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Diverse Dechlorinators and Dechlorination Genes Enriched through Amendment of Chlorinated Natural Organic Matter Fractions

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Abstract

In uncontaminated environments, chlorinated natural organic matter (Cl-NOM) can act as an electron acceptor for organohalide-respiring bacteria. It is unknown, however, whether different types of Cl-NOM are preferentially dechlorinated or whether enrichment with Cl-NOM affects the ability of bacteria to dechlorinate contaminants. In this research NOM was extracted from sediment, fractionated based on hydrophobicity, and either amended to polychlorinated biphenyl-contaminated soil directly or chlorinated and then amended to soil. Amendments of the least hydrophobic Cl-NOM fraction were dechlorinated most rapidly, followed by the moderately hydrophobic Cl-NOM fraction. Soil that had been enriched on the moderately hydrophobic fraction of Cl-NOM was also capable of faster dechlorination of the contaminants trichloroethene and tetrachlorobenzene. Community analysis of the soil during enrichment showed that some known organohalide-respiring bacteria were present and may have played a role in dechlorination; nevertheless, many bacteria appeared to be enriched during both Cl-NOM and contaminant dechlorination. In addition, the quantities of two haloalkane dehalogenase genes increased during enrichment on Cl-NOM. These results show for the first time that Cl-NOM can prime contaminant dechlorination and also suggest that hydrolytic dechlorination processes were involved in both Cl-NOM and contaminant dechlorination.

Environmental Significance Statement

This research suggests that the remediation of chlorinated organic contaminants can be stimulated by amending soil with fractions of naturally derived chlorinated natural organic matter. Culturing anaerobic communities in the presence of chlorinated natural organic matter resulted in increases in the concentration of multiple populations, including those capable of non-respiratory hydrolytic dehchlorination. Remediation approaches that focus on stimulating a range of bacteria may therefore be useful in improving remediation efficiency.

Introduction

Biological dechlorination can be effective for the remediation of chlorinated contaminants, but there are still problems during its implementation, including the stalling of dechlorination before clean-up is complete[1,2]. This could be a result of many factors, including the chemistry or hydrogeology of the site, but it could also be a result of lowered concentrations of contaminants being inadequate to sustain the activity and growth of organohalide-respiring bacteria, bacteria that are thought to be critical actors in the dechlorination of chlorinated contaminants[3–5]. Priming the respiration of chlorinated contaminants with alternative electron acceptors, such as non-toxic chlorinated or brominated compounds, can be an effective method to stimulate dechlorination *in situ*[4]. Unfortunately, the chemicals that have thus far been found to be effective primers are also toxic[4]. Finding primers that are not toxic should help prevent organohalide respiration, and therefore bioremediation, from stalling prior to reaching clean-up goals.

Organohalide-respiring bacteria, including *Dehalococcoides mccartyi*, *Geobacter spp., Desulfitobacterium spp., Dehalobacter restrictus, and Dehalogenimonas spp.*, and organisms that dechlorinate contaminants via hydrolytic dechlorination have both been a focus of bioremediation research[6–14]. Different organohalide-respiring bacteria make use of multiple reductive dehalogenase (RDase) enzymes to conserve energy during dechlorination; these bacteria can dechlorinate multiple contaminants, including trichloroethene (TCE)[15,16], polychlorinated biphenyls (PCBs)[17,18], trichloroethane[19], chlorinated benzenes[20,21], and chlorinated phenols[6]. Contaminants can also be dechlorinated via the activity of hydrolytic dehalogenase enzymes[22–24]. Bacteria make use of hydrolytic dehalogenase enzymes to remove the chlorine atom from chlorinated organics, freeing the organic base molecule for use as a carbon source for growth [10,25]. These enzymes include the hydrolytic haloalkane dehalogenases, which have been found to dechlorinate over 20 chlorinated chemicals for subsequent use as a source of carbon for growth[10,25]. Research on bioremediation has not focused on the environmental conditions that favor dechlorination via reductive versus hydrolytic processes. In

addition, it is unknown whether primers can have a stimulatory effect on reductive and hydrolytic dechlorination processes.

Uncontaminated environments rich in chlorinated natural organic matter (Cl-NOM) could provide a source of potentially non-toxic primers, as well as a niche for microbial populations containing both RDase and hydrolytic dehalogenase enzymes. Indeed, bacteria capable of degrading anthropogenic chemicals may also be able to degrade and grow on Cl-NOM[26,27]. Several thousand chlorinated chemicals are produced naturally and might offer an opportunity to enrich bacteria capable of contaminant dechlorination without the addition of toxic primers[28]. Understanding the presence and enrichment patterns of RDase vs. hydrolytic dehalogenase genes on Cl-NOM could also enable the engineering of remediation systems to enrich bacteria that utilize both reductive and non-reductive processes during dechlorination, depending on the contaminant type, contaminant concentration, and the bacteria present at the site.

In this research we tested the hypothesis that particular fractions of Cl-NOM would be preferentially dechlorinated by a mixed anaerobic community and that the amendment and subsequent dechlorination of Cl-NOM would enrich for bacteria that could dechlorinate the contaminants TCE and tetrachlorobenzene (TeCB). We also tested the hypothesis that both RDase and hydrolytic dehalogenase genes would be enriched during the dechlorination of Cl-NOM, TCE, and TeCB. The specific enrichment of various genera was also explored during both the dechlorination of Cl-NOM and dechlorination of the contaminants TCE and TeCB. This research highlights the potential for Cl-NOM to serve as a primer for contaminant dechlorination, pending suitable toxicity testing of course, while further illustrating the variety of bacteria involved in dechlorination and the likely involvement of both respiratory reductive and non-respiratory hydrolytic processes in dechlorination.

Materials and Methods

Preparation of Cl-NOM

The dechlorination of different fractions of Cl-NOM was studied through the addition of both extracted NOM, which was expected to contain some natural Cl-NOM, and additional laboratory-generated Cl-NOM. Briefly, organic-rich lake sediment was dried and sequentially extracted into three fractions with (1) water ("least hydrophobic"), (2) methanol ("moderately hydrophobic"), and (3) hexane:acetone (50:50) ("most hydrophobic") using an accelerated solvent extractor (ASE) (Thermo Scientific). Sequential extraction with the ASE on the same sediment helped to limit cross over of specific chemical structures between the fractions. These fractions contained NOM from the sediment and any Cl-NOM naturally present in that sediment, and are referred to herein as "NOM". Additional Cl-NOM (herein referred to as "Cl-NOM") was generated from each individual NOM fraction via the addition of hypochlorous acid[29,30]. The extraction and chlorination methods are described in detail in the Supporting Information. All fractions, including the three NOM fractions and the three Cl-NOM fractions, were cleaned via extraction through C_{18} columns and were resuspended into methanol. The quantity of Cl-NOM present in each fraction could not be measured; therefore, the total quantity of Cl-NOM added with each fraction was unknown and cannot be assumed to be equal.

Incubation of Soil with NOM and Cl-NOM of Different Hydrophobicities

Experimental details are provided in the Supporting Information. Briefly, enrichment cultures (140-ml) were set up in an anoxic chamber (Coy) and contained 5 g of soil, 100 ml of reduced anaerobic mineral medium (RAMM)[31] modified to have a low chloride content (see Supporting Information), and 25 µl of different fractions of concentrated NOM or Cl-NOM. The soil was provided by a consulting company from a PCB contaminated site with approximately 40 mg/kg of total PCBs or 200 µg of total PCBs/bottle. This soil was chosen because previous metagenomic analysis in our laboratory had identified a large number of genes encoding for putative dehalogenases in the soil, suggesting that the microbial community present had the potential to dechlorinate a variety of compounds.[32] Treatments consisted of bottles to which the

least, moderately, or most hydrophobic NOM or Cl-NOM fractions were added, for a total of 6 different treatments, each in triplicate. Sterile negative controls were also set up with autoclaved soil and contained a mixture of the three different fractions of the Cl-NOM. No external electron acceptors beyond Cl-NOM/NOM and HCO_3^- were added to the enrichment cultures; nevertheless, other oxidized compounds that could serve as an electron acceptor, such as Fe^{3+}, could have been present in the soil. The headspace contained 5% hydrogen as a potential electron donor in addition to NOM and the methanol in which the NOM/Cl-NOM was dissolved in. No other carbon sources were added. The only difference between the enrichment cultures was the presence of Cl-NOM vs. NOM; therefore, differential growth between the different enrichments was attributed to Cl-NOM.

Once started via the addition of the NOM or Cl-NOM fractions, enrichment cultures were sampled every 2-4 weeks for DNA analysis and chloride analysis. The release of chloride was assumed to result from the dechlorination of either the amended Cl-NOM or the Cl-NOM naturally present in the NOM amendments, given the low concentrations of PCBs initially in the soil. The sterile controls were sampled to monitor for abiotic chloride release. The amount of chloride released in the different treatments was compared using the Kruskal-Wallis test to determine if the chloride released was different among the treatments. Treatments were compared pair-wise using the Dunn's multiple comparison test in GraphPad Prism.

Enrichment with Contaminants

After enrichment with the different NOM or Cl-NOM fractions for 115 days, the triplicate serum bottles for each treatment were mixed together, then subdivided into 6 new serum bottles. The total volume of the new serum bottles was increased with the low-chloride RAMM (Supporting Information) to either 70 ml for the TCE dechlorination experiment or 100 ml for the 1,2,3,5-tetrachlorobenzene (TeCB) dechlorination experiment. TCE and TeCB were added to separate bottles to a concentration of 100 and 20 μM, respectively. Both TCE and TeCB were added as methanol stocks. Less than 5 μl of methanol was added in an effort to minimize the effects of

the methanol. Additionally the same amount of methanol was added to all of the treatments. Because TeCB is much less soluble in water, a second addition of TeCB to a final concentration of 20 μM was added after 20 days. The loss of the parent compounds (TCE and TeCB) and the formation of possible daughter products (1,1-dichloroethene (1,1-DCE), *cis*-DCE, *trans*-DCE, 1,2,3-trichlorobenzene (TCB), 1,3,5-TCB, 1,3-dichlorobenzene (DCB), and 1,2-DCB) were analyzed over time. The analytical method that was used could not reliably quantify vinyl chloride or ethene, so these additional dechlorination daughter products were not analyzed. The experiment lasted 65 days and samples were taken for chemical and DNA analysis over time. Abiotic controls were set up in the same manner but with the autoclaved enrichment cultures. The first-order degradation rate coefficients for TCE and TeCB were calculated, as were the zero-order formation rate coefficients for *cis*-DCE and 1,2,3-TCB. The rate coefficients were compared using the Kruskal-Wallis test and Dunn's multiple comparison tests were used to determine which treatments were significantly different from one another (performed in GraphPad Prism).

DNA Analysis Methods

Samples taken for DNA extraction were centrifuged for 15 min at 8000 g. The supernatant was discarded and the pellets were extracted with the FastDNA Extraction Kit (MP Biomedicals) according to the manufacturer's protocol. The DNA was analyzed by both quantitative polymerase chain reaction (qPCR) and Illumina sequencing of the 16S rRNA gene sequence. qPCR was used to quantify three putative hydrolytic dehalogenase genes, one putative RDase gene, and the total number of 16S rRNA genes, as described in the Supporting Information. The functional hydrolytic dehalogenase and RDase genes targeted were originally developed from metagenomics analysis of the PCB contaminated soil used in this study (Supporting Information).[32] Indeed, several qPCR primer sets were developed and tested to amplify genes capable of dechlorination, but only four were found to be free of non-target amplification and to be above the qPCR detection limit. Table S1 shows these primer sequences. For bacterial community analysis, Illumina paired end sequencing (2x300) was completed on the V4-V6 region of the 16S rRNA gene. Relative fractions of the populations in each

sample were calculated (see Supporting Information for details), and these values were then converted to a numerical value (number of 16S rRNA genes for the different populations) based on the total number of 16S rRNA genes present in the sample, as obtained via qPCR. This conversion from relative abundance to estimated absolute abundance has been verified in the literature as a way to account for changes in biomass[33–36]. Amplification and sequencing were completed at the University of Minnesota Genomics Center. Details are also provided in the Supporting Information. The community data were analyzed using multiple statistical methods, described in detail in the Supporting Information; in all cases a $P<0.05$ was considered significant after the P-value was adjusted by the false discovery rate (FDR) two-stage step-up method of Benjamini, Krieger and Yekutiele[37] in GraphPad Prism to take into account the number of statistical tests being performed. DNA sequences are available on NCBI under BioProject PRJNA484929.

Analytical Methods

Chloride concentration was measured with ion chromatography. Centrifuged samples were injected into a Metrohm 930 Compact IC Flex with an eluent of carbonate buffer (3.2 mM Na2CO3 and 1.0 mM $NaHCO_3$). Concentrations were determined via an external calibration curve. The detection limit was 0.01 mg Cl^-/l. TCE, TeCB, and their degradation products were measured via a gas chromatograph (HP 5890) coupled to an electron capture detector (GC-ECD). TeCB and its degradation products were extracted from 1 ml of the sample slurry with hexane, which was injected on the GC-ECD and TCE was measured in the headspace, samples of which were injected on the GC-ECD. Details for both methods are provided in the Supporting Information. The detection limit of TeCB and each of the TCB congeners (1,2,3-TCB, 1,3,5-TCB) was 50 nM and that of each DCB congener (1,3-dichlorobenzene (1,3-DCB), and 1,2-DCB)) was 100 nM. The detection limits of the DCE congeners (1,1-DCE, *trans*-DCE, and *cis*-DCE) and TCE were 1 μM and less than 100 nM, respectively. First-order degradation rate coefficients for TCE and tetra-CB were calculated during the time of active dechlorination and excluded the lag time. The formation of DCE and TCB congeners were best fit to a zero-order rate expression. The zero-order rate coefficients for DCE and TCB congener production were

calculated over the same periods of active dechlorination by dividing the quantity of product generated over a given period of time by the number of days in that period of time.

Results and Discussion

Cl-NOM dechlorination and use as a primer

The three different Cl-NOM-amended treatments all released significantly different amounts of chloride (Kruskal-Wallis $P = 0.004$), with bacteria releasing significantly more chloride from the least hydrophobic Cl-NOM fraction compared to the moderately hydrophobic fraction ($P = 0.022$, Figure 1) or the most hydrophobic fraction ($P= 0.007$). The moderately hydrophobic Cl-NOM fraction was also dechlorinated significantly more than the most hydrophobic fraction ($P=0.040$, Figure 1); no dechlorination occurred in treatments amended with the most hydrophobic Cl-NOM fraction (Figure 1). This preferential dechlorination of the least hydrophobic Cl-NOM fraction could be a result of several factors: more chlorination of this fraction of NOM may have occurred resulting in a larger quantity of Cl-NOM available for dechlorination, the compounds in this fraction may have been more bioavailable, and/or the less hydrophobic Cl-NOM may have been degraded by a greater variety of microorganisms. Similar trends with respect to dechlorination were observed in the treatments amended with the three different NOM fractions, but lower total quantities of chloride were released in these treatments when compared to those amended with the Cl-NOM fractions. This was expected, as the NOM extracts likely contained (natural) Cl-NOM, only in much lower concentrations.

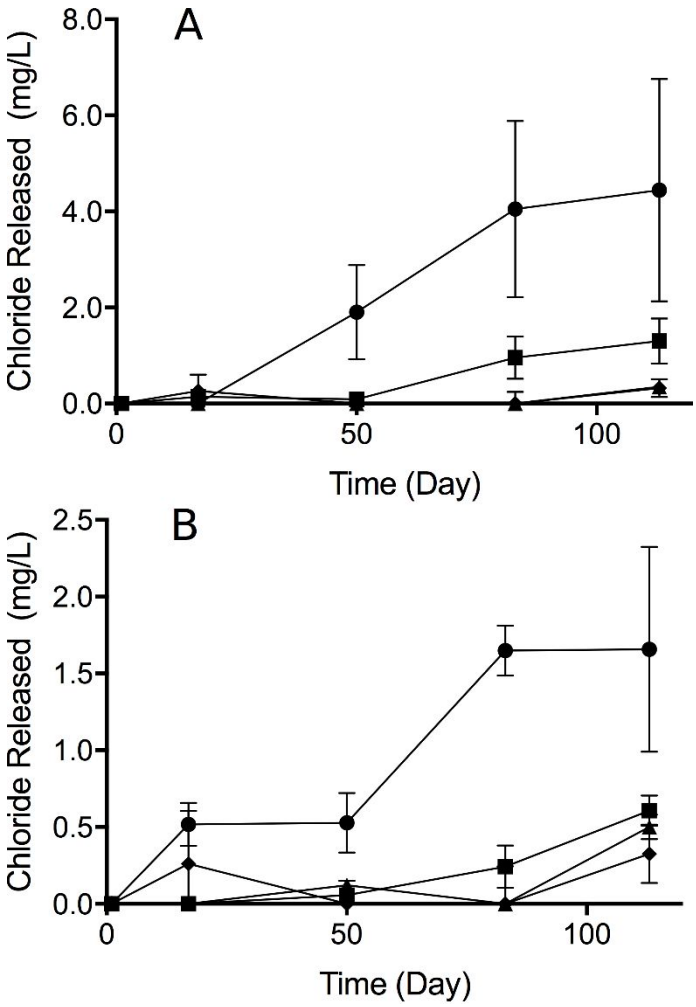

Figure 1 Panels A and B show the release of chloride with time in the treatments amended with (A) HOCl-generated Cl-NOM and (B) NOM. Results from the treatments amended with the least hydrophobic NOM or Cl-NOM fractions are shown with circles (●), those amended with the moderately hydrophobic NOM or Cl-NOM fractions are shown with squares (■), and those amended with the most hydrophobic NOM or Cl-NOM fractions are shown with triangles (▲). Diamonds (◆) show the chloride released in the sterile negative controls amended with a mixture of the three fractions of NOM or Cl-NOM. Error bars represent standard deviation. Note: the y-axes of panels A and B are on different scales to better show the differences in chloride released.

Perhaps more important than the dechlorination of the Cl-NOM fractions was the fact that certain fractions of Cl-NOM acted as primers, stimulating the dechlorination of subsequent amendments of both TCE and TeCB. Parent compound degradation rate coefficients of TCE and daughter product formation rate coefficients (*cis*-DCE and 1,2,3-TCB) are presented in Table 1. TeCB degradation coefficients were not calculated because additional TeCB was added during the experiment and the slow dissolution of TeCB further complicated the determination

of TeCB degradation rates. The treatments previously enriched with Cl-NOM fractions dechlorinated TCE to a much greater extent and had shorter lag periods prior to *cis*-DCE formation when compared to those previously enriched with NOM (Figures S1 and S2). In addition, the TCE degradation rate coefficients were greater in the treatments previously enriched with the least hydrophobic Cl-NOM fractions (P = 0.065) or the moderately hydrophobic Cl-NOM fractions ($P = 0.026$) when compared to those rates of TCE degradation in treatments previously enriched with the equivalent NOM fractions (Table 1). The *cis*-DCE formation rate coefficients showed similar trends; nevertheless, only the treatments previously enriched with moderately hydrophobic Cl-NOM fractions had significantly higher *cis*-DCE formation rate coefficients when compared to those in treatments previously enriched with the equivalent NOM fraction ($P = 0.007$, Table 1). Vinyl chloride and ethene were not analyzed and mass balances indicated that additional dechlorination products were present in the treatments previously enriched with the least and moderately hydrophobic Cl-NOM fractions (Figures S1 and S2), possibly explaining why the TCE degradation coefficient was higher in the treatments previously enriched with the least hydrophobic Cl-NOM fractions while the *cis*-DCE formation coefficient was not (Table 1). Previous enrichment with the moderately hydrophobic fraction of Cl-NOM also appeared to stimulate the degradation of TeCB, with 1,2,3-TCB formation only detected in the treatments previously enriched with the moderately hydrophobic Cl-NOM and NOM fractions and the least hydrophobic Cl-NOM fraction (Figure S3). There was no significant difference between the rate of 1,2,3-TCB formation in treatments enriched with the moderately hydrophobic Cl-NOM vs. NOM fractions ($P = 0.58$), either because of the large variation in the rates of 1,2,3-TCB formation or because enrichment of bacteria capable of dechlorination occurred in both treatments, with both the amended Cl-NOM and the natural Cl-NOM present in the NOM extracts enriching or stimulating dechlorinating microorganisms similarly. The rate coefficient for 1,2,3-TCB formation was statistically greater in the treatments previously enriched on the moderately hydrophobic Cl-NOM fractions compared to those enriched with the least hydrophobic Cl-NOM fractions ($P = 0.022$), with degradation observed after 21 days in TeCB-amended treatments enriched with moderately hydrophobic Cl-NOM and

NOM, but not until Day 36 in treatments previously enriched with the least hydrophobic Cl-NOM fraction. No 1,2,3-TCB or *cis*-DCE formation was observed during the 65-day experiment in the sterile treatments or in the treatments previously enriched with the most hydrophobic Cl-NOM or NOM fractions.

Previous work has shown that bacteria enriched with halogenated chemicals more quickly dechlorinate contaminants[4,38,39], with halogenated primers effectively stimulating native dechlorinating bacteria.[38] Indeed, specific alternative (halogenated) electron acceptors, such as congeners of pentachloronitrobenzenes, chlorinated phenols, and chlorinated benzoates, have been found to be more effective at priming the dechlorination of weathered PCBs than others.[4,39] Although the chemical structures in the Cl-NOM fractions tested herein are unknown, results suggest that different Cl-NOM chemical structures also have different abilities to prime the dechlorination of TCE and TeCB. It is unclear whether a class of chemicals within the moderately hydrophobic fraction of Cl-NOM, or a few specific chemicals within this fraction are preferentially priming or enriching bacteria capable of dechlorinating TCE and TeCB. It is also important to note that this research was performed with only one soil, a soil contaminated by PCBs; the effect of this prior contamination or the effect of the soil chemistry itself on dechlorination in these enrichments is therefore unknown.

Microbial Community Structure and the Presence of Multiple Putative Dechlorinators

Possible organohalide-respiring bacteria were detected with 16S Illumina sequencing in both the initial Cl-NOM enrichment cultures and in subsequent TCE- or TeCB-dechlorinating enrichment cultures. The genera *Geobacter*, *Dehalobacter*, and *Anaeromyxobacter*, all of which contain known organohalide-respiring bacteria,[8,11-13,40] increased in estimated absolute abundance in all of the NOM- and Cl-NOM-amended treatments over time (Figure 2). Among these organisms, the comparative increase in estimated absolute abundance of *Geobacter* spp. in the Cl-NOM-amended vs. NOM-amended treatments was the largest (P = 0.030), with the treatments amended with moderately hydrophobic Cl-NOM showing the most growth overall

(Figure 2) as well as more growth than the moderately hydrophobic NOM-amended treatments (P = 0.024). *Geobacter* spp. also increased in estimated absolute abundance in the TCE dechlorinating treatments previously enriched on the moderately hydrophobic Cl-NOM (ρ=0.95, $P < 0.001$), but not in any of the other TCE- or TeCB-amended treatments. Although it is possible that the *Geobacter* spp. detected were organohalide-respiring, it is also quite possible that these organisms were generating energy through the use of other electron acceptors, as *Geobacter* spp. are metabolically flexible.[e.g., 12]

Table 1 First-order degradation coefficients for TCE (day^{-1}) and zero-order formation rate coefficients for *cis*-DCE (μM/day) and 1,2,3-TCB (nM/day).

	Degradation or formation coefficients (average ± standard deviation)		
Treatment/Amendment	**TCE (day^{-1})**	***cis*-DCE (μM/day)**	**1,2,3-TCB (nM/day)**
Least Hydrophobic Cl-NOM	0.120 ± 0.059	1.91 ± 0.41	0.59 ± 0.20
Moderately Hydrophobic Cl-NOM	0.240 ± 0.092	4.67 ± 0.30	10.31 ± 5.78
Most Hydrophobic Cl-NOM	0.006 ± 0.020	0.00 ± 0.00	0.00 ± 0.00
Least Hydrophobic NOM	0.042 ± 0.040	2.28 ± 0.21	0.00 ± 0.00
Moderately Hydrophobic NOM	0.018 ± 0.002	2.49 ± 0.09	3.49 ± 3.23
Most Hydrophobic NOM	0.001 ± 0.006	0.00 ± 0.00	0.00 ± 0.00
Sterile Negative Control	0.007 ± 0.008	0.00 ± 0.00	0.00 ± 0.00

Anaeromyxobacter spp. also showed patterns of differential growth between the NOM- versus Cl-NOM-amended treatments (P = 0.029), with more growth observed in the treatments amended with the least hydrophobic Cl-NOM (P = 0.011), and to some extent, those amended with the most hydrophobic Cl-NOM (P = 0.10) when compared to their NOM-amended counterparts. Differential growth was not observed between the treatments amended with the moderately hydrophobic Cl-NOM vs. NOM (P = 0.41), although growth of *Anaeromyxobacter* spp. did occur in both. *Anaeromyxobacter* spp. also increased in estimated absolute abundance in the TCE-amended treatments originally enriched with the least hydrophobic Cl-NOM (ρ=0.94, $P < 0.0001$) and the moderately hydrophobic Cl-NOM (ρ = 0.95, $P < 0.001$). Again, as with *Geobacter* spp., *Anaeromyxobacter* spp. can respire organohalides along with a variety of electron acceptors;[e.g.,40] therefore, it is possible that the growth of *Anaeromyxobacter* spp. was not a result of organohalide respiration.

Dehalobacter also increased in estimated absolute abundance in both the Cl-NOM- and NOM-amended treatments; there were, however, no statistical differences between them (P = 0.89). *Dehalobacter* increased in estimated absolute abundance in the TCE-amended treatments previously enriched with the least hydrophobic Cl-NOM (ρ=0.95, P<0.001), moderately hydrophobic Cl-NOM (ρ=0.94, P<0.001), and moderately hydrophobic NOM (ρ=0.95, P<0.001) fractions. Growth was less significant in the TCE-amended treatments previously enriched with the most hydrophobic Cl-NOM fraction (ρ=0.79, P=0.085). No *Dehalobacter* growth was observed in any of the TeCB-amended treatments.

The commonly studied organohalide-respiring bacterium *Dehalococcoides mccartyi* was not detected in any of the treatments by Illumina sequencing; qPCR using *Dehalococcoides mccartyi*-specific primers was not attempted. *Desulfitobacterium*, another genus containing organohalide-respiring bacteria, was detected but did not increase in estimated absolute abundance over the enrichment period in any of the Cl-NOM- or NOM-amended enrichment cultures (P=0.56); significant growth of *Desulfitobacterium* was observed, however, in the

TCE-amended treatments previously enriched with the least hydrophobic Cl-NOM fractions (ρ=0.86, *P*=0.005).

It is possible that these genera were stimulated by Cl-NOM, either naturally present in NOM fractions[33] or HOCl-generated, and played a role in dechlorination; nevertheless, if Cl-NOM was able to serve as an electron acceptor during organohalide respiration, one would have expected these populations, including *Dehalobacter*, to grow substantially more in the treatments amended with additional Cl-NOM. Although this was observed with *Geobacter* and *Anaeromyxobacter* in some of the Cl-NOM-amended treatments, the lack of consistent enrichment of possible organohalide-respiring bacteria, including the obligate organohalide-respiring *Dehalobacter*, concomitant with dechlorination may suggest that organohalide respiration was not the primary mechanism for Cl-NOM dechlorination in this particular soil, and non-respiratory hydrolytic dechlorination may have also been occurring. Additionally, the growth patterns observed for these organohalide-respiring bacteria were not consistent with the patterns of chloride evolution observed. For example, although *Geobacter* spp. did grow more in the treatments amended with the moderately hydrophobic Cl-NOM fractions, which were also those treatments with the most rapid subsequent TCE dechlorination, no significant *Geobacter* spp. growth was observed in the other treatments in which TCE dechlorination was observed. As mentioned above, these organohalide respiring bacteria are capable of using multiple electron donors and acceptors for growth other than chlorinated organic compounds,[*e.g.*, 12, 40] and, although no electron donors other than Cl-NOM, NOM, and HCO_3^- were added to the enrichment cultures, it is likely that other electron acceptors such as Fe^{3+} and humic materials were present in the soil.

Although the growth of a single analyzed organohalide-respiring bacteria could not explain all of the differences observed in the Cl-NOM- versus NOM-amended treatments in this soil, multiple other organisms also preferentially increased in estimated absolute abundance in Cl-NOM-amended treatments (Figure 3). Spearman's rank correlation analysis (Tables S2-S4)

revealed that multiple genera grew to a statistically greater extent in treatments to which Cl-NOM was added and in treatments in which the dechlorination of TCE and TeCB appeared to have been primed with Cl-NOM addition (Figures 3-5). Amendment of cultures with the least hydrophobic Cl-NOM fractions resulted in the preferential growth of more genera than amendment of either the moderately or most hydrophobic Cl-NOM fractions (Figure 3). Finally, even though there was no measurable chloride release in the treatments amended with the most hydrophobic Cl-NOM fraction, some genera did grow preferentially in those treatments when compared statistically to the corresponding NOM-amended treatments (Tables S2-S4). It is possible that these bacteria did participate in some dechlorination, but not enough to be detected via chloride measurement.

As with the results for the targeted putative organohalide-respiring bacteria, the growth of no single genus that we detected in this soil could explain the dechlorination results observed, suggesting that multiple bacteria were involved in Cl-NOM, TCE, and TeCB dechlorination. These results were unsurprising, as previous studies have also found that Cl-NOM addition enriched bacteria from multiple phyla.[26,34] The dechlorination pathways used in those previous studies were not identified,[26,34] nor were they identified in the work presented herein. Nevertheless, our results suggest that multiple dechlorination processes, both reductive and hydrolytic, were involved in dechlorination, with annotated genomes available on IMG indicating that several of the genera that grew preferentially in the Cl-NOM-amended treatments or in the primed TCE- or TeCB-dechlorinating treatments contain hydrolytic dehalogenase genes, including haloacid dehalogenases and haloalkane dehalogenases. Again, only one soil, a soil contaminated by PCBs, was studied; this could have had an effect, stimulatory or inhibitory, on the microbial community present or on particular populations within that community.

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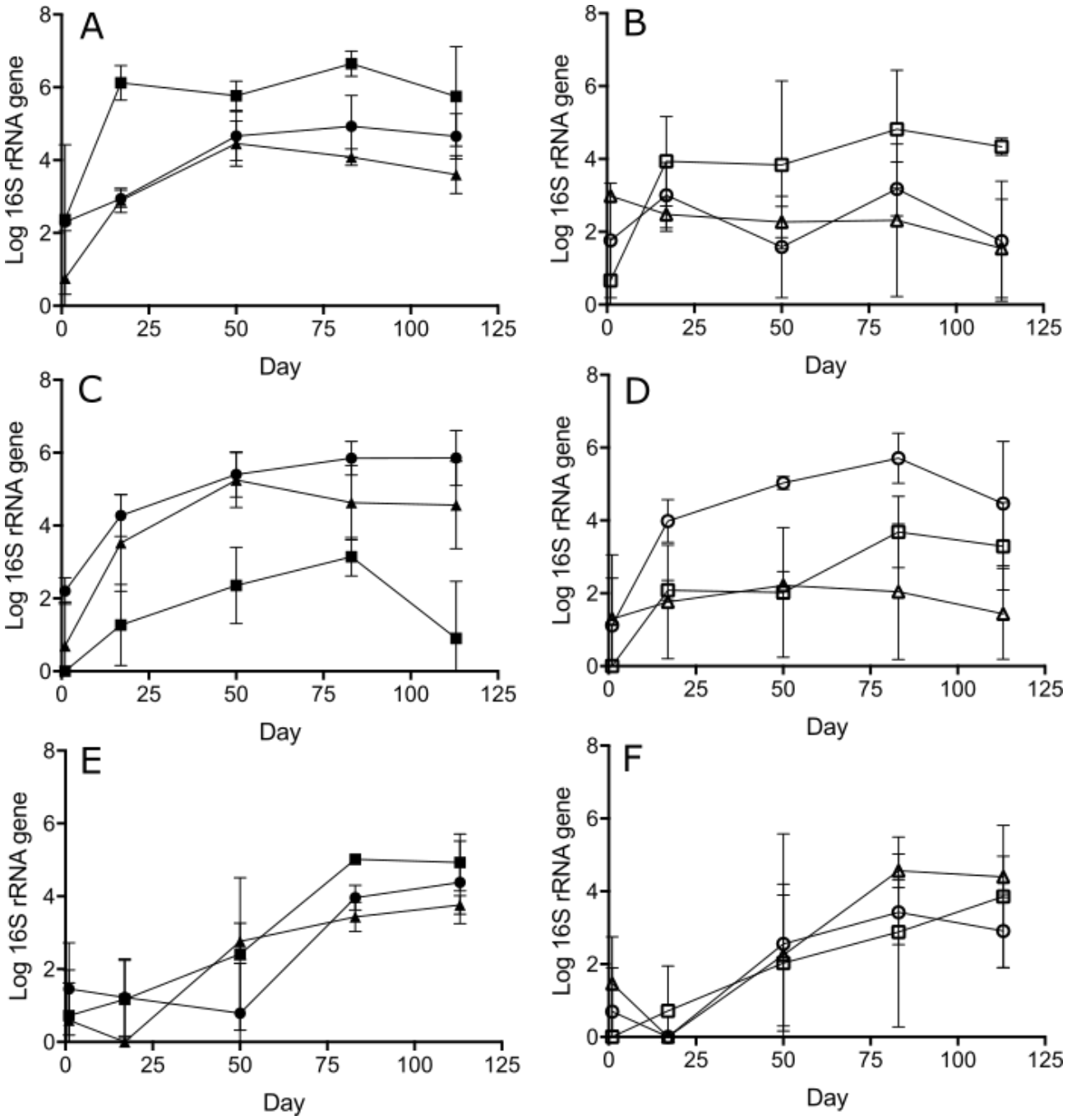

Figure 2 Estimated absolute abundance of 16S rRNA gene copies/ml slurry for the genera (A,B) *Geobacter*, (C,D) *Anaeromyxobacter*, and (E,F) *Dehalobacter* in Cl-NOM- (filled symbols) or NOM-amended treatments (open symbols) over time. Error bars represent standard deviation of triplicate bottles. Circles (● or ○) represent the treatments amended with the least hydrophobic fractions, squares (■ or □) represent those amended with the moderately hydrophobic fractions, and triangles (▲ or △) represent those amended with the most hydrophobic fractions.

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Figure 3 Organisms at the genus level that increased in estimated absolute abundance significantly more over the enrichment period in the Cl-NOM-amended treatments compared to the NOM-amended treatments, based on the Spearman's rank correlation test (false discovery rate corrected $P<0.05$). Estimated absolute abundance is defined as the relative abundance from the Illumina sequencing multiplied by the total number of 16S rRNA genes in a given sample, as measured by qPCR. The sizes of the dots indicate the increase in the log concentration of each genus in question (16S rRNA gene per ml of enrichment culture) over the experimental period (Days 1 to 113).

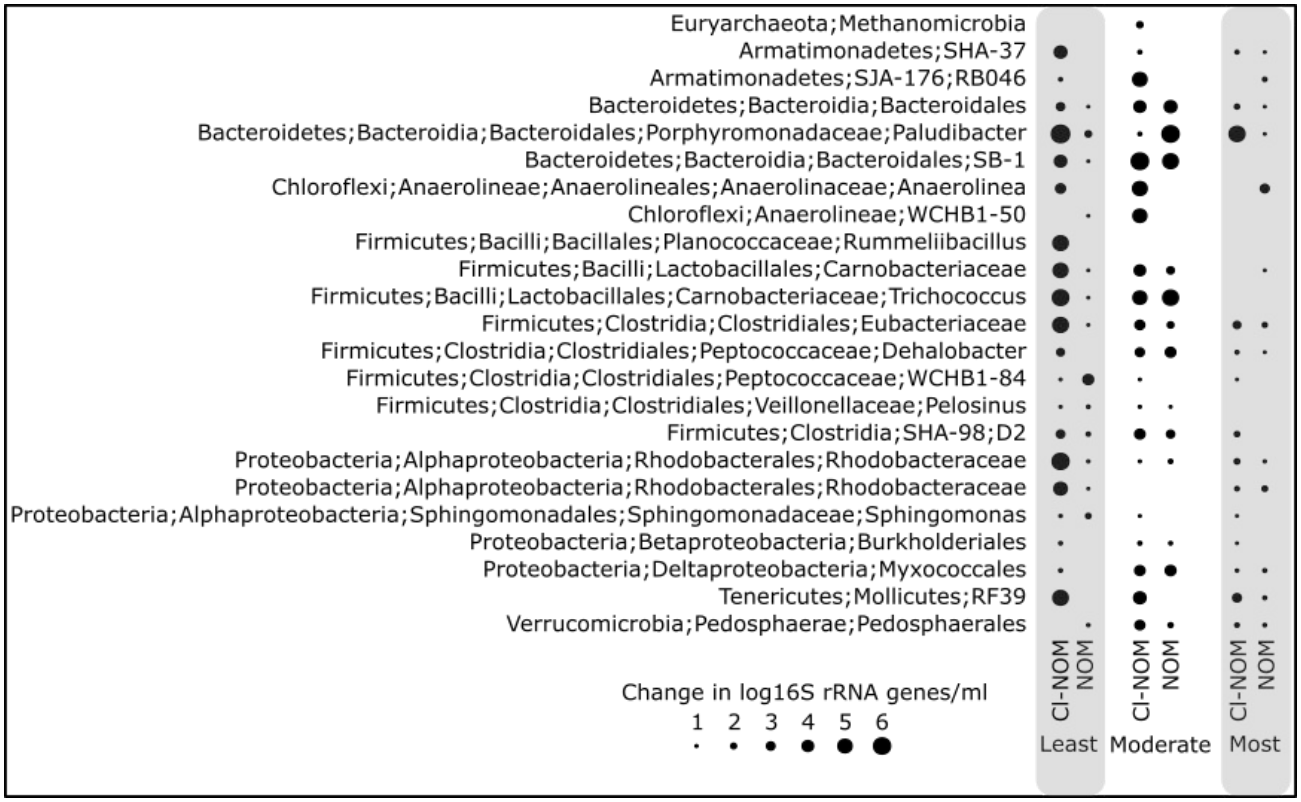

Figure 4 Genera with increase in estimated absolute abundance that significantly correlated with *cis*-DCE production over the enrichment period in the treatments, according to the Spearman's rank coefficient (false discovery rate corrected $P<0.05$). The complete phylogeny of each genera could not be determined. The sizes of the dots indicate the increase in the log concentration of each genera in question (16S rRNA gene per ml of enrichment culture) over the experimental period (Days 1 to 65).

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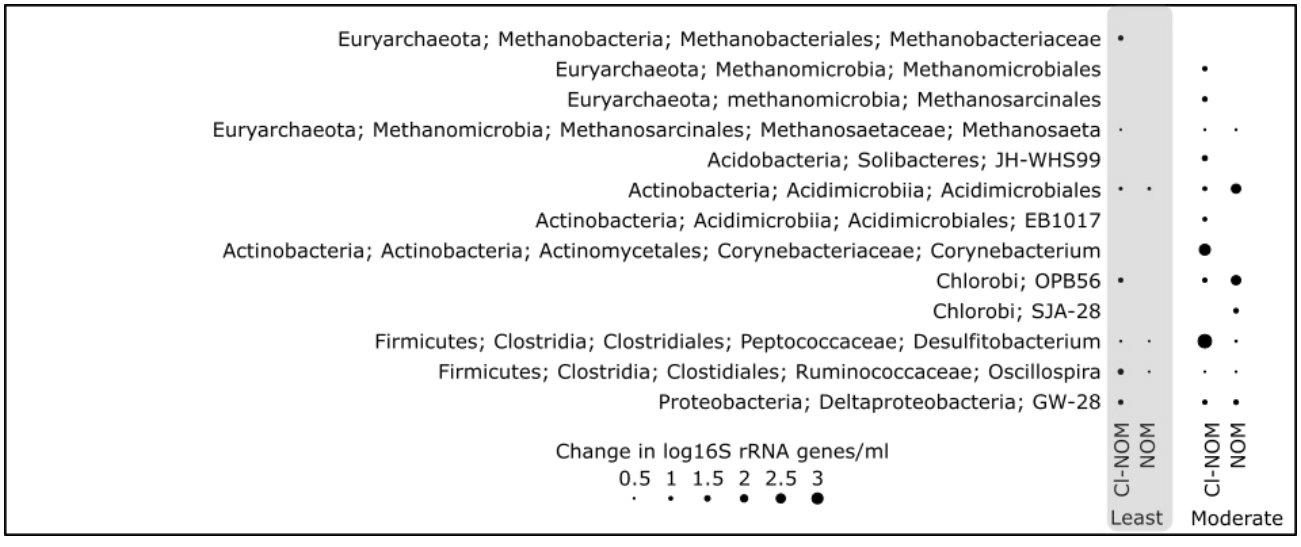

Figure 5 Genera with estimated absolute abundance values that increased significantly more (false discovery rate corrected $P<0.05$) in the treatments in which 1,2,3-TCB production was observed. No 1,2,3-TCB production was detected in the treatments amended with the most hydrophobic NOM and Cl-NOM fractions; therefore, no statistical comparisons were made in these treatments. The sizes of the dots indicate the increase in the log concentration of each genera in question (16S rRNA gene per ml of enrichment culture) over the experimental period (Days 1 to 65).

360

Dehalogenase Genes Enriched

Because the microbial community results suggested the enrichment of organisms likely to contain respiratory RDase and hydrolytic dehalogenase genes/enzymes, qPCR was used to determine whether there was evidence of hydrolytic dehalogenase or RDase genes increasing in number during dechlorination. Primers for qPCR analysis were previously developed from metagenomic analysis of the soil used in this study to target putative RDase and hydrolytic dehalogenase genes[32]; they were therefore used to quantify three putative hydrolytic and one putative reductive dehalogenase gene during enrichment on Cl-NOM, TCE, and TeCB. Of these four analyzed genes, two haloalkane dehalogenase genes increased over the period of Cl-NOM dechlorination (Figure 6). The quantities of these two putative haloalkane dehalogenase genes (PCB HAdhg1 and PCB HAdhg2) increased to a greater degree in the treatments amended with the moderately hydrophobic Cl-NOM fractions compared to those amended with the moderately hydrophobic NOM fractions (Figure 6) (P = 0.047 and 0.085 for PCB HAdhg1 and PCB HAdhg2, respectively). Both haloalkane dehalogenase genes are similar to haloakane dehalogenase genes found in the *Peptococcaceae* family (Accession number HBC92587) and *Geobacter* genus (Accession number WP_012647128). None of the genes increased to a significant degree in the treatments amended with the least hydrophobic or most hydrophobic Cl-NOM fractions compared to those amended with the corresponding NOM fractions. The same two putative haloalkane dehalogenase genes (PCB HAdhg1 and PCB HAdhg2) were present in the TCE- and TeCB-amended treatments, but did not increase over time. Other haloalkane dehalogenase enzymes have been found to be capable of dechlorinating hexachlorocylohexane and 1,2 dichloroethane.[22,24] A putative 2-haloacid dehalogenase gene (PCB 2-haloacidDhg) was detected in all of the least and moderately hydrophobic Cl-NOM- and NOM-amended treatments, but did not increase with time or with chloride evolution. 2-haloacid dehalogenases have been found in a variety of bacteria and dehalogenate 2-haloalkanoic acids to the corresponding hydroxyalkanoic acid. This enzyme is a part of a degradation pathway of 1,2-dichloroethane.[25] This specific 2-haloacid dehalogenase gene was found to be most similar to that found in a genus of *Bradyrhizobiaceae* (Accession number

WP_046827683). A putative RDase gene (PCBRDase) was also detected at Day 113 in treatments amended with the most hydrophobic Cl-NOM and NOM fractions, with average RDase quantities of $3.1x10^3 \pm 1.4x10^3$ and $3.8x10^3 \pm 3.7x10^3$ gene copies/ml slurry, respectively. This putative RDase gene was also detected in the TCE- and TeCB-amended treatments, but its dynamics did not correlate with dechlorination. This RDase gene was found to be most similar to a reductive dehalogenase gene found in a *Dehalobacter* species (Accession number WP_119776250). The increase in the two putative haloalkane dehalogenase genes suggests that they might have been involved in dechlorinating compounds present in the moderately hydrophobic Cl-NOM fraction; nevertheless, their dynamics did not explain the priming effect observed with amendment of some Cl-NOM fractions. Because, as mentioned above, others have found haloalkane dehalogenase enzymes capable of dechlorinating chlorinated compounds,[22,24] the involvement of non-respiratory hydrolytic processes in such a complex community is not surprising. The Cl-NOM fractions were not chemically characterized, however, and it is therefore not known whether haloalkanes or haloacids were present in the Cl-NOM fractions.

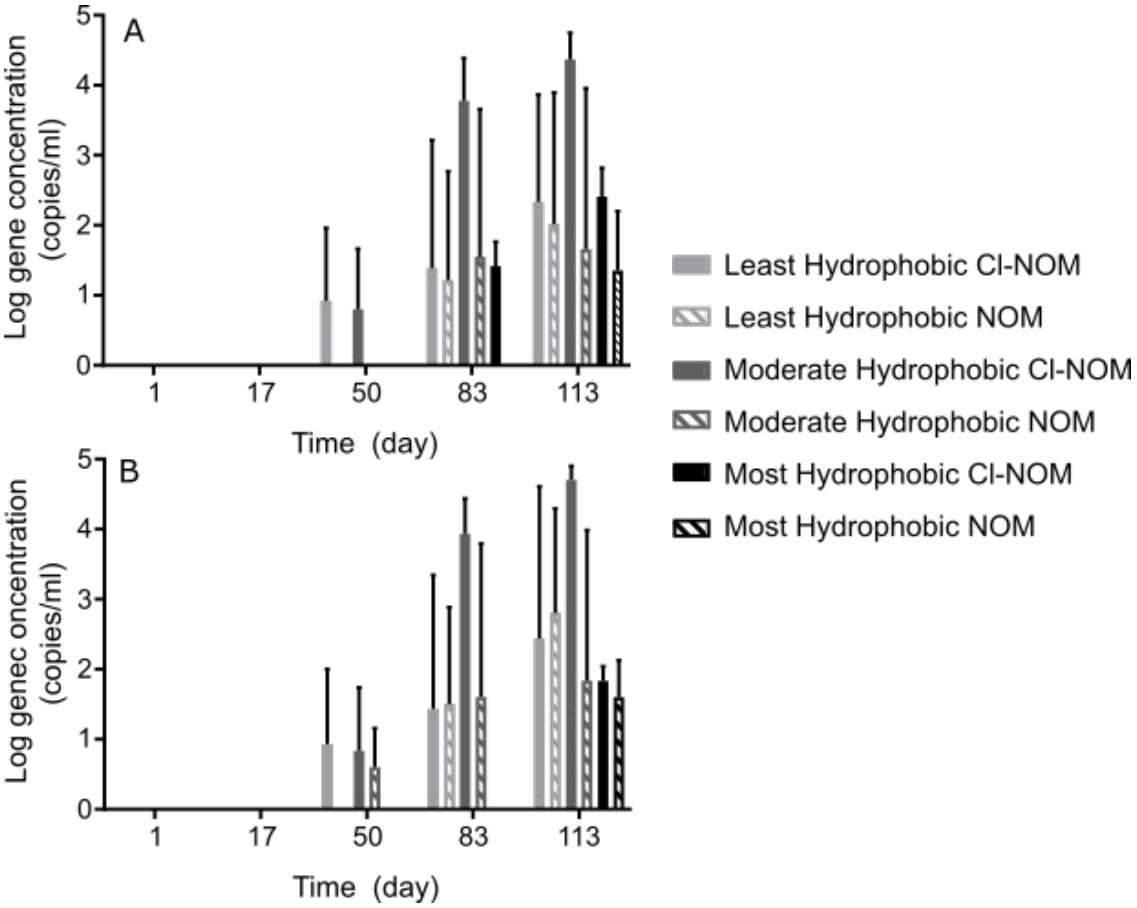

Figure 6 The number of haloalkane dehalogenase genes detected over time in treatments amended with Cl-NOM and NOM fractions of different hydrophobicities. The two panels show the number of (A) PCB HAdhg1 and (B) PCB HAdhg2 genes detected. Error bars are standard deviation of triplicate enrichment cultures.

Conclusions

The results presented herein showed for the first time that bacteria, likely a large group of both organohalide-respiring and non-respiratory dechlorinators, dechlorinated different fractions of Cl-NOM and that initial enrichment on these different Cl-NOM fractions could "prime" contaminant dechlorination. Nevertheless, the use of Cl-NOM to prime the bioremediation of weathered and more recalcitrant contaminants, such as PCBs, is unknown and should be investigated. In addition, the toxicity of Cl-NOM has not been studied and it is not known whether priming with such compounds would be advantageous from a toxicity perspective. Previous results have shown that bacteria phylogenetically similar to *Dehalococcoides* were enriched with Cl-NOM[26]. It appears, however, that both organohalide-respiring bacteria and non-respiratory dechlorinating bacteria may be active in Cl-NOM cycling and subsequent contaminant dechlorination, which was demonstrated experimentally in this research. Indeed, *Dehalococcoides mccartyi* spp. were not detected in the Cl-NOM-, NOM-, TCE-, or TeCB-amended treatments; rather, the possible organohalide-respiring bacteria that were detected were typically ones with more diverse metabolic capabilities. Taken together, these results have important environmental implications. They suggest that remediation might be stimulated with the amendment of certain fractions of naturally derived Cl-NOM, which moves the field closer to identifying non-toxic "primers" for use in remediation. They also suggest that remediation approaches that focus on stimulating a wide range of respiratory and non-respiratory dechlorinators with diverse metabolic abilities may be useful. Hydrolytic dehalogenase enzymes and bacteria utilizing non-respiratory dehalogenation processes have been found by others to be capable of dechlorinating contaminants[22,24,25,41], but are typically thought to be active only in aerobic systems, limiting interest in their use for many remediation applications. Recent

research by others, as well as the research presented herein show, however, that hydrolytic dehalogenase genes can be enriched in anaerobic environments as well[36]. This opens the door to a broader view of remediation and may help in furthering bioremediation efforts.

Conflicts of interest

There are no conflicts to declare.

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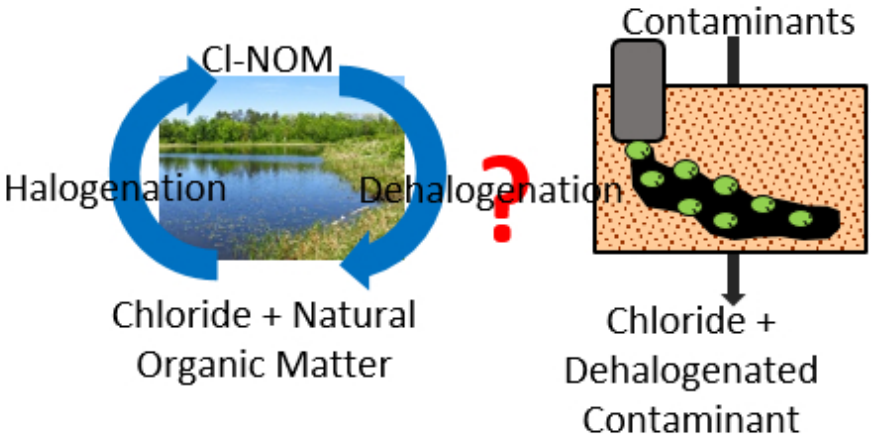

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