Novel Firmicutes group implicated in the dechlorination of two chlorinated xanthones, analogues of natural organochlorines

Firmicutes group dechlorinates chlorinated xanthones

Mark J Krzmarzick¹², Hanna R. Miller¹, Tao Yan², Paige J Novak¹#

¹Department of Civil Engineering, University of Minnesota, 122 Civil Engineering, 555 Pillsbury Drive SE, Minneapolis, MN 55455.
²Department of Civil and Environmental Engineering, University of Hawaii at Manoa, Holmes 383, 2540 Dole Street, Honolulu, HI 96822.

¹ Correspondence: 122 Civil Engineering, 555 Pillsbury Drive SE, Minneapolis, MN 55455.
Phone: 612-626-9536 Email: novak010@umn.edu

Present Address: School of Civil and Environmental Engineering, Oklahoma State University, 207 Engineering South, Stillwater, OK 74078
Abstract

Although the abundance and diversity of natural organochlorines has become well established, much is still unknown about the degradation of these compounds. Triplicate microcosms were used to determine whether, and which, bacterial communities could dechlorinate two chlorinated xanthones (2,7-dichloroxanthone and 5,7-dichloro-1,3-dihydroxylxanthone), analogues for a diverse class of natural organochlorines. According to quantitative-PCR (qPCR) results, several known dechlorinating genera were either not present or not enriched during dechlorination of the xanthones. Denaturing gradient gel electrophoresis, however, indicated that several Firmicutes were enriched in the dechlorinating cultures compared to triplicate controls amended with nonchlorinated xanthones. One such group, herein referred to as the “Gopher group,” was further studied with a novel qPCR method that confirmed enrichment of Gopher group 16S rRNA genes in the dechlorinating cultures. The enrichment of the Gopher group was again tested with two new sets of triplicate microcosms. Enrichment was observed during chlorinated xanthone dechlorination in one set of these triplicate microcosms. In the other set, two microcosms showed clear enrichment while a third did not. The Gopher group is a previously unidentified group of Firmicutes, distinct from but related to the Dehalobacter and Desulfitobacteria genera; this group also contains clones from at least four unique cultures capable of dechlorinating anthropogenic organochlorines that have been previously described in the literature. This study suggests that natural chlorinated xanthones may be effective biostimulants to enhance the remediation of pollutants and highlights that novel genera of dechlorinators likely exist and may be active in bioremediation and the natural cycling of chlorine.
Introduction

The biogeochemical importance of naturally occurring chlorinated compounds (commonly referred to as organochlorines or organochlorides) have been garnering attention as important components of the chlorine and carbon cycles (1, 2, 3, 4, 5). More than 4400 natural organohalogens have been identified (6) and the production of these compounds is becoming well-understood (1, 2, 7, 8, 9). Studies on the degradation of naturally occurring organochlorines, however, are generally lacking. Xanthones are a group of naturally occurring compounds that may be particularly abundant in some environments (10, 11, 12, 13). Xanthones are synthesized by plants, bacteria, and fungi (10, 13) and can contain a range of substitutions, including hydroxyl-, methyl- and chloro-groups (10, 11, 12). A total of 38 xanthones have been identified that contain between 1 and 4 chlorines (11, 12). Interestingly, the basic chemical structure of chlorinated xanthones resembles that of the anthropogenic chlorinated pollutants polychlorinated biphenyls (PCBs) and dibenzo-\( p \)-dioxins (dioxins) (Fig. 1).

Several bacteria referred to as organohalide respirers have been isolated that reductively dechlorinate organochlorines for energy generation. Research to date has largely focused on organohalide respirers that dechlorinate anthropogenic pollutants, and in particular, on the \textit{Dehalococcoides mccartyi}, largely because of its ability to dechlorinate a large number of pollutants (14, 15, 16, 17). Recently several organohalide respirers have been detected in uncontaminated soils and sediments (18, 19, 20), and \textit{Dehalococcoides}-like bacteria have been measured to grow in response to the amendment of enzymatically-produced organochlorines (18). Nevertheless, there remains much about organohalide respiration that is unknown, particularly with respect to those organisms whose niche lies in uncontaminated environments. Furthermore, despite the clear importance of \textit{Dehalococcoides mccartyi}, it is possible that the, at
times, narrow focus on this genus (i.e., 18) has drawn attention away from other organohalide respirers.

By investigating organohalide respiration in uncontaminated environments, we may better understand chlorine, and perhaps even carbon, cycling. We may also be able to exploit this process for the bioremediation of anthropogenic pollutants. Specifically, because chlorinated xanthones are structurally similar to pollutants such as PCBs and dioxins, these natural compounds may be useful as alternative electron acceptors, or “primers,” at contaminated sites, speeding the remediation of contaminated sediments (21, 22). With this in mind, we investigated whether two analogues of naturally occurring chlorinated xanthones, 2,7-dichloroxanthone and 5,7-chloro-1,3-dihydroxylxanthone, could be reductively dechlorinated in soils and sediments, and which organisms (Dehalococcoides-like or other bacteria) were involved in this process.

Methodology

Microcosms. Two chlorinated xanthones, 2,7-dichloroxanthone and 5,7-dichloro-1,3-dihydroxylxanthone were purchased from Specs (Delft, The Netherlands) and Princeton Biomolecular Research, Inc (Princeton, NJ, USA), respectively. Xanthone (unsubstituted) was purchased from Sigma-Aldrich, and 1,3-dihydroxylxanthone was purchased from Princeton Biomolecular Research, Inc. Soils used in experiments were from a pine-dominated forest from the Pine Barrens in New Jersey (Pine Barrens I), a maple-dominated forest from the Pine Barrens in New Jersey (Pine Barrens II), and Tilden Regional Park in California (Tilden). Sediments used in experiments came from Leech Lake in Minnesota (Leech Lake) and Palos Verdes Harbor in California (Palos Verdes). The soils and Leech Lake sediment are not known to be contaminated with anthropogenic chlorinated compounds; the Palos Verdes Harbor sediment has been...
historically contaminated with dioxins. These soils and sediments were collected for previous studies (18, 19, 23) and stored at room temperature in an anaerobic chamber (Coy) with a 3% H₂/97% N₂ headspace.

Serum bottles (160 mL) capped with Teflon-lined septa and aluminum crimps were used for microcosms. Microcosms contained 130 mL of mineral media (24) reduced with 2 µM titanium citrate, 10 mM potassium acetate, 1 mL of vitamin solution (25) and 5 g of wet soil or sediment. Microcosm headspace contained 3% H₂/97% N₂. Triplicate microcosms were amended with chlorinated xanthones and triplicate control microcosms were amended with equivalent molar amounts of non-chlorinated analogues (xanthone and/or 1,3-dihydroxylxanthone).

Triplicate autoclaved control microcosms were also set up as described for the active microcosms, seeded with 5 g of a wet mixture of forest soil collected for a previous study (18), including the “Pine Barrens I”, “Pine Barrens II”, and “Tilden” soils, and maintained with an amendment of 50 mM sodium azide. The Tilden, Palos Verdes, and autoclaved controls were maintained for 698 days; the Pine Barren I microcosms were maintained for 557 days and the Leech Lake and Pine Barrens II microcosms were maintained for 118 days. The Tilden and Palos Verdes microcosms were initially inoculated with 10 µM of 2,7-dichloroxanthone and received multiple amendments of both 2,7-dichloroxanthone and 5,7-dichloro-1,3-dihydroxylxanthone thereafter, while the Pine Barrens I microcosms were initially amended with 3 µM of 2,7-dichloroxanthone and then received multiple amendments of only 2,7-dichloroxanthone thereafter. The Pine Barrens II and Leech Lake microcosms were inoculated with 100 µM of each 2,7-dichloroxanthone and 5,7-dichloro-1,3-dihydroxylxanthone and did not receive additional amendments. The chemicals were added to the microcosms as an acetone solution (100 mM) or as dry powder. After amendment with dry powder, the microcosms were placed on
a shaker table for ten days to better incorporate the xanthones into the soil matrix. Otherwise, the microcosms were stored statically in an anaerobic glovebag at room temperature. Microcosms were sampled as previously described (23) for microbiological and/or chemical analysis.

**Analysis of Xanthones.** Silanized glassware was used when analyzing xanthones. Mixed slurry samples (1.5 mL) were taken for chemical analysis from the chlorinated xanthone-amended microcosms and the autoclaved controls. Samples were mixed with 10 g oven-baked (550°C) Ottawa sand (Sigma-Aldrich) and air-dried for 48 hours. The samples were then extracted with an ASE 350 accelerated solvent extractor (Dionex) under the EPA guidelines for accelerated solvent extraction of dioxins (EPA Method 3545A). The samples were extracted with a 50:50 mixture of acetone:hexane in three 15 minute static intervals at 100°C and 1600 psi. The solvent extract was then reduced with roto-evaporation and transferred to a vial where the remaining extract was blown down to dryness under a stream of nitrogen. Anhydrous sodium sulfate was added to the vial, and 10 mL of hexane was added to resuspend the extract. Exact amounts of slurry and hexane were determined gravimetrically. The chlorinated xanthones, and the nonchlorinated products xanthone, and 1,3-hydroxylxanthone were separated via gas chromatography (GC) on an HP-5 column (Agilent). The temperature program used for compound separation was: 40°C for 2 min, a ramp of 10°C/min to 180°C, a ramp of 15°C/min to 270°C and 15 min at 270°C for a total run time of 37 min. Substituted and unsubstituted xanthones were quantified with an electron capture detector (GC-ECD). External standards were prepared for each compound and used for quantification; standard concentrations ranged from 0.1-10 ng/mg for 2,7-dichloroxanthone and 5,7-dichloro-1,3-dihydroxyxanthone (in hexane), and 1-10 ng/mg for xanthone and 1,3-dihydroxyxanthone (in hexane), respectively. The detection limits were 0.1 µM, 0.1 µM, 1 µM and 1 µM for 2,7-dichloroxanthone, 5,7-dichloro-
1,3-dihydroxylxanthone, xanthone, and 1,3-dihydroxylxanthone, respectively. Standards and samples were also analyzed via GC coupled with mass spectrometry (GC-MS) to verify elution times (with standards) and to screen samples for the expected singly chlorinated products (the M+ peaks 230 and 232 for 2-chloroxanthone and 262 and 264 for 7-chloro-1,3-dihydroxylxanthone and 5-chloro-1,3-dihydroxylxanthone).

Recoveries of 2,7-dichloroxanthone, 5,7-dichloro-1,3-dihydroxylxanthone, xanthone, and 1,3-dihydroxylxanthone were each individually tested. Five vials (21-mL) containing 15 mL of slurry identical in composition to the microcosms described above were autoclaved. Different amounts of chemical were added to each of the five vials covering the range of concentrations expected in this study (1 µM, 5 µM, 10 µM, 20 µM, and 100 µM) and vials were placed on a shaker table for three days. Vials were then sampled, extracted, and analyzed as described above. The recoveries for 2,7-dichloroxanthone, 5,7-dichloro-1,3-dihydroxylxanthone, xanthone, and 1,3-dihydroxylxanthone were found to be 92 ± 1.3%, 89 ± 6.3%, 95 ± 3.1%, and 96 ± 2.4%, respectively (mean ± standard error of the five concentrations).

**DNA Extraction, DGGE Analysis, Cloning, and Sequencing.** DNA was extracted from microcosm slurry samples using a PowerSoil DNA kit (MoBio Laboratories). DGGE was performed similarly as described previously (23, 26). Briefly, genomic DNA was diluted 100-fold and amplified with primers 338F (5’-ACT CCT ACG GGA GGC AGC AG-3’) (27) and 518R (5’-ATT ACC GCG GCT GCT GG-3’) attached to a GC clamp (28). PCR amplicons were analyzed on a 30-55% denaturing gradient gel and electrophoresis was performed on a D-Code apparatus (BioRad) in 0.5 × TAE buffer. Electrophoresis was performed at 20 V for 15 min, followed by 200 V until the loading dye migrated to the bottom of the gel (at least 3 hours). Gels
were stained with SYBR Green I (Molecular Probes) and visualized on a UV transilluminator (either an EC3 Bioimaging system by Ultra-Violet Products or a GelDoc system by Biorad).

To determine the full 16S rRNA sequences responsible for the DGGE bands that appeared in the chlorinated xanthone-amended microcosms and not in the controls, a clone library was generated followed by an enzyme digestion to identify unique clones. Unique clones were analyzed by DGGE to identify the position of the band relative to the banding pattern of the original DNA extract. Clone libraries were developed from the DNA extracts from one of the triplicate reactors from the following chlorinated xanthone-amended microcosms: Tilden (Day 635), Palos Verdes (Day 635), Pine Barrens II (Day 118) and Leech Lake (Day 118). Nearly complete 16S rRNA genes were amplified using the bacterial primers 27F (27) and 1522R (29) as previously described (30). The amplicons were then cloned into electrocompetent *E. coli* DH5α using the pGEM-T Easy cloning kit (Promega). An enzyme digestion was performed with 2U restrictive enzyme AluI (New England Biolabs) according to the manufacturer’s recommendations. The digests were run on 1.5% agarose gels stained with GelRed (Biotium) and imaged on a UV illuminator with QuantityOne Software (BioRad). One or more representative clones from each digestion fingerprint was chosen for DGGE analysis. Clones and the DNA extract from which the clones were generated were analyzed by DGGE in parallel to identify the DGGE position of the cloned 16S rRNA gene. The clones with bands that corresponded to the position of the targeted bands (those unique to microcosms amended with chlorinated xantheines) were sequenced at the University of Minnesota Biomedical Genomics Center (BMGC) or the University of Hawaii at Manoa College of Natural Sciences Advanced Studies of Genomics, Proteomics, and Bioinformatics (ASGPB).
Bands targeted for analysis and selected bands from the control DGGE lanes, often those that were positioned near the targeted bands, were directly excised and sequenced as well (see Supporting Information for further details). Excised bands were moved to microcentrifuge tubes with ethanol-washed tweezers and 20 µL of DNase/RNase-free water was added. Gel slices were then left overnight, and the supernatant was diluted 1:100 fold and subjected to repeated rounds of PCR, DGGE, and excision until the fragment appeared clear of other fragments. The final excised fragments were then subject to PCR (for DGGE) as above, except without the GC-clamp. PCR fragments were cleaned with the GeneClean II Kit (MP Biomedical) and submitted for sequencing as above. Sequences of the excised bands from the chlorinated xanthone-amended microcosms matched well (>90% similarity) to the sequence of their corresponding clones (see Supplemental information for sequence information). Sequences of the excised bands from the controls were not closely (<90%) related to the sequences corresponding to the 12 bands of enriched bacteria with the exception of one band excised from an autoclaved control that was 94% similar in sequence to the targeted band Pine Barrens II-2 (see Supplemental Information).

**qPCR/PCR of 16S rRNA genes.** Previously-published PCR and quantitative-PCR methods were used to target the 16S rRNA genes from the reductively dechlorinating *Dehalococcoides*-like *Chloroflexi* (18), *Dehalogenimonas* spp. (31), *Desulfitobacterium* spp. (32), *Dehalobacter* spp. (32), *Desulfomonile* spp. (33), and *Anaeromyxobacter* spp. (34) (see Supplemental Information).

A novel qPCR amplification technique was developed to target a group within the *Firmicutes*, herein referred to as the “Gopher group.” The full 16S rRNA sequences from clones retrieved from NCBI’s BLAST database and this study that belong to the group were used as a
target for primer specificity. These sequences were aligned with the 16S rRNA sequences from several Firmicutes, including the closely related Desulfitobacterium spp., Dehalobacter spp., Desulfosporosinus spp. as well as representatives from more distant genera and the other 16S rRNA sequences from this study. The two qPCR primers Gfr163F (5'-TGA CCY TGG CAT CAG GGA-3') and Gfr441R (5'-TAT TTT ACA ACC CGA AGG CCT TCG-3') are specific for all Gopher group sequences (shown in FIG 4 and FIG S5) and are nonspecific for all other sequences shown in these figures. A BLAST search for these primers indicates no matches for sequences clearly belonging to other identified genera. The primers were used to form a clone library from the DNA extract of Tilden-A (Day 635) and one clone was selected for standards and prepared by an overnight culture, plasmid extraction, and plasmid quantification and dilution as previously described (18). The sequence of the plasmid insert is >95% similar to all Gopher group sequences and is provided in the Supplemental Information. Thermocycling conditions had an initial denaturation of 2.5 min at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 52°C. The amplification mixture consisted of 1X SYBR Green Mastermix (Biorad Laboratories), 100 nM of each primer, and 1 mg/L of BSA. Melting curves were run at the end of each experiment to confirm specificity of amplification and lack of primer/dimer formation.

Phylogenetic Analysis. Phylogenetic analysis was performed on nearly full-length 16S rRNA sequences with Mega5 (35) with an evolutionary history inferred using the Neighbor-Joining Method (36) with a bootstrap test (500 bootstraps) (37). Evolutionary distances were computed with the Maximum Composite Likelihood method (38), and with the scale bar as a reference, correspond to the number of base pair substitutions per site. Phylogenetic analyses included the closest matches from BLAST searches, using only those 16S rRNA sequences that
were nearly full-length, as well as the full 16S rRNA sequences from selected *Firmicutes* isolates (all enriched bands corresponded to bacteria within the *Clostridiales* order of the *Firmicutes*).

**PCR of Reductive Dehalogenase Genes.** The amplification of reductive dehalogenase genes was attempted using previously published primers and methods (39, 40, 41, 42, 43). These attempts were unsuccessful at amplifying putative reductive dehalogenase genes (see Supplemental Information).

**Accession Numbers.** The sequences from this study are deposited in NCBI’s GenBank database under accession numbers KF275148-KF275169.

**Results**

**Dechlorination of chlorinated xanthones.** Both 2,7-dichloroxanthone and 5,7-dichloro-1,3-dihydroxylxanthone were dechlorinated in the Tilden- and Palos Verdes-inoculated microcosms, with the concomitant production of the nonchlorinated daughter products xanthone and 1,3-dihydroxylxanthone (Fig. 2). There was an initial lag prior to 2,7-dichloroxanthone dechlorination and prior to 5,7-dichloro-1,3-dihydroxylxanthone dechlorination in the Palos Verdes microcosms (Fig. 2). There was no lag prior to 5,7-dichloro-1,3-dihydroxylxanthone dechlorination in the Tilden microcosms. Initial dechlorination rates varied by compound and soil/sediment, with relatively slow initial dechlorination (Fig. 2). Subsequent loss of the dechlorinated products was also observed, suggesting that these compounds were also metabolized over time. No singly chlorinated xanthones were detected in this experiment by GC-MS, suggesting that either the detection limit was too high or the dechlorination of the singly chlorinated intermediates occurred faster, or as fast, as that of the parent compounds. No significant decrease in chlorinated xanthones occurred in the control microcosms (maintained for...
698 days) or the Pine Barrens I microcosms (maintained for 557 days) (Supplemental Information; Fig. S1).

The abundance of multiple genera of organohalide-respiring bacteria (*Dehalococcoides*-like bacteria, *Desulfomonile*, *Dehalogenimonas*, *Desulfitobacterium*, *Dehalobacter*, and *Anaeromyxobacter*) was measured with qPCR to determine if these populations increased in abundance during chloroxanthone dechlorination. *Dehalococcoides*-like bacteria were detected at similar levels in the control (fed only xanthone and 1,3-dihydroxylxanthone) and chlorinated xanthone-amended microcosms during the last 100 days of microcosm operation (Fig. S2). *Dehalobacter*, *Dehalogenimonas*, *Desulfomonile*, and *Anaeromyxobacter* spp. were not detected in any of the microcosms and the qPCR method used for *Desulfitobacterium* amplified only non-targeted sequences (see Supplemental Information). Therefore, none of these organohalide-respiring bacteria were likely to be the primary organisms involved in chlorinated xanthone dechlorination.

To determine which organisms might be responsible for the dechlorination of 2,7-dichloroxanthone and 5,7-dichloro-1,3-dihydroxylxanthone, DGGE was performed on samples from the Tilden and Palos Verdes microcosms (Fig. 3). A total of 7 bands were identified that were both (1) present in the gels run with samples from the chlorinated xanthone-amended microcosms and (2) not visible in the gels run with samples from the control microcosms fed only xanthone and 1,3-dihydroxylxanthone. Clone libraries enabled identification of these 7 unique bands via nearly full-length 16S rRNA gene sequences. Of these, the sequences corresponding to bands T-3 and PV-2 were the most interesting because they were the only sequences common to both sets of microcosms and they were the most closely related to the *Desulfitobacterium* and *Dehalobacter* spp., (~91% identical and ~89% identical, respectively,
along ~1500 bp of the 16S rRNA genes. Additionally, from BLAST analysis these sequences were closely related to several clones from other dechlorinating microcosms (44, 45, 46, 47) yet not related to clones found in association with purely fermentative cultures. We named this group the “Gopher group” (Fig. 4) and developed a qPCR method to target the 16S rRNA genes of this group (see Methods). Bands in the control microcosms were also excised and sequenced for quality control (Fig. S4, Table S2, Supporting Information), with those sequenced bands from the controls having less than 90% similarity to the sequences targeted from the chlorinated xanthone-amended microcosms.

In the final 100 days of Tilden and Palos Verdes microcosm operation, the ratio of Gopher group 16S rRNA genes normalized to the number of total Bacteria 16S rRNA genes (Gopher/Bacteria) was maintained at 9.5 ± 5% and 5.0 ± 3% (mean ± standard deviation of triplicate microcosms), respectively, compared to 0.12 ± 0.08% and 0.9 ± 0.04% in the Tilden and Palos Verdes control microcosms, respectively. The overall abundance of Gopher 16S rRNA genes was also much higher in the chlorinated-xanthone amended reactors compared to the controls (8.6 ± 0.5 and 8.1 ± 0.2 logarithmic units 16S rRNA genes/g in the Tilden and Palos Verdes chlorinated xanthone amended microcosms, respectively, compared to 6.1 ± 0.4 and 7.4 ± 0.3 logarithmic units 16S rRNA genes/g for Tilden and Palos Verdes control microcosms respectively).

**Verification of Gopher group involvement.** The enrichment of Gopher 16S rRNA genes was then tested with two new inoculum sources as described in the Methods: sediment from Leech Lake in Minnesota (called Leech Lake) and additional soil from the New Jersey Pine Barrens (called Pine Barrens II microcosms). These new microcosms were amended with
chlorinated xanthones, which were subsequently dechlorinated over a period of approximately 118 days (Fig. 5A and 5B).

Figure 5C shows the enrichment of Gopher group organisms that occurred in the Leech Lake microcosms. Indeed, Gopher/Bacteria increased from 0.023 ± 0.02% to 14 ± 10%, with the abundance of Gopher 16S rRNA genes increasing from 4.8 ± 0.6 to 8.4 ± 0.1 logarithmic units 16S rRNA genes/g during dechlorination in the Leech Lake microcosms. The proportion and abundance of these organisms remained essentially constant at ≤ 0.04 ± 0.03% or ≤ 5.8 ± 0.3 logarithmic units 16S rRNA genes/g in the non-chlorinated xanthone-amended controls (Fig. 5C).

In the Pine Barrens II microcosms, the Gopher group organisms were enriched over time when amended with chlorinated xanthones in two of the three replicate microcosms, growing from 5.0 ± 0.5 to 8.0 ± 0.2 logarithmic units 16S rRNA genes/g (Fig. 5D). In the third Pine Barrens II microcosm, the Gopher group organisms failed to grow (Fig. S3); the reason for this lack of growth is unknown. Growth also occurred, though to a lesser extent (1.3 ± 0.8 logarithmic units (16S rRNA genes/g)), in the triplicate Pine Barrens II control microcosms (Fig. 5D).

The Leech Lake and Pine Barrens II microcosms were also subjected to DGGE to determine if unique organisms were enriched in addition to the Gopher group organisms (Fig. 3). The phylogenetic analysis of the full 16S rRNA genes (obtained from clone libraries) of bands uniquely enriched in the chlorinated xanthone-amended microcosms is shown in Fig. 4. Other Firmicutes were identified by DGGE that appeared to be enriched in the chlorinated xanthone-amended Leech Lake and Pine Barrens II microcosms (Fig. 4). Again, bands in the control microcosms were also excised and sequenced for quality control (Fig. S4, Table S2, Supporting
Information) and all but one of the sequenced bands from the controls were found to be less than 90% similar to the sequences targeted from the chlorinated xanthone-amended microcosms. One band, “Ctrl-PB3”, was 94.2% similar to the sequences corresponding to “PB-2” (Fig 3). This suggests that the 16S rRNA gene associated with “PB-2” is not likely to be unique to the microcosms amended with chlorinated xanthones.

Discussion

Phylogenetically, the Gopher group bacteria form a newly described group of putative organohalide respirers, unique from but related to the *Dehalobacter* and *Desulfotobacterium* in the *Firmicutes* (Fig. 4). Members within the Gopher group could be found in literature reports of at least four other cultures active in the dechlorination of chlorinated ethene (KB1 culture; (45)), chlorobenzenes (SJA culture; (44)), PCBs (Er-MLAYS and Er-LLAYS cultures; (46)), and pentachlorophenol (PCP culture; (47)). As mentioned above, the Gopher group also lacks representatives from more generic fermentative studies and therefore, their detection and presence thus far appears solely dependent on active dechlorination. Our research suggests that previously overlooked organisms belonging to the Gopher group may have participated in organohalide respiration in previously investigated cultures (i.e., 44, 45, 46, 47). These Gopher group organisms should be the focus of further directed research to clarify their niche in both contaminated and uncontaminated environments.

Additional strains of *Firmicutes* were also enriched during the dechlorination of 2,7-dichloroxanthone and 5,7-dichloro-1,3-dihydroxylxanthone (Figs. 3 and 4). Their role, if any, in the dechlorination of these two compounds is unknown. Two of the targeted DGGE bands do not appear to be associated with any previously reported dechlorinating cultures (Tilden-2 and Palos...
Verdes-3). Eight targeted bands were also closely related to bacteria from both dechlorinating and non-dechlorinating cultures (Tilden-4, Palos Verdes-1, Pine Barrens II-2, Pine Barrens II-4, the closely related sequences for Pine Barrens II-3 and Tilden-1, and Pine Barrens II-1, and Leech Lake-1). Interestingly, sequences corresponding to band Pine Barrens II-1 are closely associated with the genera *Pelosinus*; two *Pelosinus* species were recently isolated from chlorinated-solvent contaminated aquifers but upon isolation, were not capable of dechlorinating selected solvents (48). A third *Pelosinus* isolate from a dechlorinating culture (st. BXM) was deposited in GenBank but has not yet been described, and a *Pelosinus* was identified to be a dominant strain in a trichloroethene-dechlorinating enrichment culture containing *Dehalococcoides* (49). These isolates may be involved in reductive dechlorination or may be non-dechlorinating bacteria associated with organohalide-respirers. Again, further work is needed to clarify what role, if any, these non-Gopher group *Firmicutes* strains play in the dechlorination process.

**Relevance to Bioremediation and Application.** This research presents two interesting findings with relevance to bioremediation. First, the possibility that natural compounds such as chlorinated xanthones may be useful as biostimulants or “primers” to speed contaminant dechlorination exists and is intriguing. Priming with chlorinated or brominated compounds has previously been demonstrated to speed the bioremediation of chlorinated pollutants, even highly weathered pollutants (21, 22); nevertheless, currently identified primers are also considered toxic and therefore cannot be used. For chlorinated xanthones, though the dechlorination rates and lag times were relatively long at low concentrations, they were short when higher doses of 100 \( \mu \text{M} \) 2,7-dichloroxanthone and 5,7-dichloro-1,3-dihydroxylxanthone were amended (Fig. 2, Fig. 5). These compounds appear promising as primers; nevertheless, their toxicity is unknown and must
first be determined. The enrichment of the Gopher group bacteria on chlorinated xanthones is also particularly interesting, as similar organisms have been found in cultures dechlorinating anthropogenic pollutants. This further suggests that chlorinated xanthones may have the potential to enrich organisms relevant for bioremediation.

Second, this research highlights that there may exist vast, and still largely undiscovered biodiversity with respect to organohalide-respiring organisms. Indeed, recently a *Shewanella sediminis* strain was found to contain five putative reductive dehalogenases, one of which was confirmed to be functional in the dechlorination of tetrachloroethene, despite the genus never being previously associated with organohalide respiration (50). Isolates of the genera *Dehalococcoides*, *Dehalogenimonas*, *Dehalobium*, *Dehalobacter*, *Desulfitobacterium*, *Anaeromyxobacter*, *Geobacter*, *Desulfomonile*, *Desulfuromonas*, *Desulfovibrio*, *Sulfurospirillum*, *Propionibacteria*, *Clostridium* and *Desulfoluna* have been linked to the specific dechlorination activities of several compounds (51, 52, 53, 54), yet dechlorination unattributable to any of these genera has also been frequently observed (e.g. 23, 30, 46, 55,). Our results implicate a group within the *Firmicutes*, the Gopher group, in the dechlorination of 2,7-

Acknowledgments
This work was funded by the National Science Foundation (CBET-0966559). M.J.K. was partially supported by the United States Environmental Protection Agency under the Science to Achieve Results (EPA-STAR) Graduate Fellowship Program (no. 91694601).

References


Figure Legends

**FIG 1** The structure of 2,7-dichloroxanthone (A), and 5,7-dichloro-1,3-dihydroxylxanthone (B) compared to the structure of polychlorinated biphenyls (C) and dibenzo-p-dioxins (D).

**FIG 2** The concentrations of 2,7-dichloroxanthone (solid diamonds), 5,7-dichloro-1,3-dihydroxylxanthone (solid squares), xanthone (open diamonds), and 1,3-dihydroxylxanthone (open squares) over time for the Tilden microcosms (A) and the Palos Verdes microcosms (B). Errors bars represent standard error for triplicate microcosms. Arrows point to times in which chlorinated xanthones were re-amended.

**FIG 3** DGGE fingerprinting of bacterial communities from Tilden microcosms at Day 635 (A), Palos Verdes microcosms at Day 635 (B), Pine Barrens II microcosms at Day 118 (C), and Leech Lake microcosms at Day 118 (D). Triplicate (a, b, and c) microcosms are shown for sets of microcosms amended with chlorinated xanthones (ClX) and non-chlorinated xanthones (Control). Numbered bands point to those targeted for phylogenetic analysis by clone library and DGGE analysis. Selected bands from control lanes were excised and sequenced (FIG S4, Table S2).

**FIG 4** Phylogenetic tree of the nearly full length 16S rRNA genes identified from the clone libraries and DGGE analysis. Sequences from this study are in bold and the clone’s corresponding DGGE positions from FIG 3 are shown in parenthesis. Numbers at nodes indicate percent agreement from Bootstrap analysis (500 bootstraps) when over 50%. *Bacillus subtilis* was used as an outgroup to root the tree.
FIG 5 The degradation of chlorinated xanthones for microcosms inoculated with Leech Lake sediment (A) and Pine Barrens II soil (B), and the amount of “Gopher group” 16S rRNA genes per g in microcosms inoculated with Leech Lake sediment (C) and Pine Barrens II soil (D). Xanthone data is shown for the reactors amended with chlorinated xanthones (A and B): 2,7-dichloroxanthone concentrations are shown as solid circles, 5,7-dichloro-1,3-dihydroxylxanthone are shown as solid squares, unsubstituted xanthone is shown as open circles, and 1,3-dihydroxylxanthone are shown as open squares. The concentrations of “Gopher group” 16S rRNA genes per g for the chlorinated xanthone-amended microcosms are shown with dark bars and nonchlorinated xanthone-amended controls are shown with light bars. Error bars represent standard deviations for triplicate reactors, except for the “Gopher group” data from the chlorinated xanthone amended microcosms in Panel D in which case the error bars represent standard deviation for duplicate reactors.