. Rossi (CEMBL, EPFL).

Sequence recovery, bioinformatics and statistical analysis

Numerical treatment of the data gained by semiconductor sequencing was done according to a previous study (Diaby*, et al.*, 2015). Primary base calling was first performed using the Torrent Suite v3.0 software (Life Technologies). Sequencing reads were then downloaded as .sff files from the Torrent Server and processed on a Linux Ubuntu platform (BioLinux 7, Ubuntu 12.04 LTS) running on a local Dell Precision T3600 2 GHz desktop computer equipped with a 12 core processor array and 32 GB of RAM. Reads were initially processed on Mothur so as to provide the necessary .fasta and .qual files (Schloss*, et al.*, 2009).

Genome annotation and analysis

Sequencing of the SL2-1 population genome resulted in 1,580,331 reads and 426,621,911 initial bases, 346,913,840 of them with a Phred quality base score >= 20. Sequencing the SL2-2 population genome resulted in 1,150,708 reads, and 313,462,781 with a Phred quality base score >= 20. The mean read length was 269 bp for both. The assembly was done with IonGAP (Baez-Ortega*, et al.*, 2015), which relies on the assembler MIRA (Version 4.0rc4, using standard settings). The genomes of *S. multivorans* (CP007201.1), *S. halorespirans* (CP017111.1), *S. arsenophilum* (BBQF00000000.1) and the unpublished genome of *Sulfurospirillum* sp. JPD-1 were used as references in four different assemblies for each genome using the comparative genomics tool of IonGAP. The assembly with the lowest number of contigs larger than 1,000 bp (17 for SL2-1) resulted from using the genome of *S. halorespirans* as a reference. The average total coverage based on the contigs larger than 5 kbp was 135× for the SL2-1 assembly. The largest contig had a size of 938,435 bp, and the N50 contig size was 467,364 bp. No contigs larger than 500 bp for other organisms than SL2 populations (e.g. *Wolinella succinogenes*, which was found in minor amounts in the subpopulation) could be assembled. Contigs were ordered via the comparative genomics tool IonGAP according to the mentioned reference genomes and overlapping contigs were merged. By comparing all eight assemblies to each other, it was possible to generate a complete genome for the SL2-1 population, which in turn was used as a reference genome for the assembly of SL2-2 and ultimately resulted in an assembly with one scaffold that consisted of 3 contigs and a coverage of 95×. Automatic annotation was done via RAST (Aziz*, et al.*, 2008), the organohalide-respiration region and other oxidoreductases were checked manually by comparison with those of *S. multivorans* (Goris*, et al.*, 2014) and *S. halorespirans* (Goris*, et al.*, 2017). The complete genome of SL2-1 is 2,876,536 bp (accessible in GenBank with reference number CP021416). The draft genome size is 2,876,607 bp (GenBank accession number CP021979). The comparative average nucleotide identity (ANI) scores were obtained using the online tool ANI calculator (http://enve-omics.ce.gatech.edu/) (Goris*, et al.*, 2007).

Data analysis and numerical model development

An approach based on increasing levels of complexity was applied to evaluate the experimental data. A preliminary evaluation for all datasets was based on the Lineweaver-Burke and Hanes-Woolf linearization methods and the Gompertz equation. The latter is a sigmoidal function that is appropriate to evaluate bacterial growth (Zwietering*, et al.*, 1990) and was used to systematically fit the evolution of the bacterial population in each batch from the measured chloride data (see **Section S1** of the supplementary material). The bacterial substrate utilization rate and substrate affinity were further investigated in a representative subset of the experimental batches. This more detailed evaluation was based on a numerical modeling approach, which allowed for quantitative description of both bacterial and physical processes occurring in the experimental system. Nonlinear, numerical modeling methods have proven to be useful tools for investigation and quantitative interpretation of

organic contaminant biodegradation (Fennell & Gosset, 1998, Yu*, et al.*, 2005, Jin & Rolle, 2014) and microbial community dynamics (Song*, et al.*, 2014, Wade*, et al.*, 2017). Two such models were developed to simulate the degradation of the chlorinated compounds and the dynamics of the OHRB consortia observed in our experiments. The first model focused on the SL2-1 mediated dechlorination of PCE to TCE, and the second was developed to describe the SL2-2 mediated dechlorination of PCE to *cis*-DCE. Dechlorination of PCE to *cis*-DCE by SL2-2 was modeled as a one-step rather than two-step process (**Figure S1**) based on the insubstantial TCE accumulation during SL2-2 batch cultivation (Buttet*, et al.*, 2013), which indicates that PCE dechlorination is the rate-limiting step. The mechanisms behind this observation are still unknown, but we speculate that the TCE produced from PCE dechlorination remains attached to or in the proximity of the dechlorinating enzyme, where it is readily converted to *cis*-DCE as in an equivalent one-step reaction. Both models are based on the same physical system and account for the main physical, chemical and biological processes occurring in the experimental setup. Specifically, the models include: (i) Monod kinetics for the degradation of chlorinated compounds and the formation of chloride in the aqueous phase; (ii) microbial biomass dynamics expressed in terms of gene copies; (iii) interphase mass transfer of volatile organic compounds (PCE, TCE and *cis*-DCE) between the organic, aqueous and gas phases present in the experimental setup (Aeppli*, et al.*, 2009, Jin*, et al.*, 2013); and (iv) the removal of solute mass and the change of volume in both the aqueous and gas phases during each sampling event (Buchner*, et al.*, 2017). More details about data analysis, including governing equations and key modeling parameters, are provided in **Section S2** of the supplementary material.

Results and Discussion

This study aimed at elucidating the long-term coexistence (more than ten years of uninterrupted culture transfers) of two closely related populations of *Sulfurospirillum* sp. enriched in the PCE dechlorinating consortium SL2-PCEb (Maillard*, et al.*, 2011).

Genomes of Sulfurospirillum sp. SL2-1 and SL2-2 and designation of a new species

Both SL2 consortia were highly enriched with the respective *Sulfurospirillum* populations (at least 99%), as confirmed by their genome analysis. Indeed, no contigs larger than 500 bp for other bacteria could be assembled. The genomes of populations SL2-1 and SL2-2 revealed a very high degree of similarity with each other (ANI mean of 99.95%), but shared significantly lesser homology to the genomes of other *Sulfurospirillum* spp. (78-83%), as illustrated for the comparison between SL2-1 and *S. multivorans* (**Figure S2**). Therefore, SL2-1 and SL2-2 belong to a new species of *Sulfurospirillum* that we propose to name '*Candidatus* Sulfurospirillum diekertiae' (shortened as *S. diekertiae*). General features of the genomes of *S. diekertiae* SL2-1 and SL2-2 were compared to the available genomes of *Sulfurospirillum* spp. (**Table S2**). The genomes of the SL2 populations seem to be slightly smaller than those of *S. multivorans* and *S. halorespirans*, the two other OHR bacteria within the genus. A phylogenetic analysis of *Sulfurospirillum* spp. based on the 16S rRNA genes identified in the genomes is presented in **Figure 1**, revealing that *S. diekertiae* clusters phylogenetically with the other PCE-respiring *Sulfurospirillum* spp. The OHR region of the *S. diekertiae* genomes, as defined for *S. multivorans* (Goris*, et al.*, 2014) consists of the *rdh* gene cluster and the gene cluster responsible for the biosynthesis of corrinoids. DNA sequence alignment of the *S. diekertiae* SL2 populations' overall *rdh* gene clusters compared to that of *S. multivorans* revealed a high degree of sequence identity, with pairwise alignments between 99.3 and 99.7% (**Figure 2**). The largest difference is the insertion of a 106-bp fragment in both SL2 genomes, as compared to the genome of *S. multivorans*. The

fragment is located in the intergenic region between the *napH*-like gene, encoding the membrane subunit of a putative quinol dehydrogenase and *cbiB* (adenosylcobinamide-phosphate synthase). The consequence of this insertion remains unknown. The additional 106 bp, however, are also found in *S. halorespirans* (Goris*, et al.*, 2017). Furthermore, in both SL2 genomes, a *tetR* transcriptional regulatory gene downstream of the corrinoid biosynthesis cluster appears to be functional, in contrast to the *S. multivorans* genome, where the *tetR* gene is disrupted by a transposase (Goris*, et al.*, 2014). An intact *tetR* gene has also been shown in the genome of *S. halorespirans* (Goris*, et al.*, 2017), suggesting that it is not a specific feature of *S. diekertiae*. As previously identified (Buttet*, et al.*, 2013), the nucleotide sequence identity of *pceA* genes in OHR members of *Sulfurospirillum* spp. clearly indicates that it is a hotspot for mutations and most likely represents the driving force for the divergence of *Sulfurospirillum* OHR metabolism. A molecular fingerprinting method targeting the small differences in the *pceA* genes has been developed for studying the dynamics of both populations in the parental consortium SL2-PCEb (Buttet*, et al.*, 2013). However, a second reductive dehalogenase gene cluster (*rdhAB*) located downstream of *pceAB* has not diverged, and exhibits 100% sequence identity between the four *Sulfurospirillum* genomes. The *rdhAB* gene cluster was likely not exposed to any evolutionary pressure, as is evident by the lack of expression in both *S. diekertiae* and *S. multivorans* observed so far for this cluster (Buttet*, et al.*, 2013, Goris*, et al.*, 2014).

The designation of a new *Sulfurospirillum* species invited us to investigate gene clusters outside the OHR region; comparing these gene clusters to those from other members of this genus may be useful to characterize the physiology of this new species. The genomes of both *S. diekertiae* populations encode proteins for the oxidation and reduction of a large variety of organic and inorganic substrates. Similar to most other *Sulfurospirillum* spp., arsenate is likely to be reduced by this species with an arsenate reductase (SL2-1 loci Sdiek1_1006-1008); additionally, both populations contain an arsenite oxidase for the catabolic oxidation of arsenite (Sdiek1_1026-1028). Nitrate could be reduced by the Nap/Nir-system, and several molybdopterin oxidoreductases are found which might be involved in the oxidation and reduction of e.g. sulfur compounds (Goris & Diekert, 2016). Similar to *S. halorespirans*, a complete nitrous oxide reductase cluster is found in *S. diekertiae* (Sdiek1_967-982), suggesting the reduction of the greenhouse gas nitrous oxide by *S. diekertiae*. Five hydrogenase gene clusters are found in the *S. diekertiae* genomes, one periplasmic membrane-bound hydrogenase (MBH) used for the oxidation of H_2 as an energy source (Sdiek1_1468-1471) and two cytoplasmic hydrogenases (Sdiek1_1364-1369; Sdiek1_1466-1467) with unknown function (Kruse*, et al.*, 2017). The fourth hydrogenase (Sdiek1_2191-2201) gene cluster is likely to encode a membrane-bound H_2-evolving enzyme facing the cytoplasmic side similar to that of *S. multivorans* (Kruse*, et al.*, 2017). Interestingly, the fifth hydrogenase (Sdiek1_1064-1073) is most likely part of a formate hydrogen lyase (FHL) complex, as it is co-located with genes encoding a cytoplasmic formate dehydrogenase (Sdiek1_1063) and a formate channel protein (Sdiek1_1074). For *E. coli*, an FHL complex was proposed to diminish intracellular formate produced by a pyruvate formate lyase during fermentation (Sawers, 1994). Since the latter enzyme is not encoded in the genome of *S. diekertiae*, an intracellular accumulation of formate is unlikely. Instead, the *S. diekertiae* FHL complex might contribute to growth on formate as found for syntrophic *Moorella* spp. or specialized Archaea (Dolfing*, et al.*, 2008, Kim*, et al.*, 2010).

Dechlorination as a measure for growth of SL2 consortia

Since the genomes of both SL2 populations were found to be extremely similar, the reason for their long-term coexistence was then investigated at the level of their dechlorination kinetics. To this

purpose, batch cultures of both SL2 consortia were performed and monitored under various initial aqueous PCE concentrations. When chlorinated compounds are used as the sole terminal electron acceptor, chloride release is often used as a proxy for the growth of OHRB (Holliger*, et al.*, 1993, Cupples*, et al.*, 2003, Prat*, et al.*, 2011). This was verified for the two *Sulfurospirillum* populations present in the consortia SL2-PCEc and SL2-TCE. To this purpose, *rpoB*, the β subunit of the RNA polymerase, was chosen to monitor growth, as both populations harbor a single copy of this gene with full sequence conservation in their genomes. Indeed, chloride concentration and *rpoB* gene copy number were monitored in triplicate cultures, and the data obtained show that the increase of *rpoB* gene copy number of both SL2 populations aligns with the chloride release (**Figure S3**). The apparent yield on PCE (gene copy number per mole of PCE consumed) is approximately the same for both populations. However, the gene copy number yield per mole of chloride released of SL2-1 is approximately twice that of SL2-2, since the latter dechlorinates TCE further to *cis*-DCE. This unexpected result was further examined using the numerical model (see below).

PCE dechlorination kinetic parameters of individual S. diekertiae SL2 populations

To evaluate the kinetic parameters of individual *S. diekertiae* SL2 populations and determine whether one population presents a distinct advantage over the other in growth rate and/or affinity for PCE, more than 70 batch cultures (including replicates) with initial aqueous PCE concentrations of 2 to 200 μM were monitored for chloride release. In a first attempt to estimate the dechlorination-based growth rates (μ_{Cl}) of individual batch cultures, the obtained chloride data were fitted with the Gompertz equation (Zwietering*, et al.*, 1990) (see **Table S3** for the complete fitting parameters), and plotted against the initial aqueous PCE concentration (**Figure 3**). The apparent growth rate of the SL2-1 population clearly increased faster than that of SL2-2 in the initial part of the plot, suggesting that the former shows a higher affinity for PCE than the latter. However, above 50 and 80 μM PCE, SL2-1 and SL2-2 populations displayed a decrease in growth rate, respectively, indicative for inhibitory effects of PCE or of daughter products. This observation is to be compared to what has been reported for *S. multivorans,* which can dechlorinate PCE at concentrations lower than 330 μM but not at concentrations above 540 μM (Amos*, et al.*, 2007), suggesting that *S. diekertiae* is slightly more sensitive than *S. multivorans* to inhibition by chloroethenes. Lineweaver-Burk and Hanes-Woolf linearization procedures of the growth rates obtained at non-inhibitory PCE concentrations allowed the estimation of the apparent maximal growth rates ($\mu_{max,Cl}$) and affinity constants for PCE ($K_{S,PCE}$) for both SL2 populations (**Figure S4** and **Table S4**). While the two populations seem to display a similar maximal growth rate of approximately 0.1 h^{-1}, the apparent affinity for PCE of *S. diekertiae* SL2-1 is 5 to 6-fold higher compared to SL2-2. The difference in affinity for PCE in both populations could explain why TCE accumulation was observed in the parental consortium. Indeed, when cultivated at PCE concentrations lower than 50 μM, the SL2-1 population is able to dechlorinate PCE at a faster pace relative to SL2-2 (**Table S3**), resulting in the production of TCE, which can in turn be dechlorinated to *cis*-DCE by SL2-2.

A numerical model was also developed to describe the batch system and determine the kinetic parameters of dechlorination. Unlike the Gompertz model, the numerical model considers the distribution and transport of PCE and daughter products in the three-phase anaerobic batch system (two-liquid phases and the gaseous head space) and the effect of PCE removal from the system through successive sampling events. The model was tested on chloride datasets of ten batches, selected in order to span the range of initial aqueous PCE concentrations used in the experiments.

Batches #2 and #6 (**Table 3**), used to validate chloride release as a proxy for growth and displaying both chloride and *rpoB* gene copy number time series data, were included in the numerical simulations. The model was able to capture the development in biomass and chloride for all 10 datasets on which it was tested. Simulations for batches #2 and #6 are presented in **Figure 4**, and show very good agreement between the measured and the simulated data for both populations. The goodness of fit is also substantiated by the normalized root mean squared error (NRMSE), which was calculated as a quantitative metric for all the tested batches (**Table 3**). In **Figure 4** the dechlorination rate is visualized, which shows when PCE is transformed most effectively by SL2-1 and SL2-2 populations in the two selected systems. The detailed simulated results for the selected datasets corroborate the $K_{S,PCE}$ values determined for both SL2-1 and SL2-2 for PCE dechlorination to their respective end product, with approximately an 8-fold difference. The best fit for each was determined to be 7.84 and 65.82 μM for SL2-1 and SL2-2, respectively, with ranges of 0.96-15.42 μM and 63.01-89.60 μM (**Table 3**). These values differ slightly from the $K_{S,PCE}$ values obtained using the Gompertz equation and the linearization procedures (**Table S4**), but most probably better reflect the complexity of the three-phase system and the limitation imposed by mass transfer at low substrate concentrations, which is explicitly considered in the three-phase numerical model (see **Section S2** of the supplementary material). Furthermore, dechlorination rates were found to decrease with increasing initial aqueous PCE concentration.

Growth yield factor and additional insights from the numerical modeling of dechlorination data

In addition to understanding of the kinetic parameters, the numerical model can provide insights to other physical and biological parameters of the three-phase batch culture system. The apparent similar growth yield of both SL2 populations cultivated on PCE was estimated and a yield factor of $1.32 \cdot 10^8$ *rpoB* gene cn·(μmol PCE)$^{-1}$, in agreement with published data on *Sulfurospirillum* spp. (Scholz-Muramatsu*, et al.*, 1995, Aeppli*, et al.*, 2009), was used to fit all dechlorination data successfully. To dissect the observed yield factor of population SL2-2 for individual dechlorination steps, two duplicate batches of population SL2-2 were cultivated with TCE rather than PCE as the electron acceptor. Fitting with the numerical model (for details see supplementary material) revealed that dechlorination of TCE to *cis*-DCE accounted for approximately half of the biomass produced by SL2-2 when PCE was provided (**Table S5**, **Figure S5**). The comparison with the yield factor of SL2-1 on PCE (one step dechlorination) suggests that single dechlorination steps in SL2-2 only generate half of the corresponding SL2-1 biomass. This observation needs further investigation that goes beyond the scope of the present study.

The numerical modeling exercise allowed us to quantitatively interpret some important aspects of the dynamic interplay between physical, chemical and biodegradation processes in the experimental three-phase system (see the description in **Section S2** of the supplementary material). For instance, via the model simulations, the PCE concentration below which the system is mass-transfer limited could be elucidated (**Figure S6**) and the effect of sample size on the change in phase volume could be visually depicted (**Figure S7**). The model was also used to explore the possible bacterial growth patterns with similar kinetic parameters for each strain. If only the affinity for PCE ($K_{S,PCE}$) had differed in the two populations, but the maximum specific dechlorination rate kept identical, the coexistence of the two strains would have been due only to their different electron acceptor spectra (**Figure S8**).

The succession of S. diekertiae SL2-1 and SL2-2 depends on the initial PCE concentration

Knowing that each SL2 population has a different affinity for PCE, batch cultures with both populations together were established to show the effect of the initial PCE concentration on the sequence of SL2-1 and SL2-2 populations in PCE and TCE dechlorination, respectively. The two separate consortia were first cultivated at 6 and 30 μM [PCE_{aq}], then a mix of equal population proportion was used as inoculum to start cultures amended with the same initial concentrations of PCE. Care was taken to avoid PCE concentrations leading to inhibition. The cultures were monitored for the relative concentration of chlorinated ethenes, chloride release, and the abundance of population-specific *pceA* genes (**Figure 5**). At an aqueous PCE concentration of 6 μM, a clear accumulation of TCE was observed between 48 and 96 h (**Fig. 5A**), while the SL2-1 population initially grew at a faster rate than SL2-2 (**Fig. 5B**). Once most PCE was dechlorinated, the population size of SL2-1 stalled and then decreased, while SL2-2 took over and was responsible for TCE dechlorination. Starting at 30 μM aqueous PCE, TCE did not significantly accumulate (**Fig. 5C**) and both populations grew at approximately the same rate during the first dechlorination step (PCE to TCE), thus reflecting the simultaneous action of both populations (**Fig. 5D**). After 40 h of incubation, *cis*-DCE started to appear and was accompanied by a significant increase in SL2-2 population size. The two growth patterns observed here clearly highlight the distinct and concentration-dependent PCE dechlorination rate by the individual SL2 populations. The coexistence of both populations in SL2-PCEb was made possible by the relatively low frequency of culture transfer allowing the SL2-2 population to maintain a substantial population size in SL2-PCEb. These kinetic considerations together with the relaxed time frequency of culture transfers maintained throughout the years explain the long-term coexistence of both SL2 populations in the parental consortium.

Conclusions

During several years of cultivation of the SL2-PCEb bacterial consortium that dechlorinates PCE successively to TCE and *cis*-DCE, two distinct populations of PCE-dechlorinating *S. diekertiae* were able to coexist. Such a long-term coexistence raised questions about the nature and details of the interplay between these two closely related populations. As demonstrated in the first part of this study, both *S. diekertiae* populations are highly similar at the genome sequence level. Only their respective *pceA* genes show significant differences and appear as a hotspot for mutations, which may represent a possible driving force for the evolution and fine-tuning of *Sulfurospirillum* OHR metabolism. Amino acid changes in the sequence of $PceA_{TCE}$ (present in population SL2-1) could be one of the reasons for the specialization of the SL2-1 population in the exclusive dechlorination of PCE to TCE. Besides the fact that only the SL2-2 population can use TCE, the maintenance of the two populations within the parental consortium is likely explained by the different kinetic PCE dechlorination parameters observed for the individual SL2 populations, the relatively low PCE concentration routinely used in cultivation, and the relaxed time frame of batch culture transfer throughout the years.

The ecological implications of these findings remain to be investigated, but it could be imagined that similar OHRB interplay is likely to occur in environments that display PCE concentration gradients. In order to predict the dechlorination rate of relevant OHRB at polluted sites, it is therefore of great importance to identify the kinetic parameters of individual cultures and their reductive dehalogenases. In addition, (self-)inhibition of dechlorination by PCE and daughter products should be further investigated to expand the applicability of the proposed model.

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Conflict of interest

The authors declare that they have no conflict of interest.

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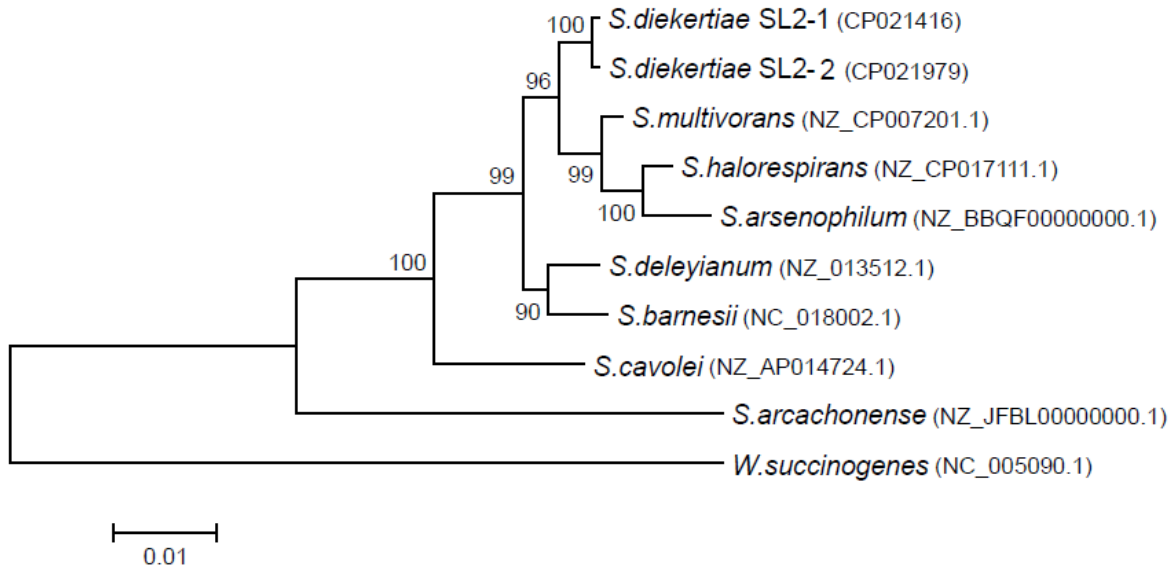

Figure 1. Phylogenetic tree based on the 16S rRNA genes from *Sulfurospirillum* spp. genomes.

The 16S rRNA genes were taken from available *Sulfurospirillum* spp. genomes (one 16S rRNA gene copy for each strain, accession number given in brackets) and aligned with ClustalX2.0 (Larkin*, et al.*, 2007). The tree was drawn using MEGA (version 6) (Tamura*, et al.*, 2013). Bootstrap values are indicated next to the nodes. The 16S rRNA gene of *Wolinella succinogenes* was used as outgroup.

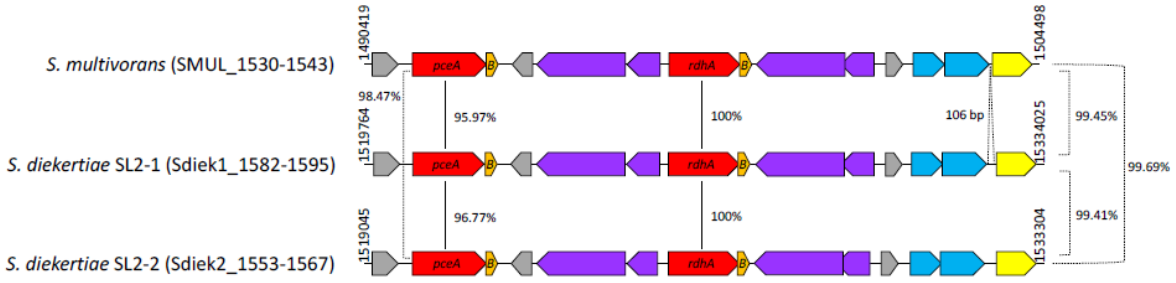

Figure 2. Comparison of the *rdh* gene cluster of *S. diekertiae* populations SL2-1 and SL2-2 with *S. multivorans*.

The *rdh* gene cluster of *S. multivorans* was aligned with the corresponding region in the genomes of *S. diekertiae* populations SL2-1 (GenBank CP021416) and SL2-2 (CP021979). The coordinates are indicated at the beginning and end of each region. Color-code: red, reductive dehalogenase genes *rdhA*; orange, membrane anchor gene *rdhB*; purple, transcriptional regulator genes; blue, *napGH*-like genes; yellow, *cbiB*, the first gene of the corrinoid biosynthesis gene cluster; grey, unassigned function.

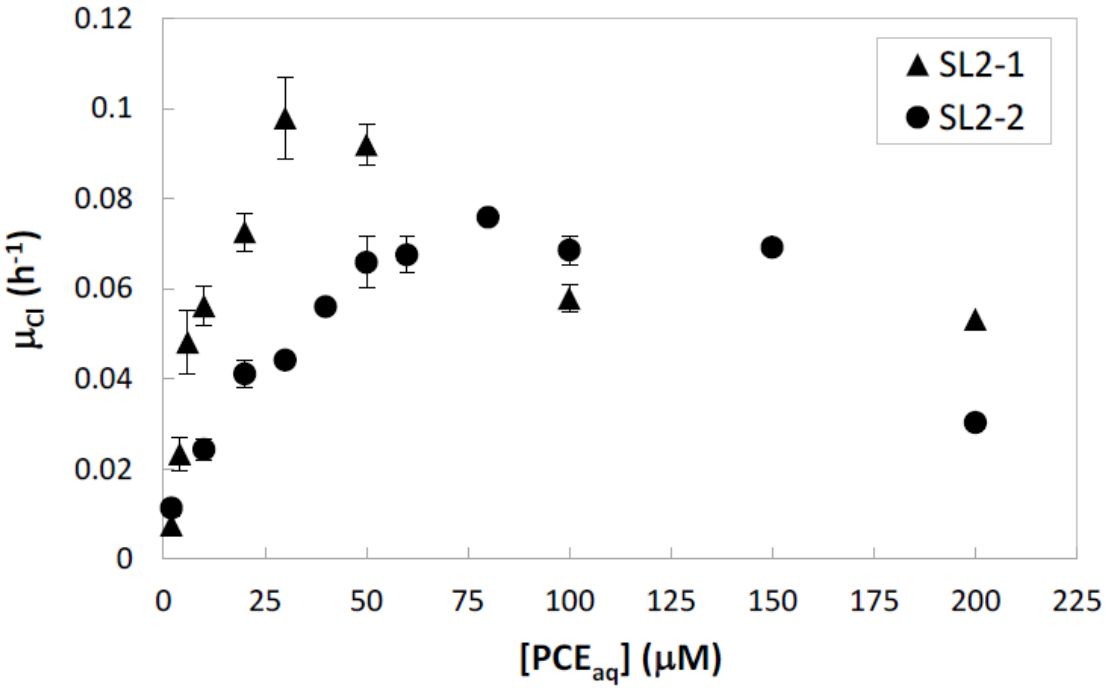

Figure 3. Dechlorination-based growth rates of *S. diekertiae* populations SL2-1 and SL2-2 as function of the initial aqueous PCE concentration. The dechlorination data of all batch cultures were fitted with the Gompertz equation (see **Table S3**). Average growth rates (μ_{Cl}) were obtained for *S. diekertiae* SL2-1 (triangles) and SL2-2 (circles) cultivated at various initial aqueous concentrations of PCE. Only the batch cultures for which the sum of squared errors (SSE) was < 0.1 were considered here. Note that for SL2-1 at 8 and 200 μM $[PCE_{aq}]$ and for SL2-2 at 2, 150 and 200 μM $[PCE_{aq}]$, the growth rates were determined from single culture replicates.

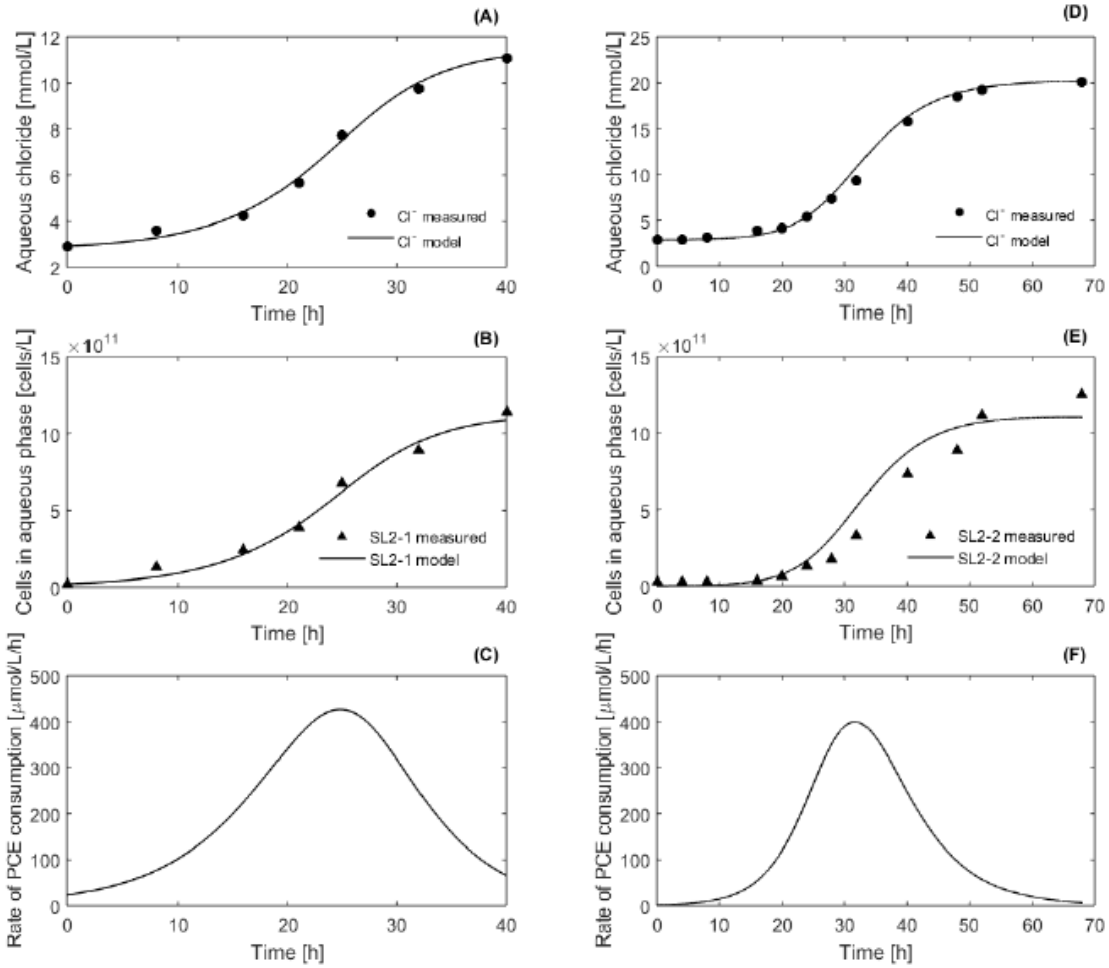

Figure 4. Data, and simulation thereof, of representative batch cultures of *S. diekertiae* SL2-1 and SL2-2 populations. The evolution of measured chloride concentration (A and D) and *rpoB* gene copy number (B and E) are displayed together with the corresponding simulated data for the consortia SL2-PCEc (harboring *S. diekertiae* SL2-1) (A and B) and SL2-TCE (*S. diekertiae* SL2-2) (D and E) cultivated at 20 μM initial aqueous PCE concentration. The simulated PCE consumption rate is also displayed for SL2-PCEc (panel C) and SL2-TCE (panel F). The fitting parameters obtained from the model for both batch cultures are given in **Table 3**.

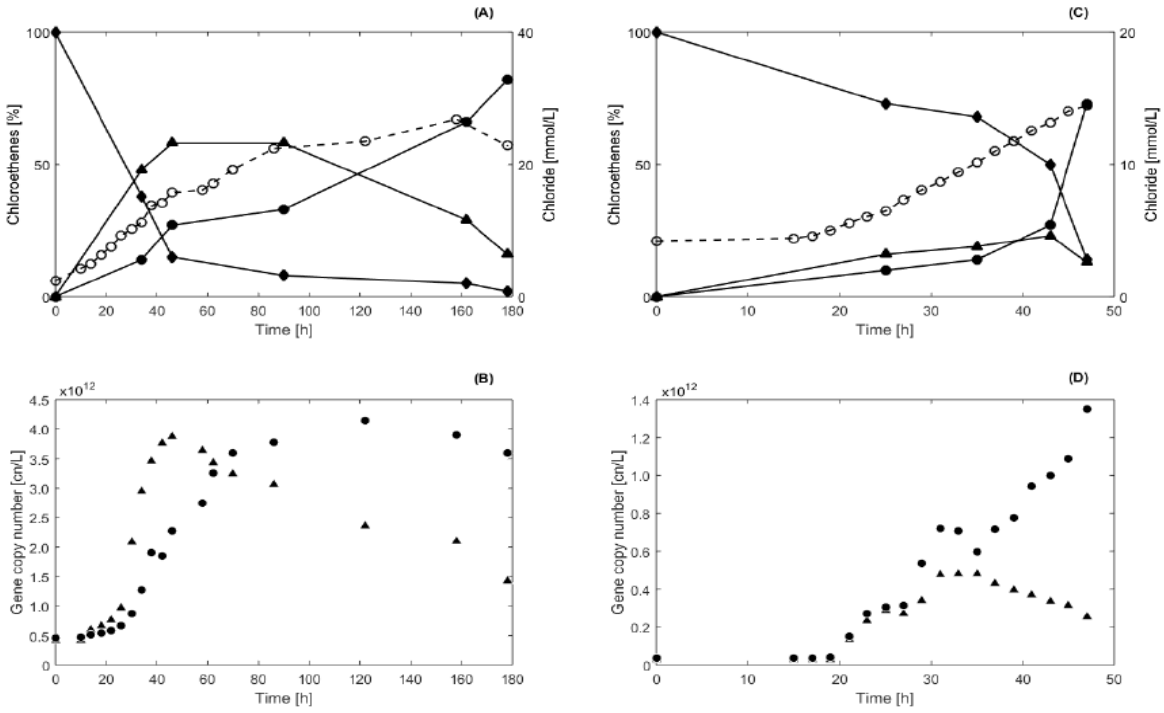

Figure 5. Growth pattern of *S. diekertiae* SL2-1 and SL2-2 populations at two different initial PCE concentrations. The two consortia were mixed together and cultivated at 6 μM [PCE_{aq}] (A and B) or 30 μM [PCE_{aq}] (C and D). The dechlorination of PCE (diamonds) to TCE (triangles) and *cis*-DCE (black circles) is shown alongside with the chloride release (white circles) in panels A and C. The gene copy number of $pceA_{TCE}$ (SL2-1, triangles), $pceA_{DCE}$ (SL2-2, circles), is presented in panels B and D. Please note the different time scale of experiments in both cultures. The other replicate cultures followed the same trend (data not shown).

Table 1. Bacterial strains and consortia, and plasmids used in this work.

Bacteria	**Description**	**References**
SL2-PCEc	Bacterial consortium selected from SL2-PCEb containing a *Sulfurospirillum* population displaying PCE to TCE dechlorination	(Maillard*, et al.*, 2011, Rouzeau-Szynalski*, et al.*, 2011, Buttet*, et al.*, 2013)
SL2-TCE	Bacterial consortium selected on TCE from SL2-PCEb containing a *Sulfurospirillum* population displaying PCE to *cis*-DCE dechlorination	(Maillard*, et al.*, 2011, Rouzeau-Szynalski*, et al.*, 2011, Buttet*, et al.*, 2013)
E. coli DH5α	F^- *endA1 glnV44* thi-1 *recA1 relA1 gyrA96 deoR nupG* Φ80d*lac*ZΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*(r_K^- m_K^+), λ–	Invitrogen
Plasmids	**Description**	**References**
pT1P-T_Q	pGEM-T harboring a fragment of $pceA_{TCE}$	(Buttet*, et al.*, 2013)
pT1P-D_Q	pGEM-T harboring a fragment of $pceA_{DCE}$	(Buttet*, et al.*, 2013)
pRPOB	pGEM-T harboring a fragment of *Sulfurospirillum rpoB*	This study

Table 2. Oligonucleotides used in this study.

Name	Target gene	Sequence (5' →3')	Reference
T1-PTQ-f	*pceA*$_{TCE}$	CTTTGGAGGTAACTTTGGAGGTTA	(Buttet*, et al.*, 2013)
T1-PTQ-r		CTTTAGGCCAAGATTGTTCATCT	
T1-PDQ-f	*pceA*$_{DCE}$	GTAACTATACCAGCTGACGTACC	(Buttet*, et al.*, 2013)
T1-PDQ-r		CATAGCGATACCTGCAACGA	
rpoB-f	*rpoB*	GATGCTAGGAATTTTATTGA	This study
rpoB-r		AACTCTTCAACGTTAACAC	

Table 3. Kinetic parameters of *S. diekertiae* SL2 populations obtained after simulation of the measured dechlorination data.

Population	Batch #	Initial aqueous PCE conc.	Vol. of organic phase	$k_{max,PCE}$	$K_{S,PCE}$	Chloride NRMSE[a]	Gene cn NRMSE[a]
		(μM)	(mL)	(gene cn ·(μmol PCE·h)$^{-1}$)	(μM)	(%)	(%)
SL2-1	1	10	10	$2.12 \cdot 10^{-09}$	0.96	2.79	
	2	20	5	$1.70 \cdot 10^{-09}$	7.84	2.51	4.15
	3	30	3.33	$2.61 \cdot 10^{-09}$	15.42	2.04	
	4	50	2	$1.22 \cdot 10^{-09}$	1.47	4.48	
	5	100	1	$9.15 \cdot 10^{-10}$	8.29	3.23	
SL2-2	6	20	5	$8.88 \cdot 10^{-09}$	65.82	2.20	7.45
	7	50	2	$2.24 \cdot 10^{-09}$	64.51	4.66	
	8	50	2	$3.17 \cdot 10^{-09}$	63.01	2.72	
	9	100	1	$2.46 \cdot 10^{-09}$	89.60	2.71	
	10	150	0.67	$1.27 \cdot 10^{-09}$	84.59	2.96	

[a] NRMSE: normalized root mean squared error.