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1 **Water table fluctuations affect dichloromethane biodegradation in lab-scale**
2 **aquifers contaminated with organohalides**

3
4 Maria L. Prieto¹, Sylvain Weill¹, Benjamin Belfort¹, Emilie E.L. Muller², Jérémy Masbou¹,
5 François Lehmann¹, Stéphane Vuilleumier², Gwenaël Imfeld^{1, *}

6
7 ¹ Université de Strasbourg, CNRS/EOST, ITES UMR 7063, Institut Terre et Environnement de
8 Strasbourg, Strasbourg, France

9 ² Université de Strasbourg, CNRS, GMGM UMR 7156, Génétique Moléculaire, Génomique,
10 Microbiologie, Strasbourg, France

11
12
13 *Corresponding author:

14 Email address: imfeld@unistra.fr (G. Imfeld)

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18 Manuscript for Water Research

19 **Highlights**

- 20 • Larger DCM removal (>90%) upon water table fluctuations than under static conditions
- 21 • Water table fluctuations alter redox conditions and potentially DCM-associated taxa
- 22 • Dual CSIA revealed distinct DCM degradation pathways under fluctuating conditions
- 23 • DCM degradation in groundwater evidenced by combining CSIA and biomolecular tools
- 24 • Accounting for dynamic environmental conditions to improve bioremediation strategies

25

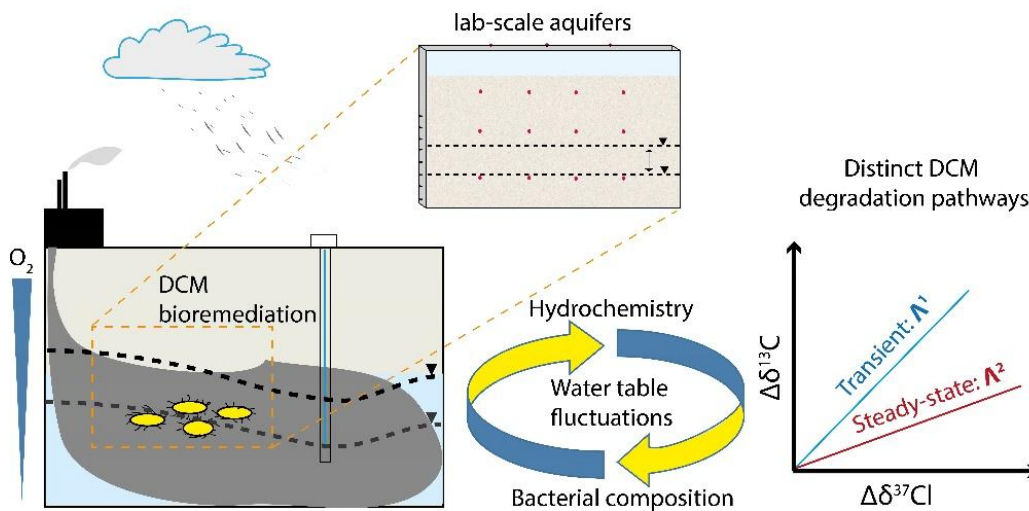
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33 **Abstract**

34 Dichloromethane (DCM) is a toxic industrial solvent frequently detected in multi-contaminated
35 aquifers. It can be degraded biotically or abiotically, and under oxic and anoxic conditions. The
36 extent and pathways of DCM degradation in aquifers may thus depend on water table
37 fluctuations and microbial responses to hydrochemical variations. Here, we examined the effect
38 of water table fluctuations on DCM biodegradation in two laboratory aquifers fed with O₂-
39 depleted DCM-spiked groundwater from a well-characterized former industrial site.
40 Hydrochemistry, stable isotopes of DCM ($\delta^{13}\text{C}$ and $\delta^{37}\text{Cl}$), and bacterial community composition
41 were examined to determine DCM mass removal and degradation pathways under steady-state
42 (static water table) and transient (fluctuating water table) conditions. DCM mass removal was
43 more pronounced under transient (95%) than under steady-state conditions (42%). C and Cl
44 isotopic fractionation values were larger under steady-state ($\epsilon_{\text{bulk}}^{\text{C}} = -23.6 \pm 3.2\text{‰}$, and $\epsilon_{\text{bulk}}^{\text{Cl}} =$
45 $-8.7 \pm 1.6\text{‰}$) than under transient conditions ($\epsilon_{\text{bulk}}^{\text{C}} = -11.8 \pm 2.0\text{‰}$, and $\epsilon_{\text{bulk}}^{\text{Cl}} = -3.1 \pm 0.6\text{‰}$).
46 Dual C-Cl isotope analysis suggested the prevalence of distinct anaerobic DCM degradation
47 pathways, with $\Lambda^{\text{C/Cl}}$ values of 1.92 ± 0.30 and 3.58 ± 0.42 under steady-state and transient
48 conditions, respectively. Water table fluctuations caused changes in redox conditions and oxygen
49 levels, resulting in a higher relative abundance of *Desulfosporosinus* (*Peptococcaceae* family).
50 Taken together, our results show that water table fluctuations enhanced DCM biodegradation,
51 and correlated with bacterial taxa associated with anaerobic DCM degradation. Our integrative
52 approach allows to evaluate anaerobic DCM degradation under dynamic hydrogeological
53 conditions, and may help improving bioremediation strategies at DCM contaminated sites.

54 **Keywords: DCM degradation, laboratory aquifers, dynamic conditions, multi-element**
55 **CSIA, 16S rRNA**

56 1. Introduction

57 Dichloromethane (DCM, CH₂Cl₂) is a toxic, persistent and halogenated volatile organic
58 compound (VOC) widely used in industrial settings (Schlosser et al., 2015). Due to accidental
59 spills and improper storage at industrial sites, DCM is commonly detected in contaminated
60 aquifers along with other VOCs (EPA, 2020; Hermon et al., 2018; Shestakova & Sillanpää,
61 2013). DCM is included in the list of priority pollutants of the U.S. Agency for Toxic
62 Substances and Disease Registry (ATSDR, 2019), and of the European Commission (European
63 Commission, 2013).

64 Monitored natural attenuation (MNA) has become a promising remediation strategy to
65 detoxify contaminated aquifers (Pope et al., 2004; Smets & Pritchard, 2003). MNA relies on an
66 integrative approach, which includes (i) monitoring contaminant concentrations in the field, (ii)
67 laboratory assays with microorganisms from the field, and (iii) evidence of *in situ*
68 biodegradation potential using stable isotope analysis and/or biomolecular methods (NRC,
69 1993). Monitoring contaminant concentrations is not sufficient to identify contaminant
70 transformation, as concentrations alone reflect both non-destructive (e.g., dilution, sorption) and
71 destructive dissipation processes (e.g., biodegradation). Compound-specific isotope analysis
72 (CSIA) is increasingly used to measure the extent of contaminant transformation *in situ*
73 (Hunkeler et al., 2009). CSIA relies on changes in stable isotope ratios (e.g., ¹³C/¹²C) of an
74 organic contaminant undergoing a (bio)degradation reaction. Typically, molecules containing
75 light isotopes (e.g., ¹²C) are degraded preferentially compared to those containing heavy
76 isotopes (e.g., ¹³C). This generally results in a change of stable isotope ratios in the remaining
77 contaminant mass, which may be specific of the transformation pathway (Elsner & Imfeld,
78 2016). The stable isotope fractionation can be used for quantitative estimations of contaminant

79 transformation *in situ* by using isotope fractionation values (ϵ) derived from reference laboratory
80 experiments (Fischer et al., 2016).

81 Dual-isotope analysis, involving the follow-up of changes in isotope ratios of two elements
82 (e.g., $^{13}\text{C}/^{12}\text{C}$ and $^{37}\text{Cl}/^{35}\text{Cl}$), is more informative and robust over a single isotope element
83 approach to evaluate transformation pathways of organic contaminants (Ojeda et al., 2020).
84 When the stable isotope ratios of two elements are compared in a dual plot, the slope (Δ)
85 provides a quantitative parameter of the corresponding transformation pathway (Elsner, 2010;
86 Ojeda et al., 2020). For DCM, the transformation pathway of methylotrophic bacteria, featuring a
87 glutathione-dependent DCM dehalogenase, was the first to be examined by dual C-Cl CSIA
88 under oxic conditions ($^{13}\text{C}/^{12}\text{C}$ and $^{37}\text{Cl}/^{35}\text{Cl}$) (Heraty et al., 1999; Torgonskaya et al., 2019).
89 Recently, dual C-Cl CSIA provided evidence of distinct anaerobic DCM pathways for
90 *Dehalobacterium formicoaceticum*, and for mixed cultures containing DCM-degrading
91 organisms such as *Candidatus* Dichloromethanomonas elyunquensis (Chen et al., 2018;
92 Kleindienst et al., 2019), and for a bacterial consortium featuring a *Dehalobacterium* strain
93 (Blázquez-Pallí et al., 2019; Trueba-Santiso et al., 2017).

94 So far, DCM transformation pathways have been examined in groundwater microcosms
95 under static conditions. However, the interplay of hydrochemical and hydrogeological dynamics
96 on DCM biodegradation in contaminated aquifers has not yet been addressed. Water table
97 fluctuations are known to affect (i) mass transfer of VOCs (e.g., DCM) from groundwater to the
98 unsaturated zone (Jeannotat & Hunkeler, 2013; McCarthy & Johnson, 1993), (ii) redox
99 conditions due to redistribution of terminal electron acceptors (e.g., O_2) (Haberer et al., 2012;
100 Seybold et al., 2002), and (iii) bacterial community composition due to changes in nutrients,
101 redox conditions and exposure to pollutants, (Peralta et al., 2014; Rühle et al., 2015). The effect

102 of water table fluctuations on DCM biodegradation is difficult to probe *in situ* as it requires a
103 high spatial and temporal monitoring resolution (Zhang & Furman, 2021). In this context,
104 laboratory aquifers under near-natural settings may prove useful, as flow fields can be controlled
105 and mass fluxes can be established (Schürner et al., 2016).

106 The purpose of the present study was to examine the effect of water table fluctuations on the
107 hydrochemistry, bacterial community composition, and DCM degradation extent and pathways
108 under controlled conditions. Two laboratory aquifers, fed with contaminated groundwater from
109 a well-characterized former industrial site (Hellal et al., 2021; Hermon et al., 2018), were set up
110 to examine the reactive transport of DCM under transient (i.e., induced water table fluctuations)
111 and steady-state (i.e., static water table) conditions. Concentrations of chloroethenes *cis*-DCE
112 and VC in the contaminated groundwater were also monitored. The objectives of the present
113 study were (i) to examine DCM dissipation processes in groundwater under steady-state and
114 transient conditions, (ii) to infer DCM degradation pathways using dual C-Cl CSIA, and (iii) to
115 analyse bacterial community composition associated with DCM biodegradation by sequencing
116 the 16S rRNA gene.

117

118 **2. Materials and methods**

119 **2.1 Chemicals**

120 DCM, *cis*-DCE and VC standards were purchased from Sigma-Aldrich (St: Louis, MO,
121 USA; analytical grade purity: >99%). A second DCM standard was purchased from VWR
122 (Radnor, Pennsylvania, USA; analytical grade purity: >99%). Stock solutions of standards were
123 prepared in methanol at 1 g L⁻¹ and diluted in water. Aliquots were stored at 4 °C. DCM

124 standards from Sigma-Aldrich and VWR are referenced here as DCM_{#1} and DCM_{#2},
125 respectively.

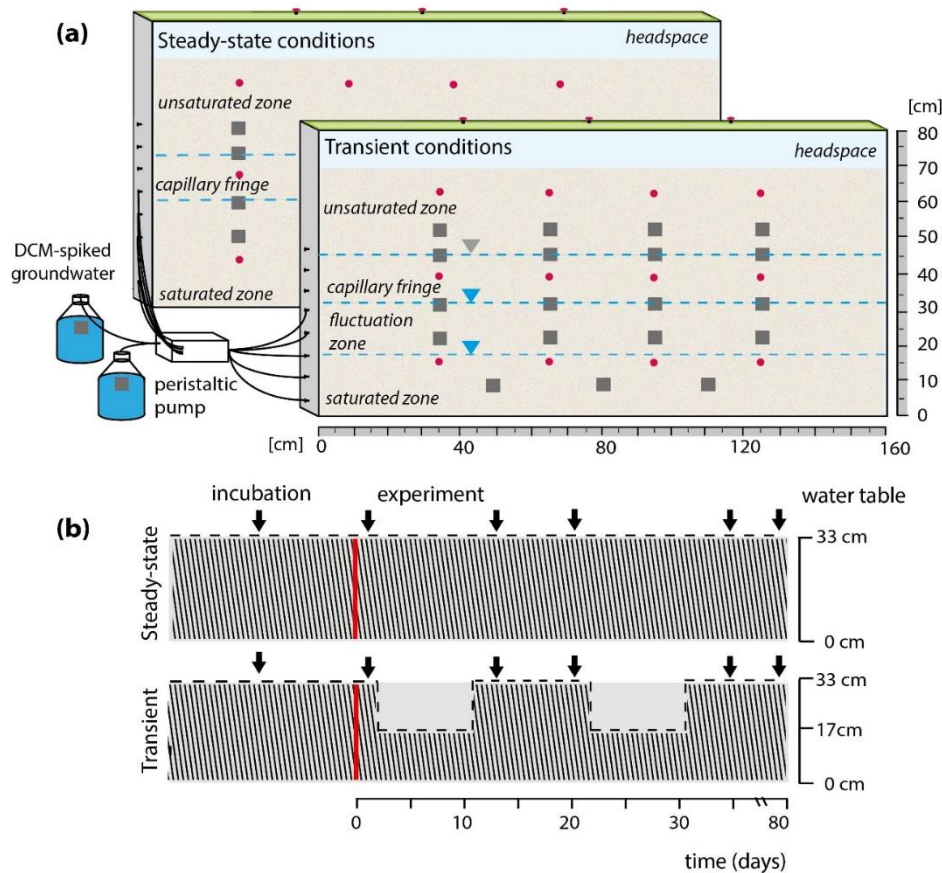
126 **2.2 Groundwater**

127 Groundwater was collected from the source zone of a well-characterized contaminated
128 aquifer (well Pz-28; Hellal et al., 2021; Hermon et al., 2018), and used as inflow water in both
129 laboratory aquifers. Isotope and biomolecular analyses evidenced *in situ* anaerobic DCM and *cis*-
130 DCE biodegradation in the source zone of the contaminated aquifer, as well as bacterial genera
131 associated with organohalide respiration (OHR) such as *Geobacter* and *Dehalococcoides* (Hellal
132 et al., 2021; Hermon et al., 2018). Field groundwater sampling and hydrochemical characteristics
133 are provided as Supporting Information (SI, Sections A and F).

134 **2.3 Experimental setup**

135 Experiments were conducted in two laboratory aquifers with inner dimensions of 160 cm ×
136 80 cm × 7 cm (length × height × width) and filled with sterile quartz sand (grain size: 0.4 - 0.6
137 mm; depth: 70 cm). A headspace zone of 10 cm above the sand compartments was covered by a
138 top glass mounted with 3 sampling ports equipped with active charcoal cartridges and opened to
139 the atmosphere. A detailed description of the experimental setup is provided in SI (Section B).
140 Experiments were conducted in a temperature-controlled room at 18 ± 1 °C. Both aquifers were
141 continuously fed with O₂-depleted contaminated groundwater (O₂ < 1 mg L⁻¹) at a rate of 430 mL
142 d⁻¹, corresponding to an average water velocity of 0.02 m d⁻¹. Groundwater was supplied from
143 two sterile and gas-tight 10 L glass reservoirs connected to each aquifer (Figure 1). Each
144 reservoir was kept under constant N₂-flux and consisted of a mixture of groundwater and
145 sterilized O₂-depleted distilled water at a ratio of 1:3. This allowed to avoid clogging of the pipes
146 feeding the aquifers due to mineral precipitation. Pure DCM was directly spiked and mixed into

147 the reservoirs to reach aqueous concentrations of 0.47 mM L^{-1} , equivalent to typical
 148 concentrations at contaminated sites (Hermon et al., 2018, Wright et al., 2017).



149
 150 **Figure 1.** Lab-scale aquifers fed with DCM-spiked groundwater under steady-state and transient
 151 conditions. (a) Schematic overview of the aquifers (flow path from left to right) indicating the
 152 saturated zone (SZ), unsaturated zone (UZ), headspace, position of sampling ports (red dots),
 153 oxygen foils (grey squares) and top glass (green) covering the aquifers along with three sampling
 154 ports opened to the atmosphere. (b) Operations to establish steady-state and transient conditions
 155 in the laboratory aquifers and sampling. Water table level is indicated in dotted lines. The end of
 156 the incubation period (total 70 days) is marked with a red line. Black arrows show sampling
 157 events of water and gas samples. Sampling within the incubation period took place 35 days prior
 158 to the experiment.

159 **2.4 Laboratory aquifer operations**

160 Both aquifers were operated simultaneously. Prior to the experiments, an incubation period
161 of 70 days was established by operating both aquifers with a continuous flow of DCM-spiked
162 groundwater to reach initial steady-state conditions and allow bacterial adaptation. Monitoring of
163 DCM concentrations and carbon stable isotope ratios during the incubation period (70 days)
164 evidenced DCM biodegradation capacity in both aquifers (data not shown). The water table was
165 positioned at a depth of 33 cm and a capillary fringe of approximately 12 cm was determined
166 visually. During the experimental phase (day 0 to 88), one aquifer remained under steady-state
167 conditions while the second aquifer underwent two water table fluctuation events (Figure 1).
168 Under steady-state conditions, a constant horizontal water flow was established using a
169 peristaltic pump (IPC 8, ISMATEC, Glattbrugg, Switzerland). Transient conditions were
170 established by (i) lowering the water table by 16 cm for 24 h (day 3), (ii) continuous horizontal
171 flow at a low water level for 6 d (up to day 10), and (iii) raising the water table to its initial
172 position for 24 h (day 11). A second identical water table fluctuation event was performed from
173 day 21 to 32 (Figure 1).

174 **2.5 Sampling**

175 Sampling was carried out in both aquifers before and after each water table fluctuation event
176 on days 0 and 13 (first water table fluctuation), and at days 20 and 35 (second water table
177 fluctuation). Pore water samples were collected for hydrochemical analysis, VOCs concentration
178 measurements, carbon and chlorine isotope analysis, and bacterial diversity and community
179 composition. Pore water samples were collected using gas-tight syringes (Hamilton Bonaduz
180 AG, Bonaduz, Switzerland) from inlet and outlet reservoirs, and sampling ports located at depths
181 of 15 and 40 cm from the bottom, and at 35, 65, 95 and 125 cm from the inflow (Figure 1). For

182 quantification and CSIA, 20 mL glass vials (Interchim, Montluçon, France) were filled with 1
183 mL of pore water sample and 1 mL of saturated salt solution (triplicate measurements). Vials
184 were immediately crimped with a Teflon septum with a magnetic crimp (Interchim, Montluçon,
185 France), and stored upside down at 4 °C until further analysis. Gas-phase samples (volume: 2
186 cm³) were collected from the unsaturated zone (z = 65cm) and headspace (z = 80 cm), and stored
187 similarly as liquid samples.

188 Water samples for DNA analysis were collected from inlet and outlet reservoirs (8 mL) and
189 from sampling ports located at depths of 15 and 40 cm (pooling 2 mL in total per single height).
190 Additionally, pore water samples were collected during the incubation period (35 days prior to
191 the experiments, see Figure 1), and prior to the core sampling (day 80) for DNA and
192 hydrochemical analysis. Both aquifers were fully drained and sand samples from four cores
193 (length: 70 cm, and inner diameter: 5 cm) were collected for DNA analysis into sterile
194 polyethylene tubes, and stored at -20 °C until further analysis. Core sand samples covering the
195 full depth of the aquifers were retrieved at 40, 70, 100, and 130 cm from the inflow. Three core
196 subsamples representing the saturated zone (SZ), capillary fringe (CF) and unsaturated zones
197 (UZ) were obtained by cutting under sterile conditions each frozen sand core at depths of 25 and
198 50 cm, respectively.

199 **2.6 Analytical methods**

200 **2.6.1 Hydrochemistry**

201 Oxygen (O₂) concentrations were monitored *in situ* by O₂ sensitive optode foils (PreSens
202 GmbH, Regensburg, Germany) located at the inlet reservoirs and across the sand compartments
203 (Figure 1). Under transient conditions, O₂ concentrations were monitored hourly during water
204 table fluctuation events. Redox potential (Eh), pH and electrical conductivity were monitored

205 prior to sampling events using laboratory probes (SCHOTT® Instruments). Major ions were
206 measured by ion chromatography (Dionex ICS-5000, Thermo Scientific, USA). Fe²⁺ was
207 measured by the BAP method (Tamura et al., 1974). Total organic carbon (TOC), dissolved
208 organic carbon (DOC) and dissolved inorganic carbon (DIC) were analyzed by a TOC analyzer
209 (TOC-V-CPH Shimadzu, NF EN 1484).

210 **2.6.2 VOCs concentrations and DCM C-Cl CSIA analysis**

211 A detailed description of analytical methods is given in the SI (Sections C, D). Briefly,
212 DCM, *cis*-DCE and VC were quantified by analyzing 200 µL of headspace sample using a gas
213 chromatograph (GC, Trace 1300, Thermo Fisher Scientific) coupled with a mass spectrometer
214 (MS, ISQ™, Thermo Fisher Scientific), as described elsewhere (Hermon et al., 2018).

215 Stable carbon isotope composition of DCM, *cis*-DCE and VC was determined by gas
216 chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), with a gas
217 chromatograph (Trace 1310) coupled via a GC/Conflow IV interface to an isotope ratio mass
218 spectrometer (Delta V plus, Thermo Fisher Scientific) (Hermon et al., 2018). In-house standards
219 of DCM, *cis*-DCE and VC were prepared daily and analyzed prior to sample measurements.
220 Reproducibility of triplicate measurements was ≤0.2‰ (1σ) within the linearity range (0.5-50 mg
221 L⁻¹). Carbon isotope ratios were reported in δ notation as parts per thousand (‰) relative to the
222 international reference material Vienna Pee Dee-Belemnite (V-PDB) (Coplen et al., 2006).

223 Chlorine isotope composition of DCM was determined by GC-qMS based on the two most
224 abundant fragment ion peaks [³⁵Cl¹²C₁H₂]⁺ (m/z 49) and [³⁷Cl¹²C₁H₂]⁺ (m/z 51), as suggested
225 elsewhere (Heckel et al., 2017; Jin et al., 2011). The detailed GC-qMS setup is provided as SI
226 (Section D). Chlorine isotope ratios were reported in δ notation in parts per thousand [‰]
227 relative to the Standard Mean Ocean (SMOC) (Kaufmann et al., 1984). Chlorine isotope ratios

228 were corrected by an external two-point calibration with pure DCM in-house standards
229 ($\delta^{37}\text{Cl}_{\text{DCM}\#1} = 3.68 \pm 0.10\text{‰}$ and $\delta^{37}\text{Cl}_{\text{DCM}\#2} = -3.35 \pm 0.12\text{‰}$) characterized at Isotope Tracer
230 Technologies Inc., Waterloo, Canada by IRMS after conversion to CH_3Cl (Holt et al., 1997), and
231 at the Departament de Mineralogia, Petrologia I Geologia Aplicada, University of Barcelona
232 using GC-qMS. Reported uncertainties include both accuracy and reproducibility based on long-
233 term measurements and standard deviations. Typical reproducibility was 0.5‰ (1σ) within the
234 tested linearity range (0.5-20 mg L⁻¹). Chlorine isotope compositions of native *cis*-DCE and VC
235 were not analyzed.

236 **2.6.3 DNA extraction from pore water and sand samples**

237 Pore water samples were filtered through sterile 0.22 μm membrane filters (Swinnex holder,
238 13 mm, Millipore, Bedford, USA) and stored at $-20\text{ }^\circ\text{C}$ until DNA extraction, as described
239 previously (Hermon et al., 2018). Sand core subsamples were retrieved from both aquifers at the
240 end of the experiments (day 88). Under sterile conditions, the bottom and top first 1 cm of the
241 core samples were removed. Each core subsample was thoroughly mixed and a subsample of 1 g
242 of sand was used for DNA extraction. For both pore water and core samples, DNA was extracted
243 using the DNeasy Power Water kit according to the manufacturer's protocol (Qiagen, Hilden,
244 Germany). Extracted DNA was quantified using Qubit fluorometric quantification with the Qubit
245 dsDNA HS Assay kit (ThermoFischer Scientific, MA, USA)

246 **2.6.4 DNA sequencing**

247 The V4-V5 hypervariable region of the 16S rRNA gene was PCR amplified by an optimized
248 and standardized amplicon library preparation protocol (Metabio®[®], GenoScreen, Lille,
249 France), including a positive (mock community) and negative (blank) control (Hermon et al.,
250 2018). Libraries were sequenced by paired-end Illumina MiSeq 2x250 bases. Demultiplexing

251 and trimming was followed by paired read assembly (minimum overlap 30 nt, minimum identity
252 of 97%), resulting in a total of 3,614,309 sequences. Denoising, chimera checking, generation of
253 operational taxonomic units (OTUs), taxonomic classification using Greengenes (v13.8 as
254 reference), and alpha-diversity metrics were performed using a custom-scripted bioinformatics
255 pipeline of GenoScreen (Hermon et al., 2018). The percentage of 16S rRNA gene sequences of
256 taxa featuring known DCM-degrading strains (identity >97%) was determined.

257 **2.7 Data analysis**

258 **2.7.1 Evaluation of isotopic data**

259 The average isotope value of the residual non-degraded fraction of DCM was derived
260 according to the Rayleigh equation (Elsner, 2010):

$$261 \quad \ln\left(\frac{R_{t,E}}{R_{0,E}}\right) = \ln\left(\frac{C_{t,E}}{C_{0,E}}\right) \cdot \frac{\varepsilon_{bulk}^E}{1000} \quad (1)$$

262 where $R_{t,E}/R_{0,E}$ is the isotope ratio of element “E” (i.e., $^{13}\text{C}/^{12}\text{C}$ and $^{37}\text{Cl}/^{35}\text{Cl}$) and $C_{t,E}/C_{0,E}$ are the
263 concentrations at a given time (t) and at the initial time (0). Carbon and chlorine isotopic
264 composition were reported in delta notation ($\delta^h\text{E}$) following $\delta^h\text{E} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$
265 (Elsner, 2010). Apparent isotope fractionation values (ε_{bulk}^E , in ‰) were obtained by least
266 squares linear regression without forcing the slope through the origin. The uncertainty
267 corresponds to the 95% confidence interval (C.I.) and the error was determined using ordinary
268 linear regression (Elsner et al., 2007).

269 Changes of carbon *versus* chlorine isotope signatures were plotted to derive the $\Lambda^{C/Cl}$ value
270 from the slope of the linear regression using the least-squares algorithm of the York method
271 (Höhener & Imfeld, 2021, Ojeda et al., 2020), and using the IsoplotR package in R (Vermeesch,
272 2018).

273
$$\Delta^{C/Cl} = \frac{\ln[(\delta^{13}C_t/1000+1)/(\delta^{13}C_0/1000+1)]}{\ln[(\delta^{37}Cl_t/1000+1)/(\delta^{37}Cl_0/1000+1)]} \approx \frac{\varepsilon_{bulk}^C}{\varepsilon_{bulk}^{Cl}} \quad (2)$$

274 DCM biodegradation was estimated based on changes in carbon and chlorine isotope ratios
 275 over time and across the flow path (i.e., at different observation points). The extent of DCM
 276 biodegradation (B , in %) was estimated using the Rayleigh model (Hunkeler et al., 2005;
 277 Thullner et al., 2012). A range of B was obtained from reported ε^C and ε^{Cl} values for both
 278 aerobic and anaerobic bacterial DCM degradation (Torgonskaya et al., 2019, Chen et a., 2018,
 279 Lee et al., 2015).

280
$$B (\%) = 1 - f = \left[\frac{\delta^h E_t + 1000}{\delta^h E_0 + 1000} \right]^{\frac{1000}{\varepsilon^E}} \cdot 100 \quad (3)$$

281 **2.7.2 Bacterial community composition**

282 Sequencing data from pore water and sand samples were deposited to the ENA archive,
 283 BioProject accession number PRJEB43379. Multivariate statistical analysis of relative OTU
 284 abundance was performed with R (R Core Team, 2019). Non-metric multidimensional scaling
 285 (NMDS) based on Bray-Curtis dissimilarities of log-transformed data was performed to visualize
 286 dissimilarities between bacterial taxa associated with DCM degradation (Hellal et al., 2021).

287

288 **3. Results and discussion**

289 **3.1. Water table fluctuations affect hydrochemical conditions**

290 Hydrochemical variations are summarized in the SI (Sections E and F). Overall, saturated O_2
 291 concentrations (8.4 mg L^{-1}) were observed in the unsaturated zone (UZ) in both aquifers ($z = 60$
 292 cm), while O_2 -depleted levels were established in the saturated zone (SZ; $z = 0\text{-}33$ cm; $O_2 < 1.0$
 293 mg L^{-1}). The increase of redox potential over time (Eh ranged from -100 to $+0$ mV at days 0 and
 294 35, respectively) was consistent with O_2 dynamics in the fluctuation zone ($z = 17\text{-}33$ cm, SI

295 Section F) (Pronk et al., 2020). Concentrations of O₂ rapidly increased up to 1.5 mg L⁻¹ during
296 drainage periods (z = 25 cm, SI, Section E). During the first imbibition period, water moved in
297 an upward direction, O₂ concentrations decreased slightly but did not return to initial
298 concentrations (1.3 mg L⁻¹). From the second imbibition period until the end of the experiment,
299 O₂ levels slowly decreased indicating that the system progressively returned to initial conditions
300 (O₂ < 1 mg L⁻¹) (Haberer et al., 2012). Re-oxygenation of groundwater below the water table was
301 likely associated with entrapped air serving as a source of O₂ to the underlying O₂-depleted water
302 (Williams & Oostrom, 2000). Redox potential and O₂ varied upon water table fluctuation.
303 Nevertheless, the high concentration of Fe²⁺ in both aquifers (up to 2.8 mg L⁻¹) suggested
304 prevailing reducing conditions, in line with reducing conditions observed *in situ* for the
305 groundwater used in this study (Hellal et al., 2021; Hermon et al., 2018) (SI, Section F). No other
306 reduced species were detected in both aquifers (<D.L.).

307 *Cis*-DCE and VC, the two main chloroethenes in the groundwater source of this study (Hellal
308 et al., 2021; Hermon et al., 2018), were also followed to further examine the established
309 hydrochemical conditions in the laboratory aquifers. Only *cis*-DCE was detected at the inflow of
310 both aquifers with average concentrations of 15 mg L⁻¹ and δ¹³C_{DCE} values of -23.5 ± 0.4‰ (SI,
311 Section G). Up to 90% of *cis*-DCE dissipated at the outflows after 35 days. Values of δ¹³C for
312 *cis*-DCE were smaller (Δδ¹³C_{DCE} < 2‰) at the outflows than δ¹³C values observed within the first
313 65 cm from inflow (Δδ¹³C_{DCE} > 10‰). Similarly, VC concentrations of up to 6 and 12 mg L⁻¹
314 were detected within the first 65 cm from inflow under steady-state and transient conditions,
315 respectively, and average δ¹³C_{VC} values of -39.2 ± 0.8‰ (n = 20) were formed along the flow
316 path (δ¹³C_{VC, standard} = -29.5 ± 0.2‰) (SI, Section G). Together with the detection of OHRB (see

317 below), this suggests that reductive dechlorination occurred in both aquifers, in agreement with
318 observations at the groundwater source (Hellal et al., 2021, Hermon et al., 2018).

319 **3.2 Water table fluctuations affect DCM mass dissipation and C and Cl isotope** 320 **fractionation**

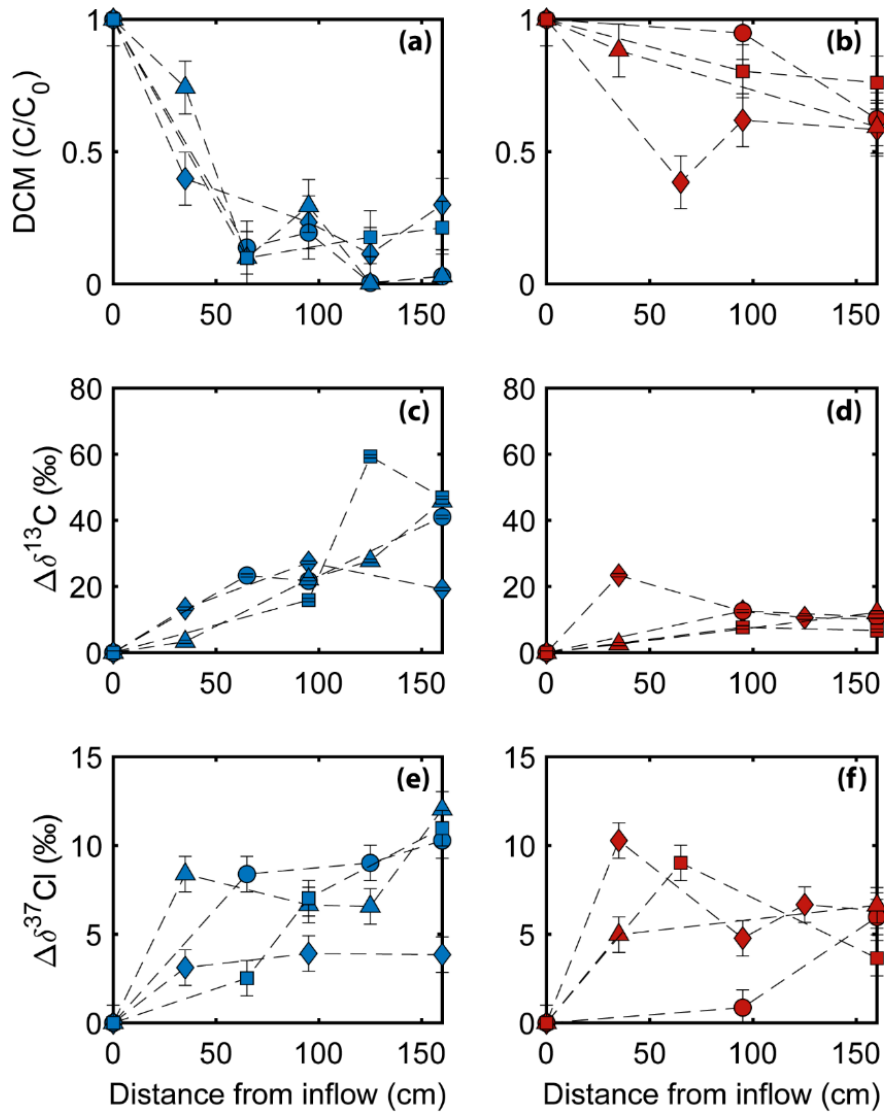
321 The absence of gas-phase DCM (gas-phase Q.L. = $0.13 \mu\text{g L}^{-1}$, sampling volume: 2 cm^3) in
322 the UZ ($z = 65 \text{ cm}$) and headspace ($z = 80 \text{ cm}$) of both aquifers throughout the experiment, and
323 inlet-outlet DCM mass balance indicated that volatilization did not contribute significantly (<10
324 %) to the overall DCM dissipation in both aquifers. Steep concentration gradients are generally
325 formed across the capillary fringe due to slow diffusion in water and small vertical dispersivity
326 (Jeannotat & Hunkeler, 2013). Previous studies showed that mass transport of PCE and TCE
327 from the SZ to the UZ (i.e., within the 15 cm above the water table) led to 90% smaller gas-
328 phase concentrations with respect to the aqueous-phase concentrations at the water table under
329 steady-state (Jeanottat & Hunkeler, 2013, McCarthy and Johnson, 1993). While variations of
330 VOCs concentrations in the gas-phase increase in the UZ when the water table drops as a result
331 of residual water being in contact with the gas, concentrations decrease again when the water
332 table is raised, returning to concentration equilibrium (Jeannotat & Hunkeler, 2013; McCarthy
333 & Johnson, 1993). Hence, undetected gas-phase DCM concentrations in the UZ ($z = 65 \text{ cm}$) and
334 headspace ($z = 80 \text{ cm}$) is likely due to the low non-dimensional Henry's coefficient of DCM of
335 0.0549 at $18 \text{ }^\circ\text{C}$ (Gossett, 1987; PCE and TCE of 0.495 and 0.265, respectively), the continuous
336 replenishment of the aqueous phase by low horizontal groundwater flow, and measurements
337 carried out before and after each water table fluctuation event.

338 In the SZ, DCM concentrations decreased along the flow path in both aquifers. After 35 days,
339 a more pronounced DCM mass dissipation in the aquifer outlets was observed under transient

340 (95%) than under steady-state (42%) conditions (Figure 2). $\delta^{13}\text{C}$ and $\delta^{37}\text{Cl}$ values of DCM in the
341 inflow water remained constant throughout the experiments ($-46.3 \pm 0.5\text{‰}$ and $-3.5 \pm 0.12\text{‰}$,
342 respectively). Under both transient and steady-state conditions, DCM became significantly
343 enriched in both ^{13}C and ^{37}Cl (Figure 2). C and Cl isotope data for DCM degradation showed a
344 good fit to the Rayleigh model (eq. 1) under both hydraulic regimes ($R^2 > 0.92$) (SI, Section H).
345 The apparent C and Cl isotope fractionation values ($\epsilon_{\text{bulk}}^{\text{C}}$ and $\epsilon_{\text{bulk}}^{\text{Cl}}$) of $-23.6 \pm 3.2\text{‰}$ and $-8.7 \pm$
346 1.6‰ , respectively, were larger under steady-state than under transient conditions ($\epsilon_{\text{bulk}}^{\text{C}}$ and $\epsilon_{\text{bulk}}^{\text{Cl}}$
347 values of $-11.8 \pm 2.0\text{‰}$ and $-3.1 \pm 0.6\text{‰}$, respectively; SI, Section H). In previous reports, DCM
348 volatilization was associated with low C and Cl fractionation ($\epsilon^{\text{C}} = +0.65\text{‰}$ and $\epsilon^{\text{Cl}} = -0.48\text{‰}$)
349 compared to DCM biodegradation (Huang et al., 1999). Thus, the magnitude of $\epsilon_{\text{bulk}}^{\text{C}}$ and $\epsilon_{\text{bulk}}^{\text{Cl}}$
350 values under both hydraulic regimes suggest that DCM biodegradation prevailed in the aquifers.

351 The extent of DCM biodegradation (B) along the flow path was calculated based on reported
352 ϵ^{C} values ranging from -71‰ to -15.5‰ and ϵ^{Cl} values from -7‰ to -5.2‰ (Torgonskaya et al.,
353 2019, Chen et al., 2018, Lee et al., 2015). The range of ϵ^{C} and ϵ^{Cl} values was defined based on the
354 assumption that both aerobic and anaerobic DCM degradation pathways co-occur in both
355 aquifers, which is supported by hydrochemical variations and micro-oxic environments in the
356 groundwater source (Hermon et al., 2018). Values of B under steady-state and transient
357 conditions ranged from 22% to 55%, and from 22% to 90%, respectively (Table 1),
358 corresponding to the DCM mass dissipation observed under both hydraulic regimes. Time-
359 dependent first-order biodegradation rate constants (λ_t) were calculated according to the
360 Rayleigh model, as described in the SI of Hermon et al., 2018, with values of $3.4 \times 10^{-3} \text{ d}^{-1}$ and
361 $5.7 \times 10^{-3} \text{ d}^{-1}$ under steady-state and transient conditions, respectively. Worthy of note, similar *in*

362 *situ* λ_t were estimated at the groundwater source of this study (Hermon et al. 2018), thus
 363 demonstrating the established near-natural settings within the laboratory aquifers.



364
 365 **Figure 2.** DCM concentrations under steady-state (red) and transient (blue) conditions (a, b),
 366 carbon isotope values (c, d) and chlorine isotope values (e, f) over distance from inflow. Symbols
 367 represent observations at depth $z = 15$ cm and at different times: day 0 (diamonds), day 13
 368 (circles), day 20 (triangles) and day 35 (squares). Error bars associated with DCM concentrations
 369 and stable isotope values represent standard errors ($3 \leq n$).

370

371

372 **Table 1.** DCM biodegradation (B [%]) under transient and steady-state conditions from days 0 to
 373 35. A range of B [%] was estimated using the full range of ϵ^C and ϵ^{Cl} values reported so far for
 374 both aerobic and anaerobic bacterial degradation of DCM. Reported values range from -27‰
 375 (${}^C B_{\min}$) to -15.5‰ (${}^C B_{\max}$), and ϵ^{Cl} value of -7‰ (${}^{Cl} B_{\min}$) to -5.2‰ (${}^{Cl} B_{\max}$) (Torgonskaya et al.,
 376 2019, Chen et al., 2018, Lee et al., 2015).

Condition	Days	$\Delta\delta^{15}C$ [‰]	$\Delta\delta^{37}Cl$ [‰]	${}^C B_{\min}$ [%]	${}^C B_{\max}$ [%]	${}^{Cl} B_{\min}$ [%]	${}^{Cl} B_{\max}$ [%]
Transient	0	17.6	4.8	22	66	48	58
	13	31.8	9.2	36	85	73	83
	20	40.3	10.3	33	86	80	85
	35	49.2	7.0	48	91	66	75
Steady-state	0	17.6	5.6	22	66	55	66
	13	11.7	5.9	16	54	34	43
	20	12.1	5.8	14	48	56	67
	35	11.6	6.3	15	51	57	66

377

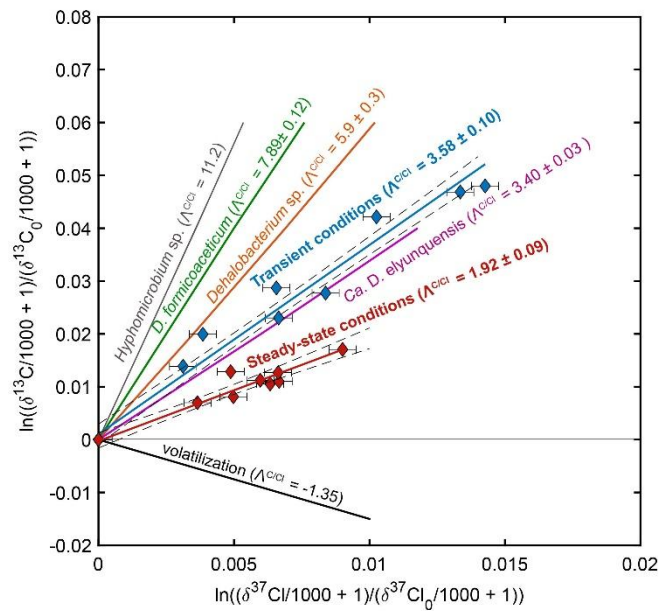
378 3.3 DCM transformation pathways under transient and steady-state conditions

379 Estimated $\Lambda^{C/Cl}$ values were lower under steady-state (1.92 ± 0.30 , $p < 0.03$) than under
 380 transient conditions (3.58 ± 0.42 , $p < 0.01$) (Figure 3). This suggests distinct DCM C-Cl bond
 381 cleavage mechanisms under dynamic hydrogeological conditions. The calculated $\Lambda^{C/Cl}$ values
 382 under transient conditions fall within the range of reported $\Lambda^{C/Cl}$ values during anaerobic DCM
 383 degradation (3.40-7.89) and are similar to $\Lambda^{C/Cl}$ values reported for *Ca. Dichloromethanomonas*
 384 *elyunquensis* (3.40 ± 0.03) (Chen et al., 2018), which suggests the prevalence of anaerobic DCM
 385 pathways. Most strikingly, the lower $\Lambda^{C/Cl}$ value determined under steady-state conditions (1.92
 386 ± 0.30) has not been reported previously. Still, this value likely also reflects anaerobic DCM
 387 degradation, given that so far higher $\Lambda^{C/Cl}$ values (8.1 to 11.2) are rather associated with aerobic
 388 DCM degradation pathways (Heraty et al., 1999; Torgonskaya et al., 2019) (SI, Section I).

389 To date, anaerobic DCM degradation by bacteria affiliated with the *Peptococcaceae* family
 390 has been examined using C-Cl CSIA. Two distinct anaerobic degradation pathways were

391 reported for DCM fermentation by *Dehalobacterium formicoaceticum* ($\Lambda^{C/Cl} = 7.89 \pm 0.12$) and
392 DCM mineralization by *Ca. Dichloromethanomonas elyunquensis* ($\Lambda^{C/Cl} = 3.40 \pm 0.03$), and
393 proposed to be associated with the Wood-Ljungdahl pathway (WLP) (Chen et al., 2018, 2020,
394 Kleindienst et al., 2019). In addition, Blázquez-Pallí et al. (2019) reported a distinct $\Lambda^{C/Cl}$ value
395 (5.9 ± 0.3) for anaerobic DCM degradation by a *Dehalobacterium*-containing culture, differing
396 from that associated with *Dehalobacterium formicoaceticum* ($\Lambda^{C/Cl}$ value = 7.89 ± 0.12),
397 although the two strains belong to the same genus. Different characteristics of the bacterial cell
398 envelope or enzyme locations may result in distinct C and Cl isotope fractionation (Trueba-
399 Santiso et al., 2017).

400 The differences in $\Lambda^{C/Cl}$ values under steady-state and transient conditions may thus reflect
401 distinct C-Cl bond cleavage reactions and distinct anaerobic DCM degradation pathways.
402 Further, our findings suggest that the prevalence of a given pathway depend on hydrochemical
403 and hydrogeological dynamics in aquifers. Nevertheless, both laboratory aquifers likely feature a
404 variety of DCM-degrading microorganisms, with the possibility of simultaneous operations of
405 several different aerobic and anaerobic degradation pathways (Van Breukelen, 2007). In
406 particular, the slight increase in O₂ levels in the fluctuation zone under transient conditions may
407 be indicative of micro-oxic environments (SI, Sections E and F). However, the $\Lambda^{C/Cl}$ values
408 obtained in our aquifers rather suggest the dominance of different anaerobic DCM degradation
409 pathways, which is further supported by the presence of taxa associated to anoxic conditions (see
410 below).



411
 412 **Figure 3.** Dual plot of $\Delta^{13}\delta\text{C}$ versus $\Delta^{37}\delta\text{Cl}$ for the degradation of DCM under steady-state (red
 413 squares) and transient (blue diamonds) conditions. The continuous lines represent the linear
 414 regression to derive the $\Lambda^{C/Cl}$ values and dashed lines represent the 95% confidence interval
 415 (C.I.), according to the York method. Reported values for DCM degradation by
 416 *Hyphomicrobium* strain MC8b (Heraty et al., 1999), *Dehalobacterium formicoaceticum* and *Ca.*
 417 *D. elyunquensis* (Chen et al., 2018), and *Dehalobacterium* sp. (Blázquez-Pallí et al., 2019) were
 418 added for comparison. Black line represents 99% of DCM volatilization (Huang et al., 1999).

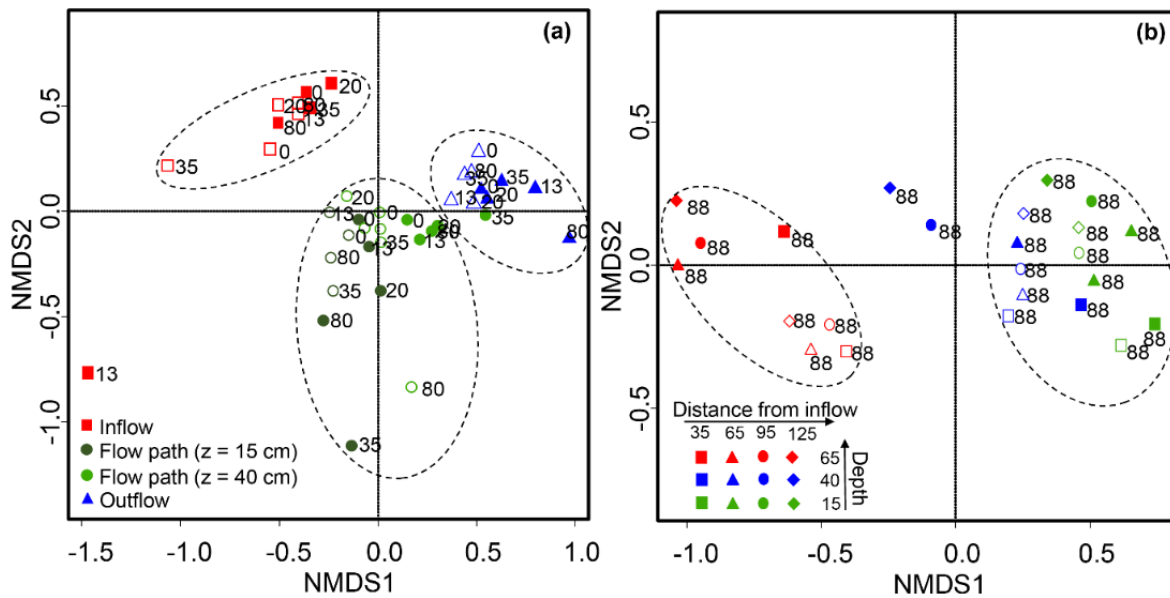
419
 420 **3.4 Water table fluctuations affect bacterial community composition and distribution of**
 421 **DCM-associated taxa**

422 In total, 3,649,087 high-quality sequences were obtained, from which 69% and 31%
 423 corresponded to pore water (n=56) and sand samples (n=24), respectively. Rarefaction curves of
 424 diversity indices reached asymptotes with increasing sequencing depth, indicating sufficient

425 sequencing efforts to capture the biodiversity extent of bacterial communities in both pore water
426 and sand samples (SI, Section J).

427 Changes in bacterial diversity were analyzed using NMDS ordination of relative OTU
428 abundance. Diversity in sand samples (day 88) varied mainly as a function of O₂ gradients in the
429 SZ and UZ (Figure 4). In contrast, differences in bacterial diversity in pore water samples (day 0-
430 80) between inlet and outlet reservoirs suggested bacterial adaptation to DCM contamination
431 along the flow path (Figure 4). Nevertheless, similar bacterial diversity under transient and
432 steady-state conditions for both pore water and sand samples suggested that water table
433 fluctuations did not play a major role in shaping the overall composition of bacterial
434 communities (SI, Sections K and L).

435 Dominant taxa in aquifers may be associated with pollutants degradation pathways. In both
436 aquifers, *Firmicutes* was the most abundant phylum in sand samples with up to 60% of retrieved
437 sequences at lower depths ($z = 15\text{cm}$, SI, Section L). Previous studies have shown the presence
438 of this phylum in highly contaminated aquifers with VOCs (Hellal et al., 2021; Wright et al.,
439 2017). Moreover, *Firmicutes* bacteria are known to utilize DCM as a sole carbon source under
440 anoxic conditions (Kleindienst et al., 2017). Hence, the occurrence of *Firmicutes* supports the
441 prevalence of anaerobic DCM biodegradation in both aquifers.

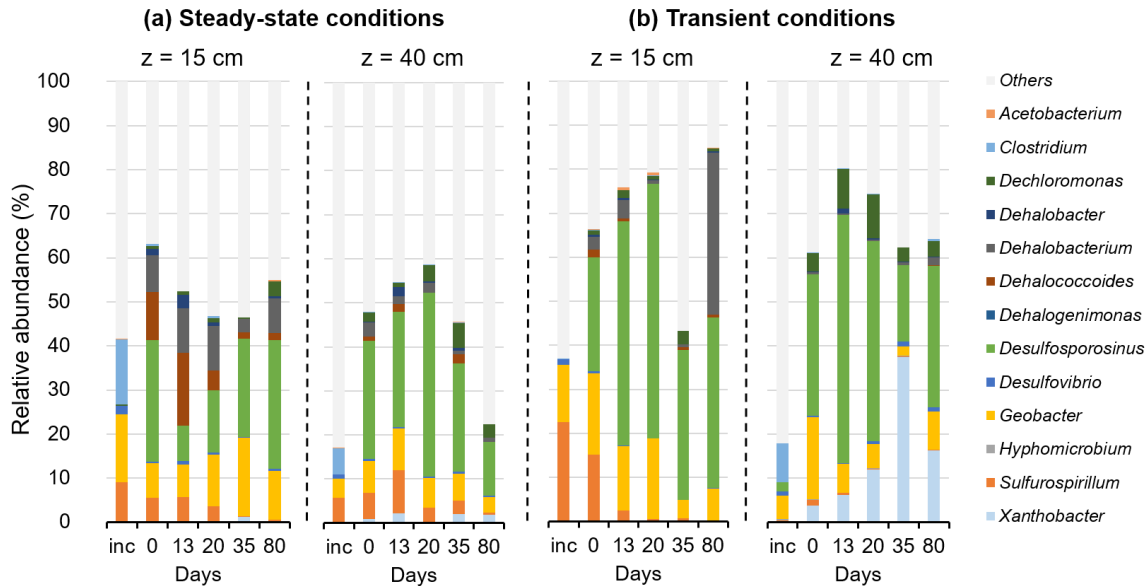


442

443 **Figure 4.** NMDS ordination plot of bacterial diversity profiles from (a) pore water and (b) sand
 444 samples. Full symbols: transient conditions, empty symbols: steady-state conditions. Numbers
 445 next to symbols represent sampling day at 0, 13, 20, 35 and 80 for pore water samples and at day
 446 88 for sand samples. Plot stress: (a) 0.11% and (b) 0.06%.

447

448 In pore water samples, the dominant OTU was associated with *Desulfosporosinus* under both
 449 steady-state and transient conditions (Figure 5). Under steady-state conditions,
 450 *Desulfosporosinus* and *Geobacter* represented on average 25% and 12% of obtained sequences
 451 in the SZ, respectively, while *Dehalobacterium* and *Dehalococcoides* represented each
 452 approximately 10% (Figure 5). Under transient conditions, in contrast, *Desulfosporosinus*
 453 increased from 25% to 58% relative abundance between days 0 and 13 in the SZ ($z = 15$ cm),
 454 corresponding to the first water table fluctuation event (Figure 5). *Geobacter* was the second
 455 most abundant genus in the SZ representing up to 15% followed by *Dehalobacterium* (4%).



456

457 Figure 5. Relative abundance of taxa associated with DCM degradation and OHR in lab-scale
 458 aquifers under (a) steady-state and (b) transient conditions in the saturated zone ($z = 15$ cm) and
 459 capillary fringe ($z = 40$ cm), and over time (from 0 to 80 days). Data includes the initial
 460 incubation period of 70 days (“inc” represents sampling 35 days prior to the experiments).
 461 Relative abundance (%) were normalized to the total sequence abundance for all defined OTUs.

462

463 Co-occurrence of *Desulfosporosinus*, *Geobacter*, *Sulfurospirillum* and *Dehalococcoides* was
 464 reported previously in contaminated aquifers with halogenated contaminants (Hellal et al., 2021;
 465 Wright et al., 2017). This parallel enrichment of different OTUs without demonstrated
 466 association with DCM degradation suggest interspecies interactions in our laboratory aquifers.
 467 Such interactions could be indicative of concomitant degradation of DCM and other halogenated
 468 contaminants, as shown recently in investigations of other contaminated sites (Hellal et al., 2021;
 469 Trueba-Santiso et al., 2020; Blázquez-Pallí et al., 2019; Hermon et al., 2018; Wright et al.,
 470 2017). Detection of bacterial genera associated with OHR such as *Dehalococcoides* further
 471 supports the concomitant degradation of DCM and *cis*-DCE in our laboratory aquifers.

472 Furthermore, reductive dechlorination of chlorinated ethenes such as *cis*-DCE relies on H₂ as
473 electron donor (DiStefano et al., 1992). Recently, it was proposed that DCM mineralization by
474 *Ca. D. elyunquensis* produces H₂ and CO₂ (Chen et al., 2020), which may sustain reductive
475 dechlorination in mixed contaminant plumes. Notably, for growth with DCM, *Ca. D.*
476 *elyunquensis* requires the presence of H₂-consuming partner populations performing H₂/CO₂
477 reductive acetogenesis, while *Dehalobacterium formicoaceticum* showed a strong dependence on
478 CO₂ (Chen et al., 2020). Concomitant degradation of DCM and chlorinated ethenes in
479 contaminated aquifers may thus result from interspecies metabolic networks associated with
480 dehalogenative metabolism.

481 More generally, dynamic environmental conditions such as water table fluctuations can
482 increase microbial metabolic activity (Pronk et al., 2020). Thus, we hypothesized that in the
483 present study, water table fluctuations promoted a larger enrichment of *Desulfosporosinus*
484 compared to steady-state conditions (Figure 5). Kleindienst et al., 2019 reported that *Ca. D.*
485 *elyunquensis* expressed similar WLP-associated enzymes as those of *Dehalobacter* and
486 *Desulfosporosinus* spp. Hence, the similar C and Cl isotope fractionation determined here for
487 DCM biodegradation under transient conditions ($\Lambda^{C/Cl} = 3.58 \pm 0.42$) and by *Ca.*
488 *Dichloromethanomonas elyunquensis* ($\Lambda^{C/Cl} = 3.40 \pm 0.03$) (Chen et al., 2018) suggest
489 *Desulfosporosinus* as a potential DCM-degrading bacterium, in line with a previous report
490 (Wright et al., 2017).

491 In contrast, the higher abundance of *Dehalobacterium* under steady-state conditions, and
492 similar C fractionation values to those of a *Dehalobacterium*-containing culture (Blázquez-Pallí
493 et al., 2019), suggest the occurrence of a fermentative DCM metabolism by as yet unknown
494 *Dehalobacterium* strains ($\Lambda^{C/Cl} = 1.92 \pm 0.30$). Worthy of note, a recent study proposed *Ca.*

495 *Formimonas warabiya* strain DCMF as a novel DCM-fermenting bacterium of the
496 *Peptococcaceae* family, capable to metabolize DCM to acetate via the WLP (Holland et al.,
497 2021) (SI, Section I). Clearly, bacteria associated with the *Peptococcaceae* family may play an
498 important role in DCM biodegradation at contaminated sites.

499 **4. Conclusions**

500 Our study examined the effect of dynamic environmental conditions such as water table
501 fluctuations on (i) hydrochemical conditions, (ii) bacterial responses, and in turn, (iii) on DCM
502 degradation pathways in multi-contaminated aquifers. Our integrative approach combining C-Cl
503 CSIA and biomolecular analyses suggested the prevalence of two distinct, possibly co-occurring,
504 anaerobic DCM degradation pathways under steady-state and transient conditions. Bacterial
505 diversity and distribution of DCM-associated taxa was similar under both conditions. However,
506 responses to water table fluctuations resulted in different bacterial community composition
507 dominated by bacteria from the *Peptococcaceae* family, in particular *Desulfosporosinus* sp. We
508 showed that environmental dynamics, which are often excluded from laboratory degradation
509 experiments, can affect *in situ* DCM transformation. While current knowledge of anaerobic
510 transformation pathways of DCM limits interpretation of DCM *in situ* biodegradation,
511 fundamental research considering dynamics of environmental conditions are needed to improve
512 bioremediation approaches at DCM contaminated sites in the future.

513

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524

525 **Associated content**

526 Supplementary data related to this article can be found at xxxxx.

527 **Author Contributions**

528 Maria L. Prieto: Investigation, Methodology, Data curation, Visualization, Formal analysis,
529 Writing - original draft, Writing - review & editing. Sylvain Weill: Conceptualization, Writing -
530 review & editing. Benjamin Belfort: Investigation, Methodology, Writing - review & editing.
531 Emilie E.L. Muller: Investigation, Writing - review & editing. Jérémy Masbou: Methodology,
532 Formal analysis, Writing - review & editing. François Lehmann: Methodology, Writing - review
533 & editing. Stéphane Vuilleumier: Conceptualization, Writing - review & editing. Gwenaël
534 Imfeld: Conceptualization, Formal analysis, Funding acquisition, Project administration,
535 Resources, Supervision, Writing - review & editing.

536

537 **Declaration of Competing Interests**

538 The authors declare that they have no known competing financial interests or personal
539 relationships that could have appeared to influence the work reported in this paper.

540

541

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