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1 **Reactive transport of micropollutants in laboratory aquifers**

2 **undergoing transient exposure periods**

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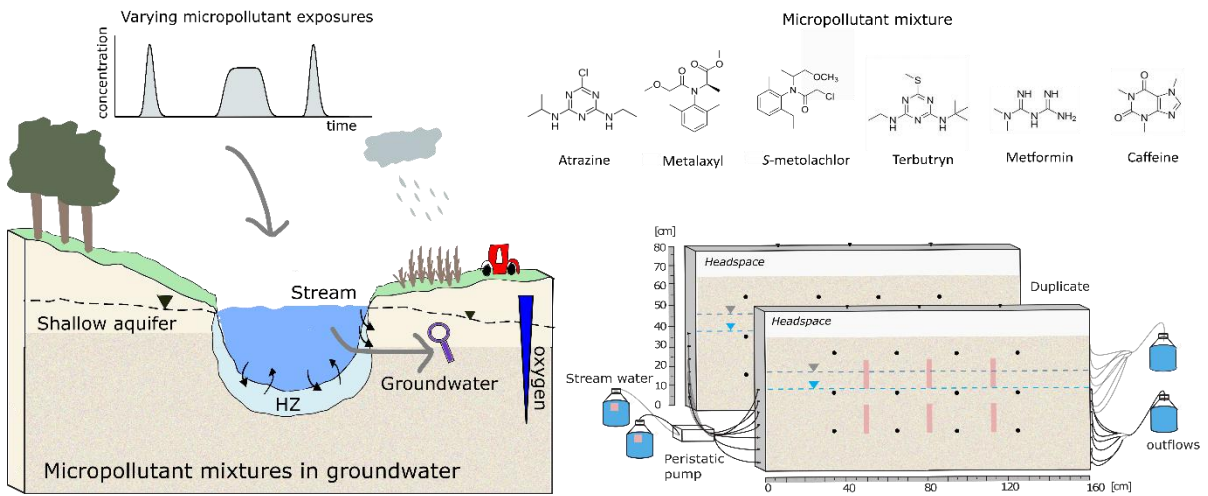
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19 **Highlights**

- 20 • Biotransformation of micropollutants in lateral stream-groundwater transition zones
- 21 • CSIA data and TPs evidenced caffeine degradation during transient exposure periods
- 22 • Model simulations highlight concentration dependency for caffeine biotransformation
- 23 • Adaptation of bacterial communities after acute and chronic exposure over 140 days
- 24 • Integrative approach for assessing micropollutant biotransformation during stream-
- 25 groundwater interactions

26

27 **Graphical abstract**



28
29

30 **Abstract**

31 Groundwater quality is of increasing concern due to the ubiquitous occurrence of
32 micropollutant mixtures. Stream-groundwater interactions near agricultural and urban areas
33 represent an important entry pathway of micropollutants into shallow aquifers. Here, we
34 evaluated the biotransformation of a micropollutant mixture (i.e., caffeine, metformin, atrazine,
35 terbutryn, *S*-metolachlor and metalaxyl) during lateral stream water flow to adjacent
36 groundwater. We used an integrative approach combining concentrations and transformation
37 products (TPs) of the micropollutants, compound-specific isotope analysis ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$),
38 sequencing of 16S rRNA gene amplicons and reactive transport modeling. Duplicate laboratory
39 aquifers (160 cm \times 80 cm \times 7 cm) were fed with stream water and subjected over 140 d to three
40 successive periods of micropollutant exposures as pulse-like (6000 $\mu\text{g L}^{-1}$) and constant (600 μg
41 L^{-1}) injections under steady-state conditions. Atrazine, terbutryn, *S*-metolachlor and metalaxyl
42 persisted in both aquifers during all periods (<10% attenuation). Metformin attenuation (up to
43 14%) was only observed from 90 d onwards, suggesting enhanced degradation over time. In
44 contrast, caffeine dissipated during all injection periods (>90%), agreeing with fast degradation
45 rates ($t_{1/2}$ <3 d) in parallel microcosm experiments and detection of TPs (theobromine and
46 xanthine). Significant stable carbon isotope fractionation ($\Delta\delta^{13}\text{C}\geq 6.6\text{‰}$) was observed for
47 caffeine in both aquifers, whereas no enrichment in ^{15}N occurred. A concentration dependence of
48 caffeine biotransformation in the aquifers was further suggested by model simulations following
49 Michaelis-Menten kinetics. Changes in bacterial community composition reflected long-term
50 bacterial adaptation to micropollutant exposures. Altogether, the use of an integrative approach
51 can help to understand the interplay of subsurface hydrochemistry, bacterial adaptations and
52 micropollutants biotransformation during stream-groundwater interactions.

53 **Keywords: micropollutants, biodegradation, CSIA, stream-groundwater interactions,**
54 **bacterial diversity, modeling**

55 **1. Introduction**

56 The ubiquitous release of micropollutants in the environment, typically in the ng L^{-1} to μg
57 L^{-1} range, may result in the deterioration of water quality, aquatic life and human health (Barbosa
58 et al., 2016; Schwarzenbach et al., 2010). Micropollutants cover a wide variety of substances,
59 including pesticides, pharmaceuticals and consumer products. Pesticides are extensively used in
60 agriculture to prevent and control harmful pests and crop yield losses worldwide (Damalas &
61 Koutroubas, 2016). Pharmaceuticals and consumer products, however, are ubiquitously released
62 into aquatic ecosystems, often from inefficient wastewater treatment facilities (Richardson &
63 Kimura, 2016). The frequent and continuous release of micropollutants and their transformation
64 products (TPs) results in their simultaneous presence as complex mixtures in surface water
65 (Bradley et al., 2021; Bradley et al., 2016; Hildebrandt et al., 2008) and groundwater (aus der
66 Beek et al., 2016; Baran et al., 2021; Loos et al., 2010; Masoner et al., 2019; Seiler et al., 1999).
67 However, the transformation of micropollutants in mixtures and their effects on non-target
68 microorganisms in shallow aquifers are still poorly understood.

69 Natural attenuation in shallow aquifers relies on the ability of microorganisms to break
70 down contaminants into less toxic chemicals (National Research Council, 2000). Micropollutants
71 attenuation results from various biotic and abiotic transformation processes involving biological
72 and chemical reactions, as well as non-degrading processes such as sorption or dilution (Fenner
73 et al., 2013). Biodegradation mainly contributes to the overall contaminant removal in aquifers
74 (Fenner et al., 2013; Meckenstock et al., 2015). However, current concentration measurements
75 are generally not sufficient to infer the contribution of biodegradation to the overall
76 micropollutant removal due to the simultaneous occurrence of both degradative and non-
77 degradative processes. In this context, compound-specific isotope analysis (CSIA) may be used

78 to evaluate micropollutant degradation by measuring changes of isotope ratios (e.g., $^{13}\text{C}/^{12}\text{C}$ or
79 $^{15}\text{N}/^{14}\text{N}$) in the remaining non-degraded molecules. Changes of isotope ratios are related to
80 kinetic isotope effects favoring the cleavage of molecules containing light isotopes compared to
81 heavy isotopes. This results in an isotope fractionation effect in which the remaining contaminant
82 is enriched in heavy isotopes (Elsner, 2010). The resulting change in isotope ratios may be
83 related to micropollutants degradation extent, mechanisms and pathways, although
84 micropollutant CSIA is still an emerging field (Elsner and Imfeld, 2016) in comparison with
85 other industrial pollutants (Ojeda et al., 2019).

86 In shallow aquifers, the hyporheic zone (HZ), i.e., the transition zone between surface
87 water and groundwater, is located beneath the stream bed and the two sides of the riparian zone
88 (Zhang et al., 2017). The HZ represents a natural biogeochemical barrier against groundwater
89 contamination (Boulton et al., 2010; Lewandowski et al., 2011). However, knowledge on the
90 interplay of dynamic hydrological and biogeochemical conditions with respect to pollutant
91 transformation in the HZ is currently limited. Recently, it has been suggested that HZs sustain
92 micropollutants biotransformation, particularly at the sediment-water interface (SWI) beneath the
93 stream bed (Droz et al., 2021; Mechelke et al., 2020; Peter et al., 2019; Schaper et al., 2018). The
94 reactivity of this zone is driven primarily by hydraulic residence times, surface-groundwater
95 exchange, mass transfer rates, nutrient turnover and bacterial diversity (Conant et al., 2019;
96 Drouin et al., 2021; Peter et al., 2019; Posselt et al., 2020). Because oxygen gradients are
97 established across the HZ, anoxic conditions are usually observed at the bottom of the SWI. This
98 may lower micropollutants degradation rates (Droz et al., 2021), and thus increase their potential
99 mobilization and accumulation in shallow groundwater (Hintze et al., 2020; Iker et al., 2010;
100 Jakobsen et al., 2019; Lesser et al., 2018; Loos et al., 2010). In conditions where the stream level

101 is higher than the adjacent groundwater, e.g., due to seasonal hydrological changes (Winter et al.,
102 1998), the losing streams may laterally flow into groundwater (Ghysels et al., 2021; Winter et al.,
103 1998), and further mobilize micropollutants into oxygen-rich zones in shallow aquifers (Chen &
104 Chen, 2003; Derx et al., 2010; Hancock, 2002). This lateral transition zone represents a yet
105 poorly understood mixing zone of stream-groundwater biogeochemical dynamics. In addition,
106 shallow aquifers in connection to streams are subjected to seasonal variations of micropollutants
107 inputs, resulting in acute and chronic exposure periods possibly affecting micropollutants
108 biotransformation.

109 Laboratory aquifers have proven advantageous to study the main drivers of
110 micropollutants biotransformation under controlled conditions, while considering the spatial and
111 temporal gradients formed across porous media (Bauer et al., 2009; Prieto-Espinoza et al., 2021;
112 Schürner et al., 2016; Sun et al., 2021a; Ye et al., 2015). Recently, the use of CSIA in laboratory
113 aquifers has shed light on micropollutant biotransformation, emphasizing the role of sorption and
114 degradation kinetics (Schürner et al., 2016), diffusion and transverse-dispersion (Sun et al.,
115 2021b), and mass-transfer limitations (Sun et al., 2021a). However, an integrative understanding
116 of the attenuation of micropollutant mixtures and the long-term change of groundwater bacterial
117 diversity in response to environmental perturbations at the lateral stream-groundwater interface
118 is yet required.

119 The purpose of this study was thus to examine the reactive transport of a micropollutant
120 mixture in laboratory aquifers continuously fed with stream water mimicking stream-
121 groundwater interactions dominated by lateral stream water flow to adjacent groundwater. The
122 micropollutant mixture was composed of the herbicides atrazine, terbutryn and *S*-metolachlor,
123 the fungicide metalaxyl, the consumer product caffeine, and the anti-diabetic drug metformin.

124 The selected micropollutants represent different classes of substances with described degradation
125 pathways and TPs, distinct half-lives and which may co-occur in surface water and groundwater
126 (Bradley et al., 2021; Masoner et al., 2019). Duplicate laboratory aquifers underwent a series of
127 consecutive pulse (acute) and constant (chronic) exposure periods (over 140 days). The pulse-
128 like and constant exposure periods represented factors affecting micropollutant trends, such as
129 (acute) hydrological events (e.g., flooding events, surface runoff) (Chow et al., 2020) and
130 (chronic) seasonal applications (Baran et al., 2021). We hypothesized that transient exposures to
131 micropollutants may affect bacterial communities and thus micropollutants degradation in
132 shallow groundwater adjacent to streams. Micropollutants and their TPs concentrations, C- and
133 N-CSIA, and DNA analysis (i.e., 16S rRNA amplicons) were performed to evaluate the
134 biotransformation of the micropollutant mixture. Concentrations and CSIA data were further
135 interpreted using a simple reactive transport model. In addition, parallel biotic and abiotic
136 microcosm experiments with stream water were conducted under oxic conditions to evaluate
137 degradation kinetics of the micropollutants.

138

139 **2. Materials and methods**

140 **2.1 Stream water collection**

141 Stream water was collected from the Souffel sub-catchment (3.6 km² out of 120 km² of
142 total catchment area) located 30 km northeast of Strasbourg (Bas-Rhin, France; 48°40'09.4"N
143 7°33'51.5"E). With conventional agriculture accounting for 80% of the land use, of which 12%
144 corresponds to urban areas and 8% forests, the Souffel catchment is categorized as severely
145 impacted by pesticide contamination. Corn and sugar beets are the main crops, receiving
146 pesticide applications as observed in nearby and similar agricultural catchments (Alvarez-

147 Zaldívar et al., 2018; Droz, 2020; Lefrancq et al., 2017). For this study, freshwater was collected
148 every two months from January to September 2020 in three sterile 50 L stainless steel containers,
149 which were stored at $18 \pm 1^\circ \text{C}$ until further use. Total organic carbon was $2.84 \pm 0.67 \text{ mg L}^{-1}$ (n
150 $= 5$) and pH was 7.1 ± 0.2 in the Souffel stream water. Background concentrations of
151 micropollutants were measured at the beginning of the sampling campaign, where only caffeine
152 (4.4 ng L^{-1}) and *S*-metolachlor (43 ng L^{-1}) were detected. For hydrochemical and bacterial
153 diversity analyses, the stream water was collected in sterile 1 L glass bottles, transported on ice
154 and stored immediately at $4 \pm 1^\circ \text{C}$ until further analyses.

155 **2.2 Micropollutants**

156 A detailed description of chemical reagents of the micropollutant mixture, corresponding
157 TPs, and their physicochemical properties is provided in the Supporting Information (SI, Section
158 A). The micropollutant mixture consisted of atrazine, caffeine, metalaxyl, *S*-metolachlor and
159 terbutryn purchased from Sigma-Aldrich (St: Louis, MO, USA; analytical grade purity: >99%).
160 A stock solution containing a mixture of these chemicals was prepared in acetonitrile (ACN) at 5
161 g L^{-1} and stored at -20°C . Metformin was purchased from Sigma-Aldrich (St: Louis, MO, USA;
162 analytical grade purity: >99%) and prepared in water at 5 g L^{-1} and stored at 4°C . In addition,
163 the following TPs were purchased: desethylatrazine, deisopropylatrazine, hydroxyatrazine,
164 theobromine, paraxanthine, xanthine, carboxylic acid metalaxyl, demethylmetalaxyl, metolachlor
165 ESA, metolachlor OXA, desethylterbutryn, desethyl-2-hydroxy-terbutryn, terbutryn-2-hydroxy,
166 1,3,5-triazine, and guanylurea. Stock solutions of the TPs were prepared in MeOH at 1 g L^{-1} and
167 stored at -20°C .

168 **2.3 Degradation kinetic experiments**

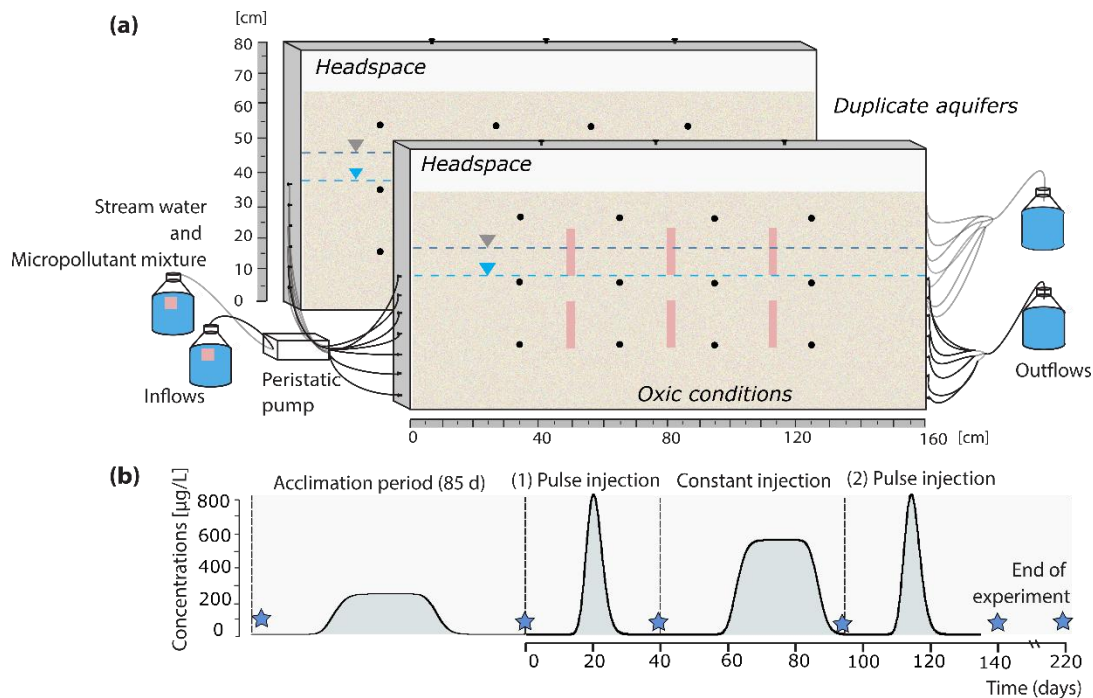
169 Biodegradation microcosm experiments were performed to examine micropollutant
170 attenuation in the stream water serving as inflow water of both laboratory aquifers. Biotic and
171 abiotic microcosms were conducted under oxic conditions in a sacrificial mode at room
172 temperature ($20 \pm 2^\circ\text{C}$) and in dark at natural pH (7.3 ± 0.4). Microcosms were prepared in 20
173 mL headspace vials crimped with butyl/PTFE caps (Interchim®) with a total volume of 15 mL
174 of stream water. Micropollutants were spiked to reach the final concentration of 6 mg L^{-1} per
175 compound, representing the maximum concentrations established at the inflow of both laboratory
176 aquifers. High micropollutant concentrations were needed to allow reliable C- and N-CSIA
177 (Droz et al., 2021). The solutions were stirred under sterile conditions until complete ACN
178 evaporation. To maintain oxic conditions ($4\text{--}9 \text{ ppm O}_2$), limit water loss and avoid microbial
179 contamination, a $0.2 \text{ }\mu\text{m}$ PTFE syringe filter (Rotilabo®) was mounted on a syringe tip, which
180 was stuck through the vial cap (Droz et al., 2021). All microcosms were kept under constant
181 shaking (orbital shaker at 120 rpm). Biotic experiments were prepared in triplicates and
182 sacrificed on days 0, 2, 5, 15, 50, 80, 100 and 200. Abiotic experiments (autoclaved triplicate
183 experiments) and blanks were sacrificed on days 0, 5, 50 and 200. A 2 mL aliquot was used for
184 hydrochemical analysis, while a 1 mL aliquot was collected to quantify metformin and TPs. The
185 remaining volume (12 mL) was used for micropollutants quantification and C- and N-CSIA
186 (i.e., $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$). Metformin CSIA was not performed due to its high polarity and low
187 volatility hampering GC-IRMS measurements.

188 **2.4 Experimental setup and operation of laboratory aquifers**

189 Experiments were conducted in two parallel laboratory aquifers with dimensions of 160
190 $\text{cm} \times 80 \text{ cm} \times 7 \text{ cm}$ (length \times height \times width) and filled with sterile quartz sand (grain size:
191 $0.4\text{--}0.6 \text{ mm}$; depth: 70 cm) (Figure 1). A detailed description of the experimental setup is

192 provided in the SI (Section B). Both aquifers were setup as duplicate and continuously fed in
 193 parallel with stream water at a rate of 1.15 L d^{-1} . The water table was positioned at 43 cm from
 194 the bottom. All injection ports from each aquifer were connected to 10 L reservoirs with
 195 continuously stirred inflow water (Figure 1). The inflow water remained oxygenated (8.6 mg L^{-1})
 196 and an atmospheric exchange from the top ports of the aquifers maintained oxic conditions
 197 throughout the experiments. The outflow reservoirs consisted of sterile 5 L bottles connected to
 198 all outflow sampling ports of each aquifer. Results from hydrochemistry, DNA, concentrations
 199 and CSIA thus represent depth-integrated water samples (i.e., mixing of all flow paths). The
 200 saturated zone (SZ, $z = 0\text{--}43 \text{ cm}$) was characterized by mean dissolved O_2 concentrations of 4.4
 201 $\pm 0.8 \text{ mg L}^{-1}$. Experiments were conducted in a temperature-controlled room at $18 \pm 1 \text{ }^\circ\text{C}$, and
 202 both aquifers were protected from light. The injection of a saline conservative tracer (NaCl ; 1 L
 203 solution at 1 g L^{-1}) prior to the experiments enabled to estimate the average pore water velocity
 204 at 0.08 m d^{-1} (SI, Section C).

205



206

207 **Figure 1.** Laboratory aquifers fed with stream water and exposed to a micropollutant mixture. (a)
208 Schematic overview of the laboratory aquifers. Stream-groundwater flows from left to right of
209 the aquifers. Water samples were retrieved from sampling ports (black dots) across the sand
210 compartment, and the outflow reservoirs (depth-integrated outflow bottles). Oxygen
211 concentrations were followed across the porous media (pink rectangles). Upper ports of the
212 aquifers, consisting in 0.2 μm PTFE syringe filter mounted on a Luer-Lock valve, remained open
213 to allow gas exchange with the atmosphere. (b) Aquifer operations, including an acclimation
214 period prior to the experiments, and subsequent periods of pulse and constant injection of
215 micropollutants. Dotted lines represent micropollutant injections and blue stars indicate sampling
216 for bacterial diversity analysis. Breakthrough curves of the micropollutants were monitored at
217 the outflow reservoirs.

218

219 After injection of the conservative tracer, the aquifers were fed with stream water for 60 d
220 (i.e., at least two pore water volumes) to ensure complete NaCl removal. An acclimation period
221 was then established by injecting a pulse of the micropollutant mixture (1 L solution at 6 mg L⁻¹)
222 for 85 d to support adaptation of the bacterial community and favor micropollutants
223 biodegradation (Poursat et al., 2019a; Prieto-Espinoza et al., 2021; Sun et al., 2021a). No
224 samples were collected during the acclimation period. For the experiment, three consecutive
225 injection periods were performed in each aquifer over 140 d (Figure 1), mimicking acute and
226 chronic micropollutants inputs driven by seasonal applications and hydrological events (Baran et
227 al., 2021; Chow et al., 2020). At the beginning of the experiment (i.e., day 0; Figure 1), a first
228 pulse injection of the micropollutant mixture (1 L at 6 mg L⁻¹ for each micropollutant) was
229 conducted. From days 40 to 61, the micropollutant mixture at 10 times lower concentrations (600
230 $\mu\text{g L}^{-1}$) was constantly injected. Finally, a second pulse injection (1 L at 6 mg L⁻¹) was performed
231 at day 93. Unspiked stream water fed the aquifers after each injection period (Figure 1), and from
232 140 to 220 d to flush out micropollutants prior to collection of core sand samples. To limit

- 233 micropollutants degradation in the inflow reservoirs during the constant injection phase, the
- 234 inflow reservoirs were replaced daily by freshly prepared spiked solutions.

235 **2.5 Sampling procedure**

236 For micropollutant concentration analysis, TPs and CSIA (i.e., $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), 1 L water
237 samples were collected from the outflow reservoirs (Figure 1), immediately filtered on 0.45 μm
238 NC membranes and stored at 4 °C until further analysis. Sampling resolution during the pulse
239 and constant injection periods was approximately every 24 h and 48 h, respectively. For TPs
240 analysis, a 1 mL water aliquot was used. Only samples from the second pulse injection (90-140
241 d) were screened due to evidence of several micropollutants degradation only after 90 d (see
242 below). For hydrochemical analysis, a 30 mL aliquot was collected every 5 d from the inflow
243 and outflow reservoirs. For DNA extraction, 200 mL were also collected from the inflow and
244 outflow reservoirs at the beginning of the acclimation period, and during the experimental period
245 at 0, 38, 90, 140 and 220 d (Figure 1). In addition, two depth-integrated pore water samples (10
246 mL) were collected from each aquifer at 45 cm and 105 cm from the inflows prior to the
247 acclimation period, and at 38, 140 and 220 d. Water samples were filtered through sterile 0.22
248 μm membrane filters (Swinnex holder, 13 mm, Millipore, Bedford, USA) and stored at $-20\text{ }^{\circ}\text{C}$
249 until DNA extraction. At the end of the experiments (220 d), four integrative sand cores were
250 retrieved from each aquifer for DNA analysis at 45 cm and 105 cm from the inflow, and at
251 depths of 15 cm and 45 cm. Each subsample core was thoroughly mixed. A subsample of 1 g of
252 sand was used for DNA extraction.

253 **2.6 Analytical methods**

254 **2.6.1 Hydrochemistry**

255 Oxygen (O_2) concentrations were monitored *in situ* by O_2 sensitive optode foils (PreSens
256 GmbH, Regensburg, Germany) located at the inlet reservoirs and across the aquifers (Figure 1).
257 Redox potential (Eh), pH and electrical conductivity were monitored using laboratory probes

258 (SCHOTT® Instruments). Major ions were measured by ion chromatography (Dionex ICS-5000,
259 Thermo Scientific, USA). Total organic carbon (TOC), dissolved organic carbon (DOC) and
260 dissolved inorganic carbon (DIC) were analyzed by a TOC analyzer (TOC-V-CPH Shimadzu,
261 NF EN 1484).

262 **2.6.2 Micropollutants extraction and quantification**

263 Solid phase extraction (SPE) of pesticides and caffeine was carried out using SolEx C18
264 cartridges (1 g, Dionex®, CA, USA) and an AutoTrace 280 SPE system (Dionex®, CA, USA),
265 following an in-house method described previously (Droz et al., 2021; Elsayed et al., 2014). A
266 detailed description of micropollutants extraction and quantification methods is provided in the
267 SI (Section D). All pesticides and caffeine were quantified by gas chromatography (GC, Trace
268 1300, Thermo Fisher Scientific) coupled with a mass spectrometer (MS, ISQ™, Thermo
269 Scientific). Metformin was quantified by liquid chromatography (UHPLC, Ultimate 3000,
270 Thermo Fisher Scientific) coupled with a tandem mass spectrometer (MS/MS, TSQ Quantiva,
271 Thermo Fisher Scientific).

272 **2.6.3 Compound-specific isotope analysis (CSIA)**

273 A detailed description of the analytical methods is provided in the SI (Section E). Stable
274 carbon and nitrogen isotope composition of pesticides and caffeine were determined by gas
275 chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS), with a gas
276 chromatograph (Trace 1310) coupled via a GC/Conflow IV interface to an isotope ratio mass
277 spectrometer (Delta V plus, Thermo Fisher Scientific). In-house standards were prepared daily
278 and analyzed prior to sample measurements. Carbon and nitrogen isotope ratios were reported in
279 δ notation as parts per thousand (‰) relative to the international reference material Vienna Pee
280 Dee-Belemnite (V-PDB) (Coplen et al., 2006) and air standards, respectively. The total

281 analytical uncertainty (1σ) of measurements was $\leq 0.5\%$ for $\delta^{13}\text{C}$ and $\leq 1\%$ for $\delta^{15}\text{N}$ values,
282 within the linearity range (6–300 ng for C and 40–300 ng for N) (Droz et al., 2021). The average
283 isotope value of the residual non-degraded fraction of the micropollutants was derived according
284 to the Rayleigh equation (Elsner, 2010):

$$285 \quad \ln\left(\frac{R_{t,E}}{R_{0,E}}\right) = \ln\left(\frac{C_{t,E}}{C_{0,E}}\right) \cdot \varepsilon_{bulk}^E \quad (1)$$

286 where $R_{t,E}/R_{0,E}$ is the isotope ratio of element “E” (i.e., $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$), and $C_{t,E}$ and $C_{0,E}$ are
287 the concentrations at a given time (t) and at the initial time (0), respectively. Carbon and nitrogen
288 isotopic composition were reported in delta notation ($\delta^h\text{E}$) following $\delta^h\text{E} = [(R_{\text{sample}}/R_{\text{standard}}) - 1]$
289 $\times 1000$ (Elsner, 2010). Apparent isotope fractionation values (ε_{bulk}^E , in ‰) were obtained by
290 least-squares linear regression without forcing the slope through the origin. The uncertainty
291 corresponds to the 95% confidence interval and the error was determined using ordinary linear
292 regression (Elsner et al., 2007).

293 **2.7 DNA extraction and sequencing**

294 For both pore water and sand core samples, DNA was extracted using the DNeasy Power
295 Water kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Extracted DNA
296 was quantified using Qubit fluorometric quantification with the Qubit dsDNA HS Assay kit
297 (Thermo Fischer Scientific, MA, USA). A detailed description of DNA sequencing is given in
298 the SI (Section F). Sequencing data from pore water and sand samples were deposited to the
299 ENA archive, BioProject accession number PRJEB52684. Multivariate statistical analysis of
300 relative observed taxonomical units (OTU) abundance was performed with R (Hellal et al.,
301 2021). Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of
302 log-transformed data was performed to visualize dissimilarities between bacterial taxa (Hellal et
303 al., 2021). Analysis of similarities (ANOSIM, $n=31$ samples) was used to infer statistical

304 differences between groups of community profiles (Torabi et al., 2020). Datasets obtained from
 305 Illumina sequencing were also used to compute α -diversity indices and for rarefaction analysis
 306 (SI, Section F). In total, 1,861,511 high-quality sequences were obtained ($n=31$ for pore water
 307 and $n=8$ for sand samples). Rarefaction curves of diversity indices reached asymptotes with
 308 increasing sequencing depth, indicating sufficient sequencing efforts to capture the biodiversity
 309 extent of bacterial communities in all samples (SI, Section F).

310 **2.8 Reactive transport modeling**

311 Carbon isotope fractionation was only considered for the model description because
 312 significant nitrogen isotope fractionation was not observed in this study (see Section 3.3). The
 313 model was simplified to a one-dimensional domain in accordance with the experimental setup
 314 mirroring transient exposure periods in a shallow aquifer (Figure 1). Oxygen transport was not
 315 considered due to a lack of temporal high-resolution oxygen data. The simple reactive transport
 316 model considering equilibrium sorption was described as (Eckert et al., 2013):

$$317 \quad {}^{12}R \frac{\partial c^{12}}{\partial t} + v \frac{\partial c^{12}}{\partial x} = D \frac{\partial^2 c^{12}}{\partial x^2} + r_{deg}^{12} \quad (2)$$

$$318 \quad {}^{13}R \frac{\partial c^{13}}{\partial t} + v \frac{\partial c^{13}}{\partial x} = D \frac{\partial^2 c^{13}}{\partial x^2} + r_{deg}^{13} \quad (3)$$

319 Here, $c_{tot}=c^{12}+c^{13}$ ($\mu\text{g L}^{-1}$) is the total concentration of the two most abundant
 320 micropollutant light (^{12}C) and heavy (^{13}C) isotopologues. R (-) is the retardation factor
 321 accounting for equilibrium sorption as $R = 1 + \frac{\rho_b}{n} K_d$, in which ρ_b (kg L^{-1}) is the dry bulk
 322 density of the sediment, n (-) is the porosity assumed as 0.37 (-), and K_d (L kg^{-1}) is the linear
 323 distribution coefficient between water and sediment phases of the isotopologues. v (m s^{-1}) is the
 324 average water velocity, and D ($\text{m}^2 \text{s}^{-1}$) is the hydrodynamic dispersion value. The degradation
 325 rates r_{deg} ($\mu\text{g L}^{-1}\text{s}^{-1}$) of the two most abundant light (^{12}C) and heavy (^{13}C) isotopologues,

326 assuming Michaelis-Menten kinetics (Eckert et al., 2013; Van Breukelen & Prommer, 2008), are
 327 given by:

$$328 \quad r_{deg}^{12} = -r_{max}^{12} \frac{c^{12}}{K_m^{12} \left(1 + \frac{c^{12}}{K_m^{12}} + \frac{c^{13}}{K_m^{13}} \right)} \quad (4)$$

$$329 \quad r_{deg}^{13} = -r_{max}^{13} \frac{c^{13} \cdot \alpha_B}{K_m^{13} \left(1 + \frac{c^{12}}{K_m^{12}} + \frac{c^{13}}{K_m^{13}} \right)} \quad (5)$$

330 where r_{max} is the maximum degradation rate and K_m is the half-saturation concentration ($\mu\text{g L}^{-1}$)
 331 of the light (^{12}C) and heavy (^{13}C) isotopologues. To compute degradation rates of the
 332 isotopologues, K_m is assumed to be identical for both isotopologues, as suggested previously
 333 (Eckert et al., 2013; Van Breukelen & Prommer, 2008). The isotope fractionation factor α_B (-) is
 334 thus defined as:

$$335 \quad \alpha_B = \frac{r_{deg}^{13}/c^{13}}{r_{deg}^{12}/c^{12}} \quad (6)$$

336 Isotope fractionation by transverse dispersion was not considered, since insignificant
 337 isotope effects by transverse dispersion have previously been shown for micropollutants reactive
 338 transport in flow-through laboratory aquifers (Schürner et al., 2016; Sun et al., 2021b). Although
 339 sorption is not expected to be significant in our experimental setup (clean quartz sand as the solid
 340 matrix), isotope fractionation affected by equilibrium sorption was modeled considering that
 341 light isotopes tend to sorb slightly more than heavier isotopes (i.e., $^{12}\text{K}_d > ^{13}\text{K}_d$) (Kopinke et al.,
 342 2005). Isotope fractionation affected by sorption is thus described as (Schürner et al., 2016):

$$343 \quad \alpha_{sorp} = \frac{^{13}R-1}{^{12}R-1} = \frac{^{13}K_d}{^{12}K_d} \quad (7)$$

344 The breakthrough curve (BTC) of the conservative tracer was fitted to retrieve the
 345 average velocity v and the longitudinal dispersion coefficient D . To avoid disturbing *endogenous*
 346 bacterial communities of the injected stream water during transient micropollutant exposures, a

347 conservative tracer could not be injected during the experiments. Conservative transport
348 according to transport parameters of the NaCl tracer was thus simulated for all injection periods
349 and compared to the micropollutant BTCs. All model results are presented for the depth-
350 integrated outflow reservoirs ($x = 160$ cm). The fitted transport parameters, the fitting procedure
351 and a description of the modeled scenarios are provided in the SI (Section G). Briefly, the
352 coupled system of one-dimensional reactive-transport equations is solved in a fully implicit and
353 fully coupled mode using a cell-centered Finite Volume Method combining a Lax-Wendroff 2nd
354 order scheme of the advective term for spatial discretization. All model parameters are assumed
355 to be spatially uniform. Finally, isotope fractionation values associated with micropollutants
356 biotransformation (ε_B , ‰) and sorption (ε_{sorp} , ‰) were calculated as $\varepsilon = (\alpha - 1) \times 1000$.

357

358 **3. Results and discussion**

359 **3.1 Kinetics of micropollutants biotransformation in stream water microcosms**

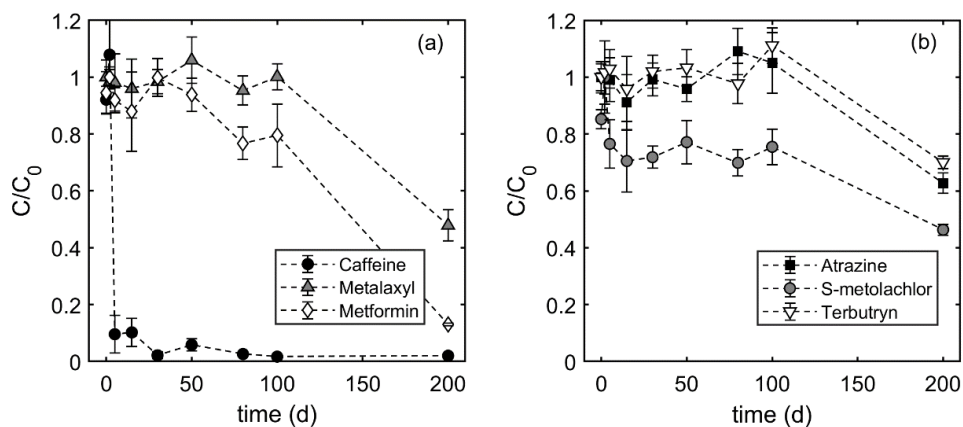
360 Microcosm experiments confirmed contrasted biotransformation potential of the selected
361 micropollutants in the Souffel stream water. In biotic experiments under oxic conditions, a
362 gradual decrease of nitrate concentrations suggested co-occurrence of nitrate reduction after 50 d
363 (SI, Section H). The hydrochemical conditions did not change significantly under abiotic
364 conditions (SI, Section H). Under abiotic conditions, less than 10% of the initial mass dissipated
365 after 200 d, corresponding to analytical uncertainties. This indicates insignificant abiotic
366 reactions, such as hydrolysis, and that biodegradation prevailed under biotic conditions.

367 Under biotic conditions, caffeine rapidly dissipated ($t_{1/2} = 3$ d; Figure 2), agreeing with
368 previous studies showing rapid half-lives of caffeine ($t_{1/2} < 4$ d) in river water microcosms (Lam
369 et al., 2004; Nödler et al., 2014). Accordingly, caffeine $\delta^{13}\text{C}$ values increased from -31.4 ± 0.1 ‰

370 up to $-26.6 \pm 0.2\text{‰}$ (SI, Section I). The estimated ϵ_c value of caffeine of $-1.1 \pm 0.3 \text{‰}$ agreed
371 with previous observations in a SWI batch experiment where slight but significant fractionation
372 ($\Delta\delta^{13}\text{C} > 2\text{‰}$) occurred after $>90\%$ of caffeine degradation ($\epsilon_c = -0.7 \pm 0.1 \text{‰}$) (Droz, 2020).
373 However, no changes in $\delta^{15}\text{N}$ values were observed. Caffeine biodegradation via the *N*-
374 demethylation pathway has been suggested as the most common caffeine biodegradation
375 pathway in at least 80% of the reported isolates characterized worldwide (Summers et al., 2015),
376 with a sequential transformation of caffeine to theobromine to xanthine (Dash & Gummadi,
377 2007; Yu et al., 2009). The detection of theobromine and xanthine in the biotic stream water
378 microcosms further supported caffeine biodegradation via the *N*-demethylation pathway (SI,
379 Section H).

380 Contrastingly, *S*-metolachlor dissipated by 20% in the first 5 d, while overall attenuation
381 was up to 60% after 200 d ($t_{1/2} = 187$ d; Figure 2). This agrees with reported half-lives of *S*-
382 metolachlor ranging from 56 to 182 d in agricultural soils and SWI microcosms (Drouin et al.,
383 2021; Torabi et al., 2020). Metformin and metalaxyl started dissipating after 50 d and 100 d with
384 estimated half-lives of 189 d and 147 d, respectively (Figure 2). Previous studies reported half-
385 lives of metalaxyl ranging from 9 to 127 d in agricultural soils (Buerge et al., 2003), while
386 metformin has been classified as a not readily biodegradable substance with half-lives ranging
387 from 28 to 98 d in activated sludge (Straub et al., 2019). On the other hand, terbutryn and
388 atrazine dissipated to up to 30% and 38% of their initial mass at 200 d, respectively (Figure 2).
389 Half-lives of atrazine and terbutryn, assuming pseudo-first order degradation, were estimated as
390 225 d and 290 d, respectively, agreeing with observed long half-lives for terbutryn ($t_{1/2} = 177$ -
391 349 d) in groundwater microcosms under oxic conditions (Talja et al., 2008).

392 Carbon and nitrogen isotope fractionation of atrazine, terbutryn, metalaxyl and *S*-
 393 metolachlor were not observed after 200 d, confirming limited biodegradation (SI, Section I).
 394 However, TPs formation confirmed that biodegradation was the main attenuation process (SI,
 395 Section H). Overall, our results suggest the occurrence of a lag-phase of 50 d before slow
 396 biotransformation of metformin, and of 100 d for atrazine, terbutryn, and metalaxyl in stream
 397 water microcosms (Figure 2). Such lag-phase has also been observed for atrazine and
 398 metolachlor degradation in biotic groundwater microcosms (Cavalier et al., 1991; Schwab et al.,
 399 2006), and may be attributed to early stages of bacterial adaptation to chemical exposure
 400 (Poursat et al., 2019a), and to the effect of chemical mixtures on bacterial growth (Reardon et al.,
 401 2002). However, the degradation kinetics of the selected micropollutants in the stream water
 402 microcosms were similar to those previously reported individually and at lower concentration
 403 ranges (e.g., Cavalier et al., 1991). Hence, the degradation kinetics in the stream water
 404 microcosms may be related to the physicochemical properties of each micropollutant (SI, Section
 405 A), where the faster kinetics were observed for the most soluble compounds.



406
 407 Figure 2. Biodegradation of micropollutants in biotic (oxic) stream water microcosms. (a)
 408 Caffeine, metalaxyl and metformin, and (b) atrazine, *S*-metolachlor and terbutryn. Error bars
 409 represent the total uncertainty of measurements ($n=3$).

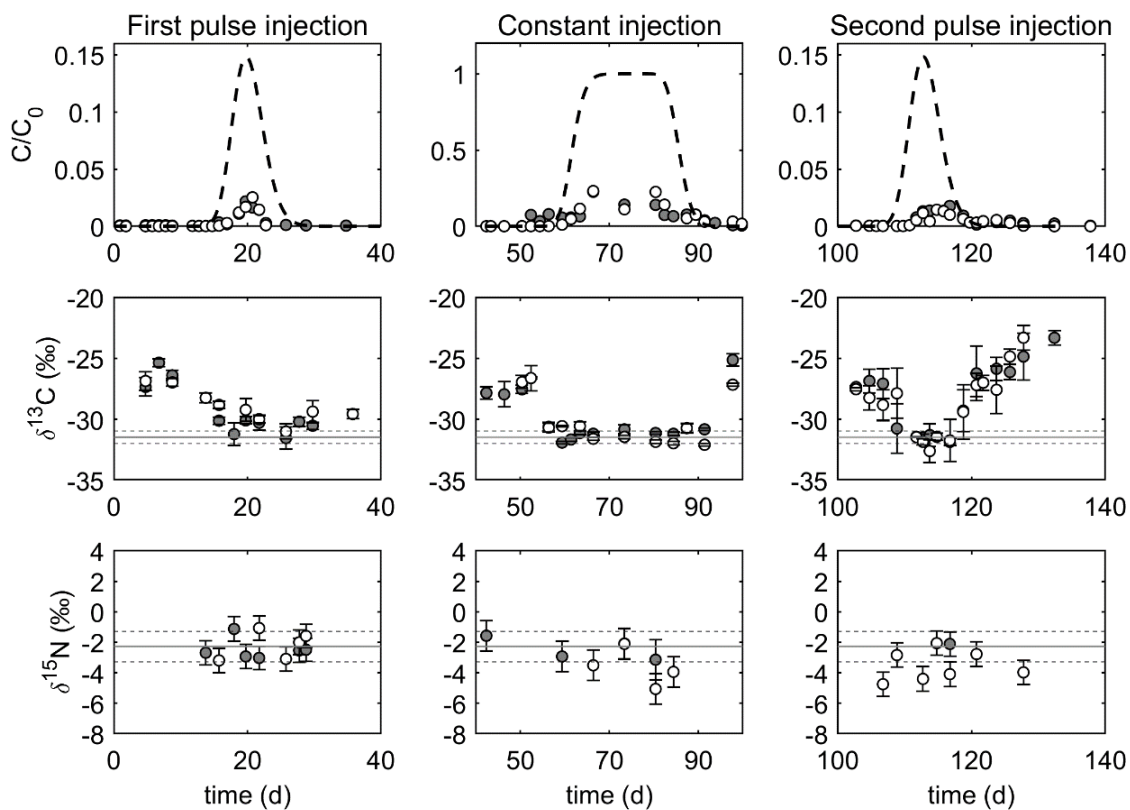
410

411 **3.2 Micropollutants biotransformation in flow-through laboratory aquifers**

412 The dynamics of hydrochemical conditions and micropollutant concentrations in both
413 aquifers were similar, supporting the reproducibility of the duplicate laboratory aquifers. At the
414 end of the 85-days acclimation period, background concentrations of micropollutants ranged
415 from 1 to 10 $\mu\text{g L}^{-1}$ at the outflows. Overall, hydrochemical conditions in both aquifers remained
416 constant throughout the experiment (0–140 d) (SI, Section J). Oxic conditions (mean dissolved
417 O_2 concentration: $4.4 \pm 0.8 \text{ mg L}^{-1}$) prevailed in both aquifers over time and across the depth of
418 the SZ. However, nitrate concentrations decreased at end of the constant and second pulse
419 injection periods (from 70-90 d and 130-140 d, respectively), while ammonium concentrations
420 increased, suggesting nitrate reduction in the aquifers (SI, Section H). This suggests the
421 formation of sub-anoxic micro-zones near the aquifer outlets, e.g., due to the formation of
422 biomass hot spots or sand clogging, resulting in faster oxygen depletion in these zones. However,
423 oxygen consumption during micropollutants BTCs could not be evidenced due to a lack of
424 temporal high-resolution oxygen data (SI, Section H), while nitrate-reducing bacteria in the
425 aquifers were not observed (SI, Section O).

426 Mass recoveries of each micropollutant are provided in SI (Section K). *Caffeine*
427 biotransformation in the laboratory aquifers occurred during all examined injection periods
428 (0–140 d), and readily dissipated compared to the other micropollutants (Figure 3, upper panel).
429 This is in agreement with the fast caffeine biodegradation kinetics in the stream water
430 microcosms (Figure 2) and previous lysimeter experiments (Koroša et al., 2020). Caffeine
431 biodegradation was the main attenuation process as abiotic transformation was insignificant in
432 the stream water microcosms (SI, Sections H and I) and sorption is expected to be insignificant
433 due to low organic content ($<0.01\%$) in the quartz sand matrix of the aquifers. Caffeine

434 biodegradation in the aquifers was also supported by the detection of caffeine TPs, i.e.,
 435 theobromine (up to 1460 $\mu\text{g L}^{-1}$) and xanthine (up to 328 $\mu\text{g L}^{-1}$), during the second injection
 436 period with similar retentions times as the parent compound (SI, Section L). These high TP
 437 concentrations suggest that further caffeine mineralization did not occur in the aquifers compared
 438 to the stream water microcosms (SI, Section H), likely due to shorter retention times. Overall,
 439 fast caffeine attenuation may be associated with microorganisms adapted to constant and long
 440 periods of caffeine inputs into surface waters (Summers et al., 2015). At least 71 bacterial strains
 441 from many different habitats worldwide are involved in caffeine biodegradation, possibly
 442 sustained by ancient metabolic pathways for methylxanthines (Summers et al., 2015; Vega et al.,
 443 2021).



444
 445 Figure 3. Caffeine concentrations (upper panel) and carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope
 446 ratios (middle and lower panels) during the transient exposures of micropollutants. Full and

447 empty symbols represent data sets from the duplicate aquifers. Dashed back lines represent
448 modeled BTCs of the saline conservative tracer (upper panel). Error bars correspond to the total
449 analytical uncertainty, which incorporates both accuracy and reproducibility of $n=3$
450 measurements. Grey horizontal lines (middle and lower panels) indicate the reference isotope
451 signatures of caffeine standards measured by EA-IRMS for $\delta^{13}\text{C}$ ($-31.40 \pm 0.02 \text{ ‰}$) and $\delta^{15}\text{N}$
452 ($-2.28 \pm 0.06 \text{ ‰}$). Dashed horizontal lines (middle and lower panels) represent the total
453 uncertainty of carbon $\pm 0.5 \text{ ‰}$ and nitrogen $\pm 1 \text{ ‰}$ isotope analysis.

454

455 *Metformin* attenuation in the laboratory aquifers increased over time. During the first
456 pulse injection (0–40 d), the BTC of metformin and the conservative tracer were similar (SI,
457 Section K), and no significant metformin attenuation was observed during the constant injection
458 period (40–90 d). In contrast, during the second pulse injection (90–140 d), only 82 ± 5 % of the
459 initial mass of metformin was recovered (SI, Section K). This indicates that metformin
460 biodegradation in the aquifers occurred mostly from 90 to 140 d. Metformin biodegradation is
461 also supported by results of the stream water microcosms (Figure 2) and the detection of its main
462 TP guanylurea in the aquifers (Poursat et al., 2019b) (SI, Section L). Overall, an increase in
463 metformin attenuation over time suggests a gradual adaptation or an increase of active bacterial
464 degraders following the acclimation and subsequent periods (from 0 to 90 d) of metformin
465 exposure. A similar bacterial adaptation has been suggested previously in chemostat systems,
466 where a 2-month pre-exposure period enhanced metformin degradation, correlating with an
467 increase in bacterial community diversity, composition and activity (Dalmijn et al., 2021).

468 Contrastingly, *S-metolachlor* showed similar BTCs as those of the conservative tracer
469 throughout the whole experiment (SI, Section K), suggesting limited *S-metolachlor*
470 biodegradation in the aquifers. The limited *S-metolachlor* biodegradation mirrored the slow *S*-
471 metolachlor degradation kinetics after 100 days in the stream water microcosms (Figure 2). This
472 contradicts the idea that *S-metolachlor* degraders in the inflow stream water may be already
473 adapted to long periods of *S-metolachlor* applications in the field (Lefrancq et al., 2017).
474 However, a previous study of oxic groundwater microcosms amended with a herbicide mixture
475 (up to $5 \mu\text{g L}^{-1}$) consisting of alachlor, metolachlor and propanil showed that metolachlor
476 persisted during the first 12 months, while significant (up to 46 %) biodegradation occurred only
477 after 18 months (Cavalier et al., 1991). Minimal *S-metolachlor* degradation was also observed

478 during the early stage (first 20 y) of a groundwater plume ($1\text{--}820\ \mu\text{g L}^{-1}$) in an anaerobic
479 fractured dolostone aquifer near an agrochemical facility (Parker et al., 2019). The persistence of
480 *S*-metolachlor in the laboratory aquifers thus indicates that long-term groundwater contamination
481 by *S*-metolachlor may occur, even under oxic conditions during stream-groundwater interactions.

482 Similarly, *metalaxyl*, *atrazine* and *terbutryn* proved persistent in both aquifers. *Metalaxyl*
483 attenuation was low to insignificant during all injection periods (SI, Section K). Mass recoveries
484 were $93 \pm 4\%$ during both pulse injections (0–40 d and 90–40 d), while during the constant
485 injection period (40–90 d) metalaxyl concentrations followed those of atrazine and terbutryn (SI,
486 Section K). *Atrazine* and *terbutryn* also showed similar BTCs as the saline conservative tracer
487 (SI, Section K), indicating insignificant degradation or sorption over time. Atrazine is a highly
488 persistent substance and its accumulation in shallow groundwater has been proven even after
489 decades of its ban in 1992 in Germany (Vonberg et al., 2014). Moreover, the persistence of the
490 urban biocide terbutryn in both laboratory aquifers also suggests its accumulation in shallow
491 groundwater from entry pathways such as leaching of facades and urban stormwater infiltration
492 systems (Hensen et al., 2018).

493 Overall, the absence of significant biodegradation of metalaxyl, atrazine and terbutryn in
494 both laboratory aquifers agrees with observations from the stream water microcosms, where
495 biotransformation only occurred after 100 d (Figure 2). However, detection of TPs of metalaxyl
496 (demethylmetalaxyl), atrazine (deisopropylatrazine) and terbutryn (terbutryn sulfoxide, 2-
497 hydroxy-terbutryn and desethyl-terbutryn) suggested that the low attenuation (<10%) of these
498 micropollutants in the aquifers from 90 to 140 days was mainly associated with biodegradation
499 (SI, Section L).

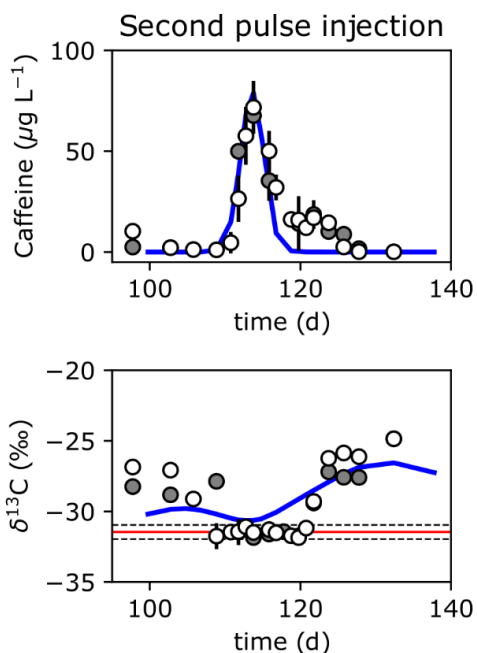
500 3.3 C and N isotope fractionation of micropollutants

501 The insignificant stable isotope fractionation ($\Delta\delta^{13}\text{C}$ and $\Delta\delta^{15}\text{N} < 2\text{‰}$) for atrazine,
502 terbutryn, metalaxyl and *S*-metolachlor confirmed their limited biodegradation in both aquifers
503 (SI, Section K). However, $\delta^{13}\text{C}$ of caffeine featured a U-shaped trend during each injection
504 period (Figure 3, middle panel), in particular during the second pulse injection (90–140 d). At
505 low concentrations ($< 20\ \mu\text{g L}^{-1}$), i.e., at the front and tail of the BTCs, significant enrichment in
506 ^{13}C of the non-degraded caffeine indicated the occurrence of caffeine biodegradation. In contrast,
507 at peak concentrations, $\delta^{13}\text{C}$ values were similar to the source caffeine signature ($\delta^{13}\text{C}_0 = -31.4 \pm$
508 0.1‰) although significant mass removal occurred (Figure 3, upper panel).

509 A concentration dependence following Michaelis-Menten kinetics has been associated
510 with the U-shaped of $\delta^{13}\text{C}$ values (Eckert et al., 2013; Schürner et al., 2016). This suggests that
511 first-order kinetics may rather occur at low concentrations followed by a transition to zero-order
512 kinetics at peak concentrations. The Michaelis-Menten type kinetics has thus been associated
513 with an increase of the stable isotope fractionation at low pollutant concentrations, e.g., at a
514 contaminant plume fringe (van Breukelen and Prommer, 2008). This agrees with the U-shaped
515 $\delta^{13}\text{C}$ values observed for caffeine BTCs (Figure 3, middle panel). A similar stable isotope pattern
516 was observed in a toluene pulse injection in a flow-through aquifer setup filled with natural
517 sediment and groundwater (Eckert et al., 2013; Qiu et al., 2013). In the case of toluene in
518 organic-rich sediment, the combination of both biodegradation and sorption resulted in U-shaped
519 $\delta^{13}\text{C}$ trends slightly skewed toward the tail of the BTC (Qiu et al., 2013). This was interpreted as
520 an effect of pollutant sorption to organic matter, which may be slightly stronger for pollutants
521 with light isotopes than for those with heavy isotopes, leading to faster breakthrough of the
522 heavy isotopologues (i.e., $^{12}\text{K}_d > ^{13}\text{K}_d$ and $^{14}\text{K}_d > ^{15}\text{K}_d$) (Kopinke et al., 2005). This would result

523 in an enrichment in ^{13}C at the contaminant front, while the BTC of ^{12}C isotopologues is expected
524 to cause a counteracting effect of the ^{13}C enriched signatures at the tail of the contaminant BTC
525 (Eckert et al., 2013).

526 Model simulations were performed to elucidate the U-shaped trend of $\delta^{13}\text{C}$ values
527 observed during caffeine biotransformation. Results from simulations of all injected periods are
528 presented in Section G of the SI. Overall, the simple RTM was able to capture the U-shaped
529 trend of $\delta^{13}\text{C}$ values. A good model fitting was observed for both caffeine BTC and $\delta^{13}\text{C}$ values,
530 particularly for the pulse-like injection periods (Figure 4 and SI, Section G). For the constant
531 injection period (40-90 days), however, concentrations and $\delta^{13}\text{C}$ values were difficult to predict
532 (SI, Section G). This could be explained by the scattered concentration data observed between
533 80-90 days, possibly due to clogging effects altering the flow paths, and the sensitivity towards
534 the biokinetic parameter for fitting the BTC fringes (Eckert et al., 2013). Nevertheless, similar
535 $\epsilon_{c,\text{bio}}$ values were estimated in all the injection periods ranging from -0.32 to -0.40 ± 0.10 ‰ and
536 are close to the ϵ_c value reported for caffeine degradation in an oxic SWI microcosm ($\epsilon_c = -0.7 \pm$
537 0.1 ‰) (Droz et al., 2021). The computed $\epsilon_{c,\text{bio}}$ values for caffeine biotransformation in the
538 laboratory aquifers may also be indicative of distinct competing degradation pathways (van
539 Breukelen, 2007) compared to the stream water microcosms, giving the wide range of bacteria
540 co-occurring in the laboratory aquifers (see below). Finally, the effect of sorption on the U-
541 shaped trend of $\delta^{13}\text{C}$ values was negligible (for all periods $\epsilon_{c,\text{sorb}} < 0.06$ ‰), confirming
542 insignificant caffeine sorption in the aquifers (Figures 3 and 4), in agreement with the absence of
543 significant shifts to higher retention times.



544

545 Figure 4. Simulated caffeine BTC (top) and carbon isotope signatures ($\delta^{13}\text{C}$; bottom) during the
 546 second pulse injection (93–140 d). Blue lines represent simulated reactive transport of caffeine.
 547 Grey and empty symbols represent datasets from both laboratory aquifers. Error bars correspond
 548 to the total analytical uncertainty accounting both accuracy and reproducibility of measurements
 549 ($n=3$). The red horizontal line (bottom) represents caffeine EA values of carbon ($-31.46 \pm$
 550 0.02‰) isotopes. Dashed horizontal lines (bottom) represent the total uncertainty of carbon \pm
 551 0.5‰ isotope analysis.

552

553 Overall, model simulations confirmed that the U-shaped $\delta^{13}\text{C}$ trend was associated with a
 554 concentration dependence upon caffeine biodegradation. This suggests that modeling Michaelis-
 555 Menten kinetics could realistically represent degradation behavior of micropollutants. The fitting
 556 of the caffeine BTC and $\delta^{13}\text{C}$ values was highly dependent on the biokinetic parameters, i.e., the
 557 r_{max} and K_m , resulting in significant stable isotope fractionation at the BTC fringes at low
 558 caffeine concentrations (Figure 4). A simple model formulation can thus result in good model
 559 predictions based on concentrations and stable isotopic data only. Nevertheless, Eckert et al.

560 (2013) pointed out that a trade-off between goodness of fit of both concentrations and stable
561 isotopes should be considered, limiting a unique and reliable set of biokinetic parameters. Since
562 biokinetic parameters are linked to the occurrence and evolution of bacterial degraders, an
563 explicit formulation of the evolution of their biomass may ultimately improve model predictions.
564 Moreover, modeling the reactive transport of micropollutant mixtures should also account for the
565 inhibition of maximum degradation rates due to potential toxicity effects and competition among
566 potential degraders. Further, the U-shaped trend of $\delta^{13}\text{C}$ values during micropollutant
567 biodegradation in aquifers highlights the need for appropriate monitoring strategies to capture
568 isotopic shifts in field scenarios. This would require an understanding of stream-groundwater
569 flow interactions to predict micropollutants loadings in shallow aquifers (Hintze et al., 2020) and
570 capture micropollutants BTC.

571

572 **3.4 Gradual bacterial adaptations during transient micropollutants exposure**

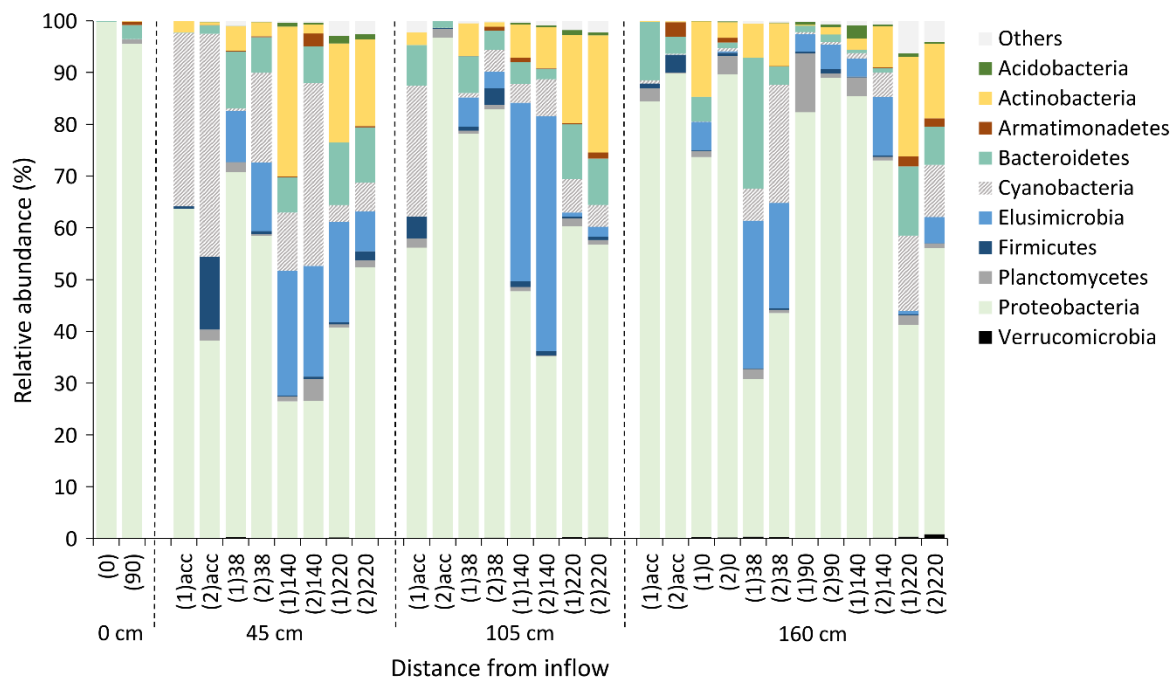
573 A gradual increase in bacterial diversity was observed in both aquifers over time and
574 according to the transient exposure periods. NMDS ordination of the relative OTUs abundance
575 showed that bacterial diversity mainly varied with the exposure periods (SI, Section M). A first
576 shift in the bacterial diversity occurred from 0 to 38 days, following the acclimation period and
577 the first pulse injection of micropollutants (i.e., acute exposure at 6 mg L^{-1}). This may be due to
578 the pre-exposure of bacteria to micropollutants, possibly leading to a series of enzyme induction
579 processes triggering pollutant biotransformation in the aquifers (Poursat et al., 2019b). This
580 degradation potential within the aquifers was also demonstrated for caffeine degradation during
581 the first injection period (Figure 2 and SI, Section L).

582 A second change in the bacterial diversity occurred from 90 to 140 d, following the
583 constant and second pulse injection periods, respectively. This indicates the development of a

584 distinct bacterial community diversity following the chronic exposure at $600 \mu\text{g L}^{-1}$, which may
585 coincide with a selective pressure of the micropollutant mixture for 21 consecutive days (SI,
586 Section M). Hence, a long-term exposure to the micropollutants may drive bacterial adaptation in
587 aquifers, possibly inducing changes at the community level or individual cells, e.g., by increasing
588 competitive strains (Kovářová-Kovar & Egli, 1998; Murínová & Dercová, 2014). Interestingly, a
589 slight shift of the bacterial diversity occurred at 220 d, while no micropollutant exposure
590 occurred from 140 to 220 d. This suggests the establishment of a well-adapted bacterial
591 community (SI, Section M), which was also observed in the sand cores at the end of the
592 experiments (SI, Section N). This idea is supported by the gradual increase in species richness
593 from 0 to 220 d, as emphasized by the increase in α -diversity (SI, Section M).

594 Accordingly, the bacterial community composition also varied over time. Differences
595 among the duplicate aquifers were observed in pore water samples, particularly within the
596 aquifers, possibly due to integrated pore water samples at different depth distances. However, the
597 trends in bacterial dynamics in both aquifers were consistent over time (Figure 5). Proteobacteria
598 dominated in pore water samples during all exposure periods (Figure 5). However, the change in
599 bacterial community composition (phylum level) after the first pulse injection (0–40 d) likely
600 corresponds to a first community response, which may be associated with the micropollutant
601 inflow in both aquifers. The abundance of Actinobacteria, Bacteroidetes and Elusimicrobia
602 increased to up to 25%, 8% and 45%, respectively, after the constant and the second pulse
603 injection periods (90 to 140 d) (Figure 5). At the end of the experiments (at 220 d),
604 Actinobacteria phylum remained as the second most abundant in pore water samples (up to
605 22%). This is in agreement with a previous study showing an increase in Actinobacteria and
606 associated genera in sediments of the HZ mimicked in river-simulating flumes (Posselt et al.,

607 2020). Although the bacterial diversity did not vary spatially over distance from inflow (SI,
 608 Section M), the bacterial community composition near the inflow (x=45 cm; Figure 5) differed
 609 from that at the outflow (x=160 cm). This is likely due to the vicinity of the source zone of
 610 micropollutants and thus higher exposure to micropollutants at the aquifer inflow (Figure 5).
 611 This was also observed for the attached bacteria retrieved from sand cores at the end of the
 612 experiments (220 d; SI, Section N), supporting the development of adapted bacterial
 613 communities even after the cease of transient micropollutant exposures.



614
 615 Figure 5. Relative abundance of bacterial phyla in pore water samples during transient exposures
 616 to the micropollutant mixture in the laboratory aquifers. Labels in brackets represent samples
 617 collected from the duplicate laboratory aquifers (1 and 2). Pore water samples were collected
 618 prior to the experiments (acc; acclimation period); after acclimation period (0 d), at the end of
 619 the first pulse injection (38 d), constant injection (90 d), and second injection periods (140 d).
 620 Samples were collected in the aquifer at 45 cm and 105 cm from the inflow, and from the outlet
 621 (x = 160 cm). 220 d correspond to the end of the experiment prior to core sand sampling.
 622

623 The phylum Actinobacteria has been associated with pesticide biodegradation and
624 proposed for pollutant cleanup applications (Mawang et al., 2021). For instance, *Arthrobacter* sp.
625 and *Rhodococcus* sp. have been described for atrazine biodegradation in agricultural soils
626 (Sagarkar et al., 2014) and inoculated chemostats (Ehrl et al., 2019; Kundu et al., 2019).
627 Although atrazine biodegradation was not significant (<5%) in both aquifers, the increase in
628 Actinobacteria suggests a gradual increase of competent bacteria degraders in the aquifers. This
629 gradual increase in competent bacteria coincides with the lag-phase (up to 100 d) observed for
630 the degradation of most micropollutants in the stream water microcosm experiments (Figure 2).
631 Hence, bacterial tolerance and adaptation to long-term micropollutant exposure may be
632 established in both aquifers. Although caffeine degradation may be associated with
633 *Pseudomonas* sp. (Summers et al., 2015), *Pseudomonas* taxa accounted for <3% of the total
634 sequences (SI, Section O). This indicates a wide array of yet unexplored taxa potentially
635 involved in micropollutant degradation, notably for caffeine and metformin, urging for
636 fundamental studies on the identification of active micropollutant degraders and their metabolic
637 pathways.

638

639 **4. Conclusion**

640 The ubiquitous release of diverse micropollutant mixtures, from urban and/or agricultural
641 sources, into surface and groundwater has urged for improved environmental studies
642 representing more realistic field conditions. Current research issues in the field of micropollutant
643 transformation in polluted shallow aquifers include (i) how the contribution of degradative
644 processes can be evaluated, and (ii) what are the factors controlling micropollutant
645 biotransformation. Integrative studies such as ours, based on high resolution sampling from

646 laboratory aquifers mimicking stream water interactions with adjacent groundwater, may help to
647 address these issues and enable more robust and long-term assessment of *in situ* transformation
648 of micropollutants under transient exposure periods associated with seasonal applications and
649 hydrological dynamics across stream-groundwater interfaces.

650 Specifically, our study highlighted that biotransformation of micropollutants as chemical
651 mixtures largely varies, independently on the acute or chronic level of exposure to
652 micropollutants. For instance, caffeine biotransformation, evaluated with carbon and nitrogen
653 CSIA and the TPs formation, was observed during all micropollutants injection periods. In
654 contrast, only moderate or negligible biotransformation of metformin, atrazine, terbutryn, *S*-
655 metolachlor and metalaxyl occurred in the laboratory aquifers, indicating their persistence. CSIA
656 of micropollutants can reinforce the evaluation of degradation or persistence of micropollutants
657 in groundwater and identify ‘hot and cold periods’ of *in situ* biotransformation for assessing
658 natural attenuation at the stream-groundwater interface. The integration of biomolecular markers
659 such as 16S rRNA enabled to examine long-term acclimation and adaptation of bacterial
660 communities and may be used as indicators of micropollutant biotransformation in groundwater.
661 Taken together, our results highlight the relevance of laboratory aquifers fed with natural stream
662 water and integrating DNA- and isotopic analyses to study and improve the assessment of
663 emerging pollutants in shallow groundwater connected to streams.

664

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683

684 **Declaration of Competing Interests**

685 The authors declare that they have no known competing financial interests or personal
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