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Microbial ecology of chlorinated solvent biodegradation

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Summary

This study focused on the microbial ecology of tetrachloroethene (PCE) degradation to trichloroethene, *cis*-1,2-dichloroethene and vinyl chloride to evaluate the relationship between the microbial community and the potential accumulation or degradation of these toxic metabolites. Multiple soil microcosms supplied with different organic substrates were artificially contaminated with PCE. A thymidine analogue, bromodeoxyuridine (BrdU), was added to the microcosms and incorporated into the DNA of actively replicating cells. We compared the total and active bacterial communities during the 50-day incubations by using phylogenic microarrays and 454 pyrosequencing to identify microorganisms and functional genes associated with PCE degradation to ethene. By use of this integrative approach, both the key community members and the ecological functions concomitant with complete PCE degradation could be determined, including the presence and activity of microbial community members responsible for producing hydrogen and acetate, which are critical for *Dehalococcoides*-mediated PCE degradation. In addition, by correlation of chemical data and phylogenic microarray data, we identified several bacteria that could potentially oxidize hydrogen. These results demonstrate that PCE degradation is dependent on some microbial community members for production of appropriate metabolites, while other members of the community compete for hydrogen in soil at low redox potentials.

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Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) are pollutants found in large quantities in industrial areas around the world, and they or their metabolites can be toxic to humans and animals. These pollutants can, however, be degraded by microorganisms in a variety of ways, including reductive dechlorination under anaerobic conditions. During reductive dechlorination, PCE is converted stepwise to ethene, losing a chlorine atom at each step. In this process, the chlorinated ethene can serve as the terminal electron acceptor for some bacteria. Reductive dechlorination can be induced at polluted sites by the introduction of compatible organic substrates and/or competent dechlorinating bacteria (Rabah and Lekmine, 2002). The organic substrate addition serves two purposes: (i) to scavenge oxygen (O_2) by aerobic microbes present at the site, thus creating anoxic conditions, and (ii) to provide an organic substrate for the anaerobic microbial community responsible for fermentation and hydrolysis of the organic substrates through several intermediates, such as organic acids, to acetate and hydrogen (H_2). The acetate and hydrogen can subsequently be used as substrates by dechlorinating bacteria, methanogens, sulfate reducers and other anaerobic microorganisms.

Bacteria responsible for carrying out PCE dechlorination under anoxic conditions have been well characterized, both with respect to their physiology and their genetics, and several strains have been successfully cultivated (Löffler *et al*., 2005). Under anaerobic conditions, these bacteria can use PCE as the terminal electron acceptor, H_2 or acetate as electron donor (Löffler *et al*., 2000) and acetate as a carbon source. When PCE is completely degraded, all chlorine atoms are replaced by H_2 and the final product is the non-toxic molecule ethene. This simplified view excludes environmental interactions that might control the dechlorinating process in soil and groundwater. For example, organic substrates not only provide a carbon source, but also enrich and select for specific members of the indigenous microbial community that will be involved in the relative production of acetate

and H_2 as well as the redox potential variation. The relative electron acceptor strengths of the different chlorinated ethenes during the dechlorination process might also affect the degree of dechlorination observed as a function of their redox potentials. This combined with the availability of reduced compounds derived from the microbial community metabolism of other organic substrates will ultimately impact the PCE degradation capacity of the system. Thus, differences in the capacity of added organic substrates to induce dechlorinating activity in the subsurface will most likely depend on the ecological and energetic capacities of the microbial communities present. In fact, each step of the dechlorination reaction might require different syntrophic interactions with different bacteria because of the relative decrease in electron acceptor strength as the reaction progresses. Some members of the microbial community and/or specific functional genes might also be sensitive to this shift in redox conditions during the dechlorination process as previously demonstrated for other environments.

The objective of this study was not only to understand and define the ecology surrounding the dechlorination process, but also to identify the members of the community that play an active role in the process. To address these aims, we established soil microcosms with different organic substrate additions: lactate, molasses and soybean oil; plus a control with no reduced organic substrate addition. During the incubations, we measured chlorinated ethenes and ethene concentrations to monitor the progress of the dechlorination reactions. Methane (CH_4) and CO_2 concentrations were monitored as well. Active microbial taxa were identified through the use of the thymidine analogue, bromodeoxyuridine (BrdU), which is incorporated into replicating DNA. This method has previously been demonstrated as an approach to identify growing bacteria in soil by immunocapture of the BrdU-labelled DNA and profiling of the captured 16S rRNA genes (Rabah and Lekmine, 2002; Löffler *et al*., 2005; Hjort *et al*., 2007; Robbins *et al*., 2011). In this study, we compared the composition of the total bacterial community in the microcosms with that of the actively growing community members using phylogenetic microarrays. In addition, we identified specific functional genes in the microcosms by metagenomics sequencing of total DNA extracted from the microcosms. Few studies to date have studied microbial community structures in laboratory enrichments (Löffler *et al*., 2000; Richardson *et al*., 2002; Brisson *et al*., 2012) and in field groundwater sites during PCE and TCE degradation (Rahm *et al*., 2006; Lee *et al*., 2012). This study constitutes the first monitoring of the active microbial community structure and function during dechlorination in soil. Using these techniques, we demonstrate connections between dechlorinating activity, active members of the microbial community and functional genes involved in carbon metabolism.

Results

Gas analyses

PCE degradation was monitored in soil microcosms that were amended with different organic substrates: lactate, soybean oil and molasses. The intermediates that were detected indicated that biodegradation progressed according to the classical reductive dechlorination pathway, with the sequential appearance of TCE, *cis*-1,2-dichloroethene (*cis*-DCE), vinyl chloride (VC) and ethene. The concentration of PCE in each microcosm amended with an organic substrate decreased by 58.9% (± 1.3%) to 76.9% (± 17.3%) over the 50-day incubation period. In addition, metabolites of PCE degradation were observed in the control microcosms without any added organic substrates, but their production was not maintained as long as in the microcosms with added organic substrates (Fig. 1). Differences in metabolite production rates were found for the different substrate treatments. The metabolites appeared more rapidly in molasses-amended microcosms compared with those amended with soybean oil and the lactate (Fig. 1). The production of metabolites was comparable between amended treatments for the first 35 days of the experiment. In the microcosms amended with molasses, however, there was a notable production of ethene by the end of the 50-day incubation period. Higher amounts of dechlorination were observed in the molasses treatments compared with the other substrate conditions based on the greater ethene concentrations in those samples.

The nitrogen (N_2) gas levels in the headspace remained relatively stable throughout the experiment, while carbon dioxide (CO_2) levels increased slightly (Fig. 2); with the exception of the samples incubated with molasses, which had a significant increase in CO_2 levels (56.5 μM) after 3 to 6 days of incubation and a slight dip in N_2 levels. This observation suggests that molasses resulted in a greater degree of respiration than the other substrates. O_2 was only detected in the microcosms without any organic addition (Fig. 2) consistent with the organic substrate degradation maintaining anoxic conditions (detection limit was 0.3 μM O_2). The data obtained on the last sampling date show the re-appearance of O_2 in the control. This is likely due to a repeated puncturing of the bottle seal. The amount of chlorinated ethene metabolites that accumulated during the incubation was inversely correlated to the presence of O_2. The presence of reduced compounds, such as CH_4 and H_2, in the headspace were also consistent with anaerobic conditions. CH_4 was detected in all of the microcosms, although it was only detected after 20

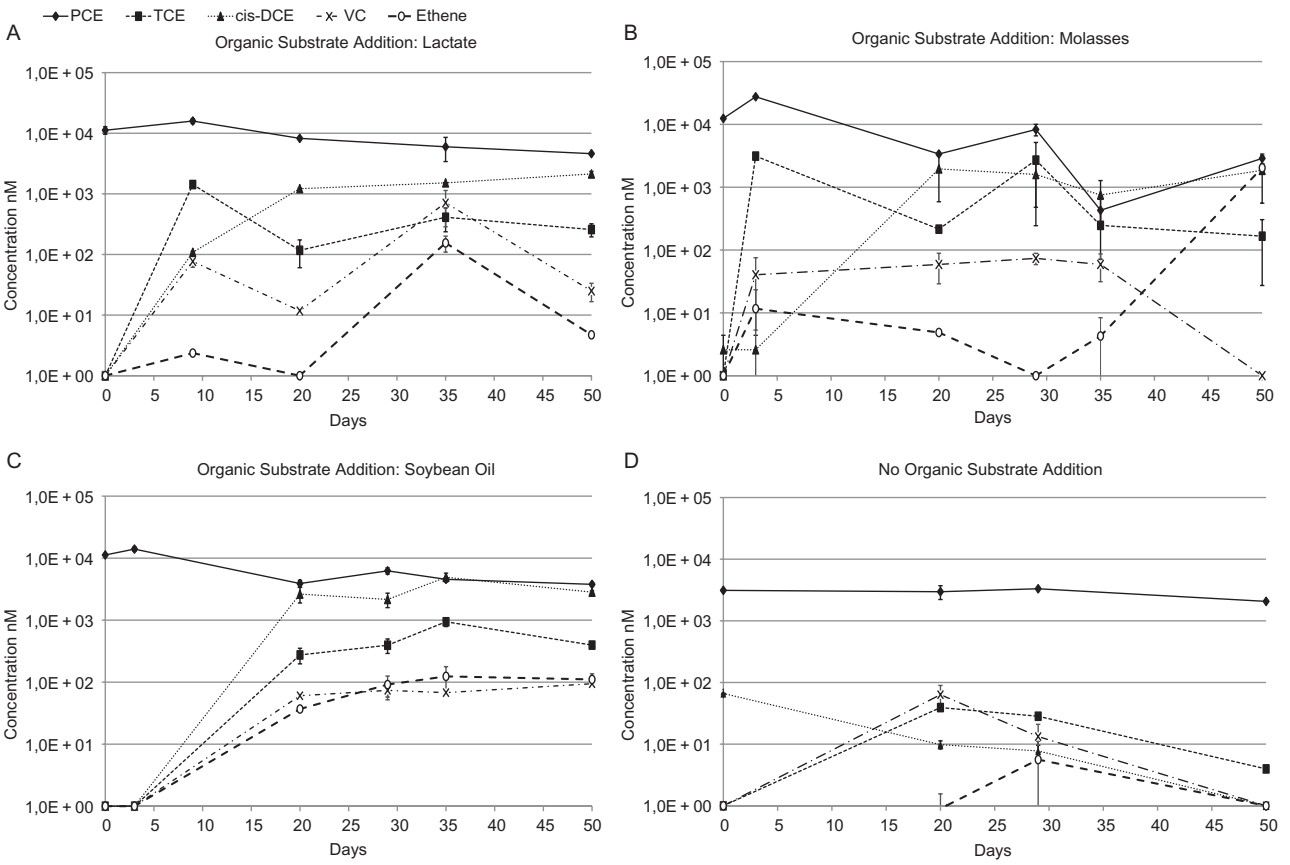


Fig. 1. Concentration of chlorinated ethenes in 2 g soil microcosms amended with 0.32 g of PCE and lactate (A, 1145 mg), molasses (B, 1158 mg), soybean oil (C, 348 mg), or without any additional carbon substrate (D) during an anaerobic incubation over 50 days. The error bars represent standard deviations calculated using three experimental replicates.

days in the unamended microcosms compared with less than 10 days for the organic substrate-amended microcosms. Increases in CH_4 concentrations corresponded to the appearance of ethene, which is associated with complete PCE dechlorination. The amounts of CH_4 that were measured were highest for those microcosms amended with molasses, followed in decreasing order by those amended with soybean oil, lactate and with no addition (Fig. 2). The microcosms amended with lactate showed a continuous increase in H_2 gas concentrations, while those amended with molasses had an even greater increase during the first 6 days of incubation. By contrast the H_2 concentration was relatively stable and low in the unamended samples and those amended with soybean oil (Fig. 2).

Chemical data combination analyses

A between group analysis (BGA) was performed to ordinate the samples based on the chemical data. BGA is based on carrying out an ordination of groups of samples using a standard multivariate statistical approach [here a principal component analysis (PCA)], rather than ordination of the individual samples (Dolédec and Chessel, 1987; Dray *et al.*, 2007). For visualization, the PCA axes were rotated to maximize the total distance between the inertial centre of each group (molasses, lactate, soybean oil and no amendment) instead of maximizing the total inertia between individual samples. A permutation test (10 000 permutations) was performed to determine the significance of the group structure (the $P = 9.999E\text{-}5$). BGA of the chemical data demonstrated that O_2 levels were associated with the microcosms that had no organic substrate addition (Fig. 3). All of the substrate additions had a relatively positive influence on the extent of complete reductive dechlorination of PCE when compared with the microcosms without organic substrate addition. The vector representing an incomplete degradation of the PCE (ratio of *cis*-DCE, VC and ethene to PCE and TCE on Fig. 3) is correlated with the microcosms without organic substrate addition and reflects the accumulation of intermediate metabolites. When the

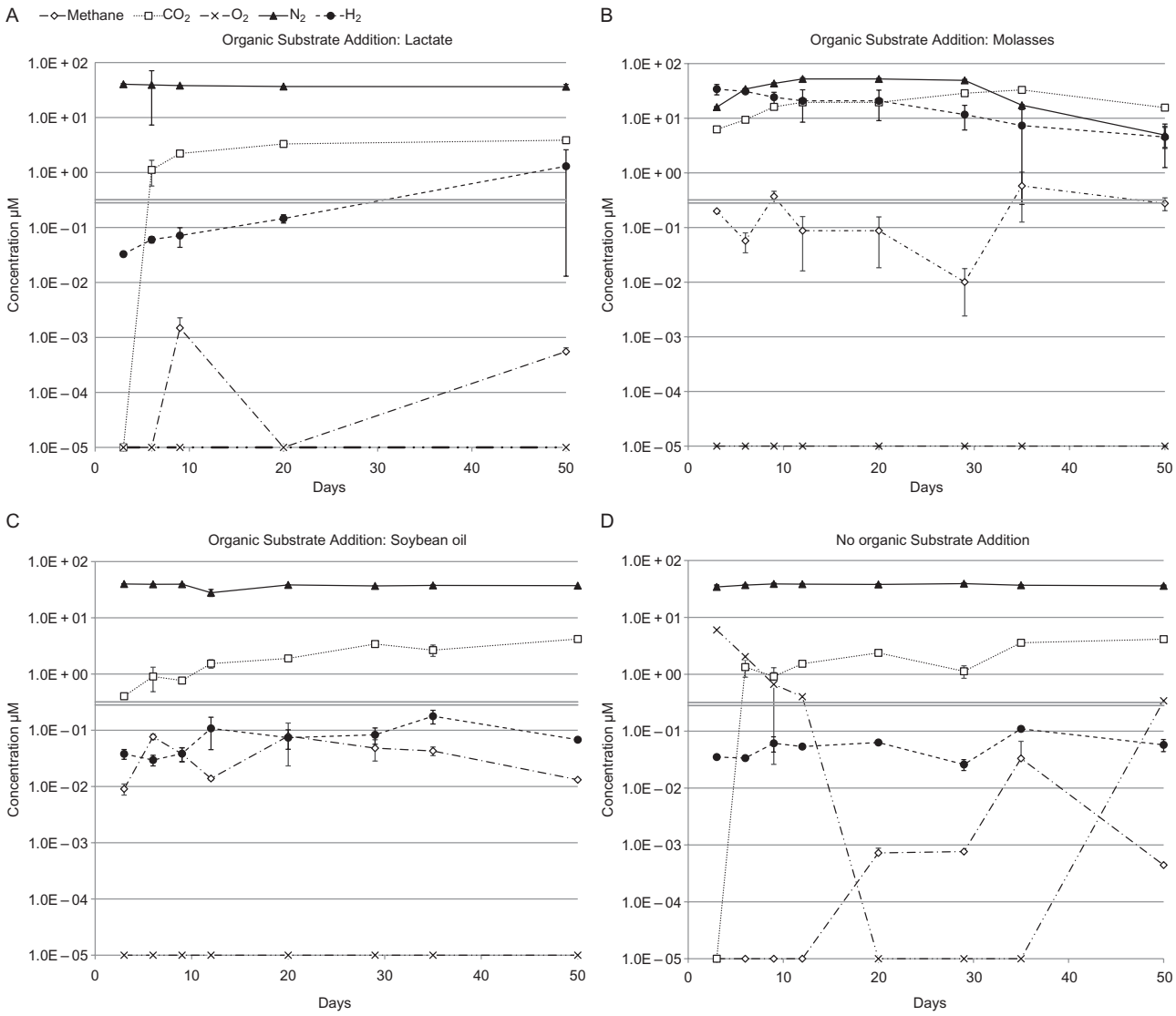


Fig. 2. CH_4, CO_2, N_2, O_2 and H_2 gas concentrations in soil microcosm headspace as a function of time. The error bars represent standard deviations calculated using three experimental replicates. O_2 quantification limit was at 0.3 µM. The panels A, B, C and D show the gas concentration for the microcosms amended respectively with lactate, molasses, soybean oil, and no addition.

ratios of products (ethene or VC) to substrates (sum of *cis*-DCE, TCE and PCE) were included in the analyses, the reductive dechlorination activities were all correlated with the microcosms amended with molasses (Fig. 3), thus, indicating that this substrate may stimulate a more complete dechlorination than the other organic substrate additions.

Finally, the gases, H_2, CO_2 (not visible in Fig. 3) and CH_4 are all more closely correlated with the microcosms amended with molasses (Fig. 3). Together these results suggest that the molasses amendment had the greatest impact on the dechlorination process. Therefore, we focused on the molasses-amended microcosms in more depth to gain a better understanding of the impact of the substrate addition on the microbial community composition and functional genes within the soil community.

Active bacterial community structure

To determine which members of the bacterial community were actively growing during PCE degradation via the reductive dechlorination pathway, the microcosms were amended with different organic substrates as described previously and with BrdU, a thymidine analogue that is incorporated into the DNA during replication. Both total community DNA and BrdU-labelled DNA that had incorporated BrdU were extracted and hybridized separately onto phylogenetic microarrays, or 'phylochips' (Delmont

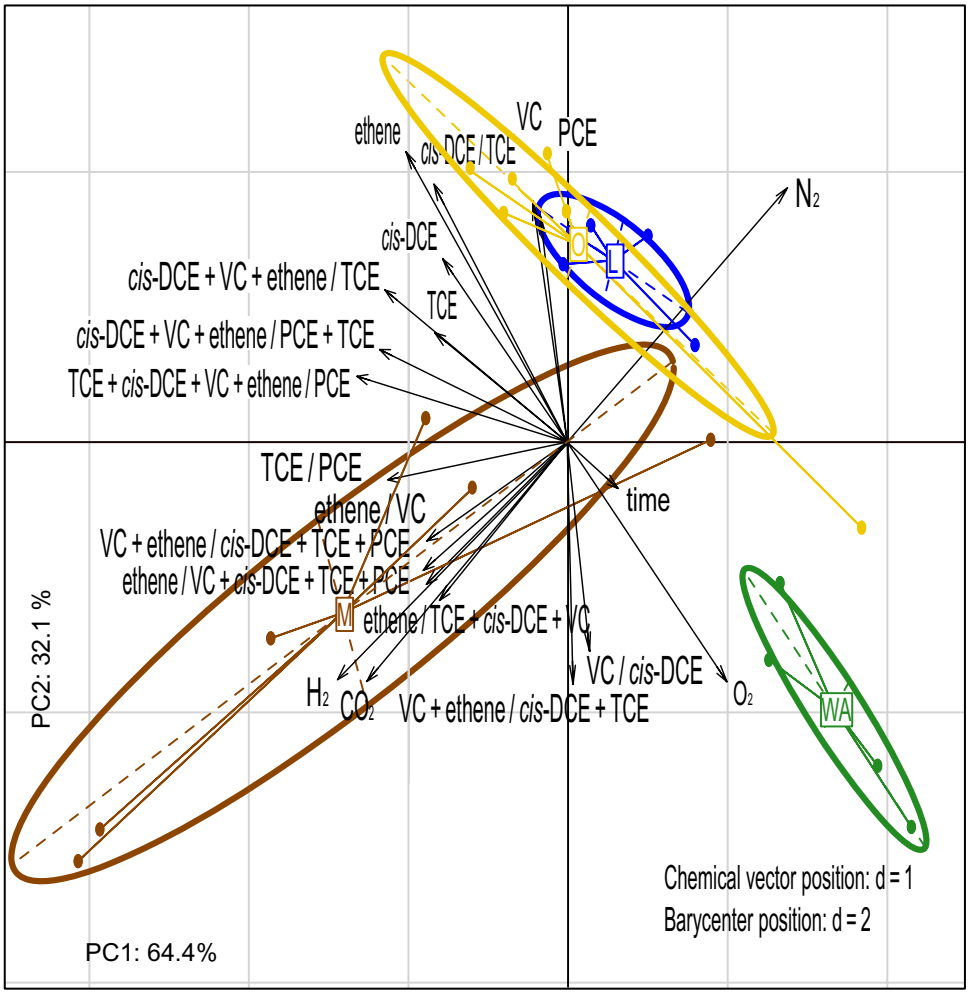


Fig. 3. BGA of microcosms incubated with different substrates based on the chemical concentrations analyzed and the ratios of products to substrates observed during the reductive dechlorination of PCE to ethene. The figure shows the barycentre position of each class for each organic substrate addition: molasses (M), lactate (L), soybean oil (O), and no organic substrate addition; only water added to same volume as other amendments (WA). The arrows indicate the chemical variable vectors responsible for the barycentre positions. The ratios of products to substrates were included in the analyses in order to correlate the organic substrate added with the reductive dechlorination activities (i.e. ethene divided by the chlorinated metabolites PCE, TCE, *cis*-DCE and VC), (d) indicate the scale of the BGA.

et al., 2011). BrdU addition alone had no significant impact on the structure of the bacterial community based on control experiments (Supporting Information Fig. S1). A BGA was performed to illustrate the differences between the hybridization groups, called classes, consisting of the different organic substrates and the differential extraction methods (active-BrdU-extracted DNA designated by 'B' and total community DNA designated by 'T') (Fig. 4). The circled clusters ('centroids') observed in Fig. 4 are based on the relative fluorescence signal differences for each oligonucleotide probe on the microarray. The probes that had the most significant changes in signal intensities between samples are those that are plotted the furthest from centre ('barycentre') of each centroid. The probes targeting genera that differentiated between the total and active communities in Fig. 4 were also plotted separately to identify the key community members (Supporting Information Fig. S2). This information is summarized below.

Significant differences were seen between the total community and the active community compositions, although those from the lactate-amended microcosms only shifted on component 2 (6.9% of the variance) (Fig. 4). The total communities in the unamended, molasses-amended and soybean oil-amended microcosms shifted laterally over time towards the active communities (Fig. 4). In addition, no obvious trend for the incubation time was apparent for the lactate-amended microcosms. Except for the lactate-amended microcosms, the total microbial communities were relatively similar on the first sampling date (3 days) (far right side in Fig. 4), but the active communities were already dissimilar

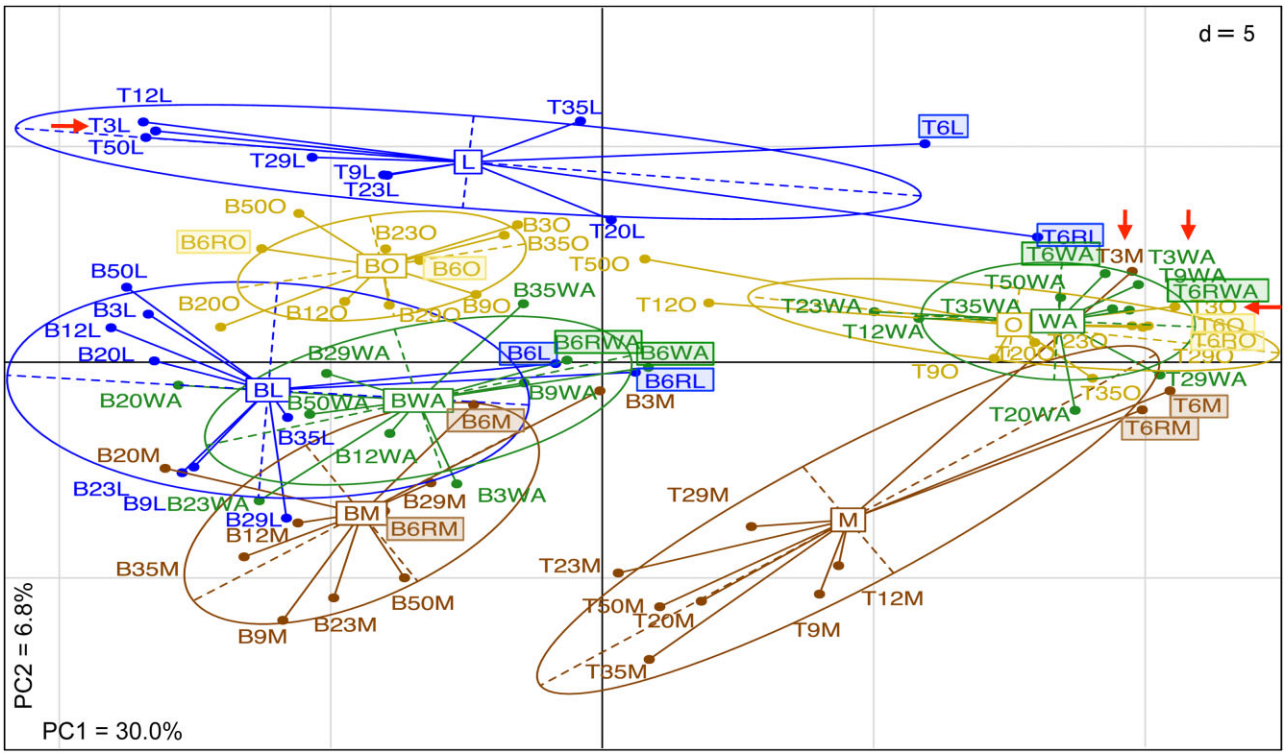


Fig. 4. BGA of microbial community profiles in the microcosms based on phylochip hybridization of total DNA corresponding to the total community and BrdU-extracted DNA corresponding to the active community. Total DNA in treatments with molasses (M, brown), lactate (L, blue), soybean oil (O, yellow) and no substrate addition (WA, green); BrdU-extracted DNA in treatments with molasses (BM, brown), lactate (BL, blue), soybean oil (BO, yellow) and no substrate addition (BWA, green). The results represent the average of three technical replicates. Some biological replicates are also included from the day 6 sampling date (indicated with shaded, boxed text). The first sampling date (day 3) for all treatments is indicated with red arrows. Each circled region, or 'centroid', represents a defined category based on organic substrate amendment and the centre is the 'barycentre', which represents the mean location of all the points for a given amendment. The first axis explains 30% of the variability of the position of the points observed on the figure. D stands for the value of the graduation.

by this date. The first sampling date is 3 days after the beginning of the incubation so that BrdU can be incorporated into the cell nucleic acid.

Microbial community profiles cluster according to treatment and activity

Another analysis of the data was performed to highlight the genera that showed the most variability according to the substrate addition (Fig. 5). In total, there were 126 genera, which varied as a function of the substrate used during the incubations. Some genera were only detected in the total community analyses [e.g. *Pelagibaca*, *Mathanomethylovorans* (light brown) and *Lonepinella* (brown)], and others were only detected in the active community (BrdU) analyses. *Roseococcus* (dark blue) was found in the active DNA fraction in all microcosms regardless of whether substrate was added or not, whereas *Sedimentibacter* (light green) was in all active communities of substrate-amended microcosms, but not found in the microcosms without substrate addition. Representatives of *Ignavigranum* (yellow), *Pedomicrobium* (purple) and *Desulfobacca* (pink) were only detected in the lactate-amended microcosm communities. However, *Thermoanaerobacter* (light blue) *Stappia* (red) and *Mogibacterium* (blue) were found in both the molasses and lactate-amended microcosm communities.

We were particularly interested in the genera that were actively growing in the molasses amendments because those treatments showed the most rapid rates of dechlorination. One candidate that might have a role in enhancing PCE degradation or be an indicator of optimal conditions (either directly or indirectly) is *Methanococcus* (orange, Fig. 5) that was abundant and active (i.e. found in the BrdU fraction) in the microcosms amended with molasses. Closer investigation of the data shown in Figs. 4 and 5 indicate some potentially interesting correlations. For example, the representative of the genus *Stappia* (red, Fig. 5) was active and detectable after 3 days of incubation in the lactate-amended microcosms (B1L) but it took longer to be detected in the other microcosms. This and other similar examples may explain the relatively close placement of the total and active commu-

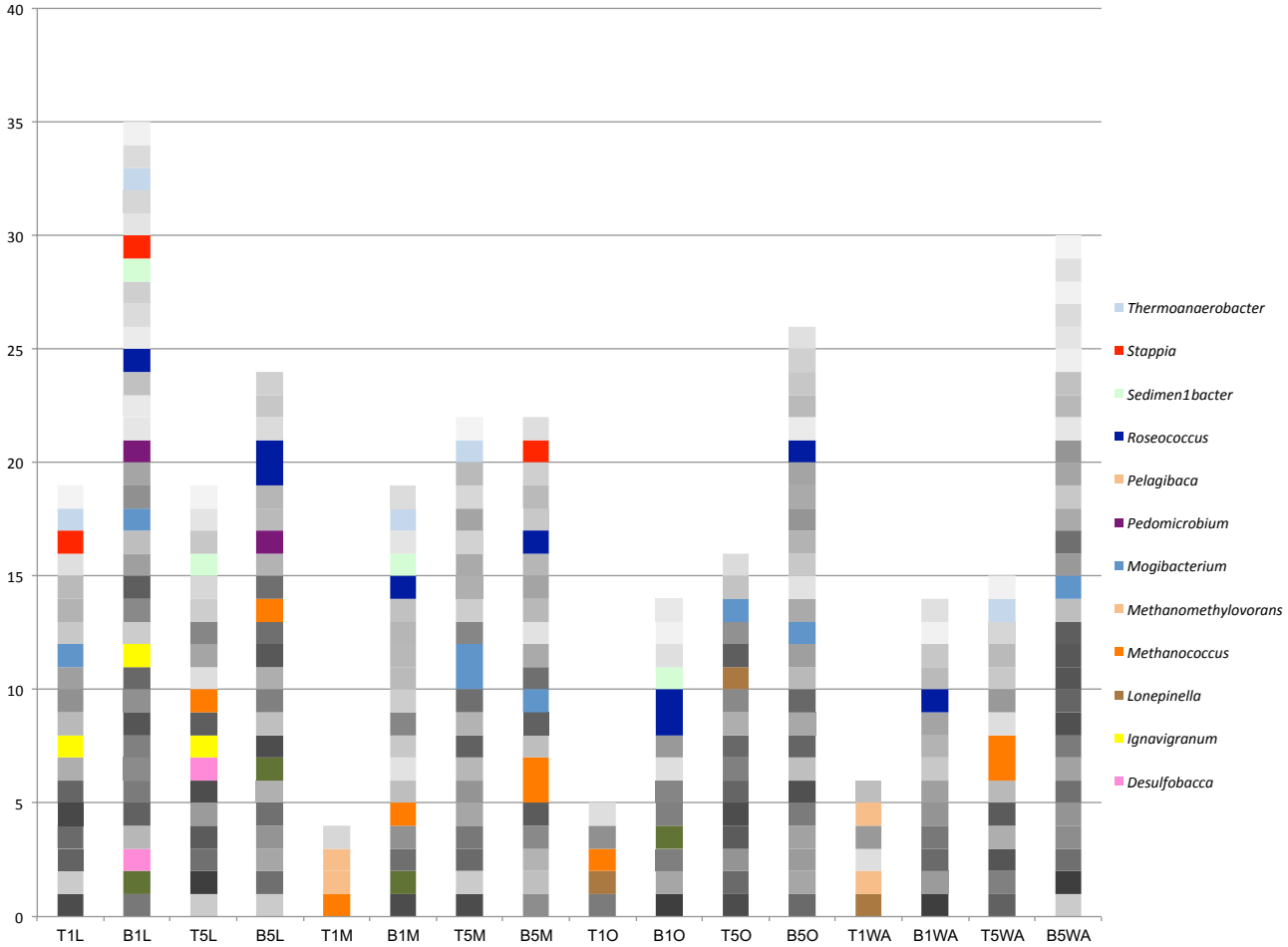


Fig. 5. Representation of the most variable genera in the microcosms based on the phylochip data. The probes were selected when the ratio of their intensity in a given sample was 30 times greater than the average intensities for the same probe in all other samples. Grey-shaded genera are not cited because of their more uniform appearance in most microcosms at most times. T1 and T5 represent total DNA at 3 and 20 days respectively. B1 and B5 represent active community DNA extracted using BrdU immunocapture after 3 and 20 days respectively. Symbols refer to the following amendments: L, lactate; M, molasses; O, soybean oil; and WA, no carbon substrate added.

nity structure in the lactate-amended microcosms on the PC1 axis of the BGA plot (Fig. 4), because there was apparently a faster community response to lactate compared with the other substrates.

The best recognized route for PCE degradation under anaerobic conditions is that carried out by microorganisms that use H_2 as an electron donor, acetate as a carbon source and PCE as an electron acceptor (Maymó-Gatell *et al.*, 1999), such as members of the genus *Dehalococcoides*. In our study, we found several candidate microorganisms that appear to be linked to this process based on their relative abundances and activities in the different treatments. These bacteria were grouped into two functional categories: potential H_2 users and versatile fermenters. The potential H_2 users were found in both the total and active communities. These included strict anaerobes and/or bacteria able to grow under auto- or lithotrophic conditions. The genera found in the total DNA extracts included *Geothermobacterium* (an iron reducer), *Methanosarcina* (a methanogen), *Cupriavidus* (a H_2 oxidizer) and *Pyrobaculum* (an iron and nitrate reducer) (Fig. 5). Three genera that were represented in the active community were members of the Clostridiales (e.g. *Clostridium*, *Eubacterium* and *Ruminococcus*). Versatile fermenters were found in both the total and active fractions, but were particularly well represented in the active community. These included representatives of *Larkinella*, *Methylobacterium*, *Roseomona, Chryseobacterium*, *Caldilinea* and *Roseospirillum*. Our analyses also highlighted several representatives of N_2-fixing genera (*Rhizobium* and *Bradyrhizobium*), denitrification (*Rhodoplanes*) and nitrification (*Nitrobacter*). That may be explained by the use of N_2 in the headspace during the incubations.

Correlation probes/chemicals analyses

To correlate the different results developed above, several linear multivariate correlations were performed between the phylogenetic probes themselves or between the probes and the chemical analyses. These correlations were supported by a Pearson test with an alpha risk set at 0.05. To take into account the multiple tests, *P*-values were corrected by controlling the false discovery rate. (Supporting Information Fig. S3). Overall, the chlorinated solvent concentrations were correlated among themselves. In addition, the ratio of metabolite to substrate for each dechlorination step was correlated to the CO_2 concentration, and this CO_2 concentration was correlated with the H_2 concentration ($r^2 = 0.72$).

Some probes that target anaerobic bacteria were correlated with dechlorination from the beginning of the degradation pathway [ratio (*cis*-DCE)/(TCE)]. In addition, probes representative of H_2-oxidizing bacteria were correlated with the last two steps of degradation during *cis*-DCE and VC degradation. For example, the strict anaerobe *Thermotoga* was correlated with CO_2 levels and to a lesser degree with the H_2 concentration. In addition, *Desulfuromonas*, an iron reducer, was correlated to the degradation of PCE as defined by the ratios of PCE degradation metabolites to PCE degradation. The facultative anaerobic phototroph, *Rhodospirillum*, was also correlated with the degradation of PCE as well as to ethene and CO_2 concentrations. Photosynthesis may provide a system for the conversion of some organic compounds into biomass and H_2 (Carlozzi and Lambardi, 2009). The family *Blautia*, belonging to the *Clostriales* was correlated with the last two steps of degradation during *cis*-DCE and VC degradation, to the ethene and CO_2 concentration. The members of this family produce acetate and succinate as end-products of metabolism. Some *Blautia* species use H_2/CO_2 as main energy sources (Liu *et al*., 2008).

Metagenome sequencing

Total metagenomic DNA from each soil microcosm was also sequenced using the 454 pyrosequencing platform after 20 days of incubation. The sequences (average 450 bp) were analyzed using the MG-RAST SEED annotation (Meyer *et al*., 2008) and classified into metabolic subsystems. The reads were normalized by the total number of annotated reads and submitted to Integrated Microbial Genome and Metagenome (IMG/M) for annotation (Markowitz *et al*., 2006) and classification by enzyme category (EC) number. Each EC number is represented in Fig. 6 by a sphere. The size of the sphere represents the sum of the relative abundance of reads, and the coordinates of each sphere in the tetrahedron represent the relative abundance of reads in each of the four substrate conditions. In addition, a colour code shows the corresponding Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolism pathway (Kanehisa and Goto, 2000). The supplementary program (Supporting Information Computer Program S1) allows anyone to rotate the tetrahedron in 3D and spot the function that is more abundant in a given sample.

Most of the metabolic subsystems studied were more dominant in the substrate-amended microcosms compared with the controls (Fig. 6). For example, the genes coding for the enzymes involved in fatty acid metabolism were present in the microcosms amended with lactate and soybean oil. The relative abundance of genes belonging to the TCA cycle showed that glycolysis and pyruvate metabolism were the main pathways present in the microcosms amended with molasses. The entire pathway for CH_4 metabolism was observed in the microcosms amended with lactate and soybean oil, but the gene specifically responsible for methanogenesis (highlighted by white circles; Fig. 6) was present in all of the microcosms. Finally, the genes involved in chlorinated compound degradation (indicated in grey; Fig. 6) were more abundant in the substrate-amended microcosms.

Among these subsystems, those associated with carbohydrate metabolism and electron donor reactions were investigated further because of their importance in PCE degradation. The molasses-fed microcosms (molasses is composed of sucrose and proteins) had relative increases in functional gene sequences associated with polymer hydrolysis and mixed organic acid fermentation (Fig. 7). Lactate-fed microcosms exhibited increases in methanogenesis, and fermentation associated with pyruvate as well as glycolysis. Globally, MG-RAST analyses showed that microcosms amended with all three substrates had more sequences associated with acetyl-CoA metabolism, such as glycolysis and gluconeogenesis, than the non-amended microcosms (Fig. 7). Both molasses and lactate had common functional gene class increases for gluconeogenesis: the acetyl-coA, which can also feed into the glyoxylate cycle, resulting in the accumulation of oxaloacetate, which can be transformed by phosphoenolpyruvate carboxylase to phosphoenolpyruvate. In addition, the soybean oil and lactate-amended microcosms had increases in genes involved in fatty acid oxidation metabolism. Therefore, although the type and extent of metabolism differed in the soil microcosms amended with different substrates, they all had enhanced carbon degradation capacities and increases in acetate and H_2 production capabilities; processes known to be critical for successful PCE degradation by *Dehalococcoides* sp. (Freeborn *et al*., 2005).

Although the dominant indigenous microbes in the soil we used in these experiments were non-dechlorinators, we were still able to detect rare members of the community

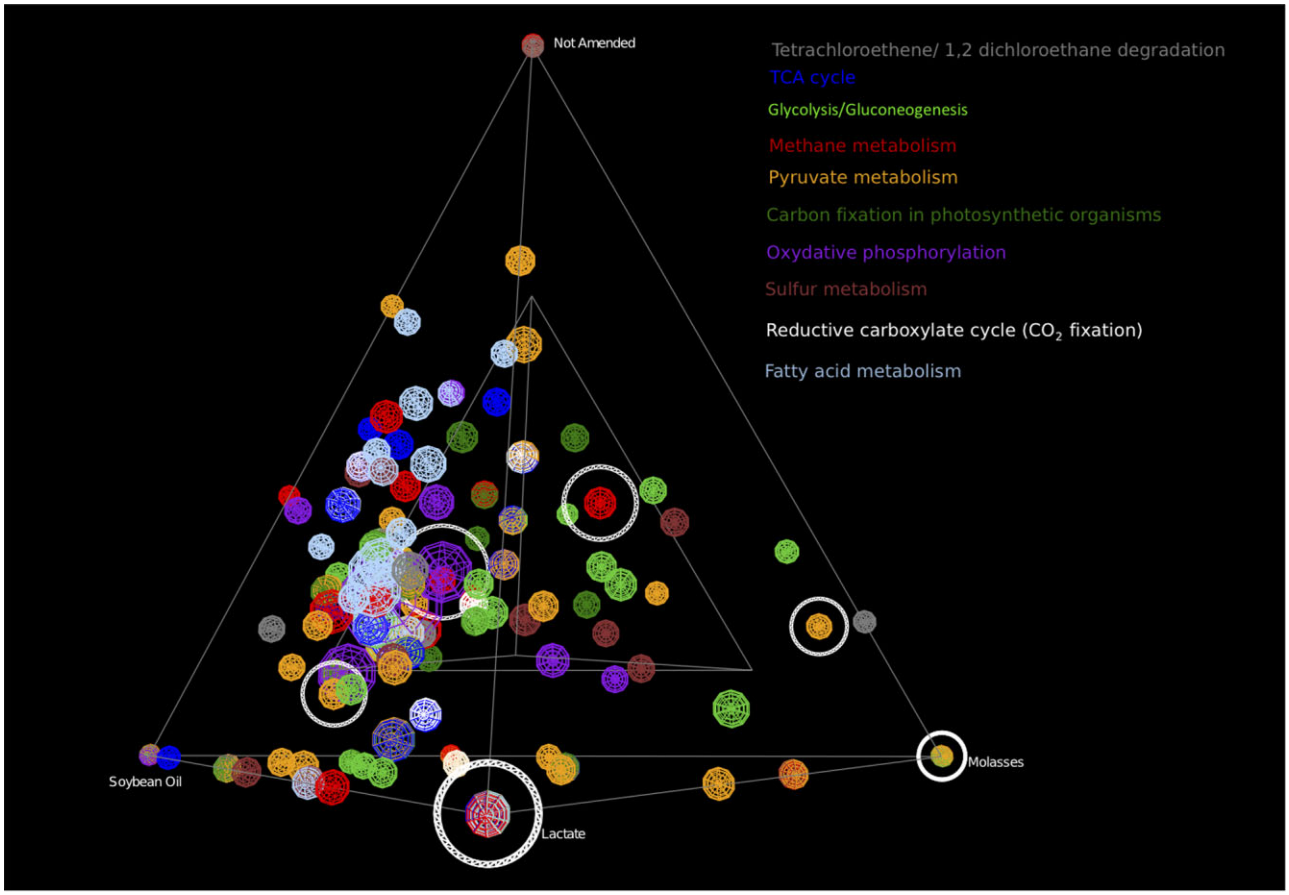


Fig. 6. Relative abundances of metabolic pathways in the different treatments. Each sphere represents the frequency of occurrence of a given EC number as log of the mean of the number of reads in the four microcosms and is plotted using the relative abundance in each sample as a coordinate in the tetrahedron. The KEGG pathway to which each EC number belongs is indicated by colour. In addition, the white circles indicate the genes involved in the methanogenesis but not classified by KEGG in the CH_4 metabolism. The program is available for Apple computers at http://www.genomenviron.org/Research/DeNovo.html

that are typical dechlorinating populations. MG-RAST analyses detected seven strains that have previously been demonstrated to be capable of dehalorespiration: 100 reads were associated to *Desulfitobacterium hafniense* strains DCB-2 & Y 51 (amendments lactate: 18, molasses: 47, soybean: 17, no amendment: 18) (Nonaka *et al*., 2006; Kim *et al*., 2012) and *Dehalococcoides* strains 195, CBDB1, BAV1, GT and VS (amendments lactate: 16, molasses: 12, soybean: 2, and no amendment: 8). (Maymó-Gatell *et al*., 1999; Krajmalnik-Brown *et al*., 2004; Muller *et al*., 2004; Sung *et al*., 2006) (Supporting Information Table S1).

Discussion

Reductive dechlorination of chlorinated solvents in the subsurface environment is dependent on the activity of bacteria capable of mediating this process. Some of the bacteria capable of PCE and metabolite degradation have already been characterized and shown to be dependent on the concentration and availability of the chlorinated compound as the electron acceptor, H_2 as the electron donor and acetate as the source of carbon (Maymó-Gatell *et al*., 1999). The source of chlorinated compounds has been in part satisfied by their industrial production and release into the environment. The availability of H_2 and acetate in many environments depends on the microbial activity of other members of the microbial community and a source of fermentable organic matter. In addition, H_2 and acetate can be utilized by members of natural microbial communities, including both bacteria and archaea that are not specifically involved in chlorinated solvent dechlorination, but their indirect contribution to the dechlorination process is largely unknown. Methanogens, sulfate reducers and dechlorinators potentially compete for the H_2 produced by other microorganisms in the community (Behrens *et al*., 2008) but the complexity of their interactions is also poorly understood. This study therefore contributes new knowledge about how a soil microbial community participates in the reductive dechlorination

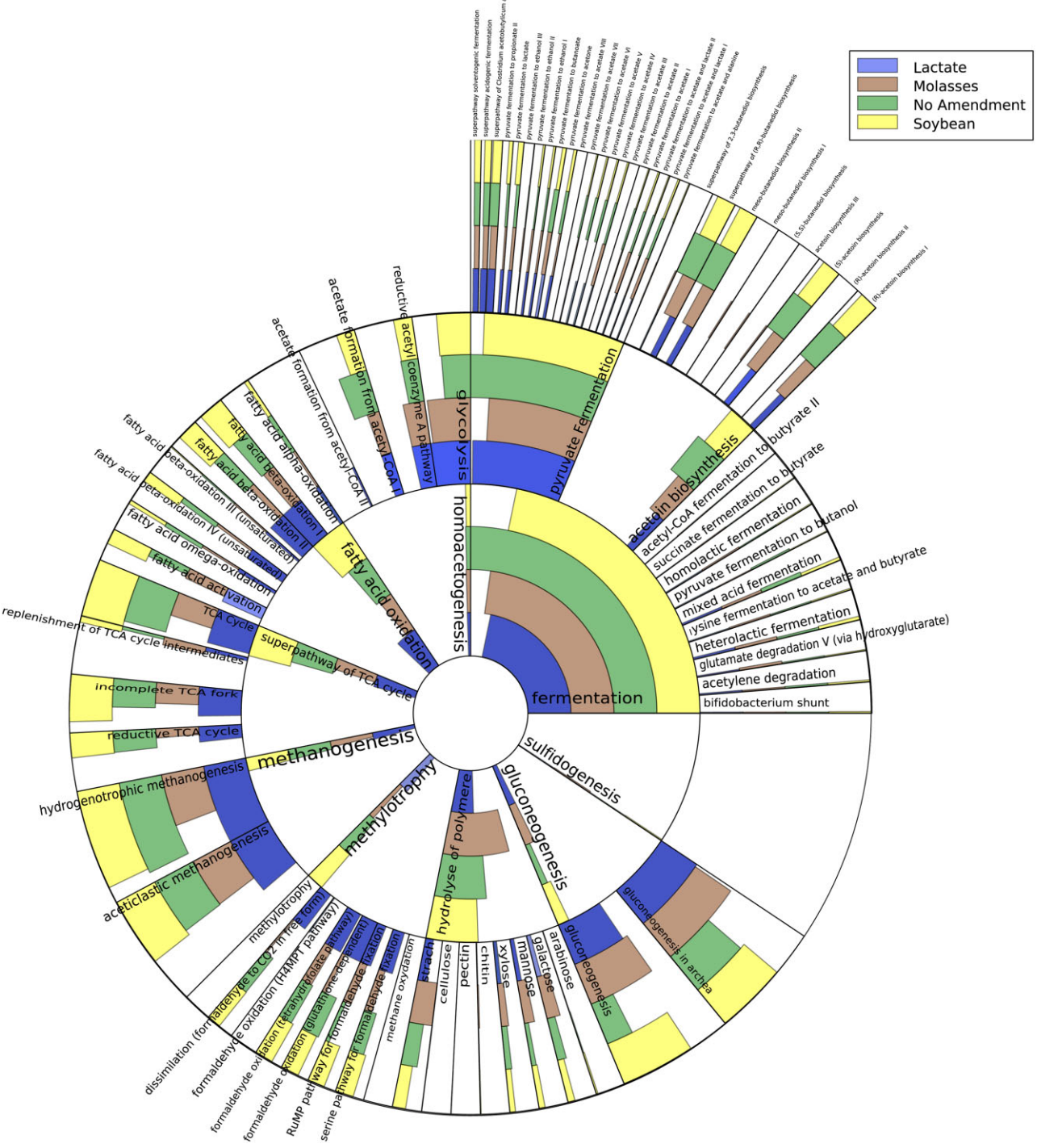
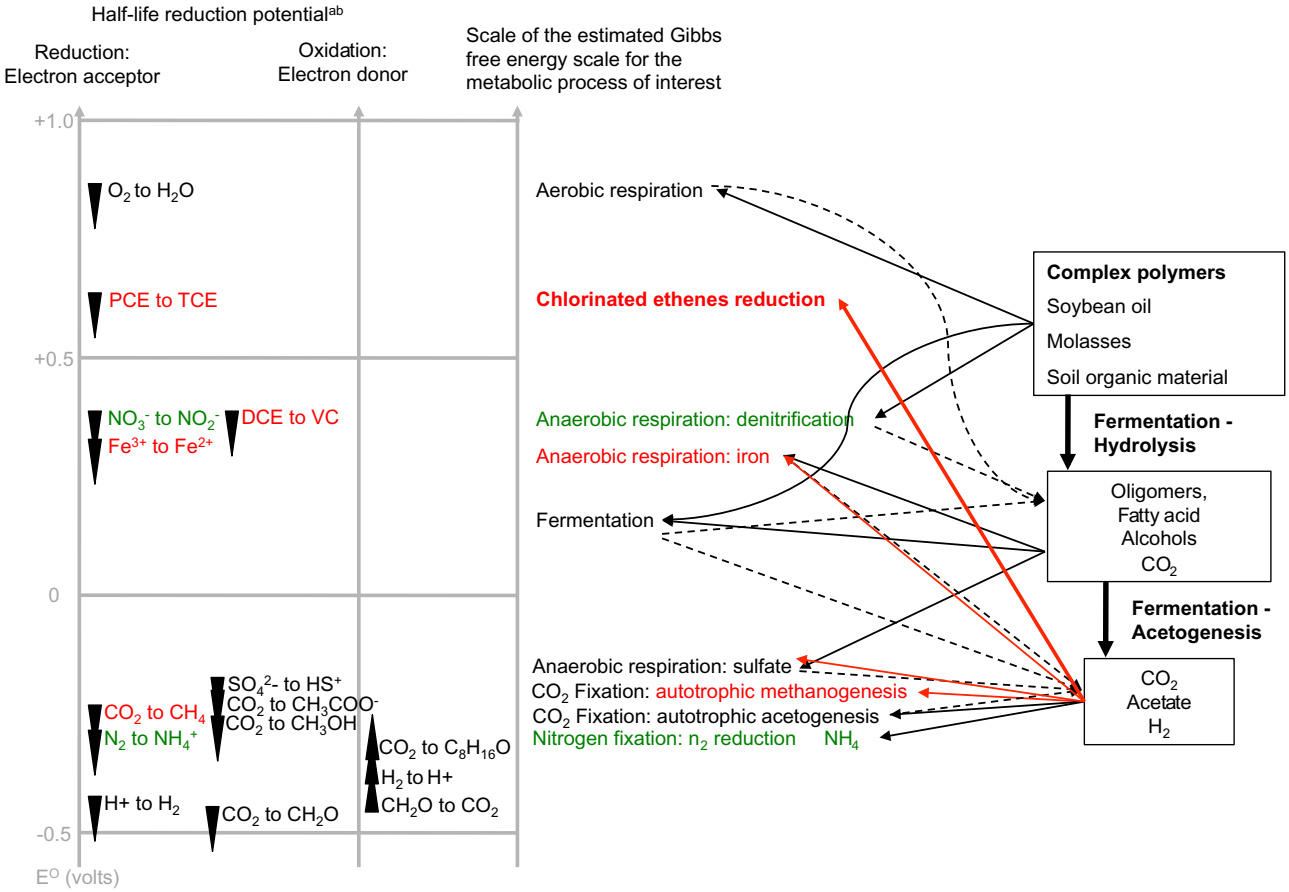


Fig. 7. Comparison of functional read assignments from the metagenome data from microcosms incubated with PCE and different amendments after 20 days of incubation: blue, lactate; brown, molasses; green, no amendment; yellow, soybean oil. The figure shows the percentage of reads from selected MG-RAST subsystems at different levels of resolution.

of PCE with various substrate amendments by combining data describing the total and active microbial community structure and community function using a combination of metagenomic sequencing and phylogenetic microarrays.

Soil microcosms were established with PCE alone as a control to compare with soil microcosms where reductive dechlorination was stimulated by the addition of lactate, molasses or soybean oil, which are commonly used to



[a] Half reaction at 25°C and pH7
[b] Base of arrow align with potential of half-reaction shown in volt

Fig. 8. Microbial trophic network related to chlorinated ethene reductive degradation. The microbial mediated processes involved in the N_2 cycle are indicated in green. Processes that can potentially interact with the reductive dechlorination, because of the use of acetate and H_2, are in red. Compounds derived from the different metabolic processes are indicated by the dashed arrows.

stimulate *in situ* cleanup during anaerobic bioremediation processes. Although the headspaces of the microcosms were purged with N_2 gas at the beginning, the PCE control was never strongly anoxic mirroring and might have even been microaerophilic at times what often happens in untreated PCE contaminated sites. During the incubations, the microcosms were monitored for PCE and its chlorinated metabolites, the non-chlorinated metabolite: ethene, as well as atmospheric gases associated with microbial respiration (O_2, H_2, CO_2, CH_4 and N_2). Some reductive dechlorination occurred in all the microcosms, even the live control, but the most effective microcosms for PCE degradation were those with molasses addition (Fig. 1). Variations in the degradation rates and the relative compound concentrations were correlated to the microbial community structure and function to identify possible mechanistic links. An overall conceptual model of electron flow in these reduced environments was developed to pinpoint where different community members might participate (Fig. 8).

The microbial community structure was analyzed with a phylogenetic microarray. The BGA analysis identified the microbial genera that were correlated to PCE degradation and that could be used as potential bioindicators of this process. For example, several strict anaerobes (potential H_2 users) were enriched in the total community: e.g. *Clostridium*, *Eubacterium*, *Ruminococcus*, *Geothermobacterium* and *Methanosarcina* and were associated with low redox environments. Therefore, they are both potential indicators of favourable redox environments for PCE reductive dechlorination as well as potential competitors of PCE degraders for H_2. Although these bacteria were identified in the total community as being correlated to complete PCE degradation, they were not detected as active. This can be explained either because they were actually not growing, or otherwise not able to incorporate

BrdU. However, examination of the active community revealed another population that could potentially oxidize H_2 (*Cupriavidus*) and an iron-reducing member of the *Archaea* (*Pyrobaculum*). Most of the other genera that were identified as active during PCE degradation were typical versatile fermentaters as observed previously (Freeborn *et al*., 2005). Our results suggest that *Pyrobaculum* might be a good bioindicator of favourable conditions for complete PCE degradation. However, we did not find a strong correlation between specific fermentaters and specific H_2 oxidizers using inter-probe correlations on the phylochip. This suggests that there might be functional redundancy in the community for H_2 production and uptake.

The complete degradation of PCE is particularly challenging because one of the key metabolites, VC, is more toxic that the parent compound and is often generated in high quantities in the environment during PCE degradation. In addition, at many field sites, there is an accumulation of *cis*-DCE (Kean *et al*., 2003). The phylochip data revealed potential H_2-oxidizing bacteria that were correlated to the last two of steps of degradation (*cis*-DCE and VC degradation): *Blautia* and *Desulfuromonas*. *Desulfuromonas* in particular is a potential bioindicator of PCE degradation because its abundance was correlated with PCE degradation to VC, and subsequently to ethene. This bacterium is an iron-reducing strain, characteristic of reduced environments and able to use H_2 as a source of reducing power (Kerin *et al*., 2006). This observation suggests that other microorganisms that compete with PCE dechlorinators for H_2 as an electron source might also serve as positive bioindicators of PCE degradation. This hypothesis is supported by the fact that as H_2 (electron acceptor during the dechlorinating process) was not correlated with PCE degradation, it may also be used by autotrophic bacteria. The positive correlation between pollutant degradation and competitors for electron sources is consistent with PCE-reducing bacteria outcompeting other members of the soil community by recovering more energy from the same electron donor because the potential redox favours reductive dechlorination compared with the other respiration processes (Fig. 8). On the other hand, with sufficient substrate (electron donor) added, no rate-limiting competition should occur for the use of the electron and carbon donor (i.e. H_2 and acetate) in the microcosms.

The overall model for dechlorination of chlorinated solvents under anaerobic conditions requires the participation of community members similar to those operating under sulfate-reducing or methanogenic conditions (Fig. 8). However, if the dechlorinating species are to successfully compete for the H_2 and acetate produced, the other members of the community will need to resist any toxic effects of the chlorinated solvent and the hydrochloric acid produced during degradation. For optimal degradation, members of the soil community should provide acetate and H_2 to the dechlorinators. Depending on the physical distance between the community members providing these critical substrates and those that use them, the concentrations of substrates in the bulk soil will vary. Thus, the bulk environmental concentrations of acetate and H_2 are not necessarily good indicators of localized dechlorinating activity and the metabolic activity of the microorganisms involved in the degradation process could provide a more contextual evidence of dechlorination activity.

The variations in the biochemical pathways identified according to different substrate amendments to soil provide some insight into the overall microbial functioning during the reductive dechlorination of PCE and its metabolites. Different classes of metabolic reactions were dominant with different substrate amendments, but many of these differences were related to the degradation of the added substrates themselves. For example, organic acid metabolism was higher for the PCE control and the soybean oil plus PCE microcosms than for PCE plus lactate or PCE plus molasses microcosms. The other surprising result was the low and similar quantity of sequences associated with methanogenesis (Fig. 7). Although no significant sulfate was present, methanogenesis did not dominate the microcosms, yet some CH_4 production did occur (Fig. 2).

If the added substrates were able to feed acetate and H_2 into the dechlorination process, then electron-donating reactions should be influenced. All three substrate additions resulted in a slight increase in the percentage of electron reducing reaction sequences in the microcosms. Therefore, the general picture painted by the relative proportion of sequences in different metabolic classes supports the concept of increased acetate and H_2 availability. The eventual pathway the organic substrates take through the metabolic network varies depending on the substrate, but they all enhanced carbon degradation leading to varying degrees of acetate and H_2 production and improved PCE degradation as a result. One potentially useful application of this study was the finding of several microbial strains that were correlated with successful PCE degradation and could be used as bioindicators of this process.

Material and methods

Microcosms

The soil used for this study was collected at the Hopland Field Station (University of California). Soil samples of 1 kg were collected at 1 m distances along a 10 m transect at the site, avoiding the rhizosphere by removal of plant debris and roots. The soil samples were mixed

together to form a composite sample. Different soil microcosms (126 in total) were prepared in 5 ml serum bottles containing 2 g of Hopland soil and 2 ml of sterile distilled water saturated with PCE (Sigma-Aldrich, St Louis, MO, USA) to result in a final concentration in the microcosms of 160 mg L^{-1} (0.97 10^{-3} M PCE). Four treatments were established with organic substrate additions as follows: (i) soybean oil (labelled SO), (ii) lactate (labelled L), (iii) molasses (labelled M) and (iv) no addition (control) (labelled WA). The quantity of organic substrate added was calculated on the basis of 8 mmol of electrons necessary for the total degradation of the PCE added, i.e. per bottle (with a margin of error of 20 ×): 1158 mg of molasses (Safeway, Berkeley, CA, USA), 1145 mg of lactate (Sigma-Aldrich, St Louis, MO, USA) and 348 mg of soybean oil (Sigma-Aldrich, St Louis, MO, USA). Finally, the O_2 was flushed from the microcosms by purging the headspace with N_2 gas before sealing the serum bottles. BrdU (Roche, Mannheim, Germany) was injected with a syringe into the sealed bottles in an anaerobic hood. Nine microcosms in triplicate (27 microcosms) were established per treatment to correspond to nine sampling times and used for analyses of the concentrations of chlorinated ethene and ethene at several sampling points. Each microcosm was supplemented 3 days before sampling with 200 µl of 200 mM BrdU in water (Roche) and incubated at room temperature in the dark during these last 3 days only to avoid BrdU depletion during the 50 days. Additional samples were prepared as controls for possible confounding effects after 6 days of incubation as follows: one treatment without BrdU but with water added at the same volume (200 µl) and soil alone performed at each sampling time (18 more microcosms).

Chemical analyses

Chlorinated ethene and ethene analyses were performed on 1 ml headspace samples that were taken from the sealed microcosms (serum bottles with Teflon-tape-coated butyl rubber stoppers) using Precision Lock gas-tight syringes (Alltech). Initial concentrations of all substrates were confirmed by gas chromatography (GC) GC SYSTEM 7890A with a flame ionization detector and a 30 m GS-GasPro (Agilent) 0.32 mm internal-diameter column. The oven temperature programme was as follows: 1 min at 45°C, ramping up at 100°C min^{-1} to 200°C, hold for 3 min (total run time 5.55 min); with a N_2 carrier gas rate of 4.0 ml min^{-1}. The monitoring of chlorinated ethenes and ethene was performed on the headspace of all replicates at each sampling time.

The concentrations of CH_4, O_2, CO_2 and N_2 (+ Argon) in influent and effluent gas samples were measured by GC headspace analysis: one gas sample (0.20 ml) was injected through a 0.5 ml gas-tight pressure lock Dynatech-Precision syringe (Alltech) into a Hewlett Packard HP 5890 series II gas chromatograph equipped with a thermal conductivity detector and a CTR1 column (Alltech) to analyze the four gases. H_2 was quantified by GC using a reductive gas detector (Trace Analytical, Menlo Park, CA, USA) by sampling 300 µL of the headspace. The CH_4, H_2, N_2, CO2 and O_2 were quantified in the headspace in each microcosm. O_2 detection limit was at 0.3 µM.

Nucleic acid extraction

Total DNA and RNA extraction was performed as previously described (Griffiths *et al*., 2000). Briefly, 0.25 g of soil were placed in a bead-beating tube (Ozyme, Saint Quentin en Yvelines, France), with 0.6 ml of extraction buffer (120 mM K_2PO_4 buffer pH 8.0 with 5% hexadecyltrimethylammonium bromide) and 6 ml of phenol/chloroform/isoamyl alcohol: 24/24/1 (supplier Sigma-Aldrich, St-Quentin Fallavier, France). The tubes were shaken in a bead beater at a speed setting of 5.5 for 30 s. After centrifugation, the supernatant was washed using chloroform/isoalmyl alcohol: 24/1. Nucleic acids were precipitated by the addition of two volumes of 1.6 M NaCl and 30% PEG 6000 (polyethene glycol: 6000), washed with 70% ethanol, and resuspended in 50 µL of sterile water.

DNA that had incorporated BrdU was purified from the total extracted DNA by immunocapture using antibodies against BrdU as previously described (Artursson and Jansson, 2003; Edlund and Jansson, 2006; 2008). The efficacy of BrdU immunocapture was confirmed by PCR amplification using primers 27F and R1492 that target the small subunit ribosomal RNA gene (Lane *et al*., 1985) and comparing visible bands on agarose gels with control microcosms to which no BrdU had been added. No PCR-amplified product was visible in the controls without BrdU.

Phylochip

The phylochip used in this study was first described by Delmont *et al*. (2011). Probes were designed using the ARB software package and PHYLARRAY software (Militon *et al*., 2007) to target the *rrs* gene and to cover a wide portion of the *Bacteria* and *Archaea* phylogeny. The probes were 20 mers with melting temperatures of 65 ± 5°C and with a weighted mismatch less than 1.5. Our design included oligonucleotide probes at different taxonomic levels as previously described (Nemir *et al*., 2010). The probes are available at http://www.genomenviron.org/Research/Microarrays.html

The DNA was labelled without prior amplification using the label IT microarray Cy3/Cy5 Labelling kit from Mirus

(Euromedex, Souffelweyersheim, France), following the manufacturer's instructions. Cy3 dye was used for the total DNA extracted, and Cy5 for BrdU-containing DNA. The labelled DNA was purified on a G50 Microspin column (GE Healthcare, Orsay, France), following the manufacturer's instructions, and performed two times at 3000 r.p.m. After purification, the labelled DNAs were fragmented, using the procedure provided by the IT microarray Cy3/Cy5 Labelling kit from Mirus (Euromedex). The quantity of DNA and the amount of Cy3 and Cy5 labelling was determined using a nanophotometer IMPLEN (Serlabo, Entraigues, France). The rest of the process was described by Delmont *et al*. (2011).

Microarray hybridizations were performed using the Agilent hybridization kit (Agilent, Massy, France). After hybridization, the microarrays were read at 532 nm and 635 nm. Several test hybridization reactions were performed to determine whether BrdU influenced the labelling or hybridization efficiency, to avoid any bias due to stearic overcrowding by this molecule, but no significant differences were observed.

An Innoscan 700 Microarray scanner (Innopsys, Toulouse, France) was used for scanning microarray slides according to the manufacturer's instructions. Scanned images were then processed with the software MAPIX (Innopsys, Toulouse, France). Raw hybridization fluorescence signals for each spot were determined based on the signal-to-noise ratio (SNR), which was calculated using the following formula: SNR = (signal intensity – background) / standard deviation of background. Hybridization fluorescence signals for all probes including negative controls were transformed by calculating the $\log_2$ of the signal. Because at least three replicates existed for all oligonucleotide probes, outliers were eliminated when any individual spot was greater than two standard deviations from the average of all replicates. Data was subsequently normalized by using the quantile approach. This normalization aimed to align the average of the quantile of each hybridization replicate (Bolstad *et al*., 2003). The value of the negative control plus the confidence interval was removed from the probe intensity value. Each resulting probe sequence was searched using BLAST (Altschul *et al*., 1997) on the NCBI database. When assigning taxonomic identities to probes of interest, all the probes that were positive for the same phylogenic subgroup were examined. Accordance to the International Union of Pure and Applied Chemistry, the detection threshold used was the negative controls replicate mean plus Three times its standard deviation. After subtraction of this threshold, the positive probes were designated with the number 1, and those under the threshold by the number 0. If several probes targeting the same genus were positive, the sum was taken for those probes and used in the analysis. Probe hybridization results for different samples were compared using BGA or by comparing the intensities of the same probe in different extracted DNA pools.

454 Sequencing

DNA (1 μg/50 μL) extracted from environmental samples was sequenced by GATC (Constanz, Germany) using a Roche 454 Titanium pyrosequencing instrument. Four 454-sequencing runs were performed during this study: one for each treatment after 20 days of incubation, i.e. PCE-polluted soil amended with molasses, lactate, soybean oil and without organic substrate additions. For the PCE-polluted soil amended with molasses, 5.8 Mbp were generated with 15 329 reads of average 382 bp lengths, ranging from 23 to 603 bp. For the PCE-polluted soil amended with lactate generated 9.6 Mbp, with 24 321 sequences of average 394 bp in length, ranging from 31 to 584 bp, ranging from 31 to 584 bp. The PCE-polluted soil amended with soybean oil generated 8.9 Mbp, with 22 531 sequences of average 396 bp in length, ranging from 24 to 583 bp. The PCE-polluted soil without organic substrate addition generated 4.7 Mbp with 12 011 sequences of average 395 bp in length, ranging from 28 to 589 bp.

The sequence data were subsequently analyzed using IMG/M (Markowitz *et al*., 2006) and MG-RAST (Meyer *et al*., 2008), which binned the reads into different microbial metabolic subsystems (SEED subsystem) based on their similarity to known functional genes, and classified the proteins that would match them. For this study, an *e*-value cut-off of 10^{-5} was used. The percentage of sequence reads identified for each sample was 37.53%, 37.89%, 43.39% and 39.26% for the PCE-polluted soil without organic substrate addition, amended with lactate, molasses and soybean oil respectively.

For treatment comparisons, the normalized numbers of reads in each subsystem were compared. The relative abundance values for fragments detected in each treatment were determined by summing all fragments from all samples that were classified in a subsystem and dividing by the number of samples included in the comparison. Fig. 6 was coded using C++ and was a picture taken from an angle of the supplementary program (computer program is available at http://www.genomenviron.org/Research/DeNovo.html).

Statistical analyses and thermodynamic calculations

The microarrays were normalized using the MATLAB program, version 7.9. Remaining statistical analyses, such as correlation probabilities, principal component analyses and Pearson tests were performed using R software, version 2.9.2 (http://www.r-project.org/). The

thermodynamic calculations were based on those published by Vogel *et al*. (1987).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Principal component analysis to control the BrdU impact on the total bacteria community structure.
Fig. S2. Plot of the variable projections, here the probes, in the sample space from the between group analysis presented Fig. 4. Only the probes with a projection on the first component of the between group analysis greater than 0.3 or less than – 0.1 are plotted below.
Fig. S3. The parameters (chemical analyses, and probes) with the highest correlation coefficients between chemical concentrations, ratio of products to substrate ratios, and phylogenetic probes during the PCE reductive dechlorination pathway. No data with correlation coefficients between 0.50 and -0.50 are shown. The probe names were replaced by the corresponding genus or family names. The genera are colour coded based on their known primary metabolism: grey, versatile fermenters; dark green, phototrophs; blue aerobic bacteria; green, denitrifiers; light green, involved in the nitrogen cycle; yellow, can use H2 or acetate as electron donor; orange, strict anaerobes involved in sulfate reduction.
Table S1. MG-RAST annotation details of the reads associated with strains that have previously been demonstrated to be capable of dehalorespiration.
Table S2. Numeric data of concentrations of PCE and of its catabolites from the endpoints of the experiments (50 days).
Computer Program S1. 3D representation of the relative abundance of metabolic pathways in the different microcosms.