Occurrence of carbamazepine, diclofenac, and their related metabolites and transformation products in a French aquatic environment and preliminary risk assessment

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Abstract

With questions emerging on the presence and risks associated with metabolites and transformation products (TPs) of organic contaminants in the aquatic environment, progress has been made in terms of monitoring and regulation of pesticide metabolites. However, less interest is shown for pharmaceutical residues, although their pseudo-persistence and adverse effects on non-target organisms are proven. This study provides original knowledge about the contamination of ten sites located along three French rivers (water, sediments, biofilms, clams) by pharmaceutical metabolites and TPs, as well as a preliminary environmental risk assessment. Studied compounds included carbamazepine with five metabolites and TPs, and diclofenac with three metabolites and TPs. Results show that metabolites and TPs are present in all studied compartments, with mean concentrations up to 0.52 µg L^{-1} in water, 229 ng g^{-1} in sediments, 2153 ng g^{-1} in biofilms, and 1149 ng g^{-1} in clams. QSAR estimations (OECD toolbox) were involved to predict the studied compounds ecotoxicities. QSAR models showed that diclofenac and its metabolites and TPs could be more toxic than carbamazepine and its metabolites and TPs to three aquatic species representing green algae, invertebrates, and fish. However, real ecotoxicological effects are still to be determined. The environmental risk assessment showed that hydroxydiclofenac, 2-[(2-chlorophenyl)-amino]-benzaldehyde and

dibenzazepine could present a greater risk than other studied compounds for aquatic organisms. In addition, the risk associated with a mixture of diclofenac and its related metabolites and TPs has been found to be greater than that of the compounds considered individually.

Keywords

Pharmaceuticals, metabolites and transformation products, ecotoxicity, risk assessment, rivers

Highlights

- Pharmaceutical degradation products are found in several aquatic compartments.
- Levels of degradation products are comparable with those of their parent compounds.
- By-products have similar or greater risk than pharmaceuticals in water and sediment.
- Some mixtures have a greater risk than pharmaceuticals considered individually.

1. Introduction

Pharmaceuticals enter into rivers mainly through wastewater discharge (wastewater treatment plants – WWTPs or septic tanks) and, to a lesser extent, from livestock (Daughton & Ternes, 1999; Fent *et al.*, 2006). These compounds are biologically active and are selected for their specific actions on the human or animal organism. Therefore, an exposure outside the medical or veterinary setting may lead to unwanted effects (toxic or disruptive effects) on living organisms. Many studies have already reported developmental disorders in frog tadpoles (Foster *et al.*, 2010), fish feminization (Petrovic *et al.*, 2002), changes in diversity and abundance of microbial communities (Liebig *et al.*, 2010), *etc.* related to the exposure to pharmaceutical residues (at concentrations ranging from ng L^{-1} to µg L^{-1}) *via* the aquatic

environment. In the long term, all these effects could have major consequences on biodiversity and water use (bathing, drinking water resources) in relation to human health: e.g. exposure to endocrine disruptors or resistant pathogenic microorganisms (Amarasiri *et al.*, 2020; Gonsioroski *et al.*, 2020).

In the environment, pharmaceuticals are found not only as active molecules (i.e. parent compounds), but also as metabolites and biotic or abiotic transformation products (TPs) (Patel *et al.*, 2019). Pharmaceuticals are first transformed in the human or animal body after uptake. They are partially metabolized into more polar and soluble forms (pharmacologically active or not) by a variety of oxidative and conjugative enzymes (glucuronide conjugation, sulfoconjugation, acetylation, amino acid conjugation, glutathione conjugation and methylation) (Jančová & Šiller, 2012). These transformations facilitate their elimination through urine and faeces. Other transformation pathways occur in wastewaters during their treatment in WWTPs. Pharmaceutical compounds and their metabolites are transformed by biodegradation and physicochemical reactions (e.g. hydrolytic cleavage of amide bonds, ether cleavage, demethylation, hydroxylation…) during secondary treatment (Quintana *et al.*, 2005; Barra Caracciolo *et al.*, 2015). Once released in the environment, pharmaceutical compounds and their metabolites are further transformed by direct photodegradation initiated by sunlight, or indirect photodegradation in the presence of free radicals or singlet oxygen (generated by the action of UV rays on natural organic matter) (Andreozzi *et al.*, 2003). Chemical hydrolysis and oxydo-reduction reactions can also lead to their transformation (as a function of pH and temperature). Another main transformation route in the aquatic environment involves biodegradation by different families of microorganisms if the molecules toxicities do not inhibit microbial activity (Barra Caracciolo *et al.*, 2015).

While the presence of pharmaceutical compounds in natural waters has been demonstrated since the 1980s (Patel *et al.*, 2019), many studies report an accumulation of these

contaminants in other aquatic compartments (Kümmerer, 2004). Thus, sediments can accumulate pharmaceutical compounds or their metabolites and TPs (Li *et al.*, 2014; Tamtam *et al.*, 2011) but also favor microbial degradation processes (Kunkel & Radke, 2008). Several studies have reported significant accumulation of pharmaceuticals in benthic organisms: river biofilms (Aubertheau *et al.*, 2017; Huerta *et al.*, 2016) and invertebrates (Burket *et al.*, 2019; de Solla *et al.*, 2016; Du *et al.*, 2015; Xie *et al.*, 2019), as well as in fish (Brooks *et al.*, 2005; Huerta *et al.*, 2013; Xie *et al.*, 2019). Only aquatic plants seem not to accumulate these compounds, or only weakly (Wilkinson *et al.*, 2018). Such a widespread presence of pharmaceuticals makes it evident that metabolites and TPs of pharmaceutical residues could also be present in many of these compartments. The issue is all the more important because metabolites and TPs can exhibit toxicity to living organisms, which can be lower, similar, or even higher than the parent compounds (Bleeker *et al.*, 1999; Bort *et al.*, 1999; Bourgeois & Wad, 1984; Schulze *et al.*, 2010). However, the risks associated with these metabolites and TPs, as well as their presence in the environment, are still very little studied due to their very large number and the absence of analytical standards for their quantification.

Questioning relating to the presence of organic contaminants metabolites and TPs in natural waters and drinking water are emerging more and more worldwide. This is largely due to the knowledge gained on pesticide metabolites and their incidence on health. In France and other European countries, the presence of pesticide residues and their metabolites in water intended for human consumption is regulated by Council Directive 98/83/EC which sets quality limits for pesticides and their relevant metabolites of 0.1 µg L^{-1} per individual substance and 0.5 µg L^{-1} for the sum of these molecules (European Council, 1998). To date, no regulation relating to the quality of natural waters includes pharmaceutical residues. However, six molecules or families of molecules (17-alpha-ethinylestradiol, 17-beta-estradiol, estrone, macrolide antibiotics, amoxicillin, ciprofloxacin) are included on the Watch list of substances for Union-

wide monitoring established in 2018 by Implementing Decision (EU) 2018/840 (European Commission, 2018).

The issue of risk assessment is today very important because of the rise of all sorts of emerging pollutants that form an "environmental soup" of a multitude of compounds, in large majority at low concentrations. Indeed, it has been shown that organic pollutants may have unpredictable biochemical interactions when mixed together, leading to different effects of what might be observed when evaluating the toxicity of a single molecule (Filby *et al.*, 2007; Sumpter & Johnson, 2005).

The present work draws up a first assessment of the contamination of the aquatic environment by pharmaceutical metabolites and TPs. Thus, two "iconic" pharmaceutical compounds (carbamazepine – CBZ and diclofenac – DCF) and eight of their associated metabolites and TPs were quantified in water, sediments, biofilms, and clams (*Corbicula fluminea*) from rivers of Western France. Then, the QSAR toolbox (OECD & European Chemicals Agency, 2020) was used to predict the missing toxicity data for these compounds considered individually or in mixtures. This allowed a first environmental risk assessment in French waters and sediments and then to open the discussion about the relevance of considering pharmaceutical metabolites and TPs for future guidelines and regulations.

2. Material and methods

2.1.Target compounds

Carbamazepine, Diclofenac, and the metabolites and TPs considered in this work are presented in Table 1. Analytical grade standards of carbamazepine (CBZ), 3-hydroxycarbamazepine (3OH-CBZ), 10,11-epoxycarbamazepine (CBZ-epox), dibenzazepine (Dibenz), acridone (Acrid), diclofenac (DCF), 4'-hydroxydiclofenac (4'OH-DCFBZ) and 5-

hydroxydiclofenac (5OH-DCF) were purchased from Sigma-Aldrich (Darmstadt, Germany), while standards of 10,11-dihydro-10,11-trans-dihydroxycarbamazepine (Trans-CBZ) and 2-[(2-chlorophenyl)-amino]-benzaldehyde (Benz) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany) and Synchem UG & Co. KG (Altenburg, Germany), respectively. Individual stock solutions were prepared in LC-MS grade methanol (Carlo Erba Reagents, Val de Reuil, France) at 200 mg L^{-1} and stored at -20 °C.

2.2.Study area and sampling strategy

A sampling campaign was conducted in September 2018 along three rivers (Vienne, Clain and Thouet) located in the same part of Western France (North of the Vienne and South of the Deux-Sèvres French departments) (Figure 1). Their watersheds present close rural/urban occupation, agricultural/socio-economic activities, and hydro-climatic conditions. They are typical of this part of France. The sampling took place at the end of summer, during the low-water flow period, thus facilitating accessibility to the studied compartments.

Ten different sampling sites were selected based on their location: upstream or downstream from urban areas (Figure 1). On each site, a surface of 20 m^2 was prospected to collect samples of all four compartments of interest. Thus, 2 L of water were grab sampled in a high-density polyethylene (PEHD) bottle. Biofilms were collected from ten rocks chosen randomly at a depth of 50 to 100 cm. For that, rock surface was scrapped with a clean toothbrush and ultrapure water (Milli-Q IQ 7000, Merck KGaA, Darmstadt, Germany). Bulk sample of surface sediment was collected as a composite sample, with a shovel, by pooling three samples at each location, and sieved on site to < 2 mm. Approximately 100 clams (*C. fluminea*) with a size > 16 mm were collected by hand. All samples were transferred to clean PEHD bottles and stored in a cool box until the end of the sampling day.

2.3.Extraction of target compounds

Target analytes were extracted from solid matrices (i.e. biofilms, sediments and clams) using pressurized liquid extraction (PLE) (ASE 350, Thermo Scientific Inc., Waltham, USA) followed by solid phase extraction (SPE) (Autotrace 150, Thermo Scientific Inc., Waltham, USA). The method developed by Aubertheau *et al.* (2017) was used for biofilms and sediments, while the method developed by Alvarez-Muñoz *et al.* (2015) was adapted for *C. fluminea*. Tables SI-1 and SI-2 summarize the conditions used by matrix. For each sample, two extraction cells were prepared. Each of the two obtained extracts was diluted with ultrapure water to 500 mL for biofilms and *C. fluminea*, and to 1 L for sediments, and loaded on two SPE cartridges. Then, the four obtained extracts were evaporated to dryness under a gentle stream of nitrogen at 30 °C (TurboVap LV, Biotage AB, Uppsala, Sweden). Finally, they were each recovered in methanol/water (10/90; v/v) spiked with increasing concentrations of a standards mixture (final concentrations of 0, 0.02, 0.05 and 0.1 mg L^{-1}) for standard addition quantification.

Recoveries were determined by spiking each matrix with all analytes to a concentration of 400 ng g^{-1} dry weight and allowing it to dry overnight in the dark at room temperature, before performing extraction and analysis. This procedure was repeated three times. Recoveries were between 19-123 % for biofilms, 30-190 % for sediments, and 37-142 % for clams (see Table SI-3).

2 L of each water sample were filtered through a 0.45 µm PVDF membrane (Durapore, Merck KGaA, Darmstadt, Germany) before SPE extraction of compounds on Oasis HLB (6 cc, 200 mg) cartridges (Waters Corporation, Milford, USA). Table SI-4 shows the extraction conditions. Samples were each loaded on four cartridges, and the four obtained extracts underwent evaporation and restitution with the same conditions as solid matrices.

2.4.Quantification of target compounds

Compounds were separated using ultra high performance liquid chromatography (UHPLC) (UltiMate 3000, Thermo Scientific Inc., Waltham, USA) on an Acquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm; Waters Corporation, Milford, USA) kept at 30 °C. A flow rate of 0.4 mL min^{-1} was used with a mobile phase composed of water and methanol, both acidified with 0.1 % formic acid. A gradient ranging from 10 % to 99 % of methanol in 13 min was applied.

The UHPLC system was coupled with a hybrid quadrupole – time-of-flight (Q-ToF) mass spectrometer (Impact HD QTOF, Bruker GmbH, Champs sur Marne, France) equipped with an electrospray ion source (ESI) and operated in broadband collision-induced dissociation (bbCID) mode. The mass range was from 150 to 500 m/z, the capillary tension was set to 2700 V, the dry gas flow rate was 4.0 L min^{-1}, and the dry temperature 250 °C. Table SI-5 lists the retention times and the exact ionized masses used for identifying all compounds. 4′OH-DCF and 5OH-DCF were subsequently grouped together as "OH-DCF" due to poor chromatographic separation.

Quantification by standard addition was chosen to normalise for matrix effects. In addition, the analytical method's performance was assessed *via* linearity, limits of detection and quantification, repeatability and reproducibility, according to the French AFNOR XP T90-210 standard (AFNOR, 1999) (detailed methods are presented in SI-6 and Table SI-7). All limits of detection and quantification are summarized in Table SI-8.

2.5.Metabolites and TPs ecotoxicity

2.5.1. Prediction of ecotoxicity data

Ecotoxicity data of pharmaceutical metabolites or transformation products are often rare or even not available at all. For this reason, the OECD Quantitative Structure Activity

191 Relationship (QSAR) Toolbox (version 4.4) was used to generate environmental toxicity
192 endpoints. This toolbox was developed by the Laboratory of Mathematical Chemistry (OASIS
193 LMC, Bourgas, Bulgaria) for the OECD, in collaboration with the European Chemicals
194 Agency (ECHA). Its main objective is to use QSAR methodologies to group chemicals into
195 categories depending on their structure and/or modes of action. This helps to fill data gaps for
196 a given compound based on the data available for its analogues, by read-across (used for
197 qualitative endpoints), trend analysis (used for quantitative endpoints if a high number of
198 analogues with experimental results are identified) or QSAR models. Blázquez *et al.* (2020)
199 determined the acute toxicity for aquatic organisms of a biocidal active substance and its
200 metabolites to assess the suitability of available QSAR models to predict the obtained values.
201 The advantages of the OECD QSAR Toolbox were also detailed by Schultz *et al.* (2018).

202 In this study, the trend analysis approach was used to estimate acute toxicities of individual
203 compounds with respect to the green algae *Pseudokirchneriella subcapitata* (EC_{50}, growth
204 rate, 96 h), the invertebrate *Daphnia magna* (LC_{50}, mortality, 48 h), and the fish *Pimephales*
205 *promelas* (LC_{50}, mortality, 96 h). This approach involves modeling the relationship between
206 the considered toxicity endpoint and a physicochemical property (by default, the Log K_{OW}),
207 using the experimental data available for the compound's analogues.

208 The toxicity estimations obtained for the individual molecules were also used for the
209 prediction of the acute toxicity of a mixture of parent molecule and its associated metabolites
210 and TPs, respecting the molar fractions observed from the average concentrations measured in
211 the studied waters (OECD, 2020). For the two mixtures considered (CBZ and its metabolites
212 and TPs; DCF and its metabolites and TPs), it appears that the compounds exhibit different
213 modes of action according to the acute aquatic toxicity classification by Verhaar (Modified),
214 the ECOSAR (ECOSAR is the Ecological Structure Activity Relationships predictive model
215 developed by the US-EPA) classification for aquatic toxicity, and the acute aquatic toxicity

modes of action classification by OASIS (OASIS is the laboratory that developed the QSAR Toolbox). Consequently, trend analysis calculations were performed considering an independent modes of action approach (OECD, 2020).

2.5.2. Environmental risk assessment

According to the ECHA guidelines (European Chemicals Agency, 2008), experimental or calculated toxicity endpoints cannot be directly used for an environmental risk assessment because natural conditions are different than laboratory test conditions. Indeed, ecosystems are more sensitive to chemical compounds than individual organisms in a laboratory environment. For this reason, these endpoints (LC_{50} or EC_{50}) are used for the estimation of predicted no effect concentrations in water ($PNEC_{water}$). $PNEC_{water}$ are obtained by dividing the lowest ecotoxicity value by an appropriate assessment factor. The scarcer the available data, the higher is the applied assessment factor. In this study, only short-term toxicity data were available. The estimation of effects on organisms throughout their life cycle was therefore less realistic than with long-term data. Hence, a high assessment factor of 1000 was applied (European Chemicals Agency, 2008).

Then, the hazard quotient (HQ) was calculated by dividing the maximal environmental concentration (MEC) measured in water by the calculated $PNEC_{water}$. When the HQ is less than 0.1, the concerned compound does not present any risk for aquatic organisms. An HQ between 0.1 and 1 indicates a medium risk, while a value above 1 means that the studied molecule exhibits a high risk for the aquatic ecosystem (Straub, 2002).

For the sediment, no ecotoxicity parameter could be predicted by the QSAR Toolbox. Therefore, the equilibrium partitioning method (EPM), described in the European Chemicals Bureau technical guidance document (European Chemicals Bureau, 2003), was used to derive a $PNEC_{sediment}$ from the $PNEC_{water}$. Thus, $\boldsymbol{PNEC_{sediment} = (0.783 + 0.0217 * K_{OC}) *}$

240 $\boldsymbol{PNEC_{water}}$ where $\boldsymbol{K_{OC}}$ represents the organic carbon/water partition coefficient, hence
241 providing an estimate of the ability of a compound to be adsorbed on the organic matter of the
242 sediment. Indeed, a Log K_{OC} greater than 3 indicates potentially significant adsorption on the
243 sediment (Tissier *et al.*, 2005). Therefore, the $PNEC_{sediment}$ value is associated with the
244 presence of organic matter in the sediment. The MEC is expressed in micrograms of pollutant
245 per gram of dry sediment, without consideration of organic carbon content, which is of 1 %
246 on average for this study's sediments. For the calculation of HQ, the corrected MEC was used
247 to consider the part of organic carbon present in the sediment.

248 **3. Results and discussion**

249 **3.1.Occurrence of pharmaceutical metabolites and TPs in rivers**

250 All studied metabolites and TPs and their parent compounds were found in all samples from
251 the three rivers. This finding highlights the large occurrence of pharmaceutical metabolites
252 and TPs in the aquatic environment. Figure 2 and Figure 3 show mean concentrations (±
253 standard deviation) of DCF and CBZ with their associated metabolites and TPs in waters,
254 biofilms, sediments, and clams (N.B.: full data are presented in Table SI-9).

255 For the CBZ family, mean concentrations in water range from 0.18 ± 0.09 µg L^{-1} for Trans-
256 CBZ to 0.52 ± 0.13 µg L^{-1} for CBZ. In biofilms, concentrations are between 258 ± 181 ng g^{-1}
257 for Trans-CBZ and 1735 ± 811 ng g^{-1} for Dibenz. In sediments, concentrations are between 48
258 ± 25 ng g^{-1} for 3OH-CBZ and 128 ± 56 ng g^{-1} for Dibenz. Finally, in clams, levels range from
259 345 ± 218 ng g^{-1} for Trans-CBZ to 1066 ± 566 ng g^{-1} for Acrid. These results show that mean
260 concentrations of Dibenz are higher than those of CBZ in most matrices. Acrid is also found
261 at levels close to those of CBZ. The other metabolites and TPs are generally less concentrated.

262 For the DCF family, the data show that metabolites and TPs are more concentrated than the
263 parent compound. Thus, mean concentrations in waters range from 0.19 ± 0.27 µg L^{-1} for

Benz to 0.26 ± 0.15 µg L^{-1} for OH-DCF. In biofilms, levels vary from 348 ± 349 ng g^{-1} for DCF to 2153 ± 1532 ng g^{-1} for Benz. In sediments, concentrations range from 51 ± 55 ng g^{-1} for DCF to 229 ± 250 ng g^{-1} for Benz. Finally, in clams, concentrations are between 493 ± 428 ng g^{-1} for DCF and 1149 ± 916 ng g^{-1} for OH-DCF.

It is worth noting that CBZ and its metabolites and TPs are generally present at higher concentrations than DCF and its metabolites and TPs in water and sediments, while DCF and its metabolites and TPs are more accumulated than CBZ and its metabolites and TPs in biofilms (especially Benz) and clams.

As shown in Table 2, in waters, CBZ concentrations are close to the levels determined by Koba *et al.* (2018) in a Czech pond used for the tertiary treatment of wastewater effluent (290 – 560 ng L^{-1}), but higher than the concentrations measured by Du *et al.* (2014) in a watercourse impacted by WWTP releases in central Texas, United States (370 ± 14 ng L^{-1}). DCF levels are of the same order of magnitude as those determined by Koba *et al.* (2018) (22 – 870 ng L^{-1}), or by Wilkinson *et al.* (2017) in three rivers of southern England (<0.96 – 253 ng L^{-1}), but higher than those measured by Du *et al.* (2014) (86 ± 55 ng L^{-1}). Then, in biofilms, the levels of CBZ are higher than those measured by Aubertheau *et al.* (2017) in the Vienne River. For example, at the downstream site of the Châtellerault WWTP, the CBZ concentration in this study is 1095 ± 28 ng g^{-1}, while in the study by Aubertheau *et al.* (2017) it was 583.5 ng g^{-1}. Likewise for the DCF concentration, which amounts to 64 ± 26 ng g^{-1} at the downstream site of the Châtellerault WWTP in this study, and to 37.2 ng g^{-1} in the study by Aubertheau *et al.* (2017). In addition, in sediments, Koba *et al.* (2018) determined much lower levels of CBZ (5.1 – 16 ng g^{-1}) and DCF (2.6 – 30 ng g^{-1}). Finally, CBZ was not detected in clams from Taihu Lake in China (Xie *et al.*, 2015), and DCF concentrations (1.41 – 5.42 ng g^{-1}) were lower than those measured in this study (493 ± 428 ng g^{-1}).

Metabolites and TPs are much less studied than their related parent pharmaceuticals (Table 2). Stülten *et al.* (2008) detected 4'OH-DCF and 5OH-DCF in WWTP effluents in Germany, at concentrations higher than the levels measured in this study, up to 0.71 µg L^{-1} and 0.45 µg L^{-1}, respectively. In their study of a Canadian river, Miao & Metcalfe (2003) did not detect 3OH-CBZ or CBZ-epox in any sample, but determined concentrations of Trans-CBZ of 2.2 ± 0.3 ng L^{-1}, much lower than those measured in this study. Koba *et al.* (2018) detected CBZ-epox and Trans-CBZ in a lagoon water at concentrations up to 71 ng L^{-1} (lower than in this study) and 490 ng L^{-1} (higher than in this study), respectively, and in the sediment at concentrations lower than the limit of quantification. Finally, Aubertheau *et al.* (2017) detected CBZ-epox in the Vienne River biofilms with a maximal concentration estimated at 5.3 ng g^{-1} which is lower than those determined in this study.

The distributions observed for the different compartments can be explained by several parameters/conditions which probably interact between them. First, the contamination of biofilms, sediments, and clams depends on the compounds carried by water. However, the composition of water can change rapidly (Ort *et al.*, 2010) while that of solid matrices is constrained by sorption/desorption kinetics (Gonzalez *et al.*, 2012). Therefore, concentrations measured in water samples represent an image of the contamination over a short period of time while those determined in solid matrices are the result of accumulation over a certain exposure period. This could explain the significant difference observed in the distribution of compounds in water compared to the other matrices, especially for the DCF family for which sediments and biofilms are characterized by a larger presence of Benz in comparison with water. The differences are less significant for CBZ and its metabolites and TPs – especially between water and sediment – but some compounds like Dibenz may have significant variations in distributions.

Another important parameter to consider is the partitioning of molecules which is generally linked to their hydrophobicity (expressed *via* the octanol/water partition coefficient - K_{OW}). K_{OW} is frequently used to predict the adsorption of pollutants to solids and thus their bioaccumulation. Rogers (1996) provided a general rule for the application of K_{OW} to the estimation of sorption: a Log K_{OW} lower than 2.5 indicates a low sorption potential, a Log K_{OW} between 2.5 and 4 indicates a medium sorption potential, and a Log K_{OW} greater than 4 a high sorption potential. Therefore, the significant presence of DCF and its metabolites and TPs in benthic organisms (biofilms, clams) is consistent with their higher Log K_{OW}. Those compounds are more hydrophobic than CBZ and its metabolites and TPs which exhibit higher levels in water and sediments. Dibenz presents an exception (Log K_{OW} = 4.06) and this is reflected in its high accumulation in biofilms.

Finally, the presence of metabolites and TPs in the aquatic environment is also linked to pharmaceutical compounds degradation pathways. For example, Phototransformation is the main degradation pathway for DCF in the environment (Boreen *et al.*, 2003), and it takes place rapidly ($t_{1/2}$ = 9.6 ± 1.2 h (Poirier-Larabie *et al.*, 2016)), with Benz identified as the most stable product (Eriksson *et al.*, 2010). This could explain the low proportion of DCF compared to its metabolites and TPs. Moreover, it is worth noting that Benz is more abundant in matrices exposed to sunlight (biofilms, sediments) than in clams' flesh. CBZ, on the other hand, is known to be persistent in the environment (Loos *et al.*, 2009). This compound is one of the least degraded/eliminated in wastewater treatment processes (32.7 % ± 17.9 % *(Luo et al.*, 2014); < 0 – 23 % (Jekel *et al.*, 2015)), but leads to the formation of Dibenz and Acrid (Kosjek *et al.*, 2009). These properties may explain the similarity between the distributions.

3.2.Individual compounds ecotoxicities

Ecotoxicity of pharmaceutical metabolites and TPs was assessed for three different aquatic trophic levels: algae (*Pseudokirchneriella subcapitata*), invertebrates (*Daphnia magna*) and

fish (*Pimephales promelas*) (Figure 4). Values obtained from the QSAR Toolbox highlight that DCF and its metabolites and TPs (Benz and OH-DCF) have similar toxicities towards green algae (EC_{50} ~ 6.01 mg L^{-1}). For Daphnia, OH-DCF (LC_{50} = 2.3 mg L^{-1}) is found to be about 40 times more toxic than DCF (LC_{50} = 80.1 mg L^{-1}), while an abnormally high EC_{50} value was found for Benz (2.88 10^{15} mg L^{-1}) demonstrating the actual limitations of QSAR models to predict the toxicity of all compounds for all organisms (Boxall *et al.*, 2004). Benz is 4 times more toxic (LC_{50} = 2.71 mg L^{-1}) for *P. promelas* than its parent compound (LC_{50} = 11.2 mg L^{-1}), while OH-DCF has the same toxicity than DCF (LC_{50} = 10.7 mg L^{-1}).

In the CBZ family, Dibenz stands out from all the other compounds, being the most toxic for the three studied species. The other metabolites and TPs exhibit toxicities that are slightly higher, of the same order of magnitude or lower than that of the parent compound. Hence, for green algae, 3OH-CBZ is as toxic as CBZ (EC_{50} ~ 19.55 mg L^{-1}), while the other compounds are 10 to 70 times less toxic. Likewise, for *P. promelas*, CBZ-epox exhibits an LC_{50} value of the same order of magnitude as the parent compound (~ 44.7 mg L^{-1}), while the other metabolites and TPs are 3 to 6 times less toxic. On the other hand, for daphnia, 3OH-CBZ and CBZ-epox (LC_{50} ~ 36.5 mg L^{-1}) are about 3 times more toxic than CBZ, Acrid is as toxic (LC_{50} ~ 105.45 mg L^{-1}) and Trans-CBZ 65 times less toxic.

All these results are in agreement with the study of Pereira *et al.* (2020). Indeed, in their systematic review on the experimental and estimated toxicities of selected pharmaceuticals in different aquatic compartments, these authors found that DCF and its metabolite 4OH-DCF have similar toxicities to invertebrates and fish. In addition, they highlighted the higher toxicities of anti-inflammatory drugs, including DCF, as compared to antiepileptics such as CBZ. However, results differ regarding the most sensitive species. Indeed, while in our study results show that green algae is the most sensitive, followed by fish and daphnia, Pereira *et al.* (2020) observed that the most sensitive species were fish, followed by invertebrates and algae.

362 Nevertheless, their observation was explained by the fact that part of the experimental data for
363 fish was obtained through cell line or tissue testing, making it difficult to extrapolate the
364 values to the entire organism.

365 **3.3.Mixtures ecotoxicities**

366 QSAR predictions were also used to assess mixtures ecotoxicities (including parent
367 compound and its corresponding metabolites and TPs) since field data revealed their
368 simultaneous presence in water and benthic organisms (biofilms, clams). Figure 4 also reports
369 the predicted toxicities of mixtures of parent compounds with their associated metabolites and
370 TPs.

371 The results show that the toxicity values of DCF or CBZ and their metabolites and TPs
372 mixtures are overall in the middle range of the individual values predicted for the compounds.
373 Thus, for *Pimephales promelas*, the mixture of CBZ and metabolites and TPs (LC_{50} = 41.5
374 mg L^{-1}) is approximately 12 times less toxic than Dibenz alone (LC_{50} = 3.27 mg L^{-1}).
375 However, the mixture is 3 to 5 times more toxic than the other metabolites and TPs, when
376 considered individually. According to the QSAR model, the mixture's toxicity is lower than
377 CBZ's individual toxicity for *Pimephales promelas* (41.5 mg L^{-1} *versus* 37.3 mg L^{-1}) and
378 especially for the algae *P. subcapitata* (37 mg L^{-1} *versus* 10.4 mg L^{-1}). On the contrary, the
379 model predicts a higher toxicity of the mixture for daphnids. This highlights the importance of
380 completing experimental ecotoxicological data to confirm mixture effects and hence the
381 interest of considering metabolites and TPs along with their associated parent pharmaceutical
382 compounds in the assessment of ecological risks. It is now known that effects resulting from
383 the exposure to a mixture of organic contaminants can be very different from observations
384 when evaluating the toxicity of a single compound, as it was already demonstrated for
385 endocrine disruptors for example (Filby *et al.*, 2007; Sumpter & Johnson, 2005). Until now,
386 few studies have been interested in this mixture effect with many pharmaceutical compounds,

and even less with metabolites and TPs. Cleuvers (2003) showed that a mixture of CBZ and a lipid lowering agent, clofibric acid, exhibited a higher toxicity than the single compounds at the same concentration during immobilization tests of *D. magna*. The same author (Cleuvers, 2004) observed acute toxicity of a mixture of anti-inflammatories including DCF, at lower concentrations than for the individual chemicals.

Wang *et al.* (2020) were the first to develop a QSAR model to predict mixture ecotoxicities of fluoroquinolone antibiotics with their photodegradation products for *Escherichia coli*. Their results showed that the mixture toxicity of fluoroquinolones derivatives is a concentration addition of their individual toxicities. Qin *et al.* (2018) developed a QSAR model to predict acute mixture ecotoxicities of two antibiotics and four pesticides towards *Aliivibrio fischeri*. The 45 studied mixtures exhibited additive, synergistic, and antagonistic effects. The authors also showed that, compared to traditional concentration additive and independent action models, their QSAR model better predicted mixture toxicities.

3.4.Comparison with pesticide metabolites

Ecotoxicity values were also predicted for a common pesticide (atrazine – ATZ) and some of its main metabolites (desethylatrazine – DEA, deisopropylatrazine – DIA, desethyldeisopropylatrazine – DEDIA, hydroxyatrazine – OH-ATZ, and aniline) to compare with CBZ, DCF and their metabolites and TPs (Figure 4). Atrazine was banned in France in 2001 but is still found in waters and river sediments. Moreover, ATZ and its metabolite DEA are frequently responsible for the downgrading of surface waters or groundwater quality. The comparison with ATZ and its metabolites shows that although DCF and CBZ are less toxic to the three species of interest than this pesticide, their metabolites and TPs exhibit EC_{50} and LC_{50} values broadly comparable to those of ATZ metabolites.

As mentioned before, in France, the presence of pesticide residues and their metabolites in waters is regulated by European Directive 98/83/EC (European Council, 1998) relating to the quality of waters intended for human consumption. This text sets quality limits at 0.1 µg L^{-1} per individual substance and 0.5 µg L^{-1} for the sum. Water quality controls are increasingly revealing situations where regulatory quality limits are exceeded for metabolites. Thus, the French Directorate General of Health approached the ANSES to define a methodology for identifying relevant metabolites (ANSES, 2019). Considering the possible effects of certain pharmaceutical metabolites and TPs, this approach should be transposed to identify the most relevant metabolites and TPs for human and/or environmental health.

3.5.Risk assessment of target compounds in the studied rivers

A methodology was developed to associate both ecotoxicity and persistence data to assess the risk related to the presence of pharmaceutical metabolites and TPs in the sampled rivers. Therefore, $PNEC_{water}$ were calculated from the predicted ecotoxicity endpoints for each individual compound as well as for mixtures, then HQs were obtained by dividing the MEC observed in waters during this study by the $PNEC_{water}$ (N.B.: a mixture's MEC is expressed as the parent molecule's mass and corresponds to the sum of the individual compounds MECs).

Persistence was evaluated with the ultimate biodegradation index obtained with the EPISUITE Biowin3 Survey Model included in the QSAR Toolbox (Environmental Protection Agency, 2012). It should be noted that in this case, only biodegradation is considered in the persistence, while other processes (hydrolysis, photolysis, *etc.*) may also transform/degrade the compounds. For mixtures, the ultimate biodegradation index was obtained by calculating the mixture's constituents' indexes weighted average. Biodegradation indexes vary between 2.25 and 2.75 for the CBZ and DCF degradation compounds, which means half-life of a few weeks to a few months, with the exception of Trans-CBZ (between 2.75 and 3.25) that presents a slightly faster biodegradation than the other molecules.

435 Figure 5 provides a classification of compounds according to their associated risk (HQ) for
436 the studied rivers and their biodegradation index. Target compounds can be divided in 4
437 groups: (1) “compounds already worrying” regarding their persistence and their high
438 concentrations: OH-DCF, Benz and Dibenz. These compounds have an HQ lower than 1 (~
439 qualified as medium risk for aquatic organisms) and are therefore of concern only when
440 compared to the other studied compounds. The same applies for the notion of persistence in
441 water; (2) “compounds to watch out for” because of their persistence in the dissolved phase.
442 These molecules could become of concern if their levels tend to increase: DCF, CBZ, 3OH-
443 CBZ, CBZ-epox and Acrid. (3) “compounds to watch out for” because of how far they have
444 exceeded the $PNEC_{water}$: no compound identified in this study; and (4) “compounds not of
445 concern” including compounds with a level of contamination below $PNEC_{water}$ (HQ < 0.1)
446 and low persistence in dissolved phase: Trans-CBZ.

447 The same methodology was also involved to assess risk regarding the sediment compartment.
448 $PNEC_{sediment}$ were derived from the $PNEC_{water}$ by the equilibrium partitioning method. The
449 K_{OC} of a mixture is its constituents K_{OC} values weighted average. The data thus calculated, as
450 well as the MEC values measured in the sediments of the three sampled rivers, were used to
451 deduce the HQs of each compound with respect to benthic organisms. Figure 6 presents the
452 classification of compounds according to their associated risk in the sediments of the studied
453 rivers and their biodegradation index. Consequently, OH-DCF and Benz are classified as
454 “compounds already worrying”; DCF, CBZ, 3OH-CBZ, CBZ-epox, and Dibenz are
455 “compounds to watch out for – group 2”; and Trans-CBZ is considered as a “compound not of
456 concern”. Comparison between risks in water and sediments suggests that Dibenz is of higher
457 concern for aquatic species than for benthic organisms. The rest of the compounds present the
458 same risk in sediment and in water. In addition, the same behaviour is observed for the

mixtures, in particular that of DCF and its metabolites and TPs which is of more concern than the parent compound alone.

The results also show the importance of considering mixtures and not only the parent compounds, as illustrated by the case of DCF. Indeed, this compound – considered individually – does not seem to represent a risk (i.e. group 2, Figures 5 and 6), while its mixture with its metabolites and TPs is already worrying for the aquatic environment because of their persistence and their high levels. However, this remark is not fully generalized since CBZ alone and its mixture with metabolites and TPs show the same level of risk. This finding suggests that it would be necessary to only identify compounds for which it is relevant.

4. Conclusion

Unsurprisingly, pharmaceutical metabolites and TPs have been found in waters, sediments, biofilms, and clams of the studied rivers, obviously in varying concentrations, but in all samples. This widespread distribution and occurrence presumably requires special attention, at least as much as the presence of pesticide metabolites. Indeed, the use of QSAR models made it possible to address the lack of experimental ecotoxicological data and to highlight that some of CBZ or DCF metabolites and TPs could be more toxic than their parent compounds for aquatic species. Thus, OH-DCF, Benz and Dibenz could represent a risk for aquatic wildlife because of their relatively high hazard quotients and their relatively slow biodegradation. The large number of possible metabolites and TPs and the possibility of individual toxicity also raises questions about a "cocktail effect". Indeed, compounds may have unpredictable biochemical interactions when considered in a mixture, resulting in different (cumulative or not) effects than individual molecules. As shown by QSAR predictions of mixtures, it appears that the risk for the aquatic environment is higher when one considers DCF along with its metabolites and TPs. Nevertheless, our results also show that

this is not generalizable for all parent compounds, e.g. CBZ. Consequently, it is urgent to identify the most relevant metabolites and TPs – as is done for pesticides – to better prioritize risk assessment.

For future works, it is important to complete the collected data by conducting sampling campaigns at different times of the year and not only in summer. This would help better understand the impact of seasonal variations on pharmaceutical molecules degradation pathways and the presence and distribution of their metabolites and TPs in the aquatic environment. Furthermore, it is important to acknowledge that QSAR modelling constitutes a first estimation of pharmaceutical metabolites and TPs ecotoxicities as well as mixture effects. Experimental data still need to be generated to confirm these calculations. Our findings constitute a starting point for further research aimed to determine the risks coming from the presence of pharmaceutical compounds along with their metabolites and TPs in the aquatic environment, with the purpose of potentially including them in water management policies.

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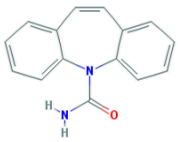
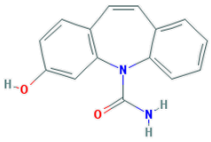
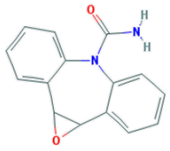
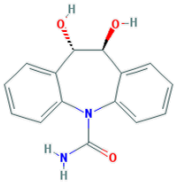
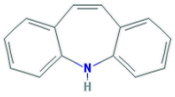
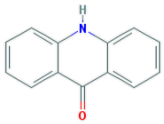
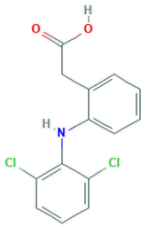
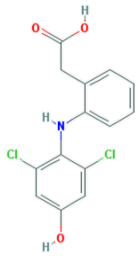
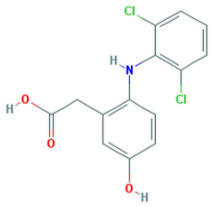
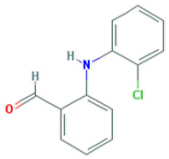
Table 1: Properties of selected compounds

Compound	Class	CAS number	pKa*	Log K_{OW}*	Chemical structure
Carbamazepine (CBZ)	**Parent compound**	298-46-4	16.00	2.45	
3-Hydroxycarbamazepine (3OH-CBZ)	Metabolite	68011-67-6	9.10	1.42	
10,11-Epoxycarbamazepine (CBZ-epox)	Metabolite	36507-30-9	16.00	0.95	
10,11-dihydro-10,11-trans-dihydroxycarbamazepine (Trans-CBZ)	Metabolite	58955-93-4	12.20	-0.21	
Dibenzazepine (Dibenz)	Metabolite + WWTP degradation product	256-96-2	19.50	4.06	
Acridone (Acridone)	Metabolite + WWTP degradation product	578-95-0	0.32	1.69	
Diclofenac (DCF)	Parent compound	15307-86-5	4.05	4.51	
4'-Hydroxydiclofenac (4'OH-DCF)	Metabolite	64118-84-9	3.76	3.70	

5-Hydroxydiclofenac (5OH-DCF)	Metabolite	69002-84-2	3.81	3.18	
2-[(2-chlorophenyl)-amino]-benzaldehyde (Benz)	Photodegradation product	71758-44-6	8.18	3.65	

*Values predicted by the QSAR Toolbox (version 4.4)

754 **Table 2: Mean concentrations and comparison with literature.**

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	Compound	Measured concentration	Literature (ng L^{-1})
Water (µg L^{-1})	CBZ	0.52 ± 0.13	290 – 560 ng L^{-1} [tertiary treatment pond] (Koba *et al.*, 2018) 370 ± 14 ng L^{-1} [stream impacted by WWTP] (Du *et al.*, 2014)
	DCF	0.20 ± 0.14	22 – 870 ng L^{-1} [tertiary treatment pond] (Koba *et al.*, 2018) <0.96 – 253 ng L^{-1} [surface water] (Wilkinson *et al.*, 2017) 86 ± 55 ng L^{-1} [stream impacted by WWTP] (Du *et al.*, 2014)
	4'OH-DCF	0.26 ± 0.15	710 ng L^{-1} [WWTP effluents] (Stülten *et al.*, 2008)
	5OH-DCF		450 ng L^{-1} [WWTP effluents] (Stülten *et al.*, 2008)
	3OH-CBZ	0.23 ± 0.08	n.d. [surface water] (Miao & Metcalfe, 2003)
	CBZ-epox	0.30 ± 0.07	n.d. [surface water] (Miao & Metcalfe, 2003) 71 ng L^{-1} [tertiary treatment pond] (Koba *et al.*, 2018)
	Trans-CBZ	0.18 ± 0.09	2.2 ± 0.3 ng L^{-1} [surface water] (Miao & Metcalfe, 2003) 490 ng L^{-1} [tertiary treatment pond] (Koba *et al.*, 2018)
Biofilm (ng g^{-1})	CBZ	722 ± 219	583.5 [Châtellerault WWTP downstream] (Aubertheau *et al.*, 2017)
	DCF	348 ± 349	37.2 [Châtellerault WWTP downstream] (Aubertheau *et al.*, 2017)
	CBZ-epox	329 ± 133	5.3 [Vienne River] (Aubertheau *et al.*, 2017)
Sediment (ng g^{-1})	CBZ	119 ± 54	5.1 – 16 [tertiary treatment pond] (Koba *et al.*, 2018)
	DCF	51 ± 55	

			2.6 – 30 [tertiary treatment pond] (Koba *et al.*, 2018)
	CBZ-epox	100 ± 47	<LoQ [tertiary treatment pond] (Koba *et al.*, 2018)
	Trans-CBZ	57 ± 39	<LoQ [tertiary treatment pond] (Koba *et al.*, 2018)
Clams (ng g^{-1})	CBZ	962 ± 381	<LoD [lake] (Xie *et al.*, 2015)
	DCF	493 ± 428	1.41 – 5.42 [lake] (Xie *et al.*, 2015)

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Figure 1: Sampling sites; Up. = upstream, Down. = downstream; Black stars indicate important cities located along the watersheds; Yellow marks indicate the locations of sampling sites.

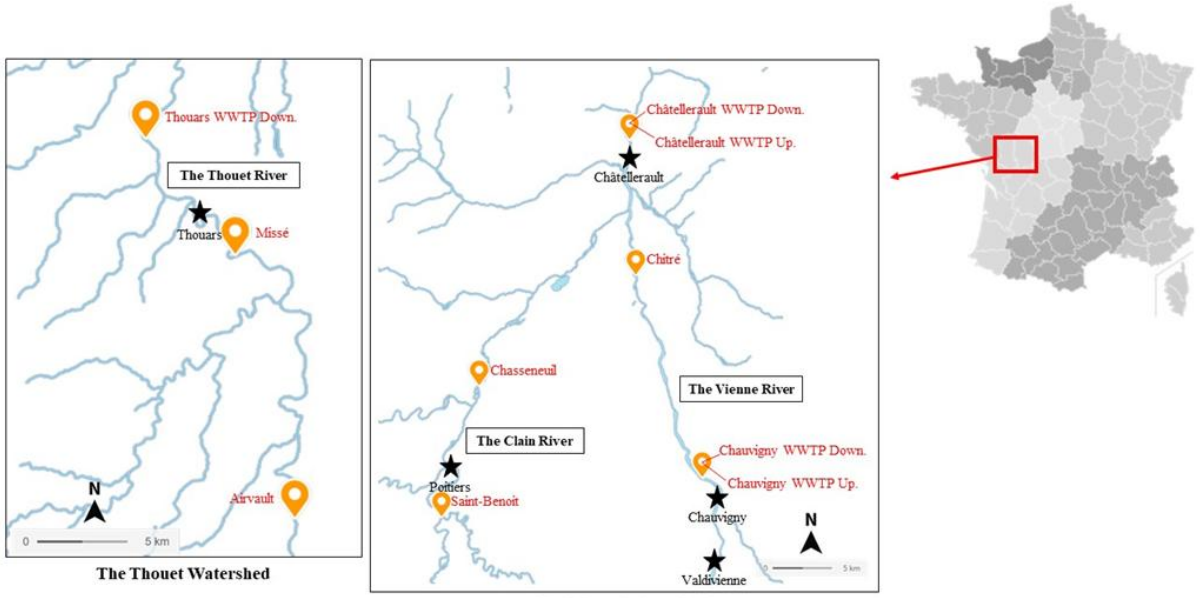


Figure 2: Mean levels (± standard deviation) of DCF and its associated metabolites and TPs in the compartments of interest, along the three studied rivers.

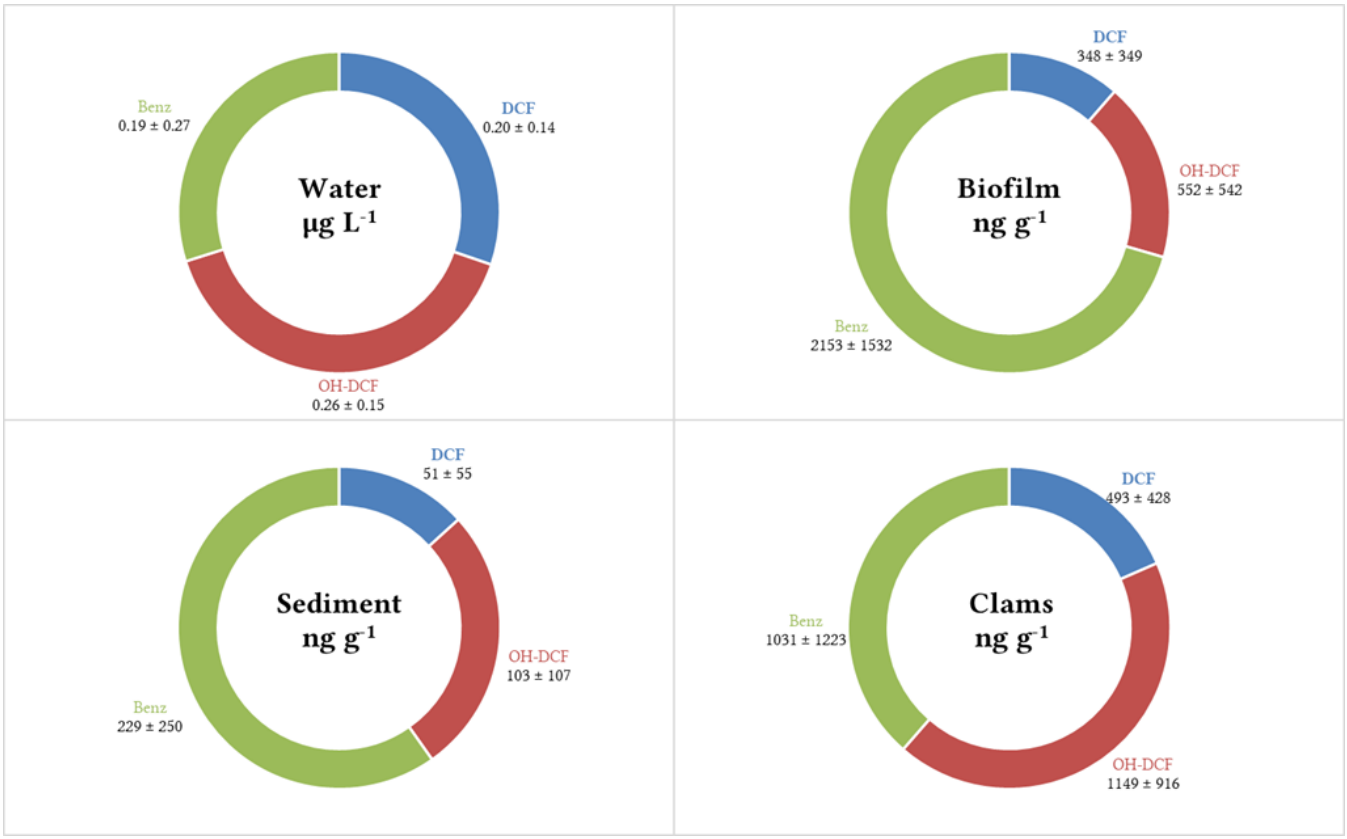


Figure 3: Mean levels of CBZ (± standard deviation) and its associated metabolites and TPs in the compartments of interest, along the three studied rivers.

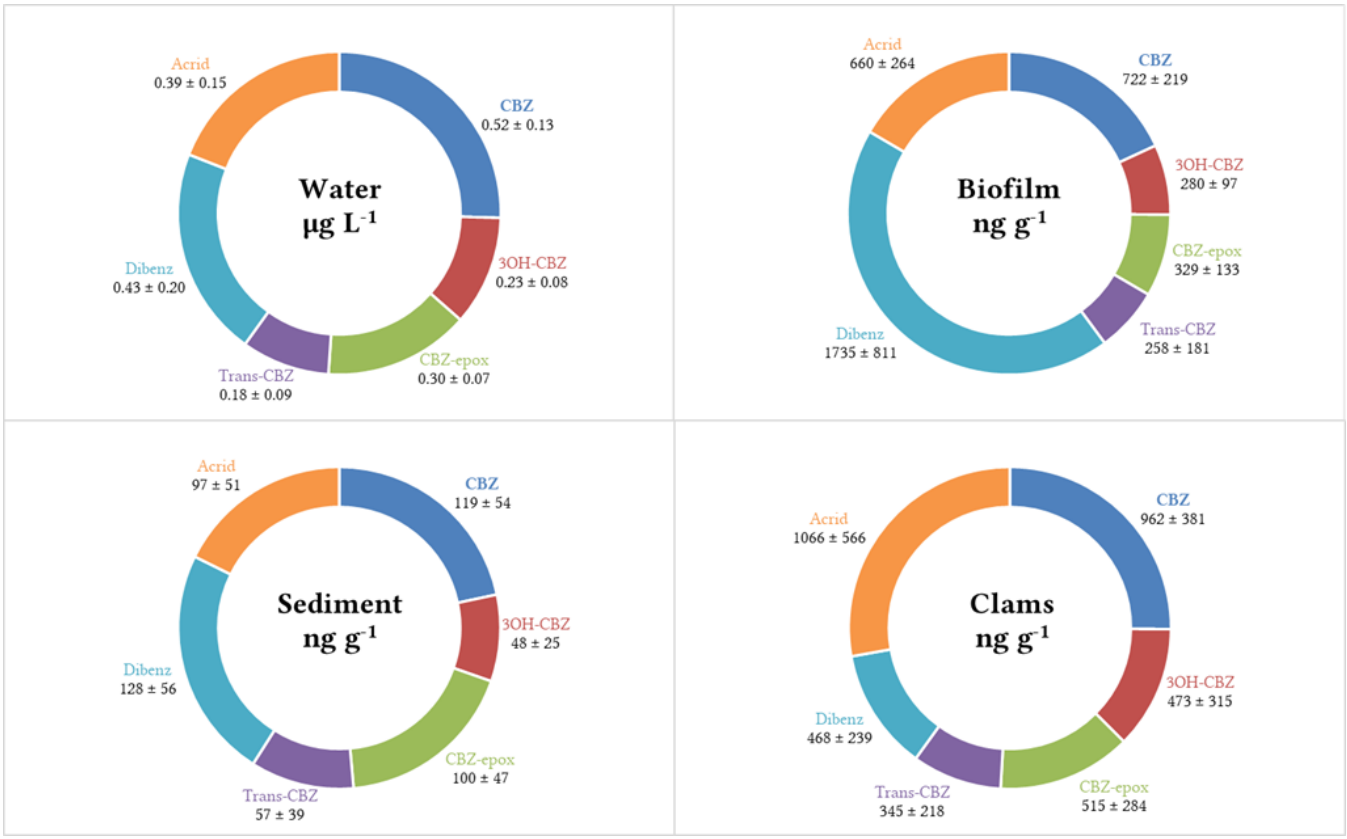
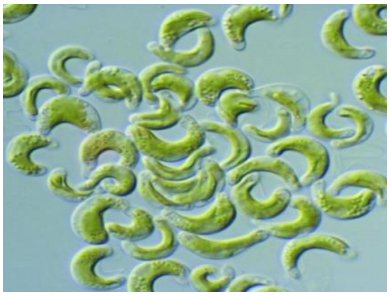


Figure 4: Ecotoxicity of targeted compounds and mixtures with respect to *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Pimephales promelas*. The values were calculated using the QSAR Toolbox trend analysis approach. The lowest toxicity endpoints are highlighted in red, and the highest in green, indicating the most and the least toxic product from each studied family towards each species. Ecotoxicities of individual compounds were used to estimate mixture ecotoxicities by trend analysis, considering independent modes of actions and molar fractions observed from the average concentrations measured in the studied waters.

	EC_{50} (mg L^{-1}) Growth rate t = 96 h
DCF	6
OH-DCF	3.42
Benz	8.62
Mixture	5.63

CBZ	10.4
3OH-CBZ	28.7
CBZ-epox	107
Trans-CBZ	707
Dibenz	5.29
Acrid	278
Mixture	37

	EC_{50} (mg L^{-1}) Growth inhibition t = 72 h
ATZ	0.14
DEA	1.96
DIA	1.7
DEDIA	22.8
OH-ATZ	0.26
Aniline	17

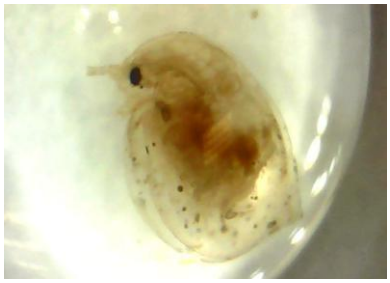


Pseudokirchneriella subcapitata

	LC_{50} (mg L^{-1}) Mortality t = 48 h
DCF	80.1
OH-DCF	2.3
Benz	2.88E+15
Mixture	1710000

CBZ	111
3OH-CBZ	35.4
CBZ-epox	37.6
Trans-CBZ	7110
Dibenz	1.58
Acrid	99.9
Mixture	40.8

	LC_{50} (mg L^{-1}) Mortality t = 48 h
ATZ	6.9
DEA	97.3
DIA	152
DEDIA	464
OH-ATZ	3.41
Aniline	0.08



Daphnia magna

	LC_{50} (mg L^{-1}) Mortality t = 96 h
DCF	11.2
OH-DCF	10.7
Benz	2.71
Mixture	6.67

CBZ	37.3
3OH-CBZ	120
CBZ-epox	52.1
Trans-CBZ	206
Dibenz	3.27
Acrid	233
Mixture	41.5

	LC_{50} (mg L^{-1}) Mortality t = 96 h
ATZ	4.1
DEA	15.6
DIA	28
DEDIA	120
OH-ATZ	19.5
Aniline	32

Pimephales promelas

Figure 5: Highlighting problematic compounds in the waters of the Vienne, the Clain and the Thouet Rivers according to the associated risk (HQ) but also their level of persistence (ultimate biodegradation half-life) (inspired by Mazellier *et al.* (2018))

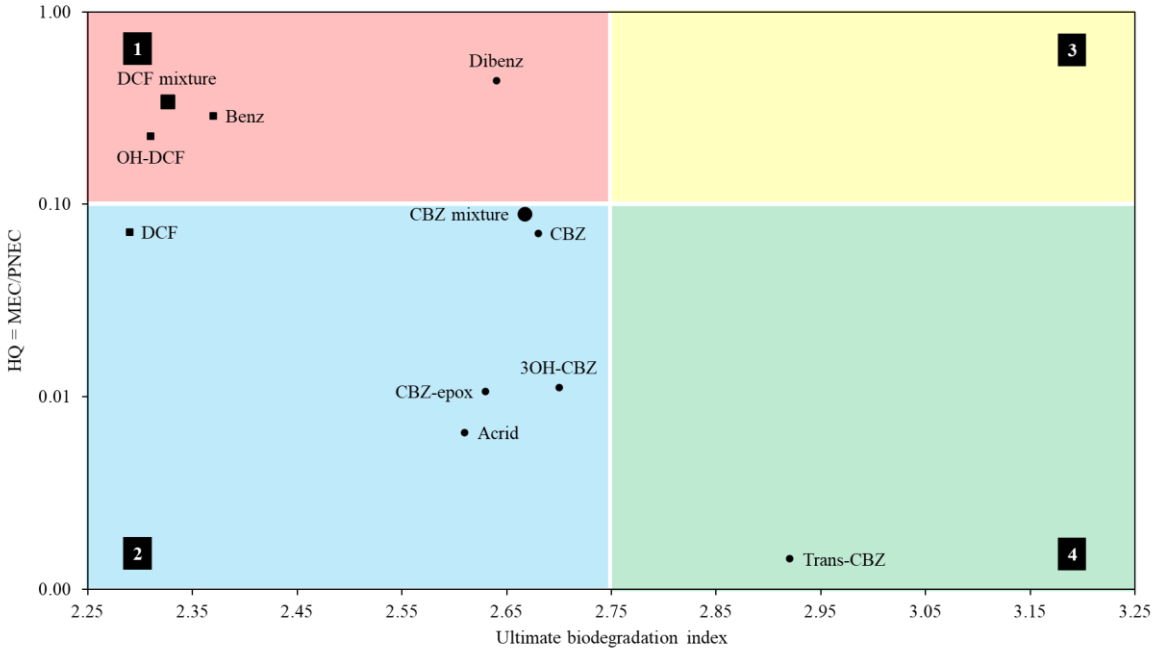


Figure 6: Highlighting problematic compounds in the sediments of the Vienne, the Clain and the Thouet Rivers according to the associated risk (HQ) but also their level of persistence (ultimate biodegradation half-life)