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HYDROCHEMICAL AND MICROBIAL EVOLUTION IN MICROCOSM EXPERIMENTS OF SITES CONTAMINATED WITH CHLOROMETHANES UNDER BIOSTIMULATION WITH LACTIC ACID

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HYDROCHEMICAL AND MICROBIAL EVOLUTION IN MICROCOSM EXPERIMENTS OF SITES CONTAMINATED WITH CHLOROMETHANES UNDER BIOSTIMULATION WITH LACTIC ACID

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Abstract

The aim of our research is to identify the sequence of degradation processes that lead to the selective enrichment of microorganisms involved in the degradation of carbon tetrachloride and chloroform under conditions of natural attenuation and lactic acid biostimulation. To this end, a comparative study using microcosm experiments were conducted to analyze these two scenarios. Microcosms were carried out with water and sediment from a field site located at a petrochemical complex. A significant finding of our work was the abiotic degradation of carbon tetrachloride induced by the biogenic activity of *Dechlorosoma suillum*. Although this is an abiotic degradation, the metabolism of this microorganism generates green rust precipitates, which in turn favor the abiotic reductive dechlorination of carbon tetrachloride. Another result was the identification of the biotic reductive dechlorination of chloroform by a bacterium of the *Clostridiales* order. Our study showed that the biostimulation with lactic acid produced faster degradation rates of carbon tetrachloride and chloroform. Lactic acid acted as an electron donor promoting the decrease in other electron acceptors such as nitrate and sulfate competing with chloromethanes. Biostimulation could, for this reason, be an efficient remediation strategy at sites contaminated by chloromethanes, especially in cases where a complex pollution history results in a rich hydrochemical background that potentially reduces natural attenuation.

Key terms: Microcosm, chloromethanes, natural attenuation, biostimulation, lactic acid, biotic and abiotic degradation, DGGE, *Dechlorosoma suillum sp, Clostridiales* order.

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1. Introduction

Carbon tetrachloride (CT) and chloroform (CF) are chlorinated solvents that have been widely used in metal degreasing, dry cleaning, and as refrigerants. These compounds are toxic, carcinogenic, and harmful to the ozone layer. Given their high density, 1.59 and 1.49 g/cm^3, respectively (Pankow and Cherry, 1996), these compounds can accumulate on the bottom of the aquifers. Their prolonged use on a large scale has resulted in many soil and groundwater contamination episodes (Penny et al., 2010).

Although these contaminants are very recalcitrant and pollute subsurface over long periods, they can be biologically degraded (McCarty and Semprini, 1994). For example, Criddle et al. (1990) reported degradation of CT under conditions of denitrification. Moreover, biostimulation, which promotes the optimal environmental conditions for the selective enrichment of indigenous microorganisms, has been used to degrade CT and CF. Numerous laboratory and field studies have been designed to examine the influence of substrate type, increase in nutrients (Devlin et al., 2000), and concentration of electron donors and electron acceptors (USEPA, 2004). The first study was conducted by Semprini et al. (1992), who used acetate as a substrate for growth and as an electron donor along with the nitrate and sulfate as electron acceptors. The experiment led to efficient *in situ* biodegradation of CT. Other biostimulation studies have demonstrated that CT and CF can be dechlorinated under anaerobic conditions in methanogenic (Mun et al., 2008), acetogenic (Egli et al., 1988), fermenting (Galli and McCarty, 1989), sulfate-reducing (Chung and Rittmann, 2008), and iron-reducing cultures (Picardal et al., 1993). In such experiments, CT and CF were sequentially reduced, giving rise to CF, dichloromethane (DCM), and even chloromethane (CM), CO_2, and CS_2 (carbon disulfide) (Hashsham et al., 1995).

Additionally, abiotic degradation of chloromethanes in the presence of iron-bearing soil minerals with high intrinsic reductive capacity has also been studied, as these minerals

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have been widely used for abiotic reductive degradation of organic contaminants in groundwater. Thus, surface associated Fe(II), magnetite (Fe_3O_4), FeS (mackinawite) and FeS_2, (pyrite), which can be common electron donors in the aquifer, have shown to enhance chloromethane degradation (Kriegman-King and Reinhard, 1994; Butler and Hayes, 2000; O'Loughlin et al., 2003; Danielsen and Hayes, 2004; Elsner et al., 2004a,b; McCormick and Adriaens, 2004; Maithreepala and Doong, 2005; Hanoch et al., 2006; Shao and Butler, 2009; Liang and Butler, 2010). Additionally, natural green rust minerals (GR) are other iron-bearing soil components with high intrinsic reductive ability. GR are layered double hydroxides with positively charged Fe(II)/Fe(III) hydroxide sheets interlayered with water molecules and anions (Hansen et al., 1996; Abdelmoula et al., 1998). Natural GR is formed by bioreduction of iron oxides (Ona-Nguema et al., 2002; Berthelin et al., 2006; O'Loughlin et al., 2007) and microbial biooxidation of Fe(II), as produced by *D. suillum* (Lack et al., 2002).

Compound specific isotope analysis (CSIA) has proved to be a powerful tool for characterizing the processes of biotic and abiotic degradation of chlorinated solvents (USEPA, 2008). In general, the degradation of these compounds is accompanied by a preferential degradation of molecules containing exclusively light carbon isotopes (i.e. ^{12}C). The result is a relative enrichment in heavy isotopes (i.e. ^{13}C) in the remaining contaminant pool. The enrichment factors in the abiotic degradation of CT, in the presence of iron complexes, have been well characterized by Zwank et al. (2005) and Elsner et al. (2004a). Of the few studies conducted on isotopic fractionation of CF owing to biotic dechlorination to form DCM that by Chan et al. (2012) is noteworthy.

Of the many laboratory studies on biotic and abiotic mechanisms of degradation of CT and CF, stand out those addressing the abiotic degradation of CT in the presence of GR (Liang and Butler, 2010) and the biogenic formation of GR (Lack et al., 2002). However, none of these two works or any other dealing with this subject, addresses the coupling of both mechanisms. Our study seeks to fill this gap in the literature by: i)

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examining whether this coupling can occur under natural attenuation conditions and, ii) assessing to what extent biostimulation can accelerate the biogenic formation of GR, and consequently the abiotic degradation of CT. On the other hand, to date, only the study of Chan et al. (2012) deals with the isotope fractionation during biodegradation or abiotic degradation of CF. Furthermore, these authors showed how a particular population of *Dehalobacter* (*Clostridiales* order) was able to biodegrade CF. Nevertheless, that study was focused on the evolution of CF as a single parent contaminant and, in addition, water or sediment from a real site were not used in the experiments, in contrast to our study. Therefore, results may not be fully representative of the natural conditions occurring in real sites. In such cases, the interactions that occur between chloromethanes and other electron acceptors could pose a problem when interpreting the results. For instance, although the reduction potential of CT and CF is higher than that of sulfates (Rijnaarts et al., 1998; de Best, 1999; de Best et al., 1999), inhibitory effects caused by competition for bioavailable electron donors between the dechlorinating and the sulfate-reducing populations can result in a high bioavailability of sulfates (Semprini et al.,1992; Picardal et al., 1993).

The purpose of our study was to identify the sequence of degradation processes leading to the selective enrichment of indigenous microbial communities involved in the degradation of CT and CF under DO conditions of reductive dechlorination. To this end, we have undertaken microcosm experiments with groundwater and sediment from a field site located at a petrochemical complex in which conditions throughout the year are reducing. Contaminants of diverse origin co-exist in this site (CT and CF as parent compounds) along with a rich hydrochemical background in nitrates and sulfates that potentially reduces natural attenuation of chloromethanes. The experiments have been centered on two scenarios: i) natural attenuation and ii) biostimulation of indigenous microbial communities to develop a rapid and selective enrichment of communities able to degrade CT and CF. The importance of studying these scenarios lies in the fact that

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the integrated study of these processes would help to better assess the potential of applying lactic acid biostimulation at field scale in contexts characterized by a hydrochemical background rich in nitrate and sulfate.

A significant finding of our work was the coupling between the biogenic formation of GR by *D. suillum* and the abiotic degradation of CT, which is a novelty of our study. Another novelty is that *D. suillum* used CT as electron acceptor at the end of the experiment to form CF. An additional finding was the apparent absence of isotopic fractionation of CF when was biodegraded in a context in which the concentration of this compound increased by the reductive dechlorination of CT.

2. Materials and Methods

2.1. Site description

Water and sediment used in the microcosm experiments were taken in an unconfined aquifer of Quaternary alluvial fan deposits outcropping in the La Pineda petrochemical complex (Tarragona, Spain), 100 km south of Barcelona. This petrochemical complex initiated its activities in stages, starting in 1960. A complex hydrogeochemical background (Table 1) characterizes the aquifer.

Table 1

From the source zone, the CT and CF free-phase of dense non-aqueous phase liquid (DNAPL) descended vertically. In this descent, free-phase left a trail of residual DNAPL in the sandy gravels and sands in the vadose zone and the saturated zone. As the free-phase descended, pools accumulated on discontinuous interlayered levels of low conductivity (reddish silts and clays), and eventually migrated towards the southeast

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owing to a slight dip in the sediments in this direction. Furthermore, Puigserver et al. (2013) showed that the chloromethane contamination affected not only the aquifer but also an underlying aquitard at this site. Despite the substantial reduction in concentrations in groundwater between 1997 and 2009 (Table S1 in the SD [Supplementary Data]), pollution continues to exceed the European groundwater quality standards.

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2.2. Microcosm experiments

2.2.1. Design of the experiments

Two microcosm experiments were conducted. The first simulated natural biotic and abiotic degradation of chloromethanes, i.e. the natural attenuation (NA experiment). The second simulated biostimulation of the indigenous microorganisms through addition of lactic acid (BLA experiment). A total volume of 10 mL of lactic acid was added as five additions of 2 mL (Sigma Aldrich, 85 % lactic acid).

Each of the two experiments consisted of two active tests (i.e. in which microorganisms were living) and two control tests (i.e. in which microorganisms were killed).

An autoclave (Selecta Model Autester 75 E DRY-PV) was used (in periods of 30 minute for two hours at a temperature of 121 °C, a pressure of 1 atm, and at saturated vapor conditions) to sterilize the control microcosm bottles containing sediment and 50 mL of stock solution 147 mM $HgCl_2$ (Riedel-Deha, Mercury II chloride puriss pa) as a bactericide, according to Trevors (1996). An autoclave (Selecta Model Autester 75 E DRY-PV) was used to sterilize the control microcosm bottles containing sediment. These bottels also contained 50 mL of stock solution 147 mM $HgCl_2$ (Riedel-Deha, Mercury II chloride puriss pa) as a bactericide, according to Trevors (1996). Autoclaving was performed for periods of 30 minute for two hours at a temperature of 121 °C, a pressure of 1 atm, and at saturated vapor conditions.

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Methanol (MeOH, Merck, ISO Pro analysis) was employed to clean and sterilize the remaining materials. Experiments were conducted in an anaerobic chamber (Coy Laboratory Products Inc.).

2.2.2. Sediment and groundwater used in the experiments

The sediment used in the experiments was obtained from cores recovered from boreholes drilled in the plume at the study site. Sediment cores were homogenized to obtain a fine sand with a silty matrix (f_{oc} = 0.03 %). Groundwater for the experiments was sampled in a piezometer located 2.5 m from the borehole. This groundwater initially showed oxidizing conditions, with dissolved oxygen (DO) concentration of 2.08 mg/L. This DO content was reduced to 0.70 mg/L by purging with N_2 gas (as described by Chen et al., 2008) for 60 min in series of 15 min to develop the most favorable conditions for the reductive dechlorination of chloromethanes. As the experiments were performed by adding pure phase of CT and CF (see section 2.3.3.), CT and CF in the groundwater sample were drastically decreased to have an only source of these compounds. This decrease was conducted during the same process of decline of DO. Thus, after purging, the concentrations of CT, CF, and DCM of the groundwater sample (14.2, 440.0 and 5.4 µg/L, respectively) were depleted to values below the Limit of Quantification.

2.2.3. Set up of the experiments

Each bottle was filled with 300 g of homogenized sediment and 1100 mL of groundwater, which represents 9.4 % and 55.0 % of the total volume of the bottle, respectively. As the bottles had a capacity of 2000 mL, the remaining 35.6 % was the anaerobic atmosphere of the chamber (95 % N_2 and 5 % H_2). To better reproduce the contamination at the site (i.e. the presence of pools of CT and CF, which slowly

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dissolve to give rise to the plume), we added pure phase of CT and CF. Thus, 10 μL of CT and 10 μL of CF (Sigma-Aldrich, Reagent grade, 99.9 %) were added at the start of the experiment. The isotopic compositions of $\delta^{13}C$ of CT and CF in groundwater used in microcosms before purging with N_2 gas were -39.3 ± 0.1 ‰ and -43.6 ± 0.1 ‰ for CT and CF, respectively. The isotopic compositions of $\delta^{13}C$ of pure phase of CT and CF added were -42.4 ‰ and -46.8 ‰, respectively. Bottles were sealed with Minivert® valves (SUPELCO analytical) and insulating tape. in addition, in the anaerobic chamber, all the bottles were arranged horizontally on shelves and covered by a thick black cloth to preserve maximum conditions of darkness.

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2.2.4. Water sampling for chemical and isotope analyses

Water samples from the two microcosm experiments were collected to study the time evolution of pH. Measurements were carried out inside the anaerobic chamber using a benchtop pH-Meter BASIC 20, Crison Instruments. Concentrations of the main inorganic electron acceptors in the experiments (sulfate, nitrate, and nitrite), acetate, lactate, CS_2, CT, CF, DCM, CM and the $\delta^{13}C$ of CT and CF were also determined. The low concentrations of DCM and the fact that all concentrations of CM were below the Limit of Quantification prevented us to ascertain the $\delta^{13}C$ of these compounds.

Sodium azide (N_3Na Fluka) was added to microcosm water samples immediately after being collected to inhibit bacterial activity, according to Trevors (1996). Before analyses, the vials containing the samples were stored in a cold chamber at 4 ºC in total darkness.

2.3. Compound specific isotope analysis

The determination of $\delta^{13}C$ of dissolved chloromethanes was carried out in duplicate by using the CSIA technique, which allows us to determine the isotopic signature of carbon by measuring the two stable isotopes, ^{12}C and ^{13}C. This relationship is

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expressed as $\delta^{13}C$ (in ‰ units) = $(R_{sample} / R_{standard} - 1) \times 1000$, where R_{sample} is the $^{13}C/^{12}C$ ratio in a given sample, and $R_{standard}$ is the $^{13}C/^{12}C$ ratio in the international standard V-PDB. Since molecules with light isotopes tend to react more rapidly, the isotopic ratio changes over time, which leads to an isotopic fractionation (α): $\alpha = R_a / R_b = (1000 + \delta^{13}C_a)/(1000 + \delta^{13}C_b)$, where R_a is the isotopic ratio of the compound at a particular time (t) or a compound in a well downstream from the source, and R_b is the isotopic ratio of the compound at time zero (t_0) or a compound in the contaminant source. For many organic pollutants, isotopic fractionation during biotic and abiotic degradation can be described as a Rayleigh process: $R_a = R_b \cdot f^{(\alpha-1)}$, where f is the relative concentration C/C_0 (normalized concentration), where C is the concentration of a compound at a given time, and C_0 is the concentration at time zero. Also, f = exp $(\delta^{13}C_{gw} - \delta^{13}C_{source})$, where $\delta^{13}C_{gw}$ is the isotopic composition of the organic compound in groundwater, and $\delta^{13}C_{source}$ is the isotopic composition of the organic compound in the source. Enrichment factor (ε): $\varepsilon = (\alpha - 1) \cdot 1000$ is, in a first approximation, a function of broken bonds during the process of degradation and can be used to distinguish reaction mechanisms (VanStone et al., 2007), pathways (Hirschorn et al., 2004) and kinetics of reactions (Sherwood Lollar et al., 2010).

2.4. Analytical techniques and protocols for chemical and isotope analyses

Concentration and isotope determinations were conducted at the laboratories of the Scientific and Technical Services of the Barcelona University (accredited by ISO 9001:2000). Sulfate, nitrate, and nitrite were analyzed following the EPA 9056 protocol; acetate according to Furlani et al. (2006); and VOCs by gas chromatography-mass spectrometry (GC-MS). To determine the $\delta^{13}C$ of chloromethanes the protocol used was based on the extraction of VOCs by direct adsorption from the aqueous phase. The extraction was made by inserting an adsorbent fiber (Supelco; SPME Fiber Assembly 75 um Carboxen PDMS) in the water sample, stored in a 100 mL amber glass bottle (SUPELCO analytical) closed with a silicone septum and maintained in agitation for 30 min to adsorb the chloromethanes. The determination of $\delta^{13}C$ was

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carried out by Gas Chromatography Combustion Isotope Ratio Mass Spectrometry (GC-C-IRMS) in accordance with the protocol described in Palau et al. (2007) and using a Delta C Finnigan MAT IRMS spectrometer.

2.5. Bacterial community analysis

Denaturing gradient gel electrophoresis (DGGE) analyses of water samples were also performed. In addition, DGGE analyses of sediment samples were undertaken at the start and the end of the experiments (see SD for detailed information). In the case of the BLA experiment, identification of microbial populations in the microcosm at the start and end of the experiment was carried out. Microbial bacterial population studies were performed by DGGE and clone library analyses. DGGE electrophoresis of PCR-amplified 16S rRNA genes were run in denaturing acrylamide gels and stained prior to photography according to standardized methodologies (see SD for further details). Clone libraries of PCR-amplified 16S rRNA genes of the whole bacterial populations were performed in pGEM-T vector according to standard methodologies. The number of analyzed clones was limited for practical reasons to 28 and 27, at the start and the end of the experiment, respectively. Rarefaction curves indicated that most of the bacterial population was represented by clone library, although saturation was not achieved (see section 4.7. in the SD). Inserts in clones were sequenced and assigned to microbial taxons by DNA sequence comparisons in genetic databanks (see SD for further details).

Additional information about analytical techniques and instrumentation for microbiological analyses of water and sediment samples is found in the SD.

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3. Results and Discussion

3.1. Degradation mechanisms of CT and CF in the NA and BLA experiments

The initial concentration of DO in the experiments (0.70 mg/L) agreed with the reducing conditions throughout the year at field scale in the plume (0.77 mg/L in average). In addition, the absence of oxygen in the anaerobic chamber where the experiments were carried out resulted in a rapid decrease of the initial concentration. Average values of 0.04 and 0.03 mg/L were attained in the active tests of the NA and BLA experiments, respectively, and 0.05 and 0.04 mg/L in the control tests, respectively. Thus, as regards the DO, conditions for reductive dechlorination were favorable throughout the experiments.

Considering that the experiments were developed in a reducing environment, the degradation mechanisms of CT and CF would have to fit these conditions. In this regard, Davis et al. (2003) reported the different mechanisms involved in the degradation of CT and CF in reducing environments and classified them into three categories. Studies conducted by other investigators thereafter (e.g. Elsner et al., 2004a; Maithreepala and Doong, 2005; Hanoch et al., 2006) have shown the validity of the classification of Davis et al. (2003). In summary form, this classification is as follows: 1) biologically-mediated reductive dechlorination of CT to form CF and DCM and reductive dechlorination of CT in abiotic systems to form, at least, CF. This mechanism is facilitated by Fe^{+2} in the presence of goethite and also in the presence of iron reduced minerals, including natural GR. 2) abiotic degradation by hydrolysis, which may generate CS_2 as an intermediate prior to CO_2 formation in reducing environments. 3) Reductive hydrolysis of CT to form CO and/or formic acid, which needs the formation of successively dechlorinated radical intermediates.

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3.1.1. Degradation mechanisms of CT

The CT pure phase added in the NA and BLA experiments progressively dissolved and reached the maximum concentrations, after which a decrease was observed in both experiments. Thus, in the NA experiment the decline was from day 33 to day 310 in the active tests (after which concentrations were no longer detectable, Figure 1A), and from day 62 to day 360 in the control tests (Figure 1B). In the BLA experiment the decline was prolonged from day 15 to day 260 in the active tests (after which concentrations were no longer detectable, Figure 1C), and from day 124 to day 360 in the control tests (Figure 1D). Based on the analysis of the evolution of CT in the active and control tests in the NA and BLA experiments, the decrease in CT can be mainly attributed to two degradation mechanisms. The first mechanism fitted the first category of the above classification and occurred in the active tests of both experiments, where increase in pH was registered (Figure S1B in the SD), indicating microbial activity. By contrast, pH remained constant in the control tests, with the exception of the initial decline due to the addition of lactic acid in the BLA. This mechanism led to the formation of CF and DCM (Figure 1A, C), which suggests that it corresponds to a process of reductive dechlorination. In this case, it is an abiotic process indirectly facilitated by biogenic activity at the start of the experiments and a biotic process at the end (see sections 3.5.1. and 3.5.2., respectively, for a discussion on the process and the microorganism involved).

The second mechanism took place mainly in the control tests of both experiments. It was not accompanied by an increase in CF (Figure 1B, D), and CS_2 was not generated (determinations were in all cases below the Limit of Quantification, i.e. 1.22 μg/L). The fact that no CF was formed is consistent with the third category, suggesting the mechanism involved being the reductive hydrolysis of CT to form CO and/or formic acid.

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Figure 1

3.1.2. Degradation rate constants and percentage of transformation of CT into CF

Once the corrections of the partition processes and mass-loss inherent in sampling were made (see section 2. in the SD for details), the decrease in CT, from the time when the added pure phase of CT was dissolved, aligned fairly well with first-order degradation kinetics. The degradation rate constants (K_{deg}) were lower in the NA experiment than in the BLA. Thus, K_{deg} were 0.034 (standard error R^2 = 0.96) and 0.032 (R^2 = 0.97) in the active and control tests of the NA experiment, respectively; and 0.041 (R^2 = 0.99) and 0.034 (R^2 = 0.95) in the active and control tests of the BLA, respectively. Furthermore, a higher percentage of the mass of CT added at the start of the active tests in the NA than in the BLA remained at the end (0.06 % and 0.01 %, respectively; Table 2). Of the rest of the mass, the percentage degraded in the NA was lower than in the BLA (39.82 % and 43.22 %, respectively; Table 2).

Table 2

The earlier decrease in CT in the active BLA than in the active NA (initiated at days 15 and 33, respectively) along with the more rapid decline in the active tests of the BLA than in the NA (K_{deg} of 0.041 and 0.034, respectively) show that lactic acid biostimulation accelerated the degradation of CT. In addition, the degradation process was more efficient than in the case of natural attenuation, as the percentage of remnant mass of CT was 0.01% at day 260, in the active tests of the BLA, whereas it was of 0.06 % at day 310, in the active tests of the NA, Table 2.

Moreover, the more rapid decrease in CT in the active tests in the BLA than in the NA (Figure 1A, C) resulted in a significant consumption of lactate in the BLA. The reduction

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3 of this compound was 68.1 % of the total mass injected. However, lactate was also
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5 used as electron donor in other redox processes (i.e., CF dechlorination, denitrification,
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7 and sulfate-reduction, see sections 3.1.3. and 3.2.), which justifies the longer lag phase
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9 in the BLA experiment (62 days) than in the NA (33 days). Additionally, the lag phase
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11 was shorter in the active tests of both experiments (Figure 1A, C) than the time elapsed
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13 before the decline of CT in the control tests (Figure 1B, D).
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18 **3.1.3. Degradation mechanism of CF**
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20 As in the case of CT, the added CF pure phase progressively dissolved and reached a
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22 maximum concentration. After this maximum a decrease was only observed in the
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24 active tests (Figure 1A, C) as CF concentrations remained constant in the control tests
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26 throughout the experiments. Thus, in the NA experiment the decline was prolonged
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28 from day 33 to day 360 (Figure 1A), and from day 15 to day 260 (after which
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30 concentrations were no longer detectable, Figure 1C). CF was transformed into DCM,
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32 which increased in parallel to the decline of CF until day 166 in the active tests of both
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34 experiments (Figure 1A, C). After that day, DCM decreased until day 360
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36 (concentration of degradation products of this compound were below the Limit of
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38 Quantification). In contrast, concentrations were always below the Limit of
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40 Quantification in the control tests (Table S2 in the SD). Based on the analysis of the
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42 evolution of CF in the active and control tests in the NA and BLA experiments (Figure
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44 1A, B and Figure 1C, D, respectively), the decrease in CF can be attributed to an only
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46 mechanism. This mechanism fitted the first category of the aforementioned
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48 classification, and it occurred in only the active tests of both experiments (CF did not
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50 vary in the controls tests). As CF was transformed into DCM, the degradation
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52 mechanism corresponds to biologically-mediated reductive dechlorination (see section
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54 3.5 for a discussion on the microorganism involved).
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3.1.4. Degradation rate constants and percentage of transformation of CF into DCM

From the time when the added pure phase of CT was dissolved, the decrease in CF, aligned fairly well with first-order degradation kinetics (see section 2. in the SD for details). The degradation rate constants were lower in the NA experiment than in the BLA. Thus, K_{deg} were 0.031 (R^2 = 0.96) and 0.046 (R^2 = 0.97) in the active test of NA and BLA, respectively. Moreover, a higher percentage of the mass of CF added at the start of the active tests in the NA than in the BLA remained at the end (0.05 % at day 360 and 0.01 % at day 260, respectively; Table 2). Of the rest of the mass, the percentage degraded in the NA was lower than in the BLA (64.14 % and 66.76 %, respectively; Table 2).

As in the case of CT, the earlier decrease of CF in the active BLA than in the active NA (initiated at days 15 and 33, respectively), along with the more rapid decrease in the active tests of the BLA than in the NA (K_{deg} of 0.046 and 0.031, respectively) show that biostimulation with lactic acid as an electron donor accelerated the degradation of CF, which is consistent with the aforementioned percentage in lactate consumed during the experiment. Therefore, biostimulation was more efficient than in the case of natural attenuation, as the percentage of remnant-mass of CF was 0.01% at day 260, in the active tests of the BLA, whereas it was of 0.05 % at day 360 in the active tests of the NA (Table 2).

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3.2. Interactions between dechlorination and other redox reactions

In addition to CT and CF, other electron acceptors were present at the start of the experiments, i.e. nitrate and sulfate (0.29 and 2.73 mmol/L, respectively). The presence of these compounds suggests competition for available electrons between denitrifying, sulfate-reducing and microorganisms that promote dechlorination. A result

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of this would be a change in redox conditions and a variation in the microbial community composition.

Of these electron acceptors, nitrate played the biggest role in the first days of the experiments. Thus, nitrate concentrations decreased over time in the active tests of the NA and BLA (Figures S2A and S3A in the SD, respectively) owing to denitrification. Consequently, this decline was accompanied by a gradual increase in nitrite concentration until day 62 and 15 in the NA and BLA experiments. The subsequent decrease in nitrite concentration indicates that lactic acid (as demonstrated by Takahashi et al., 2009). Nitrate concentrations remained constant over time in the control tests (Figures S2B and S3B in the SD).

Sulfate evolution in the active tests of the NA was relatively constant until day 133, coinciding with the denitrification process (Figure S2A in the SD) and the decrease in CT and CF (Figure 1A). After that day, sulfate concentration significantly decreased (Figure S2A in the SD). This decline in sulfate shows that sulfate-reduction activates when denitrification processes have substantially reduced the nitrate concentration. The decline in sulfate is evidence of competition between denitrifying and sulfate-reducing microorganisms for available electrons (Laverman et al., 2012). As in the case of nitrate, the decrease in sulfate occurred much earlier in the active tests of the BLA (after day 33; Figure S3A in the SD) than in the active tests of the NA (after day 133; Figure S2A in the SD). In addition, in the active tests of the BLA, the substantial decrease in CT and CF occurred from day 62 (Figure 1C), coinciding with the sulfate-reduction process (Figure S3A in the SD). Additionally, the sulfate decrease was accompanied by an increase in acetate in the active tests in both experiments (Figure S2A and S3A in the SD). Part of this acetate proceeded from the lactate fermentation (31.9 % of the added lactate remained at the end of the experiment). The increase in acetate reveals that the fermentation processes supply electrons to the medium, favoring sulfate-reducing conditions (Liamleam and Annachhatre, 2007).

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The earlier sulfate decline in the BLA considerably improved the efficiency of CT and CF degradation (Figure 1C) given that the competition for electrons with CT and CF was minimized (see section 4.3. in the SD). Consequently, the degradation processes of CT and CF were initiated before and at faster degradation rates than in the case of the NA.

3.3. Isotopic fractionation caused by the degradation mechanisms

Results showed that reductive dechlorination of CT caused isotopic fractionation of this compound in the active and control tests of the NA and BLA (Figure 2). Although the degradation rate of CT was higher in the active tests of the BLA (K_{deg} of 0.041 and 0.034 in the BLA and NA, respectively, see section 3.1.2.), the isotope enrichment factors were similar in the active tests of the NA and BLA (about -1.8 ± 0.5 ‰ and -1.9 ± 0.2 ‰, respectively). This suggests that, though in the active tests (especially in the BLA) part of the degradation is induced by biogenic activity (see section 3.1.1.), the isotopic fractionation of CT in the active and control tests is mainly attributable to abiotic degradation.

Figure 2

As for CF, albeit our data showed biotic degradation of CF in support of previous studies (e.g., Ciavarelli et al., 2012; Chan et al., 2012, Lee et al., 2012, Lima and Sleep, 2010), the $\delta^{13}C$ of CF did not vary substantially (Figure 2). This led to the lack of isotopic fractionation, which differs from the observations of Chan et al. (2012) who, in contrast to us, used CF as the only parent compound susceptible to undergo isotopic fractionation as it biodegraded. However, as discussed below, this lack of isotopic fractionation may be apparent as for metabolites produced by reductive dechlorination

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a straightforward application of the Rayleigh equation is not strictly possible. This inapplicability of the Rayleigh equation is due to the variation of the isotope ratio in these compounds owing to: i) the combined effects of isotopic fractionation during its production from the degradation of the parent compound, and ii) its own ongoing degradation. Moreover, when a compound has different origins, it is not easy to interpret its behavior because it is necessary to ascertain whether this compound is parent or metabolite (USEPA, 2008). Thus, the degradation of CT led to the formation of CF that was lighter than its parent. Simultaneously, this CF was degraded to DCM (Figure 1A, C), which is the reason why the Rayleigh equation cannot be applied. In addition, the CF that was injected at the start of the experiment was also biodegraded to DCM, and the CF remaining in the system was isotopically enriched. The presence of these two CF of different origin and different isotopic composition can offset the isotopic enrichment of CF that was initially injected. This offset prevented us from observing the isotopic fractionation of CF in the experiments, which constitutes a significant finding of our work and contrasts with previous observations of other authors.

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3.4. Selection of microorganisms as a consequence of the evolution of chloromethanes, nitrates and sulfates

DGGE profiles showed that the number of bands was greater at the start than at the end of the experiment (Figures 3 and 4), which indicates enrichment. Thus, DGGE in the NA (Figure 3) showed that the most significant changes in the population took place between day 62 (after the maximum concentration of CT and CF occurred; Figure 1A) and day 166. By contrast, in the BLA (Figure 4), there was considerable development of microorganisms until day 62, and then selective enrichment of microorganisms occurred in parallel to the fall of CT and CF (Figure 1C). The addition of lactic acid in the BLA from the start of the experiment led to the enrichment of some

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groups of microorganisms that competed for bioavailable electron donors with microorganisms able to biodegrade chloromethanes. However, after day 62, the high concentrations of CT and CF were toxic to the non-halorespiring communities (Eastmond, 2008) in both experiments, favoring the selection of other communities. Thus, a conspicuous band, which was found throughout the active tests in water and sediment samples showed an increase in the intensity in the NA. This increase was markedly appreciable after day 166, which is evidence of selection of microorganisms. This band (which is depicted by an arrow in Figures 3 and 4 and an asterisk in Figure S6, in the SD) was subsequently found to match the electrophoretic mobility of the operational taxonomic unit 6 (OTU 6) (*Dechlorosoma suillum*) (see section 3.5. and Table 3). In addition, the changes in microbial population in the NA coincided with the decline in nitrates and sulfates (Figure S2A in the SD), appreciable after day 133. In the case of the BLA, at days 166, 260 and 360 a progressive selective enrichment of OTU 6 and OTU 15 (*Clostridiales* bacterium) was observed (Figure 4, section 3.5. and Table 3) coinciding with the decline in nitrates and sulfates (Figure S3A in the SD).

Dechlorination of CT and CF in the active NA and BLA commenced when denitrification took place (Figures 1A and S2A in the SD, and Figures 1C and S3A in the SD, respectively). However, it increased after sulfate-reduction in the active BLA (Figure 1C and Figure S3A in the SD). Consequently, the addition of lactic acid (as electron donor) accelerated the exhaustion of other electron acceptors, inducing the earlier selective enrichment of the flora that directly or indirectly reduces CT and CF (Figure 4).

Figure 3

Figure 4

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In summary, selection is a result of: i) the enrichment of halorespiring and sulfate-reducing communities. The latter disappears or remains as a minority when bioavailable sulfate is depleted, which promotes even more the enrichment of OTU 6 and OTU 15, and ii) the toxicity of CT and CF to the different communities at particular concentrations.

3.5. Microbial community structure and dynamics in the biostimulation experiment

A total of 28 clones from the clone library of the initial population at the start of the experiment (t=0 days) and 27 of the final population at the end (t=360 days) were analyzed in the BLA experiment. The rarefaction curve in Figure S5, in the SD, shows that nine of the 28 clones and eight of the 27 clones analyzed at the start and end of the experiment, respectively, were different.

DGGE profiles showed a heterogeneous variety of bands in which duplicates presented a striking similarity of bands, which indicates substantial stability of the microbial community (Figures 3 and 4). As regards the identified OTU, we describe those whose role was noteworthy at the start and end of our experiment.

3.5.1. At the start of the experiment (day 0)

At the start of the experiment, during which denitrification was observed (see section 3.2.), the dominant taxonomic group was the *Betaproteobacteria* class of bacteria (67.9 % of the clones, Table 3). The presence of *Betaproteobacteria* in nitrate reduction conditions is consistent with previous studies that showed members of the *Betaproteobacteria* to be predominant in enrichment cultures of denitrifying bacteria (Heylen et al., 2006).

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The *Methylophilaceae* family (OTU 2 and OTU 3, 39.3 %) is noteworthy in the *Betaproteobacteria* class. This family includes some, but not all of the methylotrophic bacteria, which are microorganisms that are capable of growing on chloromethanes. The presence of this family is consistent with the history of the contamination of the site that is highly abundant in chloromethanes. Minor proportions of other *Betaproteobacteria* were also found (OTU 5, OTU 6, OTU 7, and OTU 9, Table 3).

Table 3

OTU 6 (3.6 %), identified as *Azospira* (synonym: *Dechlorosoma*, a genus of the family *Rhodocyclaceae*) is noteworthy for two reasons: i) its corresponding DGGE band was present in all samples taken at different times, and ii) it became one of the most intense bands at the end of the experiment (Figure 4). The genus *Azospira* contains some perchlorate-reducing strains of bacteria isolated from a waste treatment lagoon. These strains were initially termed *D. suillum* (Achenbach et al., 2001; Tan et al., 2003). This microorganism has also been detected at field scale in groundwater contaminated by chlorinated solvents (Zemb et al., 2010). In addition, this is a respiring heterotrophic microorganism that can use different electron acceptors other than oxygen (nitrate, chlorate, and perchlorate). Additionally, it is capable of using Fe(II) as electron donor (Achenbach et al., 2001, Chaudhuri et al., 2001; Lack et al., 2002). In addition, the metabolism of this microorganism generates precipitates of GR (Lack et al., 2002). Furthermore, *D. suillum* has been found to be associated with nitrate-dependent Fe(II) oxidizing microorganisms in sediments, which use nitrate as electron acceptor (Lack et al., 2002). This association between *D. suillum* and denitrifying microorganisms is consistent with the denitrification process that occurred at the beginning of our experiments (see section 3.2.) and highlights the central role played by this microorganism in the degradation of CT. Thus, in parallel with denitrification, the

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metabolic processes of *D. suillum* would have led to the precipitation of GR (Lack et al., 2002), which in turn could favor the abiotic dechlorination of CT, according to Liang and Butler (2010). This degradation mechanism is an abiotic reductive dechlorination of CT induced by the biogenic activity of *D. suillum* that led to the formation of CF (see section 3.1.1.). This abiotic dechlorination was observed particularly in the BLA experiment, in which a higher increase in *D. suillum* than in the NA experiment was seen over time. This was a significant finding of our work as the abiotic degradation of CT in the presence of GR described by Liang and Butler (2010) couples with the biogenic formation of GR by *D. suillum*, described by Lack et al. (2002). This is the first time that this coupling process is described using samples of a real site, which is a novelty of our research.

Finally, the presence of the *Clostridia* class was also identified (OTU 4, 7.1 % of the clones, which belongs to the *Clostridiales* order of gram-positive bacteria).

3.5.2. At the end of the experiment (day 360)

The increase in the percentage of *D. suillum* at the end of the experiment (OTU 6, 11.1 %, Table 3) suggests that the addition of lactic acid favors the selective enrichment of this bacterium and promotes the abiotic degradation of CT via the formation of GR. As stated above (see section 3.5.1.), *D. suillum* would be associated with nitrate-dependent Fe(II) oxidizing microorganisms, which use nitrate, chlorate, or perchlorate as electron acceptors (Achenbach et al., 2001). However, since nitrate exhaustion occurred along the experiment, this microorganism has had to use an electron acceptor other than nitrate at the end of the experiment, with CT being the only available electron acceptor. The use of CT as electron acceptor is another novelty,

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which to date has not been reported. This biotic degradation of CT would have resulted in GR formation and increase of the percentage of *D. suillum*.

The most frequently detected group of bacteria at the end of the experiment was *Clostridiales* of the phylum *Firmicutes* (OTU 12, OTU 13, OTU 14 and OTU 15, Table 3). Notably, the reductive dechlorination of CT and CF by a respiratory process has been described in some members of *Clostridiales*, i.e., genus *Dehalobacter* (Grostern and Edwards, 2006; Grostern et al., 2010; Justicia-Leon et al., 2012; Lee et al., 2011; Chan et al., 2012). Moreover, *Clostridium* species can co-metabolically degrade CT (Galli and McCarty, 1989; Lima and Sleep, 2010). So, it is reasonable to assume that one or several of the *Clostridiales* microorganisms found in our microcosms is responsible for the reductive dechlorination of CT, and especially of CF, thus favoring its enrichment.

OTU 15 (7.4 %, the remaining OTU of the *Clostridiales* order are described in the SD) showed a high identity (98 %) with a sequence from an uncultured microorganism of an anaerobic microbial community of a tar oil contaminant plume. This OTU is identified in DGGE profiles of Figure 4 in which it appears clearly after day 166, when nitrate and sulfate concentrations become drastically reduced (Figure S3A in the SD). In parallel, CF concentrations, which sharply decreased after day 62, were accompanied by an increase in DCM (Figure 1C). This increase suggests that this bacterium of the *Clostridiales* order plays a role in the CF reductive dechlorination process to form DCM in the microcosm. CF would have to become isotopically heavier as a result of this degradation process, as seen by Chan et al. (2012). However, as discussed above in section 3.3., there was an apparent lack of isotopic fractionation.

Finally, OTU 16 (3.7 %) represent the *Brevundimonas* sp (*Alphaproteobacteria*). Krausova et al. (2006) discovered this species in a consortium consisting of DCM degrading *Pseudomonas* sp. and *Brevundimonas* sp.

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4. Conclusions

DGGE profiles showed that the number of bands was higher at the start of the experiment than at the end. This greater number of bands demonstrates the occurrence of selection, which was a consequence of the enrichment of halorespiring and sulfate-reducing communities. The latter disappeared or were reduced to a minority when bioavailable sulfate was depleted. This boosted the growth *of D. suillum* (OTU 6) and a bacterium of the *Clostridiales* order (OTU 15). Furthermore, given that CT and CF can be toxic to the non-halorespiring communities, selection of the community of halorespiring bacteria was favored.

The degradation of CT to form CF was mainly caused by abiotic reductive dechlorination of this compound induced by the biogenic activity of *D. suillum*. This implies that the abiotic degradation of CT in the presence of GR coupled with the biogenic formation of GR by this microorganism. This coupling occurred particularly in the BLA experiment, in which a higher increase in *D. suillum* than in the NA was observed over time. The addition of lactic acid, as electron donor, accelerated the exhaustion of other electron acceptors, inducing earlier enrichment of the flora that directly or indirectly reduces CT and CF and faster degradation rates. Moreover, the central role played by *D. suillum* was revealed using field samples, which represents one novelty of our work along with the fact that *D. suillum* was capable of using CT as electron acceptor when nitrate was exhausted (which to date has not been reported in the literature).

It is worth highlighting that the described processes occurred in combination: i) the biogenic formation of GR coupled with the abiotic reductive dechlorination of CT to form CF, ii) the combination of this coupling process to, in parallel, transform CF by biotic reductive dechlorination to form DCM (which in turn also degrades).

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The degradation of CF was due to biotic reductive dechlorination, which transformed it into DCM because of the respiratory process of a bacterium of the *Clostridiales* order. Biostimulation with lactic acid accelerated this degradation. However, the interactions between degradation of CT and CF in a context in which the bioavailability of CF was increased by the abiotic reductive dechlorination of CT offset the isotopic enrichment of CF. This offset resulted in the apparent lack of isotopic fractionation of this compound, which constitutes another novelty of our work and contrasts with previous observations of other authors.

Our findings have significant environmental implications in terms of the assessment of the CT and CF contamination and of the biostimulation in anaerobic subsurface environments where nitrate and sulfate are present. However, as biostimulation increases mobility of the degradation products of CT and CF at laboratory scale, to better assess the potential of applying lactic acid biostimulation at field scale, further study on the fate and transport of these metabolites in such environments is necessary.

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TABLE CAPTIONS:

Table 1: Maximum, minimum and average concentration values of the main inorganic and organic compounds constituting the hydrochemical background of the site.

Table 2: Mass fractions (%) in which CT and CF originally dissolved in the NA and BLA experiments were distributed after the end day of the experiment (360) or after day in which concentrations were no longer detectable.

Table 3: Sequence analysis of clones detected in the biostimulation experiment at the start and end times in water (day 0 and day 360, respectively). OTU = Operational taxonomic unit.

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FIGURE CAPTIONS:

Figure 1: Evolution of chloromethane concentrations measured in water in the microcosm experiments (no partition correction included). Concentrations in DCM were below the Limit of Quantification. A) active tests in the NA experiment, B) control tests in the the NA experiment, C) active tests in the BLA experiment, and D) control tests in the BLA experiment.

Figure 2: Evolution of the $\delta^{13}C$ of CT and CF in microcosm experiments. A) active tests in the NA experiment, B) control tests in the NA experiment, C) active tests in the BLA experiment, and D) control tests in the BLA experiment.

Figure 3: DGGE profiles of the amplified 16S rDNA of water samples of the active test duplicates of the NA experiment. Values at the top indicate sampling time in days after the start of the experiment. OTU = Operational taxonomic unit. OTU 6 is a recombinant clone identified as *Dechlorosoma suillum* (Table 3).

Figure 4: DGGE profiles of the amplified 16S rDNA of water samples of the active test duplicates of the BLA experiment. Sampling time in days after the start of the experiment. OTU = Operational taxonomic unit. OTU 6 (A) and OTU 15 (B) are recombinant clones identified as *Dechlorosoma suillum* and a bacterium of the *Clostridiales* order, respectively (Table 3).

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Table 1

(mg/L)	NO_3^-	NO_2^-	NH_4^+	SO_4^{2-}	Cl	TOC
Max	111.48	0.27	13.87	271.22	847.23	303.00
Min	<0.1	<0.1	<0.1	25.69	115.86	1.84
Average	48.72	0.18	4.58	140.89	368.28	101.23
(µg/L)	**1,1 DCA**	**TCE**	**PCE**	**trans-DCE**	**cis-DCE**	**VC**
Max	2429.07	7.81	5.28	2.54	379.52	20.06
Min	11.23	3.38	2.20	<0.5	2.04	<0.5
Average(*)	425.18	5.66	3.12	2.16	60.32	9.46
(µg/L)	**DCM**	**Benzene**	**Toluene**	**Ethylbenzene**	**o-xylene**	**p-xylene**
Max	5.08	2.70	1337.06	2681.48	4768.51	920.32
Min	1.96	<0.5	<0.5	<0.5	<0.5	<0.5
Average(*)	3.43	2.45	506.00	642.11	905.36	224.13
(µg/L)	**Chlorobenzene**	**Total trichlorobenzene**	**Propylbenzene**	**Total butylbenzene**	**Hexachlorobutadiene**	**Naphthalene**
Max	6.83	9.00	156.51	4.97	2.98	229.02
Min	<0.5	<0.5	<0.5	<0.5	<0.5	2.02
Average(*)	4.41	6.29	79.65	2.98	2.65	79.08

(*) Values below the Limit of Quantification were not considered in this calculation.

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Table 2

	CT	CT	CF	CF
	Active tests	Active tests	Active tests	Active tests
	NA at day 310	BLA at day 260	NA at day 360	BLA at day 260
Loss-mass inherent in sampling (%)	25.90	26.17	30.80	30.05
Remnant-mass at the end of the experiment (%)	0.06	0.01	0.05	0.01
Water-gas mass partitioned (%)	32.60	28.70	4.85	3.02
Water-soil mass partitioned (%)	1.62	1.90	0.16	0.16
Loss-mass due to degradation (%)	39.82	43.22	64.14	66.76

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Table 3

OTU*	Frequency (%)		Nearest relative in GenBank (accession number)	% identity	Taxonomic group[†]
	t= 0 days	t= 360 days			
1	21.4	n.d.[#]	Uncultured bacterium clone EDW07B001_110 (HM066260.1)	96	*P. Chlorobi*, C. *Ignavibacteria*, O. *Ignavibacteriales*
2	10.7	n.d.	Uncultured bacterium clone MA-63-I98C (HM141874.1)	99	*P. Proteobacteria*, C. *Betaproteobacteria*, O. *Methylophilales*, F. *Methylophilaceae*
3	28.6	n.d.	Uncultured beta proteobacterium clone MKC1 (EF173332.1)	99	*P. Proteobacteria*, C. *Betaproteobacteria*, O. *Methylophilales*, F. *Methylophilaceae*
4	7.1	n.d.	Iron-reducing bacterium enrichment culture clone HN-HFO91 (FJ269102.1)	94	*P. Firmicutes*, C. *Clostridia*, O. *Clostridiales*
5	7.1	n.d.	*Variovorax* sp. S24561 (D84645.2)	99	*P. Proteobacteria*, C. *Betaproteobacteria*, O. *Burkholderiales*, F. *Comamonadaceae*
6	3.6	11.1	*Dechlorosoma suillum* PS, complete genome (CP003153.1)	99	*P. Proteobacteria*, C. *Betaproteobacteria*, O. *Rhodocyclales*, F. *Rhodocyclaceae*, *Azospira*
7	14.3	n.d.	*Rhodocyclus* sp. HOD 5 (AY691423.1)	99	*P. Proteobacteria*, C. *Betaproteobacteria,* O. *Rhodocyclales*, F. *Rhodocyclaceae*
8	3.6	n.d.	*Magnetospirillum* sp. 16S rRNA gene, strain MSM-4 (Y17390.1)	98	*P. Proteobacteria*; C. *Alphaproteobacteria*; O. *Rhodospirillales*; F. *Rhodospirillaceae*; *Magnetospirillum.*
9	3.6	n.d.	*Hydrogenophaga taeniospiralis* gene for 16S rRNA, partial sequence, strain:NBRC 102512 (AB681846.1)	100	*P. Proteobacteria;* C. *Betaproteobacteria;* O. *Burkholderiales;* F. *Comamonadaceae*
10	n.d.	7.4	Uncultured bacterium clone OTU-X4- 10 16S rRNA gene (JQ668611.1)	99	*P. Chloroflexi*, C. *Anaerolineae*, O. *Anaerolineales*; F. *Anaerolineaceae*
11	n.d.	7.4	Uncultured *Bacteroidetes* bacterium partial 16S rRNA gene, clone LiM 11H12 (FN646437.1)	96	*P. Bacteroidetes,* O. *Cytophagales,* F. *Cytophagaceae, Meniscus*
12	n.d.	14.8	Uncultured bacterium clone 50 (EF644507.1)	97	*P. Firmicutes*, C. *Clostridia*, O. *Clostridiales*, F. *Syntrophomonadaceae*, *Syntrophomonas*
13	n.d.	37	Bacterium enrichment culture clone T12RRH100B11 (HQ896303.1)	98	*P. Firmicutes*, C. *Clostridia*, O. *Clostridiales*, F. *Peptococcaceae*
14	n.d.	11.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 12TCLN406 (AB637332.1)	93	*P. Firmicutes*, C. *Clostridia*, O. *Clostridiales*, F. *Clostridiaceae*, *Oxobacter*
15	n.d.	7.4	Uncultured *Clostridiales* bacterium clone D12_34 small subunit ribosomal RNA gene, partial sequence (EU266838.1)	98	*P. Firmicutes*, C. *Clostridia*, O. *Clostridiales*, *Ruminococcaceae*
16	n.d.	3.7	Uncultured bacterium clone NK-M23 16S ribosomal RNA gene, partial sequence (JN685485.1)	99	*P. Proteobacteria*, C. *Alphaproteobacteria*, O. *Caulobacterales*, F. *Caulobacteraceae*, *Brevundimonas*

* An OTU is defined by a minimum 2.5 % sequence dissimilarity to any other OTU in this work, and as a proxy of a species

[#] not detected

[†] Deduced after SINA online comparisons to the SILVA seed reference alignment (www.arb-silva.de)

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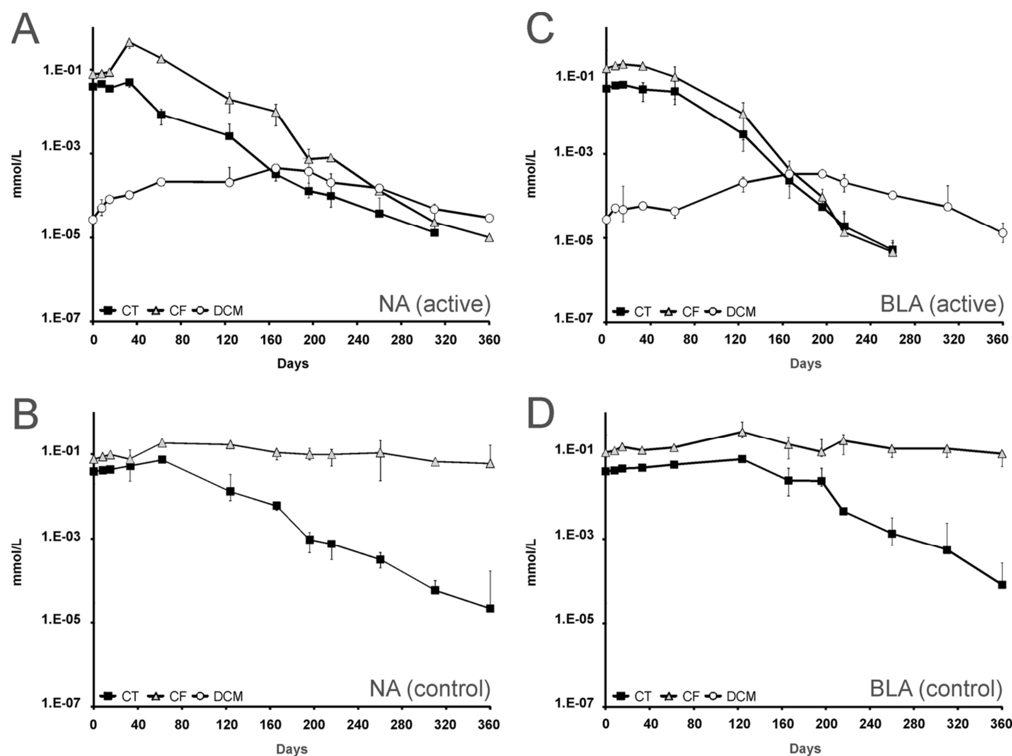

Figure 1: Evolution of chloromethane concentrations measured in water in the microcosm experiments (no partition correction included). Concentrations in DCM were below the Limit of Quantification. A) active tests in the NA experiment, B) control tests in the the NA experiment, C) active tests in the BLA experiment, and D) control tests in the BLA experiment.
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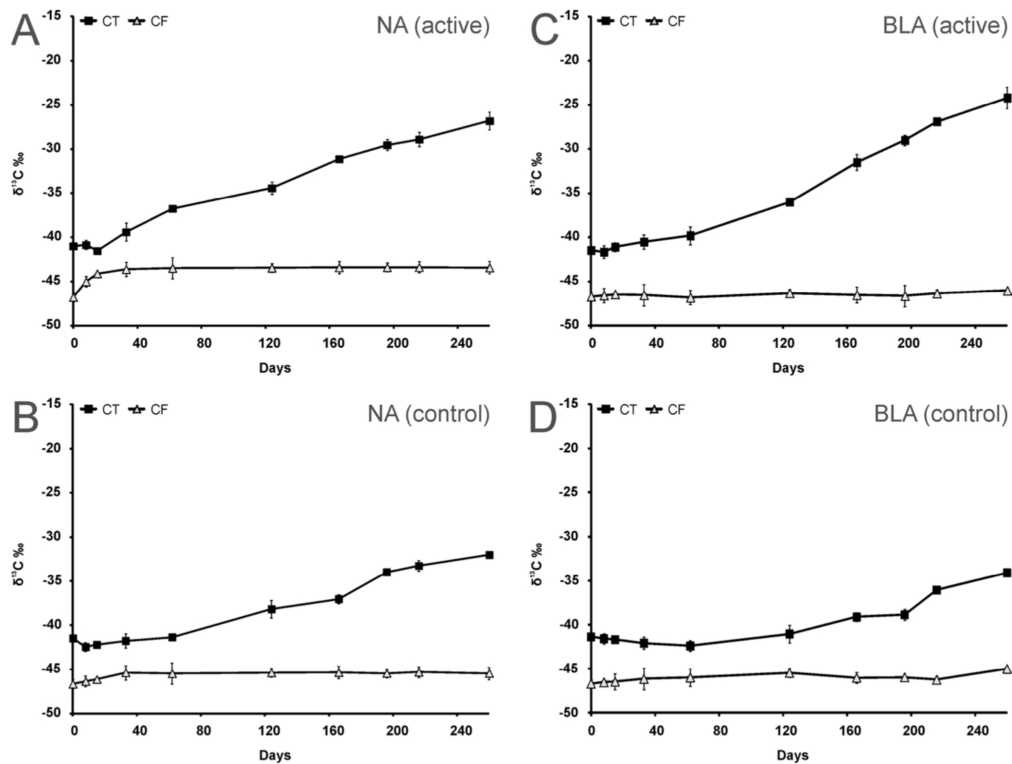

Figure 2: Evolution of the $\delta^{13}C$ of CT and CF in microcosm experiments. A) active tests in the NA experiment, B) control tests in the NA experiment, C) active tests in the BLA experiment, and D) control tests in the BLA experiment.
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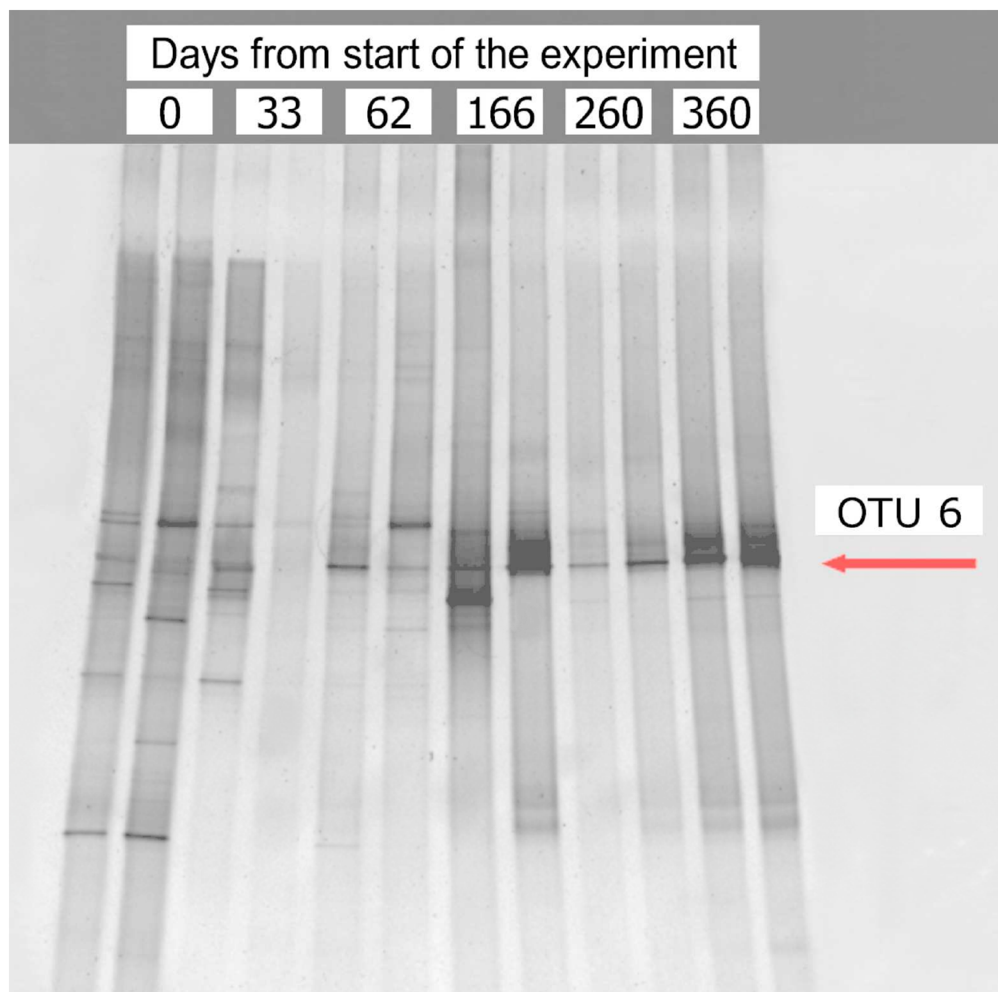

Figure 3: DGGE profiles of the amplified 16S rDNA of water samples of the active test duplicates of the NA experiment. Values at the top indicate sampling time in days after the start of the experiment. OTU = Operational taxonomic unit. OTU 6 is a recombinant clone identified as *Dechlorosoma suillum* (Table 3).
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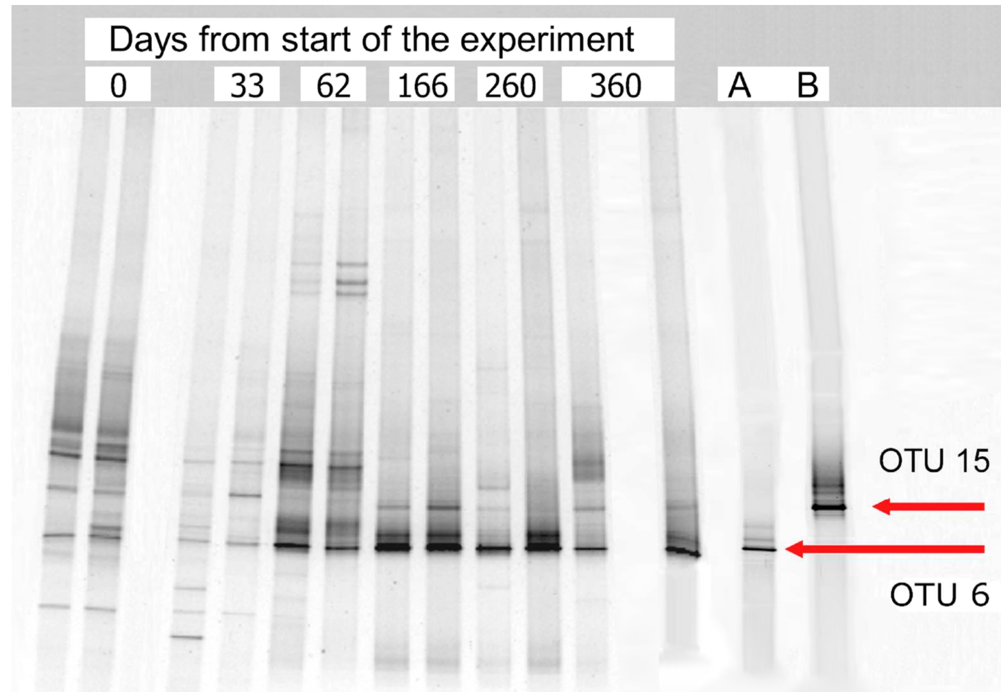

Figure 4: DGGE profiles of the amplified 16S rDNA of water samples of the active test duplicates of the BLA experiment. Sampling time in days after the start of the experiment. OTU = Operational taxonomic unit. OTU 6 (A) and OTU 15 (B) are recombinant clones identified as *Dechlorosoma suillum* and a bacterium of the *Clostridiales* order, respectively (Table 3).
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HYDROCHEMICAL AND MICROBIAL EVOLUTION IN MICROCOSM EXPERIMENTS OF SITES CONTAMINATED WITH CHLOROMETHANES UNDER BIOSTIMULATION WITH LACTIC ACID

(SUPPLEMENTAL DATA)

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1. Site description: supplementary table

Table S1: Time evolution of carbon tetrachloride (CT) and chloroform (CF) concentrations in the plume.

	CT (μg/L)				CF(μg/L)			
	1997	**2006**	**2008**	**2009**	**1997**	**2006**	**2008**	**2009**
Max	771.0	160.0	258.8	308.2	19370.0	960.0	1278.7	552.1
Min	7.0	ND	ND	ND	843.0	1.0	2.2	6.9
Average	196.0	26.0	64.9	66.3	11700.0	79.0	251.6	145.8

ND = Not detected

2. Materials and methods (evaluation of partition processes)

Part of the decrease in CT and CF is due to water phase-gas phase partition and water phase-soil phase partition as well as to the loss-mass inherent in the water sampling. The processes of partition were evaluated on the assumption that they followed models governed by Henry's Law (water-gas partition) and linear isotherm (water-soil partition). Thus, in the case of active tests of the NA and BLA experiments, the fractions in which the CT and CF dissolved were distributed can be observed in Table 2 of the main text. These fractions are are presented on day 310 (in the case of CT) and 360 (end of the experiment, for CF) in Table 2, after which concentrations were no longer detectable.

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3. Materials and methods (microbiological analyses)

3.1. DNA extraction from microbial populations in microcosms

Total DNA from the microbial populations was extracted by the Power Soil® DNA isolation kit (Mo Bio Laboratories), following the manufacturer's instructions. For sediment samples, a total of 0.25 g was used for DNA extraction. For liquid samples, 3 mL of the watery supernatant of the microcosms were filtered through 0.2 µm pore size membranes (Millipore), which were stored at -20 °C until DNA extraction. The purified DNA was finally recovered in 50 µL of sterile milliQ-water and stored at -20°C.

3.2. PCR amplification of 16S rDNA genes from microbial populations

Eubacterial 16S rRNA gene fragments were amplified by polymerase chain reaction (PCR). PCR reactions were prepared in 25 µL volume using the PuRe Taq Ready-To-Go™ mix (GE Healthcare, Little Chalfont, UK), plus 1 µL of total microbial DNA as template and 25 pmols of each primer GC40-63F (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCAGGCCTAAC ACATGCAAGTC 3') and 518R (5'ATTACCGCGGCTGCTGG 3' (El Fantroussi et al., 1999).

All the PCR amplifications were performed on an Eppendorf Mastercycler. After 5 min of initial denaturation at 95°C, 40 cycles of amplification were carried out. Each of these cycles consisted of 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 2 min of primer extension at 65°C, followed by a final primer extension of 10 min at 65°C. The PCR product was confirmed by standard 1.2 % agarose electrophoresis in TBE buffer followed by ethidium bromide staining and photography under UV light transillumination. These PCR amplicons were then used for DGGE fingerprinting analysis.

For clone library construction, 16S rRNA genes were PCR amplified by using the universal eubacterial primers 27f and 1521r (Weisburg et al., 1991) and the PCR methodology previously described. The PCR products were examined on 1.5 % agarose gels and purified with a Wizard® SV and PCR Clean-up system (Promega, Madison, WI, USA), as described by the manufacturer. The purified DNA was finally recovered in 50 µL of sterile milliQ-water and stored at -20°C.

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3.3. 16S rRNA gene clone libraries

Amplified 16S rRNA gene fragments from DNA samples of microbial populations were cloned into the plasmid vector (pGEM-T Easy Vector system II, Promega) according to the manufacturer's instructions. Initial screening of the *Escherichia coli* JM109 clones was by the blue-white method; positive clones were then analyzed by nested PCR with vector-specific pUC/M13 forward and reverse primers as indicated by the manufacturer. The PCR products were purified and used for DNA sequencing. Additionally, PCR products that gave rise to significant sequence information were used as a template for a second (nested) PCR with GC40-63f and 518r primers, as described above, for DGGE analysis. DGGE mobility profiles of PCR products obtained from members of the clone library were compared with the fingerprints of the whole microbial population of the microcosms. Profiles obtained from the total microcosm bacterial population at t=0 and t=360 days were used as markers, which enabled us to identify the bands of the community fingerprint that matched the known members of the clone library.

3.4. DGGE analysis

The PCR products from the microbial consortia and clone inserts were examined on 1.5 % agarose gels and then directly used for DGGE analysis (Muyzer et al., 1993) on 6 % polyacrylamide gels. The denaturing gradients ranged from 40 % to 60 % (100 % denaturant contained 7 M urea and 40 % formamide). Electrophoresis was performed at a constant voltage of 100 V for 16 h in 1x TAE buffer at 60ºC on an INGENYphorU-2 DGGE machine (INGENY International BV, Goes, The Netherlands). The gels were stained for 30 min with SYBR Gold nucleic acid gel stain (Molecular Probes Europe BV, Leiden, The Netherlands). Photographies were made under UV light, using a ChemiDoc XRS+ system (Bio-Rad) with Image Lab image capture and analysis software.

3.5. Sequencing and phylogenetic analysis

Sequencing was accomplished using the ABI Prism BigDye 3.1 Terminator cycle sequencing reaction kit following the manufacturer's instructions. Primers M13f or M13r were used for sequencing cloned 16S rDNA genes in clone libraries. The sequences obtained were analyzed by comparison with the SILVA 16S rRNA database by using the SINA web aligner according to the silva tree server (Pruesse et al., 2007; http://www.arb-silva.de/aligner). Percent similarities to closest neighbors were obtained

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by BLAST on-line searches (Altschul et al., 1997). The 16S rDNA sequences were deposited in the GenBanK database with accession numbers JX102499 to JX102514, respectively.

DGGE fingerprints throughout the experiments were obtained in duplicate. To identify the DGGE bands, 16S rDNA clone libraries were obtained at the start and end of the experiments. Clones were sequenced, and the sequences used to group the clones in OTUs (Operational Taxonomic Units) and for taxonomic classification of each OTU. DGGE profiles obtained from representative clones were compared with the community fingerprints at the start and end of the experiment.

4. Results

4.1. Dissolved oxygen and pH

Monitoring of pH over time allowed us to observe variations between the active and control tests and the NA and BLA experiments. pH remained constant over time in the control tests of the NA experiment (Figure S1A), whereas an increase in alkalinity was observed in the active tests (as pH varied from the initial value of 7.70 to 8.10). In contrast, a slight acidification occurred in the control tests of the BLA experiment between days 0 and 62 (Figure S1B; pH varies from 7.70 to 7.35 despite a subsequent increase). This acidification occurred as a consequence of the addition of lactic acid, whereas in the active tests an increase in pH occurred in this period. These pH values are optimal for dechlorination as they are within or slightly above the range 6.80 to 7.80 reported by Cope and Hughes (2001) and ESTCP (2004).

Microbial activity in the active test between 0 and 62 days was sufficient to prevent acidification of the medium after the first addition of lactic acid. A subsequent decline in pH (Figure S1B), coinciding with a new addition of lactic acid and with a decline in microbial diversity was observed in the active tests after day 62 (Figure 4 in the main text).

4.2. Conditions in which the reductive dechlorination of CT and CF may be hindered

Under nitrate and sulfate-reducing conditions, CT and CF are better electron acceptors than nitrates and sulfates as their standard reduction potential is lower than that of CT and CF (Rijnaarts et al., 1998; de Best, 1999; de Best et al., 1999). However, dechlorinating microorganisms also have to compete with other microorganisms for available electron donors. This competition may hinder dechlorination of

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chloromethanes (Semprini et al.,1992; Picardal et al., 1993) although the reaction is thermodynamically favorable and possible, as demonstrated by laboratory and field studies (Rijnaarts et al., 1998). For these reasons, the order of use of the different electron acceptors does not necessarily correspond to the scale of the standard reduction potential. In our case, the availability of nitrate and sulfate, which is higher than that of CT and CF at the start of the experiments, must be considered. Likewise, also the existing communities (denitrifying, sulfate-reducing and halorespiring) and their development stage have to be considered.

4.3. DCM

An increase in concentration of DCM was observed in the active tests of the NA experiment between day 33 and day 196 (Figure 1A in the main text), after which concentrations decreased. In contrast, concentrations in DCM were always below the Limit of Quantification in the control tests.

In the BLA experiment, a slow increase until day 196 in the active tests (Figure 1A, C in the main text) suggests that DCM is formed by the reductively dechlorinating microbial population. This increase in DCM is followed by a decrease, which also suggests that this compound is partially consumed by members of the anaerobic community, most probably acetogens or methylotrophic (Leisinger and Braus-Stromeyer, 1995).

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4.4. CT, CF and DCM concentrations in microcosms experiments (supplementary table)

Table S2: Time evolution of CT, CF and dichloromethane (DCM) concentrations in the microcosms experiments.

Natural Attenuation						
	Active (mmol/L)			Control (mmol/L)		
Days	**CT**	**CF**	**DCM**	**CT**	**CF**	**DCM**
0	3.9E-02	7.8E-02	2.7E-05	3.9E-02	7.6E-02	BLOQ
8	4.6E-02	8.1E-02	5.1E-05	4.1E-02	8.6E-02	BLOQ
15	3.5E-02	8.7E-02	8.3E-05	4.4E-02	9.8E-02	BLOQ
33	5.0E-02	4.5E-01	1.0E-04	5.2E-02	7.5E-02	BLOQ
62	8.5E-03	1.8E-01	2.1E-04	7.3E-02	1.9E-01	BLOQ
124	2.6E-03	1.9E-02	2.1E-04	1.3E-02	1.7E-01	BLOQ
166	3.2E-04	9.9E-03	4.5E-04	6.1E-03	1.1E-01	BLOQ
196	1.3E-04	7.3E-04	3.8E-04	9.5E-04	9.9E-02	BLOQ
216	1.0E-04	8.1E-04	2.0E-04	7.5E-04	9.8E-02	BLOQ
260	3.7E-05	1.3E-04	1.5E-04	3.2E-04	1.1E-01	BLOQ
310	1.3E-05	2.3E-05	4.8E-05	5.9E-05	6.6E-02	BLOQ
360	BLOQ	9.9E-06	3.0E-05	2.2E-05	6.0E-02	BLOQ

Biostimulation (lactic acid)						
	Active (mmol/L)			Control (mmol/L)		
Days	**CT**	**CF**	**DCM**	**CT**	**CF**	**DCM**
0	3.6E-02	1.1E-01	2.7E-05	4.2E-02	1.1E-01	BLOQ
8	4.3E-02	1.3E-01	5.1E-05	4.2E-02	1.2E-01	BLOQ
15	4.4E-02	1.4E-01	4.7E-05	4.7E-02	1.5E-01	BLOQ
33	3.4E-02	1.2E-01	5.8E-05	4.9E-02	1.2E-01	BLOQ
62	3.1E-02	6.8E-02	4.3E-05	5.7E-02	1.5E-01	BLOQ
124	2.9E-03	9.0E-03	2.0E-04	7.8E-02	3.5E-01	BLOQ
166	2.3E-04	4.3E-04	3.3E-04	2.4E-02	1.7E-01	BLOQ
196	5.5E-05	9.4E-05	3.3E-04	2.4E-02	1.2E-01	BLOQ
216	1.9E-05	1.3E-05	2.1E-04	4.5E-03	2.1E-01	BLOQ
260	5.1E-06	4.5E-06	1.0E-04	1.4E-03	1.4E-01	BLOQ
310	BLOQ	BLOQ	5.5E-05	5.5E-04	1.4E-01	BLOQ
360	BLOQ	BLOQ	1.3E-05	8.1E-05	1.0E-01	BLOQ

BLOQ: samples below the limit of quantification

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4.5. Microbial community structure

4.5.1. At the start of the experiment

At the start of the experiment (day 0), during which denitrification was observed (see section 3.2. in the main text), the dominant taxonomic group was the *Betaproteobacteria* class of bacteria. The presence of *Betaproteobacteria* in nitrate reduction conditions is consistent with earlier studies that showed members of the *Betaproteobacteria* to be predominant in enrichment cultures of denitrifying bacteria (Heylen et al., 2006). Denitrifying bacteria are capable of mineralizing DCM under denitrifying conditions (Melendez et al. 1993, Kohler et al., 1995), and of giving rise to reductive dechlorination of CT, CF and DCM under anoxic conditions as reported by Yu and Smith (2000).

The *Methylophilaceae* family (OTU 2 and OTU 3, 39.3 %) is noteworthy in the *Betaproteobacteria* class. This family includes some, but not all of the methylotrophic bacteria, which are microorganisms that are capable of growing on chloromethanes. For instance, *Methylophilus* sp DM11, a member of the *Methylophilaceae,* has been shown to grow on DCM as the only source of carbon and energy (Bader and Leisinger, 1994). The presence of this family is consistent with the history of the contamination of the site that is highly abundant in chloromethanes. This confirms the potential of methylotrophic microorganisms in the detoxification of these compounds (Doronina et al., 2000, 2001; Firsova et al., 2009; Leisinger et al., 1994; Trotsenko et al., 2003).

OTU 5 and OTU 9 belong to the family *Comamonadaceae*. OTU 9 matches *Hydrogenophaga*, an aerobic, hydrogen-oxidizing bacterium able to denitrify (Willems et al., 1989)

OTU 7, *Propionivibrio*, has also been found to include some perchlorate reducing bacterial strains (Thrash et al., 2010).

The second most frequent group of clones (21.4 %) at the start time belongs to an uncultured genus (OTU 1) affiliated with *Ignavibacteria*, a small group with few cultured members of the phylum *Chlorobi*. Clone sequences from molecular studies of dechlorinating microcosms (Genbank accession number AB186805 and AB186806, Yoshida et al., 2005) are amongst the rRNA gene sequences most similar (93 % identity) to OTU 1. Further experimental data are needed to determine the physiology of these unknown bacteria and to ascertain whether they play a role in the dechlorination process of contaminated sites.

OTU 8 (3.6% of the clones) is an *Alphaproteobacteria* (*Magnetospirillum* (Table 3 in the main text). This genus contains aerobic magnetotactic bacteria of surficial waters.

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4.5.2. At the end of the experiment

OTU 13 (sequence frequency of 37 %) showed a significant identity in the BLAST search (98 %) to a sequence obtained from bacterial populations associated with dissimilatory arsenate reduction in an industrial soil.

OTU 14, also a *Clostridiales* bacterium, showed little identity (93 %) to other sequences in the Genebank. In contrast, OTU 12 shows a high identity (98 %) to a 16s rRNA gene sequence (EF644507.1) obtained from an uncultured member of a 1,1,2,2-tetrachloroethane to ethane dechlorinating community (Rossetti et al., 2008).

OTU 10 is similar (99 %) to a sequence (AJ249113) from an uncultured microorganism of a dechlorinating mixed culture described as phylogenetically related to those of other anaerobic dechlorinating consortia (Schlotelburg et al., 2000). OTU 10 showed a 97 % identity to accession NR_041355 (*Longilinea arvoryzae* 16S ribosomal RNA, partial sequence), a microorganism of the phylum *Chloroflexi* (Yamada et al., 2007).

OTU 11 (7.4 %) is closely related to the genus *Meniscus*, whose type strain (*M. glaucopis*) is an anaerobic aerotolerant bacterium, isolated from an anaerobic digester of a wastewater treatment plant (Irgens, 1977).

4.6. DGGE profiles of sediment samples

The DGGE profiles of sediment samples at the start and the end of the experiments showed a higher phylotype diversity with respect to the profiles of the matching water samples (Figure S6). This higher diversity was in accordance with earlier observations based on water and sediment samples at field scale (Puigserver, 2010; Puigserver et al., 2013). Figure S6 also indicates lower diversity and development of bacterial communities in sediment than in water at the start of the experiment. Furthermore, and in contrast to the water samples at the end of the two experiments (day 360), the sediment samples showed greater diversity and a lower degree of microbial speciation.

The considerable reactive surface of the sediment encourages the growth of microorganisms, which could account for the greater diversity in sediment than in water. In addition, bacteria with no motility are found at the bottom of the bottle (i.e., in the sediment) owing to the absence of water flow in the microcosm experiment. In contrast, clone OTU 6 (identified as *D. suillum*, Figure S6) is the most specialized and the most abundant bacteria in water, where hydrogeochemical conditions are increasingly restrictive.

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4.7. Supplementary figures

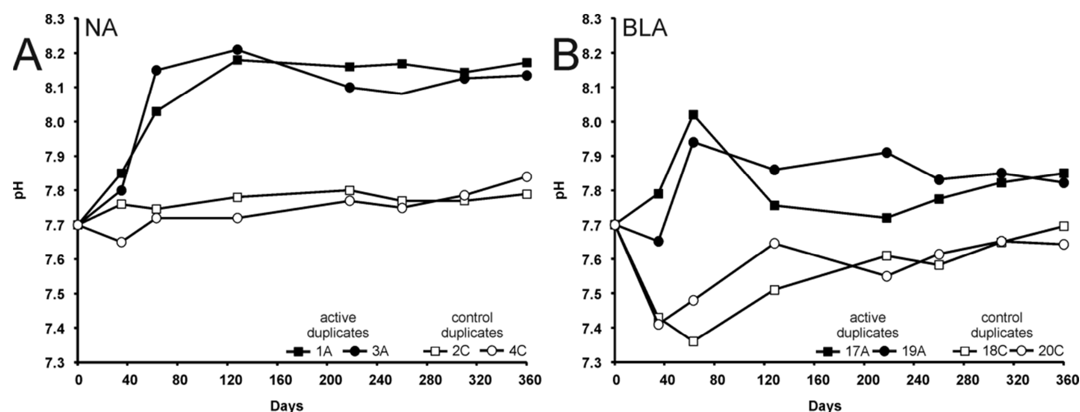

Figure S1: Time evolution of pH in water of microcosm experiments. A) natural attenuation, 1A and 3A active duplicate tests, 2C and 4C control duplicate tests. B) Biostimulation with lactic acid. 17A and 19A active duplicate tests, 18C and 20C control duplicate tests.

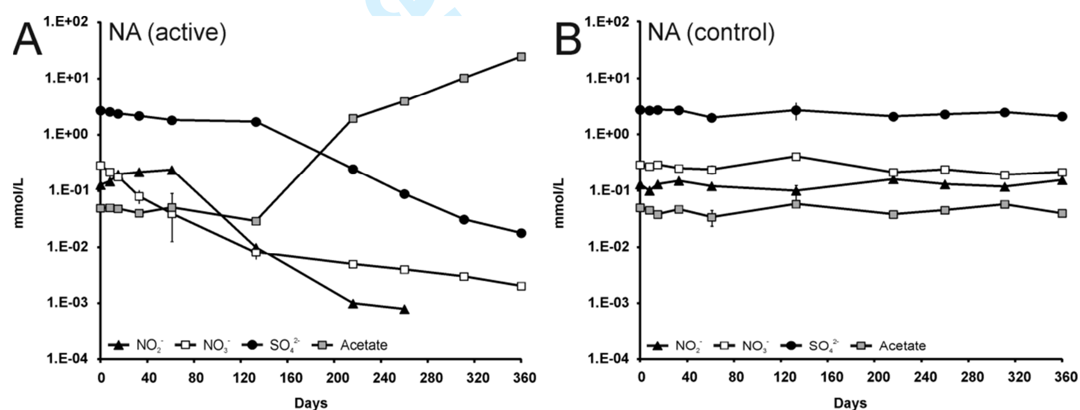

Figure S2: Time evolution of major and minor ions and acetate concentrations in the microcosm experiment to study natural attenuation (NA). A) active tests, B) control tests.

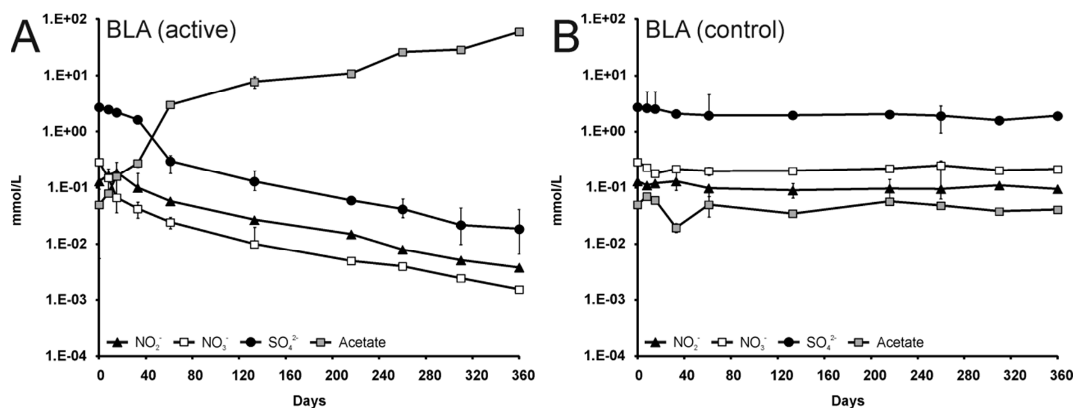

Figure S3: Time evolution of major and minor ions and acetate concentrations in the microcosm experiment to study biostimulation with lactic acid as electron donor (BLA). A) active tests, B) control tests.

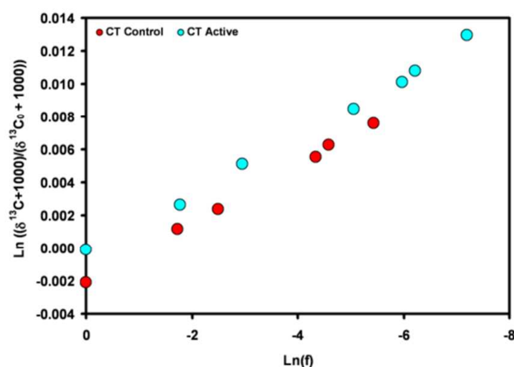

Figure S4: Rayleigh graph for the active and control tests of the microcosm experiment of natural attenuation of CT.

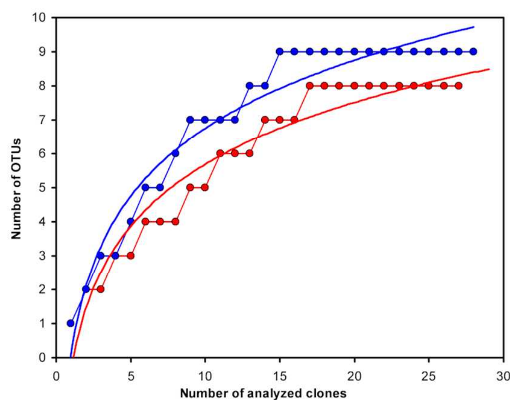

Figure S5: Rarefaction curve of the analyzed clones corresponding to the biostimulation with lactic acid microcosm experiment at the start time (day 0, blue color) and end time (day 360, red color). OTU: Operational Taxonomic Unit (Table 3 in the main text).

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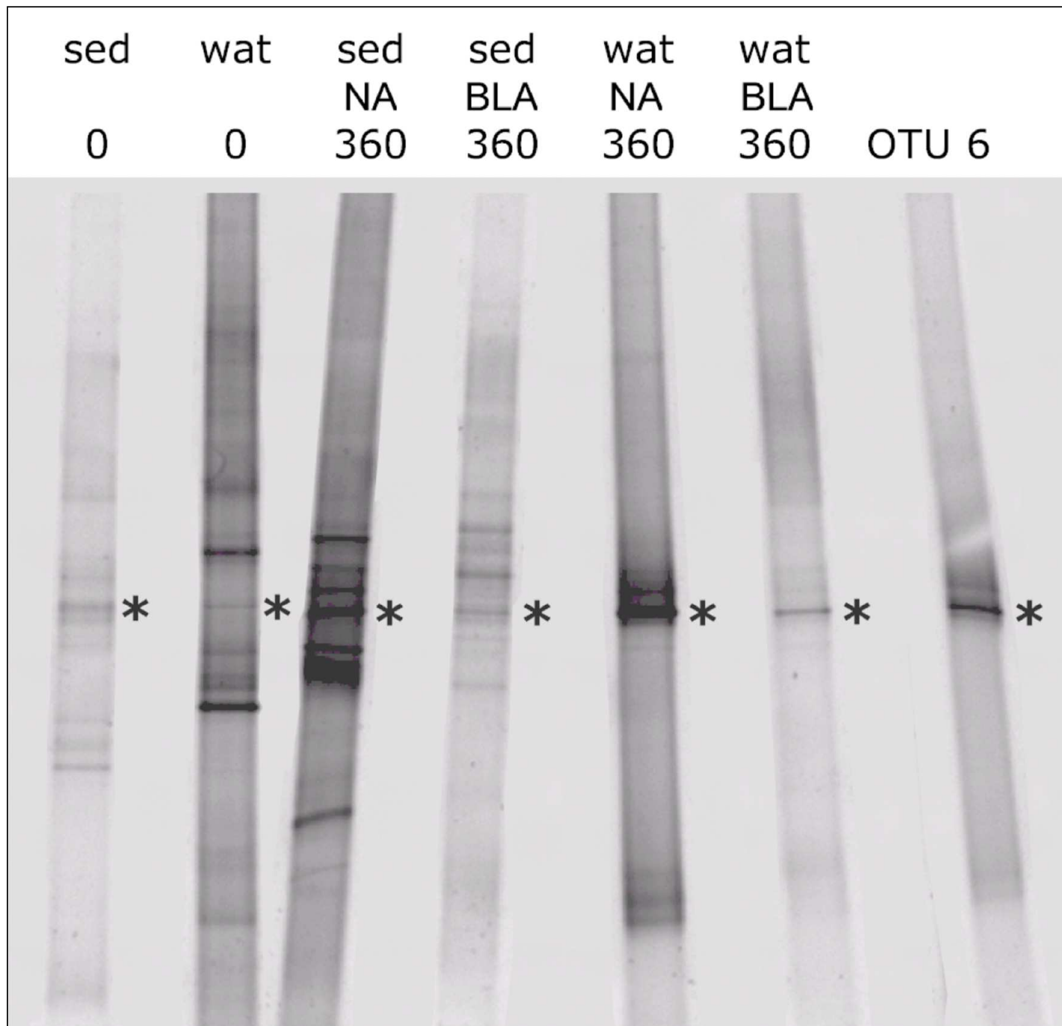

Figure S6: DGGE profiles of water and sediment in active microcosm experiments of natural attenuation and lactic acid biostimulation. In each profile, 0 = day 0 (start time), 360 = day 360 (end time), sed = sediment sample, wat = water sample, NA = natural attenuation, BLA = lactic acid biostimulation. OTU = Operational taxonomic unit. Asterisks correspond to OTU 6, which is a recombinant clone identified as *Dechlorosoma suillum* (Table 3 in the main text).

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