

## RESEARCH ARTICLE

# Novel bacterial diversity is enriched with chloroperoxidase-reacted organic matter under anaerobic conditions

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**One sentence summary:** Several novel bacteria genera grow on chloroperoxidase-reacted organic matter.

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## ABSTRACT

Fungal chloroperoxidases (CPOs) are one class of enzymes that produce natural organochlorides in soils. The microbial degradation of these organochlorides is not well known, though has implications for bioremediation, microbial ecology and natural chlorine and carbon cycling. In this study, Illumina-based 16S rRNA gene sequencing and real-time quantitative PCR (qPCR) was used to characterize the bacterial community enriched from an amendment of organic matter reacted with CPO under conditions conducive towards chlorination (CPO-OM). In total, 17 bacterial groups were enriched in triplicate microcosms inoculated with creek sediment and amended with CPO-OM. These bacterial groups were neither enriched with amendments of non-reacted organic matter extract, with or without oxidative stress induced by H<sub>2</sub>O<sub>2</sub>, nor with amendments of organic matter reacted with CPO under non-chlorinating conditions. Of these, only two represented genera with known organohalide respiring bacteria—*Dehalogenimonas* and *Dehalobacter*. The genus *Acetobacterium* was also found to be enriched but the other 14 groups of enriched bacteria do not currently have any close phylogenetically related isolates. This study highlights a gap in the current understanding of the microbiology involved in natural organochloride turnover and suggests that CPO-OM could be used for isolating and culturing strains from novel bacteria genera.

**Keywords:** Organochlorides; organohalide respiration; *Dehalococcoides*; Uncultured bacteria; biogeochemical cycling; soil organic matter

## INTRODUCTION

In terrestrial soils, the mass of organochlorides varies between 2.5 and 63 g (as Cl) m<sup>-2</sup> of area (Öberg and Grøn 1998; Öberg et al. 2005; Redon et al. 2011). Organochlorides are estimated to average approximately 2.3 mg (as Cl) g<sup>-1</sup> C in soil organic matter (SOM) and sum to 3.35 billion tons globally (Öberg 2003). More than 2200 naturally produced organochlorides have been identified (Gribble 2010). Organochlorides have been estimated to have

a turnover time in soils of 100–300 years, which is much longer than SOM on average (Öberg et al. 2005; Bastviken et al. 2007; Redon et al. 2011). Natural organochlorides are thus a substantial and uniquely acting fraction of the SOM.

Organochloride production is known to be catalyzed by heme-dependent haloperoxidases, flavin-dependent halogenases and non-heme iron-dependent halogenases, among other enzymes (Bastviken et al. 2009; Blasiak and Drennan 2009; Butler and Sandy 2009; Rohlenová et al. 2009; van Pée 2012a). The

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haloperoxidase enzymes were the first halogenating enzymes to be discovered, and they are found in a variety of plants, bacteria, fungi and animals (Butler and Sandy 2009). With free inorganic chloride and a reactive oxygen species such as hydrogen peroxide, haloperoxidases chlorinate SOM through produced hypochlorous acid or some other oxidized form of chlorine, which in turn oxidizes electron rich regions of organic compounds both within and outside of the enzyme's active site (Butler and Sandy 2009). Overall, the chlorination reaction from haloperoxidases is somewhat indiscriminant and thus catalyzes the production of a large diversity of organochlorides (van Pée 1996; Leri and Myneni 2010). In contrast, flavin-dependent halogenases are known to be regioselective as the mechanism of chlorination operates through direct interaction of chloride and amino acid residues at the enzyme's active site (Dong et al. 2005).

Perhaps the most commonly studied halogenating enzyme has been the chloroperoxidase (CPO) from the fungus *Caldariomyces fumago* that was first described in 1959 (Shaw and Hager 1959). At an acidic pH (2.75–4.50) and in the presence of free chloride and hydrogen peroxide, CPO has been found to readily chlorinate a large diversity of compounds such as antipyrine, NADH, 2-chlorodimedone, barbituric acid, catechol, a variety of phenols and 17 other aromatic compounds (Libby, Beachy and Phipps 1996; Vázquez-Duhalt, Ayala and Márquez-Rocha 2001; Reina, Leri and Myneni 2004). CPO also catalyzes the chlorination of both aliphatic and aromatic moieties of bulk SOM (Reina, Leri and Myneni 2004) and the aromatic moieties of fulvic acid (Niedan et al. 2000). While CPO predominately chlorinates when used in the presence of free chloride and low pH, it acts as an oxidizing enzyme with cytochrome P450-like activity at higher pH's (>5.0), particularly in the absence of chloride (Vázquez-Duhalt, Ayala and Márquez-Rocha 2001; La Rotta, D'Elia and Bon 2007; Díaz-Díaz et al. 2010; Liu et al. 2014; Pereira, Arends and Sheldon 2015).

The degradation of enzymatically produced organochlorides in soils is not well understood, though it is postulated that organohalide respiring bacteria (OHRB) have a large role in the turnover of natural organochlorides (i.e. Krzmarzick et al. 2012), and it is widely accepted that natural organohalides are the basis for OHRB evolution (Richardson 2013). OHRB couple growth with the dehalogenation of organohalides and have been extensively studied in the context of bioremediation of contaminated soils, sediments and aquifers. Isolated strains from several genera have been found to be OHRB such as *Dehalococcoides*, *Dehalogenimonas*, 'Dehalobium', *Dehalobacter*, *Desulfotobacterium*, *Anaeromyxobacter*, *Desulfomonile*, *Desulfuromonas*, *Geobacter* and *Sulfurospirillum* (Richardson 2013). Most strains of *Dehalobacter*, as well as all current isolates of *Dehalococcoides*, *Dehalogenimonas* and 'Dehalobium', exclusively respire organohalides for energy conservation (Justicia-Leon et al. 2012; Richardson 2013). OHRB are known to dechlorinate a large diversity of anthropogenically produced organohalides, including chlorinated ethenes (Maymó-Gatell et al. 1997) and phenols (Adrian et al. 2007) that also have known natural sources (Abrahamsson et al. 1995; Hoekstra et al. 1999). DNA-based analysis of dechlorinating cultures implicates a further phylogenetic diversity of unisolated bacteria involved in organohalide turnover (i.e. Kittelmann and Friedrich 2008; Krzmarzick et al. 2014). Additionally, some bacteria, such as *Acetobacterium* sp. strain AG, can co-metabolically dehalogenate organohalides but have not been found to use organochlorides for respiration (Ding, Chow and He 2013). A further diversity of bacteria has been linked to organochlorides because of syntrophic relationships to OHRB (e.g. *Sedimentibacter*; Maphosa et al. 2012).

Establishing the connection of these populations to specific dehalogenation activity in soils is difficult; direct analytical measurements of individual compounds at *in situ* concentrations with chromatographic methods is prohibitive due to the low concentrations of individual compounds, structural diversity of natural organochlorides, and the complexity of the background matrices of soils (Leri and Myneni 2010). The ability for bacteria in natural soils to dechlorinate has been shown through the addition and subsequent dechlorination of anthropogenic chemicals (e.g. Zlamal et al. 2017), and *in situ* dechlorination rates have been estimated through the use of  $^{36}\text{Cl}$  tracers and X-ray absorption near edge structure (XANES) of the Cl-C spectral signatures (Leri and Myneni 2010; Montelius et al. 2016). None of these studies linked specific bacterial groups to the dechlorination activities. In a study of the dechlorination of two chlorinated xanthenes, which are a part of a broad class of natural organochlorides (Elix and Crook 1992), one group of unisolated *Firmicutes*, named the 'Gopher group', was enriched during dechlorination (Krzmarzick et al. 2014). Additionally, a phylogenetically diverse group of bacteria spanning the classes *Dehalococcoidia* and *Anaerolineae* was found to grow on a CPO-reacted organic matter (CPO-OM) concomitantly with chloride release (Krzmarzick et al. 2012). A more comprehensive analysis of the bacterial diversity enriched on natural organochlorides, however, has not been completed.

In this study, microcosms were used to test the hypothesis that CPO-OM will induce the growth of known genera of OHRB who evolved using natural organochlorides such as those produced from CPO as their terminal electron acceptors. Microbial communities were analyzed over time in microcosms amended with an extract of SOM reacted with CPO under conditions favorable for chlorination. Controls were amended with either SOM (named OM-Control) to determine whether the bacteria were enriched on SOM in general, SOM reacted with CPO in the absence of free chloride ( $\text{Cl}_{\text{org}}$ -free CPO-OM Control) to determine whether the bacteria grew due to non-chlorinated substrates that may have been produced from the CPO reaction, or co-amended with SOM and hydrogen peroxide ( $\text{H}_2\text{O}_2$  + OM Control) to determine whether bacteria found enriched on the CPO-OM amendment could be enriched due to oxidative stress conditions that CPO-OM may exhibit (Liu et al. 2014). Illumina-based 16S rRNA gene sequencing and real-time quantitative PCR (qPCR) were respectively used to identify and measure the microbial communities enriched over 84 days due to the amendment of CPO-OM.

## MATERIALS AND METHODS

### Production of CPO-OM, OM-Control, $\text{Cl}_{\text{org}}$ -free CPO-OM Control and $\text{H}_2\text{O}_2$ + OM Control amendments

Approximately 2 kg of surface soil was collected from a forest with oak and hickory tree cover in Payne County, Oklahoma, USA, in March 2014 to provide SOM for the enzymatic reaction. The material was rich with detritus and collected from the top 3 cm of the soil horizon (the O and A-horizons). The material was homogenized and stored at  $-20^\circ\text{C}$  until extraction of SOM. Aliquots of 10 g of soil were added to 50 mL Falcon tubes, and the SOM was sequentially extracted with the solvents methanol, acetone, dichloromethane and then hexane (ACS Reagent grade or higher). In each sequential extraction, the aliquot of soil was amended with 10 mL of solvent and vortexed and sonicated at  $50^\circ\text{C}$  for 20 min. The solvent-soil mixture was allowed to settle, and the solvent was transferred to a silanized flask. SOM was

extracted from a total of 400 g of the soil. One set of extractions were mixed and volumetrically split into two 500-mL flasks for the CPO-OM and OM-Control amendment reactions; a second set of extractions was mixed and volumetrically split into two 500-mL flasks for the  $\text{Cl}_{\text{org}}$ -free CPO-OM control and  $\text{H}_2\text{O}_2$  + OM amendment reactions. A stream of compressed air was used to evaporate the solvent to leave behind a dry SOM extract.

A reaction of the SOM with CPO was performed under conditions found previously to be conducive to chlorination activity (Niedan, Pavasars and Öberg 2000; Ortiz-Bermúdez, Srebotnik and Hammel 2003; Reina, Leri and Myneni 2004; Krzmarzick et al. 2012). The dried SOM extract was amended with 100 mL of DI water with 0.1 M  $\text{K}_2\text{PO}_4$  and 20 mM KCl to provide chloride for chlorination. The mixture was stirred vigorously to dissolve the soluble SOM and the pH value was adjusted to 3.0 with phosphoric acid. An aliquot of 100 U of CPO (0.34 mg as crude protein as determined with the Protein Assay Kit II (Bio-Rad Laboratories, Hercules, CA, USA)) was added followed by three additions of 100  $\mu\text{L}$  of 0.1%  $\text{H}_2\text{O}_2$  in 30 min intervals. The chlorination reaction incubated statically overnight, and doses of CPO and  $\text{H}_2\text{O}_2$  were repeated once a day for three additional days. Enzymatic chlorination activity of the CPO under these conditions was verified with a parallel chlorination reaction of phenol (Fig. S1, Supporting Information). The chlorination of the SOM itself could not be verified with ion chromatography (IC) analysis (for chloride) due to the low mass of SOM in the reaction (30 mg) for likely sufficient chloride uptake above the standard deviation of replicate IC analysis ( $\pm 1 \text{ mg L}^{-1}$ ). Spectrophotometric analysis of the reactor contents was performed, which indicated a large shift in the absorbance of the SOM, thus supporting enzymatic transformation (Fig. S2, Supporting Information). The reacted contents were extracted sequentially three times into dichloromethane. The dichloromethane was transferred to silanized 160 mL microcosm bottles and the extracted contents were dried under a stream of compressed air for 12 h to allow total evaporative loss of the dichloromethane solvent.

Three control amendments were also produced and prepared. For the primary control, the SOM extract was treated and processed identically except for the absence of CPO additions during the reaction. For the  $\text{Cl}_{\text{org}}$ -free CPO-OM Control, the SOM was treated identically except for the absence of KCl in the buffer to prevent chlorination but allow other types of CPO catalyzed reactions. For the  $\text{H}_2\text{O}_2$  + OM Control, SOM extract was treated the same way as the primary control (no CPO amendments), but the extract was amended to microcosms with twice weekly 0.01  $\mu\text{M}$  additions of hydrogen peroxide. Final amended weight of amendment was  $9.3 \pm 0.4 \text{ mg}$  in each microcosm in all treatments.

## Microcosms

A 500-mL grab sediment sample was collected from a slowly running creek at Ray Harrell Nature Park (Broken Arrow, OK, USA) in March 2014 for inoculum. The sediment was collected 0.1 m deep under 0.3 m of water. The sediment was homogenized, capped and stored at  $4^\circ\text{C}$  until use in microcosms. Microcosms were constructed in silanized 160 mL serum bottles capped with Teflon® stoppers and aluminum crimps similar to previously published research (Krzmarzick et al. 2014). Each microcosm contained the respective CPO-OM or control amendment, 2.2 g of sediment (by dry weight), 130 mL of anaerobic mineral media reduced with 0.2 mM sodium sulfide and 0.2 mM cysteine (Löffler, Sanford and Ritalahti 2005), 10 mM acetate, 1 mL of vitamin solution (Wolin, Wolin and Wolfe 1963) and  $25 \mu\text{g L}^{-1}$

cobalamin that is otherwise a limiting nutrient for some OHRB (He et al. 2007). Microcosms were constructed and maintained statically in an anaerobic glovebag (Coy Laboratories) with a 3%  $\text{H}_2/97\% \text{N}_2$  headspace. Samples were collected at Days 0, 7, 14, 25, 39, 61 and 82 for DNA analysis with cut-off Pasteur pipettes as previously described (Krzmarzick et al. 2014).

## Illumina sequencing and analysis

DNA was extracted from 1.6 mL of slurry using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Concentrations of extracted DNA were determined with the QuantiFluor dsDNA System (Promega, Madison, WI, USA). For Illumina sequencing, composite DNA extracts of equal concentrations were made from portions of the DNA extracts from the triplicate OM-Control and the triplicate CPO-OM amended microcosms from Days 7, 25, 39, 61 and 82. The 10 composite DNA extracts were submitted to Molecular Research LP (Shallowater, TX, USA) for PCR amplification of 16S rRNA genes, bTEFAP® Illumina MiSeq 2 × 300bp barcoded amplicon sequencing, and analysis with a proprietary pipeline (Dowd et al. 2008). The 16S rRNA genes were amplified with primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3'). The total number of reads varied from 41 649 to 142 939 per sample. OTUs were delineated with a 97% or higher similarity with UCLUST (Edgar 2010). The OTUs were compiled as a percentage of reads for each sample. A total of 4724 OTUs were identified and annotated in the final data set. OTUs were classified using the RDP Classifier (Wang et al. 2007), and OTUs were analyzed with BLASTn (NCBI) to determine the closest related isolates.

OTU sequences recovered from the Illumina data set and closely related 16S rRNA gene sequences deposited into NCBI's GenBank were used for phylogenetic analysis of the OTUs. The sequences of 507–541 bp each were aligned using the MUSCLE alignment tool in MEGA6 (Tamura et al. 2013). Tree topology and branch lengths were obtained from a bootstrap analysis (1000 replicates) using the Neighbor-Joining method (Saitou and Nei 1987) and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei and Kumar 2004).

To identify what groups of bacteria were potentially enriched in the CPO-OM amended microcosms, the relative abundance (OTU read depth/total sample reads) of OTUs in the Illumina-based sequencing analysis was compared between CPO-OM amended microcosms and OM-Control amended microcosms. For each OTU and sampling date, the relative abundance from the OM-Control amended microcosm sample was divided by the relative abundance in the CPO-OM amended sample. The data were then ranked and the distribution is shown in Fig. S3 (Supporting Information). This measurement was used to indicate what level of enrichment in the CPO-OM amended microcosms over the OM-Control amended microcosms is beyond the background distribution. Based on this analysis, it was decided that an OTU with at least a 0.05% relative abundance level and a relative abundance that is  $50 \times$  greater in the CPO-OM amended microcosms compared to the OM-Control microcosms was of interest with regard to being enriched. This lower limit of abundance (0.05%) corresponds to greater than 21 reads in the sample with the lowest read count.

## Quantitative PCR

Because composite samples from the triplicate microcosms were used in Illumina sequencing, the reproducibility amongst the triplicate microcosms was not known from that dataset. Furthermore, because the Illumina sequencing was performed on 16S rRNA gene amplifications using universal bacteria primers, PCR biases may have been introduced, making relative abundances of reads a poor substitute for qPCR-based gene quantification. Thus, targeted qPCR assays were developed for the OTUs identified to be enriched in the CPO-OM microcosms. Novel qPCR primer sets to target these OTUs were developed from the 507 to 541 bp sequences recovered from the Illumina data set. Existing qPCR assays for *Acetobacterium* spp. were used to simultaneously target three OTUs that were closely related to these bacteria (>97% similarity) (Duhamel and Edwards 2006). Primers and qPCR methodological parameters are shown in Table S1 (Supporting Information). Additionally, previously designed qPCR assays were used to quantify 16S rRNA genes from the known OHRB *Dehalococcoides mccartyi* (Duhamel, Mo and Edwards 2004), *Dehalogenimonas* (Yan et al. 2009), *Dehalobacter* (Smits et al. 2004), *Desulfotobacterium* (Smits et al. 2004), *Sulfurospirillum* (Duhamel and Edwards 2006), *Geobacter lovleyi* (Sung et al. 2006) and *Desulfovibrio* (Fite et al. 2004) (Table S2, Supporting Information). A previously designed qPCR assay was used that targets a broader group of bacteria related to *Dehalococcoides* (Krzmarzick et al. 2013) and one that targets the putative OHRB 'Gopher Group' (Krzmarzick et al. 2014) (Table S2, Supporting Information). Each qPCR reaction (10 µl) used 1 × iTaq SyberGreen Supermix with ROX master mix (Bio-Rad Laboratories, Hercules, CA, USA), 10 µg bovine serum albumin, 300 nM of each primer and 0.5 ng of sample DNA extract. Analysis was performed on a CFX Connect Real Time System (Bio-Rad Laboratories, Hercules, CA, USA). The thermocycling protocol was 95°C for 3 min followed by 40 cycles of 95°C for 15 s and an annealing temperature of 59°C for 30 s, with the exception of the qPCR targeting *Dehalococcoides mccartyi* that used an annealing temperature of 56°C. For quality control, a melting curve analysis was performed after each qPCR assay. A small clone library analysis of the amplification from microcosm-derived DNA was used to confirm specificity of the qPCR assays for *Dehalogenimonas*, *Dehalococcoides mccartyi* and *Dehalobacter*. Clone libraries were prepared using the pGEM-T Easy Vector System II (Promega, Madison, WI, USA) and plasmids were sequenced at the Oklahoma State University DNA Sequencing Core Facility; all sequenced amplicons from these clone libraries (nine apiece) were nearly identical to their targeted genus (>99% identity along 16S rRNA genes).

Standards were produced for each respective qPCR assay. PCR amplification products were cloned into *E. coli* cells with the pGEM-T Easy Vector System II kit (Promega, Madison, WI, USA). An isolate containing plasmid was grown overnight in LB media, and the plasmids were extracted with the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Plasmid extract was quantified with a Quantus Fluorometer with the QuantiFluor dsDNA System (Qiagen, Hilden, Germany) and serially diluted to produce qPCR standards. Each sample was analyzed with qPCR in duplicate; the duplicates were log<sub>10</sub> transformed and averaged. Measurements for triplicate microcosms were averaged and these means and standard deviations were used in analysis. Values below the detection limit were given the value of the detection limit in analysis.

## Accession numbers

Illumina MiSeq data are available from NCBI under Bioproject PRJNA326398.

## RESULTS AND DISCUSSION

### Enriched bacterial groups from CPO-OM amendment

From the Illumina-based 16S rRNA gene sequencing and analysis, a total of 38 OTUs were identified to be potentially enriched in the CPO-OM amended microcosms compared to the OM-Control amended microcosms. Conversely, eight OTUs were found to be enriched in the OM-Control amended microcosms compared to the CPO-OM amended microcosms: five were closely related to the genus *Desulfosporosinus*, two were related to *Pseudomonas* and one was distantly related to an *Anoxybacter* (Table S3, Supporting Information). Two of the 38 OTUs identified to be enriched from the CPO-OM amendment were also closely related to *Desulfosporosinus* (>96% sequence identity). Since the relative abundances between the CPO-OM amended and OM-Control microcosm samples were found to be negligible for the genus as a whole (<6 × enriched for all sample dates), these *Desulfosporosinus*-related OTUs were excluded from further analysis. The reason for the differentiation in the specific strains of *Desulfosporosinus* enriched is not known. From targeted qPCR assays of the other 36 OTUs identified as enriched from CPO-OM amendment, 16 were found to be below the detection limit of their respective qPCR assay, to be inconsistently enriched between triplicate microcosms, to not be significantly enriched over time, or to not be significantly different between CPO-OM and OM-Control treatments (Data not shown). The discrepancy between the Illumina-derived data on OTU relative abundances and the qPCR results may at least be partially explained due to PCR biases likely induced in the 16S rRNA gene amplification prior to Illumina sequencing.

Groups of bacteria representing 20 OTUs were found to be reproducibly enriched in the CPO-OM amended microcosms with qPCR analysis. The relative abundances of these OTUs from the Illumina-based 16S rRNA analysis are shown in Table S4 (Supporting Information). Five of these OTUs belong to the phylum *Firmicutes*, seven belong to the phylum *Bacteroidetes*, four belong to the phylum *Cloacimonetes* and one each is associated with the phyla *Acidobacteria*, *Synergistetes*, *Spirochaetes* and candidate division BRC1 (Table 1). The phylogenetic analysis of these OTUs and closely related sequences deposited into NCBI's GenBank are shown in Fig. S4 (Supporting Information). Three OTUs were closely related (>97% identity) to isolated strains of *Acetobacterium*, which contains some strains that can dehalogenate at least co-metabolically (Ding et al. 2013). *Acetobacterium* spp. are also common co-culture species with other OHRB strains (Duhamel and Edwards 2006). The other 17 enriched OTUs from this analysis were distantly related to any isolated strains of bacteria, though 10 of these (OTUs 99, 64, 36, 4909, 48, 569, 824, 378, 933, 4241) have at least 95% identity to at least one 16S rRNA gene sequence deposited into GenBank from enrichment cultures dechlorinating anthropogenic organochlorides. Without closely cultured isolates, however, it is not known if these bacteria groups are OHRB, anaerobically degrade organochlorides by another means such as hydrolytic dehalogenation (Wang et al. 2010), or are co-culture syntrophs that benefit from organochloride degrading bacteria.

The 20 enriched OTUs were analyzed across the triplicate CPO-OM amended and control microcosms by 15 qPCR assays.

**Table 1.** Phylogenetic association of the OTUs found enriched on CPO-OM and their closest related isolate.

| OTU      | Phylogenetic association                                                      | Closest related isolate                                                    |
|----------|-------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| OTU 409  | <i>Acidobacteria</i> , unclassified genus in family <i>Holophagaceae</i>      | 94% identity to <i>Geothrix fermentans</i> [NR_063779]                     |
| OTU 71   | <i>Firmicutes</i> , unclassified class                                        | 83% identity to <i>Dehalobacter restrictus</i> [CP007033]                  |
| OTU 295  | <i>Firmicutes</i> , unclassified class                                        | 82% identity to <i>Clostridium spiroforme</i> JCM 1432 [NR_114393]         |
| OTU 443  | <i>Firmicutes</i> , genus <i>Acetobacterium</i>                               | 97% identity to <i>Acetobacterium</i> sp. HAAP-1 [AF479584]                |
| OTU 8121 | <i>Firmicutes</i> , genus <i>Acetobacterium</i>                               | 97% identity to <i>Acetobacterium wieringae</i> [NZ_LKEU01000008]          |
| OTU 69   | <i>Firmicutes</i> , genus <i>Acetobacterium</i>                               | 97% identity to <i>Acetobacterium</i> sp. HAAP-1 [AF479584]                |
| OTU 4241 | <i>Synergistetes</i> , unclassified genus in the family <i>Synergistaceae</i> | 87% identity to <i>Thermanaerovibrio velox</i> Z-9701 [NR_104765]          |
| OTU 933  | <i>Spirochaetes</i> , genus <i>Treponema</i>                                  | 90% identity to <i>Treponema calarium</i> DSM7334 [EU580141]               |
| OTU 333  | Domain <i>Bacteria</i> , unclassified phylum BRC1                             | 78% identity to <i>Desulfocurvus vexinensis</i> VNS36 [NR_043981]          |
| OTU 99   | <i>Cloacimonetes</i> , genus <i>Ca. Cloacamonas</i>                           | 88% identity to <i>Ca. Cloacamonas acidaminovorans</i> st. Evry [CU466930] |
| OTU 64   | <i>Cloacimonetes</i> , genus <i>Ca. Cloacamonas</i>                           | 95% identity to <i>Bacterium</i> W18 [DQ238245]                            |
| OTU 36   | <i>Cloacimonetes</i> , genus <i>Ca. Cloacamonas</i>                           | 94% identity to <i>Cloacimonetes</i> JGI 0000059-L07 [KJ535434]            |
| OTU 4909 | <i>Cloacimonetes</i> , genus <i>Ca. Cloacamonas</i>                           | 92% identity to <i>Cloacimonetes</i> JGI 0000059-L07 [KJ535434]            |
| OTU 306  | <i>Bacteroidetes</i> , unclassified class                                     | 90% identity to <i>Bacteroidetes</i> PPf50E2 [AY548787]                    |
| OTU 343  | <i>Bacteroidetes</i> , unclassified class                                     | 88% identity to <i>Bacteroidetes</i> PPf50E2 [AY548787]                    |
| OTU 801  | <i>Bacteroidetes</i> , unclassified class                                     | 90% identity to <i>Bacteroidetes</i> PPf50E2 [AY548787]                    |
| OTU 378  | <i>Bacteroidetes</i> , unclassified family in order <i>Bacteroidales</i>      | 86% identity to <i>Bacterium</i> YC-ZSS-LKJ7 [KP174588]                    |
| OTU 824  | <i>Bacteroidetes</i> , unclassified class                                     | 85% identity to <i>Bacterium</i> YC-ZSS-LKJ7 [KP174588]                    |
| OTU 48   | <i>Bacteroidetes</i> , unclassified class                                     | 87% identity to <i>Flavobacterium</i> sp. CC-PY-35 [KF851345]              |
| OTU 569  | <i>Bacteroidetes</i> , unclassified class                                     | 86% identity to <i>Flavobacterium</i> sp. CC-PY-35 [KF851345]              |

Three pairs of closely related OTUs (36 and 4909, 306 and 343 and 48 and 569) and the three *Acetobacterium*-related OTUs (443, 8121 and 69) were measured by single qPCR assays, respectively. The growth of these bacterial groups as measured by qPCR are shown for the CPO-OM amended and OM-Control amended microcosms in Fig. 1. These bacterial groups were not enriched or grew significantly in the  $\text{Cl}_{\text{org}}$ -free CPO-OM Control amendment, or from the  $\text{H}_2\text{O}_2$  + OM Control conditions (Fig. S5, Supporting Information). Thus, these bacterial groups are neither likely growing on non-chlorinated products that CPO may have produced nor do they benefit from an environment containing free-radical oxidative stress that the CPO reaction may have produced (Liu et al. 2014). With rare exceptions (Day 82, OTU 824 and OTU group 36/4909), the abundances of these bacterial groups were not significantly different in the  $\text{Cl}_{\text{org}}$ -free CPO-OM Control,  $\text{H}_2\text{O}_2$  + OM Control or the OM-Control amended microcosms (Student t-test,  $P > 0.05$ ).

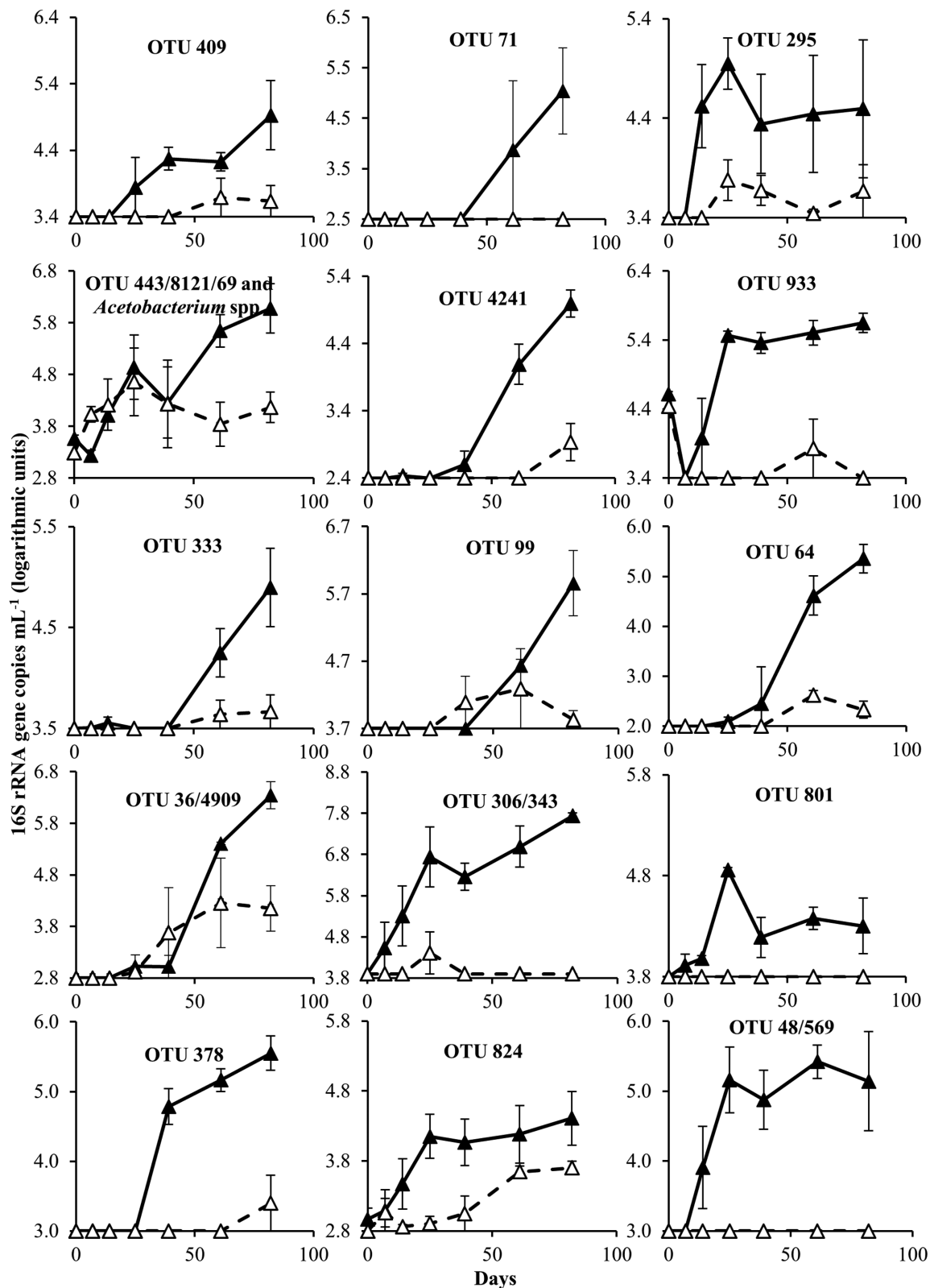
The growth of OTUs is roughly split between those that grew most significantly within the first 39 days and those that grew the most significantly only after 39 days. The five groups in the phylum *Bacteroidetes* (OTU pairs 306/343 and 48/569, and OTUs 801, 378, 824) the OTU from the phylum *Spirochaetes* (OTU 933), and one *Firmicutes* OTU (OTU 295) were enriched heavily before Day 39. One of these groups, OTU pair 306/343, exhibited the highest enrichment for any measured OTU in this study, growing to 8.8 log units of 16S rRNA genes  $\text{mL}^{-1}$  while remaining below or at the detection limit in the OM-Control amended microcosms. This OTU is closely related to an uncultured clone sequence from an anaerobic digester degrading a *Mycrocystis* bloom (98% identity) (NCBI GenBank database accession number HQ904207) (Fig. S4, Supporting Information). Cyanobacteria, such as *Mycrocystis*, are known to produce a wide range of organochlorides (Gribble 2003). The other two *Firmicutes* groups (OTU 71 and the *Acetobacterium* cluster), the three *Cloacimonetes* groups (OTU pair 36/4909, and OTUs 64 and 99), the OTU from the phyla *Synergistetes* (OTU 4241), and the OTU from candidate division BRC1 (OTU

333) became significantly enriched only after Day 39. The OTU from the phylum *Acidobacteria* (OTU 409) exhibited enrichment more consistently over time.

### Analysis of the *Dehalococcoides*-like *Chloroflexi*

The phylum *Chloroflexi* contains the well-studied OHRB strains within the genera *Dehalococcoides* and *Dehalogenimonas* as well as the strain ‘*Dehalobium chlorocoercia* DF-1’ (May et al. 2008). Additionally, molecular analysis of organochloride degrading cultures suggests that other genera within the class *Dehalococcoidia* and the closely related class *Anaerolineae* (i.e. Watts et al. 2005; Kittelmann and Friedrich 2008) likely dechlorinate organochlorides as well. Indeed, a marine *Anaerolineae* has recently been found to contain a reductive dehalogenase gene (Fullerton and Moyer 2016). In previous research, a qPCR assay of the ‘*Dehalococcoides*-like *Chloroflexi*’, which targeted some groups of *Dehalococcoidia* and *Anaerolineae*, found that these bacteria are enriched from CPO-OM (Krzmarzick et al. 2012) and correlated to the fraction of SOM that was chlorinated in natural soils. Using a slightly broader methodology to target this group (Krzmarzick et al. 2013), the *Dehalococcoides*-like *Chloroflexi* was again shown to be enriched by the CPO-OM used in this study (Fig. 2A). By Day 61, the number of 16S rRNA genes of this group of bacteria grew to  $7.9 \pm 0.2$  log copies  $\text{mL}^{-1}$ , a 1.3 log increase compared to the OM-Control amended microcosms. The *Dehalococcoides*-like *Chloroflexi* were at similar abundances in the OM-Control,  $\text{H}_2\text{O}_2$  + OM Control and  $\text{Cl}_{\text{org}}$ -free CPO-OM Control amended microcosms in the first 39 days (Fig. S6, Supporting Information), but then either maintained or decreased in concentration in the  $\text{Cl}_{\text{org}}$ -free CPO-OM Control and  $\text{H}_2\text{O}_2$  + OM Control amended microcosms, while the population rose on average 0.8 log units of 16S rRNA genes  $\text{mL}^{-1}$  in the OM-Control microcosms.

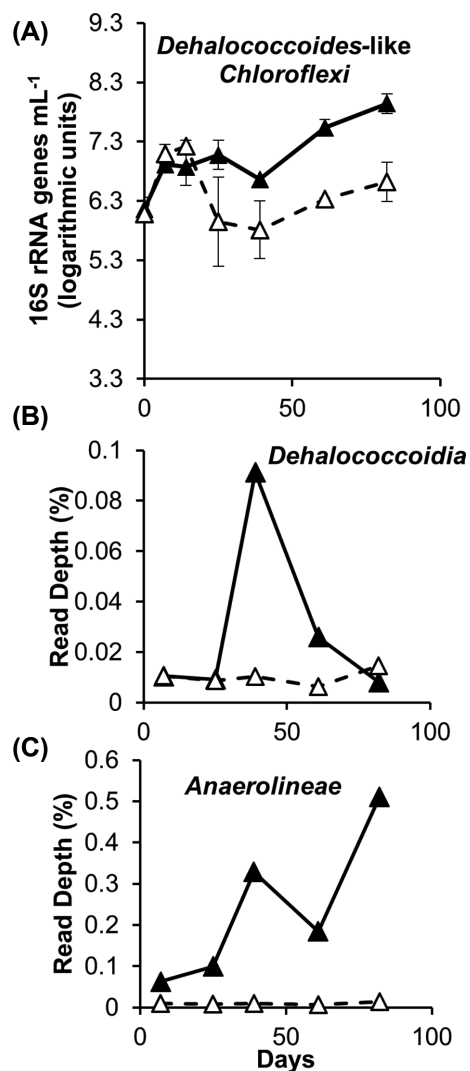
The growth of the *Dehalococcoides*-like *Chloroflexi* group in the CPO-OM amended microcosms is significantly less than the two to four orders of magnitude enrichment in the previous



**Figure 1.** The number of 16S rRNA genes  $\text{mL}^{-1}$  over time as determined by targeted qPCR assays for the enriched OTUs. The data for CPO-OM amended microcosms are shown with dark triangles, solid lines. OM-Control amended microcosms are shown with open triangles, dashed lines. Data for  $\text{Cl}_{\text{org}}$ -free CPO-OM and  $\text{H}_2\text{O}_2$  + OM Control amended microcosms are shown in Fig. S5 (Supporting Information). Error bars represent standard deviations for triplicate microcosms.

study (Krzmarzick et al. 2012), and the growth in this study

occurred over a much longer time scale (after 61 days, compared to <20 days in the earlier study). Though analysis of



**Figure 2.** The qPCR assay targeting a group of *Dehalococcoides*-like *Chloroflexi* (A) and the relative abundance (read depth/total reads) of the composite samples from the Illumina-based 16S rRNA gene analysis for *Dehalococcoidia* (B) and *Anaerolineae* (C) indicates an enrichment for these classes of the phylum *Chloroflexi* due to CPO-OM. The phylogenetic analysis of the OTUs in these two classes found to be more than 10 × enriched from the Illumina 16S rRNA gene analysis is shown in Fig. S7 (Supporting Information). The data for CPO-OM amended microcosms are shown with dark triangles, while OM-Control amended microcosms are shown with open triangles. Error bars represent standard deviations for triplicate microcosms in (A).

the organochlorides is not available between studies to conclusively compare CPO-OM mixtures used, the two studies differ significantly in their treatment of the CPO-OM produced mixture prior to amendment in the microcosms. In Krzmarzick et al. (2012), produced CPO-OM was processed through a hexane/acetone extraction and purified across a C18 column, which may have biased for lighter and smaller mass fractions of CPO-OM compared to the present study that contained no purification, used dichloromethane extraction, and used a blow-down method that likely evaporated volatiles. Other discrepancies could result from the inoculum used, the starting material the organochlorides were produced from, and the concentrations of organochlorides amended. Advances in analytical analysis of complex mixtures of natural organochlorides are needed to help elucidate these possibilities.

From the Illumina-based 16S rRNA gene analysis on the composite samples, the class *Dehalococcoidia* was shown to be enriched in the CPO-OM microcosm sample over the OM-Control amended microcosm sample according to relative abundances (Fig. 2B). The percentage of the reads belonging to this class rose from 0.01% on Days 7 and 14 to 0.091% on Day 39 in the CPO-OM amended microcosm sample, while in the OM-Control amended microcosm sample, the relative abundance was maintained at about 0.010%. From the Illumina data set, the class *Dehalococcoidia* at Day 39 included 23 unique OTUs in the family *Dehalococcoidia*; each OTU contained five or fewer reads out of 41 649 total for that sample. One OTU had 99% identity to the 16S rRNA gene of *Dehalogenimonas* strain BL-DC-9, while another had 99% identity to the 16S rRNA genes of clones within the putatively perchloroethylene-dechlorinating Lahn cluster (Kittelman and Friedrich 2008). No OTU was found to have more than 95% 16S rRNA gene identity to *Dehalococcoides mccartyi* or ‘*Dehalobium chlorocoercia*’. A phylogenetic analysis of the most significantly enriched OTUs in this class is shown in Fig. S7 (Supporting Information). Thus, though these data agree with the qPCR measurement of a broad enrichment of the class *Dehalococcoidia* from CPO-OM amendment, it suggests that no single OTU in this class became heavily enriched.

The Illumina-based 16S rRNA gene analysis also shows that the class *Anaerolineae* was enriched in the CPO-OM amended microcosms up to 33 × the relative abundance of the OM-Control amended microcosms (Fig. 2C). A total of 232 OTUs belonging to this class was recovered from the Illumina sequencing. Of these, 30 were at least 10 × enriched in the CPO-OM amended reactors over the OM-Control amended microcosms with regard to relative abundance, and comprised at least 0.01% of the relative abundance of that sample. Thus, similar to the *Dehalococcoidia*, the enrichment of the class *Anaerolineae* was across the broad class and not specific to any single OTU. BLAST queries and phylogenetic analysis indicates that many of these enriched clones were similar (>98% identity) to clones from cultures and soils dechlorinating anthropogenic organochlorides (Fig. S7, Supporting Information). Other sequences that were highly related were from petroleum degradation microcosms and soils, rice paddy soils, and insect gut microbiomes.

### Targeted qPCR of OHRB genera

Seven genera containing known OHRB and the putative OHRB ‘Gopher group’ (Krzmarzick et al. 2014) were analyzed with targeted qPCR assays (Fig. 3; Fig. S6, Supporting Information). *Dehalococcoides mccartyi* was found to grow in both the OM-Control amended microcosms and the CPO-OM amended microcosms nearly two orders of magnitude by Day 61. Though *Dehalococcoides mccartyi* sequences were not found in the Illumina data set, clone library analysis of the qPCR amplicons confirmed that the qPCR assay did amplify *Dehalococcoides mccartyi* 16S rRNA genes. Because all strains of *Dehalococcoides* thus far are obligate OHRB, it is speculated that they grew on natural organochlorides pre-existing in the sediment used in the inoculum and/or in the soil used for the CPO-OM and OM-Control amendments. Growth of this genus in non-organochloride amended microcosms is commonly reported as these bacteria are speculatively responding to the highly reduced enrichment conditions while growing on *in situ* natural organochlorides (e.g. Watts et al. 2005). *Dehalococcoides mccartyi* rose similarly in the Cl<sub>org</sub>-free CPO-OM Control-amended and OM-Control amended microcosms (5.7 ± 0.3 and 6.0 ± 0.3 log units of 16S rRNA genes mL<sup>-1</sup> by Day 61, respectively), but this growth was significantly lower in the H<sub>2</sub>O<sub>2</sub> + OM

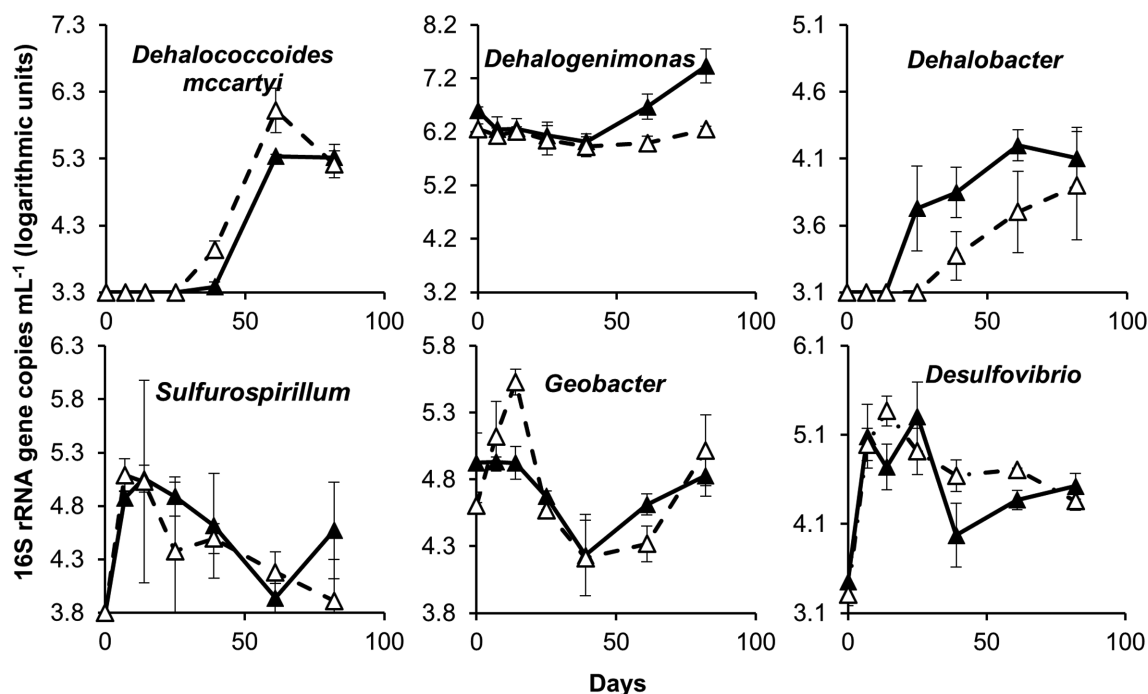


Figure 3. The number of 16S rRNA genes mL<sup>-1</sup> over time as determined by targeted qPCR assays for known organohalide respirers. The data for CPO-OM amended microcosms are shown with dark triangles, while OM-Control microcosms are shown with open triangles. Error bars represent standard deviations for triplicate microcosms. Data for Cl<sub>org</sub>-free CPO-OM and H<sub>2</sub>O<sub>2</sub> + OM Control amended microcosms are shown in Fig. S6 (Supporting Information).

Control amended microcosms (growth to  $3.7 \pm 0.1$  log units of 16S rRNA genes mL<sup>-1</sup> by Day 61). Because *Dehalococcoides mccartyi* is highly sensitive to oxygen (Amos et al. 2008), the hydrogen peroxide additions likely inhibited its growth, and its lack of growth in the CPO-OM amended microcosms could be influenced from possible oxidative stress from compounds produced from the CPO reaction.

*Dehalogenimonas* was found to be enriched in the CPO-OM amended microcosms compared to the control microcosms. This enrichment was moderate, being  $7.4 \pm 0.3$  log units of 16S rRNA genes mL<sup>-1</sup> in the CPO-OM amended microcosms by Day 84 compared to  $6.2 \pm 0.1$  log units in the OM-Control amended microcosms but was well-reproduced (Student t-test,  $P = 0.02$ ). In the Cl<sub>org</sub>-free CPO-OM Control and H<sub>2</sub>O<sub>2</sub> + OM Control amended microcosms, the levels of *Dehalogenimonas* were similar to the OM-Control amended microcosms (Student t-test,  $P > 0.05$ ). *Dehalobacter* was found to be slightly enriched from the CPO-OM amendment, growing from below detection limit to  $4.2 \pm 0.1$  copies of 16S rRNA genes mL<sup>-1</sup> by Day 61. *Dehalobacter* also grew in the OM-Control amended microcosms to similar levels by Day 82, albeit at a slower rate. Thus, the CPO-OM spurred the growth of *Dehalobacter* comparatively minimally compared to the heavily enriched bacterial groups identified above.

*Sulfurospirillum*, *Geobacter* and *Desulfovibrio* were not found to be reproducibly enriched from CPO-OM amendment. Both *Sulfurospirillum* and *Desulfovibrio* grew in both the CPO-OM amended and control microcosms during the first 7 days. *Geobacter* populations fluctuated during the study though reproducible enrichment was not observed. The genus *Desulfotobacterium* and the related 'Gopher group' were not found above the detection limit in any samples.

The lack of growth of most known OHRB on the CPO-OM amendment is not understood from these experiments. The lack of enrichment could be an inability to outcompete other

microbial groups for this substrate under the chemical/physical conditions of the experiment, or these strains may lack the ability or any competitive advantage to use CPO-OM for their physiology. Other organochlorides such as those formed from FADH<sub>2</sub>-dependent halogenases (van Pée 2012b; Aeppli et al. 2013) may provide more suitable substrates for these OHRB instead. Some evidence suggests that FADH<sub>2</sub>-dependent, O<sub>2</sub>-dependent, and nonheme iron,  $\alpha$ -ketoglutarate-dependent halogenases are more heavily responsible for organochloride production in the environment than CPOs (van Pée 2012a), and thus it is possible that the CPO did not catalyze significant fractions of the most common organochlorides in soils. Pure culture studies with OHRB could elucidate these possibilities.

In marine systems, natural organobromides are abundant, particularly in marine sponges (Gribble 1999). Though many of the well-studied OHRB such as *Dehalococcoides* have debromination abilities (Lee et al. 2011), sponges are not currently known to host such organisms (Taylor et al. 2007). Rather, a strain of a novel species of *Desulfoluna* was isolated from marine sponges that has been found to debrominate the bromophenols found in high abundances in the sponge (Ahn, Kerkhof and Häggblom 2009). Bacteria dominant in the niche dechlorinating CPO-produced organochlorides in soils may similarly be unique to those isolated in studies evaluating the dechlorination of anthropogenics.

In conclusion, a total of 17 closely related genus-level groups of bacteria, including the known OHRB genera *Dehalobacter* and *Dehalogenimonas*, were identified to be significantly enriched from the CPO-OM across triplicate microcosms compared to control microcosms during the 82-day study. These two OHRB genera, however, were enriched less than the nearly two to five orders of magnitude enrichment of *Acetobacterium* and 14 other groups of bacteria measured in this study. Additionally, the study suggests a broad heterogeneous group of *Dehalococcoidia*

and *Anaerolineae* were enriched from CPO-OM. The large number of enriched OTUs without cultured strains indicates a diversity of bacteria occupy a niche influenced by CPO-OM, whether through organohalide respiration, some other dehalogenation or degradation, or syntrophic relationships with dechlorinating species. CPO-OM potentially could provide a means for the enrichment and isolation of several novel bacterial genera. The lack of enrichment from the CPO-OM of several known OHRB such as *Dehalococcoides mccartyi* additionally suggests that a diversity of OHRB either do not occupy a natural niche centered on CPO-produced organochlorides or were physiologically outcompeted due to chemical/physical conditions and microbial community dynamics in these microcosms. The primary drivers of niche differentiation between OHRB strains are not well understood, though recent evidence suggests the chlorinated substrates may be a primary driver (Tang et al. 2016) which raises the possibility that some strains of OHRB may have evolved towards specializing on chlorinated substrates other than those produced by CPOs. Furthermore, physical-chemical parameters such as available carbon source, pH and temperature likely biased the specific groups of organisms enriched on the CPO-OM. Further experiments are needed to elucidate the specific niche favorable to the phylogenetically diverse OHRB strains.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](#) online.

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**Conflict of interest.** None declared.

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