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Organohalide Respiration with Chlorinated Ethenes under Low pH Conditions

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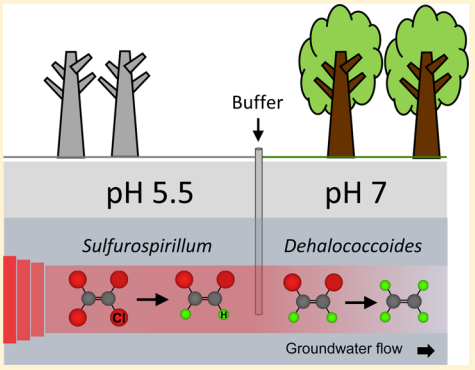
Supporting Information

ABSTRACT: Bioremediation at chlorinated solvent sites often leads to groundwater acidification due to electron donor fermentation and enhanced dechlorination activity. The microbial reductive dechlorination process is robust at circumneutral pH, but activity declines at groundwater pH values below 6.0. Consistent with this observation, the activity of tetrachloroethene (PCE) dechlorinating cultures declined at pH 6.0 and was not sustained in pH 5.5 medium, with one notable exception. *Sulfurospirillum multivorans* dechlorinated PCE to *cis*-1,2-dichloroethene (*c*DCE) in pH 5.5 medium and maintained this activity upon repeated transfers. Microcosms established with soil and aquifer materials from five distinct locations dechlorinated PCE-to-ethene at pH 5.5 and pH 7.2. Dechlorination to ethene was maintained following repeated transfers at pH 7.2, but no ethene was produced at pH 5.5, and only the transfer cultures derived from the Axton Cross Superfund (ACS) microcosms sustained PCE dechlorination to *c*DCE as a final product. 16S rRNA gene amplicon sequencing of pH 7.2 and pH 5.5 ACS enrichments revealed distinct microbial communities, with the dominant dechlorinator being *Dehalococcoides* in pH 7.2 and *Sulfurospirillum* in pH 5.5 cultures. PCE-to-trichloroethene- (TCE-) and PCE-to-*c*DCE-dechlorinating isolates obtained from the ACS pH 5.5 enrichment shared 98.6%, and 98.5% 16S rRNA gene sequence similarities to *Sulfurospirillum multivorans*. These findings imply that sustained *Dehalococcoides* activity cannot be expected in low pH (i.e., ≤ 5.5) groundwater, and organohalide-respiring *Sulfurospirillum* spp. are key contributors to in situ PCE reductive dechlorination under low pH conditions.



INTRODUCTION

Reductive dechlorination processes release hydrochloric acid, which rapidly dissociates to form chloride anions and hydronium ions in groundwater. Furthermore, the fermentation of biostimulation substrates (e.g., lactate, emulsified vegetable oil, molasses, corn cobs, newsprint, wood chips, microbial biomass) results in acidification due to the production of organic acids and protons.[1] In aquifers with low buffering capacity, pH reductions can decrease rates and even stall microbial dechlorination processes.[2] Additional undesirable secondary effects of acidification include increased solubility of toxic metals and metalloids, which may affect microbial activity and impair groundwater quality (i.e., exceed regulatory standards).[3] Extensive dechlorination and fermentation reactions may generate enough acidity to affect the groundwater pH depending on the buffering capacity of the aquifer. For instance, calcite plays an important role in buffering the pH of calcareous soils, but the amount of calcite in aquifers varies considerably.[4] A recent U.S. Geological Survey investigation reported that groundwater in aquifer systems of the Northern Atlantic Coastal Plain was commonly acidic (median pH 5.3) due to the lack of minerals (e.g., calcium carbonate) that would buffer the natural acidity. This observation suggests that remediation of sites impacted with chlorinated ethenes in this aquifer system would be challenging without pH management.[5]

A common response to groundwater pH reductions following in situ biostimulation is the addition of alkaline chemicals or compounds with buffering capacity to increase and maintain the pH in a range suitable for dechlorinating bacteria. For example, additions of sodium bicarbonate or colloidal $Mg(OH)_2$ have been used to manipulate groundwater pH in situ;[6,7] however, large amounts of bicarbonate may be required to adjust the pH of calcareous soils. Further complicating the

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issue is the fact that increased carbonate concentrations can cause precipitation rather than dissolution of calcite ($CaCO_3\downarrow \leftrightarrow Ca^{2+}+CO_3^{2-}$).[8] A low cost, self-regulating approach using silicate minerals, which exhibit pH dependent solubility and dissolution rates, was proposed to buffer groundwater, but further experimentation indicated that silicate minerals and their dissolution products inhibited microbial reductive dechlorination of chlorinated ethenes.[9] Modeling approaches have been applied to estimate the aquifer buffering capacity required for stabilizing groundwater pH;[7,8,10−14] however, the biogeochemical and hydrological complexity of in situ subsurface environments limits their widespread implementation. Although engineering approaches are feasible, in situ pH adjustment remains challenging, and all current approaches have limitations.

An alternate solution would be to rely on microorganisms that perform reductive dechlorination under low pH conditions. Enhanced in situ reductive dechlorination has shown success as a cost-effective remedy for a variety of chlorinated pollutants.[15] A number of bacterial isolates responsible for tetrachloroethene (PCE) reductive dechlorination have been identified and include members of the genera *Desulfitobacterium*, *Sulfurospirillum*, *Dehalobacter*, *Desulfuromonas*, *Geobacter*, and *Dehalococcoides* (*Dhc*).[16,17] These dechlorinating bacteria were all enriched and isolated at circumneutral pH, and subsequent characterization revealed that maximum reductive dechlorination activity was achieved at circumneutral pH. Dechlorination activities of these pure cultures were severely inhibited below pH 6.0, and no dechlorination was reported at pH < 5.5 (Table 1).[9,18−20] Based on the information reported in the literature, the known dechlorinating microorganisms are neutrophils and grow under circumneutral pH conditions. To date, no microorganisms capable of growth with chlorinated ethenes in a laboratory setting at pH 5.5 or below have been identified. Also, information is lacking on how pH shifts (e.g., pH decreases following biostimulation) affect microbial community structure and function. To fill these knowledge gaps, a series of fed-batch culture experiments was conducted to determine the pH range of existing pure and mixed cultures capable of dechlorinating chlorinated ethenes, to enrich and isolate PCE dechlorinators active under low pH conditions, and to investigate the responses of the bacterial community to different pH environments.

Table 1. Reported pH Values for Optimal Growth of Characterized Dechlorinating Bacteria

bacteria	pH range	reference
Geobacter lovleyi strain SZ	6.5−7.5	26
Desulfitobacterium sp. strain Y51	6.5−7.5	59
Desulfuromonas chloroethenica strain TT4B	6.5−7.4	23
Desulfuromonas michiganensis strain BB1	6.8−8	23
Sulfurospirillum multivorans	7−7.5	20
Dehalococcoides mccartyi	6−8	60

MATERIALS AND METHODS

Chemicals. PCE and trichloroethene (TCE) were purchased from Acros Organics (VWR international, West Chester, PA), and *cis*-1,2-dichloroethene (*c*DCE), vinyl chloride (VC) and ethene were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). HOMOPIPES (homopiperazine-1,4-bis(2-ethanesulfonic acid)) and MES (2-(*N*-morpholino)-ethanesulfonic acid) were purchased from Acros Organics. Sodium bicarbonate was purchased from Fisher Scientific (Pittsburgh, PA).

Medium Preparation. Reduced mineral salts medium was prepared with deionized water following established protocols.[21] A filter-sterilized (0.22 μm) vitamin stock solution was added by syringe after the medium had been autoclaved.[21] The mineral salts medium contained 30 mM bicarbonate and the pH was adjusted to 7.2 with CO_2. For pH 4.5 mineral salts medium, 30 mM HOMOPIPES (pK_a = 4.84 at 20 °C) replaced bicarbonate. For pH 5.5 or 6 mineral salts medium, MES buffer (30 mM, pK_a = 6.15 at 20 °C) was used. Culture bottles prepared with the Good's buffers HOMOPIPES and MES[22] were flushed with oxygen-free dinitrogen, and following the distribution of medium, 1 mM sodium bicarbonate was added from a 1 M stock solution. The medium pH values were verified after autoclaving, after inoculation, and after incubation. If necessary, pH adjustments were made by adding appropriate volumes of 1 M hydrochloric acid or 1 M sodium hydroxide from nitrogen-flushed anoxic and filtered-sterilized stock solutions.

Screening Available Cultures for Reductive Dechlorination Activity at Different pH Values. Several PCE-dechlorinating isolates including *Desulfuromonas michiganensis* strain BB1,[23] *Desulfitobacterium* sp. strain Viet1,[24] *Desulfitobacterium* sp. strain JH1,[25] *Geobacter lovleyi* strain SZ,[26] and *Sulfurospirillum multivorans*,[20] as well as the *Dhc*-containing consortium BDI (Bio-Dechlor Inoculum)[27] were tested for PCE dechlorination at pH 5.5, 6, and 7.2. The dechlorinating isolates and the PCE-to-ethene-dechlorinating BDI consortium have been maintained with PCE as electron acceptor for numerous transfers.[21] Cultivation occurred in 160 mL serum bottles containing 100 mL pH 7.2 mineral salts medium amended with 5 μL neat PCE (0.49 mM nominal concentration), 10 mL hydrogen (4.2 mM nominal concentration), and 5 mM lactate. Experimental bottles received 3% (vol/vol) inocula from actively dechlorinating cultures. Replicate bottles autoclaved after inoculation served as negative controls.

Sampling Sites, Microcosms Setup and Transfer Cultures. Solid and/or groundwater samples were obtained from 16 locations (4 pristine and 12 contaminated sites), and were used to established microcosms for enriching PCE dechlorinators at pH 5.5 and pH 7.2 (Supporting Information SI, Table S1). Groundwater, soil, and sediment samples were transported in coolers and stored at 4 °C for no longer than 1 week prior to microcosm setup. The solids and groundwater (SI Table S1) were transferred into a glovebox (97% nitrogen/3% hydrogen, vol/vol) (Coy Laboratory Products, Ann Arbor, MI). Using autoclaved spatulas, approximately 10 g (wet weight) of soil or sediment material was added to 160 mL serum bottles filled with 100 mL mineral salts medium containing 5 mM lactate. For groundwater fed-batch culture experiments, 50 mL of groundwater was added to serum bottles containing 50 mL of mineral salts medium containing 5 mM lactate. The serum bottles were closed with autoclaved black butyl rubber stoppers (Geo-Microbial Technologies Inc., Ochelata, OK) held in place with aluminum caps (Wheaton Industries Inc., Millville, NJ). After removing the serum bottles from the glovebox, neat PCE (5 μL) was added to each serum bottle using a 5 μL glass syringe (Hamilton Company, Reno, NV). The Hamilton syringe was flushed at least three times with PCE to remove any air before the needle was flame-

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sterilized, and the PCE was injected through the rubber stopper. Additionally, 10 mL pure hydrogen was added from autoclaved hydrogen gas stock bottles to ensure that electron donor was not limiting. All microcosms were established at least in duplicate and incubated at room temperature (~21 °C). Time zero measurements of chlorinated ethenes were conducted after a 24-h equilibration period. After VC and ethene were detected, the microcosms were shaken by hand for homogenization in preparation for transfer. Using nitrogen gas-flushed 3 mL plastic syringes, 3 mL aliquots of microcosm suspensions were removed and immediately transferred to new bottles with fresh mineral salts medium (pH 7.2 or 5.5) amended with 5 mM lactate, 5 μL PCE and 10 mL hydrogen. No PCE reductive dechlorination daughter products were observed in control vessels without the inoculum.

DNA Extraction. Microbial biomass was collected from 2 mL liquid culture suspensions by vacuum filtration through 0.22 μm membrane filters (Millipore GVWP025000). Filter-trapped microbial cells were suspended in the PowerSoil bead tubes (MO BIO Laboratories Inc., Carlsbad, CA) and ruptured with a high efficiency Bead Ruptor Homogenizer (Omni International, Kennesaw, GA) at a speed of 3.25 m/s for 5 min. Genomic DNA was extracted using the MO BIO PowerSoil DNA Isolation Kit following the manufacturer's recommendations. DNA concentrations were quantified with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE). DNA samples extracted from replicate cultures were pooled and stored at −20 °C.

16S rRNA Gene Amplicon Sequencing and Analysis. MiSeq 16S rRNA gene amplicon sequencing was conducted to resolve the taxonomic compositions of enrichment cultures that showed sustained reductive dechlorination at pH 5.5 and pH 7.2. Sequencing was performed using a MiSeq desktop sequencer (Illumina, Inc., San Diego, CA) available through the Center for Environmental Biotechnology at the University of Tennessee and followed established protocols.[28] Generally, amplification was performed in 50 μL assays consisting of 5 μL DNA sample, 1 μL 515F primer (10 μM), 1 μL barcoded 806R primer (10 μM),[29] and 44 μL of a mixture of 31 μL deionized molecular biology grade water (5 PRIME Inc., Gaithersburg, MD, USA), 5 μL Invitrogen Pfx50 buffer (Invitrogen, Carlsbad, CA), 1 μL dNTP mix (10 mM), 1 μL Invitrogen Pfx50 Polymerase, and 5 μL of $MgCl_2$ (25 mM) (Invitrogen, Carlsbad, CA). The following temperature cycling program was applied: denaturation at 94 °C for 3 min followed by 35 cycles at 94 °C for 45 s, annealing at 55 °C for 60 s, extension at 72 °C, and final extension at 72 °C for 10 min. The amplicon quality (size) was checked using the High Sensitivity DNA Kit on a model 2100 Bioanalyzer (Agilent, Santa Clara, CA). The relative DNA concentrations of individual samples were estimated based on the peak height of the appropriate sized amplicons compared with the upper marker peak heights with samples of known DNA concentrations. Then, individual DNA samples were pooled to contain equal quantities of DNA from each sample. Pooled samples were purified with solid-phase reversible immobilization (SPRI) magnetic beads (Beckman Coulter, Inc., Indianapolis, IN). The purified samples were analyzed again with the High Sensitivity DNA kit for quality assurance and verification of complete removal of primer dimers. The concentration of the pooled amplicons was determined using the qPCR-based Illumina Library Quantification kit (KAPA Biosystems, Boston, MA) following the manufacturer's protocol, and diluted to 10 nM prior to Illumina sequencing. Forward and reverse sequence files were paired, cleaned and analyzed using Mothur software using the analysis pipeline MiSeq SOP.[30] The trimmed and paired sequences were uploaded to the SILVAngs server for analysis based on high-quality SILVA alignments.[31] Out of a total of 172 533 sequences, 83 sequences were rejected by SILVAngs and were not analyzed (SI Table S2). The remaining sequences were analyzed using the SILVAngs data analysis service with the default parameters (www.arb-silva.de/ngs/service/file/?file=SILVAngs_User_Guide_15_12_15.pdf).

Isolation Procedures. Ten-fold dilution-to-extinction series in liquid and semisolid medium (10 mL) amended with 5 mM lactate and 1 μL neat PCE were established in 20 mL glass vials as described.[21] Semisolid medium received 1% (vol/vol) low melting agarose (MP Biomedicals, LLC., Solon, OH), and colony formation was monitored weekly by visual inspection. White colonies with an estimated diameter of approximately 0.5 mm were retrieved from dilution agarose tubes using nitrogen-flushed 1 mL plastic syringes with a 25G 260 × 7/8 BD PrecisionGlide needles (BD, Franklin Lakes, NJ), and transferred to 160 mL serum bottles with fresh pH 5.5 medium to test for PCE dechlorination.

Identification of Isolates. PCR amplicons obtained with the 16S rRNA gene-targeted general bacterial primers set 8F/1541R (8F: 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1541R: 5′-AAG GAG GTG ATC CAG CCG CA-3′)[21] were treated with the DNA Clean & Concentrator-5 kit (Zymo Research Corp., Irvine, CA). Both strands of purified amplicons were Sanger sequenced, and nearly full-length 16S rRNA gene sequences were obtained by using DNA Baser software (Heracle BioSoft SRL, Romania) to pair both strands with default settings. The 16S rRNA gene sequences were then blasted against the NCBI nucleotide (nt) database to identify phylogenetically related (>97% sequence similarity) sequences (SI Table S3). The sequences were imported into the Geneious software environment (Biomatters, Auckland, New Zealand), aligned with MAFFT,[32] and a 16S rRNA gene phylogenetic tree was constructed with Tree Builder using the software's default settings. A Zeiss Axio Imager.A2 (Zeiss, Thornwood, NY) was used to visualize the liquid cultures for microscopic examination.

Analytical Methods. The pH of the bulk liquid phase was determined by transferring 1 mL liquid aliquots from a culturing vessel into a 2 mL plastic tube, centrifuging the tube for 30 s at 18 000 *g*, then measuring the pH of the supernatant with a Fisher Scientific Accumet Glass AgCl pH electrode (Pittsburgh, PA). Total chlorinated solvent mass or concentrations of chlorinated compounds were determined by analyzing headspace gas samples on an Agilent 7890A gas chromatograph (GC) equipped with an Agilent DB-624 column and a flame ionization detector (Agilent Technologies, Santa Clara, CA). Gas samples (100 μL) were removed from the headspace of 160 mL serum bottles using a gastight 250 μL Hamilton SampleLock syringe (Hamilton, Reno, NV) and then manually injected into the GC. The concentrations of chlorinated ethenes were calculated by normalizing the peak area values to those of standard curves generated by adding known amounts of chlorinated ethenes to 160 mL serum bottles with the same headspace-to-liquid ratios. The total moles of polychlorinated ethenes per bottle were calculated according to the equation: total moles = (volume × density)/molecular weight. The retention times for different chlorinated ethenes and ethene were determined by injecting neat

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compounds into the GC, and used to assign peaks to PCE daughter products.[33,34] The average PCE dechlorination rates in pH 5.5 and pH 7.2 cultures were calculated based on the total amount of chloride released over 11-day and 7-day incubation periods, respectively, during which approximately 90% of the initial amount of PCE had been dechlorinated. Chloride release calculations were based on the gas chromatographic concentration measurements of PCE, TCE and *c*DCE, and assumed that each reductive dechlorination step liberates one chlorine substituent as chloride.

Sequence Data. The 16S rRNA gene sequences of *Sulfurospirillum* sp. strain ACS_{TCE} and strain ACS_{DCE} were deposited to GenBank under accession numbers KX101071 and KX101070. Amplicon sequencing data of pH 7.2 and pH 5.5 ACS enrichments were deposited to GenBank under accession numbers SRX2547923 and SRX2547924, respectively.

RESULTS

Screening Dechlorinating Isolates and Consortium BDI for Low pH Reductive Dechlorination Activity. *Desulfuromonas michiganensis* strain BB1 (PCE → *c*DCE), *Desulfitobacterium* sp. strain Viet1 (PCE → TCE), *Desulfitobacterium* sp. strain JH1 (PCE → *c*DCE), *Geobacter lovleyi* strain SZ (PCE → *c*DCE), and consortium BDI (PCE → ethene) containing strain SZ and *Dhc* strains GT, FL2 and BAV1 dechlorinated PCE to the expected dechlorination end products at pH 7.2; however, PCE dechlorinating activity was diminished or lost following transfers to pH 6.0 and pH 5.5 medium (Table 2). *Geobacter lovleyi* strain SZ dechlorinated 68.5 μmoles PCE to *c*DCE within 48 h at pH 7.2, but dechlorination of the same amount of PCE at pH 6.0 required more than 10 days, and no PCE dechlorination was observed at pH 5.5. Similarly, *Desulfuromonas michiganensis* strain BB1 dechlorinated PCE to *c*DCE within 1 week at pH 7.2, but it took more than 30 days to degrade PCE to *c*DCE at pH 6.0. *Sulfurospirillum multivorans* was the only tested organism able to degrade PCE to *c*DCE at pH 5.5, 6.0, and 7.2 within 4 days (SI Table S4); however, this organism could not sustain PCE dechlorination at pH 5.0.

Table 2. Screening of Isolates and Consortium BDI for PCE Degradation at Different pH Values[a]

culture	pH 5.5	pH 6.0	pH 7.2
consortium BDI	–	*c*DCE, VC	ethene
Geobacter lovleyi SZ	–	*c*DCE	*c*DCE
Desulfuromonas michiganensis BB1	–	*c*DCE	*c*DCE
Sulfurospirillum multivorans	*c*DCE	*c*DCE	*c*DCE
Desulfitobacterium sp. JH1	–	–	*c*DCE
Desulfitobacterium sp. Viet1	–	–	TCE

[a]A dash "–" indicates that no dechlorination was observed within the 50-day monitoring period.

Dechlorination Activity in Microcosms and Enrichment Cultures. PCE to ethene reductive dechlorination was observed in microcosms established at pH 5.5 and pH 7.2 with samples collected from five sampling sites (#5, 6, 7, 11, and 16 in SI Table S1). Microcosms established with the soil sample from the ACS site (#13, Table S1) degraded PCE to ethene at pH 7.2, but VC was the dechlorination end product at pH 5.5 (Figure 1). Microcosms established with acidic peat bog material from the Shady Valley location (#12, Table S1) showed PCE to *c*DCE dechlorination at pH 5.5 and pH 7.2. The tidal flat sample (#15, Table S1) degraded PCE to TCE at pH 7.2, but not at pH 5.5. No PCE dechlorination activity was detected in the microcosms established with the other site materials tested.

Attempts to establish stable PCE-to-ethene-dechlorinating enrichment cultures in pH 5.5 medium were not successful for any of the ethene-producing microcosms. PCE dechlorination activity was lost following two consecutive transfers in pH 5.5 medium, except the ACS enrichment cultures, which maintained PCE-to-*c*DCE reductive dechlorination activity at pH 5.5 (Figure 1). By comparison, transfer cultures derived from the ethene-producing pH 7.2 ACS microcosms maintained PCE-to-ethene dechlorination activity following consecutive transfers to pH 7.2 medium (Figure 1). At pH 4.5, PCE dechlorination to *c*DCE was observed in microcosms established with materials from the ACS site, but PCE dechlorination activity could not be maintained at this pH in transfer cultures (i.e., in the absence of the solid phase).

pH Effects on Community Structure. To investigate the changes in community structure in response to pH differences, and to identify the dechlorinator(s) responsible for the measured reductive dechlorination activity in enrichment cultures derived from the ACS microcosms that sustained PCE dechlorination at both pH 5.5 and pH 7.2, 16S rRNA gene amplicon sequencing was performed. After removing low quality reads, 69 030 sequences (17 441 054 total base pairs) and 103 503 sequences (26 171 881 total base pairs) were obtained from the ACS pH 5.5 and the pH 7.2 enrichments, respectively. A total of 172 409 sequences from the pH 5.5 and the pH 7.2 enrichment cultures were classified into 815 operational taxonomic units (OTUs) based on a 98% identity cutoff (SI Table S2), and only 41 sequences could not be classified according to the SILVAngs empirical threshold ((sequence identity + alignment coverage)/2 ≥ 93%).[31] Rarefaction analysis of sequences showed that the rarefaction curves did not plateau, suggesting that the sequencing effort did not capture the diversity of low abundance members in both the pH 7.2 and the pH 5.5 enrichment cultures (SI Figure S1).

Sequences representing the phyla Proteobacteria and Firmicutes were most abundant in the pH 5.5 and pH 7.2 ACS enrichment cultures, respectively (Figure 2). In the pH 5.5 enrichment culture, the phyla Proteobacteria, Firmicutes, and Bacteroidetes contributed 57.9%, 31.8%, and 7.2%, respectively, to the classified sequences, compared to 2.8%, 59.6%, and 3.0%, respectively, in the pH 7.2 enrichment. In the pH 7.2 enrichment culture, sequences of the phyla Caldiserica (4.0%), Chloroflexi (21.9%), and Spirochaetes (4.4%) were more abundant than in the pH 5.5 enrichment (Figure 2). Sequences representing the phyla Aigarchaeota, Thaumarchaeota, Chlorobi, Lentisphaerae, Nitrospirae, and Synergistetes were present in the pH 7.2 PCE-to-ethene-dechlorinating enrichment, but were not observed in the pH 5.5 enrichment, indicating their preference for circumneutral pH conditions. By comparison, sequences representing the phyla Euryarchaeota, Nanoarchaeota, Dictyoglomi, Fusobacteria, and Thermotogae were detected in pH 5.5 PCE-to-*c*DCE-degrading ACS enrichment culture, but not in the pH 7.2 enrichment. Furthermore, the most abundant genera in the pH 5.5 ACS enrichment differed from those dominating in the pH 7.2 enrichment. At pH 7.2, the 16S rRNA gene sequences of the genera *Dhc* (phylum Chloroflexi) and *Acetobacterium* (phylum Firmicutes) dominated the enrichment and accounted for

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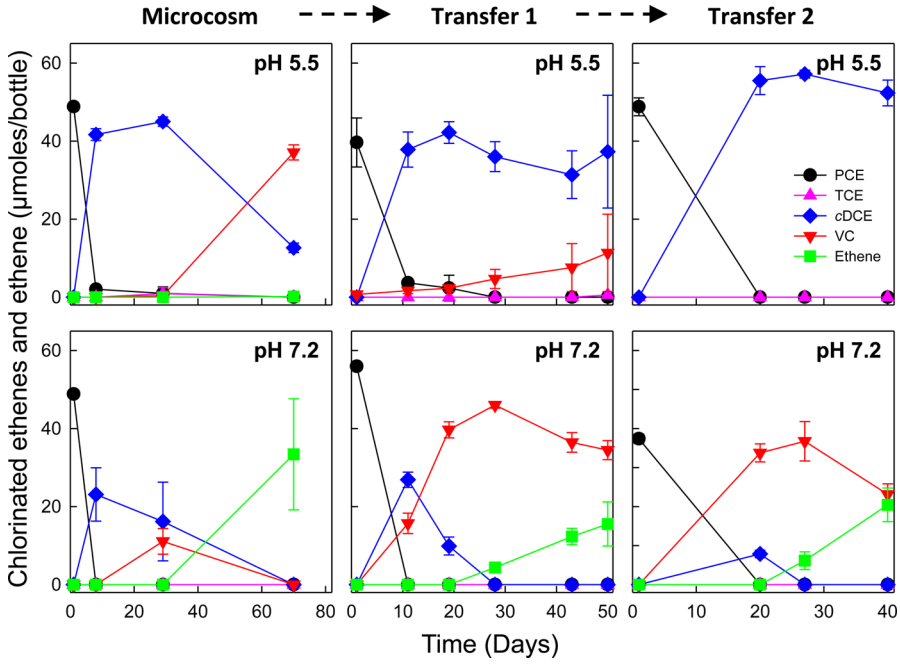
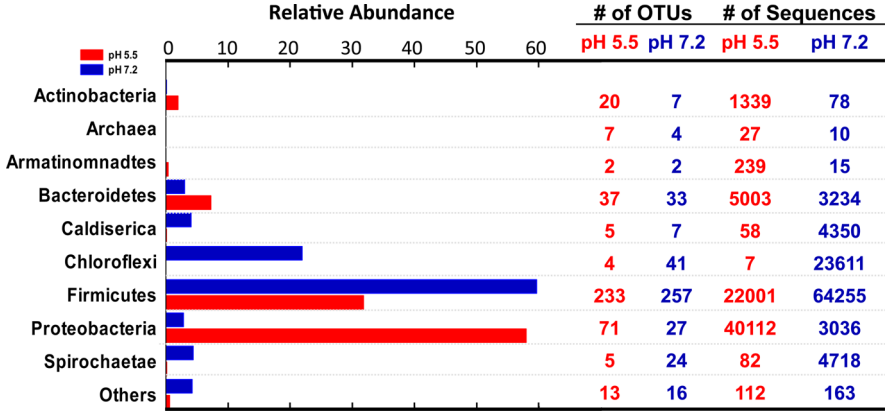


Figure 1. PCE dechlorination in ACS microcosms and 1st and 2nd generation transfer cultures at pH 5.5 (top panels) and pH 7.2 (bottom panels). The error bars represent one standard deviation below or above the average of triplicate batch cultures.



	# of OTUs		# of Sequences	
	pH 5.5	pH 7.2	pH 5.5	pH 7.2
Actinobacteria	20	7	1339	78
Archaea	7	4	27	10
Armatinomnadtes	2	2	239	15
Bacteroidetes	37	33	5003	3234
Caldiserica	5	7	58	4350
Chloroflexi	4	41	7	23611
Firmicutes	233	257	22001	64255
Proteobacteria	71	27	40112	3036
Spirochaetae	5	24	82	4718
Others	13	16	112	163

Figure 2. Relative abundance comparison of the phyla dominating the pH 5.5 and pH 7.2 PCE-dechlorinating enrichments cultures. The numbers of operational taxonomic units (OTUs) and total classified sequences within each phylum are shown on the right.

22.6% and 57.6% of all sequences, respectively. In contrast, *Desulfovibrio* (phylum Proteobacteria) (33.0%), *Sulfurospirillum* (phylum Proteobacteria) (25.2%), and *Megasphaera* (phylum Chloroflexi) (19.9%) sequences dominated the pH 5.5 enrichment (Table 3).

Low pH PCE Reductive Dechlorination by Two *Sulfurospirillum* Isolates. Isolation efforts focused on the ACS PCE-to-*c*DCE-dechlorinating enrichment that maintained dechlorinating activity for at least 10 consecutive transfers in pH 5.5 medium. Repeated dilution to extinction series and transferring individual colonies from the 10^{-4} and 10^{-5} semisolid medium dilution tubes yielded two PCE-dechlorinating isolates. Light microscopic observations revealed uniform, spirillum-shaped cells, and a single 16S rRNA gene sequence type was recovered from each culture. Both isolates grew with PCE in defined mineral salts medium at pH 5.5, and dechlorinated PCE at similar rates of 28.3 ± 2.3 μmoles Cl^- released per liter and day. Isolate ACS_{TCE} generated TCE as the dechlorination end product, whereas isolate ACS_{DCE} dechlorinated PCE and TCE to *c*DCE. Approximately 30% higher dechlorination rates were measured for both isolates at pH 7.2 (Figure 3). Isolates ACS_{TCE} and ACS_{DCE} shared highly similar 16S rRNA genes (99.7% identity) and affiliated with the genus *Sulfurospirillum* within the ε-proteobacteria class. Isolate ACS_{TCE} and isolate ACS_{DCE} shared 98.6% and 98.5% 16S rRNA gene sequence identity, respectively, with the characterized PCE-to-*c*DCE dechlorinator *Sulfurospirillum multivorans* (NR_121740.1). A phylogenetic analysis based on available *Sulfurospirillum* 16S rRNA gene sequences demonstrated that ACS_{TCE} and ACS_{DCE} isolates were most closely related to the PCE-to-*c*DCE dechlorinator *Sulfurospirillum* sp. strain JPD-1 (AY189928.1) with 99.7% and 99.6% sequence identity, respectively (Figure 4).

DISCUSSION

Microbial Reductive Dechlorination in Low pH Groundwater. Successful bioremediation of groundwater impacted with chlorinated ethenes relies on the growth of

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Table 3. Bacterial Community Structure in ACS pH 5.5 and pH 7.2 Enrichment Cultures[a]

	% of sequences	
genus	pH 7.2	pH 5.5
Dehalococcoides	22.6	0.0
Acetobacterium	57.6	0.0
Spirochaetaceae uncultured	4.6	0.1
Caldisericum	4.2	0.1
Desulfuromonadales BVA18	2.6	0.0
vadinBC27	1.1	0.0
Desulfovibrio	0.1	33.0
Sulfurospirillum	0.2	25.2
Megasphaera	0.0	19.9
Propionibacterium	0.0	1.5
Pelosinus	0.0	1.0
others	7.0	19.2
total	100.0	100.0

[a]The percentage values indicate the abundances of representative OTUs as determined by 16S rRNA gene amplicon sequence analysis.

native and/or bioaugmented dechlorinating microorganisms, especially *Dhc*. Since *Dhc* sustains reductive dechlorination activity within a narrow pH range (Table 1), adjustments to maintain a circumneutral pH environment may be necessary. An alternative solution is to rely on microorganisms capable of degrading chlorinated ethenes to ethene at low pH (i.e., pH < 6.0). The available examined *Dhc* cultures could not sustain growth at pH ≤ 5.5, and it was unexpected to observe ethene formation in pH 5.5 microcosms established with samples collected from five geographically distinct locations. Ethene formation was not observed in transfer cultures in pH 5.5 medium, consistent with the current understanding of *Dhc* growth requirements and the reported pH ranges for sustained dechlorination activity of characterized *Dhc*-containing consortia, which are pH 6.1−7.4 for consortium SDC-9,[35] pH 6.0−8.3 for consortium KB-1,[36] pH 6.0−7.5 for consortium PM,[9] and pH 5.9−7.0 for consortium SL2-PCEa.[9] Based on the information reported in the literature (Table 1) and the survey of diverse sample materials from geographically distinct locations, it is indicated that at pH values below 6.0, dechlorinators are rare, and evidence for the existence of microbes that sustain ethene formation under low pH conditions is lacking. Apparently, reductive dechlorination is performed by neutrophilic bacteria, and manipulating the in situ pH may be the only solution to implement bioremediation at sites with low pH groundwater (e.g., aquifers in the Northern Atlantic Coastal Plain). The impact of pH adjustment on key dechlorinators (e.g., *Dhc*), as well as overall microbial community structure and function, is not well understood and further investigations are warranted.

Contradictory to laboratory observations, field studies reported reductive dechlorination and ethene formation indicative of *Dhc* activity under low pH conditions (e.g., pH 2.0−4.0,[37] and pH 5.6−5.8[38]). Various factors complicate the significance and the interpretation of these findings. For instance, we observed ethene formation in pH 5.5 microcosms established with materials from five distinct sites, but this activity was lost in transfer cultures. A likely explanation is that the solid phase materials have an effect on the activity of dechlorinators. It is known that solid phase properties can affect local pH environments,[39] and organisms inhabiting the solid-aqueous interface may experience more favorable (i.e., higher) pH conditions than bulk groundwater pH measurements would indicate. Thus, the in situ geochemical and mineralogical conditions may permit the activity and growth of organohalide-respiring bacteria, such as *Dhc*, in low pH groundwater aquifers. Detailed investigations to explain these observations or provide predictive understanding of microbial activity in low pH aquifers are currently lacking. Also possible is that *Dhc* biomass

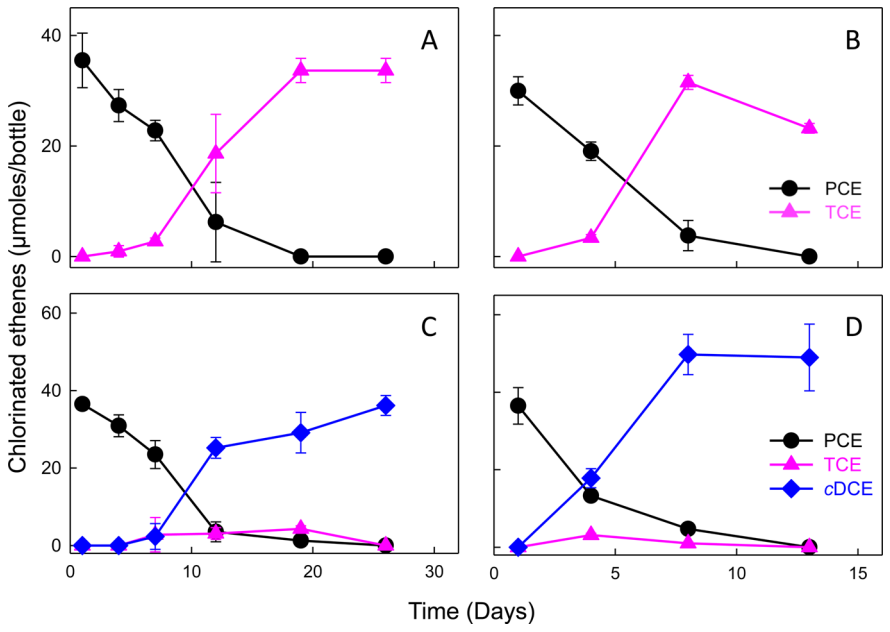

Figure 3. PCE reductive dechlorination by *Sulfurospirillum* isolates strain ACS_{TCE} and ACS_{DCE} at pH 5.5 and pH 7.2. PCE-to-TCE dechlorination by strain ACS_{TCE} at pH 5.5 (A) and pH 7.2 (B). PCE-to-*c*DCE dechlorination by strain ACS_{DCE} at pH 5.5 (C) and pH 7.2 (D). The isolates were grown with lactate as carbon source and electron donor. Error bars represent one standard deviation below or above the average of duplicate batch cultures.

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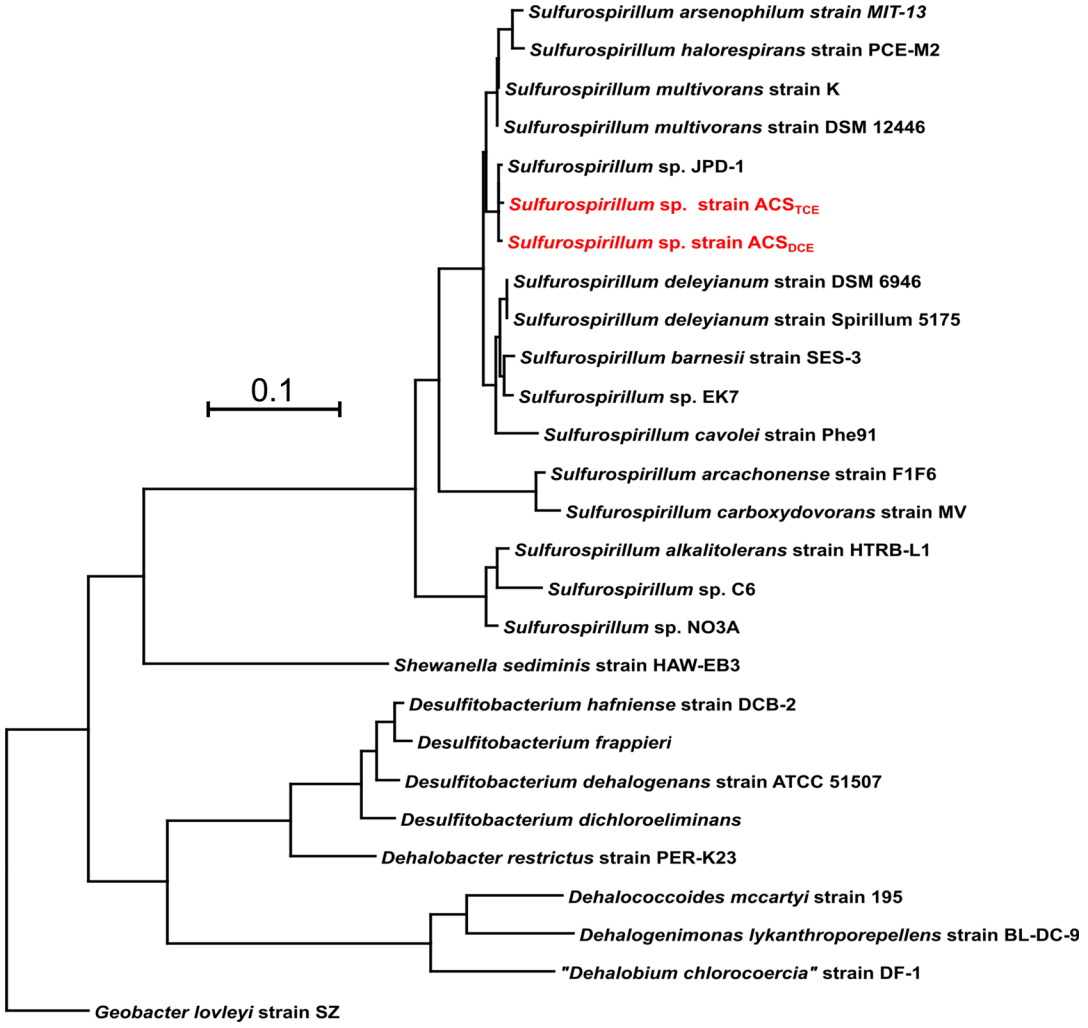

Figure 4. Phylogenetic affiliation of *Sulfurospirillum* sp. strain ACS_{TCE} and ACS_{DCE} based on 16S rRNA gene sequences. Strain ACS_{TCE} and strain ACS_{DCE} are most closely affiliated with *Sulfurospirillum* sp. strain JPD-1. Sequence accession numbers are listed in SI Table S3. The scale bar indicates nucleotide substitutions per site.

maintains residual capacity to dechlorinate chlorinated ethenes following groundwater pH reductions, but no growth occurs and *Dhc* dechlorination activity is not sustained. To complicate matters further, experiments with the *Dhc*-containing PCE-to-ethene-dechlorinating consortium SL2-PCEa demonstrated that individual reductive dechlorination steps were differently affected by pH.[9] For example, TCE-to-*c*DCE dechlorination stalled at pH 4.8, *c*DCE-to-VC dechlorination stalled at pH 5.3, and VC-to-ethene dechlorination stalled at pH 5.9.[9] These findings suggested that pH affected consecutive steps of TCE-to-ethene reductive dechlorination differently, and the VC-to-ethene dechlorination step was most sensitive to pH decreases, which can explain stalled in situ reductive dechlorination activity and elevated VC concentrations at sites with low pH groundwater.[40]

Another complicating factor that can easily lead to erroneous conclusions when interpreting in situ contaminant concentration measurements is the detection of PCE/TCE reductive dechlorination daughter products (i.e., *c*DCE, VC, ethene) in monitoring wells with low pH groundwater. Since dissolved daughter products transport with the groundwater, the location where bacterial reductive dechlorination activity occurs may not coincide with the sampling location. Daughter products generated in upstream aquifer zones with pH conditions conducive for *Dhc* activity may reach low pH zones, where their presence can lead to the erroneous interpretation of microbial dechlorination activity at the sampling location. Thus, information that suggests microbial reductive dechlorination activity should be carefully interpreted to determine the sustainability of the process (e.g., *Dhc* growth).

Members of the Genus *Sulfurospirillum* are Key Dechlorinators in Low pH Groundwater. Several *Sulfurospirillum* isolates (e.g., *Sulfurospirillum* sp. strain MV, *Sulfurospirillum multivorans*, *Sulfurospirillum halorespirans*, and *Sulfurospirillum* sp. strain JPD-1) capable of PCE reductive dechlorination have been described, and activity has been reported between pH 6.0 and 8.0.[20,41−43] *Sulfurospirillum multivorans* has served as a model organism for exploring biochemical aspects of organohalide respiration and is physiologically well characterized, with a pH optimum for growth between 7.0 and 7.5.[20] Among the PCE-dechlorinating pure cultures tested in this study, *Sulfurospirillum multivorans* was the only organism capable of sustained dechlorination activity at pH 5.5, indicating that the pH range of this organism extends to pH 5.5. Two distinct *Sulfurospirillum* isolates, ACS_{TCE} and ACS_{DCE}, that dechlorinated PCE at pH 5.5 were obtained from the ACS microcosms. Furthermore, a recent study exploring silicate minerals for pH control demonstrated

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that *Sulfurospirillum* was enriched in a PCE-to-*c*DCE-dechlorinating consortium at pH 5.5.[9] Two different *Sulfurospirillum* populations were identified in consortium SL2,[44,45] and a functional genotyping approach identified two distinct reductive dehalogenase genes responsible for PCE-to-TCE and PCE-to-*c*DCE reductive dechlorination.[46]

The ability of *Sulfurospirillum* to grow with PCE as an electron acceptor under low pH conditions distinguishes this group from other PCE dechlorinators, and has practical implications for bioremediation. The treatment of dense nonaqueous liquids (DNAPL) ganglia and pools remains a major remediation challenge, and prior work demonstrated that *Sulfurospirillum multivorans* was able to dechlorinate PCE to *c*DCE in the presence of free-phase PCE and contribute to enhanced PCE DNAPL dissolution.[33,34] The large amount of hydrochloric acid released from microbial dechlorination activity at the DNAPL-groundwater interface decreases local groundwater pH, and a sustained process requires pH-tolerant dechlorinators. Batch culture experiments demonstrated that the *Sulfurospirillum multivorans* PCE reductive dechlorination rates can exceed PCE dissolution rates, thus effectively avoiding high aqueous phase PCE concentrations and associated toxicity.[34] High dechlorination rates and activity under low pH conditions suggest that PCE-respiring *Sulfurospirillum* spp. can be relevant contributors to PCE-DNAPL dissolution and PCE reductive dechlorination in low pH groundwater. Further, these unique properties suggest that bioaugmentation approaches for achieving enhanced PCE-DNAPL dissolution would benefit from consortia comprising pH-tolerant, PCE-dechlorinating *Sulfurospirillum* spp. Of course, this process would result in a *c*DCE plume, which is not a desirable remediation outcome, and additional efforts would be required to achieve complete detoxification. For example, the installation of downstream bioreactive barriers and pH adjustment, as appropriate, can be envisaged, to stimulate reductive dechlorination based on *Dhc* activity, or aerobic oxidation, either cometabolic (e.g., methanotrophs)[47] or metabolic (i.e., *Polaromonas* sp. strain JS666).[48]

Effects of pH on Microbial Community Structure. Enrichment at pH 7.2 versus pH 5.5 had a pronounced effect on microbial community structure (Table 3). *Acetobacterium* was the most abundant genus in the pH 7.2 enrichment consistent with the known physiology of *Acetobacterium* spp. to perform H_2/CO_2-reductive acetogenesis and lactate fermentation.[49] Members of the genus *Dhc* were the most prevalent dechlorinators at pH 7.2, apparently outcompeting *Sulfurospirillum* for PCE and TCE under the specific enrichment (e.g., neutral pH) conditions at neutral pH. The relative abundance of members of the phyla Chloroflexi and Spirochaetes decreased at pH 5.5 relative to the pH 7.2 enrichment conditions. The coexistence of *Dhc* and members of the Spirochaetes has been observed in other dechlorinating communities,[50] and it was suggested that *Sphaerochaeta* (a genus within the Spirochaetes) may provide *Dhc* with substrates (e.g., acetate and H_2) or protect *Dhc* from redox stress.[51] Consistent with the pH range observed for the two *Sulfurospirillum* isolates, *Sulfurospirillum* 16S rRNA gene sequences dominated in the PCE-dechlorinating pH 5.5 ACS enrichments. *Desulfovibrio* also made up a substantial portion in the pH 5.5 enrichment culture; this organism has not been implicated in PCE reductive dechlorination, but has considerable metabolic versatility.[52−54] *Desulfovibrio* is commonly observed in low pH enrichments,[55] and may be involved in lactate fermentation. *Megasphaera*, *Pelosinus*, and *Propionibacterium* were also relatively more abundant at pH 5.5 than in the pH 7.2 enrichments. These organismal groups comprise characterized lactate fermenters that produce propionate, acetate, CO_2, and H_2.[56−58] Overall, enrichment at pH 5.5 versus 7.2 had profound effects on the composition of the overall community. *Dhc* strains were eliminated under pH 5.5 enrichment conditions, and PCE-dechlorinating *Sulfurospirillum* became the dominant dechlorinators. Although the presence of other, not yet identified PCE-dechlorinating bacteria cannot be excluded, the low pH conditions resulted in *c*DCE accumulation as the dechlorination end product, and this compound was not utilized as electron acceptor by any member of the pH 5.5 enrichment culture community.

Collectively, these findings suggest that dechlorinators capable of sustained dechlorination of chlorinated ethenes at pH 5.5 are not common in the environment. The examined members of the *Dhc* group could not grow and sustain dechlorination of chlorinated ethenes at pH 5.5, suggesting their requirement for a circumneutral pH environment for effective reductive activity and ethene formation. The efforts to find dechlorinators that sustain reductive dechlorination activity at low pH yielded *Sulfurospirillum* isolates and emphasize that organohalide-respiring members of this genus play relevant roles for PCE reductive dechlorination in low pH groundwater, including DNAPL source zones.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b01510.

The sequencing rarefaction curves (Figure S1), a table detailing the materials used for microcosm setup (Table S1), and the accession numbers of 16S rRNA gene sequences of selected bacteria (Table S3) used to construct the phylogenetic tree are provided (PDF).

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Notes

The authors declare no competing financial interest.

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