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7 **Bacterio-plankton transformation of diazepam and 2-amino-5-**
8 **chlorobenzophenone in river waters**

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29 **ABSTRACT**

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

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

57 Keywords: diazepam, 2-amino-5-chlorobenzophenone, bacteria, benzodiazepines,
58 benzophenones, river, ESI-MS

59

60 Introduction

61 Contamination of aquatic systems by human and veterinary pharmaceuticals now
62 appears to be extensive. However, there is a significant lack of knowledge of their aquatic
63 transport and fate, and effects on non-target organisms¹⁻³. The benzodiazepines (Fig. 1) are a
64 group of widely-prescribed anxiolytic/sedative pharmaceuticals with both human and
65 veterinary applications⁴. Of the 35 compounds in this group, diazepam (Fig. 1) is the second
66 most frequently prescribed⁵ and is included in the World Health Organisation Essential Drugs
67 List⁶. Diazepam is metabolized in the human body to oxazepam, temazepam and
68 nordiazepam (Fig. 1), all of which are pharmacologically-active.

69 Diazepam and its metabolites are primarily excreted in urine, either in the free form or
70 as sulphate and glucuronide conjugates; between 5 and 50 % of the administered dose of
71 diazepam is excreted⁷. Once in the wastewater stream, the glucuronide may be deconjugated⁴.
72 Of the 118 pharmaceuticals examined in urban wastewaters from four continents, diazepam
73 was observed to have one of the highest mean and maximum concentrations in influent
74 wastewaters (22 – 23 $\mu\text{g L}^{-1}$)⁸. During conventional sewage treatment, generally ≥ 80 % of
75 diazepam in the influent stream is lost to surface waters via the sewage works effluent^{8,9}. As a
76 result it has been detected in surface waters of Europe, the USA, Asia and Australia¹⁰⁻¹³.
77 Diazepam has been ranked as a high risk compound with respect to aquatic organisms⁸, while
78 ambient concentrations of its metabolite, oxazepam, can markedly alter the behaviour and
79 feeding of the wild European perch *Perca fluviatilis*¹⁴. Thus, it appears that inputs of
80 benzodiazepines to surface waters can have ecological and evolutionary consequences.

81 Within the pH range for surface waters (5-9), dissolved diazepam is a neutral
82 molecule⁷. It is stable with respect to chemical hydrolysis, and with sediment : water partition
83 coefficients $< 100 \text{ L kg}^{-1}$ for both organic-rich (sewage solids) and organic-poor particles^{7,15},

84 little sorption (< 0.1 %) of the compound will occur at suspended sediment concentrations
85 typical of low-turbidity rivers. Diazepam photo-degrades in water^{5,16}, yielding a range of
86 products, including the water-soluble 2-amino-5-chlorobenzophenone (ACB; Fig. 1), a
87 substituted benzophenone which appears relatively resistant to further photo-degradation¹⁶.
88 As the photolysis half-life for diazepam under environmentally-relevant conditions ranges
89 from 16 to 168 h^{5,16}, conversion of diazepam to ACB may be an important abiotic removal
90 process for diazepam in sunlit surface waters. However, hydroxylated benzophenones have
91 been shown to exhibit estrogenic activity and their presence in surface waters has been
92 reported^{17,18}, while concentrations of up to 130 ng L⁻¹ of benzophenone have been detected
93 in Korean rivers receiving wastewater effluent¹⁹.

94 There appear to be no published toxicity data for diazepam metabolites and
95 transformation products, including ACB⁹. Biotic (bacterio-plankton) transformation studies
96 of diazepam have largely focussed on the role of sewage treatment^{7,9}; surface water studies
97 are much rarer. In a microcosm set up to simulate aerobic and anaerobic transformations in
98 aquatic sediment systems, less than 2 % of the 0.35 μmole diazepam added was
99 biotransformed within the 100 days of the experiment¹⁵.

100 The observed or potential effects of benzodiazepines and benzophenones make the
101 understanding of their fate in aquatic environments very important⁹. The aim of the present
102 work was to investigate the biotic transformation of two representative compounds from
103 these groups (diazepam and ACB) by natural, riverine bacterio-plankton communities using a
104 specifically designed experimental protocol²⁰. Incubations were undertaken in laboratory
105 batch microcosms in the presence and absence of a readily degradable organic substrate that
106 could act as a priming agent for xenobiotic removal^{21,22}. Concentrations of the parent
107 compounds were measured and the presence of metabolites investigated after an incubation
108 period that reflected typical residence times for surface waters in these catchments. Finally, to

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

112

113 **Materials and Methods**

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

116 **Study areas**

117 The River Tamar (SW England, UK) drains a rural, agriculture-dominated, catchment of 928
118 km² and has a mean flow of 22.5 m³ s⁻¹ at its tidal limit at Gunnislake. In contrast to the
119 Tamar, the tidal limit on the River Mersey (Howley Weir, Warrington, NW England, UK) is
120 the drainage end-point of a highly urbanised region of ca. 2000 km² (mean flow 37.5 m³ s⁻¹).
121 The River Mersey was once severely polluted, but remedial measures undertaken during the
122 last three decades have significantly improved water quality. Table 1 provides a synopsis of
123 chemical data for these rivers at their tidal limits for the period 2008–2010, together with data
124 covering the times when sampling took place. Table 1 indicates that both were low turbidity
125 systems (i.e. suspended particulate matter concentration < 15 mg L⁻¹) and that the Mersey had
126 lower concentrations of dissolved oxygen, and higher concentrations of nitrate, ammonium,
127 ortho-phosphate and dissolved organic carbon, relative to the Tamar.

128 **Incubation experiments**

129 Chemicals: Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-
130 one, AR grade, Sigma-Aldrich, UK), 2-amino-5-chlorobenzophenone (ACB; AR grade,
131 Sigma-Aldrich, UK) and a tripeptide comprising glycine, leucine and tyrosine residues (≥ 98
132 % purity, Sigma-Aldrich, UK) were used for the study.

133

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

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

146 Preparation of bacterial inoculum: Bacterio-plankton concentrations were measured in water
147 samples (10⁵-10⁶ cells per mL) to ensure that the prepared bacterial inoculum was
148 representative. The bacterial inoculum was prepared using water from the same sampling
149 sites, collected within 24 h of the start of the incubations. This water was filtered through a
150 1.6 µm pore size membrane (combusted GF/A) to remove any particles²³ and then re-filtered
151 through a 0.2 µm pore diameter membrane filter (Whatman Anodisc 47). The bacterio-
152 plankton retained on the membrane was resuspended in a small volume of the 0.2 µm filtered
153 water to provide the inoculum, which was then added to the prepared incubation water to
154 produce a final, representative bacterio-plankton concentration. Water for the inocula was
155 collected on 17 March and 19 April 2009 (Tamar) and 8 and 22 February 2010 (Mersey).

156 Incubation experiments: Incubation water (60 mL) was transferred to a 125 mL screw-capped
157 amber glass bottle to which was added 15–22 µL of stock diazepam or ACB solution and 1
158 mL of the bacterial inoculum. Starting concentrations of the compounds were approximately
159 30 µM. Pre-incubation Microtox[®] assays using the bacterium *Vibrio fischeri* showed that

160 diazepam and ACB were non-toxic at these concentrations ($EC_{50} \gg 100 \mu\text{M}$, 15 min
161 exposure). In a separate set of incubations, the effect of labile DOM on the biotransformation
162 of diazepam and ACB was tested by adding the tripeptide (equivalent to $90 \mu\text{mol N L}^{-1}$ and
163 $510 \mu\text{mol C L}^{-1}$) alongside diazepam and ACB. Control incubations of prepared river water
164 containing bacterial inoculum only and diazepam/ACB only were also prepared to account
165 for sorption effects. Ortho-phosphate was added to all incubations to give ca. $1\text{-}2 \mu\text{M P}$ at $t =$
166 0.

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

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

181

182

183

184 **Chemical and microbiological analysis**

185 Analyses by ESI-MS were performed in positive mode using a Finnigan MAT LCQ MS, a
186 quadrupole ion trap mass spectrometer with an external source atmospheric pressure interface
187 capable of electro-spray ionisation. The sample matrix was 50 : 50 methanol : water amended
188 with 0.1 % (v/v) formic acid and solutions were introduced by low-flow infusion at a rate of
189 3 $\mu\text{L min}^{-1}$. Once thawed, each sample was diluted 1:1 with the mixed methanol and formic
190 acid solution. Samples were then injected into the instrument. The signal sensitivity for both
191 diazepam and ACB, in positive-ion mode, was optimised by adjustment of instrumental
192 parameters using in-built tuning procedures. Ion count integration was performed for 2
193 minutes, with 5 replicates recorded per sample, while ion count stability was recorded in real
194 time using single ion monitoring. Quantification of each analyte was achieved by generating
195 an external calibration curve using matrix-matched standards on each analytical day, and
196 bracketing individual samples with a drift matrix-matched calibration standard to account for
197 variations in instrumental sensitivity; the variation was then calculated using an algorithm²⁰.
198 The mass spectra for both diazepam and ACB contained two isotopic peaks (due to ³⁵Cl and
199 ³⁷Cl atoms). Base peaks (attributed to $[\text{M}+\text{H}]^+$) for diazepam and ACB occurred at m/z 285
200 and m/z 232, respectively; a single peak for tripeptide occurred at m/z 352. Nitrate+nitrite
201 and ortho-phosphate were determined by segmented flow and spectrophotometric detection²⁴
202 and ammonium by o-phthaldialdehyde fluorescence²⁵. Viable counts of bacterio-plankton
203 were undertaken using 100 μL aliquots from the microcosms. These were diluted in
204 phosphate-buffered saline solution and 100 μL of each dilution spread on half strength Luria
205 Bertani agar (Merck, Germany) and incubated at 30 °C for two days. Colonies were
206 enumerated as colony forming units (cfu) mL^{-1} of the original suspensions. Total counts of
207 bacterio-plankton were determined microscopically by staining water samples with DAPI²⁶.
208 The microcosms contained bacterio-plankton populations of 10^5 - 10^6 cells per mL at both 0

209 and 21 days; approximately 10 % were recoverable as viable colonies on nutrient agar
210 plates. DNA extraction followed²⁷. Each microcosm water sample was membrane filtered
211 (0.2 µm pore diameter) and the retained cells disrupted on the filter by mechanical bead
212 beating. The DNA was extracted into hexadecyltrimethylammonium bromide and phenol-
213 chloroform-isoamyl alcohol, and then resuspended in 50 µL nuclease-free water. Nested PCR
214 amplifications were performed on extracted samples using Super Taq DNA polymerase and
215 G-Storm thermal cyclers. DNA amplification was undertaken in a 50 µL sample using 1 µM
216 of the universal primers for eubacterial 16S rRNA genes (27_f and 1492_r)²⁸ with 1 unit *super*
217 Taq DNA polymerase. The amplified DNA fragments were re-amplified using forward
218 primer 341 and reverse primer 907²⁹.

219 Denaturing gradient gel electrophoresis (DGGE) analysis³⁰ was performed on GC-
220 clamped products of the second PCR amplification using the Bio-Rad D-code system to
221 separate DNA on a 8 % polyacrylamide gel in Tris acetate EDTA buffer (pH 8.0) with a 20 -
222 60 % denaturant gradient, in which 100 % denaturant was 7 M urea amended with 40 %
223 formamide. Electrophoresis was performed at 60 °C, run at 60 V (16 h) and the DNA banding
224 visualised using Sybr Green I stain with detection and image capture on a Storm 860
225 Molecular Imager. Amplified eubacterial 16S ribosomal gene DNA was pooled from
226 duplicate microcosms and cloned into *E. coli* using the PGEM vector system (Promega)
227 according to the manufacturer's instructions. Based on the data collected from the initial 21-
228 day incubations, clones (50-70) were selected at random from Tamar and Mersey water
229 microcosms incubated for 0 and 21 days in the presence and absence of ca. 30 µM ACB. The
230 clones were sequenced by GATC (Germany) and preliminary identification assigned using
231 the Ribosomal Database Project³¹.

232 **Results and Discussion**

233 Removal of both substrates was observed in at least one of the incubations, suggesting that
234 the concentration at which they were added did not affect the ability of the bacterio-plankton
235 community to utilise them³².

236

237 **Diazepam**

238 In Tamar waters after 21 days, the concentration of diazepam added (30 μM , 1.8 $\mu\text{mole total}$)
239 was unchanged in both the abiotic control ($29.8 \pm 3.8 \mu\text{M}$, mean $\pm 1\sigma$, $n = 6-10$; t-test, $p =$
240 0.93) and the biotic incubation containing diazepam and peptide ($28.8 \pm 4.4 \mu\text{M}$, $p = 0.56$). In
241 contrast, the mean concentration had decreased by 36 %, to $18.5 \pm 2.9 \mu\text{M}$, over 21 days ($p <$
242 0.001) in the biotic incubation containing diazepam only (Fig. 2a). Given the limited extent
243 of partitioning to the solid phase reported for diazepam⁷, and the very low solid particulate
244 material (SPM) concentrations in the incubations ($< 1 \text{ mg L}^{-1}$), the decrease in the dissolved
245 concentration was almost certainly due to active uptake by the bacterio-plankton, as opposed
246 to simple abiotic sorption to cell surface components. In the peptide-amended experiment, the
247 peptide was consumed by the bacteria, via ammonification, leading to an increase in
248 concentrations of ammonium from $0.8 \mu\text{M}$ at the beginning of the incubation to $42.1 \mu\text{M}$ at
249 the end (Fig. 2b). As this form of DOM is readily utilised by the riverine bacterial
250 community,^{20,33} the data suggest that the degradation of this alternative carbon/nitrogen
251 source is preferred over assimilation of diazepam.

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

256 Prediction to the second tier of biotransformation indicated that up to 5 chemical species may
257 be produced, including nordiazepam and three benzophenones (SI Fig. 1 and SI Table 1). N
258 atoms were retained throughout, meaning that each molecule should be observed in positive
259 mode ESI-MS. However, none of the predicted products were detected (Fig. 3) suggesting
260 that, if biotransformation products were produced, they were not released into solution, but
261 were further metabolised rapidly, or were present at concentrations below the limit of
262 detection under these conditions ($< 0.05 \mu\text{M}$ and $< 0.9 \mu\text{M}$ for diazepam and ACB,
263 respectively). Transformation products have been reported for diazepam, including
264 nordiazepam³⁴. However, these data were acquired in sludge-seeded bioreactors at an SPM of
265 3 g L^{-1} and, interestingly, little degradation of diazepam ($< 10 \%$) was observed over the 16
266 days duration of that experiment³⁴.

267 In the Mersey water microcosms, $26.0 \pm 2.9 \mu\text{M}$ diazepam was added. After 21 days,
268 concentrations of diazepam in the abiotic and both biotic incubations had not changed
269 significantly (t-test, p range 0.06 – 0.86; Fig. 2a). The tripeptide was again consumed when
270 added as an additional substrate (Fig. 2b). As the bacterio-plankton of an urban river might be
271 expected to be responsive, having probably encountered the molecule previously, the absence
272 of diazepam removal was surprising, particularly as River Tamar microcosms were able to
273 effect significant removal of the diazepam (Fig. 2). A contrast in the removal of another
274 xenobiotic, atrazine, was also observed in a previous study for incubations using bacterial
275 populations from the same rivers²⁰. There, 11 % removal over 21 days was observed in
276 Tamar samples, when atrazine was the only substrate added, contrasting with 0 % removal in
277 Mersey samples. However, addition of tripeptide increased removal from Mersey water from
278 0 to 37 %, while the Tamar removal value remained at 11 %. There are very few studies on
279 the bacterio-plankton compositions of unconnected rivers. In the Santa Ana River basin
280 (USA), urban impacted and rural, agriculturally impacted streams contained bacterio-

281 plankton communities that showed few differences³⁵, suggesting that bacterial response to
282 added xenobiotics might be similar. The bacterio-plankton populations in the incubations
283 were prepared to give a final concentration that matched *in situ* measurements at the time of
284 collection²⁰, so the contrast in the removal of diazepam between Tamar and Mersey waters
285 reflects inoculum composition rather than cell numbers.

286 **2-amino-5-chlorobenzophenone**

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

306 Fig. 4c, d). In summary, the removal of ACB occurred in all microcosms and was more
307 extensive in the Tamar microcosms. The presence of the peptide substrate enhanced ACB
308 removal in the Tamar microcosms but not for the Mersey. The addition of amino acids has
309 been shown to stimulate the biotransformation of phenols by a natural microbial lacustrine
310 community³⁶. The UMBBD gave two theoretical degradation pathways for ACB, and
311 prediction to the second tier of biotransformation showed that of the 9 chemical species
312 potentially produced, three retained the N atom, including one hydroxylated benzophenone
313 (SI Fig. 2). However, as for the diazepam experiments, predicted ACB biotransformation
314 products were not detected in solution (Fig. 5).

315

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

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

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

330 of a bacterial population acclimated for ACB degradation. It is our contention, therefore, that
331 at the low concentrations of ACB, or its benzodiazepine precursors, which enter surface
332 waters of urban or rural catchments¹⁶, the xenobiotic is assimilated without significantly
333 impacting the structure of the indigenous riverine microbial community.

334 **Environmental implications**

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

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

354 to the advection of diazepam to low turbidity coastal waters and its subsequent photo-
355 degradation to ACB in sunlit surface layers. This pathway may also be applicable to other
356 pharmacologically-active 1,4-benzodiazepine molecules known to degrade to ACB (e.g.
357 oxazepam, temazepam and nordiazepam), which would be significant given the reported
358 ecological effects on freshwater fish exposed to environmental concentrations of oxazepam¹⁴.

359 **Conclusions**

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

377

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387 been considerably improved as a result.

388

389 **Notes and references**

390 Electronic Supplementary Information available

391 SI Table 1. Predicted pathways for the biotransformation of diazepam and
392 2-amino-5-chlorobenzophenone (ACB) in aerobic systems, including the probability of
393 degradation by named pathways and details of the mechanisms and enzymes involved. The
394 predicted products are shown in SI Figures 1 and 2.

395

396 SI Fig. 1 The University of Minnesota Biocatalysis and Biodegradation Database
397 (UMBDD) prediction pathways (to tier 2) of the aerobic bacterial biotransformation of
398 diazepam. The benzophenone units are ringed. The 'btxxxx' annotation refers to the specific
399 enzymic reaction mechanisms stored in the UMBDD database, which are listed in
400 SI Table 1.

401

402 SI Fig. 2 UMBDD prediction pathways (to tier 2) of the aerobic bacterial
403 biotransformation of ACB. The 'btxxxx' annotation refers to the specific enzymic reaction
404 mechanisms stored in the UMBDD database, which are listed in SI Table 1.

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482 Table 1. River water flow and physico-chemical characteristics close to sample collection points, together with water quality data for 2008 – 2010.
 483

	Sampling date of bacterial inoculum	Daily mean flow (m ³ s ⁻¹)	Suspended particulate matter (mg L ⁻¹)	Dissolved oxygen (% sat.) (mg L ⁻¹)		Nitrate (µmol L ⁻¹ N)	Ortho-phosphate (µmol L ⁻¹ P)	Ammonium (µmol L ⁻¹ N)	Dissolved organic carbon (µmol 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

484 ^a 2-amino-5-chlorobenzophenone.

485 ^b Daily mean flow (DMF), gauged at Gunnislake, NGR SX 42627 72525.

486 ^c Environment Agency of England & Wales (EAEW), unpublished data. Sampling location and dates: Gunnislake, 3 March 2009 (DMF 29.2 m³ s⁻¹) and 3
 487 April 2009 (DMF 7.1 m³ s⁻¹). Data are mean values (n = 2).

488 ^d EAEW, unpublished data. Sampling location and date: Gunnislake, 23 April 2009 (DMF 6.5 m³ s⁻¹).

489 ^e EAEW, unpublished data. Sampling location and date: Gunnislake, 25 May 2010 (DMF 3.6 m³ s⁻¹).

490 ^f EAEW, unpublished data. Sampling location and date: Gunnislake, 31 Jan 2008 – 7 Sept 2010.

491 ^g DMF, gauged at Westy, NGR SJ 62834 88342 (ca. 0.15 km from Howley Weir).

492 ^h EAEW, unpublished data. Sampling location and date: Howley Weir (Warrington), 19 Feb 2010 (DMF 41.8 m³ s⁻¹).

493 ⁱ EAEW, unpublished data. Sampling location and date: Howley Weir (Warrington), 18 June 2010 (DMF no data).

494 ^j EAEW, unpublished data. Sampling location and date: Howley Weir (Warrington), 21 Jan 2008 – 13 Sept 2010.

495

496 **Figure Captions**

497 Fig. 1. Reported photo-degradation pathway for benzodiazepines to ACB¹⁶. Diazepam and
498 temazepam initially photo-degrade to form 5-chloro-2-(methylamino)benzophenone
499 which subsequently photo-degrades to 2-amino-5-chlorobenzophenone.

500 Fig. 2. Concentrations (μM) in solution at $t = 0$ and $t = 21$ days in the Tamar and Mersey
501 incubations. (a) diazepam (b) ammonium (c) nitrate+nitrite and (d) ortho-phosphate.
502 Error bars represent $\pm 1\sigma$ of the results from duplicate incubations with each sample
503 analysed 3 - 5 times ($n = 6 - 10$).

504 Fig. 3. Mass spectra of diazepam for a standard, and abiotic and bacteria inoculated samples
505 at day 21 in the Tamar. Diazepam exhibits a singly-charged adduct ($[\text{M}+\text{H}]^+$). The
506 horizontal arrow represents the range of m/z values for biotransformation products
507 predicted by the UMBBD.

508 Fig. 4. Concentrations (μM) in solution at $t = 0$ and $t = 21$ days in the Tamar and Mersey
509 incubations. (a) ACB (b) ammonium (c) nitrate+nitrite and (d) ortho-phosphate. Error
510 bars represent $\pm 1\sigma$ of the results from duplicate incubations with each sample
511 analysed 3 - 5 times ($n = 6 - 10$).

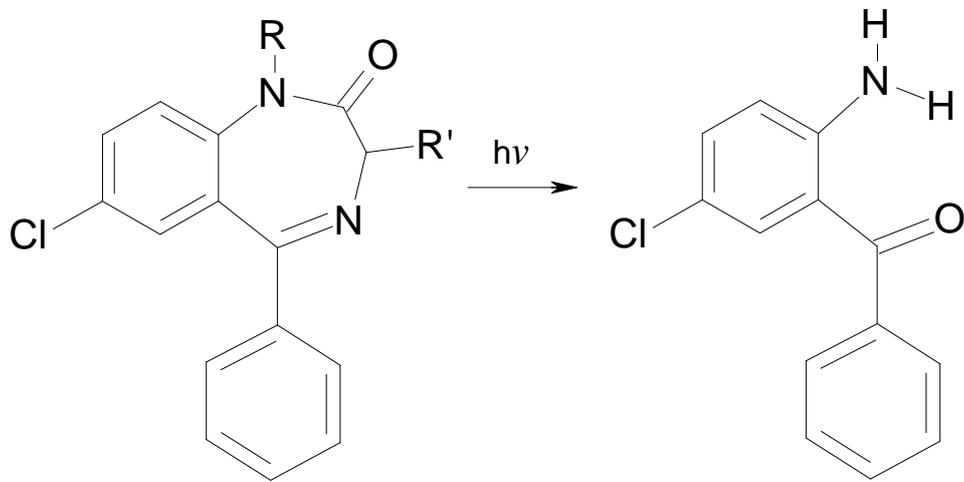
512 Fig. 5. Mass spectra of ACB for a standard, and abiotic and bacteria inoculated samples at
513 day 21. (a) Tamar (b) Mersey. ACB exhibits a singly-charged adduct ($[\text{M}+\text{H}]^+$). The
514 horizontal arrow represents the range of m/z values for biotransformation products
515 predicted by the UMBBD.

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

519 Fig. 7. A conceptual model of the transport and fate of diazepam and 2-amino-5-
520 chlorobenzophenone (ACB) along the river - estuary - coastal water continuum. The
521 pathways shown by solid lines are supported by data from the current study and
522 photo-degradation data reported by West and Rowland¹⁶. Pathways represented by the
523 dashed lines are proposed. DOM is dissolved organic matter.

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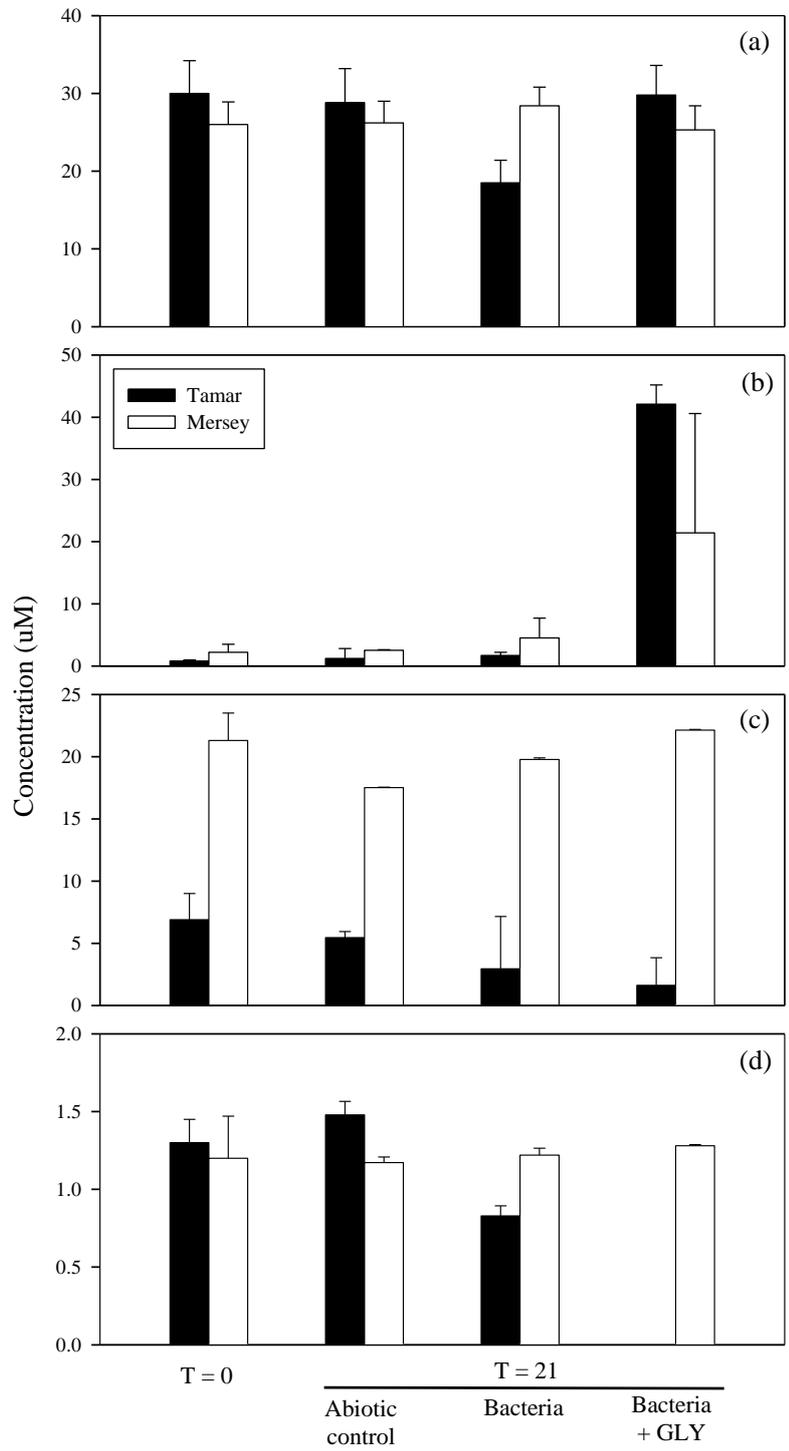
525 Fig 1
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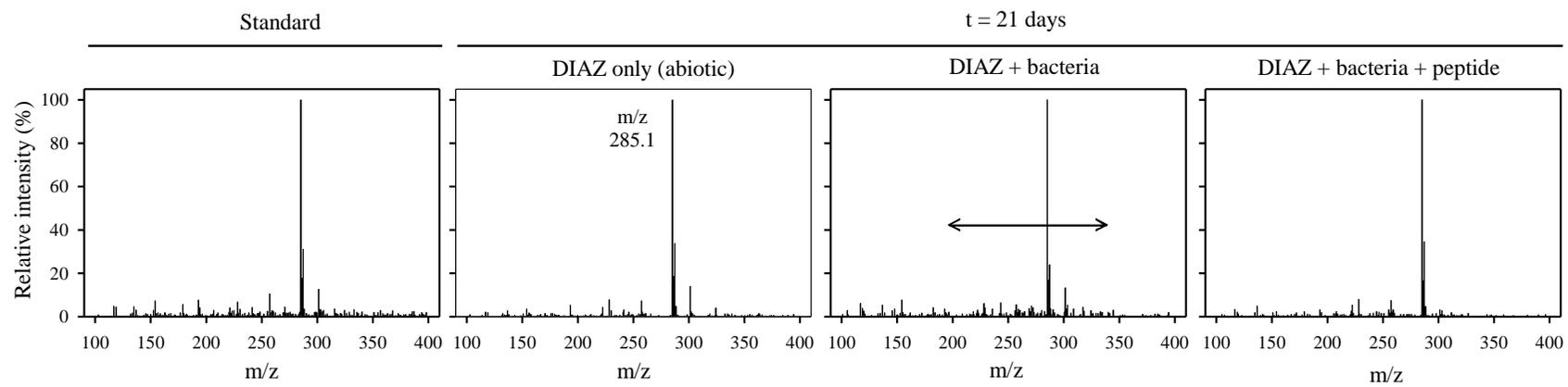
	R	R'
Diazepam	CH ₃	H
Nordiazepam	H	H
Temazepam	CH ₃	OH
Oxazepam	H	OH

2-amino-5-chloro-
benzophenone

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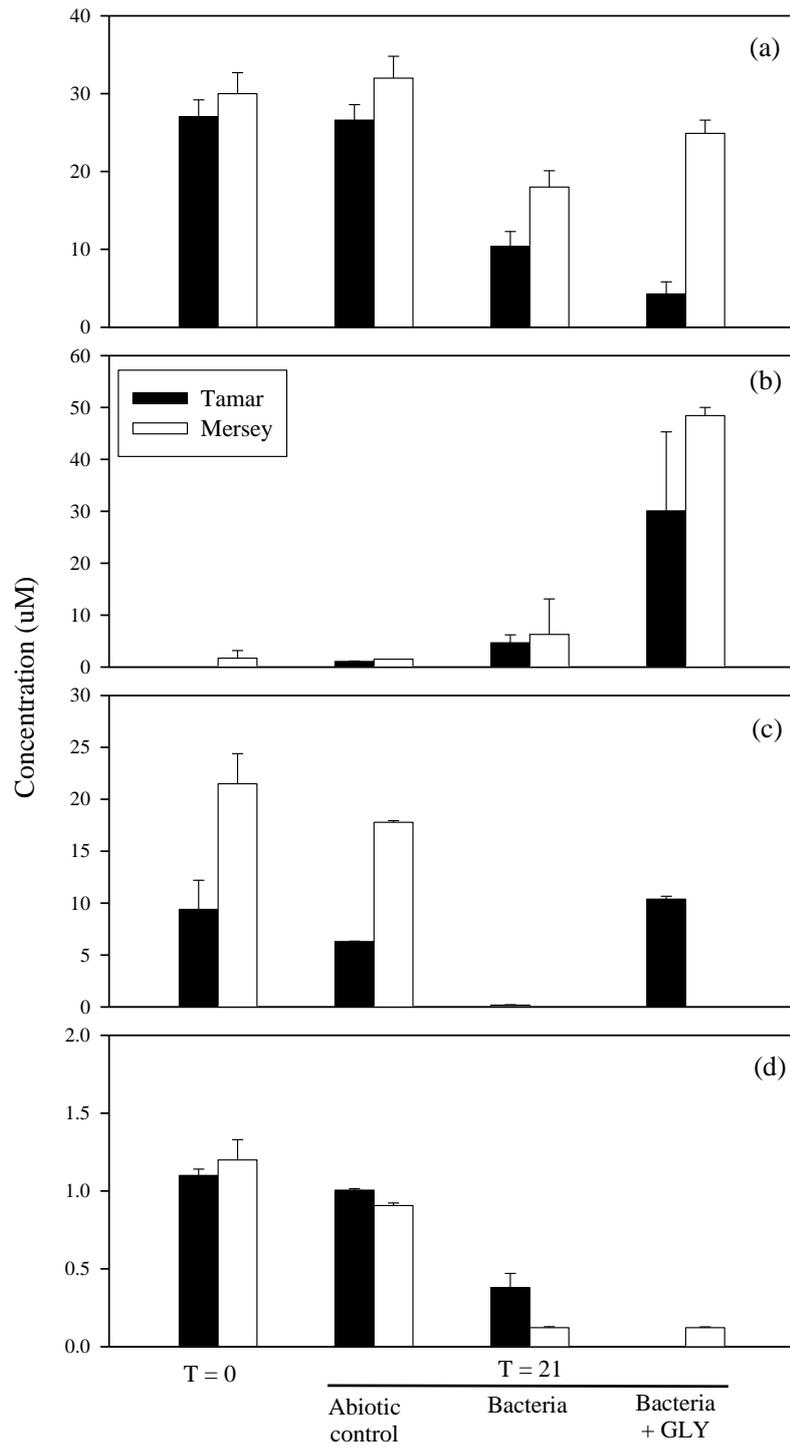


533 Fig. 3
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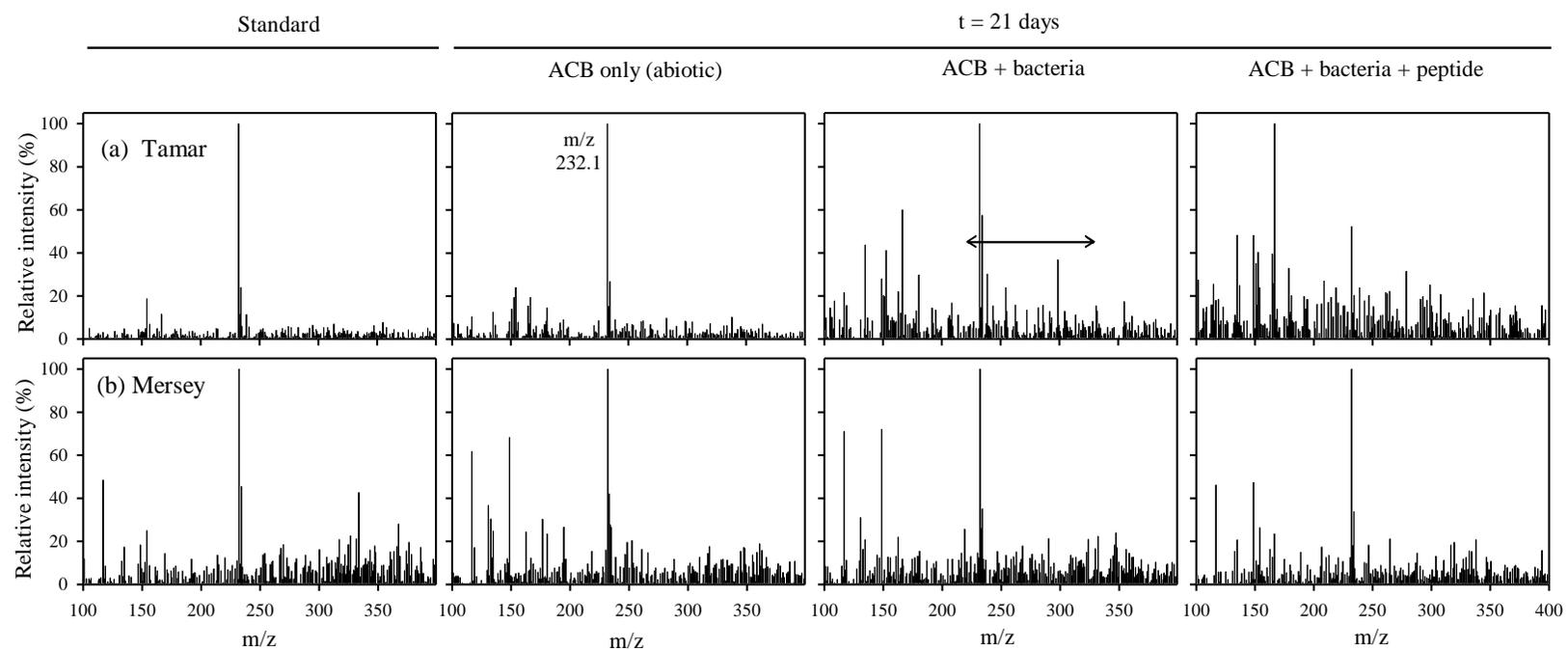


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538 Fig. 4
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543 Fig. 5
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River Tamar

River Mersey

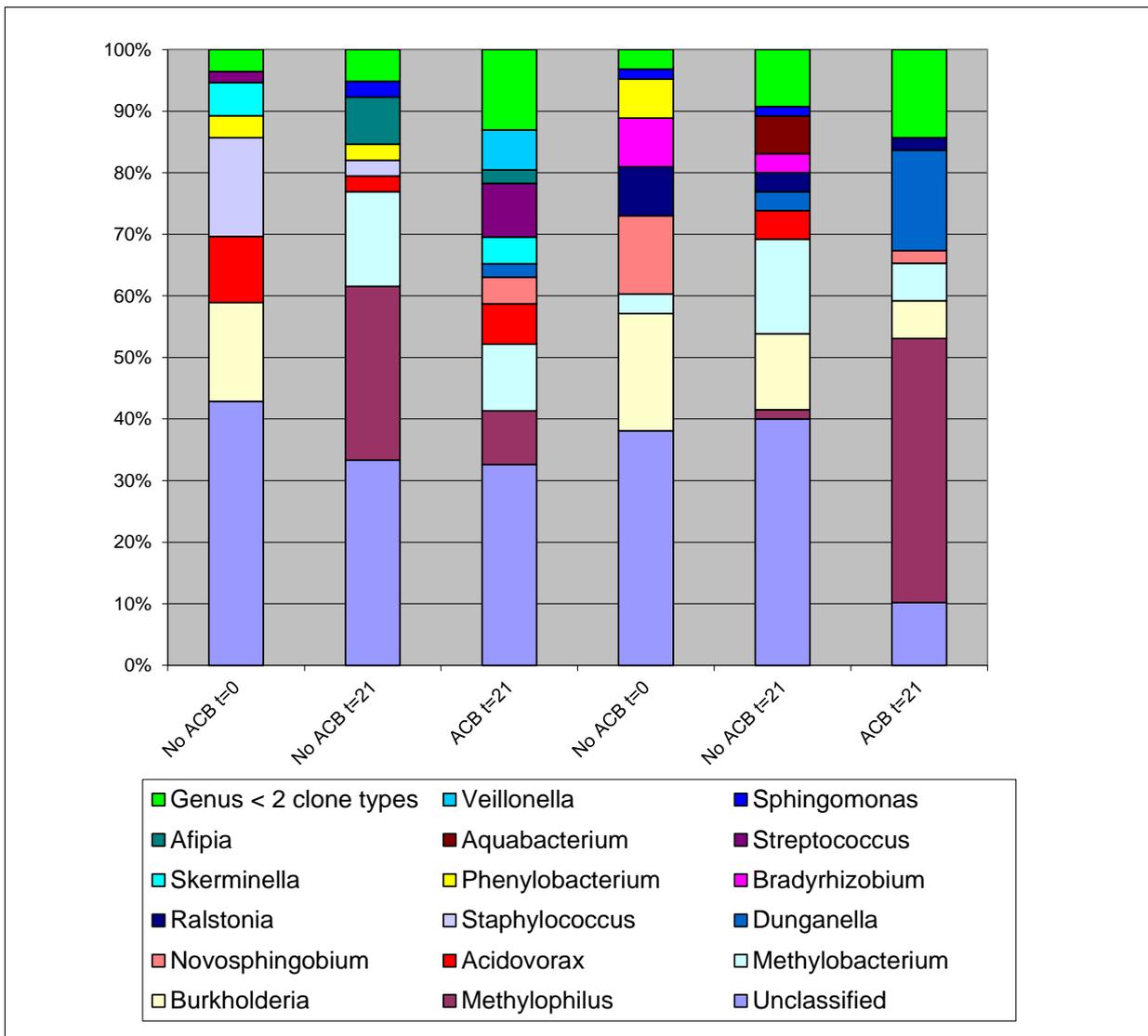
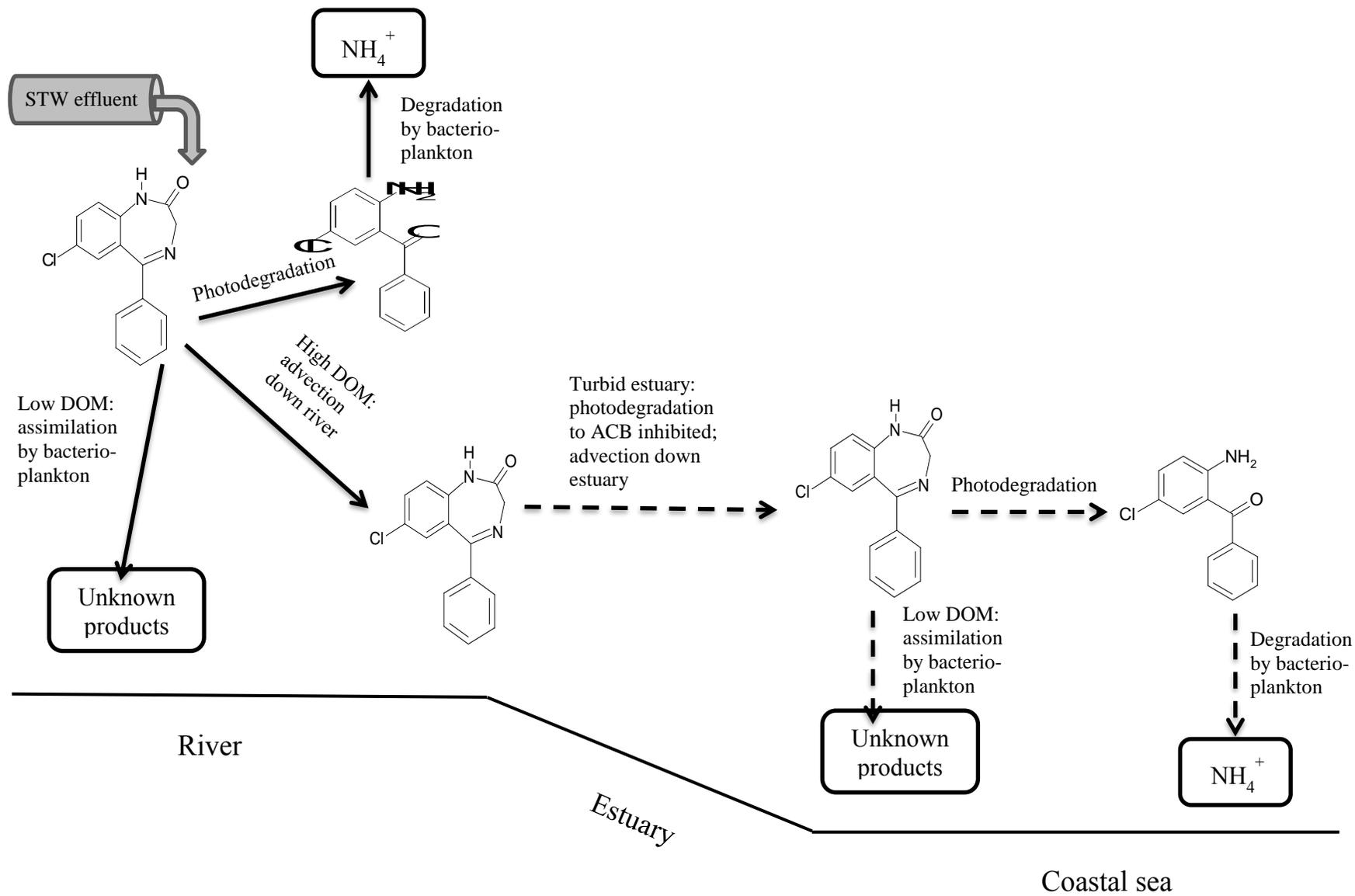


Fig 7



SI Table 1. Predicted pathways for the biotransformation of diazepam and 2-amino-5-chlorobenzophenone (ACB) in aerobic systems, including the probability of degradation by named pathways and details of the mechanisms and enzymes involved. The predicted products are shown in Appendices A and B.

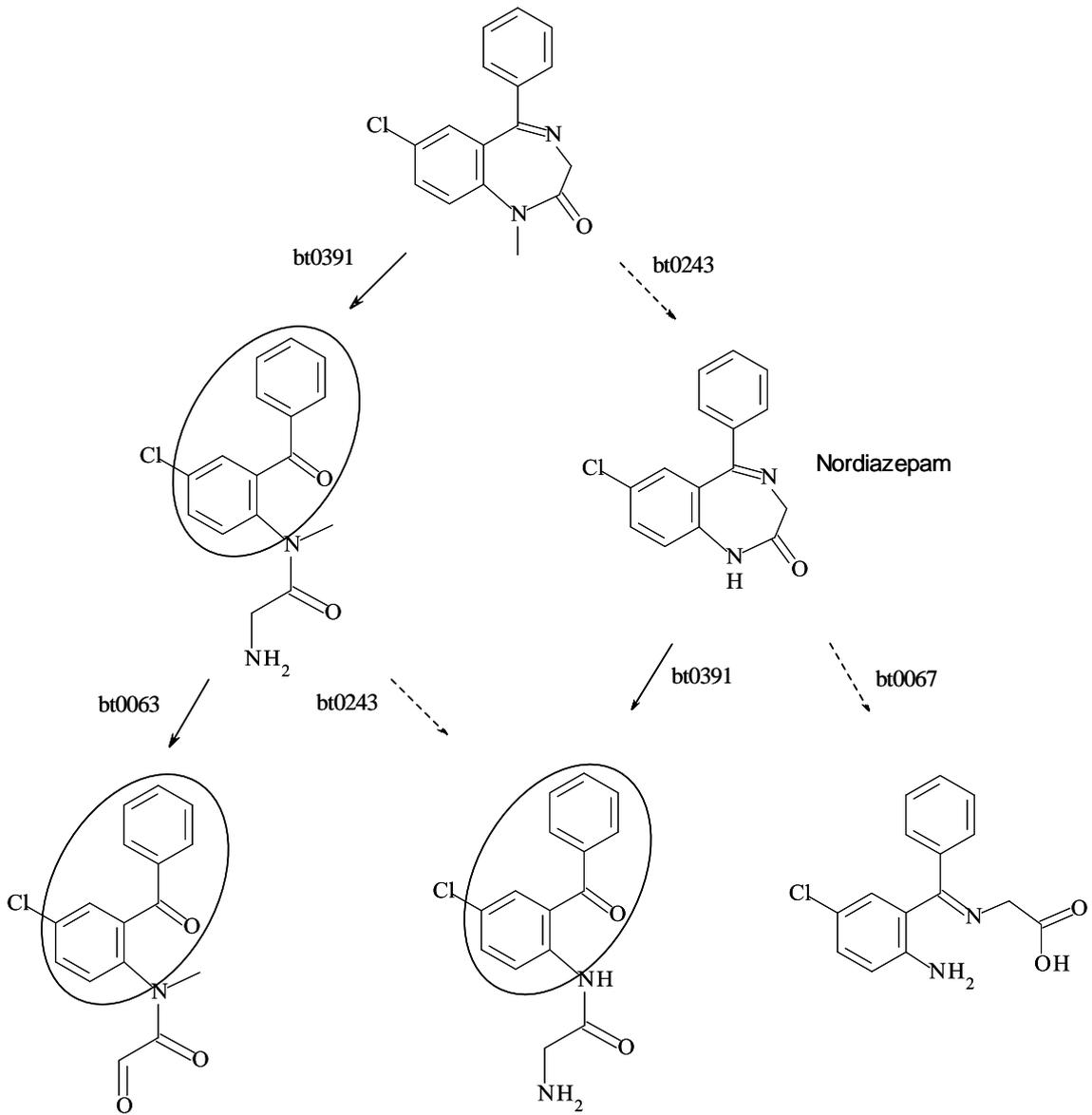
Diazepam

Rule	Probability	Comments	Enzymes
bt0391	Likely	This rule acts on all primary and cyclic imine groups not part of an aromatic system. Excludes thioamide S,S-oxide substrates	<i>trans</i> -ACOHDA hydrolase 1-aminocyclopropane-1-carboxylate deaminase 3-formiminopyruvate hydrolase 5-oxo-4,5-dihydropyrrole-2-carboxylate amidase
bt0063	Likely	Oxidative removal of an R group from an amine. An aldehyde is produced if the leaving R group is attached through a primary carbon. A ketone is produced if the leaving R group is attached through a secondary carbon. Rule will produce cis products in rings with double bonds	6-aminohexanoate transaminase, caffeine demethylase, cyclohexylamine oxidase, 2,6-diethyl-N-(methoxymethyl)aniline hydrolase, trimethylamine dehydrogenase, dimethylamine dehydrogenase, methylamine dehydrogenase, glyphosate dehydrogenase, hexadecyltrimethylammonium chloride monooxygenase, 6-hydroxy-L-nicotine oxidase, 6-hydroxy-D-nicotine oxidase, 6-hydroxypseudoxyntocine dehydrogenase, iminodiacetate dehydrogenase, methylamine dehydrogenase, N-methyltaurine dehydrogenase, heteroxanthine demethylase, nitrilotriacetate monooxygenase, nitrilotriacetate dehydrogenase, 3-nitrotyramine oxidase, 3-nitrotyrosine transaminase, paraxanthine demethylase, pyridoxamine-pyruvate transaminase, theobromine demethylase, trimethylamine N-oxide demethylase, tropine dehydrogenase, aromatic aminotransferase
bt0243	Neutral	Oxidative removal of an aliphatic R group from a secondary or tertiary urea or amide nitrogen. An aldehyde is produced if the leaving R group is attached through a primary carbon. A ketone is produced if the leaving R group is attached through a secondary carbon. Oxidative cleavage of the C-N bond in amides and ureas are covered by a single rule, since there is no chemical reason to divide them. Cleavage of urea derivatives occurs between the N and the C with the most positive partial charge. Rule will produce cis products in rings with double bonds	alachlor hydrolase, caffeine demethylase, 2-hydroxy-2',6'-diethyl-N-acetanilide hydrolase, hydroxymonomethylisoproturon dimethylaminatedehydrogenase, N-isopropylacetanilide monooxygenase, isoproturon dimethylaminatedehydrogenase, heteroxanthine demethylase, monodemethylisoproturon dimethylaminatedehydrogenase, paraxanthine demethylase, theobromine demethylase, theophylline demethylase
bt0065	Neutral	There are separate rules for amide and urea hydrolysis. However, microbial amidases have been shown to also hydrolyze environmental urea compounds	<i>p</i> -Acetamidophenol amidohydrolase, N-acetylanthranilate amidase, 6-aminohexanoate-cyclic-dimer hydrolase, 6-aminohexanoate-dimer hydrolase, <i>epsilon</i> -caprolactam lactamase, N-cyclohexylformamide amidohydrolase, N-(2,6-diethylphenyl)-2-hydroxyacetamide hydrolase, formylaminopyrimidine amidohydrolase, isonicotinic acid hydrazide hydrolase, 5-oxo-4,5-dihydropyrrole-2-carboxylate amidase

2-amino-5-chlorobenzophenone

Rule	Probability	Comments	Enzymes
bt0065	Neutral	Also handles fused rings. All fused aromatic ring products with hydroxyl at 2,3 position are excluded	acetanilide 1,2-dioxygenase, 2-aminobenzenesulfonate 2,3-dioxygenase, 4-aminobenzenesulfonate 3,4-dioxygenase (deaminating), anthranilate 3-monooxygenase, 4-aminobenzoate 3,4-dioxygenase (deaminating), aniline dioxygenase, 2-chloro-N-isopropylacetanilide 1,2-dioxygenase, N-isopropylaniline 1,2-dioxygenase
bt0353	Neutral	This rule handles the 2,3-dioxygenation of mono-substituted aromatics (bt0369) and subsequent oxidation to form the catechol derivative (bt0255). The substituents are based on "Reactions of Toluene Dioxygenase" and Hudlicky T, Gonzalez D, Gibson DT (1999) <i>Aldrichimica Acta</i> 32(2): 35-62. The aromatic hydrocarbon dioxygenases produce an activated dioxygen species that is thought to be sufficiently reactive to potentially functionalize most, if not all, aromatic ring carbon atoms	diphenyl ether 2,3-dioxygenase, 2-[(3-hydroxy(phenyl)methyl)phenyl]-propanoate dioxygenase
bt0351	Neutral	This rule handles extradiol (<i>meta</i>) ring cleavage for <i>vic</i> -dihydroxybenzenoids and 1-amino-2-hydroxybenzenoids, including, but not limited to, 2,3-dihydroxybiphenyl derivatives, many PCB congeners, 3- and 4-substituted chlorocatechols, 2-aminophenol, 2,3-dihydroxy DDT and its derivatives, 3- and 4-substituted alkyl catechols, diphenylether derivatives, and aromatized intermediates of steroid degradation (<i>e.g.</i> , testosterone)	No enzymes given
bt0254	Neutral	This rule does not handle 2,3-dihydroxy linear polyaromatics such as 2,3-dihydroxynaphthalene, 3-methylcatechol, 3-sulfocatechol, 3-fluorocatechol, 4-C-substituted catechol, 3,4-dihydroxyphenylacetate or 2,3,5-trihydroxytoluene derivatives. Though certain compounds are predicted as being cleaved exclusively by an intradiol or by an extradiol pathway, this does not mean that they can never be cleaved by the other pathway in certain environments or by certain organisms	anthracene-1,2-diol 1,2-dioxygenase, hydroxyquinol 1,2-dioxygenase, catechol 1,2-dioxygenase, 4,6-dichloro-3-methylcatechol 1,2-dioxygenase, 3,5-dichlorocatechol 1,2-dioxygenase, 3,6-dichlorocatechol 1,2-dioxygenase, 4,5-dihydroxybenzo(a)pyrene dioxygenase, protocatechuate 3,4-dioxygenase, 4,5-dihydroxypyrene dioxygenase, protocatechuate 3,4-dioxygenase type II

SI Figure 1



SI Figure 2

