

**ENHANCED REDUCTIVE DECHLORINATION  
OF CHLORINATED ALIPHATIC  
HYDROCARBONS: MOLECULAR AND  
BIOCHEMICAL ANALYSES**

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**Enhanced Reductive Dechlorination of Chlorinated Aliphatic Hydrocarbons :**

**Molecular and Biochemical Analyses**

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## **PROJECT SUMMARY**

**Title – Enhanced Reductive Dechlorination of Chlorinated Aliphatic Hydrocarbons: Molecular and Biochemical Analyses**

**Project I.D. –** WR06R002

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**Period of contract –** July 2006 to June 2008.

**Background/need –** Chloroalkenes are widespread groundwater contaminants for which enhanced reductive dechlorination (ERD) via electron donor augmentation can serve as an effective, *in situ* bioremedial strategy. A major goal of ERD is to stimulate late stage dechlorination reactions, which are needed to prevent accumulation of toxic intermediates. Establishment of methanogenic conditions is important for the onset and support of late stage dechlorination processes that involve reduction of dichloroethene (DCE) to vinyl chloride (VC), and VC reduction to the non-toxic end-point compound, ethene. However, little is known about the composition of microbial communities that are active in late-stage dechlorination. Elucidating the structure and function of microbial communities associated in the field with late stage dechlorination reactions could help establish a mechanistic foundation that would advance the science of ERD, and consequently enhance the effectiveness with which the process might be applied and managed.

**Objectives -** 1) Delineate the microbial community dynamics induced by soluble carbohydrate augmentation in field samples and during progress in ERD. 2) Elucidate the composition of communities that effect complete dechlorination of CAH in field samples.

**Methods –** Cells in the groundwater samples were collected by filtration and DNA was extracted from them to use in Automated Ribosomal Intergenic Spacer Analysis (ARISA) in order to determine microbial community diversity. PCR was done using fluorescently labeled primers, and then the profile of fluorescent peaks for each sample was determined. ARISA however provides information of community diversity but does not define community structure since organisms cannot be identified. 16S-ITS clone libraries were set up for identification of the organisms represented by major peaks on the community profiles, or were consistently present over time. The DNA extracted from the groundwater samples was used to amplify the 16S-ITS regions of the template. These were then used for ligation and transformation to set up the clone libraries. The clones were screened and the ones that contained the ARISA fragment of interest were sequenced. Sequence similarities with the 16S gene were used for organism identification. These clone libraries were used to identify some of the major peaks seen in the microbial community profiles. The time period during which this study was undertaken was September 2006 to July 2008.

**Results and Discussion** – Microbial community diversity was studied in locations that differed from each other by the stage of dechlorination. The community profile at these locations was studied over the span of a year as ERD continued. In the locations showing late stage dechlorination, the predominant species were affiliated with *Clostridia*, *Bacteroidetes*, *Mollicutes* and *Spirochaetes*. This corresponded with the other studies on microbial characterization of reductive dechlorination sites. These communities were absent in the location demonstrating only early stage processes. At this site, the predominant classes there were *Delta proteobacteria* and *epsilon proteobacteria*. Therefore a distinct community profile was associated with VC stalling conditions. The community dynamics was studied and the profile did not change substantially within a location over the period of 1 year.

**Conclusions** - Distinct community profiles seem to be associated with early and late stage of dechlorination. The species that are previously known to be affiliated with dechlorination sites are not observed in the location showing stalling of dechlorination at vinyl chloride. Late stage processes are preferred since it does not lead to the accumulation of toxic by-products. Therefore, understanding the difference in microbial communities stimulated in areas showing early and late stage processes is imperative. This study also gives an insight into populations other than dechlorinators in a field site undergoing ERD. A greater understanding of the role played by these species is needed for complete and successful ERD treatment.

**Key words** – Trichloroethylene, Bioremediation, Enhanced Reductive Dechlorination

**Funding** – University of Wisconsin System Groundwater Research Program; U.S. Geological Survey

**INTRODUCTION** - Chlorinated aliphatic hydrocarbons (CAH) such as perchloroethylene (PCE), trichloroethylene (TCE), and trichloroethane (TCA) are toxicants and pervasive groundwater contaminants. Thus, sites with CAH-impacted groundwater are one of the most (if not the most) commonly targeted locations for cleanup, for which bioremediation utilizing enhanced reductive dechlorination (ERD) mediated by anaerobic bacteria has become a major process for *in situ* treatment.

Reductive dechlorination involves sequential removal of chlorines from CAH, ultimately resulting in the non-toxic end-products ethene or ethane. Under natural conditions, the process frequently stalls, resulting in accumulation of *cis*-dichloroethylene (c-DCE) and vinyl chloride (VC). Since VC is considered more toxic than any of its parent compounds, a major goal of ERD is to establish conditions conducive to the late stage dechlorination processes (reduction of c-DCE and VC to ethene) that are essential for effective remediation. Data from field sites where ERD has been implemented indicate that the onset of late stage transformations typically coincides with establishment of methanogenic conditions [2, 3]. However, the microbial communities, interactions and activities that underlay effective ERD at these sites are poorly understood.

Because of its ability to mediate VC reduction to ethene, the chloridogenic (dehalorespiring) bacterium *Dehalococcoides ethenogenes* initially attracted much attention. But, VC reduction by *D. ethenogenes* is slow, and microcosm and field screening studies have indicated that this transformation can occur in the absence of *D. ethenogenes* [4-7]. Recently, VC-respiring strains of *Dehalococcoides* sp. that are highly efficient in VC reduction have been isolated, and genes encoding reductive dehalogenases (RDases) mediating VC reduction to ethene have been identified [8].

While VC dechlorinators and other dehalorespiring bacteria have key roles in CAH bioremediation, their activity is dependent upon that of commensalistic fermentative organisms, which generate the hydrogen, electron donors and growth factors chloridogens require. There is also growing recognition that varying composition and activities of fermentative consortia may have significant effects on the activities of dechlorinators [6], but the nature and activities of fermentative organisms that are predominant in methanogenic ERD are poorly understood. Microcosm studies with model ERD amendments (organic acids and alcohols) have shown that the nature of the electron donor has a significant effect on the structure and activity of the microbial community stimulated [6], but little is known about the microbial communities established in response to ERD amendments (*e.g.*, molasses) that are actually used in the field.

Finally, to the best of the authors' knowledge, there are only two reports that have examined microbial communities associated with CAH bioremediation in field studies [1, 9]. Both reports were significant in demonstrating that integrating data on molecular microbial community structure with biogeochemical measurements provided new insights into biodegradative processes operative at the sites, which could facilitate site management. Likewise, elucidating the structure and function of microbial communities associated in the field with late stage dechlorination reactions could help establish a mechanistic foundation that would advance the science of ERD, and consequently enhance the effectiveness with which the process might be applied and managed.

## PROCEDURES AND METHODS -

**1) Field site, ERD biostimulation and field sampling.** This study was done in collaboration with ARCADIS, an environmental consulting and engineering firm that was contracted to carry-out remediation of the study site. The site (located in southeastern Wisconsin) was developed in the early 1940's for heavy manufacturing, and operated until 1992 when the plant closed. Since 1992, the site has not been utilized. The predominant groundwater contaminants are TCE (120,000 µg/L to non-detectable (ND)), 1,1,1-trichloroethane (1,1,1-TCA; 14,000 µg/L to ND), c-DCE (19,000 µg/L to ND), and VC (4,300 µg/L to ND).

An anaerobic reactive zone was established within the groundwater plume through the addition of a carbon amendment solution (100:3.5:2 (v:v:v), potable water:granular cheese whey:blackstrap molasses). Approximately 100, 2-inch diameter injection wells were installed in the areas of impacted groundwater. Each well had an approximate depth of 15 feet, and a screened interval of 5 to 10 ft. Approximately 300 gallons of carbon amendment solution was injected into each well, or as an alternative 300 gallons of a 100:7 solution of potable water to blackstrap molasses be injected into each well. The carbon amendment injections began in April 2006.

Samples of aquifer water and sediment cores were obtained during the installation of the injection wells. Water samples were processed for chemical and microbiological analyses as described below. The progress of ERD was monitored by chemical analysis of groundwater samples acquired from a monitoring well network; groundwater being sampled quarterly during the period. Samples of groundwater used for molecular analysis were shipped to the laboratory on ice by overnight courier. Chemical analysis was also done on groundwater samples, was carried out by a certified environmental analysis laboratory contracted by ARCADIS and samples of groundwater were sent from the locations to the laboratory for further molecular analysis.

**2.) Molecular microbial community analyses.** Molecular analysis of microbial communities was applied to DNA extracted from field samples of groundwater. Groundwater samples were acquired from locations showing high levels of cDCE/VC reduction, and for comparison, a location inactive in cDCE/VC reduction was obtained. Field groundwater samples (500-mL) were acquired as discussed above, transported to lab and processed (filtration through polyethersulfone membranes, 0.2 µm pore diam). The membranes were processed for extraction of Bacterial DNA using the Mobio Soil DNA extraction kit ().

Whole community profiles were developed using Automated Ribosomal Intergenic Spacer Analysis (ARISA) with bacterial primers 1406f and 23Sr [10]. The forward primer was synthesized with a fluorochrome label (5'-FAM). PCR conditions were optimized for the present application as 94°C for 2min, followed by 30 cycles of 94°C for 15 sec, 50°C for 15 sec, 72°C for 45 sec and finally 72°C for 2 min. For each sample, fluorescent DNA fragment analysis was done in duplicate through the UW-Madison Biotechnology Center by using an ABI 3700 automated sequencer to generate electropherograms, and Genemarker v 1.5 for profile alignment. Individual peaks were treated as operational taxonomic units (OTUs). Peak areas were normalized within ARISA profiles, and expressed as fraction of the total peak area comprising the profile termed as relative intensity.

**3) 16S-ITS Clone library** - ARISA profiles are coupled with 16S rDNA-ITS clone libraries in order to yield organism identification. By doing so, ARISA peaks of interest can be tracked to the ITS contained in these clones, and identification made by sequencing of the associated 16S rDNA [11] To create the Bacterial 16S rDNA-ITS clone libraries, PCR primers

27f [12] and 23Sr were used to amplify the majority of the Bacterial 16S rDNA gene as well as the adjoining ITS. The PCR parameters were 94° (2min), followed by 30 cycles of 94°C for 15sec, 43°C for 15sec and 72°C for 2 min, finally 72°C for 7 min. Purified PCR products will be cloned ligation into pGEM-T Easy (Promega) and transformed into *E. coli* JM109 competent cells. Clone libraries were established with DNA extracted from a sediment sample obtained from a methanogenic reactive zone showing high levels of c-DCE/VC dechlorination and from locations showing stalling at c-DCE/ VC dechlorination.

The procedure of Grant and Ogilvie [13] was used to screen clones for ARISA peaks of interest for 16S rDNA sequencing. Briefly, clones were propagated in 96-well plates, and ARISA done as described above on clones pooled across rows and across columns. An ARISA peak of interest was associated with a clone by identifying the combination of reactions from a row pool and a column pool that possess the peak, and then locating the well at the intersection of the row and column. Plasmid DNA was isolated from clones of interest, and sequencing of 16S rDNA by BigDye (Applied Biosystems) cycle sequencing will be done as in the PI's previous work. Sequence similarities will be determined by BLAST-N searches against GenBank.

**4) DNA sequence analysis.** Each clone was sequenced by the dideoxy termination method by using an Applied Biosystems (Foster City, CA) automated sequencing system available at University of Wisconsin-Madison. GenBank database searches were carried out using the National Center for Biotechnology Information BLAST web server.

## RESULTS AND DISCUSSION –

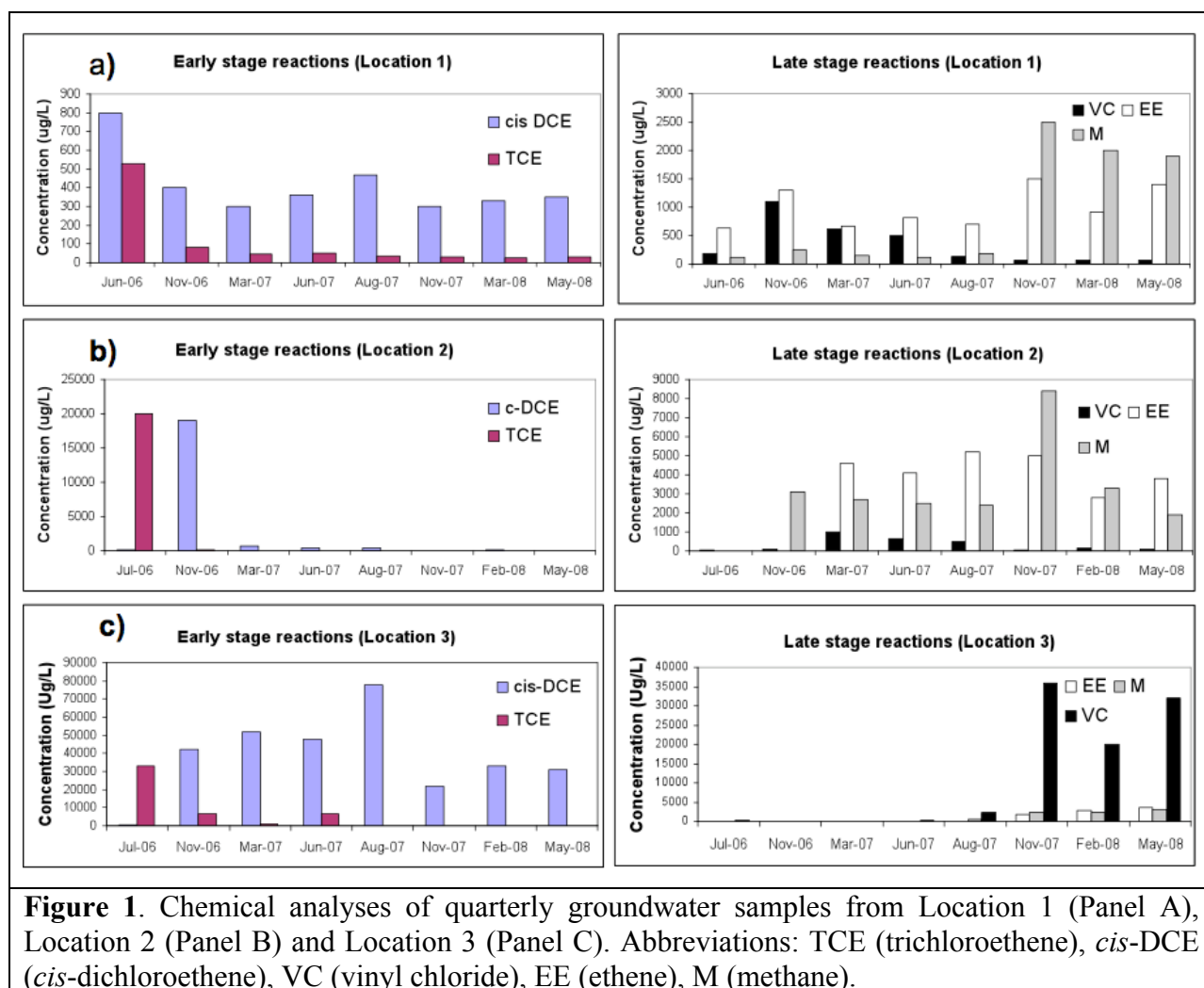
Each location had a distinct ARISA profile, and each location showed variation in ARISA profile over the course of one year (Figs. 2-4). The identities of major peaks could be determined from sequencing clones recovered from the Bacterial 16S rDNA-ITS libraries. However, many of the fragments relatively low in abundance (*i.e.*, smaller peaks in Figs. 2-4) eluded recovered in the libraries. Thus, the identifies of the organisms yielding these peaks were not determined.

At the first time point, the predominant fragments at Location 1 had the greatest sequence identity to members of the *Clostridia*, *Mollicutes* and CFB (*Cytophaga-Flavobacterium-Bacteroides*) groups of bacteria (Fig 2a, Table 1). A fragment identified as *Pseudomonas* sp. was also prominent. Over the next three time points, the diversity of the community appeared to expand (*i.e.*, increase in number of peaks, Fig. 2b) and then contract. The final profile (Fig. 2d) was similar to that developed at the first sampling point one year earlier. Notable trends were the consistent identification of *Clostridia* as prevalent organisms across the one-year time span, and the disappearance of *Pseudomonas* sp. after the first time point. At Location 2, the predominant clones at the first and second sampling times were identified as *Bacteroidetes* and *Spirochaetes* (Fig. 3a, Table 1). At the two later time points, a new peak identified as a *betaproteobacteria* was prominent. At Location 3, *deltaproteobacteria* and *epsilonproteobacteria* were initially most abundant; the former represented the single peak resolved in the final sample.

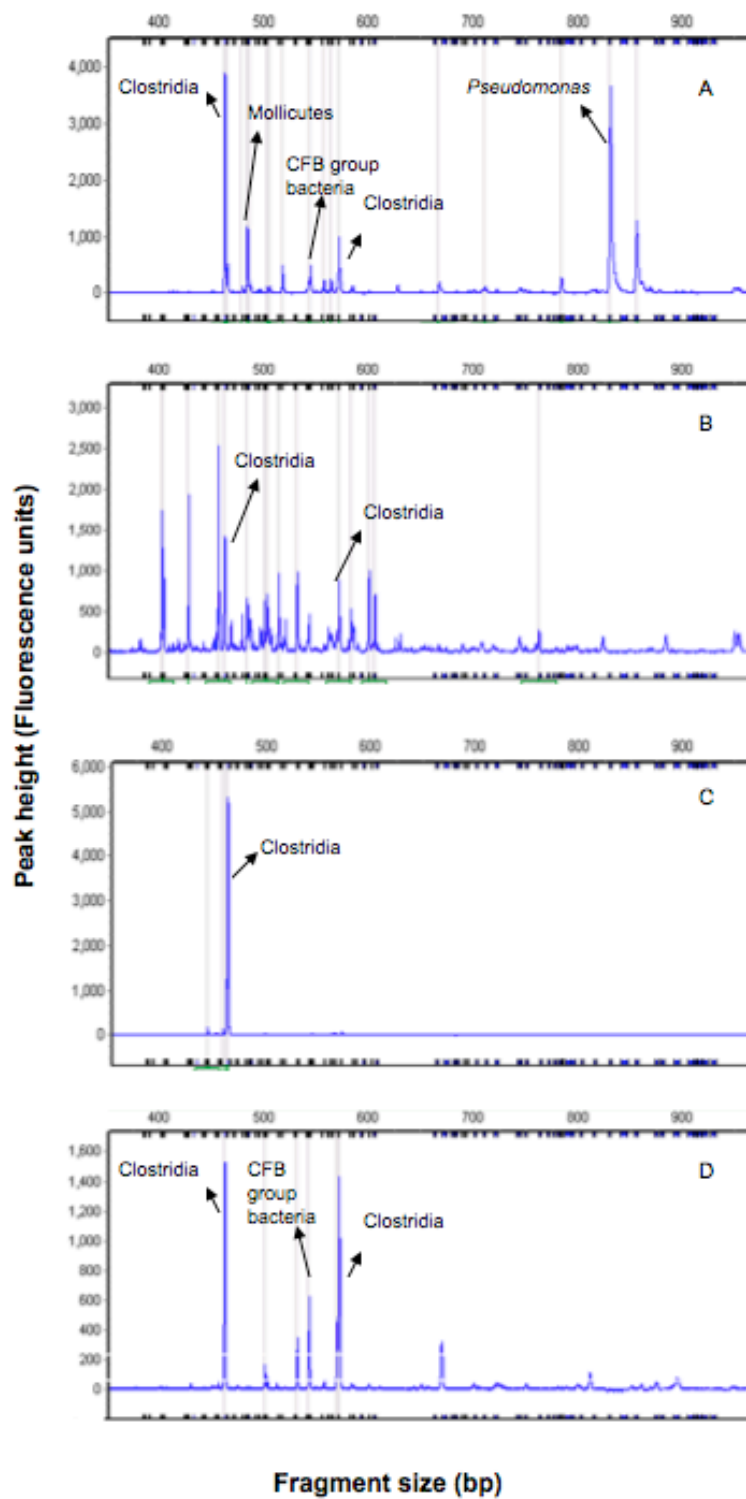
The groups predominating at the late stage locations (*Clostridia*, *Bacteroides*, *Mollicutes* and *Spirochaetes*) have been previously identified in TCE-contaminated aquifers, and in microcosms established to study CAH degradation [1, 15]. In the present study, its notable that these groups were absent form Location 3, which was stalled at the early dechlorination stage for the duration of the study. This finding indicates that late stage dechlorination requires establishment of a particular community of microbes, which enhance and/or complement the activity of chloridogenic organisms, such as *Dehalococcoides* sp.



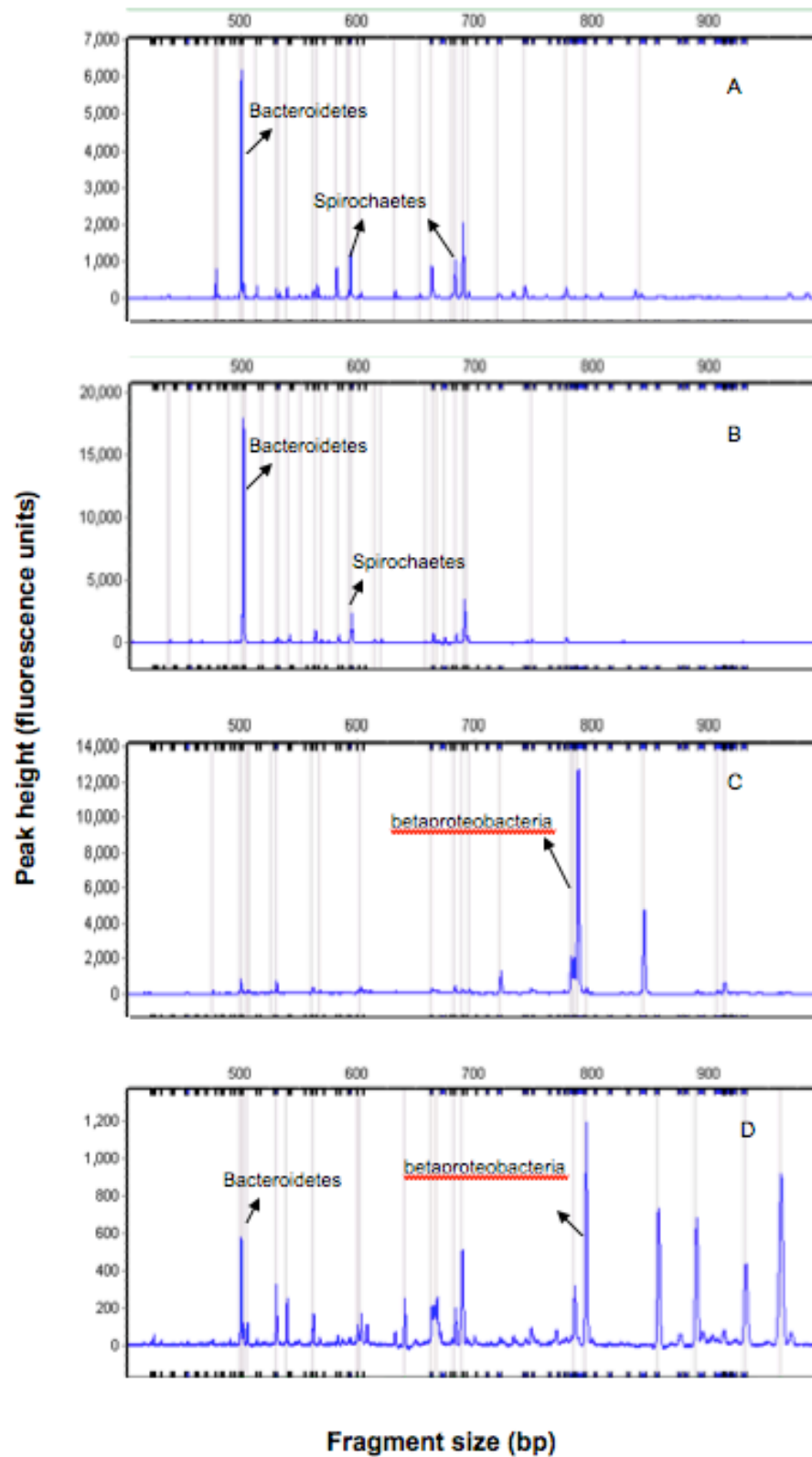
To the best of the authors' knowledge, the present study is the first to document the variation occurring in microbial community structure through the dechlorination process at an actual field site. The relative abundance of the predominant species appeared to reflect changes in the chemical profile of the groundwater. For Location 1, late stage dechlorination increased substantially between the first and second time points, and remained high thereafter (Fig. 1a). A corresponding change in community structure was documented to occur between these sampling points (Fig. 2a,b); the later time points were similar in the predominance of *Clostridia*. Location 2 showed the opposite trend, with late stage dechlorination high at the first and second time points, and then decreasing for the third and fourth (Fig. 1b). Again a corresponding change occurs in the community structure (Fig. 3a-d).



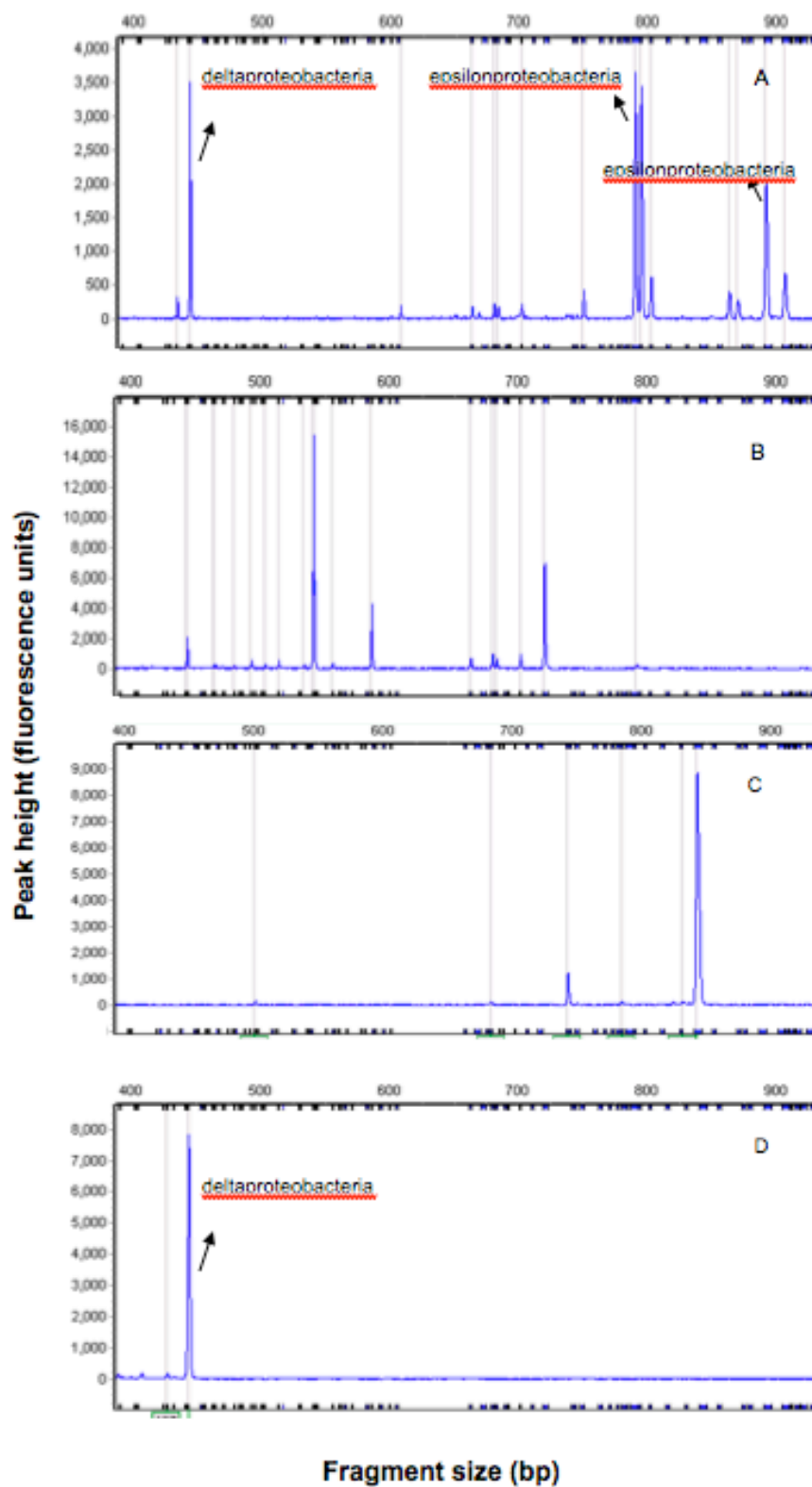
**Figure 1.** Chemical analyses of quarterly groundwater samples from Location 1 (Panel A), Location 2 (Panel B) and Location 3 (Panel C). Abbreviations: TCE (trichloroethene), *cis*-DCE (*cis*-dichloroethene), VC (vinyl chloride), EE (ethene), M (methane).



**Figure 2.** ARISA Profile of bacterial communities from Location 1. Data are from quarterly samples: Panel A., June 2007, B. November 2007, C. February 2008, D. May 2008.



**Figure 3.** ARISA Profile of bacterial communities from Location 2. Data are from quarterly samples: Panel A., June 2007, B. November 2007, C. February 2008, D. May 2008.



**Figure 4.** ARISA Profile of bacterial communities from Location 3. Data are from quarterly samples: Panel A., June 2007, B. November 2007, C. February 2008, D. May 2008.

**Table 1.** Estimated relative abundance of selected fragments in the bacterial community profiles for the three locations under study.

Location 1	Fragment size (bp)	Putative class/order	% identity	Mean Relative Intensity* $\pm$ S.D. (Relative abundance)			
				June'07	Nov'07	Feb'08	May'08
	463	<i>Clostridia</i>	90%	16.55 $\pm$ 2.05	6.79 $\pm$ 1.14	74.77 $\pm$ 0.39	19.25 $\pm$ 14.29
	484	<i>Mollicutes</i>	92%	4.91 $\pm$ 0.29	2.85 $\pm$ 0.35	ND	ND
	544	<i>CFB group</i>	92%	2.05 $\pm$ 0.49	ND	ND	12.32 $\pm$ 1.81
	572	<i>Clostridia sp.</i>	98%	4.53 $\pm$ 0.74	4.17 $\pm$ 0.04	ND	36.25 $\pm$ 0.42
	831	<i>Pseudomonas sp.</i>	97%	41.65 $\pm$ 3.88	ND	ND	ND
<b>Location 2</b>							
	501	<i>Bacteroidetes</i>	90%	34 $\pm$ 7.09	48.3 $\pm$ 7.39	1.33 $\pm$ 0.24	5.49 $\pm$ 1.99
	594	<i>Spirochaetes</i>	87%	7.3 $\pm$ 0.6	11.67 $\pm$ 1.36	ND	ND
	690	<i>Spirochaetes</i>	88%	18.9 $\pm$ 5.35	10.76 $\pm$ 3.45	ND	8.26 $\pm$ 2.38
	795	<i>Beta proteobacteria</i>	92%	ND	ND	47.59 $\pm$ 1.67	16.28 $\pm$ 3.78
<b>Location 3</b>							
	443	<i>Delta proteobacteria sp.</i>	97%	10.6 $\pm$ 0.72	4.0 $\pm$ 0.24	ND	88.04
	789/795	<i>Epsilon proteobacteria</i>	95%	21.8 $\pm$ 0.11	ND	ND	ND
	891	<i>Epsilon proteobacteria</i>	91%	16.4 $\pm$ 2.43	ND	91.92 $\pm$ 0.88	ND

\* Average of triplicate ARISA runs

ND – Peak not detected or below threshold of a peak area of 250 fluorescence units.

**CONCLUSIONS** – Distinct community profiles are associated with early and late stage of dechlorination. The species that are previously known to be affiliated with dechlorination sites are not observed in the location showing stalling of dechlorination at vinyl chloride. Late stage processes are preferred since it does not lead to the accumulation of toxic by-products. Therefore, understanding the difference in microbial communities stimulated in areas showing early and late stage processes is imperative. This study also gives an insight into populations other than dechlorinators in a field site undergoing ERD. A greater understanding of the role played by these species is needed for complete and successful ERD treatment.

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