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2 **Bacterio-plankton transformation of diazepam and 2-amino-5-**

3 **chlorobenzophenone in river waters**

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27 **ABSTRACT**

28 Benzodiazepines are a large class of commonly-prescribed drugs used to treat a variety of
29 clinical disorders. They have been shown to produce ecological effects at environmental
30 concentrations, making understanding their fate in aquatic environments very important. In
31 this study, uptake and biotransformations by riverine bacterio-plankton of the
32 benzodiazepine, diazepam, and 2-amino-5-chlorobenzophenone, ACB (a photo-degradation
33 product of diazepam and several other benzodiazepines), were investigated using batch
34 microcosm incubations. These were conducted using water and bacterio-plankton populations
35 from contrasting river catchments (Tamar and Mersey, UK), both in the presence and absence
36 of a peptide, added as an alternative organic substrate. Incubations lasted 21 days, reflecting
37 the expected water residence time in the catchments. In River Tamar water, 36 % of
38 diazepam was removed when the peptide was absent. In contrast, there was no removal of
39 diazepam when the peptide was added, although the peptide itself was consumed. For ACB,
40 61 % was removed in the absence of the peptide, and 84 % in its presence ($p < 0.001$ in both
41 cases). In River Mersey water, diazepam removal did not occur in the presence or absence of
42 the peptide, with the latter again consumed, while ACB removal decreased from 44 to 22 %
43 with the peptide present. This suggests that bacterio-plankton from the Mersey water
44 degraded the peptide in preference to both diazepam and ACB. Biotransformation products
45 were not detected in any of the samples analysed but a significant increase in ammonium
46 concentration ($p < 0.038$) was measured in incubations with ACB, confirming mineralization
47 of the amine substituent. Sequential inoculation and incubation of Mersey and Tamar
48 microcosms, for 5 periods of 21 days each, did not produce any evidence of increased ability
49 of the microbial community to remove ACB, suggesting that an indigenous consortium was
50 probably responsible for its metabolism. As ACB degradation was consistent, we propose
51 that the aquatic photo-degradation of diazepam to ACB, followed by mineralization of ACB,

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52 is a primary removal pathway for these emerging contaminants. As ACB is photo-produced
53 by several benzodiazepines, this pathway should be relevant for the removal of other
54 benzodiazepines that enter the freshwater environment.

55 Keywords: diazepam, 2-amino-5-chlorobenzophenone, bacteria, benzodiazepines,
56 benzophenones, river, ESI-MS

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58 ## Introduction

59 Contamination of aquatic systems by human and veterinary pharmaceuticals now
60 appears to be extensive. However, there is a significant lack of knowledge of their aquatic
61 transport and fate, and effects on non-target organisms[1-3]. The benzodiazepines (Fig. 1) are a
62 group of widely-prescribed anxiolytic/sedative pharmaceuticals with both human and
63 veterinary applications[4]. Of the 35 compounds in this group, diazepam (Fig. 1) is the second
64 most frequently prescribed[5] and is included in the World Health Organisation Essential Drugs
65 List[6]. Diazepam is metabolized in the human body to oxazepam, temazepam and
66 nordiazepam (Fig. 1), all of which are pharmacologically-active.

67 Diazepam and its metabolites are primarily excreted in urine, either in the free form or
68 as sulphate and glucuronide conjugates; between 5 and 50 % of the administered dose of
69 diazepam is excreted[7]. Once in the wastewater stream, the glucuronide may be deconjugated[4].
70 Of the 118 pharmaceuticals examined in urban wastewaters from four continents, diazepam
71 was observed to have one of the highest mean and maximum concentrations in influent
72 wastewaters (22 – 23 $\mu g\ L^{-1}$)[8]. During conventional sewage treatment, generally ≥ 80 % of
73 diazepam in the influent stream is lost to surface waters via the sewage works effluent[8,9]. As a
74 result it has been detected in surface waters of Europe, the USA, Asia and Australia[10-13].
75 Diazepam has been ranked as a high risk compound with respect to aquatic organisms[8], while
76 ambient concentrations of its metabolite, oxazepam, can markedly alter the behaviour and
77 feeding of the wild European perch *Perca fluviatilis*[14]. Thus, it appears that inputs of
78 benzodiazepines to surface waters can have ecological and evolutionary consequences.

79 Within the pH range for surface waters (5-9), dissolved diazepam is a neutral
80 molecule[7]. It is stable with respect to chemical hydrolysis, and with sediment : water partition
81 coefficients < 100 $L\ kg^{-1}$ for both organic–rich (sewage solids) and organic–poor particles[7,15],

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82 little sorption (< 0.1 %) of the compound will occur at suspended sediment concentrations
83 typical of low–turbidity rivers. Diazepam photo-degrades in water[5,16], yielding a range of
84 products, including the water-soluble 2-amino-5-chlorobenzophenone (ACB; Fig. 1), a
85 substituted benzophenone which appears relatively resistant to further photo-degradation[16].
86 As the photolysis half-life for diazepam under environmentally-relevant conditions ranges
87 from 16 to168 h[5,16], conversion of diazepam to ACB may be an important abiotic removal
88 process for diazepam in sunlit surface waters. However, hydroxylated benzophenones have
89 been shown to exhibit estrogenic activity and their presence in surface waters has been
90 reported[17,18], while concentrations of up to 130 ng L^{-1} of benzophenone have been detected
91 in Korean rivers receiving wastewater effluent[19].

92 There appear to be no published toxicity data for diazepam metabolites and
93 transformation products, including ACB[9]. Biotic (bacterio-plankton) transformation studies
94 of diazepam have largely focussed on the role of sewage treatment[7,9]; surface water studies
95 are much rarer. In a microcosm set up to simulate aerobic and anaerobic transformations in
96 aquatic sediment systems, less than 2 % of the 0.35 μmole diazepam added was
97 biotransformed within the 100 days of the experiment[15].

98 The observed or potential effects of benzodiazepines and benzophenones make the
99 understanding of their fate in aquatic environments very important[9]. The aim of the present
100 work was to investigate the biotic transformation of two representative compounds from
101 these groups (diazepam and ACB) by natural, riverine bacterio-plankton communities using a
102 specifically designed experimental protocol[20]. Incubations were undertaken in laboratory
103 batch microcosms in the presence and absence of a readily degradable organic substrate that
104 could act as a priming agent for xenobiotic removal[21,22]. Concentrations of the parent
105 compounds were measured and the presence of metabolites investigated after an incubation
106 period that reflected typical residence times for surface waters in these catchments. Finally, to

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107 investigate the effect of the presence of ACB on bacterio-plankton community structure and
108 the ability of species present to metabolise ACB, sequential inoculation and incubation of
109 Mersey and Tamar microcosms, for 5 periods of 21 days each, were undertaken.
110

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111 ## Materials and Methods

112 The rationale for the design, testing and validation of the incubation procedure, as well as full
113 experimental details, are provided in Tappin et al.[20].

114 ### Study areas

115 The River Tamar (SW England, UK) drains a rural, agriculture-dominated, catchment of 928
116 km^2 and has a mean flow of 22.5 $m^3 s^{-1}$ at its tidal limit at Gunnislake. In contrast to the
117 Tamar, the tidal limit on the River Mersey (Howley Weir, Warrington, NW England, UK) is
118 the drainage end-point of a highly urbanised region of ca. 2000 km^2 (mean flow 37.5 $m^3 s^{-1}$).
119 The River Mersey was once severely polluted, but remedial measures undertaken during the
120 last three decades have significantly improved water quality. Table 1 provides a synopsis of
121 chemical data for these rivers at their tidal limits for the period 2008–2010, together with data
122 covering the times when sampling took place. Table 1 indicates that both were low turbidity
123 systems (i.e. suspended particulate matter concentration < 15 mg L^{-1}) and that the Mersey had
124 lower concentrations of dissolved oxygen, and higher concentrations of nitrate, ammonium,
125 ortho-phosphate and dissolved organic carbon, relative to the Tamar.

126 ### Incubation experiments

127 Chemicals: Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-
128 one, AR grade, Sigma-Aldrich, UK), 2-amino-5-chlorobenzophenone (ACB; AR grade,
129 Sigma-Aldrich, UK) and a tripeptide comprising glycine, leucine and tyrosine residues (≥ 98
130 % purity, Sigma-Aldrich, UK) were used for the study.

131

132 Preparation of incubation water: A bulk freshwater sample was collected from the tidal limit
133 of the Tamar and Mersey rivers. The water was filtered (GF/F; 0.7 µm nominal pore size) to

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134 emove suspended particles, passed through a strong anion-exchange resin (Dowex® X-100,
135 200 mesh; water flow rate 80 mL h^{-1}) to remove nitrate, UV-irradiated (400 W medium
136 pressure Hg lamp, 6 h) to remove dissolved organic matter (DOM) and then re-filtered
137 through a 0.2 μm filter membrane (Whatman Anodisc 47, aluminium oxide) to remove any
138 remaining particulate matter. These processes reduced nitrate and DOC concentrations to <
139 15 μM and < 60 μM, respectively, ensuring that competitive carbon and nitrogen substrates
140 were, as far as possible, removed and that the river water matrix was compatible with direct
141 analysis of samples by electrospray ionisation–mass spectrometry (ESI-MS). Finally, the
142 water was sterilised by autoclaving (115 ^{o}C, 15 min). All incubation samples and standards
143 for the experiment were then matrix-matched using this water.

144 Preparation of bacterial inoculum: Bacterio-plankton concentrations were measured in water
145 samples (10^5-10^6 cells per mL) to ensure that the prepared bacterial inoculum was
146 representative. The bacterial inoculum was prepared using water from the same sampling
147 sites, collected within 24 h of the start of the incubations. This water was filtered through a
148 1.6 μm pore size membrane (combusted GF/A) to remove any particles[23] and then re-filtered
149 through a 0.2 μm pore diameter membrane filter (Whatman Anodisc 47). The bacterio-
150 plankton retained on the membrane was resuspended in a small volume of the 0.2 μm filtered
151 water to provide the inoculum, which was then added to the prepared incubation water to
152 produce a final, representative bacterio-plankton concentration. Water for the inocula was
153 collected on 17 March and 19 April 2009 (Tamar) and 8 and 22 February 2010 (Mersey).

154 Incubation experiments: Incubation water (60 mL) was transferred to a 125 mL screw-capped
155 amber glass bottle to which was added 15–22 μL of stock diazepam or ACB solution and 1
156 mL of the bacterial inoculum. Starting concentrations of the compounds were approximately
157 30 μM. Pre-incubation Microtox® assays using the bacterium *Vibrio fischeri* showed that

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158 diazepam and ACB were non-toxic at these concentrations (EC_{50} >> 100 µM, 15 min
159 exposure). In a separate set of incubations, the effect of labile DOM on the biotransformation
160 of diazepam and ACB was tested by adding the tripeptide (equivalent to 90 µmol N L^{-1} and
161 510 µmol C L^{-1}) alongside diazepam and ACB. Control incubations of prepared river water
162 containing bacterial inoculum only and diazepam/ACB only were also prepared to account
163 for sorption effects. Ortho-phosphate was added to all incubations to give ca. 1-2 µM P at t =
164 0.

165 The bottles were loosely-capped, placed in a re-sealable plastic bag and transferred to
166 an orbital shaker. Incubations were performed in duplicate at ambient temperature in the
167 dark. An incubation time of 21 days was selected as a reasonable approximation of the river
168 water transit time in the Tamar and Mersey catchments. At day 0 and day 21, incubated
169 samples were filtered (combusted GF/F) and sub-samples collected for subsequent analyses
170 and stored frozen until required.

171 Based on the data from the incubations, an experiment was designed to select for
172 ACB-responsive bacteria, using the methods described above, except that they were
173 performed in triplicate and there was no addition of GLY. At the end of the initial 21 day
174 incubation period, 1 mL was used to inoculate a fresh microcosm that was then incubated for
175 21 days. This was sequentially repeated and after the fifth and final 21 day incubation,
176 samples were collected for analysis by ESI-MS. The water used for the inocula in these
177 experiments was collected on 11 May 2010 from the tidal limit of both the Tamar and Mersey
178 rivers.

179

180 **Chemical and microbiological analysis**

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181 Analyses by ESI-MS were performed in positive mode using a Finnigan MAT LCQ MS, a
182 quadrupole ion trap mass spectrometer with an external source atmospheric pressure interface
183 capable of electro-spray ionisation. The sample matrix was 50 : 50 methanol : water amended
184 with 0.1 % (v/v) formic acid and solutions were introduced by low-flow infusion at a rate of
185 3 μL min^{-1}. Once thawed, each sample was diluted 1:1 with the mixed methanol and formic
186 acid solution. Samples were then injected into the instrument. The signal sensitivity for both
187 diazepam and ACB, in positive-ion mode, was optimised by adjustment of instrumental
188 parameters using in-built tuning procedures. Ion count integration was performed for 2
189 minutes, with 5 replicates recorded per sample, while ion count stability was recorded in real
190 time using single ion monitoring. Quantification of each analyte was achieved by generating
191 an external calibration curve using matrix-matched standards on each analytical day, and
192 bracketing individual samples with a drift matrix-matched calibration standard to account for
193 variations in instrumental sensitivity; the variation was then calculated using an algorithm[20].
194 The mass spectra for both diazepam and ACB contained two isotopic peaks (due to ^{35}Cl and
195 ^{37}Cl atoms). Base peaks (attributed to $[M+H]^+$) for diazepam and ACB occurred at *m/z* 285
196 and *m/z* 232, respectively; a single peak for tripeptide occurred at *m/z* 352. Nitrate+nitrite
197 and ortho-phosphate were determined by segmented flow and spectrophotometric detection[24]
198 and ammonium by o-phthaldialdehyde fluorescence[25]. Viable counts of bacterio-plankton
199 were undertaken using 100 μL aliquots from the microcosms. These were diluted in
200 phosphate-buffered saline solution and 100 μL of each dilution spread on half strength Luria
201 Bertani agar (Merck, Germany) and incubated at 30 ^{o}C for two days. Colonies were
202 enumerated as colony forming units (cfu) mL^{-1} of the original suspensions. Total counts of
203 bacterio-plankton were determined microscopically by staining water samples with DAPI[26].
204 The microcosms contained bacterio-plankton populations of 10^5-10^6 cells per mL at both 0
205 and 21 days; approximately 10 % were recoverable as viable colonies on nutrient agar

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206 plates. DNA extraction followed[27]. Each microcosm water sample was membrane filtered
207 (0.2 µm pore diameter) and the retained cells disrupted on the filter by mechanical bead
208 beating. The DNA was extracted into hexadecyltrimethylammonium bromide and phenol-
209 chloroform-isoamyl alcohol, and then resuspended in 50 µL nuclease-free water. Nested PCR
210 amplifications were performed on extracted samples using Super Taq DNA polymerase and
211 G-Storm thermal cyclers. DNA amplification was undertaken in a 50 µL sample using 1 µM
212 of the universal primers for eubacterial 16S rRNA genes (27_f and 1492_r)[28] with 1 unit *super*
213 Taq DNA polymerase. The amplified DNA fragments were re-amplified using forward
214 primer 341 and reverse primer 907[29].

215 Denaturing gradient gel electrophoresis (DGGE) analysis[30] was performed on GC-
216 clamped products of the second PCR amplification using the Bio-Rad D-code system to
217 separate DNA on a 8 % polyacrylamide gel in Tris acetate EDTA buffer (pH 8.0) with a 20 -
218 60 % denaturant gradient, in which 100 % denaturant was 7 M urea amended with 40 %
219 formamide. Electrophoresis was performed at 60 ^{o}C, run at 60 V (16 h) and the DNA banding
220 visualised using Sybr Green I stain with detection and image capture on a Storm 860
221 Molecular Imager. Amplified eubacterial 16S ribosomal gene DNA was pooled from
222 duplicate microcosms and cloned into *E. coli* using the PGEM vector system (Promega)
223 according to the manufacturer's instructions. Based on the data collected from the initial 21-
224 day incubations, clones (50-70) were selected at random from Tamar and Mersey water
225 microcosms incubated for 0 and 21 days in the presence and absence of ca. 30 µM ACB. The
226 clones were sequenced by GATC (Germany) and preliminary identification assigned using
227 the Ribosomal Database Project[31].

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228 ## Results and Discussion

229 Removal of both substrates was observed in at least one of the incubations, suggesting that
230 the concentration at which they were added did not affect the ability of the bacterio-plankton
231 community to utilise them[32].

232

233 **Diazepam**

234 In Tamar waters after 21 days, the concentration of diazepam added (30 µM, 1.8 µmole total)
235 was unchanged in both the abiotic control (29.8 ± 3.8 µM, mean ± 1σ, n = 6-10; t-test, p =
236 0.93) and the biotic incubation containing diazepam and peptide (28.8 ± 4.4 µM, $p = 0.56$). In
237 contrast, the mean concentration had decreased by 36 %, to 18.5 ± 2.9 µM, over 21 days ($p <$
238 0.001) in the biotic incubation containing diazepam only (Fig. 2a). Given the limited extent
239 of partitioning to the solid phase reported for diazepam[7], and the very low solid particulate
240 material (SPM) concentrations in the incubations (< 1 mg L^{-1}), the decrease in the dissolved
241 concentration was almost certainly due to active uptake by the bacterio-plankton, as opposed
242 to simple abiotic sorption to cell surface components. In the peptide-amended experiment, the
243 peptide was consumed by the bacteria, via ammonification, leading to an increase in
244 concentrations of ammonium from 0.8 µM at the beginning of the incubation to 42.1 µM at
245 the end (Fig. 2b). As this form of DOM is readily utilised by the riverine bacterial
246 community,[20,33] the data suggest that the degradation of this alternative carbon/nitrogen
247 source is preferred over assimilation of diazepam.

248 The University of Minnesota Biocatalysis and Biodegradation Database (UMBBD,
249 http://umbbd.msi.umn.edu/index.html) was used to select peaks of interest in the mass
250 spectra, based on predicted biotransformation products of diazepam. The UMBBD
251 predictions are most reliable when the compound is the predominant source of C or N.

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252 Prediction to the second tier of biotransformation indicated that up to 5 chemical species may
253 be produced, including nordiazepam and three benzophenones (SI Fig. 1 and SI Table 1). N
254 atoms were retained throughout, meaning that each molecule should be observed in positive
255 mode ESI-MS. However, none of the predicted products were detected (Fig. 3) suggesting
256 that, if biotransformation products were produced, they were not released into solution, but
257 were further metabolised rapidly, or were present at concentrations below the limit of
258 detection under these conditions (< 0.05 μM and < 0.9 μM for diazepam and ACB,
259 respectively). Transformation products have been reported for diazepam, including
260 nordiazepam[34]. However, these data were acquired in sludge-seeded bioreactors at an SPM of
261 3 g L^{-1} and, interestingly, little degradation of diazepam (< 10 %) was observed over the 16
262 days duration of that experiment[34].

263 In the Mersey water microcosms, 26.0 ± 2.9 μM diazepam was added. After 21 days,
264 concentrations of diazepam in the abiotic and both biotic incubations had not changed
265 significantly (t-test, p range 0.06 – 0.86; Fig. 2a). The tripeptide was again consumed when
266 added as an additional substrate (Fig. 2b). As the bacterio-plankton of an urban river might be
267 expected to be responsive, having probably encountered the molecule previously, the absence
268 of diazepam removal was surprising, particularly as River Tamar microcosms were able to
269 effect significant removal of the diazepam (Fig. 2). A contrast in the removal of another
270 xenobiotic, atrazine, was also observed in a previous study for incubations using bacterial
271 populations from the same rivers[20]. There, 11 % removal over 21 days was observed in
272 Tamar samples, when atrazine was the only substrate added, contrasting with 0 % removal in
273 Mersey samples. However, addition of tripeptide increased removal from Mersey water from
274 0 to 37 %, while the Tamar removal value remained at 11 %. There are very few studies on
275 the bacterio-plankton compositions of unconnected rivers. In the Santa Ana River basin
276 (USA), urban impacted and rural, agriculturally impacted streams contained bacterio-

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277 plankton communities that showed few differences[35], suggesting that bacterial response to
278 added xenobiotics might be similar. The bacterio-plankton populations in the incubations
279 were prepared to give a final concentration that matched *in situ* measurements at the time of
280 collection[20], so the contrast in the removal of diazepam between Tamar and Mersey waters
281 reflects inoculum composition rather than cell numbers.

282 **2-amino-5-chlorobenzophenone**

283 The ACB was biodegraded to a much greater extent than diazepam, probably because,
284 as a primary aromatic amine, it contains nitrogen that is more accessible to enzyme attack,
285 relative to the amide and imine nitrogen in the diazepam molecule (Fig. 1). Although removal
286 occurred in all incubations, it was significantly greater in the rural River Tamar than in the
287 urban-influenced River Mersey microcosms (Fig. 4a). For the incubations with Tamar water,
288 there was no significant difference in the concentration of ACB (27.0 ± 2.2 μM) in the abiotic
289 control after 21 days (mean ± 1σ, n = 6-10; p = 0.76). In the presence of bacteria there was a
290 61 % decrease in concentration by day 21 ($p < 0.001$), while ACB in the tripeptide-amended
291 incubation, decreased by 84 % ($p < 0.001$), with concomitant disappearance of the peptide.
292 After 21 days in Mersey water, the concentration of ACB added (30.0 ± 2.7 μM) was
293 unchanged in the abiotic control relative to t = 0 (p = 0.18), while concentrations had
294 decreased by 44 % in the presence of bacterio-plankton ($p < 0.001$) and by 22 % in the
295 presence of both bacterio-plankton and peptide ($p < 0.001$). The loss of ACB from solution in
296 the Tamar bacterio-plankton only incubations was accompanied by a significant increase in
297 concentrations of dissolved ammonium from 1.1±0.1 to 4.7±1.5 μM ($p < 0.038$), while there
298 was also an increase from 1.9±0.7 μM to 11.1 μM in one of the Mersey replicates (Fig. 4b).
299 This pattern is consistent with the hydrolytic de-amination of the primary aromatic amine as
300 predicted by the UMBBD (SI Figure 2 and SI Table 1). Concurrent reductions in ortho-
301 phosphate and, in three out of four cases, nitrate+nitrite were observed (t–test, all $p < 0.001$;

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302 Fig. 4c, d). In summary, the removal of ACB occurred in all microcosms and was more
303 extensive in the Tamar microcosms. The presence of the peptide substrate enhanced ACB
304 removal in the Tamar microcosms but not for the Mersey. The addition of amino acids has
305 been shown to stimulate the biotransformation of phenols by a natural microbial lacustrine
306 community[36]. The UMBBD gave two theoretical degradation pathways for ACB, and
307 prediction to the second tier of biotransformation showed that of the 9 chemical species
308 potentially produced, three retained the N atom, including one hydroxylated benzophenone
309 (SI Fig. 2). However, as for the diazepam experiments, predicted ACB biotransformation
310 products were not detected in solution (Fig. 5).

311

312 **Effect of ACB on bacterio-plankton community structure**

313 The DGGE profiles of the amplified eubacterial 16S rRNA genes did not exhibit reproducible
314 differences that could be equated with the presence of ACB. The taxonomic composition of
315 the microcosm communities was therefore examined by sequencing clone libraries (50-70
316 clones each) from pooled microcosms (Fig. 6). Although 10-40 % of sequences could not be
317 classified, all microcosms contained representatives of a range of bacterial genera, including
318 those from the α-proteobacteria, β-proteobacteria and Firmicutes groups previously reported
319 as occurring in freshwaters[35,37,38]. Similarities in the composition of the starting bacterio-
320 plankton compositions in the two river waters, and their subsequent influence on xenobiotic
321 removal, are difficult to ascertain from these data (cf. section 3.2.1); however, members of
322 the genera represented in Fig. 6 are capable of degrading xenobiotics[39,40].

323 In the experiment where microcosms were sequentially sub-cultured through five
324 passages, ACB removal over the 21 day period of the final incubation set was 26 % and 44 %
325 for the Tamar and Mersey, respectively, demonstrating the complete absence of the selection

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326 of a bacterial population acclimated for ACB degradation. It is our contention, therefore, that
327 at the low concentrations of ACB, or its benzodiazepine precursors, which enter surface
328 waters of urban or rural catchments[16], the xenobiotic is assimilated without significantly
329 impacting the structure of the indigenous riverine microbial community.

330 **Environmental implications**

331 As a result of this study and previous work, the photo-degradation of diazepam and
332 complete biotransformation (mineralization) of its photo-degradation product, ACB, is
333 proposed as a realistic removal pathway for these emerging contaminants in aquatic systems.
334 Photo-degradation of diazepam to ACB has been demonstrated under environmentally-
335 realistic surface water conditions, suggesting that bacterio-plankton within a riverine
336 consortium have the capacity to remove and mineralize ACB entering surface waters or
337 formed *in-situ* through photo-chemical transformation of diazepam. As ACB is a persistent
338 photo-degradation product of several 1,4-benzodiazepines, photo-chemical -
339 biotransformation coupling may be an important removal pathway in surface waters for this
340 group of molecules. It is noteworthy that the enhanced removal of ACB in the presence of
341 tripeptide, a source of labile dissolved organic matter, in the Tamar incubations, supports
342 recent hypotheses of a priming effect for DOM biodegradation in both fresh and oceanic
343 waters[21,22].

344 A schematic representation of how diazepam could be transported across the river-
345 estuary continuum to reach coastal waters is proposed in Fig. 7. Bacterio-plankton removal of
346 diazepam could occur if background labile DOM concentrations are low (i.e. absence of
347 competitive substrates). If physical conditions facilitate photodegradation (direct and/or
348 indirect)[16] of diazepam to ACB, then the ACB will be mineralized by bacterio-plankton.
349 However in turbid rivers and estuaries, photo-degradation to ACB could be inhibited, leading

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350 to the advection of diazepam to low turbidity coastal waters and its subsequent photo-
351 degradation to ACB in sunlit surface layers. This pathway may also be applicable to other
352 pharmacologically-active 1,4-benzodiazepine molecules known to degrade to ACB (e.g.
353 oxazepam, temazepam and nordiazepam), which would be significant given the reported
354 ecological effects on freshwater fish exposed to environmental concentrations of oxazepam[14].

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355 ## Conclusions

356 The biotransformation of some human and veterinary pharmaceuticals has previously
357 been reported, usually during wastewater treatment or in surface waters dominated by
358 wastewater effluent. It is only very recently (5-10 years) that studies using laboratory
359 incubations or *in situ* measurements have revealed the potential for xenobiotic
360 transformations under conditions relevant to natural surface waters. Furthermore, while
361 coupled abiotic-biotic degradation pathways for some pharmaceuticals have been proposed,
362 the current study is one of the few to provide a conceptual transformation model for surface
363 waters based on experimental data. From this, and other studies, it is clear that some human
364 and veterinary pharmaceuticals, including benzodiazepenes and their metabolites, are
365 significantly degraded on the same timescales as hydraulic residence times of surface waters
366 in small to medium sized catchments. More refractory molecules, including diazepam it
367 would appear, may transfer to estuaries and coastal waters where their fate and effects are
368 currently unknown. Global manufacture and usage of the benzodiazepine group of drugs is
369 unlikely to decrease in the near future, and given the recent evidence of the effects of
370 oxazepam on fish behaviour, further systematic research into the transport, fate and
371 ecotoxicological effects of benzodiazepenes and benzophenones in the aquatic environment is
372 recommended.

373

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381 studies were undertaken in an ISO 9000:2001 accredited laboratory. Finally, we are grateful
382 to two reviewers for their thoughtful and incisive comments on the manuscript, which has
383 been considerably improved as a result.

384

385 **Notes and references**

386 Electronic Supplementary Information available

387 SI Table 1. Predicted pathways for the biotransformation of diazepam and
388 2-amino-5-chlorobenzophenone (ACB) in aerobic systems, including the probability of
389 degradation by named pathways and details of the mechanisms and enzymes involved. The
390 predicted products are shown in SI Figures 1 and 2.
391
392 SI Fig. 1 The University of Minnesota Biocatalysis and Biodegradation Database
393 (UMBDD) prediction pathways (to tier 2) of the aerobic bacterial biotransformation of
394 diazepam. The benzophenone units are ringed. The 'btxxxx' annotation refers to the specific
395 enzymic reaction mechanisms stored in the UMBDD database, which are listed in
396 SI Table 1.
397
398 SI Fig. 2 UMBDD prediction pathways (to tier 2) of the aerobic bacterial
399 biotransformation of ACB. The 'btxxxx' annotation refers to the specific enzymic reaction
400 mechanisms stored in the UMBDD database, which are listed in SI Table 1.
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Table 1. River water flow and physico-chemical characteristics close to sample collection points, together with water quality data for 2008 – 2010.

	Sampling date of bacterial inoculum	Daily mean flow ($m^3 s^{-1}$)	Suspended particulate matter ($mg L^{-1}$)	Dissolved oxygen (% sat.)	Dissolved oxygen ($mg L^{-1}$)	Nitrate ($\mu mol L^{-1}$ N)	Ortho-phosphate ($\mu mol L^{-1}$ P)	Ammonium ($\mu mol L^{-1}$ N)	Dissolved organic carbon ($\mu mol L^{-1}$)
Tamar									
ACB[a]	17 March 2009	13.9[b]	< 3.0[c]	101[c]	11.7[c]	181[c]	1.20[c]	< 2.1[c]	146[c]
Diazepam	19 April 2009	7.0[b]	3.4[d]	110[d]	11.3[d]	138[d]	1.50[d]	< 2.1[d]	163[d]
ACB (selection experiment)	11 May 2010	4.9[b]	3.9[e]	99[e]	9.6[e]	144[e]	3.84[e]	6.71[e]	242[e]
2008-2010[f] (x ± 1σ; n=33)	-	-	16.5 ± 30.0	99 ± 4	10.7 ± 0.9	178 ± 40	1.87 ± 1.23	2.71 ± 2.36	240 ± 115
Mersey									
Diazepam	9 February 2010	32.3[g]	12.6[h]	65[h]	8.3[h]	356[h]	13.0[h]	80.7[h]	428[h]
ACB	21 February 2010	38.0[g]							
ACB (selection experiment)	11 May 2010	no data	8.5[i]	72[i]	6.8[i]	643[i]	34.5[i]	61.0[i]	515[i]
2008-10[j] (x ± 1σ; n=37)	-	-	14.0 ± 3.0	79 ± 9	8.6 ± 1.7	423 ± 174	20.6 ± 10.5	54.5 ± 24.2	505 ± 54

[a] 2-amino-5-chlorobenzophenone.
[b] Daily mean flow (DMF), gauged at Gunnislake, NGR SX 42627 72525.
[c] Environment Agency of England & Wales (EAEW), unpublished data. Sampling location and dates: Gunnislake, 3 March 2009 (DMF 29.2 $m^3 s^{-1}$) and 3 April 2009 (DMF 7.1 $m^3 s^{-1}$). Data are mean values (n = 2).
[d] EAEW, unpublished data. Sampling location and date: Gunnislake, 23 April 2009 (DMF 6.5 $m^3 s^{-1}$).
[e] EAEW, unpublished data. Sampling location and date: Gunnislake, 25 May 2010 (DMF 3.6 $m^3 s^{-1}$).
[f] EAEW, unpublished data. Sampling location and date: Gunnislake, 31 Jan 2008 – 7 Sept 2010.
[g] DMF, gauged at Westy, NGR SJ 62834 88342 (ca. 0.15 km from Howley Weir).
[h] EAEW, unpublished data. Sampling location and date: Howley Weir (Warrington), 19 Feb 2010 (DMF 41.8 $m^3 s^{-1}$).
[i] EAEW, unpublished data. Sampling location and date: Howley Weir (Warrington), 18 June 2010 (DMF no data).
[j] EAEW, unpublished data. Sampling location and date: Howley Weir (Warrington), 21 Jan 2008 – 13 Sept 2010.

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491 **Figure Captions**

492 Fig. 1. Reported photo-degradation pathway for benzodiazepines to ACB[16]. Diazepam and
493 temazepam initially photo-degrade to form 5-chloro-2-(methylamino)benzophenone
494 which subsequently photo-degrades to 2-amino-5-chlorobenzophenone.

495 Fig. 2. Concentrations (μM) in solution at t = 0 and t = 21 days in the Tamar and Mersey
496 incubations. (a) diazepam (b) ammonium (c) nitrate+nitrite and (d) ortho-phosphate.
497 Error bars represent ± 1σ of the results from duplicate incubations with each sample
498 analysed 3 - 5 times (n = 6 - 10).

499 Fig. 3. Mass spectra of diazepam for a standard, and abiotic and bacteria inoculated samples
500 at day 21 in the Tamar. Diazepam exhibits a singly-charged adduct ($[M+H]^+$). The
501 horizontal arrow represents the range of *m/z* values for biotransformation products
502 predicted by the UMBBD.

503 Fig. 4. Concentrations (μM) in solution at t = 0 and t = 21 days in the Tamar and Mersey
504 incubations. (a) ACB (b) ammonium (c) nitrate+nitrite and (d) ortho-phosphate. Error
505 bars represent ± 1σ of the results from duplicate incubations with each sample
506 analysed 3 - 5 times (n = 6 - 10).

507 Fig. 5. Mass spectra of ACB for a standard, and abiotic and bacteria inoculated samples at
508 day 21. (a) Tamar (b) Mersey. ACB exhibits a singly-charged adduct ($[M+H]^+$). The
509 horizontal arrow represents the range of *m/z* values for biotransformation products
510 predicted by the UMBBD.

511 Fig. 6. Genus identification of clones created from riverine incubations with or without 2-
512 amino-5-chlorobenzophenone (ACB) at day 0 and day 21 for the rivers Tamar and
513 Mersey.

514 Fig. 7. A conceptual model of the transport and fate of diazepam and 2-amino-5-
515 chlorobenzophenone (ACB) along the river - estuary - coastal water continuum. The
516 pathways shown by solid lines are supported by data from the current study and
517 photo-degradation data reported by West and Rowland[16]. Pathways represented by the
518 dashed lines are proposed. DOM is dissolved organic matter.

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Fig 1

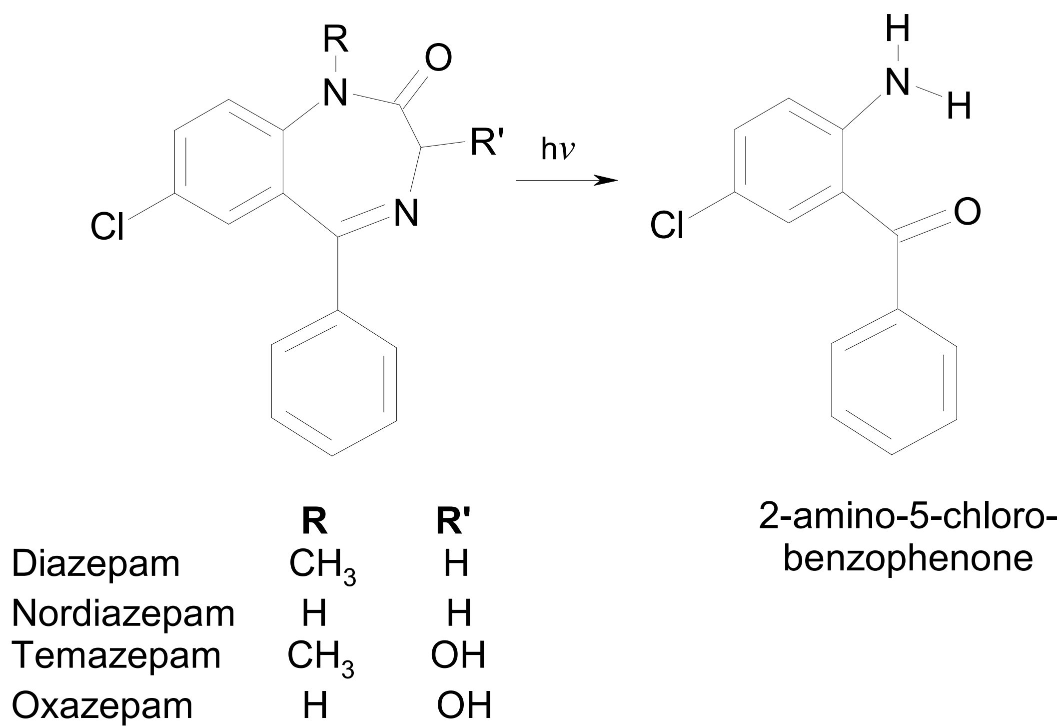

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Fig. 2

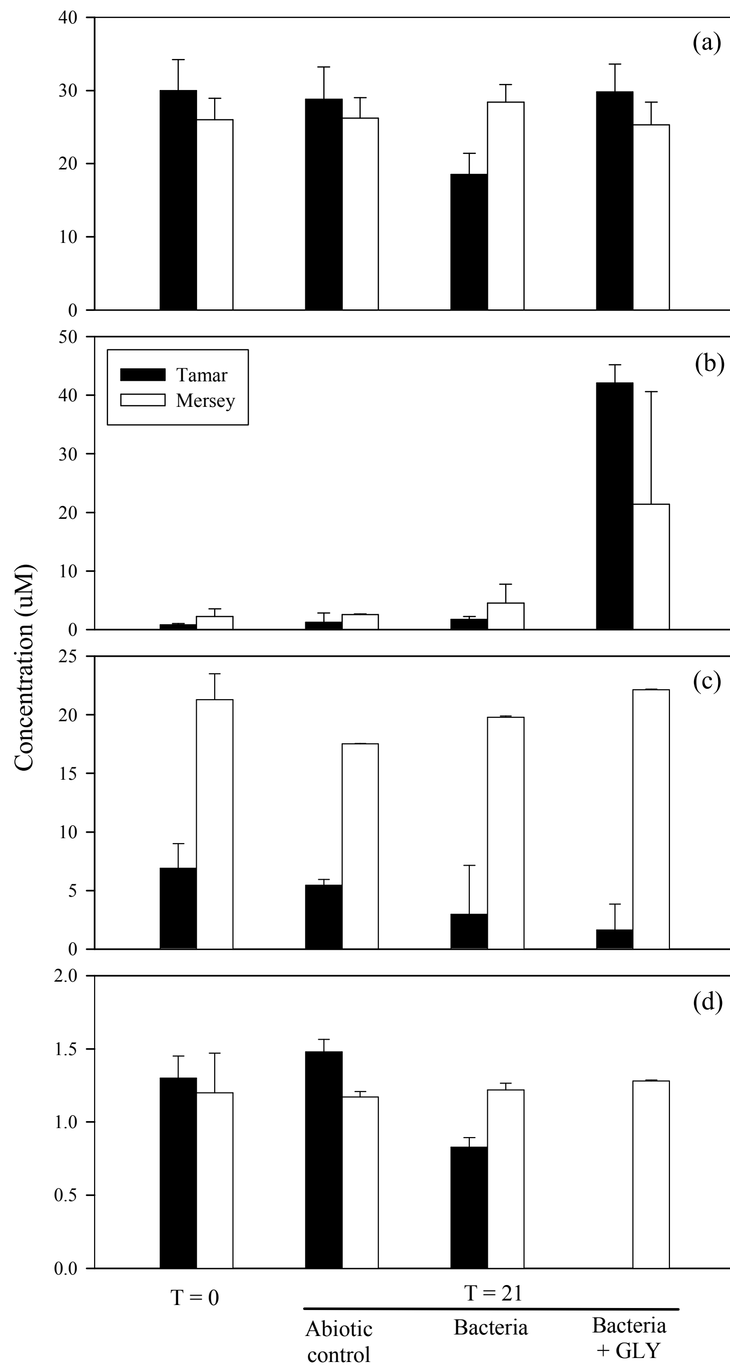

Fig. 3

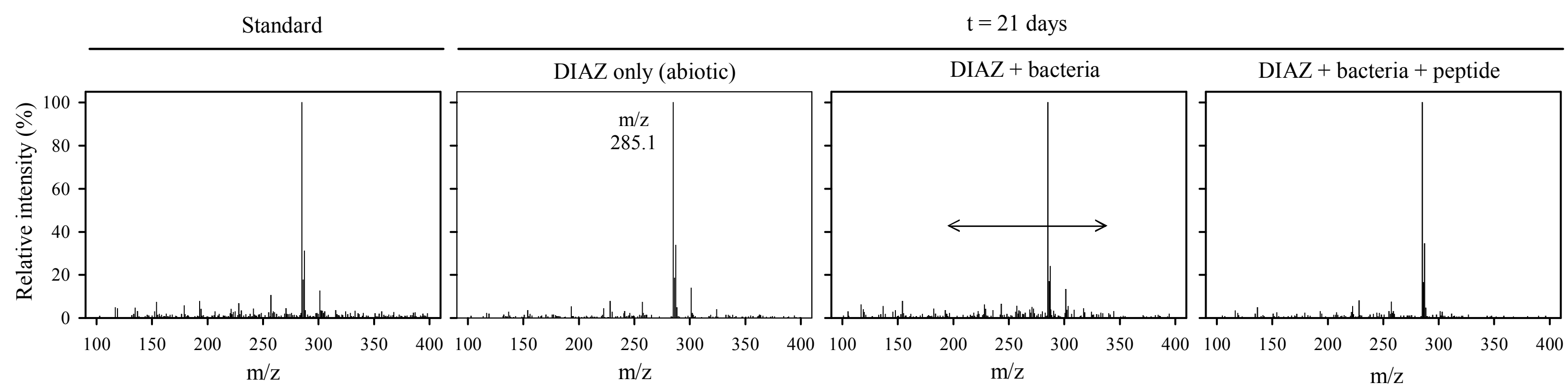

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Fig. 4

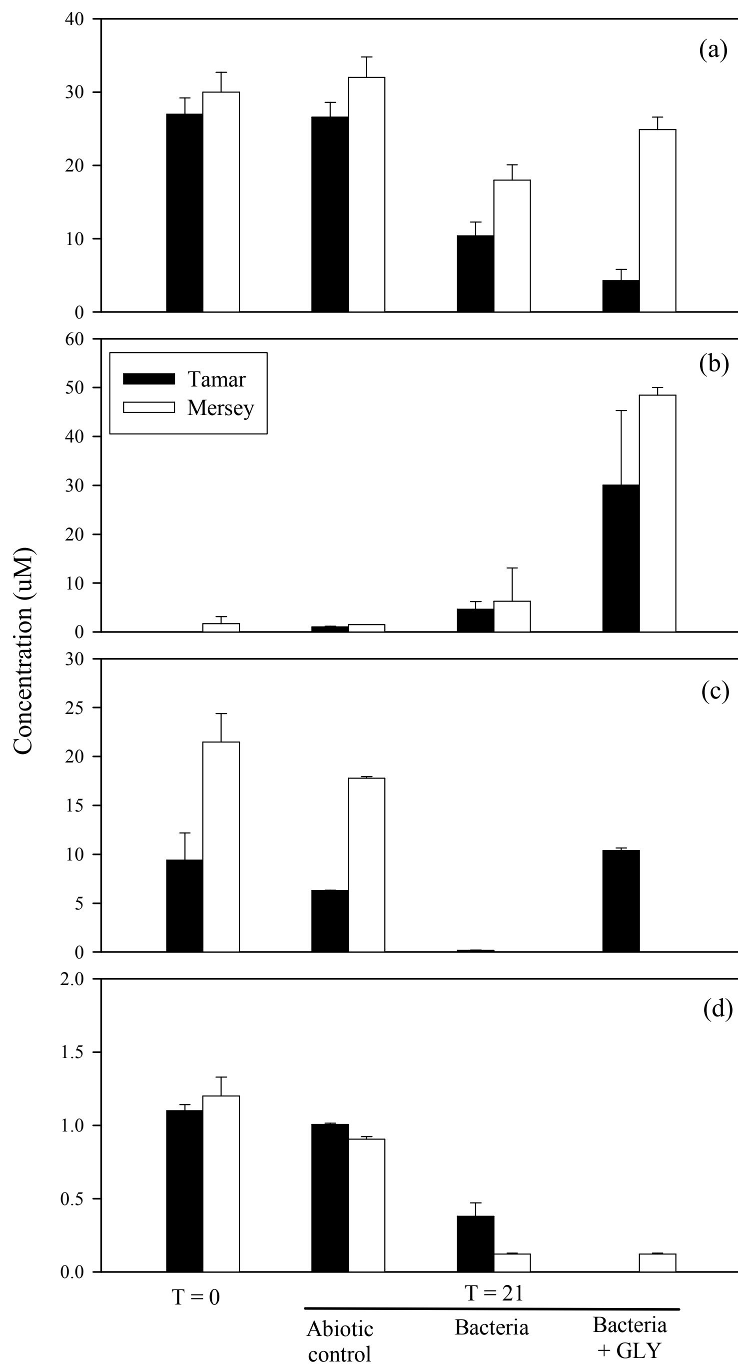

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Fig. 5

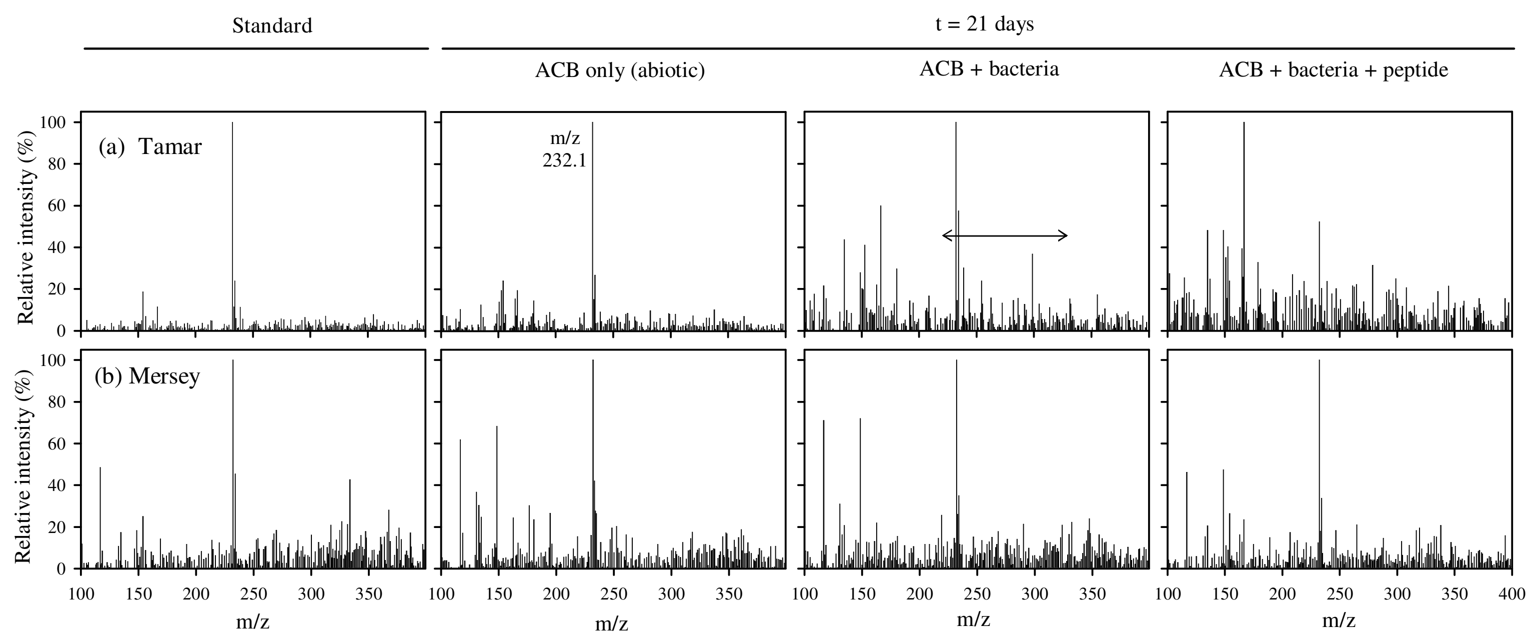

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Fig. 6

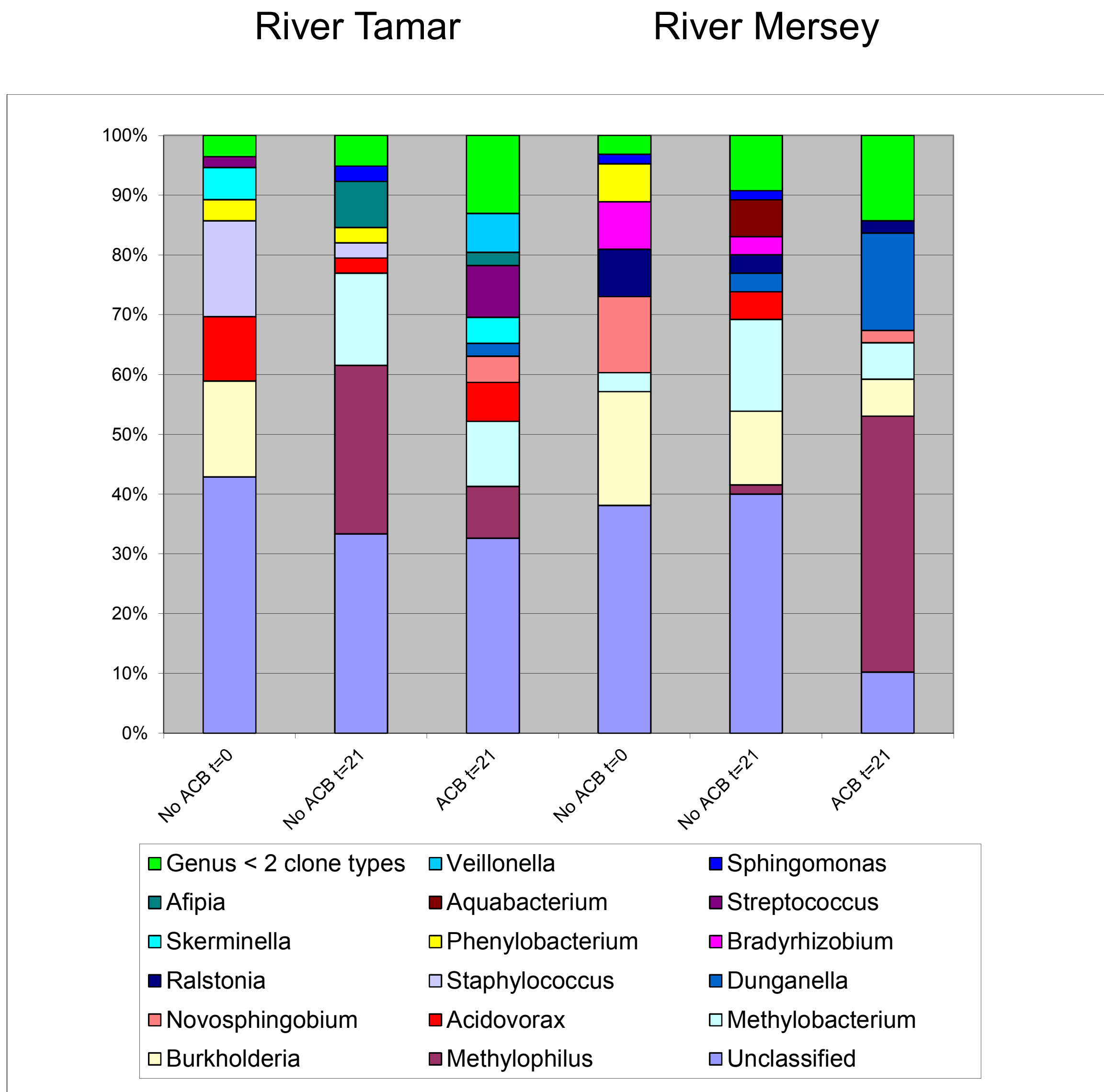

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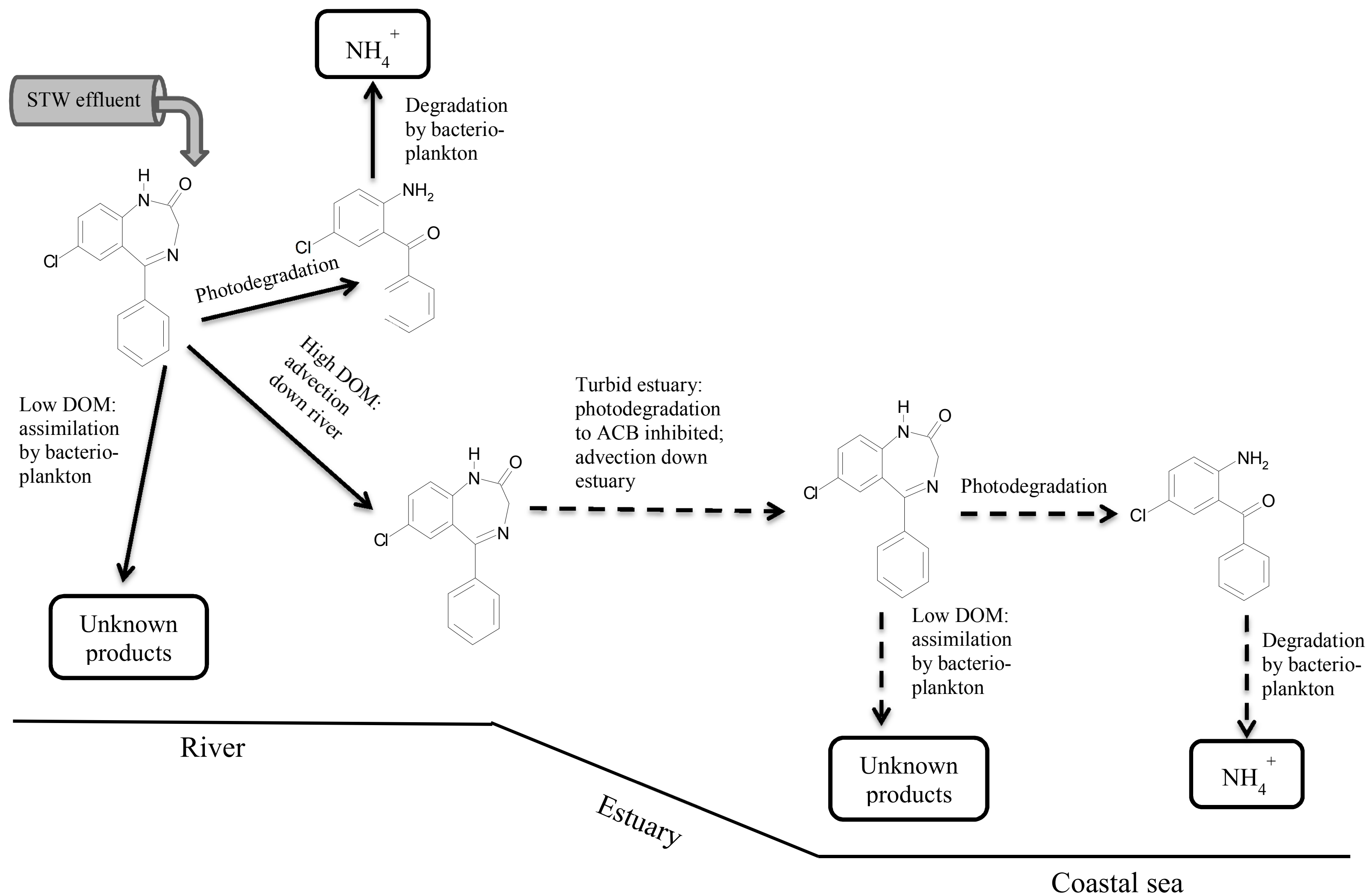
Fig 7



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