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Chlorinated ethene biodegradation and associated bacterial taxa in multi-polluted groundwater: insights from biomolecular markers and stable isotope analysis

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16

17 **Abstract**

18 Chlorinated ethenes (CEs) are most problematic pollutants in groundwater. Dehalogenating bacteria,
19 and in particular organohalide-respiring bacteria (OHRB), can transform PCE to ethene under
20 anaerobic conditions, and thus contribute to bioremediation of contaminated sites. Current
21 approaches to characterize *in situ* biodegradation of CEs include hydrochemical analyses,
22 quantification of the abundance of key species (e.g. *Dehalococcoides mccartyi*) and dehalogenase
23 genes (*pceA*, *vcrA*, *bvcA* and *tceA*) involved in different steps of organohalide respiration by qPCR,
24 and compound-specific isotope analysis (CSIA) of CEs. Here we combined these approaches with
25 sequencing of 16S rRNA gene amplicons to consider both OHRB and bacterial taxa involved in CE
26 transformation at a multi-contaminated site. Integrated analysis of hydrogeochemical characteristics,
27 gene abundances and bacterial diversity shows that bacterial diversity and OHRB mainly correlated
28 with hydrogeochemical conditions, suggesting that pollutant exposure acts as a central driver of
29 bacterial diversity. CSIA, abundances of four reductive dehalogenase encoding genes and the
30 prevalence of *Dehalococcoides* highlighted sustained PCE, DCE and VC degradation in several wells
31 of the polluted plume. These results support the notion that bacterial taxa associated with OHR play
32 an essential role in natural attenuation of CEs, and also suggest that representatives of taxa including
33 *Dehalobacterium* and *Desulfovibrio* co-occur with *Dehalococcoides*. Overall, our study emphasizes
34 the benefits of combining several approaches to evaluate the interplay between the dynamics of
35 bacterial diversity in CE-polluted plumes and *in situ* degradation of CEs, and to contribute to a more
36 robust assessment of natural attenuation at multi-polluted sites.

37 **Keywords:** Chlorinated ethenes, Bacterial biomolecular markers, Reductive dechlorination,
38 Organohalide-respiring bacteria, Dehalogenases, CSIA.

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40

41 **1 Introduction**

42 Extensive use of chlorinated ethenes (CEs) as solvents has resulted in their accumulation and
43 persistence in polluted aquifers worldwide, representing serious risks for human health and the
44 environment. Among remediation strategies for CEs, natural attenuation and bio-stimulation are two
45 promising options as organohalide-respiring bacteria (OHRB) can reduce tetrachloroethene (PCE)
46 and trichloroethene (TCE) to harmless ethene. However, this is often accompanied by an
47 accumulation of toxic dichloroethene (DCE) and vinyl chloride (VC) under reducing conditions. A
48 first step in polluted site management is effective assessment of pollutant fate. Operational protocols,
49 such as the 1998 US-EPA protocol (Wiedemeier et al., 1998), provide a framework to characterize
50 pollutant dynamics and transformation based on chemical data analysis and modelling to determine
51 the occurrence of biodegradation in groundwater. In addition, ongoing research on OHRB may help
52 develop and implement integrative approaches to assess *in situ* natural attenuation of CEs (Adrien
53 and Löffler, 2016).

54 Microbial and molecular methods to study bacterial populations in polluted aquifers include culturing
55 approaches, DNA-based methods, analytical chemistry and compound-specific stable isotope
56 analysis (CSIA). Microbial and molecular approaches for characterization of polluted sites have been
57 reviewed (Weiss and Cozzarelli, 2008), as well as techniques to elucidate halogenated compounds in
58 anoxic environments (Ding and He, 2012). Detection of genes involved in degradation pathways or
59 targeting OHRB, such as *Dehalococcoides* spp., has been used to examine the relationships between
60 gene abundance and degradation rates or potentials (Clark et al., 2018; Hermon et al., 2019; Lu et al.,
61 2006; Rahm and Richardson, 2008a; Rahm and Richardson, 2008b). Bacterial diversity in polluted
62 sites has been studied to assess pollutant impact using fingerprinting approaches, such as DGGE
63 (Dennis et al., 2003; Govender et al., 2011), T-RFLP (Tarnawski et al., 2016), and DNA sequencing

64 (Brisson et al., 2012; David et al., 2015; Imfeld et al., 2011; Jin et al., 2020; Kao et al., 2016; Lowe et
65 al., 2002; Kotik et al., 2013; Wright et al., 2017). However, interpretation of microbial diversity
66 datasets in relation to complex pollutant and hydrochemical dynamics remains challenging, and often
67 neglects interactions between taxa and their environment. In this context, compound-specific isotope
68 analysis (CSIA) can directly evidence *in situ* biodegradation of CEs, and can be coupled to diversity
69 data. In particular, CSIA can help to evaluate pollutant degradation with respect to non-degradative
70 dissipative processes, including sorption or dilution (e.g., Carreón-Diazconti et al., 2009; Courbet et
71 al., 2011; Hunkeler, 2016; Kaown et al., 2016; Kuntze et al., 2020; Thullner et al., 2012).

72 It is promising to couple these approaches to better evaluate and predict the degradation of
73 chlorinated solvents by OHR *in situ* (Carréon-Diazconti et al., 2009; Courbet et al., 2011; Hunkeler
74 et al., 2011; Hermon et al., 2018; Imfeld et al., 2011; Kaown et al., 2016). This can be extended by
75 specific follow-up of OHRB biomarkers selected for their specificity and relevance to identified
76 questions (Maphosa et al., 2010). For example, Lee et al. (2016) have proposed a data-mining
77 approach that uses a predictive model coupling geochemical data and gene abundances that could be
78 used as a decision-making tool. Similarly, Tarnawski et al. (2016) validated an integrative method to
79 follow-up reductive dechlorination of CEs in polluted aquifers based on the occurrence and potential
80 of reductive dechlorination as well as the identification of reasons for incomplete reductive
81 dechlorination.

82 Recent work is moving towards more integrative approaches to assess *in situ* biodegradation of CEs
83 and establishing generic protocols including biomarkers for polluted site assessment (Clark et al.,
84 2018; Kaown et al., 2016; Tarnawski et al., 2016; Lee et al. 2016; Weiss et al., 2008). This brought up
85 new and as yet unresolved questions: What are the biological and/or geochemical drivers of diversity
86 and distribution of OHRB? How do degradation of organohalide pollutants and the abundance of

87 bacterial taxa associated with dehalogenation vary in relation to *in situ* environmental conditions? To
88 date, few studies have combined high-throughput sequencing methods with CSIA, geochemical and
89 gene abundance assessment to address these questions.

90 The purpose of our study was to examine degradation potential of CEs in a multi-polluted aquifer *in*
91 *situ*, by integrating hydrogeochemical, CSIA and DNA sequence data (16S rRNA taxonomy and
92 selected dehalogenase genes relevant to OHR of PCE) obtained over a period of two years on a
93 multi-contaminated former industrial site. This integrative approach enables to identify zones of the
94 groundwater aquifer where natural attenuation of CEs actively occurs, as well as the main factors
95 driving the distribution and activity of OHRB *in situ*, for improvement of site assessment and future
96 remediation.

97 **2 Materials and Methods**

98 **2.1 Study site, groundwater sampling and water parameter analyses**

99 Groundwater samples were taken from the former industrial site of Themeroil (Varenes-le-Grand,
100 France, GPS coordinates, 46.701141 N, 4.843919 E), characterized by intensive oil and solvent
101 processing activities from 1971 until it was liquidated in 2009. Halogenated solvents and BTEX were
102 released into the aquifer due to improper storage (BRGM, 1998). Monitoring of the site showed high
103 concentrations of VOCs (mainly CEs) and BTEX, accumulated within DNAPL in the groundwater of
104 the site, and a pollutant plume extending over an area of about 300 m² to the South-South-East
105 (BRGM, 2011). The DNAPL plume is confined to a small, well-characterized, captive sandy-clayey
106 aquifer with low permeability ($K_{\text{sat}} < 10^{-5} \text{ m s}^{-1}$).

107

108

Figure 1

110 Groundwater from the site was sampled at 12 previously installed monitoring wells, in September
111 2015 and 2016, and in February 2016 and 2017 (Figure 1). Groundwater was pumped from an
112 average depth of 6 m using Twister pumps (Proactive, Bradenton, USA) and collected after well
113 purging and stabilization of hydrogeochemical parameters. Water temperature, electrical conductivity
114 (standardized to 25°C), pH and redox potential (Eh) were measured on site using IDS multi-
115 parameter probes (WTW, Germany) according to recommendations of the French standard FD X31-
116 615. Well depths and water level data are provided in SM-E.

117 Groundwater samples were also collected for chemical analysis of total dissolved organic (DOC),
118 inorganic carbon (DIC), total organic carbon (TOC), cations (NH_4^+) and anions (NO_3^- , NO_2^- , HS^- ,
119 SO_4^{2-}) and Fe^{2+} , as described previously (Hermon et al., 2018). Samples for chlorinated ethenes were
120 stored in Teflon-capped glass vials according to recommendations of Weissling, the Cofrac-
121 accredited private laboratory in charge of analyses on-site. Collected BTEX and hydrocarbon
122 concentration data, although beyond the scope of the present study, were also considered in data
123 analysis. Samples for CSIA were collected in 20 mL glass vials (Interchim, Montluçon, France),
124 filled with 13 mL of groundwater, immediately stabilized by adding 2 mL of a saturated salt solution,
125 and capped with a Teflon septum. Samples were conserved at 4°C and analyzed within a week, as
126 described below. Finally, groundwater samples for DNA extraction and molecular analyses were
127 collected in two sterile 2L PE bottles and conserved at 4°C. Samples were filtered in the laboratory
128 within 24 h, and filters frozen at -20°C until further analysis.

129 **2.2 Carbon stable isotope analysis of CEs**

130 Carbon isotope composition of CEs was analyzed using a GC-C-IRMS system consisting of a
131 TRACE™ Ultra Gas Chromatograph (ThermoFisher Scientific) coupled *via* a GC IsoLink/Conflow

132 IV interface to an isotope ratio mass spectrometer (DeltaV Plus, ThermoFisher Scientific), as
133 described previously (Hermon et al., 2018). The procedure for CSIA is detailed in SM-A. Isotope
134 signatures of PCE, TCE, DCE, and VC were calculated as an isotope balance, as previously
135 described (Imfeld et al., 2011; Stelzer et al., 2009). For all samples, standard deviation of isotope
136 balance was $\leq 1\%$ (SM-A).

137 **2.3 DNA analysis**

138 **2.3.1 DNA extraction, qPCR analysis of 16S *rrs* and dehalogenase coding genes**

139 Total DNA was extracted from membrane filters after groundwater filtration, as described in Hermon
140 et al. (2018). Quantification of bacterial 16S *rrs*, *pceA*(Dhc), *pceA*(Dsb), *vcrA*, *tceA* and *bvcA* genes
141 in groundwater was achieved using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad,
142 France). Primers and PCR conditions, no-template controls and calibration curves were as described
143 in Hermon et al. (2019). Gene *pceA*(Dhb) is involved in the organohalide respiration of PCE to *cis*-
144 DCE by strains of the *Dehalobacter* and *Desulfitobacterium* genera (Regeard et al., 2004) whereas
145 *pceA*(Dhc) is involved in the organohalide respiration of PCE to *cis*-DCE by strains of the
146 *Dehalococcoides* genera. Genes *vcrA*, *tceA* and *bvcA* are dehalogenase genes known only to be
147 carried by strains of the *Dehalococcoides* genera that code for dehalogenases that degrade TCE, *cis*-
148 DCE and VC to ethane.

149 **2.3.2 Illumina Miseq sequencing and bioinformatics**

150 Sequences of the V4-V5 hypervariable region of the 16S *rrs* gene (Claesson et al., 2010) were PCR
151 amplified using an optimized and standardized amplicon library preparation protocol (Meta-biote®,
152 GenoScreen, Lille, France), as described in SM-B. Denoising, chimera checking, generation of
153 operational taxonomic units (OTUs) and taxonomic classification were performed using the custom-

154 scripted bioinformatics pipeline of GenoScreen, also described in SI section J in Hermon et al.
155 (2018).

156 Analysis of bacterial diversity for the 12 monitoring wells and across the four sampling campaigns
157 yielded a total of 2,710,996 sequences. SChao1 diversity indices for the entire bacterial community
158 reached asymptotes with increasing sequencing depth (SM-C), indicating sufficient sampling. The
159 OTU database generated across the 12 wells and 4 sampling campaigns was interrogated for
160 sequences of interest by BLAST, applying a genus-level cut-off of 97% identity for taxonomic
161 assignment.

162 **2.4 Data analysis**

163 To compare relative abundances of 16S *rrs*, *pceA*, *vcrA*, *tceA* and *bvcA* genes, Kruskal-Wallis tests
164 and post-hoc multi-comparison tests were performed with R Studio (Version 1.0.153) using the
165 *agricolae* package (De Mendiburu and Simon, 2015). Multivariate statistical analysis was carried out
166 using R (version 3.5.3) (R Development Core Team, 2017). To visualize variations in bacterial
167 diversity and in particular bacterial taxa associated with dechlorination, two-dimensional nonmetric
168 multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of log-transformed data was
169 performed using R software. Correlations between diversity profiles and hydrogeochemical variables
170 were evaluated by fitting environmental vectors *a posteriori* onto the NMDS. Significance of the
171 fitted vectors was assessed by conducting a 1000-step Monte-Carlo permutation test. Only variables
172 with $p < 0.05$ were considered significant.

173 Datasets of overall bacterial diversity, of taxa associated with dechlorination, and physico-chemical
174 datasets including pollutant concentrations and hydrogeochemical parameters were jointly analyzed
175 and compared by multi-factor analysis (MFA, SM-D) (Bécue-Bertaut and Pagès, 2008; Escofier and
176 Pagès, 1994).

177 Datasets obtained from Illumina sequencing were also used to compute α -diversity indices and for
178 rarefaction analysis. Shannon's diversity index (H'), calculated as $H' = -\sum p_i \ln p_i$, provides an
179 estimate of community heterogeneity, and increases with increasing heterogeneity. H' is maximal
180 when sequences are equally distributed across all OTUs. The inverse Simpson diversity index (S)
181 was computed as $S = 1/D$, with $D = \sum p_i^2$ and p_i the relative abundance of OTU i . Simpson's index
182 gives the probability that two individual sequences picked at random do not belong to the same OTU.
183 Thus, S also increases with increasing diversity. The Chao1 richness estimate was calculated as
184 $S_{chao1} = S_{obs} + \frac{f_1^2}{2f_2}$, where S_{obs} is total number of OTUs in a sample, f_1 is the number of OTUs with
185 only one sequence (i.e., "singletons"), and f_2 is the number of OTUs with only two sequences (i.e.,
186 "doubletons"). Chao1 provides an estimate of the number of total OTUs in the sample, based on the
187 number of detected OTUs represented by singletons and doubletons.

188 **3 Results**

189 **3.1 CEs and hydrogeochemical conditions on site**

190 Patterns of distribution of CEs and hydrogeochemical conditions varied widely along the plume, but
191 did not fluctuate much over time. PCE and TCE concentrations were high only in the source zone.
192 Concentrations of *cis*-1,2-DCE and VC remained high over time in wells of the source and plume
193 zones but did not increase in the boundary wells. This suggests that the plume is not expanding over
194 time, although residual pollutants continuously feed it from the source area. Accordingly, the twelve
195 monitored wells were assigned to three zones: source (Pz26, Pz27, Pz28 and Pz29), plume (Pz3,
196 Pz6(10), Pz5 and Pz20) and boundary (Pz15, Pz17, Pz18 and Pz11) (Figure 1 and SM-E).

197 Although PCE was the main CE initially used on site, it was only found in high concentrations (up to
198 33 mg·L⁻¹) in the source zone (Pz26 and Pz27), as was TCE (up to 76 mg·L⁻¹). The highest

199 concentration of *cis*-1,2-DCE was also detected in the source zone, in Pz29, reaching 260 mg·L⁻¹ in
200 Pz29 in September 2016 (Figure 1; SM-E). However, high VC and *cis*-1,2-DCE concentrations (up
201 to 27 mg·L⁻¹) were also monitored in the plume (Figure 1, SM-E). In boundary wells, concentrations
202 of *cis*-1,2-DCE were always below 0.7 mg·L⁻¹. While detection of VC clearly suggests that OHR
203 takes place, persistence of less chlorinated transformation products of PCE suggests that conditions
204 are not optimal for OHRB to complete full degradation of CEs to ethene.

205 Hydrogeochemical conditions did not differ significantly between February and September
206 campaigns, although groundwater temperatures were systematically higher in September. The
207 presence of ferrous iron (Fe²⁺) and sulfide in the source and plume zones down to Pz6(10) was
208 consistent with anoxic conditions. In contrast, wells in the boundary zone all featured aerobic
209 conditions (see SM-E). Analysis of hydrogeochemical data and pollutant concentrations (SM-F),
210 including CEs, BTEX and hydrocarbons, revealed distinctive features for some wells within the
211 source, plume and boundary zones. For example, conditions at Pz20 were similar to those of the
212 boundary zone despite the high concentrations of CEs detected. In contrast, Pz3, within the plume,
213 clustered with Pz28 and 29 from the source area, whereas Pz26 (source zone) grouped with Pz5 and
214 Pz6(10) from the plume zone. Finally, Pz27, while clustering with the other wells from the source
215 zone, was characterized by higher DOC and TOC concentrations due to the presence of hydrocarbons
216 (data not shown).

217 **3.2 $\delta^{13}\text{C}$ of CEs in groundwater**

218 Overall, isotopic data suggest important spatial variability in the degradation of CEs across the
219 plume, with preferential zones of degradation. Indeed, isotopic signatures of CEs at the source
220 ($\delta^{13}\text{C}_{\text{source}}$) differed significantly from those of the plume, which were themselves distinct from those
221 of the boundary zone. Differences in isotopic composition between the source and the plume

222 ($\Delta\delta^{13}\text{C}_{\text{source-plume}}$) ranged from 2 to 12 ‰ depending on the compound (data not shown). This suggests
223 the occurrence of *in situ* biodegradation of CEs, as their degradation by acid- or base-catalyzed
224 hydrolysis is unlikely under the conditions encountered in the aquifer (Washington, 1995). For
225 example, *cis*-1,2-DCE was enriched in ^{13}C from 1 to 10 ‰ between the source zone and the
226 boundary. With regard to *cis*-1,2-DCE concentrations and isotopic values, it appears that Pz26, Pz28
227 and Pz29 represent potential source wells for *cis*-1,2-DCE. Judging by isotopic mass balance (Stelzer
228 et al. 2009), the average signature of the source was estimated at -30.5 ‰. On the other hand, *cis*-1,2-
229 DCE was enriched in ^{13}C in Pz27, suggesting that the area of this well, although located in the source
230 zone, was not a contributing source of CEs.

231 The lowest $\delta^{13}\text{C}_{\text{CE}}$ value (well Pz26) was considered as the isotopic composition of the source, under
232 the reasonable assumption that CEs were less degraded at this location. Change in isotopic ratios of
233 total CEs ($\Delta\delta^{13}\text{C}_{\text{CE}} > 2$ ‰) relative to the source (Pz26) indicated that biodegradation occurred in
234 wells Pz27, Pz28, Pz6(10) and Pz3, but not in Pz29 and Pz5. Biodegradation varied little over time
235 for all the wells (SM-G). The isotopic mass balance of CEs accounted for the reductive
236 dechlorination chain from PCE to VC (SM-G), assuming that under prevailing physicochemical
237 conditions, reductive dehalogenation of CEs to VC was the dominant process. Overall, isotopic mass
238 balance analysis allowed to identify preferential zones of biological degradation, and appeared most
239 pronounced in well Pz6(10). However, this analysis could not be performed for wells in the boundary
240 because of the low concentrations of CEs in this zone.

241 **3.3 Bacterial diversity**

242 Bacterial diversity was higher in the boundary zone than in the source and plume (SM-H). Up to
243 seven times less OTUs were found in source wells (Pz26, 27, 28 and 29) compared to boundary
244 wells. Thus, diversity was lowest in the more polluted wells, where several OTUs were highly

245 abundant and dominated, as indicated by the low inverse Simpson index (between 4 and 20) in the
246 source and plume areas compared to the boundary zone (up to 120). The Shannon index also
247 indicated a more homogeneous distribution of OTU abundance in the boundary zone than in the
248 source zone (Figure 2, SM-H). Indeed, taxa with high relative abundance generally dominated in the
249 source and plume zones, while rare taxa prevailed in Pz18 and Pz11.

250 Analysis of OTUs shared between different zones showed that for the 29516 total OTUs that were
251 defined, only 7.5% were found in all zones of the plume (SM-I). The boundary zone comprised the
252 highest number of specific OTUs, followed by the plume and the source. The boundary and plume
253 shared more OTUs than with the source.

254

255

Figure 2

256

257 *Proteobacteria* represented up to 60% of obtained sequences in Pz11, and up to 80% in Pz20. They
258 generally dominated in boundary wells (Figure 3), as previously observed in both pristine (Shirokova
259 and Ferris, 2013) and PCE-polluted groundwaters (Kotik et al., 2013). Other dominant phyla in the
260 boundary zone were *Nitrospirae* OP3 and OP11. Upstream of Pz20, *Proteobacteria* were
261 significantly less abundant, with *Firmicutes*, *Tenericutes*, *Spirochaetes* and *Chloroflexi* detected at
262 high levels in the plume (i.e. Pz6(10), Pz5 and Pz3). These phyla were also found in source wells
263 Pz27, Pz28 and Pz29, although in variable proportions, together with *Bacteroidetes* in Pz27 and
264 Pz28. Pz26 differed from the other source wells, with 40% *Proteobacteria* and proportionally more
265 *Spirochaetes*.

266

267 **Figure 3**

268

269 Changes in bacterial diversity over time and across wells, analyzed using NMDS ordination of
270 relative sequence abundances for each OTU (Figure 4), showed that bacterial diversity mainly varied
271 according to zones (boundary, plume and source). This is consistent with the Venn diagram (SM-I)
272 and histogram of phyla (Figure 3). Worthy of note, the bacterial diversity in Pz27 was similar to
273 other source wells, although hydrogeochemical characteristics of Pz27 clearly differed (SM-E). In
274 addition and as mentioned above for hydrogeochemical parameters (SM-H), the Pz20 bacterial
275 diversity profile clustered with that of boundary rather than plume wells. Pz11 differed from other
276 boundary wells. Again, this demonstrated that bacterial diversity can differ although hydrochemical
277 characteristics are similar.

278 *A posteriori* fitting of hydrogeochemical variables onto the NMDS ordination showed that bacterial
279 composition correlated best with changes in pollutant concentration (CEs, BTEX and other
280 hydrocarbons) (Figure 4). Pollutant concentrations may thus exert a strong selective pressure on
281 bacterial diversity, whereas changes in redox potential, ammonium/nitrate and ferrous iron may have
282 a lesser effect. As already noted above, total groundwater bacterial diversity did not change
283 significantly across the four sampling campaigns. These results showed that bacterial diversity was
284 stable in a hydrogeochemically stable polluted aquifer, and that similar conditions did not
285 systematically imply similar bacterial communities. This highlights the necessity to extend analysis
286 to bacterial parameters for correct assessment of the site.

287

288 **Figure 4**

290 Only a small proportion of sequences could not be affiliated at the phylum level, whereas this
291 proportion was higher at the genus level. The five most abundant genus-level OTUs represented up to
292 45% of sequences in the source and plume zones, but only 20% in the boundary zone (SM-J).
293 *Treponema* and *Geobacter* genera dominated groundwater bacterial communities of Pz26 with 25
294 and 23% relative abundance, respectively. *Sphaerochaeta*, a genus previously identified in
295 dechlorinating microbial consortia (Chung et al., 2008; Gu et al., 2004) was the most abundant genus
296 in Pz27, with 15% relative abundance. In Pz28, dominant OTUs were affiliated to *Treponema* and
297 *Paludibacter* (25 and 22%, respectively). Although previously found at polluted sites (Kotik et al.,
298 2013; Lee et al., 2015), *Treponema* is not known to be involved in chlorinated ethene degradation.
299 *Paludibacter* features strictly anaerobic chemo-organotrophic strains, and was detected in TCE-
300 polluted groundwater (Dong et al., 2011), and in an aquifer contaminated with chloroform (Kim et
301 al., 2019). The dominant OTU in wells Pz29 and Pz5 was assigned to *Desulfosporosinus*, with 11 and
302 20% relative abundance, respectively. *Dehalococcoides*, the only genus with strains known to fully
303 reduce PCE and TCE to ethene, represented 45 and 24% of relative sequence abundance on average
304 in plume wells Pz3 and Pz6(10), respectively. *Hydrogenophaga*, a facultative anaerobe with
305 representatives capable of co-metabolically degrading TCE (Kuo et al., 2014; Liang et al., 2011), was
306 the dominant genus in wells Pz20, Pz17 and Pz15, representing 28, 21 and 15% total relative
307 abundance, respectively. In Pz18, 88% of sequences could not be affiliated to a genus. Among these,
308 a *Proteobacteria* affiliated to the *Nitrospirales* order was the most abundant (7% of total sequences).
309 In Pz11, the most abundant genus was *Gallionella*, representing 7% of total sequences (see SM-J).
310 Taken together, bacterial diversity analysis showed that genera associated with CE degradation (i.e.
311 OTUs with >97% identity in 16S *rrs* gene sequence) were particularly abundant in wells Pz26, Pz3,
312 Pz5 and Pz6(10). This warranted a more detailed study of their distribution.

313 3.4 Genera associated with organohalide respiration

314 The relative abundances of bacterial genera associated with OHR in the source zone clearly differed
315 from those of the plume and boundary ($p < 0.05$), but did not change significantly on a seasonal or
316 yearly basis ($p < 0.05$) (Figure 5). Variations in relative abundance correlated best with concentrations
317 of CEs ($p < 0.05$; see SM-K). More specifically, OTUs related to *Geobacter* were particularly
318 abundant in source wells. *Geobacter* features several strains of *G. lovleyi* that possess gene
319 *pceA*(Dsb) and reduce PCE to cDCE (Atashgahi et al., 2016). In contrast, OTUs most closely related
320 to *Dehalococcoides* were mainly found in the plume zone (wells Pz3 and Pz6(10)), where DCE and
321 VC were also found. Several strains of *Dehalococcoides* belonging to *D. mccartyi* or other species
322 have been found to possess one or several dehalogenases that dechlorinate PCE to ethene (i.e. *pceA*,
323 *vcrA*, *tceA* or *bvcA*) (Dolinová et al., 2016; Zinder, 2016). *Sulfurospirillum*, known to reduce PCE
324 and TCE (Maillard et al. 2011), was found in low abundance in Pz26. Strains of *Clostridium* and
325 *Acetobacterium* were also shown to dechlorinate CEs in either pure or mixed cultures (Dolinová et al.
326 2016). Sequences assigned to *Clostridium* were found in low abundance in all wells of the source and
327 plume, while *Acetobacterium* was detected as one of the most abundant OHR-associated genera in
328 Pz27 and Pz28 (Figure 5).

329

330

Figure 5

331

332 3.5 Distribution of CE dehalogenases

333 Four reductive dehalogenase genes were chosen as key biomarkers for this study (Liang et al., 2017;
334 Hermon et al., 2019). Figure 6 shows a synthetic representation of the mean log gene copies/ng DNA

335 in the four sampling campaigns. Gene copy numbers are presented in SM-L. Overall, dehalogenase
336 gene abundances were comparable to those in other CE-contaminated groundwaters (Lee et al., 2006,
337 Matturro et al., 2012). Relative abundance of the *pceA*(Dsb) dehalogenase gene was highest in Pz5
338 and Pz26 (Figure 6), and correlated with occurrence of *Geobacter*. NMDS analysis of bacterial taxa
339 associated with OHR showed that gene *pceA*(Dsb) correlated with Pz26, where *Geobacter* dominated
340 (see SM-M). In contrast, relative abundance of *pceA*(Dhc) and *vcrA* genes, specific to
341 *Dehalococcoides*, was up to two orders of magnitude higher in wells Pz5, Pz3 and Pz6(10) compared
342 to other wells. Analysis showed that typical *Dehalococcoides* dehalogenase genes (*pceA*(Dhc), *vcrA*,
343 *tceA* and *bvcA*) correlated with dehalogenation-associated taxa in wells Pz6(10), Pz3 and Pz29 where
344 *Dehalococcoides* was dominant (SM-M). On the other hand, gene *bvcA* was only detected in Pz3,
345 while *tceA* was only detected in Pz3 and Pz28, and at relative abundances lower than for the other
346 dehalogenase genes that were investigated. These results suggest that the Pz3 area in the plume
347 shows the highest genetic potential for dehalogenation of VC and DCE.

348

349

Figure 6

350

351 4 Discussion

352 In this study, potential for *in situ* reductive dechlorination of chlorinated ethenes (CEs) at an
353 emblematic contaminated site in France was newly evaluated by combining (i) hydrogeochemical
354 parameters, (ii) CSIA data, (iii) bacterial diversity (Illumina MiSeq) and (iv) dehalogenase gene
355 abundances (*pceA*, *vcrA*, *tceA* and *bvcA*). This integrative approach enabled us to identify potential
356 drivers of the diversity and distribution of bacterial taxa associated with dechlorination of CEs. It also

357 demonstrated the benefit of combining these methods to evaluate the potential for *in situ* degradation
358 of CEs.

359 **4.1 Contamination as the main driver of total bacterial diversity at the Themeroil site**

360 Pollutant concentration was the main driver of bacterial diversity and the taxa associated with CE
361 degradation, while CE dehalogenase distribution was related to OHRB abundance. These
362 relationships were confirmed by multiple factor analysis (MFA) of bacterial diversity and
363 hydrogeochemical data (SM-N). MFA showed that groundwater samples were mainly discriminated
364 by contamination level, with source and plume samples clearly distinct from boundary samples (SM-
365 K). Overall, our results suggest that pollutant level exerts a significant selective pressure on bacterial
366 diversity, as previously seen in similar highly polluted environments (Lowe et al., 2002; Imfeld et al.,
367 2011). Correlation analysis between bacterial diversity, specific OHRB and hydrogeochemical data
368 clearly shows a similar overall response of both OHRB and non-OHRB taxa to hydrogeochemical
369 variations across the pollution plume (Figure 4, SM-K, SM-O). Although pollutant pressure was
370 indeed suspected to be a major driver of microbial diversity, OHRB might be more specifically
371 correlated with the presence of electron donors, redox conditions or available carbon sources. In the
372 NMDS ordination of bacterial taxa associated with OHR (SM-K), parameters such as ferrous iron
373 (Fe^{2+}) and Eh were significantly associated with differences in OHR between wells. For instance,
374 Fe^{2+} was particularly high in Pz3 and Pz6(10), characterized by high pollutant concentrations and
375 highest OHR activity and degradation potential as assessed by CSIA (SM-K). Our observation
376 contrasts with the recent report by Jin et al. (2020) who showed that redox potential, dissolved
377 oxygen, temperature and methane concentration were major drivers of bacterial and archaeal
378 diversity, whereas concentration of chlorinated solvents had no significant impact. In that study,
379 however, selective pressure on bacterial diversity from CE toxicity may have been much less
380 pronounced, since maximal concentrations of *cis*-1,2-DCE and VC were significantly lower than in

381 our study (0.27 and 8.6 mg·L⁻¹, compared to 260 and 27 mg·L⁻¹ for *cis*-1,2-DCE and VC,
382 respectively).

383 Also worthy of note in this context, the number of OTUs increased along the plume, and were
384 inversely correlated with pollutant concentrations. For instance, the number of OTUs was one order
385 of magnitude higher in Pz18 (boundary) than in Pz27 and Pz28 (source), paralleling values of inverse
386 Simpson index (SM-H). Shannon index also remained low in both source and plume zones, with an
387 uneven distribution of sequences among OTUs in these zones presumably due to predominance of
388 pollutant-resistant taxa. Indeed, the source zone shared the least OTUs with other zones (see SM-I,
389 Venn diagram). Taken together, our observations all support the idea that high pollutant
390 concentrations negatively affect bacterial diversity in aquifers, as found in previous studies, e.g. by
391 Lowe et al. (2002) at a TCE-polluted Superfund site.

392 **4.2 Taxa associated with organohalide respiration as indicators of biodegradation**

393 That variations in bacterial diversity and pollutant concentrations were correlated was not
394 unexpected. Beyond that, we also explored whether abundance data of specific OHRB taxa could be
395 used as biomarkers to evaluate CE biodegradation potential in the investigated aquifer.

396 Sequences associated with OTUs affiliated to known OHRB were extracted from the sequence
397 database (Figure 5). They were affiliated to *Dehalococcoides*, *Geobacter*, *Acetobacterium*,
398 *Sulfurospirillum*, *Clostridium* and *Desulfomonile*. Most represented were *Dehalococcoides* and
399 *Geobacter*. The genus *Dehalococcoides* includes strict anaerobes that use H₂ as electron donor and
400 organohalides as respiratory terminal electron acceptors (Zinder, 2016). So far, only one known
401 species of *Geobacter*, *G. lovleyi*, has ability for OHR (Atashgahi et al., 2016). The presence of
402 *Dehalococcoides* in organohalide-polluted sites worldwide makes it one of the most important
403 players for natural attenuation of CEs to date (Zinder, 2016), and reflects its frequent use as a

404 biomarker in this context (Lu et al., 2006; Rouzeau-Szynalski et al., 2011; Saiyari et al., 2018).
405 Interestingly, the 16S rRNA amplicon sequence analysis performed here also highlights the presence
406 of genera that can degrade organohalides co-metabolically, such as *Acidovorax* and *Hydrogenophaga*
407 (Kuo et al., 2014; Liang et al., 2011).

408 A wider knowledge of OHRB as well as of global bacterial distribution and diversity in
409 contamination plumes may provide a better-informed view for efficient bioremediation strategies,
410 that also includes other associated, non-OHRB populations. Indeed, focusing on OHRB and their
411 diversity and metabolic capacities may overlook the potential effect and importance of other taxa for
412 organohalide respiration activity at a given contaminated site, since activities of interest rely on
413 interactions between bacteria with different metabolic regimes within complex overall energy-flow
414 pathways (David et al., 2015; Miura et al., 2015). For example, anaerobes that provide OHRB with
415 electron donors could be just as important to the process of OHR as OHRB themselves. Conversely,
416 some types of metabolism may actually be detrimental to OHR, and taking the occurrence of
417 corresponding taxa may also help in implementing efficient bioremediation. In the present study, for
418 instance, sulfate-reducing bacteria and methanogens, which can compete with *Dehalococcoides*
419 (Futagami et al., 2011), were also found in varying relative abundance in Themeroil groundwater.

420 Specifically, a Spearman's similarity coefficient matrix from relative abundances showed that
421 *Dehalococcoides* significantly correlated with occurrence of *Dehalobacterium* and
422 *Desulfosporosinus* sp. in our study ($p < 0.001$) (SM-N). Notably in this context, Rouzeau-Szynalski et
423 al. (2011) found frequent co-occurrence of *Dehalococcoides* with *Desulfitobacterium*, but not with
424 these two genera. Systematic co-occurrence of known OHR-associated taxa also suggests potential
425 synergies between OHRB involved at different stages of the dechlorination sequence of PCE to
426 ethene. While further data on co-occurrence will be needed to confirm positive metabolic
427 interactions, our data show that analysis of overall bacterial diversity could provide additional

428 insights for prediction of dechlorination efficiency *in situ*. This is of particular interest in a context
429 where stalling of CE dehalogenation, with concomitant accumulation of DCE and VC, is frequently
430 observed (Daprato et al., 2007; Ding and He, 2012; Maphosa et al., 2010).

431 **4.3 Key dehalogenases and dehalogenation-associated taxa: an imperfect correlation**

432 Our results also showed that relative abundance of dehalogenase genes and OHRB did not
433 systematically correlate (SM-L). Based on relative abundances of known OHRB, Pz26, Pz3 and
434 Pz6(10) had highest dehalogenation potential. In Pz3 in particular, 45% of obtained 16S rRNA
435 sequences were affiliated to *Dehalococcoides* (Figure 5). In a complementary analysis, we analyzed
436 the abundance of four key reductive dehalogenase genes of the dehalogenation sequence for PCE
437 (*pceA*, *tceA*, *vcrA*, *bvcA*), that are all frequently used as biomarkers in bioremediation assessment at
438 sites polluted with CEs (Carreón-Diazconti et al., 2009; Clark et al., 2018; Liang et al., 2017;
439 Matturro et al., 2012; Lee et al., 2008). Pz3 was the only well where *tceA* and *bvcA* were found in
440 addition to *Dehalococcoides* (Figure 6). Also worthy of note, the highest abundance of *pceA*(Dsb)
441 was observed in Pz26 and Pz5, while *pceA*(Dhc) and *vcrA* were most abundant in wells Pz3, Pz6(10)
442 and Pz5 (Figure 6).

443 **4.4 Hydrogeochemical data and biomarkers suggest but do not prove dehalogenation**

444 Although the presence of OHRB in the ground waters of the source and plume zones, along with high
445 abundances of key dehalogenase genes, suggests that the potential is present for CE reduction, they
446 do not prove that dehalogenation is actively occurring.

447 At the onset of our study, hydrogeochemical data already suggested that OHR was occurring on site,
448 as PCE and TCE were found in the source zone in residual concentrations only, whereas DCE and
449 VC were the dominant CEs. However, the nature and concentrations of CEs alone did not pinpoint

450 wells where OHR was active. Dehalogenase gene abundance and diversity of known OHRB taxa
451 suggested areas around Pz28, Pz29, Pz3, Pz5 and Pz6(10) as key DCE degradation hotspots.
452 Moreover, Pz3 and Pz6(10) had the highest relative abundance of *Dehalococcoides*-associated
453 sequences. Nevertheless, inhibition processes may be occurring on-site that limit OHR. For example,
454 in wells Pz28, Pz29, Pz3, Pz5 and Pz6(10), high DCE concentrations might inhibit VC organohalide
455 respiration, as shown in previous reports (e.g. Lai and Becker, 2013). These authors suggested that
456 concentrations as low as 17 mg·L⁻¹ DCE could have an impact. In our study, this could contribute to
457 explain the accumulation of VC on-site, although degradation potential was present.

458 In summary, integration of hydrogeochemical and biomarker data is suggestive of synergistic and
459 antagonistic interactions on the site of Themeroil. Yet, without actual confirmation of occurrence and
460 extent of OHR, it remains difficult to conclude on the range of conditions for OHRB to operate
461 successfully. This is where stable isotope analysis (CSIA) shows its utility, by allowing evaluation of
462 the occurrence of biodegradation *in situ*.

463 **4.5 CSIA as key approach to evidence biodegradation of chlorinated ethenes at the Themeroil** 464 **site**

465 While occurrence of OHRB and dehalogenases may underline OHR potential, a strong asset of CSIA
466 is the *in situ* confirmation of OHRB activity. Changes in carbon isotope composition ($\delta^{13}\text{C}$) indicated
467 biodegradation in wells Pz27, Pz28, Pz3 and Pz6(10), and appeared particularly significant in well
468 Pz6(10). On the one hand, our results are consistent with biomarker data for Pz3 and Pz6(10), which
469 showed the highest proportions of sequences affiliated to *Dehalococcoides* and the highest
470 abundances of genes *vcrA* and *pceA*(Dhc) (Figures 5 and 6). In addition, Pz3 was the only well
471 where *bvcA* and *tceA* genes were also found in significant abundance, and the Pz3 area was thus
472 expected to be the most actively degrading DCE and VC. However, degradation of DCE and VC may

473 actually be limited in Pz3 compared to Pz6(10), due to concentration-dependent inhibition, with DCE
474 concentration higher in Pz3 ($130 \text{ mg}\cdot\text{L}^{-1}$ compared to $30 \text{ mg}\cdot\text{L}^{-1}$ in Pz6(10), SM-E). Alternatively, it
475 is also possible that degradation rate in Pz3 was highest, but that steady inflow of DCE reduced the
476 degraded fraction, thereby leading to underestimating degradation rate. On the other hand, molecular
477 biomarkers did not identify Pz27 as a well with degradation activity, whereas CSIA clearly identified
478 the occurrence of biodegradation in this well. Thus, occurrence of OHR may be overlooked if several
479 complementary approaches are not combined and jointly interpreted. It should also be mentioned that
480 the relatively high intrinsic quantification threshold of CSIA for CEs limited integration of CSIA and
481 biomolecular data in wells with low concentrations of CEs, or in which extensive *in situ*
482 biodegradation had likely occurred, such as those in the boundary zone. In addition, CSIA of CEs
483 based on carbon alone may not be sufficient to distinguish different pathways of transformation of
484 CEs, such as dehalogenation under aerobic and/or co-metabolic conditions and OHR. In the future,
485 multi-element CSIA of CEs ($\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{37}\text{Cl}$) could be applied for this purpose (Hunkeler, 2016;
486 Vogt et al., 2016).

487 **4.6 Conclusion: the case for integrative approaches in assessment of organohalide-** 488 **contaminated sites**

489 Current research issues in the field of *in situ* degradation of organohalides include (i) how
490 interactions of dechlorinating taxa with their environment modulate their activity and (ii)
491 identification of factors that prevent complete degradation of CEs to ethene, resulting in
492 accumulation of DCE and VC. It is our contention that integrative studies such as ours may help to
493 address these questions, and enable increasingly precise assessment of complex polluted sites over
494 time. Specifically, our study has highlighted that bacterial biomarkers of CE dehalogenation are best
495 used in combination, and that their occurrence in groundwater is only partly correlated with observed

496 changes in CE concentrations. Thus, combination of bacterial biomarkers with hydrochemistry and
497 CSIA data can reinforce the evaluation of *in situ* CE degradation at site scale, and help identify
498 hotspots of *in situ* biodegradation for practical application. Here, we could identify areas of the
499 contamination plume where natural attenuation was actively occurring, thereby allowing to better
500 focus bioremediation efforts on these areas, and also to try to establish similar conditions at other
501 areas of the site. Our results also provide new insights on factors driving distribution and activity of
502 organohalide-respiring bacteria as well as of potentially useful co-occurring taxa on-site. Overall, our
503 study illustrates the usefulness of integrating different analytical chemical and biomolecular
504 approaches for more robust assessment and remediation of sites polluted with organohalides.

505 **5 Conflict of Interest**

506 *The authors declare that the research was conducted in the absence of any commercial or financial*
507 *relationships that could be construed as a potential conflict of interest.*

508 **6 Credit Author Statement**

509 **Jennifer Hellal:** conceptualization, verification, formal analysis, investigation, writing-original draft
510 preparation. **Catherine Joulian:** investigation, verification, writing - review & editing. **Charlotte**
511 **Urien:** investigation, data curation. **Stephanie Ferreira:** verification. **Jérémie Denonfoux:**
512 investigation, data curation. **Louis Hermon:** investigation. **Stéphane Vuilleumier:** writing - review
513 & editing. **Gwenaél Imfeld:** conceptualization, verification, formal analysis, investigation, writing -
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