Temporal hydrochemical and microbial variations in microcosm experiments from sites contaminated with chloromethanes under biostimulation with lactic acid

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\textbf{ABSTRACT}

The aim of this research was to identify the sequence of degradation processes that leads 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 was conducted to analyze these two scenarios. The authors used groundwater and sediment collected from a field site located at a petrochemical complex to create the microcosms. Chemical, compound-specific isotope and microbial analyses were performed. A significant finding of this work was the abiotic degradation of carbon tetrachloride. Another result was the identification of biotic reductive dechlorination of chloroform by a bacterium of the Clostridiales order. This study showed that biostimulation with lactic acid produced faster degradation rates of carbon tetrachloride and chloroform. Lactic acid acted as an electron donor and promoted a decrease in the concentration of other electron acceptors such as nitrate and sulfate, which competed with chloromethanes. Thus, biostimulation could be an efficient remediation strategy for sites contaminated with chloromethanes, especially when a site’s complex pollution history results in chemical background concentrations that are high in compounds that could potentially reduce natural attenuation.

\textbf{KEYWORDS}

Biostimulation with lactic acid; biotic and abiotic degradation; chloromethanes; Clostridiales order; compound-specific isotope analysis (CSIA); Dechlorosoma suillum sp.; DGGE; microcosm; natural attenuation

\textbf{Introduction}

Carbon tetrachloride (CT) and chloroform (CF) are dense non-aqueous-phase liquids (DNAPLs). In particular, they are chlorinated solvents that are widely used in metal degreasing, dry cleaning, and refrigeration. These compounds are toxic, carcinogenic, and harmful to the ozone layer. Given their high densities of 1.59 and 1.49 g/cm\textsuperscript{3} as DNAPLs, respectively (Pankow and Cherry 1996), these compounds can accumulate at the bottom of aquifers. Their prolonged use on a large scale has resulted in many soil and groundwater contamination episodes (Penny, Vuilleumier, and Bringel 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 the degradation of CT under denitrification conditions. Moreover, biostimulation, which promotes 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 types, increase in nutrients (Devlin, Eedy, and Butler 2000), and concentration of electron donors and electron acceptors (US Environmental Protection Agency [EPA] 2004). Semprini et al. (1992) conducted the first study by using acetate as a substrate for growth and as an electron donor, along with 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, Ng, and He 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 and generated CF, dichloromethane (DCM), and even chloromethane (CM), CO₂ (carbon dioxide), and CS₂ (carbon disulfide) (Hashsham, Scholze, and Freedman 1995).

Additionally, abiotic degradation of chloromethanes in the presence of iron-bearing soil minerals with high intrinsic reductive capacities have also been studied, and these minerals have been widely used for the abiotic reductive degradation of organic contaminants in groundwater. Surface-associated Fe(II), magnetite (Fe₃O₄), FeS (mackinawite), and FeS₂ (pyrite), which are common electron donors in aquifers, have been shown to enhance abiotic chloromethane degradation (Kriegman-King and Reinhard 1994; Butler and Hayes 2000; O’Loughlin, Kenner, and Burris 2003; Danielsen and Hayes 2004; Elsner et al. 2004a, 2004b; McCormick and Adriaens 2004; Maithreepala and Doong 2005; Hanoch, Shao, and Butler 2006; Shao and Butler 2009). Natural green rust minerals are also iron-bearing soil components with high intrinsic abiotic reductive abilities of CT (Liang and Butler 2010).

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 subsequently conducted by other investigators (e.g., Elsner et al. 2004a; Maithreepala and Doong 2005; Hanoch, Shao, and Butler 2006) have validated the classification system of Davis et al. (2003). In short, the categories of the classification system are 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 iron reduced minerals. (2) Abiotic degradation by hydrolysis, which may generate CS₂ as an intermediate prior to forming CO₂ in reducing environments. (3) Reductive hydrolysis of CT to form CO and/or formic acid, which needs the formation of successively dechlorinated radical intermediates.

Compound-specific isotope analysis (CSIA) is a powerful tool used for characterizing the biotic and abiotic degradation processes of chlorinated solvents (US EPA 2008). In general, the degradation of these compounds is accompanied by a preferential degradation of molecules that exclusively contains light carbon isotopes (i.e., ¹²C). The result is a relative enrichment in heavy isotopes (i.e., ¹³C) in the remaining contaminant dissolved in the aquifer. 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). However, few studies have been conducted on the isotopic fractionation of CF due to biotic dechlorination that results in the formation of DCM, but the study published by Chan et al. (2012) is noteworthy.

The paper by Chan et al. (2012) is the only study that has addressed isotope fractionation during the biotic degradation of CF. The authors showed how a particular population of Dehalobacter (Clostridiales order) was able to biodegrade CF. In contrast to our study, Chan and coworkers focused on the evolution of CF as a single parent contaminant and did not use water and sediment from a real site for the experiments. Therefore, the results may not be fully representative of the natural conditions that occur in real sites, where the interactions between chloromethanes and other electron acceptors could be problematic for data interpretation. For instance, although the reduction potentials of CT and CF are higher than that of sulfates (Rijnaarts et al. 1998; de Best 1999; de Best et al. 1999), inhibitory effects caused by competition between the dechlorinating and the sulfate-reducing populations for bioavailable electron donors could result in 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 that leads to the selective enrichment of indigenous microbial communities involved in the degradation of CT and CF under dissolved oxygen (DO) conditions of reductive dechlorination. To this end, we conducted microcosm experiments with groundwater and sediment from a field site located at a petrochemical complex where conditions throughout the year were reducing. Contaminants of diverse origins coexist in this site (CT and CF as parent compounds), and there are high background levels of nitrates and sulfates that potentially reduce the natural attenuation of chloromethanes. The experiments focused on two scenarios: (i) natural attenuation and (ii) biostimulation of indigenous microbial communities to develop rapid and selective enrichment of communities that could
degrade CT and CF. An integrated study of these scenarios and processes could help assess the potential for applying lactic acid biostimulation at a field scale in contexts characterized by high nitrate and sulfate chemical background concentrations.

For the first time, we found that abiotic and biotic degradation of CT to form CF occurred simultaneously. Additionally, we observed that D. suillum and a bacterium of the Clostridiales order were enriched (selected) at the end of the study. This enrichment occurred with the near-complete exhaustion of nitrates and sulfates (and disappearance of denitrifying and sulfate-reducing microorganisms) and with the degradation of CT and CF to form DCM. This indicates that the addition of lactic acid favors the selective enrichment of these microorganisms and suggests that lactic acid plays a possible role in the degradation of CT and CF.

**Materials and methods**

**Site description**

Water and sediment used in the microcosm experiments were collected from an unconfined aquifer of Quaternary alluvial fan outcropping in the La Pineda petrochemical complex (Tarragona, Spain), 100 km south of Barcelona. This petrochemical complex initiated its activities in stages, beginning in 1960. The aquifer is characterized by sediment with a complex mixture of organic compounds, which includes DNAPLs (e.g., chlorinated solvents), light non-aqueous-phase liquids (LNAPLs; e.g., gasoline), and inorganic compounds (e.g., nitrate and sulfate) (Table 1A). The pollution episode studied was detected in 1996 in one of the plants in the petrochemical complex, but it is unknown when the episode started. The two main pollutants associated with this episode are CT and CF. Both compounds were used in refrigerant production and were stored independently in two tanks. Spillages of various durations occurred repeatedly in tank auxiliary structures, such as valves, pipes, and pumps, which were installed directly above the ground and a few meters away from the tanks. The depth of the water table oscillated between 5.5 and 9 m below ground level, and the average maximum water table oscillation was 1.5 m throughout the year.

From the source zone, the CT and CF free phase descended vertically. During this descent, the CT and CF free phase left a trail of residual DNAPL in the sandy gravel and sand of the vadose and saturated zones. As the free phase descended, pools accumulated on discontinuous interlayered levels of low conductivity (reddish silts and clays), and eventually migrated toward the southeast due to a slight dip in the sediments. Currently, these pools are aged DNAPL sources. Puigserver et al. (2013) showed that chloromethane contamination affected not only the aquifer but also an underlying aquitard at this site. Despite a substantial reduction in groundwater contaminant concentrations between 1997 and 2009 (Supplemental Material, Table S1), pollution continues to exceed the contaminant levels established by European groundwater quality standards. Several other organic and inorganic compounds that exist at the site could have had inhibitory effects or served as competing electron donors and/or acceptors at the field scale. However, total inhibition did not occur because isotope fractionation of dissolved CT occurred, with $\Delta^{13}C$ values of 5.52‰, between wells located along a flow line starting at the source of pollution to wells located downgradient in the plume at distances of 180 m. This fractionation demonstrated that the degradation of CT, in this case to form CF, was one of the mechanisms contributing to the natural attenuation of this compound. Degradation of CF also occurred at the field scale. In this case, isotopic fractionation was not observed. However, this lack of fractionation was only apparent because this compound had two different origins: (1) the degradation of CT spilled from the CT storage tank, and (2) as the parent compound spilled from the CF storage tank. The same effect is described in “Isotopic Fractionation Caused by Degradation Mechanisms” at the scale of the conducted microcosm experiments.

**Microcosm experiments**

**Design of the experiments**

Two microcosm experiments were conducted. The first simulated the natural biotic and abiotic degradation of chloromethanes, i.e., natural attenuation (NA experiment). The second simulated the biostimulation of indigenous microorganisms through the addition of lactic acid (BLA experiment). A total volume of 10 ml of lactic acid was added in five additions of 2 ml (85% lactic acid; Sigma Aldrich, Madrid, Spain).
Each experiment was performed in duplicate and 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 (model Autester 75 E DRY-PV; Selecta, Barcelona, Spain) was used to sterilize the control microcosm bottles containing sediment. These bottles also contained 50 ml of a 147 mM HgCl₂ stock solution (mercury II chloride, puriss. p.a.; Riedel-Deha, Seelze, Germany) as a bactericide, following procedures reported by Trevors (1996). These bottles were autoclaved for periods of 30 min for a total of 2 h at a temperature of 121°C, a pressure of 1 atm, and at saturated vapor conditions.

Methanol (MeOH; ISO Pro Analysis; Merck, Darmstadt, Germany) was used to clean and sterilize the remaining materials. Experiments were conducted in a flexible vinyl anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA).

Sediment and groundwater used in the experiments
The sediment used in the experiments was obtained from cores recovered from a borehole drilled in the plume at 180 m from the DNAPL pools of CT and CF.

Table 1. (A) Maximum, minimum, and average concentration values of the main inorganic and organic compounds constituting the chemical background of the site; and (B) chemical composition of groundwater used in the experiments (before being purged to decline DO content).

(A) Concentration values (mg/L) of the main inorganic and organic compounds constituting the chemical background of the site

| Compound | Max | Min | Average |
|----------|-----|-----|---------|
| NO₃⁻     | 111.5 | <0.1 | 48.72 |
| NO₂⁻     | 0.27  | <0.1 | 0.18   |
| NH₄⁺     | 13.87 | <0.1 | 4.58   |
| SO₄²⁻    | 271.2 | <0.1 | 140.9  |
| Cl⁻      | 847.2 | 25.69 | 368.3  |
| TOC      | 303.0 | 115.9 | 101.2  |

(B) Chemical composition of groundwater used in the experiments

| Compound | Max | Min | Average |
|----------|-----|-----|---------|
| NO₃⁻     | 17.98 | <0.1 | 6.19   |
| NO₂⁻     | 4.60  | <0.1 | 5.40   |
| NH₄⁺     | 6.19  | <0.1 | 1.78   |
| SO₄²⁻    | 262.08 | <0.1 | 2.43   |
| Cl⁻      | 271.4 | <0.1 | 1.75   |
| TOC      | 24.34 | <0.1 | 1.75   |

Note. BLOQ = below the limit of quantification.
Each bottle had a capacity of 2000 ml and was filled with 300 g of homogenized sediment and 1100 ml of groundwater, which represented 9.4% and 55.0% of the total volume of the bottle, respectively. The remaining 35.6% of the 2000-ml bottle capacity was filled with the inert atmosphere (100% N2) in the chamber when the bottles were filled with water and sediment for the experiments (see Supplemental Material for a detailed protocol for filling the bottles for experiments and setting up of the anaerobic chamber). To better reproduce contamination at the site (i.e., the presence of pools of CT and CF, which slowly dissolved to give rise to the plume), 10 μl of CT and 10 μl of CF (reagent grade, 99.9%; Sigma-Aldrich) were added at the start of the experiment. The isotopic compositions of 13C of CT and CF in groundwater used in microcosms before purging with N2 gas were −39.3 ± 0.1‰ and −43.6 ± 0.1‰ (Vienna Pee Dee belemnite international standard, V-PDB), respectively. The isotopic δ13C compositions of pure phase of CT and CF added were −42.4‰ and −46.8‰, respectively. Bottles were sealed with Mininert valves for sampling (Supelco Analytical, Madrid, Spain) and insulating tape. Additionally, all the bottles were arranged horizontally on the shelves of the anaerobic chamber and covered with a thick black cloth to maximize darkness.

**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, L’Hospitalet de Llobregat, Barcelona, Spain). Concentrations of the main inorganic electron acceptors in the experiments (i.e., sulfate, nitrate, and nitrite), acetate, lactate, CS2, CT, CF, DCM, CM, and the δ13C of CT and CF were also determined. The low concentrations of DCM and the fact that all concentrations of CM were below the LOQ prevented us from determining the δ13C of these compounds.

Sodium azide (N3Na; Fluka, Tres Cantos-Madrid, Spain) was added to the microcosm water samples immediately upon collection to inhibit bacterial activity, following procedures reported by Trevors (1996). The vials containing the samples were stored in a cold chamber at 4°C in total darkness until they were analyzed.

**Compound-specific isotope analysis**

The determination of δ13C of dissolved chloromethanes was carried out in duplicate using the CSIA technique, which determined the isotopic signature of carbon by measuring the two stable isotopes, 12C and 13C.
This relationship was expressed as \( \delta^{13}C \) (in \(^\%\) units) = \( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \) \times 1000, where \( R_{\text{sample}} \) was the \( ^{13}C/^{12}C \) ratio in a given sample, and \( R_{\text{standard}} \) was the \( ^{13}C/^{12}C \) ratio in the international standard V-PDB. Because molecules with light isotopes tend to react more rapidly, the isotopic ratio changed over time, which led to an isotopic fractionation factor (\( \alpha \)): \( \alpha = \frac{R_t}{R_0} = \left( \frac{1000 + \delta^{13}C_0}{1000 + \delta^{13}C_b} \right) \), where \( R_t \) was the isotopic ratio of the compound at a particular time (\( t_0 \)) or a compound in a well downstream from the source, and \( R_0 \) was the isotopic ratio of the compound at time zero for the contaminant source. For many organic pollutants, isotopic fractionation during biotic and abiotic degradation can be described through the Rayleigh process: \( R_t = R_0 \cdot f^{(\alpha - 1)} \), where \( f \) is the relative concentration \( C/C_0 \) (normalized concentration), \( C \) is the concentration of a compound at a given time, and \( C_0 \) is the concentration at time zero. Additionally, \( f = \exp \left( \frac{\delta^{13}C_{\text{gw}} - \delta^{13}C_{\text{source}}}{1000} \right) \), where \( \delta^{13}C_{\text{gw}} \) is the isotopic composition of the organic compound in groundwater, and \( \delta^{13}C_{\text{source}} \) is the isotopic composition of the organic compound in the source. Enrichment factor (\( \varepsilon \)): \( \varepsilon = (\alpha - 1) \times 1000 \) is, to a first approximation, a function of broken bonds during the degradation process and can be used to distinguish reaction mechanisms (Van-Stone et al. 2007), pathways (Hirschhorn et al. 2004), and kinetics of reactions (Sherwood Lollar et al. 2010).

**Analytical techniques and protocols for chemical and isotope analyses**

Concentration and isotope analyses were conducted at the Scientific and Technical Services laboratory at Barcelona University (accredited by ISO 9001:2000). Sulfate, nitrate, and nitrite concentrations were analyzed following the EPA 9056 protocol, acetate concentrations were analyzed according to procedures reported by Furlani et al. (2006), and VOCs (including CS$_2$) were measured by gas chromatography–mass spectrometry (GC-MS). A protocol based on the extraction of VOCs by direct adsorption from the aqueous phase was used to determine the \( \delta^{13}C \) of chloromethanes. The extraction was conducted by inserting an adsorbent fiber (SPME fiber assembly 75-\( \mu \)m Carboxen/polydimethylsiloxane (PDMS); Supelco, Madrid, Spain) in the water sample, which was stored in a 100-ml amber glass bottle (Supelco Analytical), closed with a silicone septum and agitated for 30 min to adsorb the chloromethanes. The determination of \( \delta^{13}C \) was carried out using 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 (a previous name of Thermo Fisher Scientific, Inc.) MAT IRMS spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA).

**Bacterial community analysis**

Denaturing gradient gel electrophoresis (DGGE) analyses of water samples of the microcosms were performed in duplicate. In addition, DGGE analyses of sediment samples of the microcosms were conducted at the start and the end of the experiments (see Section 4 in Supplemental Material for more detailed information). In the case of the BLA experiment, microbial populations in the microcosm were identified at the start and end of the experiment. Microbial bacterial population studies were performed by DGGE and clone library analyses. DGGE electrophoresis of polymerase chain reaction (PCR)-amplified 16S rRNA genes were run in denaturing acrylamide gels and stained prior to photography following standard methodologies (see Section 4 in Supplemental Material for further details). Clone libraries of PCR-amplified 16S rRNA genes for all the bacterial populations were generated with pGEM-T vectors 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 the clone library, although saturation was not achieved. Inserts in clones were sequenced and assigned to microbial taxons by DNA sequence comparisons in genetic databanks (see Section 4 in Supplemental Material for further details). Additional information on the analytical techniques and instrumentation used for the microbiological analyses of water and sediment samples can be found in Supplemental Material.

**Results and discussion**

**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...
observed at the field scale in the plume (0.77 mg/L in average) throughout the year. Average values of 0.04 and 0.03 mg/L in DO 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, the DO experimental conditions were favorable for reductive dechlorination.

**Degradation mechanisms of CT**

The CT pure phase added in the NA and BLA experiments progressively dissolved and reached maximum CT concentrations, after which a decrease in concentration was observed in both experiments. In the NA experiment, there was a concentration decline from day 33 to day 310 (after which concentrations were no longer detectable; Table 2A and Figure 1A) in the active tests, and from day 62 to day 360 (end of the experiment; Figure 1B) in the control tests. In the BLA experiment, the decline was prolonged from day 15 to day 260 (after which concentrations were no longer detectable; Table 2A and Figure 1C) in the active tests, and from day 124 to day 360 (end of the experiment; Figure 1D) in the control tests. Based on the analyses of CT evolution in the active and control tests in the NA and BLA experiments, the decrease in CT was mainly attributed to two degradation mechanisms. The first mechanism fit the first classification category proposed by Davis et al. (2003) (see “Introduction”), i.e., biotic and/or abiotic reductive dechlorination to form CF, and occurred in the active tests of both experiments (Figure 1A, C) where increase in pH was registered (Figure S1A, B), indicating microbial activity. In contrast, pH remained constant in the control tests, with the exception of an initial decline due to the addition of lactic acid in the BLA experiment (Figure S1A, B). This mechanism led to the formation of CF and DCM (Figure 1A, C), which confirms that it corresponded to the process of reductive dechlorination.

The second mechanism of degradation of CT occurred mainly in the control tests of both experiments. It was not accompanied by an increase in CF (Figure 1B, D), and CS₂ was not generated (determinations of this compound were in all cases below the LOQ, i.e., 1.22 μg/L). The fact that no CF was formed is consistent with the third classification category proposed by Davis et al. (2003) (see “Introduction”), i.e., reductive hydrolysis of CT to form CO and/or formic acid.

**Degradation rate constants and percentages of transformation of CT to CF**

Once corrections of the partition processes of CT and mass-loss inherent in sampling were made (see Section 3 in Supplemental Material for details), the

| CT | CF |
|----|----|
| (A) Range of values in percentage | (A) Range of values in percentage |
| Mass-loss inherent in sampling | 25.885–25.915 | 28.684–28.715 |
| Remnant-mass in water at the end of the experiment (dissolved) | 0.0499–0.0691 | 0.014–0.019 |
| Water-gas mass partitioned (volatilized) | 32.573–32.635 | 4.821–4.882 |
| Water-soil mass partitioned (sorbed) | 1.604–1.635 | 0.141–0.178 |
| Mass loss due to degradation | 39.803–39.843 | 64.123–64.165 |
| (B) Average values in percentage/month | (B) Average values in percentage/month |
| Mass-loss inherent in sampling | 2.81 | 2.83 |
| Remnant-mass in water at the end of the experiment (dissolved) | 0.0064 | 0.0045 |
| Water-gas mass partitioned (volatilized) | 3.53 | 0.45 |
| Water-soil mass partitioned (sorbed) | 0.18 | 0.01 |
| Mass loss due to degradation | 4.31 | 5.88 |

aAfter this day concentrations were no longer detectable.
bEnd of the experiment.
cOr when concentrations were no longer detectable.
decrease in this compound, from the time when the added pure phase of CT was dissolved, aligned fairly well with first-order degradation kinetics. The degradation rate constants ($k_{\text{deg}}$) of the NA experiment were lower than those in the BLA experiment. $k_{\text{deg}}$ values were 0.034 day$^{-1}$ (standard error $R^2 = .96$) and 0.032 day$^{-1}$ ($R^2 = .97$) in the active and control tests of the NA experiment, respectively, and 0.041 day$^{-1}$ ($R^2 = .99$) and 0.034 day$^{-1}$ ($R^2 = .95$) in the active and control tests of the BLA experiment, respectively. Furthermore, a lower average percentage per month of the mass of CT added at the start of the active tests in the NA experiment than in the BLA experiment (4.31%/month and 5.29%/month, respectively; Table 2B) was degraded at the end (Table 2A). A decline in CT was observed from day 33 to day 310 in the case of the NA experiment and from day 15 to day 260 in the BLA experiment (Table 2A).

The earlier decrease in CT in the active BLA experiment compared with the active NA experiment (initiated at days 15 and 33, respectively; Table 2A) along with the more rapid decline in the active tests of the BLA experiment ($k_{\text{deg}}$ of 0.041 day$^{-1}$) compared with the NA experiment ($k_{\text{deg}}$ of 0.034 day$^{-1}$), in which CT still was present in the water on day 310 whereas CT values in the BLA samples were below LOQ on day 260 (Table 2B), show that lactic acid biostimulation accelerated the degradation of CT. In addition, the degradation process was more efficient and faster than in the case of natural attenuation, as the percentage of degraded mass of CT was higher at day 260 (after which values were below LOQ) in the active tests of the BLA experiment than in the active tests of the NA experiment at day 310 (in which CT was still detected) (Table 2A, B).

Moreover, the CT concentration in the active tests in the BLA experiment decreased more rapidly than in the NA experiment (Figure 1A, C), resulting in a significant consumption of lactate in the BLA experiment. The reduction of this compound was 68.1% of the total mass injected. However, lactate was also used as an electron donor in other redox processes (i.e., CF dechlorination, denitrification, and sulfate reduction, see “Degradation Mechanism of CF” and “Interactions Between Dechlorination and Other Redox Reactions”), which justifies the longer lag phase in the BLA experiment (62 days) than in the NA experiment (33 days).

**Degradation mechanism of CF**

As in the case of CT, the added CF pure phase progressively dissolved and reached a maximum
concentration. After this maximum was reached, a decrease in concentration was only observed in the active tests (Figure 1A, C) and CF concentrations remained almost constant in the control tests throughout the experiments; this indicated that this compound did not degrade. In the NA experiment, the decline was prolonged from day 33 to day 360 (end of the experiment; Table 2A and Figure 1A), and the decline was prolonged from day 15 to day 260 (after which concentrations were no longer detectable; Table 2A and Figure 1C) in the BLA experiment. CF was transformed into DCM, which increased in concentration parallel to the decline of CF until day 166 in the active tests of the NA and BLA experiments (Figure 1A, C). Subsequently, DCM decreased until day 360. CM was also determined in the active tests of the NA and BLA experiments, but concentrations of this compound were below the LOQ (1.63 μg/L; Table S2). In contrast, concentrations of DCM were always below the LOQ in the control tests (1.77 μg/L; Table S2), which was in agreement with the lack of CF degradation observed in the control tests. Based on the analysis of the evolution of CF in the active and control tests in the NA and BLA experiments (Figure 1A, B and Figure 1C, D, respectively), the decrease in CF can only be attributed to a single mechanism that occurred exclusively in the active tests of both experiments (CF did not vary in the controls tests) where CF degraded to DCM. This mechanism fits the first category of the classification system proposed by Davis et al. (2003) (see “Introduction”). The degradation mechanism where CF was transformed into DCM corresponds to biologically mediated reductive dechlorination.

Degradation rate constants and percentages of transformation of CF to DCM

Similarly to CT, the decrease in CF aligned fairly well with first-order degradation kinetics. The degradation rate constants were lower in the NA experiment than in the BLA experiment. The \( k_{\text{deg}} \) were 0.031 day\(^{-1}\) \((R^2 = .96)\) and 0.046 day\(^{-1}\) \((R^2 = .97)\) in the active tests of NA and BLA experiments, respectively. Moreover, there was a lower average percentage of degradation per month of the mass of CF added at the start of the active tests (and also produced by degradation of CT) in the NA experiment than in the BLA experiment at the end (5.88%/month and 8.17%/month, respectively; Table 2B). A decline in CF was observed from day 33 to day 360 in the case of the NA experiment and from day 15 to day 260 for the BLA experiment (Table 2A).

The earlier decrease of CF in the active BLA experiment (initiated on day 15; Table 2A) than in the active NA experiment (initiated on day 33; Table 2A), along with the more rapid decrease in the active tests of the BLA experiment (\(k_{\text{deg}}\) of 0.046 day\(^{-1}\)) than in the NA experiment (\(k_{\text{deg}}\) of 0.031 day\(^{-1}\)) show that biostimulation with lactic acid as an electron donor accelerated the degradation of CF. These observations are consistent with the aforementioned consumption of lactate during the experiment (see “Degradation Rate Constants and Percentages of Transformation of CT to CF”). Therefore, biostimulation was more efficient and faster than natural attenuation, as the percentage of degraded mass of CF was higher at day 260 (after which values were below LOQ) in the active tests of the BLA experiment than in the active tests of the NA experiment at day 360 (in which CF still was detected) (Table 2A, B).

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 suggested competition for available electrons between denitrifying and sulfate-reducing compounds and microorganisms that promoted dechlorination.

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

Sulfate evolution in the active tests of the NA experiment was relatively constant until day 133, coinciding with the denitrification process (Figure S2A) and the
decrease in CT and CF (Figure 1A). After that day, sulfate concentrations significantly decreased (Figure S2A). This decline in sulfate demonstrated that sulfate reduction activated after denitrification processes substantially reduced the nitrate concentration. The decline in sulfate was 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 experiment (after day 33; Figure S3A) than in the active tests of the NA experiment (after day 133; Figure S2A). In addition, in the active tests of the BLA experiment, the substantial decrease in CT and CF that occurred from day 62 (Figure 1C) coincided with the sulfate reduction process (Figure S3A). Additionally, the decrease in sulfate was accompanied by an increase in acetate in the active tests of both experiments (Figure S2A and S3A). Part of this acetate was generated from lactate fermentation (31.9% of the added lactate remained at the end of the experiment). The increase in acetate revealed that the fermentation processes supplied electrons to the medium, favoring sulfate-reducing conditions (Liamleam and Annachhatre 2007). However, the steeper increase in acetate in the NA experiment than in BLA experiment (Figure S2A and Figure S3A, respectively) indicated that higher acetate consumption occurred in the BLA experiment than in the NA experiment. This acetate originated from two sources: from the fermentation of background organic matter and from the fermentation of supplied lactate.

The earlier decline in sulfate in the active BLA experiment compared with the active NA experiment (sulfate was constant in the controls) considerably improved the efficiency of CT and CF degradation (Figure 1C). Given that the competition for electrons with CT and CF was minimized, this observation is in agreement with the fact that the degradation processes of CT and CF were initiated earlier and at faster rates in the BLA experiment than in the NA experiment (see “Degradation Rate Constants and Percentages of Transformation of CT to CF,” “Degradation Rate Constants and Percentages of Transformation of CF to DCM,” and Table 2A).

Isotopic fractionation caused by 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 experiments (Figure 2). This suggests that abiotic and biotic degradation occurred simultaneously. In addition, the degradation rate of this compound was higher in the active tests of the BLA experiment than in the NA experiment ($k_{\text{deg}}$ of 0.041 and 0.034 day$^{-1}$, respectively; see “Degradation Rate Constants and Percentages of Transformation of CT to CF” and Table 2A), which highlights the role of lactic acid biostimulation before simple abiotic degradation was observed in the controls of the NA and BLA experiments.

Although 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 differed from the observations of Chan et al. (2012) who, in contrast to this work, used CF as the only parent compound susceptible to undergo isotopic fractionation as it biodegraded. However, this low isotopic fractionation might not be real. Thus, following US EPA (2008), procedures for measuring for metabolites of reductive dechlorination, the isotope ratio in these compounds would vary due to the combined effects of isotopic fractionation during its production from the degradation of the parent compound and 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 the compound is parent or metabolite (US EPA 2008).

Thus, in this work, the degradation of CT injected as pure phase at the start of the experiment led to the formation of CF that was lighter than its parent compound. Simultaneously, this CF was degraded to DCM (Figure 1A, C). In addition, the CF that was injected was also biodegraded to DCM (Figure 1A, C) and the CF remaining in the system was isotopically enriched. The presence of these CF of different origin and isotopic composition offset the isotopic enrichment of CF that was initially injected. This offset prevented us from observing the isotopic fractionation of CF in the experiments.

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

The 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 indicated enrichment. Thus, DGGE in the NA experiment (Figure 3) showed that the most significant changes in the population occurred between day 62 (after the maximum concentration of CT and CF occurred; Figure 1A) and day 166. In contrast, there was considerable development of microorganisms in the BLA experiment (Figure 4) until day 62, and selective enrichment of microorganisms occurred in parallel to the fall of CT and CF (Figure 1C). The addition of lactic acid from the start of the BLA experiment led to the enrichment of groups of microorganisms that competed for biodegradable electron donors with microorganisms that were able to biodegrade chloromethanes. After day 62, selection occurred because some of the initial communities thrived after they were transferred to a new environment in which lactic acid was added as an electron donor at levels not observed in the original environment.

Thus, a conspicuous band, which was found throughout the active tests in water and sediment samples, exhibited an increase in intensity for the NA experiment. This increase was markedly appreciable after day 166, which was evidence of the selection of microorganisms. This band (which is depicted by an arrow in Figures 3 and 4 and an asterisk in Figure S6) was subsequently found to match the electrophoretic mobility of the operational taxonomic unit 6 (OTU 6) (Dechlorosoma suillum) (see “Microbial Community Structure and Dynamics in the Biostimulation Experiment” and Table 3). In addition, the changes in microbial population in the NA experiment coincided with the decline in nitrates and sulfates (Figure S2A) and were appreciable after day 133. In the case of the BLA experiment, a progressive selective enrichment of OTU 6 and OTU 15 (Clostridiales bacterium) was observed (Figure 4, “Microbial Community Structure and Dynamics in the Biostimulation Experiment,” and Table 3) at days 166, 260, and 360, which coincided with the decline in nitrates and sulfates (Figure S3A).

Dechlorination of CT and CF in the active NA and BLA experiments commenced when denitrification occurred (Figures 1A and S2A and Figures 1C and S3A, respectively). However, this dechlorination increased after sulfate concentrations decreased in the active BLA experiment (Figure 1C and Figure S3A). Consequently, the addition of lactic acid (as an electron donor) accelerated the exhaustion of other electron donor.
Figure 3. DGGE profiles of the amplified 16S rDNA of water samples (in duplicate) 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 (in duplicate) 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).
acceptors different from chloromethanes, inducing the earlier selective enrichment of the flora, which coincided with the decline in CT and CF (Figure 4).

In summary, selection could have been a result of the following: (i) enrichment in the active NA tests of sulfate-reducing and halorespiring communities; the former disappeared or remained as a minority when bioavailable sulfate was depleted, which promoted further enrichment of OTU 6 and OTU 15; and (ii) because some of the initial communities in the active BLA tests thrived after being transferred to a new environment in which lactic acid as an electron donor was added at levels not observed in the original environment.

### 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 | Frequency (%) | Nearest relative in GenBank (accession number) | % Identity | Taxonomic group |
|-----|---------------|-----------------------------------------------|------------|----------------|
|     | t = 0 days    | t = 360 days                                  |            |                |
| 1   | 21.4          | n.d.                                          | 96         | P. Chlorobi, C. Ignavibacteria, O. Ignavibacteriales |
| 2   | 10.7          | n.d.                                          | 99         | P. Proteobacteria, C. Betaproteobacteria, O. Methylophilales, F. Methylophilaceae |
| 3   | 28.6          | n.d.                                          | 99         | P. Proteobacteria, C. Betaproteobacteria, O. Methylophilales, F. Methylophilaceae |
| 4   | 7.1           | n.d.                                          | 94         | P. Firmicutes, C. Clostridia, O. Clostridiales |
| 5   | 7.1           | n.d.                                          | 99         | P. Proteobacteria, Beta proteobacteria, O. Burkholderiales, F. Comamonadaceae |
| 6   | 3.6           | 11.1                                          | 99         | P. Proteobacteria, C. Betaproteobacteria, O. Rhodocyclales, F. Rhodocyclaceae, Azospira |
| 7   | 14.3          | n.d.                                          | 99         | P. Proteobacteria, C. Betaproteobacteria, O. Rhodocyclales, F. Rhodocyclaceae |
| 8   | 3.6           | n.d.                                          | 98         | P. Proteobacteria, C. Alphaproteobacteria, O. Rhodospirillales, F. Rhodospirillaceae, *Magnetospirillum* |
| 9   | 3.6           | n.d.                                          | 100        | P. Proteobacteria, C. Betaproteobacteria, O. Burkholderiales, F. Comamonadaceae |
| 10  | n.d.          | 7.4                                           | 99         | P. Chloroflexi, C. Anaerolineae, O. Anaerolineales, F. Anaerolineaceae |
| 11  | n.d.          | 7.4                                           | 96         | P. Bacteroidetes, O. Cytophaga, F. Cytophagaceae, *Meniscus* |
| 12  | n.d.          | 14.8                                          | 97         | P. Firmicutes, C. Clostridia, O. Clostridiales, F. Syntrophomadaceae, *Syntrophomonas* |
| 13  | n.d.          | 37                                            | 98         | P. Firmicutes, C. Clostridia, O. Clostridiales, F. Peptococcaceae |
| 14  | n.d.          | 11.1                                          | 93         | P. Firmicutes, C. Clostridia, O. Clostridiales, F. Clostridiales, *Oxobacter* |
| 15  | n.d.          | 7.4                                           | 98         | P. Firmicutes, C. Clostridia, O. Clostridiales, F. Ruminococcaceae |
| 16  | n.d.          | 3.7                                           | 99         | P. Proteobacteria, C. Alphaproteobacteria, O. Caulobacteriales, F. Caulobacteraceae, *Brevundimonas* |

*Note. P. = phylum; C. = class; O. = order; F. = family.

OTU = operational taxonomic unit.

1Not detected

1Deduced after SINA online comparisons to the SILVA seed reference alignment (www.arb-silva.de).
the microbial community (Figures 3 and 4). With regards to the identified OTU, we describe those that had noteworthy roles at the start and end of our experiment.

**Start of the experiment (Day 0)**
At the start of the experiment, denitrification was observed (see “Interactions between Dechlorination and Other Redox Reactions”) and the dominant taxonomic group was the Betaproteobacteria class of bacteria (67.9% of the clones; Table 3). The presence of Betaproteobacteria in nitrate-reducing conditions was consistent with previous studies that showed members of the Betaproteobacteria to be the predominant species in enrichment cultures of denitrifying bacteria (Heylen et al. 2006).

The Methylphilaceae family (OTU 2 and OTU 3; 39.3%) is a noteworthy member of the Betaproteobacteria class. This family includes some, but not all, methylotrophic bacteria, which are microorganisms that are capable of growing on chloromethanes. The presence of this family was 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).

OTU 6 (3.6%), identified as Azospira (synonym: Dechlorosoma, a genus of the family Rhodocyclaceae) was 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 by 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 and Reinhold-Hurek 2003). This microorganism has also been detected at the field scale in groundwater contaminated by chlorinated solvents (Zemb et al. 2010). In addition, this is a respiring heterotrophic microorganism that can use electron acceptors other than oxygen (i.e., nitrate, chlorate, and perchlorate). Additionally, it is capable of using Fe(II) as an electron donor (Achenbach et al. 2001; Chaudhuri, Lack, and Coates 2001; Lack et al. 2002). Furthermore, D. suillum has been found to be associated with nitrate-dependent Fe(II)-oxidizing microorganisms in sediments, which use nitrates as electron acceptors (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 “Interactions between Dechlorination and Other Redox Reactions”). In addition, abiotic dechlorination of CT was particularly observed in the BLA experiment, coinciding with a higher increase in D. suillum than in the NA experiment. This is the first time that the coincidence of these processes has been described using samples from a real site.

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).

**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) suggested that the addition of lactic acid favored the selective enrichment of this bacterium while continuing to coincide with the abiotic degradation of CT. As stated above (see “Start of the Experiment (Day 0)”), 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, the fact that nitrate was practically exhausted during the experiment suggests that this microorganism may have played a role in the degradation of CT.

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). The reductive dechlorination of CT and CF by a respiratory process has been described for some members of Clostridiales, i.e., genus *Dehalobacter* (Grostern and Edwards 2006; Grostern et al. 2010; Lee et al. 2011; Chan et al. 2012; Justicia-Leon et al. 2012). The *Clostridium* species has been shown to cometabolically degrade CT (Galli and McCarty 1989; Lima and Sleep 2010). Thus, it is reasonable to assume that one or several of the Clostridiales microorganisms found in our microcosms was also responsible for the reductive dechlorination of CT and CF, thus favoring its enrichment.

OTU 15 (7.4%, the remaining OTU of the Clostridiales) showed a high identity (98%) with a sequence extracted from an uncultured microorganism of an anaerobic microbial community of a tar oil contaminant plume. This OTU was identified in the DGGE
profiles shown in Figure 4 and appeared clearly after day 166 when nitrate and sulfate concentrations declined (Figure S3A). Simultaneously, CF concentrations sharply decreased after day 62, and this change in concentration was accompanied by an increase in DCM (Figure 1C). This increase suggests that this bacterium of the Clostridiales order played a role in the CF reductive dechlorination process to form DCM in the microcosm.

Finally, OTU 16 (3.7%) represented the *Brevundimonas* sp. (Alphaproteobacteria). Krausova, Robb, and Gonzalez (2006) previously discovered this species in a consortium consisting of DCM-degrading *Pseudomonas* sp. and *Brevundimonas* sp.

**Conclusions**

The 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 demonstrated the occurrence of selection, which was a consequence of the enrichment of halorespiring and sulfate-reducing communities. The latter disappeared or was reduced to a minority when bioavailable sulfate was decreased.

This depletion was accompanied by growth in *D. suillum* (OTU 6) and a bacterium of the Clostridiales order (OTU 15). The addition of lactic acid, an electron donor, accelerated the exhaustion of other electron acceptors, inducing earlier enrichment of the flora that directly or indirectly reduced CT and CF and increased degradation rates. The abiotic degradation of CT to form CF occurred particularly in the BLA experiment, in which higher increases in *D. suillum* were observed over time than in the NA experiment. Although the presence of this microorganism coincided with the abiotic degradation of CT, it is not known whether *D. suillum* played a role in this degradation process. The transformation of CF by biotic reductive dechlorination to form DCM was a result of the respiratory process of a bacterium of the Clostridiales order (OTU 15). It is worth highlighting the coincidence of the following processes: (i) the abiotic reductive dechlorination of CT; (ii) the parallel transformation of CF by biotic reductive dechlorination; and (iii) the progressive enrichment of *D. suillum* (OTU 6) and a bacterium of the Clostridiales order (OTU 15).

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

**Acknowledgments**

We are indebted to the Catalan Water Agency and members of Clariant Ibérica S.A. of Tarragona and INTECSON S.L. of Reus for their support and cooperation while carrying out this fieldwork. We also thank members of the Department of Geochemistry, Petrology, and Geological Prospecting at the University of Barcelona and particularly members of the hydrogeology group. We are grateful to members of the Scientific Technical Services at the University of Barcelona for their help in analyzing the samples.

**Funding**

This research was supported by projects CTM 2005-07824 and CGL 2008-02164/BTE of the Spanish Ministry of Education and Science and Clariant Ibérica S.A.

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