Interspecies interaction and effect of co-contaminants in an anaerobic dichloromethane-degrading culture

Alba Trueba-Santiso[a], David Fernández-Verdejo[a], Irene Marco-Rius[b], Jesica M. Soder-Walz[a], Oriol Casabella[a], Teresa Vicent[a], Ernest Marco-Urrea[a,*].

[a]Departament d'Enginyeria Química, Biològica i Ambiental, Universitat Autònoma de Barcelona (UAB), Carrer de les Sitges s/n, 08193 Cerdanyola del Vallès, Spain.

[b]Institute for Bioengineering of Catalonia. Parc Científic de Barcelona. Edifici Clúster c/ Baldiri Reixac 10-12, 08028 Barcelona, Spain.

*Corresponding author

Departament d'Enginyeria Química, Biològica i Ambiental, Universitat Autònoma de Barcelona (UAB), c/ de les Sitges s/n, 08193 Cerdanyola del Vallès, Spain. E-mail: ernest.marco@uab.cat, Phone: +34 935812694.

ABSTRACT

An anaerobic stable mixed culture dominated by bacteria belonging to the genera *Dehalobacterium*, *Acetobacterium*, *Desulfovibrio*, and *Wolinella* was used as a model to study the microbial interactions during DCM degradation. Physiological studies indicated that DCM was degraded in this mixed culture at least in a three-step process: i) fermentation of DCM to acetate and formate, ii) formate oxidation to CO_2 and H_2, and iii) H_2/CO_2 reductive acetogenesis. The 16S rRNA gene sequencing of cultures enriched with formate or H_2 showed that *Desulfovibrio* was the dominant population followed by *Acetobacterium*, but sequences representing *Dehalobacterium* were only present in cultures amended with DCM. Nuclear magnetic resonance analyses confirmed that acetate produced from ^{13}C-labeled DCM was marked at the methyl ([2-^{13}C]acetate), carboxyl ([1-^{13}C]acetate), and both ([1,2-^{13}C]acetate) positions, which is in accordance to acetate formed by both direct DCM fermentation and H_2/CO_2 acetogenesis. The inhibitory effect of ten different co-contaminants frequently detected in groundwaters on DCM degradation was also investigated. Complete inhibition of DCM degradation was observed when chloroform, perfluorooctanesulfonic acid, and diuron were added at 838, 400, and 107 µM, respectively. However, the inhibited cultures recovered the DCM degradation capability when transferred to fresh medium without co-contaminants. Findings derived from this work are of significant relevance to provide a better understanding of the synergistic interactions among bacteria to accomplish DCM degradation as well as to predict the effect of co-contaminants during anaerobic DCM bioremediation in groundwater.

KEYWORDS:

Dehalobacterium; dichloromethane; co-contaminants; inhibition; bioremediation.

1. INTRODUCTION

Dichloromethane (DCM) is a frequently detected groundwater contaminant that typically occurs in mixtures in either disposal sites or domestic well samples [1,2], and it is ranked 11th (out of 33) on the European Union priority list of pollutants [3]. Biodegradation of DCM has been described in the literature under both oxic and anoxic conditions [4], and it has become apparent that bacteria play a key role in the natural attenuation of sites impacted with DCM [5]. Most of the described DCM-degrading bacteria are aerobic methylotrophic [4], but in contaminated aquifers oxygen is oftenly very limited. Under anoxic conditions, *Dehalobacterium*, *Dehalobacter*, and *Candidatus* Dichloromethanomonas are the only known genera capable of utilizing DCM as growth substrate [6,7,8]. *Dehalobacterium formicoaceticum* is the only reported isolate [6], and previous studies suggested that it utilizes DCM as sole growth substrate and ferments this compound to acetate and formate [9]. Detailed physiological studies with *Candidatus* Dichloromethanomonas elyuquensis and *Dehalobacter*-containing cultures have shown that DCM degradation requires syntrophic partners (i.e. hydrogenotrophic homoacetogens and methanogens) present in the mixed culture, probably to prevent H_2 from reaching inhibitory levels [10,11]. The reasons why H_2 exerts and inhibitory effect on DCM degradation are still unclear, but Chen et al. [11] hypothesized that it could be provoked by formate accumulation inside the cell of *Candidatus* Dichloromethanomonas elyuquensis because this strain does not contain formate transporters and formate conversion to CO_2 and H_2 is impeded at high concentrations of H_2.

Halogenated compounds are often present in aquifers as complex mixtures from industrial, urban or agricultural practices, and in some cases may impede the accomplishment of remedial goals. In particular, chloroform (CF), chlorinated ethenes,

and chlorinated ethanes have been shown to inhibit the dechlorinating activity of organohalide-respiring bacteria [12-15]. To date, publications on the inhibitory effect of certain chlorinated solvents over DCM-degrading bacteria are scarce. For instance, previous studies showed that l,1-dichloroethane, tetrachloroethene, and trichloroethene (TCE) did not affect DCM degradation in *D. formicoaceticum*, while tetrachloromethane, CF, and chloromethane caused an immediate and complete inhibition of DCM degradation and bacterial growth [9]. A better understanding of the effect of co-contaminants in enrichment cultures under laboratory conditions may provide a basis to anticipate whether bioremediation is a feasible strategy to remediate groundwater when multiple contaminants are present.

The aim of the work presented herein is two-fold. First, we attempt to examine the bacterial interactions in a mixed culture during DCM degradation. We use a *Dehalobacterium*-containing culture derived from a membrane bioreactor operating in an industrial wastewater treatment plant that had been enriched with DCM for more than three years [16]. Previous studies indicated that this mixed bacterial culture was mainly composed by four genera (*Dehalobacterium*, *Acetobacterium*, *Desulfovibrio*, and *Wolinella*) and produced acetate and formate when grown with DCM and H_2 in a bicarbonate-buffered medium [16]. Secondly, we assessed the effect of selected halogenated contaminants on the DCM-degrading capability of this mixed culture. The list of tested compounds comprises chloroalkanes, chloroalkenes, CF, acetone, polyfluoroalkyl substances, as well as the halogenated pesticide diuron and its transformation product 3,4-dichloroaniline (3,4-DCA). The selected compounds are among the most frequently detected in contaminated groundwater, do not serve as substrates for this culture [16], and are all included in the 2017 ATSDR Priority List of Hazardous Substances [17], with the exception of 3,4-DCA.

2. MATERIALS AND METHODS

2.1 Cultivation

The experiments were performed with a stable DCM-degrading consortium composed predominantly by bacteria from the genera *Dehalobacterium*, *Acetobacterium*, *Wolinella* and *Desulfovibrio* derived from slurry samples of the membrane bioreactor of an industrial wastewater treatment plant [16]. The culture was grown in 100 mL glass serum bottles (microcosms) with 65 mL of reduced, anoxic, bicarbonate-buffered medium (pH=7) described elsewhere but lacking acetate and H_2 [16]. Briefly, this medium contained DCM, yeast extract (200 mg/L), vitamins, trace elements, tungsten (22.8 μM), selenium (24.2 μM), and $Na_2S \cdot 9H_2O$ and L-cysteine (0.2 mM each) as reducing agents. Bicarbonate (0.01 M) and resazurin (0.25 mg/L) were used as a buffer and redox indicator, respectively. The serum bottles were sealed with Teflon-coated butyl rubber stoppers and aluminium crimp caps and gassed with N_2 (0.4 bar overpressure). The bacterial consortia was maintained active by transferring a 3% (v/v) inoculum during the exponential degradation phase of 2 mM DCM doses into fresh medium. The microcosms were cultivated under static conditions (25°C) in the dark. Two controls were always included for each experiment in triplicate: i) abiotic controls consisting of anaerobic medium plus DCM to discard its abiotic transformation, and ii) biotic controls consisting of anaerobic medium plus bacterial inoculum but without DCM to evaluate production of metabolites from the components of the medium. DCM was replaced by either formate or H_2 when indicated.

To elucidate the role of the bacteria present in the mixed culture in the degradation of the daughter products of DCM, the changes in the bacterial community structure were studied after applying the dilution-to-extinction method [18] in 12-mL

vials containing the anoxic medium described above but using either formate (420 µmol) or H_2 (0.2 bar overpressure) as substrate in parallel cultures without DCM. After two extinction series, the more diluted vial showing activity against the tested substrate was selected as inoculum for serum bottle microcosms and it was amended with different substrates. The procedure followed and the vials selected are depicted in Fig. S1A and B.

2.2 Analytical methods

Volatile halogenated compounds and acetone were quantified by injecting a 0.5-mL headspace sample into an Agilent 6890N gas chromatograph (GC) fitted with an Agilent DB-624 column (30 m × 0.32 mm with 0.25 µm film thickness) and a flame ionization detector as previously described [16]. Formate and acetate were measured using a Dionex 3000 Ultimate high-performance liquid chromatography equipped with a UV detector at 210 nm, as described elsewhere [16]. Hydrogen and carbon dioxide concentration was measured on 0.1-mL headspace samples using an Agilent 7820A GC equipped with a thermal conductivity detector. Separation was achieved using a MolSieve 5A 60/80 SS (1.82 m x 2 mm, Agilent) and a Porapak Q 60/80 UM columns (1.82 m x 2 mm, Agilent). Nitrogen was the carrier gas at 138 kPa. Oven temperature was held isothermal at 40 ºC, the injector temperature at 200 ºC and the detector temperature at 250 ºC. Peak areas were calculated using Chromeleon software (Thermo Scientific). Results were presented as either nominal concentrations or total µmoles per bottle. Calibration was based on aqueous standards under the same conditions of temperature, pressure and liquid and headspace volumes as those in the microcosms.

Nuclear magnetic resonance (NMR) analysis was performed with *Dehalobacterium*-containing cultures grown with 3.6 mM of ^{13}C-DCM using the same

medium described above. Samples were taken at the initial time (when DCM was amended) and after DCM consumption, and 500 µL of each sample were mixed with 50 µL deuterium oxide and transferred to 5 mm wide NMR tubes. ^{1}H decoupled ^{13}C-NMR was performed at 25ºC in a 500 MHz vertical-bore Bruker spectrometer using a CPTCI probe (Bruker BioSpin GmBH). The acquisition parameters were the following: repetition time = 1 s, acquisition time = 0.56 s, nominal flip angle = 30º, pulse width = 5.7 µs (hard pulse), spectral width = 27.8 kHz, number of points = 32768, number of scans = 30720. Data were Fourier transformed, zero-filled (2x), baseline and phase corrected, and 1 Hz line broadening applied using TopSpin 4.0.6 (Bruker BioSpin GmBH). To confirm the chemical shift assignment of the peaks, 6.5 mg of the NMR standard 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) were added to the samples above and data acquired using the same parameters aforementioned. An additional sample containing 1 M of unlabelled methanol, 1 M of unlabelled formic acid and DSS in medium solution was also analysed by NMR to confirm the presence or not of those compounds in the bacteria-metabolised samples.

2.3 DNA extraction and 16S rRNA gene amplicon sequencing

Cells were harvested by centrifugation (7000 × g, 40 min at 10 ºC) from 65-mL samples (one microcosm) using sterile falcon tubes in an Avanti J-20 centrifuge. The pelleted cells were resuspended in sterile PBS buffer and DNA was then extracted using the Gentra Puregene Yeast/Bact kit (Qiagen) following the instructions from the manufacturer. The DNA of each of the biological replicates was extracted and analysed separately. Amplicons of the region V3-V4 for 16SrRNA genes were amplified with primers S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 [19] using the Illumina MiSeq platform at Serveis de Genòmica i Bioinformàtica from the Universitat Autònoma de

Barcelona. Gene sequences were processed using the 16S Metagenomics workflow in the MiSeq Reporter analysis software based on quality scores generated by real-time analysis during the sequencing run. Quality-filtered indexed reads were demultiplexed for generation of individual FASTQ files and aligned using the banded Smith–Waterman method [20] of the Illumina-curated version of the Greengenes taxonomic database. The output of this workflow was a classification of reads at multiple taxonomic levels. To calculate relative abundance, we divided the number of sequences belonging to a taxonomic level by the total number of sequences obtained from the specific sample.

2.4 Inhibition tests

Each co-contaminant inhibition test consisted on two groups of experimental bottles prepared in triplicates at the same time: (i) controls amended only with neat DCM and (ii) parallel sets of experimental bottles amended with different concentrations of the co-contaminant from acetone stock solutions plus neat DCM. Both experimental bottles were inoculated with the *Dehalobacterium*-containing culture (3 %, v/v) at the exponential phase of DCM degradation. Then, a time course experiment was performed, monitoring DCM concentration daily by GC. All experimental bottles amended with co-contaminants were compared to the corresponding controls amended only with neat DCM. Rates were calculated from linear (R^2>0.98, n≥3) DCM disappearance after the lag phase period.

To study the functional recovery of DCM degradation after the complete inhibition observed with some co-contaminants, cultures inhibited by CF were purged with nitrogen to remove CF, and 3.5 mL of the inhibited cultures were transferred into fresh medium spiked with neat DCM. Those cultures inhibited by other halogenated

compounds were directly transferred to fresh medium without purge. These experiments were set up at least in duplicate.

2.5 Microscope observation

Aliquots (20 µL) of *Dehalobacterium*-containing cultures were placed in slides, covered with a cover slip, and observed under a light microscope (Zeiss Axioscop) at 40 x or 100 x (adding immersion oil) objectives.

3. RESULTS AND DISCUSSION

3.1 Mass balance and fate of DCM

Dehalobacterium-containing cultures fed with DCM (153.9 ± 0.4 µmol) were initially stablished in the abovementioned defined medium without H_2 but containing CO_2 from the bicarbonate buffered medium. After a lag phase of 7 d, DCM degradation occurred at a rate of 902 ± 10 µmol d^{-1} L^{-1}, and acetate and CO_2 were produced concomitantly (Fig. 1). Formate and H_2 were potential degradation intermediates, however they were not detected (Fig. 1). After a second feeding of DCM, it was consumed without an observable lag phase, indicating that bacterial degradation of DCM is a growth-supporting process. Controls without DCM also produced acetate but at noticeable lower concentrations, presumably derived from the fermentation of the yeast extract contained in the medium (Fig. S2). Different attempts were made to remove yeast extract from the medium but always resulted in a marked decrease in DCM degradation.

Formate was provided at two different concentrations (430.9 ± 39.6 and 964.3 ± 71.7 µmol) in cultures where DCM and H_2 were omitted to ascertain whether formate was oxidized by the microbial consortia (Fig. 2). Formate degradation was accompanied

by an increase in acetate and H_2 concentration (Fig. 2, Fig. S3A). The concentration of CO_2 slightly increased in the initial stages, but after 4 d it was consumed coinciding with a steep increase of acetate in the medium (Fig. S3A). A decrease in CO_2 was not observed in controls where formate was omitted (Fig. S3B), suggesting that acetate was produced via CO_2 reduction.

The microcosms amended with 7 mL of H_2 (~268 µmol) consumed both H_2 and CO_2 and produced acetate, indicating H_2/CO_2 reductive acetogenesis (Fig. 3). Acetate was also generated in the controls without H_2 but at significantly lower concentrations (Fig. 3).

Physiological studies with the isolate *D. formicoaceticum* suggested that DCM is transformed to methylene-tetrahydrofolate, which is funnelled into the Wood-Ljunddahl pathway and disproportionated [9]. By following this pathway, two thirds of methylene-tetrahydrofolate is oxidized to formate, and the remaining third is reduced to acetate in combination with CO_2, according to Eq. 1:

$$3CH_2Cl_2 + 4H_2O + CO_2 \rightarrow 2HCOO^- + CH_3COO^- + 9H^+ + 6Cl^- \quad \text{(Eq. 1)}$$

In our study, formate was not observed as a transient product when DCM was amended to *Dehalobacterium*-containing cultures. Similarly, there was no evidence of formate production in cultures containing *Candidatus* Dichloromethanomonas elyuquensis when fed with DCM [11]. However, the fact that formate was turned over to CO_2 and H_2 (Fig. 2) provided a basis to hypothesize that formate derived from DCM was rapidly oxidized by a yet unknown bacteria from the mixed culture (Eq. 2) and evaded detection.

$$HCOO^- + H^+ \rightarrow H_2 + CO_2 \quad \text{(Eq. 2)}$$

In addition, production of acetate in microcosms containing either H_2 or formate suggested that released hydrogen served as the electron donor for CO_2 reduction to acetate (Eq. 3):

$$4H_2 + 2CO_2 \rightarrow CH_3COO^- + 2\ H_2O + H^+ \qquad \text{(Eq. 3)}$$

Stoichiometric analysis revealed that the electrons generated from H_2 oxidation in cultures growing with DCM, formate, and H_2 were mostly recovered in acetate (range between 65-87%) (Table S1). The balance did not precisely match the stoichiometric conservation of H_2 because part of the carbon derived from DCM fermentation was sequestered into the biomass and not yielded acetate.

3.2 Degradation experiment using ^{13}C-labelled DCM

The metabolites produced after ^{13}C-labelled DCM degradation were analysed by NMR. As observed in Fig. 4, acetate, CO_2, and methanol were detected after the consumption of 3.6 mM ^{13}C-DCM. Production of acetate and CO_2 had already been demonstrated by chromatographic analyses, but methanol had not been detected. Methanol was previously reported in *D. formicoaceticum* and it was considered as a side reaction when DCM is uncoupled from growth [9].

The metabolism of DCM fermentation following Eq. 1 implies that the formate and the methyl group of acetate are derived entirely from ^{13}C-DCM, whereas the carboxyl group of acetate is formed from CO_2 [9] (Fig. S4). The ^{13}C-NMR spectrum of the samples after ^{13}C-DCM consumption displayed peaks at 26.0 ppm and 184.1 ppm, corresponding to the methyl and carboxyl groups of acetate, respectively. The inserts on Fig. 4B show the splitting caused by the carbon-carbon J-coupling (~52.3 Hz) in acetate ^{13}C-labeled in both methyl and carboxyl carbons ([1,2-^{13}C]acetate). Therefore, we can conclude that acetate derived from ^{13}C-DCM degradation accumulated in form of

acetate labelled with ^{13}C at the carboxyl ([1-^{13}C]acetate), methyl ([2-^{13}C]acetate), and both ([1,2-^{13}C]acetate) positions. Since data acquisition was not done on a fully-relaxed state but under a steady-state condition, we cannot reliably quantify each species. However, we can roughly calculate that there was a ratio [2-^{13}C]acetate/[1,2-^{13}C]acetate ~ 2.5 and [1-^{13}C]acetate/[1,2-^{13}C]acetate ~ 2.1. This data suggests that two acetate producing pathways could be operating in this culture. On the one hand, acetate labelled solely in the methyl group ([2-^{13}C]acetate) is consistent with ^{13}C-DCM fermentation by *Dehalobacterium* using unlabelled bicarbonate from the buffered medium (Eq. 1, Fig. S4). This bacterium could also produce [1,2-^{13}C]acetate if using $^{13}CO_2$ released during ^{13}C-formate oxidation. Hence, $^{13}CO_2$ could be reduced to CO by CO dehydrogenase, which is encoded in *D. formicoaceticum* genome [21], and become the carboxyl group of acetate via acetyl-CoA using the Wood-Ljungdahl pathway (Fig. S4). In a previous study, NMR analysis of products formed from ^{13}C-DCM by the isolate *D. formicoaceticum* showed that carboxyl group was unlabelled because the only source of CO_2 was the carbonate-buffered medium and ^{13}C-formate was not further oxidized by other bacteria to $^{13}CO_2$ [9]. On the other hand, acetogenic bacteria could produce acetate labelled indistinctly at C1 or C2 positions using the pool of labelled and unlabelled CO_2 present in the medium, but unlike to *Dehalobacterium*, acetogens can produce acetate labelled exclusively in the carboxyl group ([1-^{13}C]acetate).

3.3. Role of bacteria in the mixed culture

We aimed to identify the community members responsible for the transformation of DCM-degrading metabolites in this mixed culture. Hence, the approach was extinction culturing by diluting the consortium using formate and H_2 as sole substrates in parallel

281 treatments (Fig. S1). The resulting cultures were tested against DCM, formate, and H_2
282 consumption, and their DNA sequenced using paired end Mi-Seq Illumina (Fig. 5).
283 No sequences representing *Dehalobacterium* were detected in the dataset
284 generated with DNA extracted from cultures grown with either formate or H_2 (Fig. 5B-
285 D). Accordingly, these cultures were not capable of consuming DCM (data not shown)
286 corroborating that *Dehalobacterium* was responsible for DCM degradation. This is in
287 agreement with previous studies showing that formate alone could not support growth
288 of *Candidatus* Dichloromethanomonas elyuquensis and *Dehalobacterium*
289 *formicoaceticum* [9,11].
290 When the cultures were enriched with formate, the bacterial community was
291 dominated by *Desulfovibrio* (69.6%) and *Acetobacterium* (26.7%) genera (Fig. 5B). In
292 cultures enriched with H_2, the most prevalent genera was again *Desulfovibrio* (76.3%),
293 and *Wolinella* (10.2%) and *Acetobacterium* (7.6%) were found at lower relative
294 abundance (Fig. 5C). When this H_2-enriched culture was tested for formate degradation,
295 it was readily consumed, and the genus *Acetobacterium* contributed up to 16.9% of the
296 total amplicons but *Desulfovibrio* was still the dominant population (Fig. 5D).
297 Interestingly, *Desulfovibrio* was also detected in previous anaerobic enrichment cultures
298 containing *Dehalobacter* and *Candidatus* Dichloromethanomonas elyuquensis capable
299 of fermenting DCM [10,11].
300 The Wood-Ljunddahl pathway allows acetogenic bacteria to convert H_2/CO_2 and
301 formate via acetyl-CoA to acetate. All members of the genus *Acetobacterium* use H_2 as
302 the electron donor for CO_2 reduction to acetate, and some species can also utilize
303 formate, which is an intermediate derived from CO_2 in acetate formation [22]. Due to
304 this versatility, *Acetobacterium* may be involved in the reactions described in Eq. 2 and
305 Eq. 3. On the other hand, the yeast extract contained in the medium is a potential source

306 of sulphate for the sulphate-reducing bacterium *Desulfovibrio* using H_2 and formate as
307 electron donors. Alternatively, *Desulfovibrio* species have also been shown that, in the
308 absence of sulphate, they can convert CO_2 and H_2 to formate ($CO_2 + H_2 \rightarrow HCOO^- +$
309 H^+) but also produce H_2 from formate ($HCOO^- + H_2O \rightarrow HCO_3^- + H_2$) [23,24].
310 *Wolinella* here plays a more uncertain role. It is typically described to require H_2 and
311 formate as electron donor substrates to drive the reduction of various electron acceptors
312 including inorganic nitrogen compounds (i.e. nitrate, nitrite) that could be provided by
313 the yeast extract [24], and the absence of *Wolinella* in formate-amended cultures (Fig.
314 5B and 5D) is likely due to lower growth rates with this substrate.

315 In previous studies, DCM degradation was severely inhibited by H_2 in a mixed
316 culture containing *Candidatus* Dichloromethanomonas elyuquensis and the presence of
317 hydrogenotrophic partners was considered mandatory to enable DCM degradation to
318 proceed [11]. Different attempts were made to study whether exogenous added H_2
319 exerted an inhibition effect on DCM degradation in our mixed culture, but inhibitory H_2
320 partial pressure could not be stablished because acetogenic bacteria consumed H_2
321 quickly in the microcosms (data not shown).

322

323 **3.4. Effect of co-contaminants on DCM degradation**

324 In this study, we assessed the effects of different organochlorines as co-contaminants on
325 the activity of DCM degradation. The results obtained with the microcosms amended
326 with chloroalkanes [1,1,2-trichloroethane (1,1,2-TCA) and 1,2-dichloroethane (1,2-
327 DCA)] and chloroalkenes [TCE and *cis*-dichloroethylene (*cis*-DCE)] showed that 1,2-
328 DCA and *cis*-DCE did not affect the rate of DCM degradation for the range of
329 concentrations tested (100-850 µM) (Fig. 6A-B). A slight decrease on DCM
330 degradation rate was observed for TCE and 1,1,2-TCA at concentrations of 850 and ≥

500 µM, respectively (Fig. 6C-D). Previous studies have shown that 1,2-DCA and *cis*-DCE provoked a decrease in the dechlorination potential of *Dehalococcoides* [13]. In agreement with our results, DCM degradation by *D. formicoaceticum* was not inhibited by TCE at 1 mM [6].

The co-contamination of groundwater with CF and DCM is a relevant scenario for this study due to the existence of anaerobic bacteria belonging to the genus *Dehalobacter* capable of transforming CF into DCM via organohalide respiration [7,26]. CF is also a known strong inhibitor for many bacterial processes, such as methanogenesis [27,28] and reductive dechlorination of chloroethenes [29,30] and orto-chlorophenol [31]. Here, the addition of 67 and 84 µM CF did slow DCM degradation but when added at 838 µM CF, no DCM degradation was observed for 90 d (Fig. 6E). Egli *et al.* [32] pointed out that anaerobic dechlorination of chlorinated methanes is frequently reported for bacteria harbouring the reductive acetyl coenzyme A (acetyl-CoA) pathway that involves the coenzyme B_{12} as a carrier for methyl groups. This pathway is utilized by *D. formicoaceticum* to insert CO_2 into the carboxyl group of acetate during DCM fermentation [9,21]. Vitamin B_{12} is known to stimulate the reductive dechlorination of some chlorinated methanes (i.e. CF and carbon tetrachloride) in microbial mixed cultures [33]. Therefore, we tested whether the addition of 10 µM of vitamin B_{12} was effective in reactivating the DCM degradation in cultures that had been exposed to 838 µM CF for 37 d, but DCM was not degraded 40 d after the addition (data not shown). Likewise, DCM fermentation by *D. formicoaceticum* and *Dehalobacter*-containing cultures was shown to be inhibited by CF at concentrations of 1 mM and 41.8 µM, respectively [6,34].

The fluorinated organic compounds perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) were amended at concentrations up to 400 and 483 µM,

356 respectively. The addition of 400 µM PFOS completely inhibited DCM degradation,
357 whereas PFOA at concentrations ≥ 241 µM allowed its full degradation but at
358 degradation rates lower than the controls (Fig. 6F-G). This is in contrast with the results
359 for *Escherichia coli*, where PFOA displayed a higher toxicity than PFOS [35]. Recently,
360 a mixture of ten polyfluoroalkyl substances (including PFOA and PFOS) was shown to
361 prevent the full reductive dechlorination of TCE in a *Dehalococcoides*-containing
362 culture [12]. However, the effect of individual PFOA and PFOS on *Dehalococcoides* sp.
363 still remains unknown.

364 We also tested the effect of the herbicide diuron and its daughter product 3,4-
365 DCA on DCM degradation. Diuron amended at 43 µM caused a marked decrease in the
366 DCM degradation rate, but when diuron was added at concentrations of 107 and 322
367 µM, DCM degradation was completely inhibited (Fig. 6H). In contrast, the addition of
368 3,4-DCA only caused a slight reduction of the DCM degradation rate at concentrations
369 above 463 µM (Fig. 6I).

370 Lastly, acetone was also tested as a co-contaminant in order to discard that the
371 described observed inhibitory effects were caused by its presence, as previous tested
372 organohalides were added in the microcosms as acetone stock solutions. Also, acetone
373 is a common groundwater contaminant, ranked 189 (out of 275) on the 2017 Priority
374 List of Hazardous Substances [17]. The concentrations of acetone tested (8-78 µM)
375 corresponded to the range added in the previous experiments. As shown in Fig. 6J, none
376 of the acetone concentrations tested had an adverse effect on DCM degradation rate.

377 **3.5. Functional recovery of DCM degradation activity after inhibitors exposure**

378 *Dehalobacterium*-containing cultures exposed for 37 d to the inhibitory CF
379 concentration of 838 µM were used as inoculum in CF-free medium and amended with

DCM. DCM degradation started after a lag phase of 4 d. DCM was depleted in all six replicates 9 d later (Fig. S5A). The same procedure was repeated with inoculum exposed to 838 µM CF for 90 d. In this case, microcosms exhibited an extended lag phase before DCM degradation was fully consumed (Fig. S5A), probably due to the decrease of viable cells caused by the longer exposition to the inhibitory concentration of CF.

Cultures that were inhibited by 107 and 322 µM diuron for 32 d were transferred in parallel to diuron-free microcosms amended with DCM. In both cases, DCM degradation activity started after 7 d of lag phase, and DCM was fully degraded two days later (Fig. S5B). Similarly, DCM degradation was recovered in cultures exposed to 400 µM PFOS for 30 d (Fig. S5C).

The fact that *Dehalobacterium sp.* are endospore-forming bacteria [21,36] could explain the recovery capacities observed in this study. Endospores are known to provide bacteria with a higher degree of resistance to several physical or chemical damage, such as those derived from wet and dry heat, UV and gamma radiation, extreme desiccation or oxidizing agents [37]. Accordingly, endospores were also observed by optical microscopy in our DCM-degrading culture (data not shown). In particular, this hypothesis seems a potential explanation for the recovery after PFOS inhibition, as it is described to disrupt bacterial cell membranes (among other cytotoxical effects), which results in cell inactivation or cell death. However, another possibility is that these pollutants act as reversible enzyme inhibitors binding via weak noncovalent bonds to *Dehalobacterium* enzymes modulating the enzyme activity by competing either directly or indirectly with DCM. Further research is required to explore the nature of this inhibitory responses and the recovery capacities of *Dehalobacterium* sp.

4. CONCLUSIONS

Acetate and formate have been identified as DCM degradation products using pure cultures of *Dehalobacterium formicoaceticum* in previous studies [9]. The physiological studies reported here indicated that formate oxidation is a feasible mechanism for H_2 generation in our *Dehalobacterium*-containing culture, although H_2 was not detected as intermediate during DCM degradation, likely due to the activity of H_2 scavengers (i.e. acetogenic bacteria). The analysis of the bacterial community structure of cultures enriched with formate and H_2 revealed a strong enrichment of *Desulfovibrio* and *Acetobacterium,* suggesting that both genera are involved in the transformation of the daughter products of DCM degradation. The balance of acetate produced from H_2 released during formate oxidation revealed that most of the electrons generated from H_2 were recovered in acetate, which is in agreement with the presence of *Acetobacterium* in the formate and H_2-grown cultures. The isotopic composition of acetate produced from ^{13}C-DCM suggests that acetate can be formed directly from DCM fermentation by *Dehalobacterium,* but also from reductive acetogenesis using CO_2 and H_2. These observations suggest interspecies H_2 transfer during DCM degradation to hydrogenotrophic partners (i.e. *Acetobacterium* and *Desulfovibrio*).

Contaminated sites typically involve complex mixtures of contaminants, the origin and fate of which are usually driven by biochemical transformations that can impact microbial attenuation. The results of this study have significant implications for bioremediation practices. We demonstrate that 1,1,2-TCA, 1,2-DCA, TCE, and *cis*-DCE barely affected DCM dechlorination at concentrations up to 850 µM. Conversely, complete inhibition of DCM was observed for CF, PFOS, and diuron in a dose-dependent manner. However, the *Dehalobacterium*-containing culture recovered its DCM degradation capability after the inhibition episodes had ceased, indicating that a

concentration reduction of these co-contaminants may completely detoxify DCM in groundwaters. This is of special relevance for CF, because DCM can derive from the organohalide respiration of CF by *Dehalobacter* sp, and co-contamination of both compounds is usually observed in CF-impacted aquifers. The concentrations of PFOS and diuron that caused complete DCM inhibition are well above to those found in environmental samples, which are usually in the range of ng/L [2,38,39,40]. Therefore, we conclude that PFOS and diuron could only affect DCM degradation by *Dehalobacterium* in a worst-case scenario. Interestingly, acetate and H_2 derived from DCM and formate degradation in this mixed culture can serve as a carbon source and electron donor, respectively, for organohalide-respiring bacteria present in the contaminated site (i.e. *Dehalococcoides*, *Dehalobacter*), which would act as "biostimulants" to enhance the remediation of halogenated co-contaminants.

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583 **FIGURE LEGENDS**

584 **Figure 1.** Time course of DCM degradation in a *Dehalobacterium*-containing culture.
585 Symbols means µmol per bottle of DCM (■), acetate (▲), CO_2 (♦), formate (▼), and
586 H_2 (●). Bars indicate standard deviation for triplicate bottles. Arrows indicate addition
587 of DCM.

588 **Figure 2**. Time course of formate degradation and concomitant production of acetate in
589 a *Dehalobacterium*-containing culture. Formate was added at two different
590 concentration in parallel treatments (open symbols and solid symbols refer to the
591 treatments with 964 and 431 µmol formate, respectively). Symbols means µmol per
592 bottle of formate (inverted triangles) and acetate (triangles). Bars indicate standard
593 deviation for triplicate bottles. Arrows indicate addition of formate.

594 **Figure 3.** Consumption of H_2 in a *Dehalobacterium*-containing culture. Symbols means
595 µmol per bottle of H_2 (●) and acetate (▲). Production of acetate in controls without H_2
596 were plotted (Δ). Bars indicate standard deviation for triplicate bottles. Arrows indicate
597 addition of H_2.

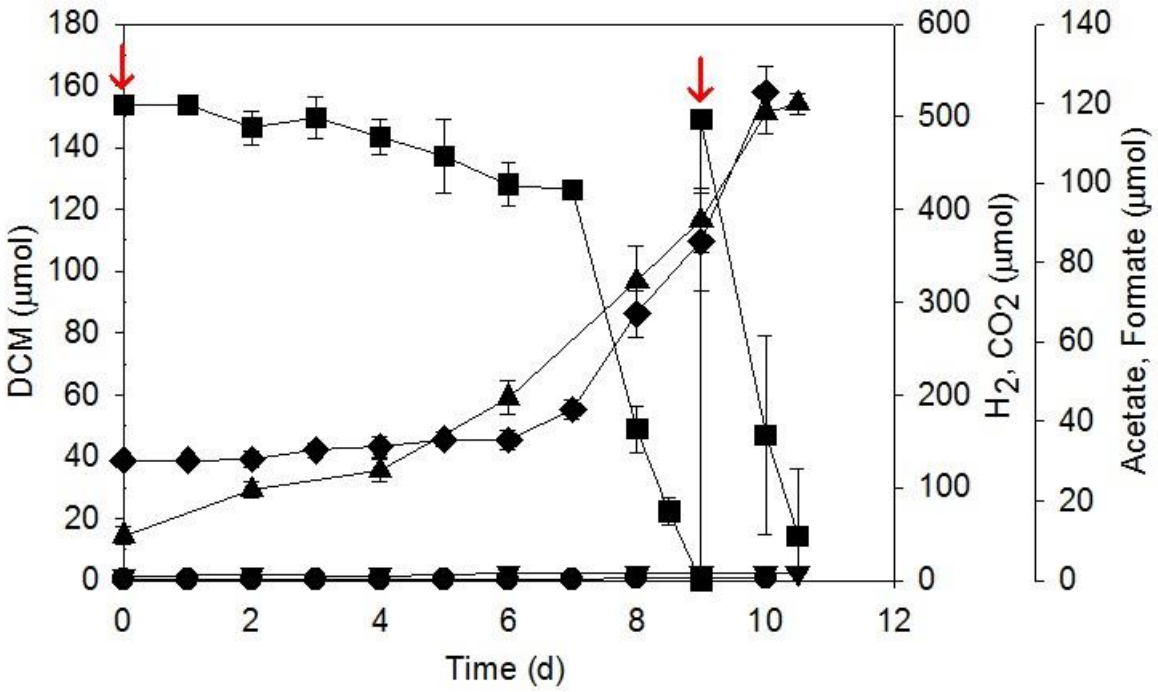
598 **Figure 4.** ^{13}C-NMR spectra of the metabolic products of ^{13}C-DCM in a
599 *Dehalobacterium*-containing culture at two different time points: immediately after the
600 addition of ^{13}C-DCM (Panel A), and after ^{13}C-DCM had been consumed (Panel B).
601 Peak resonances were confirmed by adding the NMR standard 4,4-dimethyl-4-
602 silapentane-1-sulfonic acid to the samples. The identity of methanol was confirmed by
603 adding 1 M of unlabeled methanol to the medium.

604 **Figure 5**. Relative abundance (%) of the bacterial population in the *Dehalobacterium*-
605 containing culture after enriched with DCM (A), formate (B), and H_2 (C) based on the

16S rRNA gene amplicon sequencing. Samples enriched with H_2 that were fed with formate are depicted in D. The results are average of duplicate samples. The number of quality filtered reads obtained from cultures grown with DCM, H_2, H_2+formate and formate were 93316/67640, 84389/47614, 22978/79460, 64182/74340, respectively. Only taxa with greater than 1% relative abundance are shown and reported to the lowest taxonomic rank possible.

Figure 6. Effect of different concentrations of co-contaminants on the degradation rate of DCM degradation in a *Dehalobacterium*-containing culture. Controls (C) correspond to cultures that contained DCM but no co-contaminants. 1,2-DCA (1,2-dichloroethane), cis-1,2-DCE (cis-1,2-dichloroethylene), TCE (trichloroethene), 1,1,2-TCA (1,1,2-trichloroethane), PFOS (perfluorooctanesulfonic acid), PFOA (perfluorooctanoic acid), 3,4-DCA (3,4-dichloroaniline). "*" means that degradation of DCM was completely inhibited. DCM was always amended at 2000 µM. Concentration in the *x*-axis is expressed in µM of the corresponding co-contaminant. Error bars represent the standard deviations of triplicate samples.

628 **FIGURE 1**



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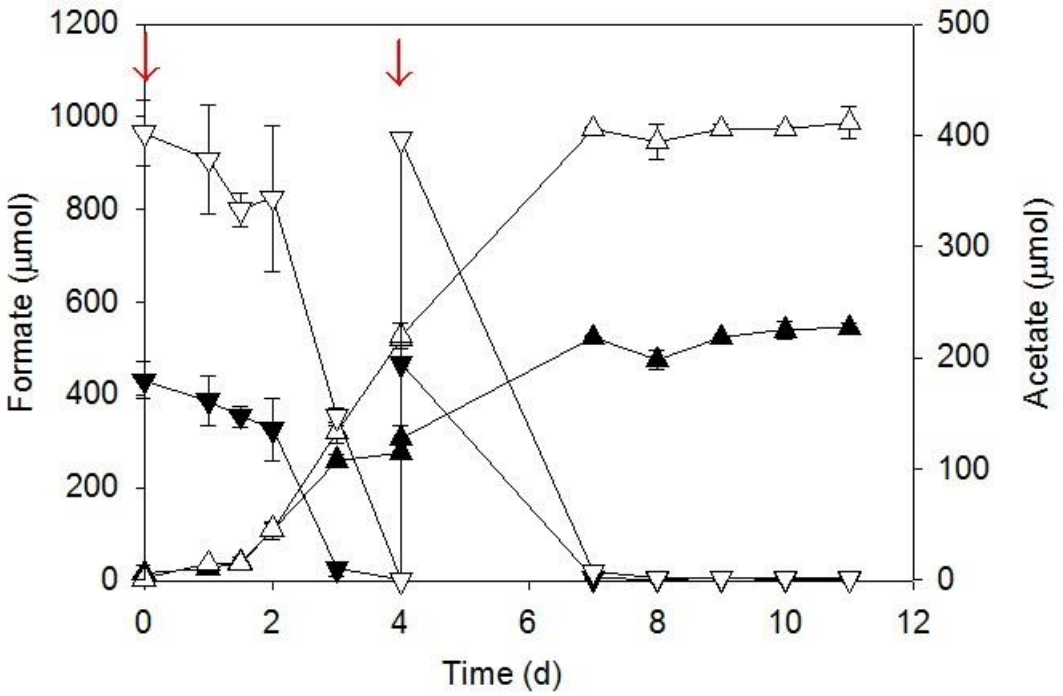
638 **FIGURE 2**



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641 **FIGURE 3**

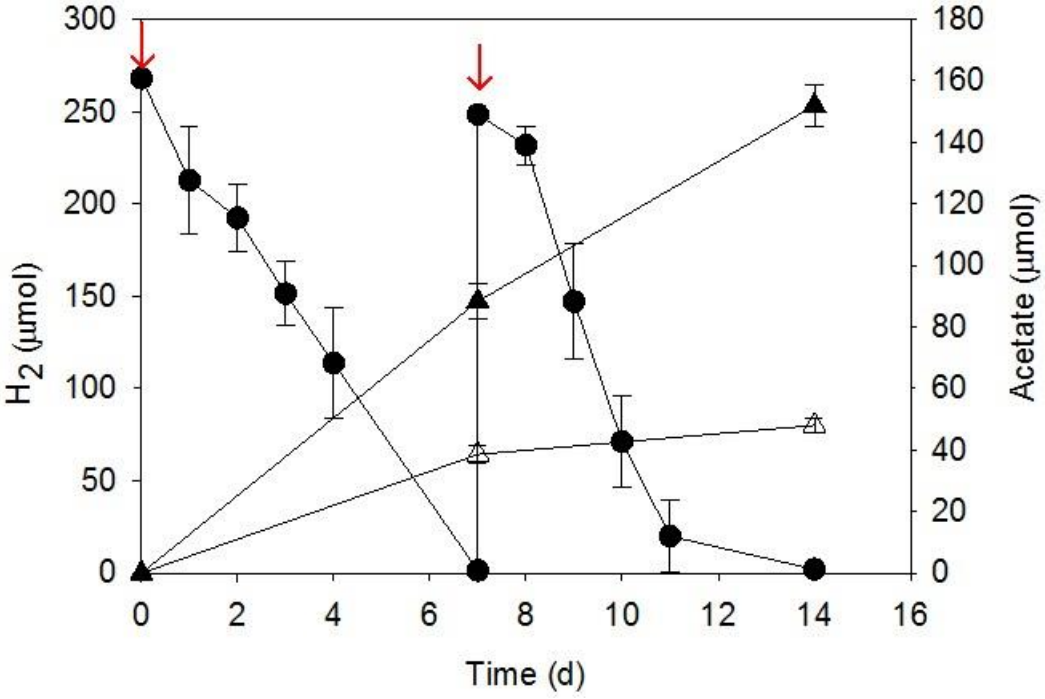

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FIGURE 4

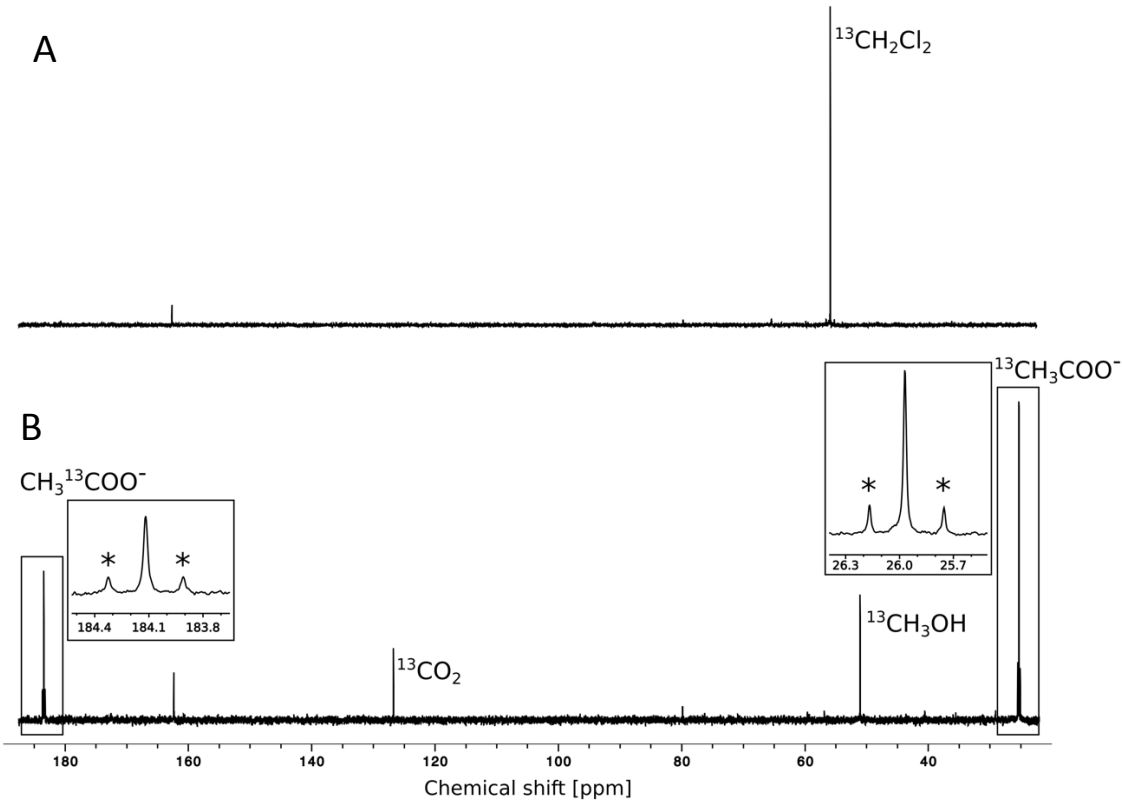
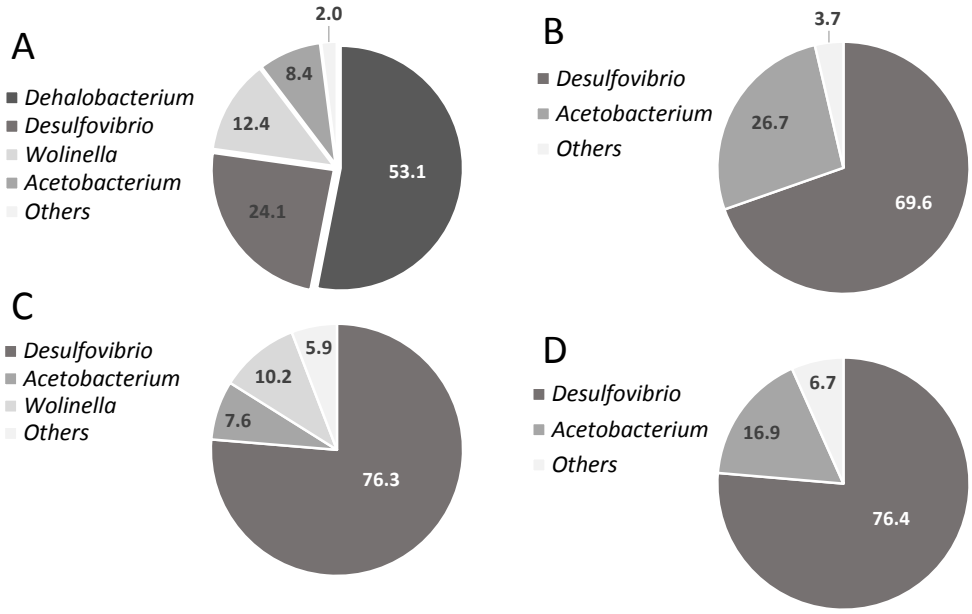
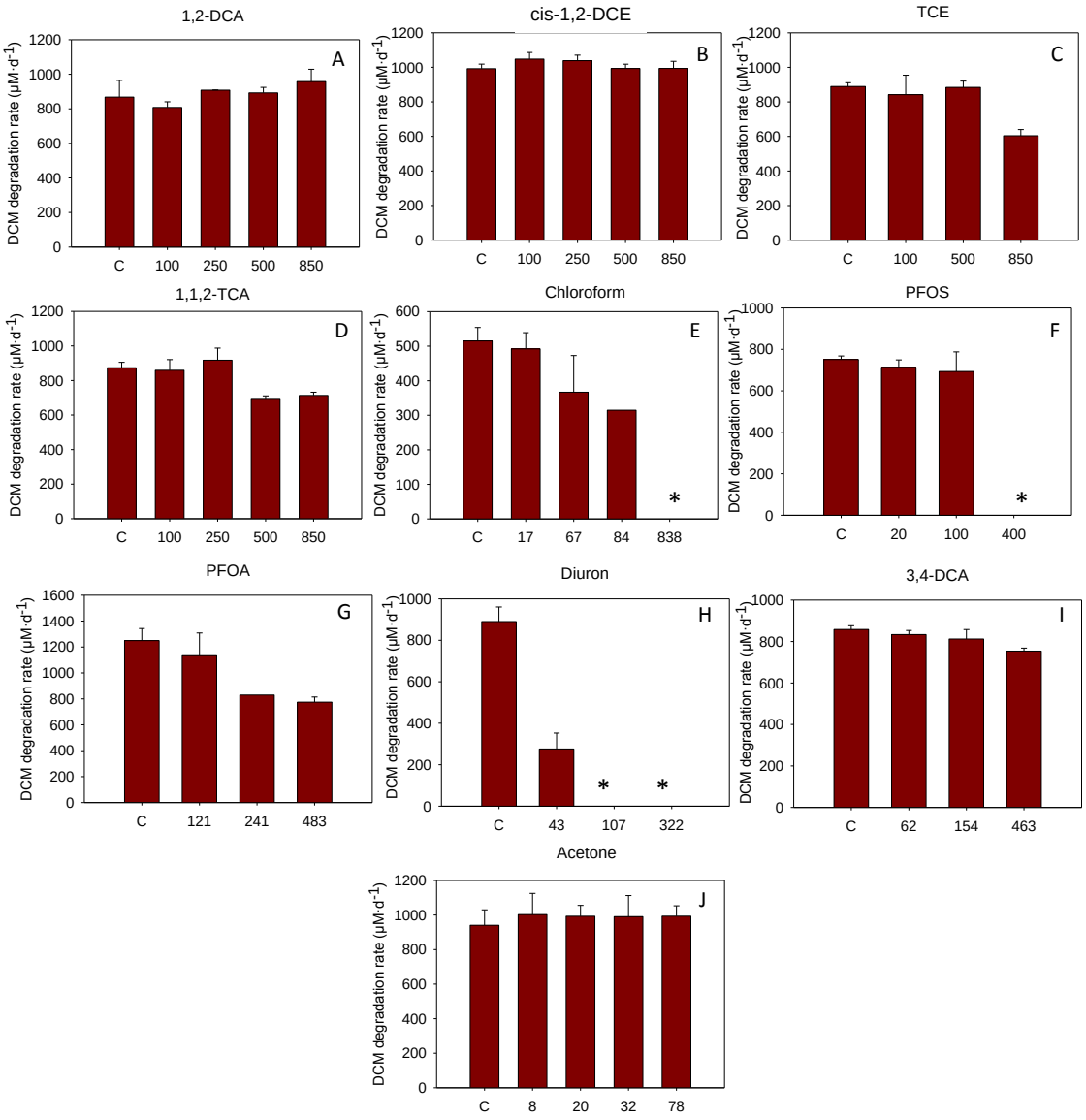

A
$^{13}CH_2Cl_2$
B
$CH_3{}^{13}COO^-$
184.4
184.1
183.8
$^{13}CO_2$
$^{13}CH_3OH$
26.3
26.0
25.7
$^{13}CH_3COO^-$
180
160
140
120
100
80
60
40
Chemical shift [ppm]

FIGURE 5


A
Dehalobacterium
Desulfovibrio
Wolinella
Acetobacterium
Others
53.1
24.1
12.4
8.4
2.0
B
Desulfovibrio
Acetobacterium
Others
69.6
26.7
3.7
C
Desulfovibrio
Acetobacterium
Wolinella
Others
76.3
7.6
10.2
5.9
D
Desulfovibrio
Acetobacterium
Others
76.4
16.9
6.7

667

FIGURE 6



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