Abundance of Chlorinated Solvent and 1,4-Dioxane Degrading Microorganisms at Five Chlorinated Solvent Contaminated Sites Determined via Shotgun Sequencing

Hongyu Dang (danghon1@msu.edu), Yogendra Kanitkar, Robert Stedtfeld, Syed Hashsham and Alison M. Cupples (Michigan State University, East Lansing, MI)
Paul Hatzinger (APTIM Federal Services, Lawrenceville, NJ)

Abstract: Shotgun sequencing was used for the quantification of key taxonomic and functional biomarkers associated with chlorinated solvent bioremediation in twenty groundwater samples, from five contaminated sites, following bioaugmentation with SDC-9 between ~ 0.5 and 6.3 years prior to evaluation. The analysis determined the relative abundance of 1) genera previously associated with chlorinated solvent degradation, 2) reductive dehalogenase (RDases) genes, 3) genes associated with 1,4-dioxane removal (*prmA, thmA, tomA3, tbuA1, tmoA, touA*), and 4) genes associated with aerobic chlorinated solvent degradation (*etnC, etnE, mmoX, pmoA*). The taxonomic analysis revealed numerous genera previously linked to chlorinated solvent degradation, including, for example, *Dehalococcoides, Desulfitobacterium* and *Dehalogenimonas*. The functional gene analysis indicated *vcrA* and *tceA* from *D. mccartyi* were the RDases with the highest relative abundance. Lower abundance levels of genes associated with reductive dehalogenation were found from *Dehalobacter* and *Desulfitobacterium*. Two aerobic solvent degradation genes, *etnC* or *etnE,* were detected in at least one groundwater sample from each site, as were *pmoA* and *mmoX*. For 1,4-dioxane biomarkers, the most abundant number of reads aligned to *Methylosinus trichosporium OB3b mmoX,* followed by *Burkholderia cepacia G4 tomA3* and *Pseudomonas pickettii PKO1 tbuA1.* Three others were detected at lower levels. The work illustrates the importance of shotgun sequencing to provide a more complete picture of the functional abilities of *in situ* microbial communities. The approach is advantageous over current methods, such as traditional qPCR, because an unlimited number of functional genes can be quantified.

INTRODUCTION

The chlorinated solvents tetrachloroethene (PCE) and trichloroethene (TCE) and their metabolites require remediation because of their risks to human health. *D. mccartyi* is a key microorganism for the complete transformation of these chemicals to the non-hazardous end product, ethene [1]. Examples of commercially available mixed cultures containing *D. mccartyi* for chlorinated solvent remediation include SDC-9 (from APTIM, formerly CB&I) and KB-1 (from SiREM) [2]. It was estimated that several hundred sites in the US have been subject to bioaugmentation with cultures containing *D. mccartyi* [3]. Following bioaugmentation, remediation professionals commonly monitor *D. mccartyi* populations, typically targeting reductive dehalogenase (RDase) genes such as *vcrA* [4] using quantitative PCR (qPCR) on nucleic acids extracted from groundwater [5].

While qPCR has been successful for documenting the occurrence and dechlorinating activity of *D. mccartyi* [6] most laboratories only have the instrumentation (bench-top real-time thermal cycler) to target a small number of functional genes. Next generation sequencing (NGS) is now becoming the tool of choice for environmental samples. For example, 16S rRNA gene amplicon NGS (16S rRNA-NGS) has been used to monitor microbial communities during chlorinated solvent natural attenuation [7, 8], following

biostimulation [6, 9 10, 11], during zerovalent iron-based [11, 12] and thermal-based [13, 14] chlorinated solvent remediation.

In contrast to 16S rRNA-NGS, shotgun (or whole genome) sequencing offers the opportunity to investigate both the taxonomic and functional characteristics of microbial communities. However, only a limited number of researchers have adopted this approach for describing chlorinated solvent groundwater microbial communities. Notably, these studies have primarily focused on taxonomic data, without specifically addressing RDases or other functional genes related to chlorinated solvent degradation [15, 16]. Others have examined dehalogenating genes in forest soils using shotgun sequencing [17]. To our knowledge, the current work represents the first study to target contaminant degrading functional genes in groundwater from chlorinated solvent contaminated sites using shotgun sequencing.

The samples included groundwater (from twenty injection or monitoring wells, post bioaugmentation with SDC-9) from five contaminated sites as well as the bioaugmentation culture, SDC-9. Although other researchers have used NGS to study *D. mccartyi* containing enrichment cultures (e.g. KB-1, D2, ANAS) [18, 19], limited data is available on SDC-9.

The specific objectives were 1) to determine the importance of chlorinated solvent degraders (aerobic and anaerobic) using taxonomic profiling and 2) to determine the relative abundance of genes associated with chlorinated solvent and 1,4-dioxane biodegradation. We propose that this approach (or a derivative) will ultimately be the method of choice for predicting biodegradation potential at contaminated sites.

METHODS

DNA Extraction from Groundwater and SDC-9. Groundwater samples from injection and monitoring wells were collected at five different chlorinated solvent sites (San Antonio TX, Tulsa OK, Edison NJ, Quantico VA, and Indian Head MD) through traditional low-flow sampling [20]. Only one of the five locations (Tulsa, OK) was known to be contaminated with 1,4-dioxane. The water was pumped into sterile amber bottles (1L), which were placed on ice and then shipped overnight to Michigan State University. All sites were previously bioaugmented with the commercially available reductive dechlorinating culture, SDC-9 [21]. The groundwater and mixed culture (SDC-9) DNA extraction methods were previously described [4].

Sequencing and Taxonomic Analysis. DNA extracts from twenty groundwater samples and the culture SDC-9 were submitted for library generation to the Research Technology Support Facility Genomics Core at Michigan State University (MSU. The Rubicon ThruPLEX low input DNA library preparation kit was used to generate libraries. Completed libraries were subject to quality control and quantified using a combination of Qubit dsDNA HS and Caliper LabChipGX HS DNA assays. The samples were loaded on one lane of an Illumina HiSeq 4000 flow cell and sequenced in a 2x150 bp paired end format. Base calling was performed by Illumina Real Time Analysis (RTA) v2.7.6 and output of the RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.18.0. Taxonomic analysis was conducted by using Meta Genome Rapid Annotation using Subsystem Technology (MG-RAST) [22].

Reference Sequences Collection, Functional Gene Analysis. Two approaches were employed to analyze the functional gene data. First, protein sequences associated with RDases for published genomes were collected from the National Center for Biotechnology Information (NCBI). Secondly, to enable a wider number of sequences to be examined, protein sequences were collected from additional sources e.g. Functional

Gene Pipeline and Repository (FunGene) [23], NCBI BLAST. The reference protein sequences were dereplicated (removing sequences with 100% identity). Low quality sequences and Illumina adapters sequences were removed by Trimmomatic [24]. DIAMOND (double index alignment of next-generation sequencing data) [25] was used as the alignment tool for all functional genes. Relative abundance values were calculated using the number of aligned reads divided by the total number of sequences for each sample, and the relative abundance values were then normalized by the number of dereplicated reference sequences for each gene to produce normalized relative abundance values.

RESULTS AND DISCUSSION

Sequencing and Taxonomic Analysis of Chlorinated Solvent Degraders. From the twenty groundwater DNA extracts, the majority (seventeen) generated between ~4 and ~6 million sequences each, post quality control. Three samples (PMW2, AW1, IW7) produced lower sequence counts (157,000, 471,513 and 1,547,247). The average sequence length varied from 226 to 241 bp (standard deviations from 34 to 41 bp). The rarefaction curves (data not shown) plateaued indicating the analysis had captured the majority of the diversity within the samples. The sequencing data for each site was examined to determine the relative abundance of genera previously associated with chlorinated solvent degradation (Figure 1). The analysis is only at the genus level and therefore, except for *Dehalococcoides*, may overestimate the abundance of possible degraders. The following chlorinated solvent degrading genera were investigated: *Anaeromyxobacter* [26], *Dehalococcoides* [2, 27], *Polaromonas* [28], *Nocardioides* [29], *Desulfitobacterium* [30, 31], *Geobacter* [32], *Sulfurospirillum* [33, 34], *Dehalobacter* [35, 36], *Desulfomonile* [37], *Desulfuromonas* [38], *Propionibacterium* [39], *Mycobacterium* [40], *Dehalobacter* [36], *Desulfomonile,* [41] and *Dehalogenimonas* [42, 43]. *Dehalobacter* and *Desulfomonile* were not detected in any of the culture or groundwater samples by MG-RAST and are not included in Figure 1.

The relative abundance of methanotrophs in the groundwater samples was also investigated (Figure 1). Methanotrophs are important because of their ability to use particulate and soluble methane monoxygenases (pMMO and sMMO) to cometabolically oxidize several chlorinated solvents [44].

Dehalococcoides, the key dechlorinating genera in SDC-9 (31% in SDC-9), was detected in every sample at every site (averages for each site ranging from 0.2 to 1.4%). *Desulfitobacterium* was detected at all five sites, although the relative abundance (average ranging from 0.1 to 0.4%) was typically less than that of *Dehalococcoides*. Except for *Dehalococcoides*, *Desulfitobacterium* was present at a higher relative abundance in SDC-9 (2.7%) compared to other dechlorinating microorganisms (<0.4%). At three sites, *Geobacter* was the most abundant genus in this group (Figure 1A, B and C) and at two sites, it was either the second or third most abundant (Figure 1D and E). Considering its low relative abundance (0.06%) in SDC-9, at four sites (San Antonio, Quantico, Edison and Indian Head), *Polaromonas* was detected at higher relative abundance levels (averages ranging from 0.8 to 3.3%) compared to many other genera in this group. *Anaeromyxobacter* was also detected at all five sites at higher levels (averages ranging from 0.3 to 0.6%) than it was observed in SDC-9 (0.06%). *Mycobacterium* was found in a similar range (from 0.3 to 0.5%) in groundwater. *Desulfuromonas, Nocardioides, Sulfurospirillum* and *Propionibacterium* were observed in the groundwater samples with averages ranging from 0.04 to 0.18%.

Methanotrophs examined were present only at low levels in SDC-9 (averages ranging from 0.006-0.035%). In groundwater samples, *Methylococcus* or *Methylosinus*

were typically the most abundant, followed by *Methylobacterium* and *Methylocella*.

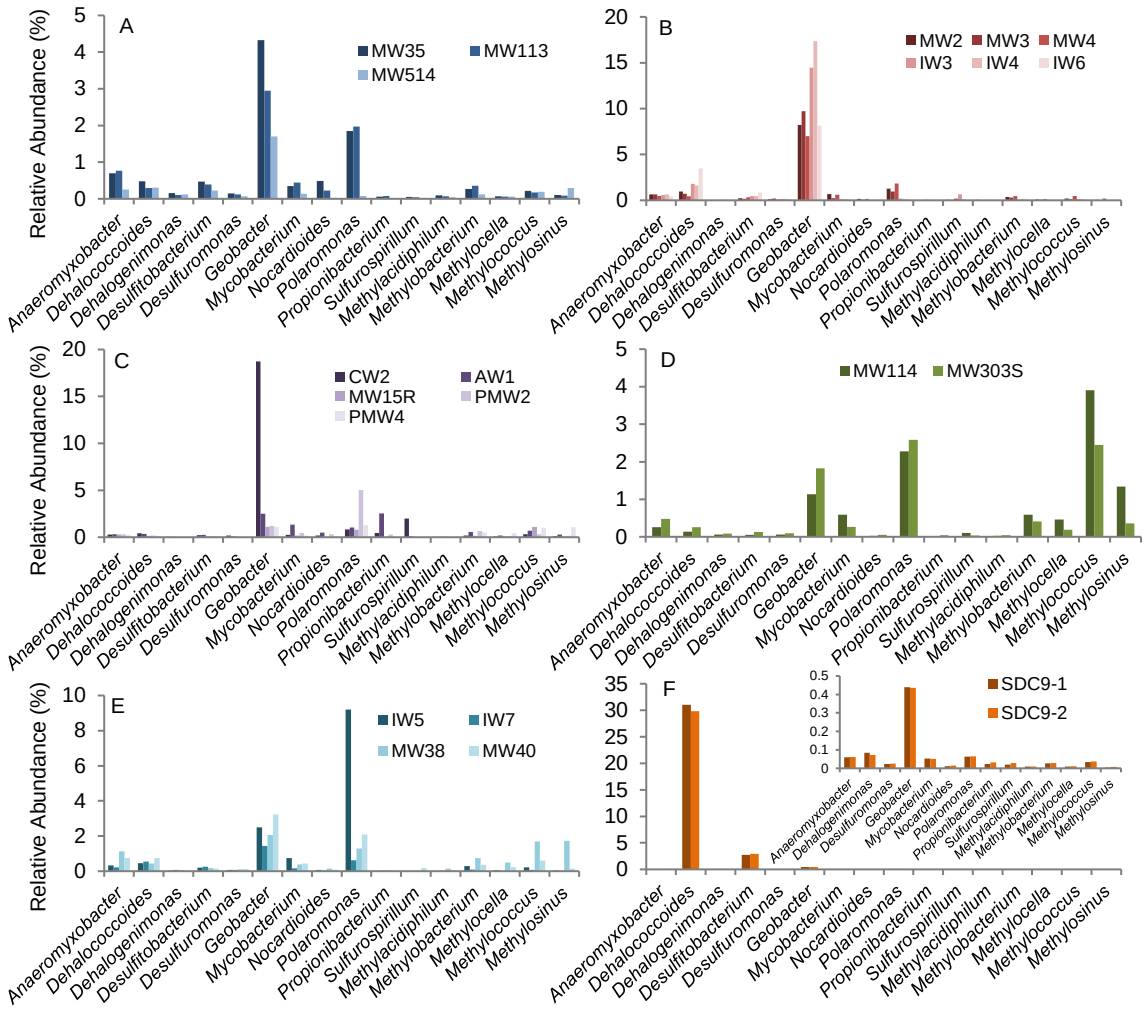

FIGURE 1. Relative abundance (%) of methanotrophs and genera associated with chlorinated solvent biodegradation in groundwater from San Antonio (A), Tulsa (B), Quantico (C), Edison (D), Indian Head (E) and SDC-9 (F). Note, MW: monitoring well and IW: injection well. The insert in F does not include *Dehalococcoides* or *Desulfitobacterium* to enable a y-axis with a different scale.

Functional Gene Analysis. Although taxonomic data is important for characterizing microbial communities *in situ*, it is well recognized that certain limitations are associated with such data. A key limitation concerns an inability to classify to the species level when short sequences are analyzed. Another related limitation concerns the inability of taxonomic data to provide in-depth information on function. To address this, the current study focused on quantifying the functional genes related to chlorinated solvent and 1,4-dioxane biodegradation.

The groundwater sequencing data were aligned to characterized RDases from *D. mccartyi* and three other genera (*Dehalogenimonas*, *Dehalobacter* and *Desulfitobacterium*) (Figure 2). RDases from *D. mccartyi* were the most abundant (Figure 2A). Samples from Tulsa illustrated some of the highest values for *tceA* and *vcrA*, a pattern perhaps caused by the higher chlorinated ethene concentrations at this site. The wells at Indian Head contained the second most abundant reads aligning to

RDases from *D. mccartyi*. These results agree with the MG-RAST analysis (Figure 1B and E).

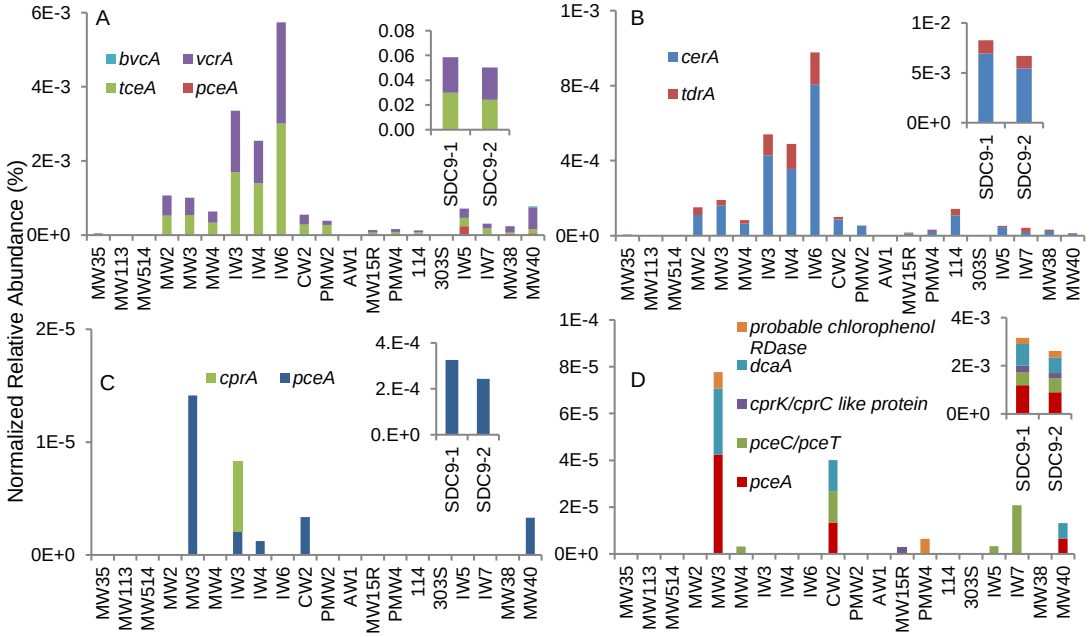

FIGURE 2. Normalized relative abundance (%) of genes associated with reductive dechlorination in *Dehalococcoides mccartyi* (A), *Dehalogenimonas* spp. (B), *Dehalobacter* spp. (C) & *Desulfitobacterium* spp. (D) in SDC-9 (inserts) and in groundwater from the five sites.

The abundance of RDases from *Dehalogenimonas*, *Dehalobacter* and *Desulfitobacterium* were found in lower numbers and the results varied between sites (Figure 2B, C, D). The majority of reads aligning with *cerA* and *tdrA* from *Dehalogenimonas* were from Tulsa, followed by Indian Head and Edison (Figure 2B). Reads aligning to RDases from *Dehalobacter* and *Desulfitobacterium* were less abundant but were found in at least one well from three of the five sites (except San Antonio and Edison) (Figure 2C, D). Although *Desulfitobacterium* was detected with the MG-RAST analysis, *Dehalobacter* was not. Additional differences between the MG-RAST and the functional gene data sets included the presence of the genera *Anaeromyxobacter* and *Sulfurospirillum* with MG-RAST, but the absence of functional genes (associated with the removal of chlorinated chemicals) from these microorganisms. Also, *Geobacter* and *Polaromonas* were present at all sites, however, reads aligning to *pceA* of *Geobacter lovleyi* and cytochrome P450 of *Polaromonas JS666* were observed from only one sample each. These findings emphasize the importance of functional gene analysis to clearly define *in situ* biodegradation capabilities.

The majority of the RDases found in SDC-9 were from *D. mccartyi,* with *tceA* and *vcrA* being the most abundant (~two orders of magnitude higher than the RDases from other species) (inserts in Figure 2). RDases from *Dehalogenimonas, Dehalobacter, Desulfitobacterium* were also present in SDC-9.

Reads aligning to the genes associated with the aerobic degradation of 1,4-dioxane [45] were also investigated (Figure 3). From the twelve genes examined, only six were identified in the groundwater samples (Figure 3A). These genes were detected in at least one sample from all five sites, despite the fact that only one of the sites (Tulsa) was known to be contaminated with 1,4-dioxane (Figure 3A). The highest abundance values were from the two wells at Edison, followed by three wells at Indian Head and four wells from Quantico. The MG-RAST taxonomic data were examined for the presence of the

genera associated with these genes (Figure 3B). From this group, *Pseudomonas* was the most dominant genus, followed by *Burkholderia, Mycobacterium, Methylosinus* and *Rhodococcus*. Similar to the functional gene data, the genus *Pseudonocardia* was not detected in any groundwater sample.

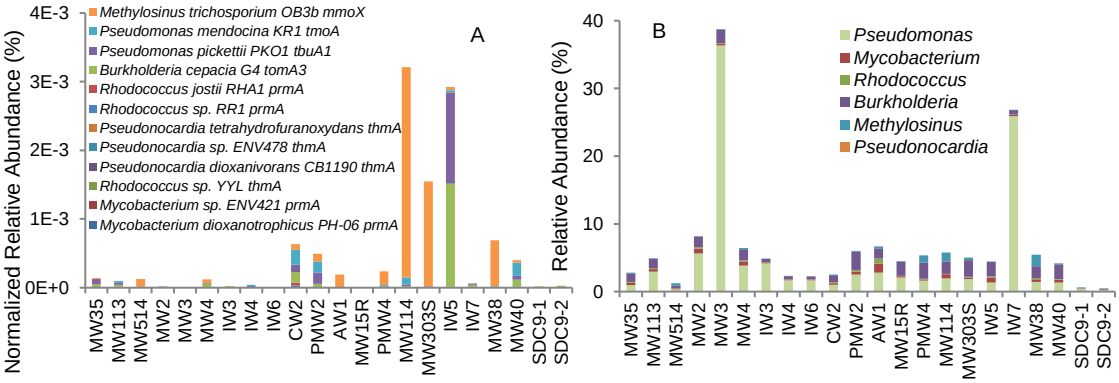

FIGURE 3. Normalized relative abundance of genes (%) (A) and relative abundance of genera (%) (B) associated with 1,4-dioxane degradation in all groundwater samples and in SDC-9. The relative abundance of *Pseudonocardia* was zero in all groundwater samples and in SDC-9.

Reads aligning with the genes associated with the aerobic degradation of the chlorinated ethenes (*pmoA, mmoX, etnC, etnE)* [44, 46] were detected in the groundwater samples (Figure 4A, B). Reads aligning with *pmoA* and *mmoX* illustrated a higher occurrence in a number of samples (PMW4, MW114, 303s, MW38) from three sites (Quantico, Edison, Indian Head), but were still considerably lower than those aligning to *vcrA* or *tceA*. The abundance of *etnC* and *etnE* was also high in MW114 (followed by MW4, MW2, MW40 and MW3 from Edison, Tulsa, Indian Head). Notably, the highest normalized relative abundance values for *etnC* and *etnE* were two orders of magnitude lower than *vcrA* or *tceA*.

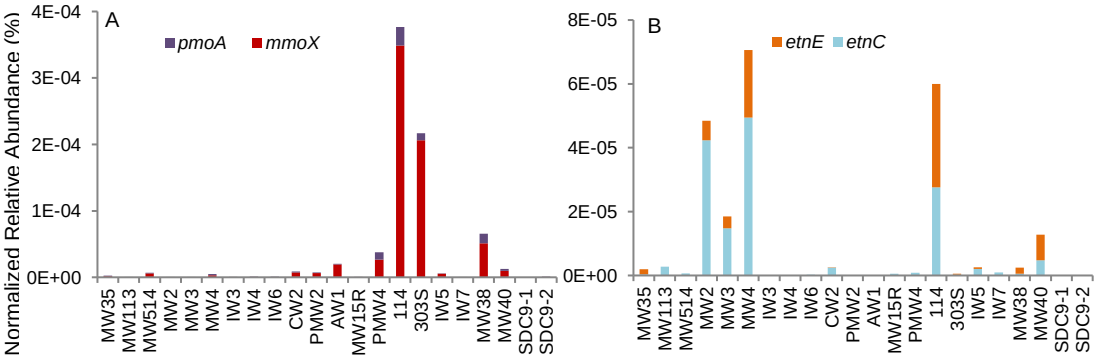

FIGURE 4. Normalized relative abundance (%) of genes associated with the aerobic degradation of the chlorinated solvents (B, C) and in groundwater from the five chlorinated solvent sites.

To our knowledge, this study represents the first analysis of the genes associated with 1,4-dioxane degradation in groundwater using shotgun sequencing. Here, from the twelve sequences investigated, the most abundant number of reads aligned to *Methylosinus trichosporium OB3b mmoX,* followed by *Burkholderia cepacia G4 tomA3* and *Pseudomonas pickettii PKO1 tbuA1.* Notably, although *mmoX* from *M. trichosporium OB3b* has been associated with 1,4-dioxane degradation at high concentrations [47], at

low, environmental relevant concentrations, no removal was observed [48]. Three others were detected at lower levels (*Pseudomonas mendocina KR1 tmoA, Rhodococcus jostii RHA1 prmA, Rhodococcus sp. RR1 prmA*). In some cases, remarkably, the normalized relative abundance values were in the same range as those for *vcrA* and *tceA,* even though 1,4-dioxane was not previously reported at 4 of the 5 sites, and reducing conditions (i.e., negative oxidation-reduction potential; nORP) generally prevailed. Reads aligning to *Mycobacterium* 1,4-dioxane degrading gene sequences (*prmA*) were not detected in the current study, even though the taxonomic MG-RAST data indicated this genus was present. This discrepancy again illustrates the importance of functional gene data to corroborate taxonomic data and assumptions about function. Further, the current work illustrates the importance of shotgun sequencing to provide a more complete picture of the potential of *in situ* microbial communities to degrade 1,4-dioxane compared to qPCR, which typically only targets a small number of genes.

In summary, methods were developed to determine the abundance of genes associated with chlorinated solvent and 1,4-dioxane biodegradation in groundwater samples from multiple samples from multiple contaminated sites. The data indicated the presence of both aerobic and anaerobic biomarkers for chlorinated solvent degradation. Not surprisingly, the taxonomic data alone was insufficient to determine the functional abilities of these communities. The approach developed will enable researchers to investigate the abundance of any contaminant degrading gene in any sample, greatly expanding the analytical toolbox for natural attenuation, biostimulation or bioaugmentation.

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