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

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ABSTRACT

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

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

Introduction

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

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

Within the pH range for surface waters (5-9), dissolved diazepam is a neutral molecule[7]. It is stable with respect to chemical hydrolysis, and with sediment : water partition coefficients < 100 L kg^{-1} for both organic–rich (sewage solids) and organic–poor particles[7,15], little sorption (< 0.1 %) of the compound will occur at suspended sediment concentrations typical of low–turbidity rivers. Diazepam photo-degrades in water[5,16], yielding a range of products, including the water-soluble 2-amino-5-chlorobenzophenone (ACB; Fig. 1), a substituted benzophenone which appears relatively resistant to further photo-degradation[16]. As the photolysis half-life for diazepam under environmentally-relevant conditions ranges from 16 to168 h[5,16], conversion of diazepam to ACB may be an important abiotic removal process for diazepam in sunlit surface waters. However, hydroxylated benzophenones have been shown to exhibit estrogenic activity and their presence in surface waters has been reported[17,18], while concentrations of up to 130 ng L^{-1} of benzophenone have been detected in Korean rivers receiving wastewater effluent[19].

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

The observed or potential effects of benzodiazepines and benzophenones make the understanding of their fate in aquatic environments very important[9]. The aim of the present work was to investigate the biotic transformation of two representative compounds from these groups (diazepam and ACB) by natural, riverine bacterio-plankton communities using a specifically designed experimental protocol[20]. Incubations were undertaken in laboratory batch microcosms in the presence and absence of a readily degradable organic substrate that could act as a priming agent for xenobiotic removal[21,22]. Concentrations of the parent compounds were measured and the presence of metabolites investigated after an incubation period that reflected typical residence times for surface waters in these catchments. Finally, to investigate the effect of the presence of ACB on bacterio-plankton community structure and the ability of species present to metabolise ACB, sequential inoculation and incubation of Mersey and Tamar microcosms, for 5 periods of 21 days each, were undertaken.

Materials and Methods

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

Study areas

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

Incubation experiments

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

Preparation of incubation water: A bulk freshwater sample was collected from the tidal limit of the Tamar and Mersey rivers. The water was filtered (GF/F; 0.7 μm nominal pore size) to emove suspended particles, passed through a strong anion-exchange resin (Dowex® X-100, 200 mesh; water flow rate 80 mL h^{-1}) to remove nitrate, UV-irradiated (400 W medium pressure Hg lamp, 6 h) to remove dissolved organic matter (DOM) and then re-filtered through a 0.2 µm filter membrane (Whatman Anodisc 47, aluminium oxide) to remove any remaining particulate matter. These processes reduced nitrate and DOC concentrations to < 15 µM and < 60 µM, respectively, ensuring that competitive carbon and nitrogen substrates were, as far as possible, removed and that the river water matrix was compatible with direct analysis of samples by electrospray ionisation–mass spectrometry (ESI-MS). Finally, the water was sterilised by autoclaving (115 °C, 15 min). All incubation samples and standards for the experiment were then matrix-matched using this water.

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

Incubation experiments: Incubation water (60 mL) was transferred to a 125 mL screw-capped amber glass bottle to which was added 15–22 µL of stock diazepam or ACB solution and 1 mL of the bacterial inoculum. Starting concentrations of the compounds were approximately 30 µM. Pre-incubation Microtox® assays using the bacterium *Vibrio fischeri* showed that diazepam and ACB were non-toxic at these concentrations (EC_{50} >> 100 µM, 15 min exposure). In a separate set of incubations, the effect of labile DOM on the biotransformation of diazepam and ACB was tested by adding the tripeptide (equivalent to 90 µmol N L^{-1} and 510 µmol C L^{-1}) alongside diazepam and ACB. Control incubations of prepared river water containing bacterial inoculum only and diazepam/ACB only were also prepared to account for sorption effects. Ortho-phosphate was added to all incubations to give ca. 1-2 µM P at t = 0.

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

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

Chemical and microbiological analysis

Analyses by ESI-MS were performed in positive mode using a Finnigan MAT LCQ MS, a quadrupole ion trap mass spectrometer with an external source atmospheric pressure interface capable of electro-spray ionisation. The sample matrix was 50 : 50 methanol : water amended with 0.1 % (v/v) formic acid and solutions were introduced by low-flow infusion at a rate of 3 µL min^{-1}. Once thawed, each sample was diluted 1:1 with the mixed methanol and formic acid solution. Samples were then injected into the instrument. The signal sensitivity for both diazepam and ACB, in positive-ion mode, was optimised by adjustment of instrumental parameters using in-built tuning procedures. Ion count integration was performed for 2 minutes, with 5 replicates recorded per sample, while ion count stability was recorded in real time using single ion monitoring. Quantification of each analyte was achieved by generating an external calibration curve using matrix-matched standards on each analytical day, and bracketing individual samples with a drift matrix-matched calibration standard to account for variations in instrumental sensitivity; the variation was then calculated using an algorithm[20]. The mass spectra for both diazepam and ACB contained two isotopic peaks (due to ^{35}Cl and ^{37}Cl atoms). Base peaks (attributed to $[M+H]^+$) for diazepam and ACB occurred at *m/z* 285 and *m/z* 232, respectively; a single peak for tripeptide occurred at *m/z* 352. Nitrate+nitrite and ortho-phosphate were determined by segmented flow and spectrophotometric detection[24] and ammonium by o-phthaldialdehyde fluorescence[25]. Viable counts of bacterio-plankton were undertaken using 100 µL aliquots from the microcosms. These were diluted in phosphate-buffered saline solution and 100 µL of each dilution spread on half strength Luria Bertani agar (Merck, Germany) and incubated at 30 °C for two days. Colonies were enumerated as colony forming units (cfu) mL^{-1} of the original suspensions. Total counts of bacterio-plankton were determined microscopically by staining water samples with DAPI[26]. The microcosms contained bacterio-plankton populations of 10^5-10^6 cells per mL at both 0 and 21 days; approximately 10 % were recoverable as viable colonies on nutrient agar plates. DNA extraction followed[27]. Each microcosm water sample was membrane filtered (0.2 µm pore diameter) and the retained cells disrupted on the filter by mechanical bead beating. The DNA was extracted into hexadecyltrimethylammonium bromide and phenol-chloroform-isoamyl alcohol, and then resuspended in 50 µL nuclease-free water. Nested PCR amplifications were performed on extracted samples using Super Taq DNA polymerase and G-Storm thermal cyclers. DNA amplification was undertaken in a 50 µL sample using 1 µM of the universal primers for eubacterial 16S rRNA genes (27_f and 1492_r)[28] with 1 unit *super* Taq DNA polymerase. The amplified DNA fragments were re-amplified using forward primer 341 and reverse primer 907[29].

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

Results and Discussion

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

Diazepam

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

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

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

In the Mersey water microcosms, 26.0 ± 2.9 µM diazepam was added. After 21 days, concentrations of diazepam in the abiotic and both biotic incubations had not changed significantly (t-test, p range 0.06 – 0.86; Fig. 2a). The tripeptide was again consumed when added as an additional substrate (Fig. 2b). As the bacterio-plankton of an urban river might be expected to be responsive, having probably encountered the molecule previously, the absence of diazepam removal was surprising, particularly as River Tamar microcosms were able to effect significant removal of the diazepam (Fig. 2). A contrast in the removal of another xenobiotic, atrazine, was also observed in a previous study for incubations using bacterial populations from the same rivers[20]. There, 11 % removal over 21 days was observed in Tamar samples, when atrazine was the only substrate added, contrasting with 0 % removal in Mersey samples. However, addition of tripeptide increased removal from Mersey water from 0 to 37 %, while the Tamar removal value remained at 11 %. There are very few studies on the bacterio-plankton compositions of unconnected rivers. In the Santa Ana River basin (USA), urban impacted and rural, agriculturally impacted streams contained bacterio-plankton communities that showed few differences[35], suggesting that bacterial response to added xenobiotics might be similar. The bacterio-plankton populations in the incubations were prepared to give a final concentration that matched *in situ* measurements at the time of collection[20], so the contrast in the removal of diazepam between Tamar and Mersey waters reflects inoculum composition rather than cell numbers.

2-amino-5-chlorobenzophenone

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

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

Effect of ACB on bacterio-plankton community structure

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

In the experiment where microcosms were sequentially sub-cultured through five passages, ACB removal over the 21 day period of the final incubation set was 26 % and 44 % for the Tamar and Mersey, respectively, demonstrating the complete absence of the selection of a bacterial population acclimated for ACB degradation. It is our contention, therefore, that at the low concentrations of ACB, or its benzodiazepine precursors, which enter surface waters of urban or rural catchments[16], the xenobiotic is assimilated without significantly impacting the structure of the indigenous riverine microbial community.

Environmental implications

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

A schematic representation of how diazepam could be transported across the river-estuary continuum to reach coastal waters is proposed in Fig. 7. Bacterio-plankton removal of diazepam could occur if background labile DOM concentrations are low (i.e. absence of competitive substrates). If physical conditions facilitate photodegradation (direct and/or indirect)[16] of diazepam to ACB, then the ACB will be mineralized by bacterio-plankton. However in turbid rivers and estuaries, photo-degradation to ACB could be inhibited, leading to the advection of diazepam to low turbidity coastal waters and its subsequent photo-degradation to ACB in sunlit surface layers. This pathway may also be applicable to other pharmacologically-active 1,4-benzodiazepine molecules known to degrade to ACB (e.g. oxazepam, temazepam and nordiazepam), which would be significant given the reported ecological effects on freshwater fish exposed to environmental concentrations of oxazepam[14].

Conclusions

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

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Notes and references

Electronic Supplementary Information available

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 SI Figures 1 and 2.

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

SI Fig. 2 UMBDD prediction pathways (to tier 2) of the aerobic bacterial biotransformation of ACB. The ‘btxxxx’ annotation refers to the specific enzymic reaction 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.)**	**(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

[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.

Figure Captions

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

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

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

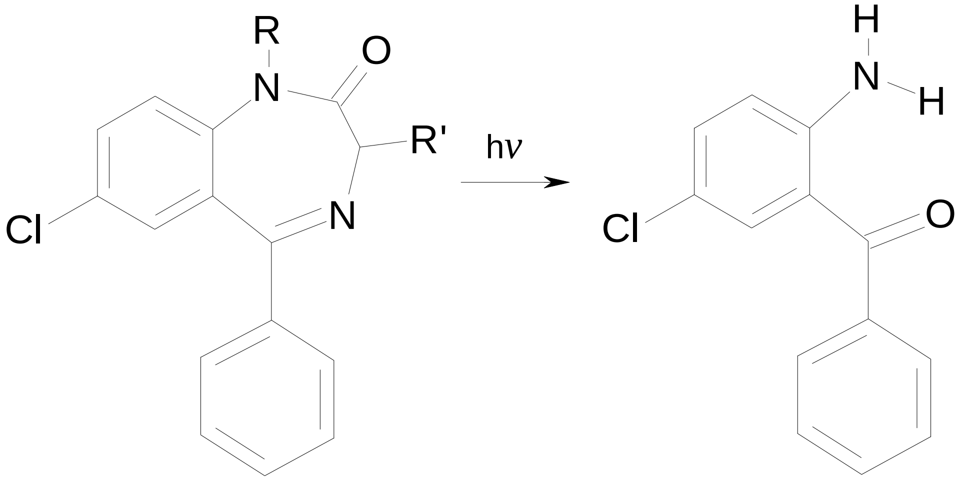
Fig. 4. Concentrations (µM) in solution at $t = 0$ and $t = 21$ days in the Tamar and Mersey incubations. (a) ACB (b) ammonium (c) nitrate+nitrite and (d) ortho-phosphate. Error bars represent ± 1σ of the results from duplicate incubations with each sample analysed 3 - 5 times ($n = 6 - 10$).

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

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

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

Fig 1



	R	R'
Diazepam	CH_3	H
Nordiazepam	H	H
Temazepam	CH_3	OH
Oxazepam	H	OH

2-amino-5-chloro-benzophenone

Fig. 2

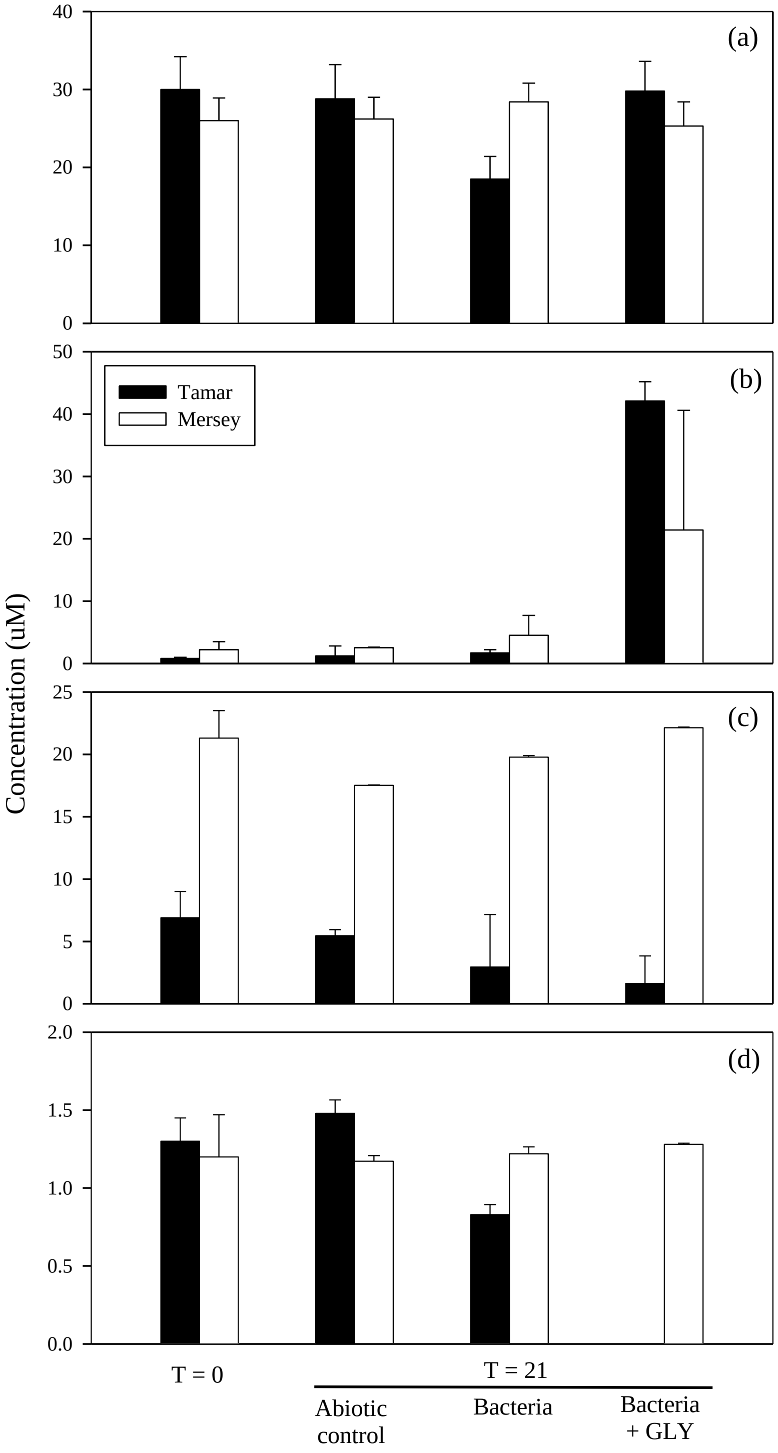

Concentration (uM)
(a)
40
30
20
10
0
(b)
50
40
30
20
10
0
Tamar
Mersey
(c)
25
20
15
10
5
0
(d)
2.0
1.5
1.0
0.5
0.0
T = 0
T = 21
Abiotic control
Bacteria
Bacteria + GLY

Fig. 3

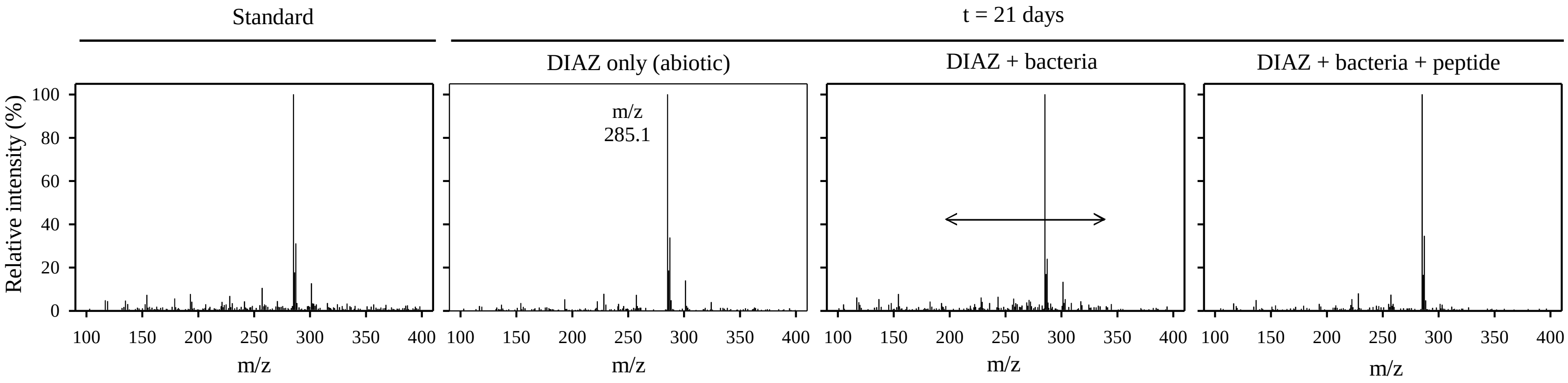

Standard
t = 21 days
DIAZ only (abiotic)
DIAZ + bacteria
DIAZ + bacteria + peptide
Relative intensity (%)
100
80
60
40
20
0
m/z
285.1
100
150
200
250
300
350
400
m/z

Fig. 4

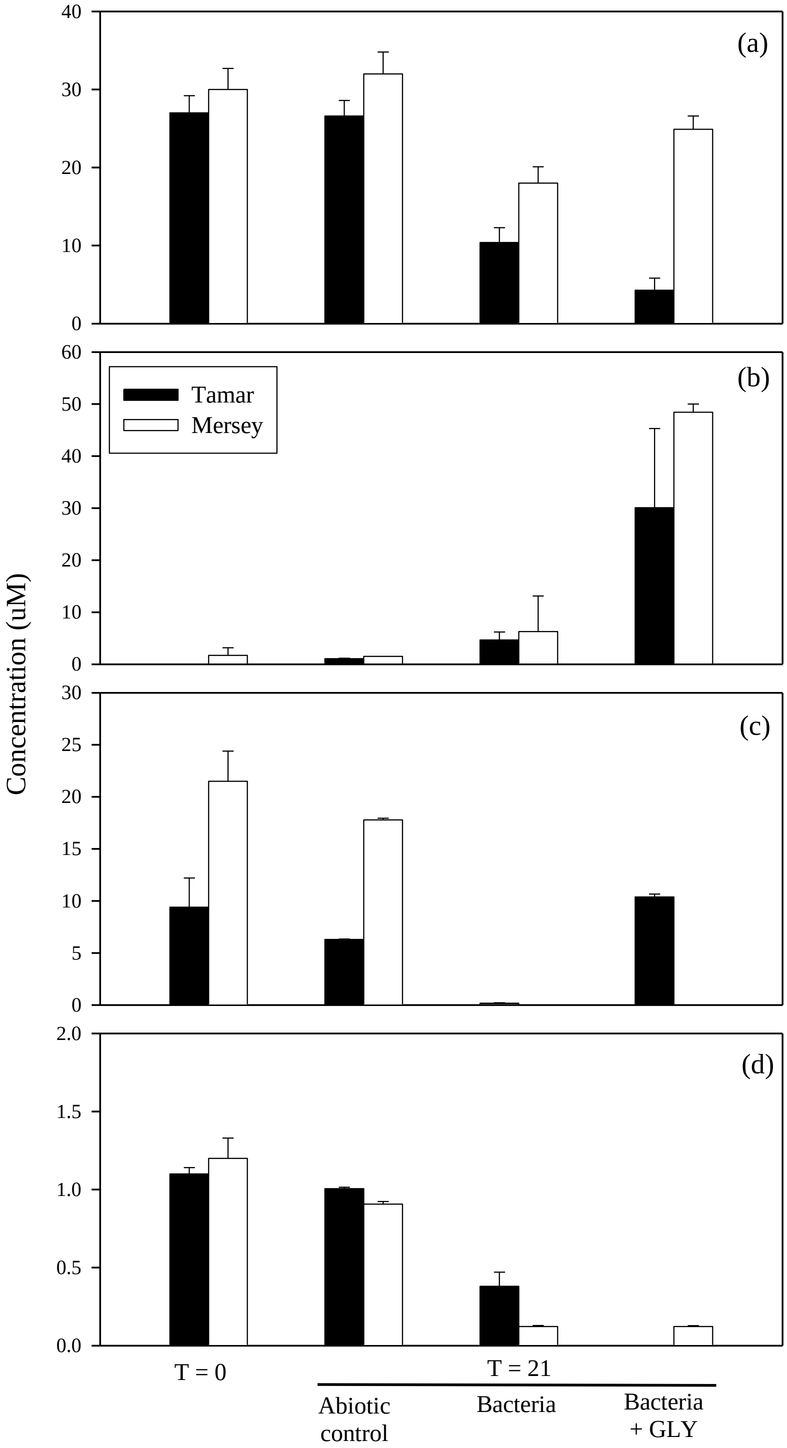

Concentration (uM)
(a)
40
30
20
10
0
(b)
60
50
40
30
20
10
0
Tamar
Mersey
(c)
30
25
20
15
10
5
0
(d)
2.0
1.5
1.0
0.5
0.0
T = 0
T = 21
Abiotic control
Bacteria
Bacteria + GLY

Fig. 5

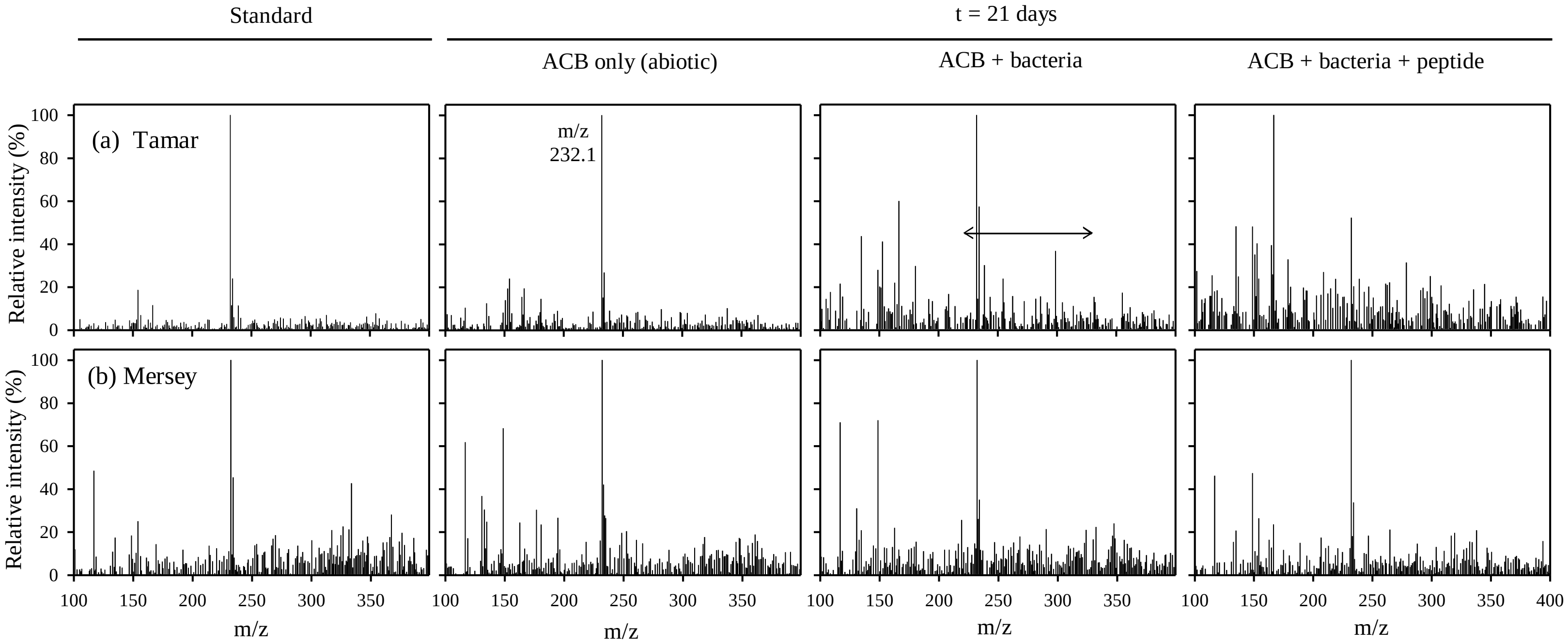

Standard
t = 21 days
ACB only (abiotic)
ACB + bacteria
ACB + bacteria + peptide
(a) Tamar
(b) Mersey
m/z
232.1
Relative intensity (%)
0
20
40
60
80
100
100
150
200
250
300
350
400
m/z

Fig. 6

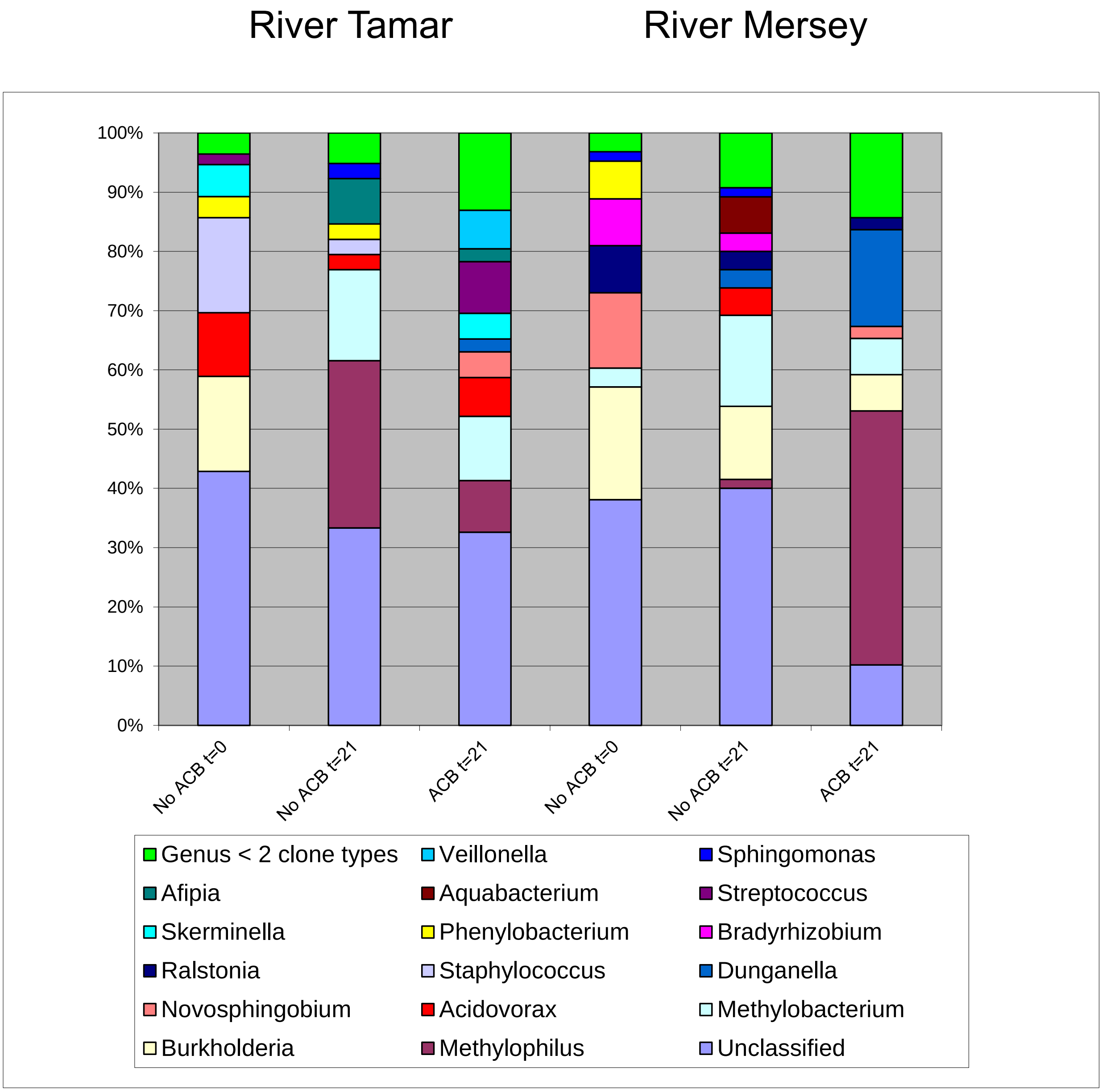
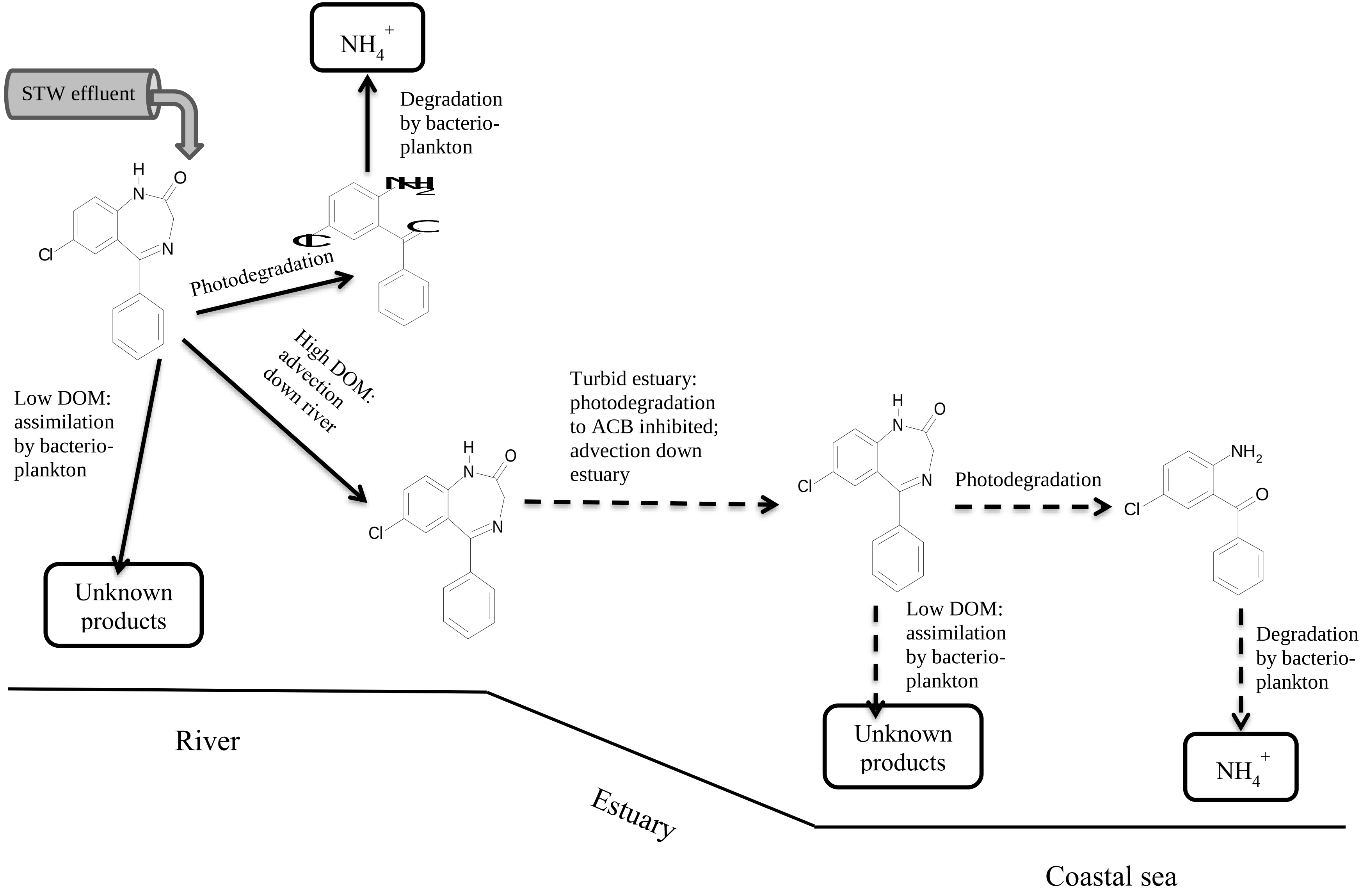

River Tamar
River Mersey
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
No ACB t=0
No ACB t=21
ACB t=21
No ACB t=0
No ACB t=21
ACB t=21
Genus < 2 clone types
Veillonella
Sphingomonas
Afipia
Aquabacterium
Streptococcus
Skerminella
Phenylobacterium
Bradyrhizobium
Ralstonia
Staphylococcus
Dunganella
Novosphingobium
Acidovorax
Methylobacterium
Burkholderia
Methylophilus
Unclassified

Fig 7


STW effluent
NH_4^+
Degradation by bacterio-plankton
Photodegradation
High DOM: advection down river
Low DOM: assimilation by bacterio-plankton
Unknown products
Turbid estuary: photodegradation to ACB inhibited; advection down estuary
Photodegradation
Low DOM: assimilation by bacterio-plankton
Unknown products
Degradation by bacterio-plankton
NH_4^+
River
Estuary
Coastal sea

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.

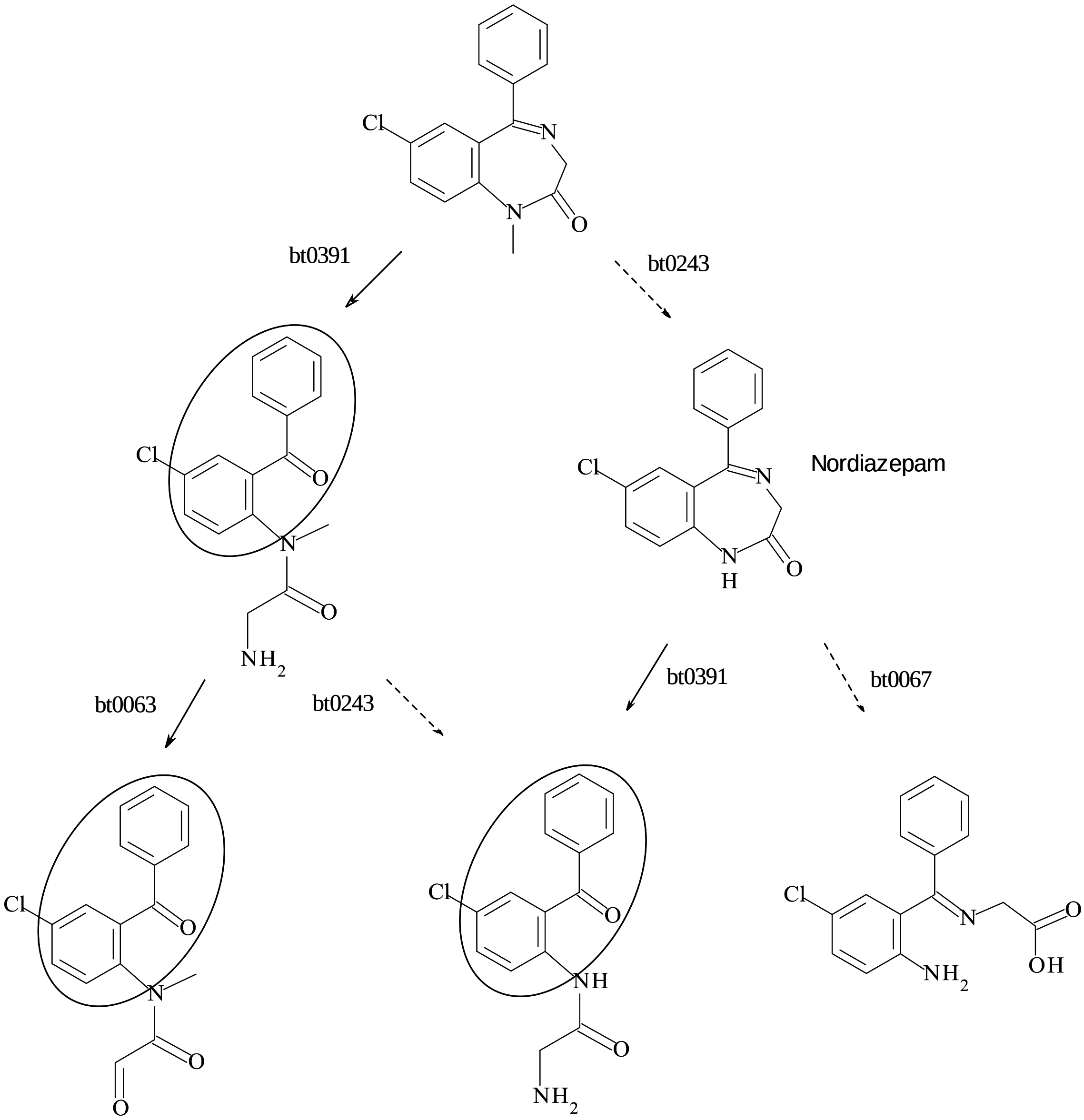
Diazepam

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	*trans*-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-hydroxypseudooxynicotine 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 dimethylaminedehydrogenase, N-isopropylacetaniline monooxygenase, isoproturon dimethylaminedehydrogenase, heteroxanthine demethylase, monodemethylisoproturon dimethylaminedehydrogenase, 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	*p*-Acetamidophenol amidohydrolase, N-acetylanthranilate amidase, 6-aminohexanoate-cyclic-dimer hydrolase, 6-aminohexanoate-dimer hydrolase, *epsilon*-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-dixoxygenase
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) Aldrichimica Acta 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 (*meta*) ring cleavage for *vic*-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 (*e.g.*, 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


Cl
N
N
O
bt0391
bt0243
Cl
O
N
O
NH2
Cl
N
Nordiazepam
N
H
O
bt0063
bt0243
bt0391
bt0067
Cl
O
N
O
O
Cl
O
NH
O
NH2
Cl
N
O
OH
NH2

SI Figure 2