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A natural niche for organohalide respiring *Chloroflexi*

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Abstract

The phylum *Chloroflexi* contains several isolated bacteria that have been found to respire a diverse array of halogenated anthropogenic chemicals. The distribution and role of these *Chloroflexi* in uncontaminated terrestrial environments, where abundant natural organohalogens could function as potential electron acceptors, has not been studied. Soil samples (116 total, including 6 sectioned cores) from a range of uncontaminated sites were analyzed for the number of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes present. *Dehalococcoides*-like *Chloroflexi* populations were detected in all but 13 samples. The concentrations of organochlorine ([organochlorine]), inorganic chloride, and total organic carbon (TOC) were obtained for 67 soil core sections. The number of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes positively correlated to [organochlorine]/TOC while the number of *Bacteria* 16S rRNA genes did not. *Dehalococcoides*-like *Chloroflexi* were also observed to increase in number with a concomitant accumulation of chloride when cultured with an enzymatically produced mixture of organochlorines. This research provides evidence that organohalide respiring *Chloroflexi* are widely distributed as part of uncontaminated terrestrial ecosystems, they are correlated to the fraction of TOC present as organochlorines, and they increase in abundance while dechlorinating organochlorines. These findings suggest organohalide respiring *Chloroflexi* may play an integral role in the biogeochemical chlorine cycle.

Introduction

Chloroflexi is a deeply branching and diverse phylum containing isolates that are aerobic and anaerobic thermophiles, filamentous anoxygenic phototrophs, and anaerobic organohalide respirers *(17, 20, 32, 39)*. *Chloroflexi* have been estimated to dominate the microbial community of some sea-floor sediments and also can make up 12% and 16% of the community in the B-horizon of temperate grasslands and alpine meadows, respectively *(9, 21, 47)*. Much of the *Chloroflexi* present in these environments have been found to form deeply branching lineages unrelated to any isolated strains of *Chloroflexi*. In addition, there is a lack of physiological data regarding the niche of these high-abundance *Chloroflexi*.

The *Chloroflexi* phylum contains several isolates that have been shown to be obligate organohalide respirers. These isolates include the genus *Dehalococcoides*, and more recently, *Dehalobium chlorocoercia* DF-1, strain *o*-17, and *Dehalogenimonas lykanthroporepellens* strains BL-DC-8 and BL-DC-9 *(10, 31, 32, 50)*. Although the *Dehalococcoides* isolates have nearly identical 16S rRNA sequence similarity, *Dehalobium*, strain *o*-17, and *Dehalogenimonas* are more distantly related, with 89-91% 16S rRNA gene sequence identity to each other, and approximately 87-90% 16S rRNA gene sequence identity to the cultured *Dehalococcoides* species *(5, 31, 50)*. Members of the genus *Dehalococcoides* have been found to dechlorinate a wide range of persistent organic contaminants, and as a part of mixed consortia, *Dehalococcoides*-like species are thought to be promising for bioremediation applications *(3, 11, 18, 32)*. All cultured organohalide respiring *Chloroflexi* have been shown thus far to be obligate organohalide respirers and share similar limited metabolic capabilities with respect to nutrients and electron donors *(19, 31, 50)*.

Although the connection between the organohalide respiring *Chloroflexi* and the dechlorination of anthropogenic contaminants in laboratory cultures is well established, additional evidence suggests that in the environment other electron acceptors, such as natural organochlorines, exist. For example, in a study of sediments contaminated with 1,2-dichloroethane (1,2-DCA), the abundance of *Dehalococcoides* did not correlate with the presence or absence of 1,2-DCA dechlorination *(45).* In another study of the halorespiration of chlorinated benzenes, the abundance of *Dehalococcoides* in contaminated river sediment did not correlate significantly with the amount of hexachlorobenzene *in situ (42)*. Furthermore, in follow-on research by the same investigators, *Dehalococcoides*-like organisms from fresh sediment grew two orders of magnitude in batch cultures before dechlorination of amended chlorobenzenes was detected *(43)*. This disconnect between the presence and growth of *Dehalococcoides*-like species and the organohalide respiration of known chlorinated contaminants suggests that much is unexplained concerning organohalide respiration in the environment.

In uncontaminated systems, naturally occurring organohalogens could potentially serve as electron acceptors for organohalide respiring bacteria. In marine environments, natural organobromine compounds are produced by a variety of species and include bromoindoles, -phenols and -pyrroles, among other molecules *(15, 44)*. Natural organobromine is ubiquitous in marine sediments and appears to be degraded during the breakdown of organic matter as part of a biogeochemical bromine cycle *(27)*. Reducing conditions are believed to promote reductive debromination of natural organobromine in the sedimentary environment *(4, 6, 33)*. Indeed, the hypothesis that bacteria indigenous to seafloor sediments may respire brominated phenols has been supported in the recent literature *(13)*.

The natural chlorine cycle has also received increasing attention as a multifaceted biogeochemical process. Plants, marine organisms, insects, bacteria, fungi, and mammals produce thousands of natural organochlorines, and many of these organochlorines closely resemble anthropogenic compounds *(14, 16, 36, 46)*. In terrestrial environments, the transformation of chloride into organochlorine compounds occurs in part via the activity of the chloroperoxidase enzyme *(38)*, resulting in organochlorine levels often exceeding those of chloride in surface soils *(36, 37)*. With soil depth, chlorine speciation changes from predominantly organic to inorganic, suggesting that the natural organochlorine in soil organic matter may undergo biogeochemical dechlorination processes as well *(29)*. The hypothesis that organohalide respiring *Chloroflexi* may use natural organochlorines as electron acceptors in uncontaminated environments has been discussed in recent literature *(2, 7, 19, 23, 24)*, though the association between organohalide respiring *Chloroflexi* and natural organochlorine in natural terrestrial environments has not been investigated.

We hypothesized that organohalide respiring *Chloroflexi* occupy a niche in terrestrial soils using natural organochlorines as terminal electron acceptors. To test this hypothesis, soils from pristine areas with different vegetative covers (uncontaminated grasslands and forests) were investigated for the presence of *Dehalococcoides*-like *Chloroflexi*. Secondly, soil cores from the (uncontaminated) New Jersey Pine Barrens were assessed for the number of *Dehalococcoides*-like *Chloroflexi* present and natural organochlorine content, and a correlation between these parameters was explored. Finally, the growth of *Dehalococcoides*-like *Chloroflexi* and the accumulation of chloride were measured in batch reactors fed an enzymatically (chloroperoxidase) produced mixture of organochlorines.

Methods

Soil collection. Both grab samples and soil cores were collected for analysis from sites with no known history of anthropogenic contamination. Grab samples were collected between 3 cm and 5 cm below the surface, generally in the upper A horizon of the soil, from four separate Minnesota State Parks (20 samples), three nature parks in Oklahoma (12 samples), a regional park in California (1 sample), and a national forest in Oregon (1 sample). Pairs of adjacent soil cores were collected from the Brendan Byrne State Forest within the New Jersey Pine Barrens in both September 2006 and January 2008, for a total of 6 pairs. Each pair came from a maple-, oak-, or pine-dominated area. One core of each pair was used for organochlorine analysis and the other was used for microbial analysis. Soil cores were 30 cm in depth with the exception of the maple-dominated core sampled September 2006 for microbial analysis, which was 14 cm deep. All soil cores for microbial analysis were split into 2-cm sections, providing a total number of 82 core samples.

Grab samples were collected with scoopulas and spoons washed with 95 percent ethanol between sampling to avoid microbial cross-contamination. Samples were packaged individually in glass jars or plastic bags, placed immediately on ice, and shipped or transported to the laboratory within 24 hours. All samples were frozen at -70°C upon arrival. Soil cores were collected in butyrate plastic sleeves. The cores for microbial analyses were shipped on ice within 24 hours of collection. Upon arrival the cores were immediately transferred into an anaerobic glove bag (Coy Laboratory Products) where the sections were cut with a cast-cutter, separated with ethanol-washed scoopulas, and frozen at -70°C until analysis.

DNA extraction. For genomic DNA extraction, each soil core section or grab sample was homogenized with a mortar and pestle washed and rinsed with 95 percent ethanol. DNA was

extracted with the FastDNA Spin Kit for Soil (MP Biomedicals) with one modification; the DNA-containing Binding Matrix was washed two times with 1.0 mL of 6.0 M BioUltra-grade guanidine thiocyanate solution (Sigma-Aldrich) to remove soil humics *(22)*. For the grab samples, 50 µL of water was used for last step of the extraction with no further cleanup. For the cores, 150 µL of water was used during the last step of the extraction, which was further cleaned using the PowerClean DNA Clean-Up kit (MoBio Laboratories). DNA was also extracted from five sediments contaminated with PCBs (from Baltimore Harbor, Maryland, Fox River, Wisconsin, and Hudson River, New York) or dioxins (Palos Verdes Harbor, California), and a trichloroethene (TCE)-contaminated aquifer material (New York) to allow the comparison of the DNA in uncontaminated samples to that in contaminated samples and to serve as a positive control for the microbial analyses. For each sample, 0.5 g of soil or sediment was used for DNA extraction.

During each round of DNA extractions, a sample of autoclaved soil was extracted in an identical manner. This served as a control to ensure that there was no exogenous contamination of samples during extraction. In addition, three separate sub-samples of three samples (the 24-26 cm section of the soil core from an oak-dominated area taken January 2008, a grab sample with pine cover taken from Mille Lacs-Kathio State Park, MN, and the TCE-contaminated aquifer material, New York) were extracted to determine reproducibility and variability of the DNA extraction. These three samples were chosen based on their representative soil consistencies, as all sections from the soil cores consisted of predominantly fine sand, and all samples from Oklahoma, Minnesota, and California consisted of clayey-loam. The Oregon and TCE-aquifer material consisted of clay interspersed with gravel. The standard deviations of the qPCR results (see below) for both *Dehalococcoides*-like *Chloroflexi* and *Bacteria* 16S rRNA gene copies of

the subsamples were less than the standard deviations obtained for replicate thermocycler runs of the same sample.

qPCR of *Dehalococcoides*-like *Chloroflexi* and *Bacteria*. *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes and *Bacteria* 16S rRNA genes were quantified for each sample using quantitative polymerase chain reaction (qPCR). Each qPCR reaction totaled 25 µL using iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories), 25 µg Bovine Serum Albumin (Roche Diagnostics), 300 nM forward primer, 300 nM reverse primer, and 1 µL of undiluted DNA extract or standard. An ABI 7000 Thermocycler (Applied Biosystems) with 7000 System Software was used with a thermocycler protocol of 50°C for 2 minutes, 95°C for 3 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. A melting curve analysis was performed after each complete run to ensure that primer-dimers were not amplified and the amplification was specific.

For qPCR to enumerate *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes, two primers, Dhc1154F (5'-CAC ACA CGC TAC AAT GGA CAG AAC-3') and Dhc1286R (5'-GAT ATG CGG TTA CTA GCA ACT CCA AC-3'), were designed using PrimerExpress software based on the *Dehalococcoides* BAV1 and the *Dehalococcoides ethenogenes* 195 genomes. When used with our samples, previously published primers *(41, 52)* were found to predominantly amplify sequences not phylogenetically related to *Chloroflexi,* as determined from clone library analysis of the amplicons. The concentration of both the reverse and forward primers and the annealing/extension temperature were optimized for specificity using melting curve analysis and gel electrophoresis. The *Chloroflexi* isolate *Herpetosiphon aurantiacus* was used as a negative control in qPCR assays. For *Bacteria* 16S rRNA gene quantification, Eub341F (5'-CCT ACG

GGA GGC AGC AG-3') and Eub534R (5'-ATT ACC GCG GCT GCT GGC-3'), were used under the same conditions described above *(34, 41)*.

Both *Bacteria* and *Dehalococcoides*-like *Chloroflexi* qPCR runs used the same set of standards. Standards were made from a frozen glycerol stock of an *Escherichia coli* clone containing a plasmid with the complete 16S rRNA gene from *Dehalococcoides* sp. strain BAV1. Clones were grown overnight and plasmids were extracted using the QIAprep Spin MiniPrep Kit (Qiagen) following the microcentrifuge protocol. Plasmid concentration was measured using Hoechst dye 33258 and a fluorometer (model TD-700, Turner Designs) with dilutions of calf thymus DNA as standards. The plasmid extract was serially diluted to achieve 12 standards containing between 10 and 2×10^9 copies of plasmid per µL. For the *Dehalococcoides*-like *Chloroflexi* 16S rRNA gene quantification, all standards were log-linear. For *Bacteria* 16S rRNA gene quantification, standards were log-linear between 2×10^5 and 2×10^9 copies per µL. All *Bacteria* 16S rRNA gene quantifications were within the linear range of the standards and above the detection limit. The 16S rRNA genes were quantified in triplicate for each sample of DNA extract, allowing the standard deviation of the qPCR assay to be calculated. The detection limits were 500 gene copies/g soil for *Chloroflexi* and 10^7 gene copies/g soil for *Bacteria*.

Quality assurance and primer specificity verification. A clone library (42 total clones) was used to verify the specificity of the qPCR primers. Thirteen unique partial 16S rRNA sequences were obtained from eight samples. The eight samples were as follows: the 24-26 cm and 28-30 cm sections from the oak-cover soil core collected in January 2008, the 4-6 cm and 12-14 cm sections from the maple-cover soil core collected in January 2008, a sample each from hardwood cover and cedar cover collected from Ray Harral Nature Park (Broken Arrow, OK), a samples from hardwood cover collected from Afton State Park (MN), and a sample from pine

cover collected from Interstate State Park (MN). Although only 86 bp were amplified by the two primers as a result of the qPCR method, the close phylogenetic relationship of these clones to other putatively organohalide respiring *Chloroflexi* (see SI) supports the specificity of our qPCR method targeting *Dehalococcoides*-like *Chloroflexi*. Using the program MatGat 2.1 *(8)*, amplified sequences ranged from 77 to 100% sequence identity to the obligately organohalide respiring *Dehalococcoides*. With the exception of the single sequence that was identical to *Dehalococcoides* sp. BAV1, BLAST searches found that the amplified sequences were most similar to uncultured unclassified bacteria and uncultured *Chloroflexi* from rhizopheric bacterial communities, freshwater and marine sediments, anaerobic sludge digesters, and contaminant-dechlorinating consortia (≥97 percent sequence identity). These primers, however, do contain mismatches with the more recently discovered *Dehalogenimonas* and *Dehalobium* sequences, and are therefore likely to exclude some organohalide respiring *Chloroflexi*.

Organochlorine and inorganic chloride analysis on soil cores. The cores for organochlorine and inorganic chloride analysis were separated into sections with a small power saw. These sections were 2 cm in depth for the first 10 cm of the core, and 4 to 6 cm in depth for the remaining core lengths. These divisions were made because of the decrease in variability of soil characteristics at deeper depths. Each section was analyzed as previously described *(28)*, with some modifications. Freeze-dried soil samples were pulverized and compressed into pellets in a matrix of ~50% by weight (poly acrylic acid), sodium salt. Chlorine 1*s* X-ray absorption near-edge structure (XANES) spectra were acquired at beamline X15B at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, Upton, NY, USA. X-ray analysis was performed in the beamline's specialized hutch box under He using a Ge fluorescence detector. Sample pellets were mounted on Kapton tape and exposed to the incoming

X-ray beam at a 45˚ angle. Sample fluorescence was measured over an energy range of 2800 to 2880 eV using a 0.25 eV step size near the chlorine K-absorption edge and 0.5-2.0 eV step sizes above and below the edge. Chlorine 1*s* XANES spectra were processed and analyzed as described previously *(28)*. The quantification of organochlorine was attempted for eight grab samples; the high mineral content of the samples, however, prohibited the acquisition of quantitative, reliable, and reproducible chlorine speciation data. In addition, the chlorine concentrations of the maple core collected in January 2008 could not be analyzed with minimal error because of X-ray beam instability at the synchrotron, resulting in poor calibration curves. Organochlorine concentrations ([organochlorine]) and inorganic chloride concentrations were therefore only obtained along the depths of 5 of the 6 soil cores. Error bars reported below indicate standard errors from multiple scans of the same sample, when performed. The minimum detection limit for total chlorine speciation was 0.03 mmol/kg of soil.

Total organic carbon analysis in soil cores. Total organic carbon (TOC) was measured in the soil core segments at the West Virginia University Division of Plant and Soil Sciences (Morgantown, WV). The samples were subjected to dry combustion on a LECO TruSpec CHN 2000 and run interspersed with blanks, as well as EDTA standards to ensure consistent measurements.

Enzymatic synthesis of organochlorines. The chlorination of organic matter was performed with a chloroperoxidase enzyme (from *Caldariomyces fumago*, Sigma-Aldrich) using a method adapted from *35, 37,* and *38*. The organic matter from the soil collected from Father Hennepin State Park in Minnesota (pine cover) was extracted with an accelerated solvent extractor (ASE 350, Dionex) using a mixture of 50:50 acetone and hexane. The extract was split

evenly by volume into two flasks, and each was blown down to dryness and resuspended in 165 mL phosphate buffer (0.1 M K_2PO_4, 20 mM KCl).

The two flasks were treated identically throughout the chlorination process (described briefly below), with the exception that the chloroperoxidase enzyme was only added to one of the flasks, allowing the organic matter in the other flask to be used as a non-chlorinated control. While both flasks were stirring, 600 units of chloroperoxidase enzyme were added to one of them. This was immediately followed by the addition of 150 µL of 0.1 M hydrogen peroxide to both flasks every 20 minutes for 1 hour. The reaction mixtures were left overnight and the addition of chloroperoxidase and hydrogen peroxide (or only hydrogen peroxide for the control) was repeated every day for four days. The pH of the reactors was maintained at 3.0-3.5 throughout the process. After the reaction was complete, the mixture was purified on a C18 column and extracted with sequential extractions of acetone, a mixture of 50:50 acetone and hexane, and hexane for use in the batch experiments described below.

Samples were taken from each flask at the beginning and end of the chlorination reaction for chloride analysis; between 7.3 and 19.4 mM chloride was consumed in the reaction with chloroperoxidase and no loss of chloride occurred in the flasks to which no chloroperoxidase was added.

Batch reactors. Batch reactors were used to test the hypothesis that *Dehalococcoides*-like *Chloroflexi* from uncontaminated environments would grow with concomitant dechlorination of organochlorines produced in a manner similar to naturally derived organochlorines (*e.g.*, generated enzymatically via the action of the chloroperoxidase enzyme). Three sets of reactors, each in triplicate, were set-up. One set received three additions of enzymatically produced organochlorines ("organochlorine amended"); a second set received two

additions of the extracted organic matter to which no chloroperoxidase enzyme was added, and one addition of the enzymatically produced organochlorines ("organic matter control"); a final set received no amendments ("unamended control"). For the amendments, the organochlorine or unchlorinated organic matter extracts from the C18 column (above) were respectively split into three equal parts by volume for the triplicate reactors. For the first amendment of organochlorines or the unchlorinated organic extract (amendment 1), the amendment was added to empty 160-mL serum bottles and the solvent was blown down to dryness. The reactors were then moved into an anaerobic glovebag with a 3% H_2/97% N_2 headspace (Coy) and the following was added: 130 mL mineral media *(40)* reduced with 2 mM titanium citrate, 10 mM potassium acetate, 1 mL of vitamin solution *(49)* and 5 grams of soil from the New Jersey Pine Barrens (Maple cover). For subsequent amendments 2 and 3, the amendment was added to new reactor bottles, blown down to dryness, and the entire content of the previous corresponding reactor was transferred to the new bottle in the glovebag. Potassium acetate (10 mM) was then added and the volume of each reactor mixture was brought up to 140 mL with fresh reduced mineral media. The unamended controls were treated the same, except they received no amendment of organochlorines or organic extract. Additionally, triplicate abiotic reactors were prepared and maintained for 132 days. The abiotic controls were prepared with an amendment of enzymatically produced organochlorines, autoclaved soil, 50 mM sodium azide, and mineral media, vitamin solution, and potassium acetate as described above. The pH of the reactors was maintained at 7.0-7.5 with H_3PO_4 and NaOH. Samples for chloride and qPCR were taken throughout the experiment as previously described *(51)*. Briefly, reactor bottles were shaken for 5 min to homogenize the contents of the reactor, and a sawed-off glass Pasteur pipette was used to transfer slurry contents to microcentrifuge tubes. For DNA extraction, 1.6 mL of slurry was

centrifuged at 5000×g for 5 min, the supernatant was removed, and the pellet was then transferred to beadbeating tubes for DNA extraction with the PowerSoil DNA Isolation Kit (MoBio Laboratories). QPCR for *Dehalococcoides*-like *Chloroflexi* and *Bacteria* was performed as described above and normalized to the volume of slurry extracted. Error bars represent the standard errors between triplicate reactors, using the means of duplicate measurements for qPCR as the measurement from each reactor. Chloride was analyzed as described below; error bars represent the standard errors between triplicate reactors.

Ion chromatography. Chloride concentrations were quantified via ion chromatography on a Metrohm 761 Compact Ion Chromatograph (Metrohm US Inc). Samples were centrifuged for 5 minutes at 10,000×g to settle particulates and the supernatant was diluted 100 fold in Milli-Q water. Diluted sample (1.4 mL) was injected onto a Metrosep A Supp5 column. An isocratic method was used with an eluent (3.2 mM Na_2CO_3; 1.0 mM $NaHCO_3$) flowrate of 0.7 mL/min. The detection limit was 0.2 mM.

Calculation of growth yield. The growth yield of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes per mol of chloride was calculated using the equation Y=N/C, where N is the maximum concentration of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes detected in a given reactor and C is the concentration of chloride that accumulated in the reactors at the time of maximum *Dehalococcoides*-like *Chloroflexi* numbers.

Statistical analysis. StataIC 10.1 software was used to analyze the relationship of the number of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes/g soil and the number of *Bacteria* 16S rRNA genes/g soil to [organochlorine]/TOC, [chloride], depth, and TOC. The nonparametric Spearman's rank coefficient correlation was used to determine whether a correlation between any two variables existed. The Wilcoxon rank-sum test was used to investigate the significance

on the variables *Chloroflexi*, *Bacteria*, TOC, and [organochlorine]/TOC due to the difference in tree cover type of the soil cores. A linear regression model was used to examine the relative contribution and significance on the number of *Chloroflexi* 16S rRNA genes/g soil from the variables [organochlorine]/TOC, depth, and whether the core was from the pine-dominated forest. Between the 5 soil cores for which paired microbial and chemical data were available, a total of 67 samples were available for analysis. If the value of a parameter was at the detection limit ("non detect"), the value of the detection limit itself was used for statistical analysis. Replicate statistical analyses were performed in which the value of the detection limit was replaced by either half or ten percent of the detection limit; the statistical significance of the results did not change as a result.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences derived from the verification of qPCR method have been deposited in the GenBank database under the accession numbers EU912597 to EU912609.

Results

Abundance of *Dehalococcoides*-like *Chloroflexi* in natural soils. All 34 grab samples from uncontaminated grasslands and forests tested positive for *Dehalococcoides*-like *Chloroflexi*, with numbers ranging from 9.4×10^3 to 4.2×10^7 16S rRNA gene copies/g soil and ranging from 4.7×10^{-6} to 3.6×10^{-3} *Chloroflexi*/*Bacteria* 16S rRNA genes (Table 1). Numbers of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes in the soil cores ranged from less than the detection limit ($<5 \times 10^2$) to 2.3×10^5 gene copies/g soil, with one sample at 6.3×10^6 gene copies/g soil (corresponding to a *Chloroflexi*/*Bacteria* 16S rRNA genes of $<6.75 \times 10^{-7}$ to 2.5×10^{-2}) (Fig. 1). For comparison, the 5 contaminated samples (PCB-, dioxin-, or TCE-

334 contaminated) contained 5.0×10^4 to 1.1×10^7 *Chloroflexi* 16S rRNA gene copies/g soil
335 (corresponding to 1.5×10^{-3} to 4.2×10^{-2} of *Chloroflexi*/*Bacteria* 16S rRNA genes). There was
336 no statistically significant difference between samples collected from contaminated and
337 uncontaminated sites with respect to the number of *Dehalococcoides*-like *Chloroflexi* 16S rRNA
338 genes present or the percent of the total community of *Bacteria* that consisted of
339 *Dehalococcoides*-like *Chloroflexi*.

340 **Correlation of *Dehalococcoides*-like *Chloroflexi* to natural organochlorines.** If these
341 widespread *Dehalococcoides*-like *Chloroflexi* are using natural organochlorine as an electron
342 acceptor for growth as has been hypothesized *(2, 7, 19, 23, 24)*, the number of these organisms
343 present in a given sample should correlate to [organochlorine] while the number of *Bacteria*
344 present should not. [Organochlorine] was observed to correlate linearly with TOC (R^2=0.66,
345 t=11.2, P<0.001); this result was expected, as TOC is the precursor of organochlorines *(28)*.
346 Therefore, to factor out the covariance of [organochlorine] with TOC, [organochlorine]/TOC
347 was used to investigate further correlations with *Dehalococcoides*-like *Chloroflexi*. The
348 nonparametric Spearman's rank test was used to determine whether such a correlation existed for
349 the 67 soil core sections in which paired microbial and organochlorine data were available. The
350 number of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes/g soil in these samples correlated
351 to [organochlorine]/TOC (ρ=0.31, P=0.012), but did not correlate with depth, TOC, or [chloride]
352 (ρ=0.10, P=0.40, ρ=-0.16, P=0.18, and ρ=-0.03, P=0.82, respectively). Indeed, it was not
353 expected that *Dehalococcoides*-like *Chloroflexi* would correlate to [chloride] (a product of
354 halorespiration) or depth (implying dissolved oxygen concentrations) because the soils
355 investigated in this study were well-drained, resulting in a lack of chloride accumulation and an
356 environment that was not predictably more reduced with depth. TOC was not expected to

correlate with *Dehalococcoides*-like *Chloroflexi*, as non-chlorinated organic matter should not exert a direct selective pressure for the growth of organohalide respirers. The number of *Bacteria*, however, were expected to correlate to TOC and were not expected to correlate to [organochlorine]/TOC; this was observed (ρ=0.82, P<0.001 for TOC and ρ=-0.15, P=0.22 for [organochlorine]/TOC). There was also a negative correlation between the number of *Bacteria* present and depth (ρ=-0.66, P<0.001), although this may be a factor of the association of depth to TOC (ρ=-0.56, P<0.001). There was no observed correlation between *Bacteria* and [chloride] (ρ=0.12, P=0.34).

To further investigate the association of *Dehalococcoides*-like *Chloroflexi* and the environmental parameters measured in the soil cores, a linear regression model was developed. The two variables depth and [organochlorine]/TOC are not statistically correlated with each other (Spearman's ρ=-0.14, P=0.11); therefore, these two parameters may be included in a linear regression model as independent variables. Additionally, the variable "vegetative cover" may be included by giving a particular cover type a value of 1 and the other cover types values of 0. In this case, "pine cover" was investigated; it is also independent from the variables depth and [organochlorine]/TOC. In a linear regression model with these three variables (Fig. 2), the correlation coefficients were 0.025, 0.33, and -0.88 for depth, log([organochlorine]/TOC), and "pine cover," respectively; all were statistically significant with respect to their correlation with log(*Chloroflexi*) (t=3.29, P=0.002, t=3.62, P<0.001, and t=-6.6, P<0.001, respectively). This linear regression analysis again supported the statistical association between the number of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes and [organochlorine]/TOC, but also highlighted an unexplained and quite strong association between tree cover and *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes. The strength of the correlation with pine

cover indicates that factors in addition to [organochlorine]/TOC affect the number of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes/g of soil. Different tree species have been observed to produce different organic exudates, including ones that are able to induce aerobic PCB-degraders *(26)*; therefore, certain tree species, such as oak and maple, may produce organochlorines that are more bioavailable, more oxidized, or otherwise more favorable for reduction and energy generation in organohalide respiring *Chloroflexi*.

Growth of *Dehalococcoides*-like *Chloroflexi* in batch reactors. The increase in *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes was measured in batch reactors to which enzymatically-produced organochlorines, organic matter, or nothing was added (Fig. 3). The organochlorine amended reactors were amended three times with enzymatically-produced organochlorines. For the organic matter control reactors, the first and third amendments were made with the organic extract, whereas the second amendment was with the enzymatically-produced organochlorines. For each amendment of enzymatically produced organochlorines the number of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes increased by 2.9±0.3 to 4.0±0.2 orders of magnitude for the triplicate reactors. The organic extract increased the number of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes, but by a significantly lower amount (0.89±0.2 and 1.7±0.3 orders of magnitude). For the unamended controls, there was an initial increase in the number of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes (0.55±0.1 orders of magnitude) and no statistically significant increase thereafter. The number of *Bacteria* 16S rRNA genes was also measured with qPCR and did not increase significantly during these experiments.

The organic extract and the soil used to seed the reactors would have contained any natural organochlorines already present in the soil, and therefore, some growth of

403 *Dehalococcoides*-like *Chloroflexi*, at least initially, was expected in all of the reactors. Indeed,
404 this increase in growth was observed. Nevertheless, the growth of *Dehalococcoides*-like
405 *Chloroflexi* (rate and total amount of genes present) was statistically greater (Student t-test,
406 $P<0.05$) in the reactors to which the enzymatically-produced organochlorines were added,
407 indicating that terrestrial organochlorines do serve as a growth substrate for organohalide
408 respiring *Chloroflexi*.

409 The accumulation of chloride was also measured in the batch reactors as well as in
410 abiotic controls. After amendment with the enzymatically-produced organochlorines, the
411 chloride increase was between 2.4±0.5 mM and 2.7±0.4 mM (Fig. 3). In the organic matter and
412 unamended controls, the increase in chloride was not significantly different from zero.
413 Additionally, in abiotic controls amended with enzymatically-produced organochlorines, no
414 increase in chloride was detected (see SI for data) indicating that the increase of chloride was not
415 abiotic. Again, this indicates that the growth of *Dehalococcoides*-like *Chloroflexi* resulted from
416 the dechlorination of organochlorines, and at a level that was detectable via chloride production.
417

418 ## Discussion

419 Recent literature has supported the concept that respiration of natural organohalides
420 occurs in the environment. For example, laboratory cultures of two different species of
421 *Dehalococcoides* have been shown to be capable of growing on several chlorinated phenols as
422 electron acceptors *(2)* and one chlorinated phenol has been shown to induce transcription of
423 several reductive dehalogenase genes *(12)*. Because chlorinated phenols can be produced
424 naturally *(37),* it was conjectured that they represent at least one class of naturally occurring
425 compounds that *Dehalococcoides* may use in uncontaminated environments. Another study

found that mixed cultures containing *Dehalococcoides*-like microorganisms from uncontaminated sediment in the North Sea could degrade tetrachloroethene to *trans*- and *cis*-dichloroethene *(24)*. Because evidence exists for the natural production of tetrachloroethene by marine algae *(1)*, tetrachloroethene could be considered a natural substrate for *Dehalococcoides*-like organisms in marine systems. Our study, however, is the first to find direct evidence of a natural niche for organohalide respiring *Chloroflexi* by showing that *Dehalococcoides*-like *Chloroflexi* are widespread in uncontaminated terrestrial environments, they correlate to the quantity of natural organochlorine compounds present in these uncontaminated samples, and they grow in the presence of enzymatically produced organochlorines while releasing chloride.

Interestingly, no lag in growth was observed in our batch experiments, suggesting that these *Chloroflexi* may constitutively dechlorinate enzymatically produced organochlorines for energy generation. Also interesting is the observation that these organisms grew rather quickly, reaching a maximum population 11-16 days after the amendment of organochlorine. The growth yield of these *Dehalococcoides*-like *Chloroflexi* was estimated to be 3×10^{11} to 1×10^{13} copies 16S rRNA genes/mol chloride, which is similar to the $\sim 10^{12}$-10^{14} cells/mol chloride determined for *Dehalococcoides* isolates respiring anthropogenic contaminants *(2, 30, 43)*. The short amount of time needed for the *Dehalococcoides*-like *Chloroflexi* to grow and putatively dechlorinate the organochlorine mixture could be a result of the likely high solubility and low molecular masses of the organochlorine mixture, as these organochlorines were produced in a phosphate buffer. In natural systems, organochlorines may also be a part of larger humic substances, which may be less bioavailable and thus more recalcitrant *(14)*. The recalcitrance of organochlorines in nature may in part explain the rather low correlation (ρ=0.31) found between the *Dehalococcoides*-like *Chloroflexi* and natural organochlorines above.

These results are encouraging because if the organisms that respire natural organochlorines can also dechlorinate compounds such as PCBs, organohalide respirers could be quickly grown to a high density on natural organochlorines *ex situ*, after which they could be added to contaminated dredge spoils, or even contaminated sediment *(25, 48)*. The environment is complicated, however, and multiple parameters not measured in this study may also affect the number of *Dehalococcoides*-like *Chloroflexi* present in a given uncontaminated environment; this was observed via the negative association between the number of *Dehalococcoides*-like *Chloroflexi* and "pine cover". Furthermore, the specific organochlorines that were dechlorinated during *Dehalococcoides*-like *Chloroflexi* growth were not determined in this study. Research of this nature would help in not only understanding the niche of these important organisms more fully, but could also aid in the development of technologies for the remediation of anthropogenic contaminants, such as PCBs.

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TABLE 1. Quantification of *Dehalococcoides*-like *Chloroflexi* in the grab samples from both uncontaminated and contaminated sites

Soil Cover	Location	*Chloroflexi* 16S rRNA genes/g soil	*Chloroflexi* 16S rRNA genes/*Bacteria* 16S rRNA genes
Grass	Redbud Valley NP, OK[a]	$(2.4 \times 10^{7}) \pm (4.3 \times 10^{6})$[b]	$(1.2 \times 10^{-3}) \pm (2.4 \times 10^{-4})$
	Afton SP[c], MN	$(4.2 \times 10^{7}) \pm (1.1 \times 10^{7})$	$(3.6 \times 10^{-3}) \pm (1.1 \times 10^{-3})$
	Interstate SP, MN	$(4.0 \times 10^{5}) \pm (1.0 \times 10^{5})$	$(5.6 \times 10^{-4}) \pm (1.4 \times 10^{-4})$
Hardwood	Redbud Valley NP, OK	$(9.9 \times 10^{6}) \pm (2.5 \times 10^{6})$	$(3.7 \times 10^{-4}) \pm (1.1 \times 10^{-4})$
	Redbud Valley NP, OK	$(1.1 \times 10^{5}) \pm (2.3 \times 10^{4})$	$(8.4 \times 10^{-5}) \pm (1.7 \times 10^{-5})$
	Ray Harral NP, OK[d]	$(2.0 \times 10^{7}) \pm (3.5 \times 10^{6})$	$(9.0 \times 10^{-4}) \pm (1.6 \times 10^{-4})$
	Ray Harral NP, OK	$(8.6 \times 10^{6}) \pm (6.9 \times 10^{4})$	$(2.9 \times 10^{-4}) \pm (1.4 \times 10^{-5})$
	Ray Harral NP, OK	$(8.9 \times 10^{5}) \pm (1.6 \times 10^{5})$	$(2.4 \times 10^{-4}) \pm (4.5 \times 10^{-5})$
	Ray Harral NP, OK	$(1.2 \times 10^{6}) \pm (1.4 \times 10^{5})$	$(2.3 \times 10^{-4}) \pm (3.1 \times 10^{-5})$
	Afton SP, MN	$(4.5 \times 10^{6}) \pm (2.6 \times 10^{5})$	$(4.5 \times 10^{-4}) \pm (5.6 \times 10^{-5})$
	Afton SP, MN	$(7.4 \times 10^{6}) \pm (7.7 \times 10^{5})$	$(2.1 \times 10^{-4}) \pm (3.4 \times 10^{-5})$
	Interstate SP, MN	$(9.0 \times 10^{6}) \pm (6.9 \times 10^{5})$	$(1.4 \times 10^{-4}) \pm (5.9 \times 10^{-5})$
	Interstate SP, MN	$(5.5 \times 10^{5}) \pm (1.2 \times 10^{5})$	$(5.4 \times 10^{-4}) \pm (1.2 \times 10^{-4})$
	Father Hennepin SP, MN	$(3.1 \times 10^{5}) \pm (7.0 \times 10^{4})$	$(9.7 \times 10^{-5}) \pm (2.2 \times 10^{-5})$
	Tilden Regional Park, CA	$(3.3 \times 10^{5}) \pm (1.0 \times 10^{4})$	$(4.3 \times 10^{-4}) \pm (2.0 \times 10^{-5})$
Pine	McClellan-Kerr WMA[e], OK	$(3.0 \times 10^{7}) \pm (6.8 \times 10^{6})$	$(2.2 \times 10^{-3}) \pm (6.1 \times 10^{-4})$
	Afton SP, MN	$(4.4 \times 10^{6}) \pm (9.2 \times 10^{5})$	$(4.7 \times 10^{-6}) \pm (2.5 \times 10^{-6})$
	Afton SP, MN	$(1.2 \times 10^{7}) \pm (5.2 \times 10^{5})$	$(8.9 \times 10^{-4}) \pm (2.4 \times 10^{-4})$
	Interstate SP, MN	$(3.3 \times 10^{6}) \pm (1.3 \times 10^{5})$	$(3.3 \times 10^{-5}) \pm (3.0 \times 10^{-5})$
	Interstate SP, MN	$(1.7 \times 10^{6}) \pm (7.5 \times 10^{4})$	$(5.3 \times 10^{-4}) \pm (9.0 \times 10^{-5})$
	Interstate SP, MN	$(8.2 \times 10^{5}) \pm (1.3 \times 10^{5})$	$(6.3 \times 10^{-4}) \pm (1.1 \times 10^{-4})$
	Mille Lacs-Kathio SP, MN	$(2.3 \times 10^{5}) \pm (9.4 \times 10^{4})$	$(1.1 \times 10^{-4}) \pm (4.5 \times 10^{-5})$
	Mille Lacs-Kathio SP, MN	$(5.1 \times 10^{5}) \pm (8.6 \times 10^{4})$	$(2.1 \times 10^{-4}) \pm (3.5 \times 10^{-5})$
	Banning SP, MN	$(1.7 \times 10^{4}) \pm (3.2 \times 10^{3})$	$(1.5 \times 10^{-5}) \pm (3.0 \times 10^{-6})$
	Father Hennepin SP, MN	$(3.3 \times 10^{5}) \pm (2.2 \times 10^{4})$	$(1.4 \times 10^{-4}) \pm (1.6 \times 10^{-5})$
	Father Hennepin SP, MN	$(2.2 \times 10^{5}) \pm (1.1 \times 10^{4})$	$(8.8 \times 10^{-5}) \pm (6.0 \times 10^{-6})$
	Father Hennepin SP, MN	$(5.3 \times 10^{5}) \pm (2.1 \times 10^{5})$	$(2.6 \times 10^{-4}) \pm (1.1 \times 10^{-4})$
	Father Hennepin SP, MN	$(3.4 \times 10^{6}) \pm (1.1 \times 10^{6})$	$(1.5 \times 10^{-4}) \pm (5.1 \times 10^{-5})$
	Mount Hood National Forest, OR	$(9.4 \times 10^{3}) \pm (1.5 \times 10^{3})$	$(2.0 \times 10^{-5}) \pm (3.3 \times 10^{-6})$
Cedar	Ray Harral NP, OK	$(6.4 \times 10^{6}) \pm (1.2 \times 10^{6})$	$(4.9 \times 10^{-4}) \pm (1.0 \times 10^{-4})$
	Ray Harral NP, OK	$(2.5 \times 10^{7}) \pm (9.7 \times 10^{6})$	$(1.8 \times 10^{-3}) \pm (7.1 \times 10^{-4})$
	Ray Harral NP, OK	$(6.2 \times 10^{6}) \pm (1.4 \times 10^{6})$	$(1.8 \times 10^{-4}) \pm (4.8 \times 10^{-5})$
	Ray Harral NP, OK	$(2.3 \times 10^{6}) \pm (8.6 \times 10^{5})$	$(1.1 \times 10^{-4}) \pm (5.2 \times 10^{-5})$
	Afton SP, MN	$(1.4 \times 10^{7}) \pm (5.0 \times 10^{6})$	$(1.1 \times 10^{-3}) \pm (4.0 \times 10^{-4})$
Contaminated	Solvent-contaminated aquifer, NY	$(4.7 \times 10^{5}) \pm (2.3 \times 10^{4})$	$(1.2 \times 10^{-2}) \pm (7.2 \times 10^{-4})$
	Hudson River, NY	$(5.0 \times 10^{4}) \pm (6.9 \times 10^{3})$	$(1.5 \times 10^{-3}) \pm (2.5 \times 10^{-4})$
	Baltimore Harbor, MD	$(1.1 \times 10^{5}) \pm (3.8 \times 10^{4})$	$(1.7 \times 10^{-3}) \pm (6.9 \times 10^{-4})$
	Fox River, WI	$(1.1 \times 10^{7}) \pm (5.9 \times 10^{5})$	$(4.2 \times 10^{-2}) \pm (2.4 \times 10^{-3})$
	Palos Verdes Harbor, CA	$(1.1 \times 10^{6}) \pm (4.5 \times 10^{5})$	$(5.3 \times 10^{-3}) \pm (2.4 \times 10^{-4})$

[a] Redbud Valley Nature Preserve, Catoosa, OK
[b] Standard deviation
[c] State Park
[d] Ray Harral Nature Park, Broken Arrow, OK
[e] Wildlife Management Area

FIGURE LEGENDS

676

677 **Fig. 1.** The distribution of *Dehalococcoides*-like *Chloroflexi* (solid symbols) and total
678 [organochlorine]/TOC (open symbols) in the soil cores taken from dominantly oak (A), pine (B),
679 and maple (C) forests in September 2006 (left) and January 2008 (right).

680

681 **Fig. 2.** Linear regression fit of the variable Log(*Chloroflexi*) and a model including the
682 independent variables depth (cm), [organochlorine]/TOC, and "pine cover" (samples with pine
683 cover are given a value of 1 and maple and oak cover are given a value of 0).

684

685 **Fig. 3.** The increase of 16S rRNA gene sequences of *Dehalococcoides*-like *Chloroflexi* (top) and
686 increase in chloride concentrations (bottom) during amendments 1 (left), 2 (center) and 3 (right).
687 Symbols are: organochlorine amended (■), organic matter control (▲) (received
688 organochlorines only for amendment 2 and organic matter for amendments 1 and 3), and
689 unamended (●). The amount of organic matter used for each of the amendments was equivalent,
690 regardless of whether it was treated with chloroperoxidase. Error bars are the standard error
691 between mean values of triplicate reactors.

FIG. 1.

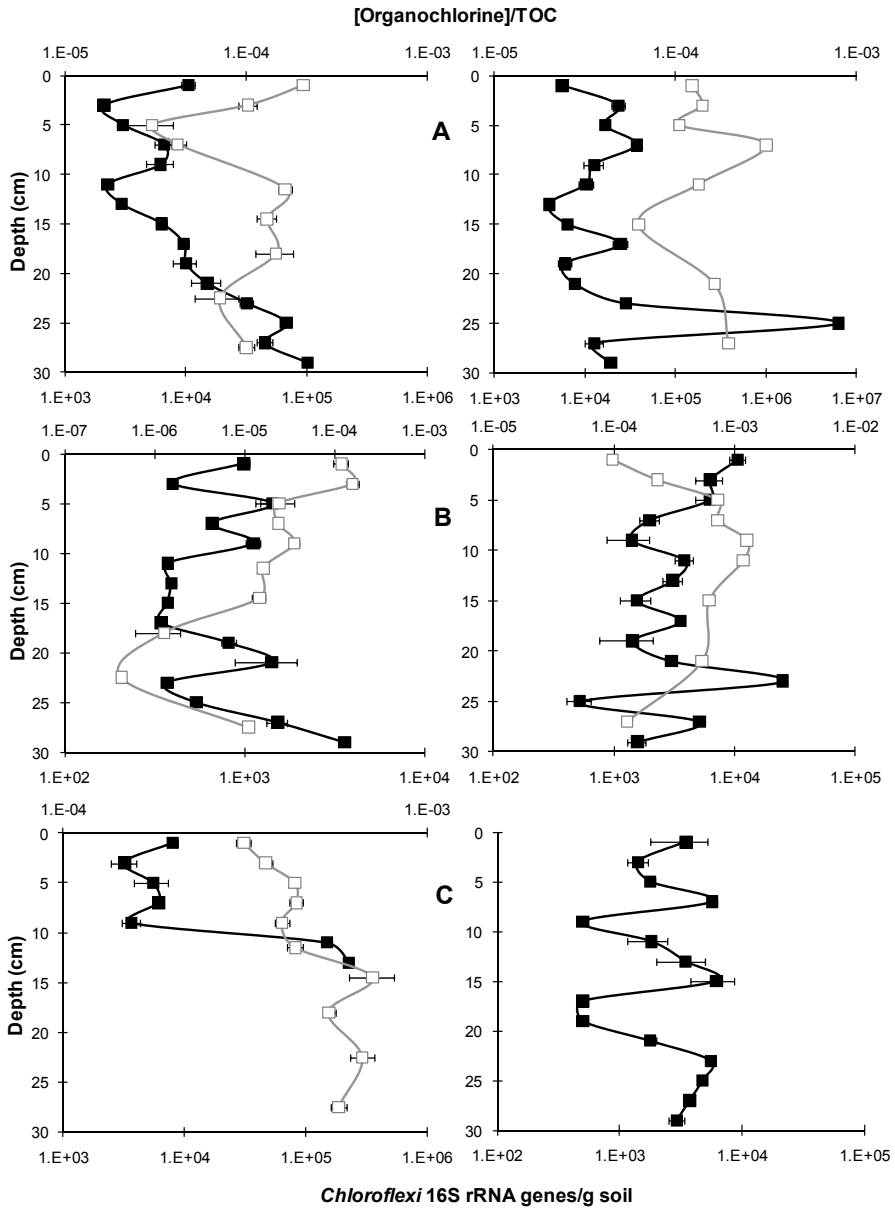

FIG. 2.

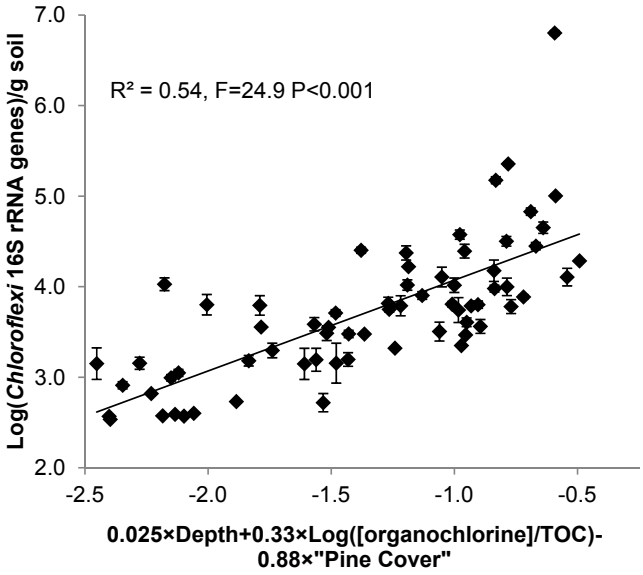

FIG. 3.