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Bioaccumulation and bioconcentration of carbamazepine and other pharmaceuticals in fish under field and controlled laboratory experiments. Evidences of carbamazepine metabolization by fish

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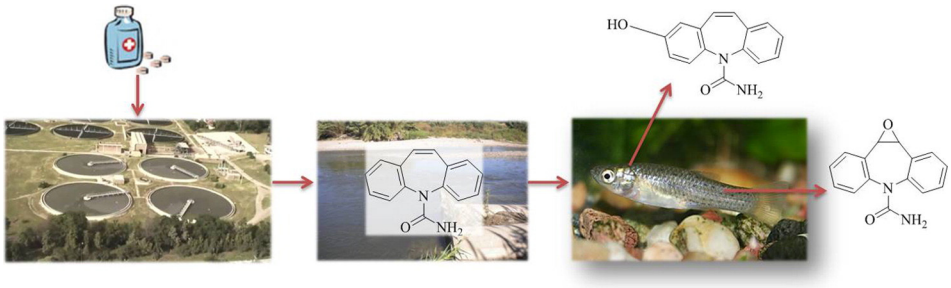
HIGHLIGHTS

- Wild *G. affinis* and *J. multidentata* bioaccumulated 20 pharmaceuticals.
- Laboratory exposed fish biotransformed CBZ to 2-OH-CBZ and CBZ-EP.
- CBZ and 2-OH-CBZ were preferentially bioconcentrated in brain and liver.
- CBZ-EP was only detected in gills and muscle.

GRAPHICAL ABSTRACT



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ABSTRACT

There is a growing interest in evaluating the presence of pharmaceutical residues and their metabolites in aquatic biota. In this study, twenty pharmaceuticals, including carbamazepine (CBZ) and two metabolites, were analyzed in homogenates of two fish species (*Gambusia affinis* and *Jenynsia multidentata*) captured in polluted areas of the Suquía River (Córdoba, Argentina). The twenty target pharmaceuticals were found in *G. affinis*, while only fifteen were detected in *J. multidentata*. We observed a noticeable difference in the accumulation pattern of both fish species, suggesting different pathways for the bioaccumulation of polar pharmaceuticals in each fish.
In order to investigate uptake and tissue distribution of pharmaceuticals, a detailed study was performed under controlled laboratory conditions in *J. multidentata*, exposed to CBZ. CBZ and two of its metabolites (carbamazepine-10,11-epoxide – CBZ-EP and 2-hydroxycarbamazepine – 2-OH-CBZ) were monitored in five organs of fish under laboratory exposure. To our knowledge, this is the first report on the presence of CBZ and its metabolite 2-OH-CBZ in gills, intestine, liver, brain and muscle of fish, while the metabolite carbamazepine-10,11-epoxide (CBZ-EP) was detected in gills and muscle. A ratio CBZ-EP/CBZ close to 0.1 suggests that gills and muscle of *J. multidentata* could metabolize CBZ through the CBZ-EP pathway.

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Our results reinforce the need of analyzing multiple species to account for the environmental impact of pollutants, negating the simplification of a single, "representative model" during ecotoxicological biomonitoring. To our knowledge, the biotransformation of CBZ to its metabolites (CBZ-EP, 2-OH-CBZ) in fish, under controlled laboratory *in vivo* exposures, is reported for the first time.

1. Introduction

Contaminants of emerging concern are chemicals that show potential risks to the environment or even to human health but are not yet subjected to regulatory criteria (Sauvé and Desrosiers, 2014). Pharmaceuticals are considered emerging contaminants, due to their universal use, their chemical-physical properties and their incomplete removal in wastewater treatment plants (WWTP) (Zenker et al., 2014). Since pharmaceuticals are biologically active compounds, designed to interact with specific pathways and processes in humans and animals, alterations in similar metabolic pathways in non-target organisms exposed to them cannot be discarded. Therefore, over the past 15 years, a substantial amount of work has been done to determine the occurrence, fate, effects, and risks of pharmaceuticals in the environment (Boxall et al., 2012).

Recent studies have revealed residues of psychiatric drugs, analgesics and anti-inflammatories, β-blockers, antiplatelet agents, pharmaceuticals for asthma treatment, for high blood pressure, antihistaminic, lipid regulators, antibiotics and contraceptives in fish and invertebrates captured downstream from WWTPs, indicating that these compounds tend to accumulate in the biota (Brooks et al., 2005; Chu and Metcalfe, 2007; Ramirez et al., 2009; Wille et al., 2011; Du et al., 2012; Huerta et al., 2013; Du et al., 2014; Liu et al., 2015; Ruhí et al., 2015; Xie et al., 2015; Moreno-González et al., 2016). It has been proposed that bioaccumulation should be regarded as a hazard criterion itself, since some effects may only be recognized in a later phase of life, have multi-generation effects, or are manifested only in higher members of a food-web (van der Oost et al., 2003).

Among psychiatric drugs, CBZ is one of the most commonly found pharmaceuticals in urban impacted surface waters and it has been proposed as a marker of anthropogenic pollution (Clara et al., 2004). This drug is used for the treatment of epilepsy, trigeminal neuralgia, bipolar depression and mania (Brunton, 2012). In humans, CBZ is almost completely absorbed from the gastro-intestinal tract, with the major percentage of the dose being recovered from the urine (72%) (Cunningham et al., 2010). The urinary excretion of CBZ is mainly composed by hydroxylated and conjugated metabolites, with only 2% of the dose as unchanged CBZ. The non-absorbed CBZ (28%) is recovered from feces, where ca. 14% is in the form of metabolites, and the rest remains as the parent compound (Cunningham et al., 2010). The accumulation of CBZ has been reported in periphyton, algae, zooplankton, invertebrates, fish and birds organs/tissues in both field monitoring and laboratory experiments (Ramirez et al., 2007; Ramirez et al., 2009; Lajeunesse et al., 2009; Vernouillet et al., 2010; Wille et al., 2011; Du et al., 2012; Garcia et al., 2012; Huerta et al., 2013; Klosterhaus et al., 2013; Martínez Bueno et al., 2013; Wang and Gardinali, 2013; Almeida et al., 2014; Du et al., 2014; Tanoue et al., 2014; Álvarez-Muñoz et al., 2015; Almeida et al., 2015; Boillot et al., 2015; Freitas et al., 2015a; Freitas et al., 2015b; Tanoue et al., 2015; Xie et al., 2015; Freitas et al., 2016; Moreno-González et al., 2016). However, to our knowledge, only one study has investigated the presence of CBZ metabolites in biota (marine mussels), experimentally exposed to CBZ (Boillot et al., 2015). The study on the presence of metabolites is important to properly assess the risk of pharmaceuticals in biota because some of them have been detected in water in higher amounts than parent compounds and they can also exhibit pharmacologic activity (López-Serna et al., 2012).

Even though pharmaceuticals in the environment has been a research topic of increasing concern worldwide, to the author's knowledge, there are not yet any reports on pharmaceuticals accumulation by aquatic biota in South America. In Córdoba, Argentina, the Suquía River is an urban impacted river, mainly due to discharges from the WWTP of Córdoba city (1.4 million inhabitants) (Pesce and Wunderlin, 2000; Wunderlin et al., 2001). The presence of pharmaceuticals has been reported in water of this river downstream from the WWTP, including CBZ (up to 113 ng L^{-1}), diclofenac (up to 145 ng L^{-1}) and atenolol (up to 581 ng L^{-1}) as the most frequently found pharmaceuticals (Valdés et al., 2014).

Therefore, the objectives of this work were: 1) to evaluate the differential bioaccumulation of CBZ and other pharmaceuticals in two wild fish species (*J. multidentata* and *G. affinis*), captured from polluted areas in the Suquía River basin; 2) to evaluate the uptake, bioconcentration and probable biotransformation of CBZ in the native fish *J. multidentata*, experimentally exposed to CBZ.

2. Materials and methods

2.1. Chemicals and materials

High purity grade (>95%) pharmaceutical standards of diclofenac, codeine, carbamazepine, citalopram, diazepam, lorazepam, atenolol, sotalol, propanolol, nadolol, carazolol, hydrochlorothiazide, clopidogrel, salbutamol and levamisole were acquired from Sigma–Aldrich. Sertraline and venlafaxine were purchased from the European Pharmacopeia (EP). Metabolites 2-hydroxycarbamazepine (2-OH-CBZ) and carbamazepine-10, 11-epoxide (CBZ-EP) were purchased from Toronto Research Chemicals (TRC). Metoprolol was obtained from the US Pharmacopeia (USP). Isotopically labeled compounds, used as internal standards, ibuprofen-d_3, diazepam-d_5, ronidazole-d_3 and fluoxetine-d_5 were acquired from Sigma–Aldrich. Atenolol-d_7, carbamazepine-d_{10}, hydrochlorothiazide-d_2, and citalopram-d_4 were purchased from CDN isotopes. Venlafaxine-d_6 was from TRC. Stock solutions of individual standards were prepared in methanol at 1000 mg L^{-1}. Mixed standard solutions were prepared by proper dilution in methanol at 20 mg L^{-1} and 1 mg L^{-1}. Working standard solutions for the calibration curve (at 0.1, 0.5, 1, 5, 10, 50, 100 μgL^{-1}) were prepared in methanol/water 10:90 (*V/V*) before each analytical batch. HPLC grade methanol, dichloromethane and ultrapure water (Lichrosolv) were purchased from Merck (Darmstadt, Germany). Oasis HLB cartridges (60 mg/3 mL), used for solid phase extraction, were acquired from Waters Corporation (Milford, MA, USA).

2.2. Field study

2.2.1. Sampling and monitoring campaigns

The Suquía River basin is located in a semi-arid region of the province of Córdoba, Argentina (Fig. 1), with a mean annual rainfall of 700–900 mm. Its watershed covers approximately 7700 km^2, of which almost 900 km^2 correspond to the Córdoba city drainage area and represent the middle-lower basin (Vázquez et al., 1979). The Suquía River begins at the San Roque Dam and it flows mainly from west to east for about 200 km, discharging its waters into Mar Chiquita Lagoon. The river crosses Córdoba city and, near the eastern edge of the city, receives the WWTP discharge. Downstream, the river crosses small towns, which add their sewage and run-off inputs. In this area, the river water is sometimes used for crop irrigation. The basin has a high flow period during the wet season (November to April) with an average flow of 14.4 m^3s^{-1}, whereas during the dry season (May to October)

Fig. 1. Map of the Suquía River Basin with sampling stations downstream the wastewater treatment plant of Cordoba city (WWTP): S1: Capilla de los Remedios; S2: Río Primero.

its estimated average flow is 4.5 m^3s^{-1}. Current treatment capacity of the WWTP reaches 8 m^3s^{-1}, serving approximately 800,000 inhabitants from Córdoba city (data from the official web site of Córdoba city hall, CC, 2015). Thus, during the dry season, the river is highly impacted.

Fish (*G. affinis* and *J. multidentata*) were captured by fish net during wet and dry seasons (April-wet season- and July-dry season-2012). Approximately 30 female adults of each species were captured at each monitoring site. This study focused on females because they are bigger than males in size, enabling a better analysis of samples (whole fish/organs/tissues). Monitoring sites were located close downstream from the WWTP of Cordoba city. Monitoring points include: Chacra de la Merced and Villa Corazón de María (5.5 and 16 km downstream the WWTP, respectively). There was absence of fish in these two monitoring stations. A third station, Capilla de los Remedios (Site 1, S1: 31°26′5.3″ S; 63°49′ 54.1″ W, Fig. 1), located 35 km downstream from the WWTP, showed presence of *G. affinis* individuals (total weight: 0.4 ± 0.1 g; standard length: 28 ± 3 mm) only during the wet season. Finally, a fourth station, Río Primero, was located 70 km downstream from the WWTP (Site 2, S2: 31°20′20.5″ S; 63°36′35.2″ W, Fig. 1), where both *G. affinis* (total weight: 0.4 ± 0.2 g; standard length: 28 ± 3 mm) and *J. multidentata* (total weight: 0.7 ± 0.4 g; standard length: 30 ± 5 mm) individuals were collected. It is reported that both species were abundant along the Suquía River basin, in polluted as well as unpolluted sites (Hued and Bistoni, 2005); however, our current sampling campaign evidenced absence of fish in more polluted areas (close to the WWTP). *G. affinis* is an introduced species, very resistant to unfavorable conditions (Pyke, 2005) and proposed as a bioindicator of invaded Neotropical basins (Rautenberg et al., 2015). *J. multidentata* is a native species, previously proposed as bioindicator of pollution in the basin (Monferrán et al., 2011; Maggioni et al., 2012). Fish were anesthetized with ice and sacrificed by transecting the spinal cord at the moment of the capture, washed with ultrapure water, wrapped in aluminum film and transported to the laboratory at 4 °C. Upon arrival to the laboratory, fish were weighed (wet weight, w.w.), their size was measured and they were kept at −20 °C until analysis. Before analysis, fish were freeze-dried, weighed (dry weight, d.w.) and triturated with a pestle in a mortar, making one composite sample of 10 individuals of *G. affinis*, or 6 individuals of *J. multidentata* in one plastic tube (affording a total weight of approximately 1 g d.w. of fish per tube). Half of the weight (0.5 g) was used to analyze pharmaceuticals and the other half for lipids content analysis. All procedures are in compliance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 2011).

2.2.2. Biota analysis

Homogenates of wild fish captured were analyzed according to Huerta et al. (2013). Briefly, 0.5 g of triturated freeze-dried pooled fish from each composite sample (each tube) were extracted by pressurized liquid extraction in an ASE 350® (Thermo Scientific Dionex, USA), with methanol as extraction solvent (4 extraction cycles of 5 min each at 50 °C). Final extracts were evaporated to dryness under a gentle stream of nitrogen and dissolved in 1 mL methanol. A high pressure liquid chromatography system (Agilent Technologies, USA, model 1260 Infinity), equipped with a diode array detector (PDA), was used for clean-up by gel permeation chromatography (GPC). Five hundred microliters of the methanolic extract were injected in an EnviroPrep column (300 mm × 21.2 mm × 10 μm pore size), coupled to a PLgel Guard column (50 mm × 7.5 mm) (Agilent Technologies, USA), using dichloromethane/methanol 90:10 as mobile phase at 5 mL min^{-1}. Purified fractions, containing target compounds (between 13.5 and 26.5 min), were collected, evaporated to dryness and reconstituted with 1 mL methanol/water (10:90, *V/V*), adding 50 μL of a 1 mg L^{-1} mixture solution containing internal standards (ibuprofen-d_3, diazepam-d_5, ronidazole-d_3, fluoxetine-d_5, atenolol-d_7, carbamazepine-d_{10}, hydrochlorothiazide-d_2,citalopram-d_4 and venlafaxine-d_6). The liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) method applied was the one developed by Gros et al. (2012) and Huerta et al. (2013), using a Waters Acquity Ultra-Performance™ liquid chromatograph, coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer, with an ESI source (Applied Biosystems, USA, model ABSciex 5500-QTRAP). Two SRM transitions between the precursor ion and the two most abundant fragment ions were monitored for each compound. The first transition was used for quantification purposes, whereas the second one was used to confirm the identity of the target compounds. Besides the monitoring of the SRM transitions, the relative abundance of the two SRM transitions in the sample were compared with those in the standards, and the relative abundances in the samples must be within ±20% of the two SRM ratios in the analytical standards. Quantification was done by internal sample calibration. Recovery percentages were verified by triplicate using a mixture of homogenates from both species in the same proportion spiked at 100 ng g^{-1} d.w. Recovery was calculated as the ratio of the area of spiked sample relative to the internal standard, compared to the ratio of area of standard relative to the internal standard. The calibration curve was prepared using the same homogenate matrix (range 0.01–50 μg L^{-1}, $R^2 > 0.999$). Matrix effects percentages were calculated using Eq. (1):

$$\text{Matrix effect } (\%) = \left(\frac{A_{spikedmatrix} - A_{matrix}}{A_{solvent}} - 1\right) \times 100 \quad (1)$$

where $A_{spiked\ matrix}$ corresponds to the area of the analyte in the spiked matrix; A_{matrix} corresponds to the area of the analyte naturally occurring in the matrix; and $A_{solvent}$ is the area of the analyte dissolved in mobile phase, respectively. Positive values indicate ion enhancement, whereas

negative values indicate ion suppression. Two intermediate concentrations of the calibration curve (5 and 12.5 μg L^{-1}) were used for matrix effect calculation and the average between both values is reported. Method detection limits (MDL) and method quantification limits (MQL) are those reported by Huerta et al. (2013). Method validation criteria can be found in Supporting Information (Table S1). Concentrations (d.w.) are reported as mean ± standard deviation of 3 pooled samples. For mean calculation, a "nd" value was considered = 0 and concentrations <MQL were replaced by the MDL value. Concentrations of each pool can be found in supporting information (Table S2). Frequencies were calculated from mean values for all sampling sites, species and season (5 total observations), considering a positive value every analyte detected (concentrations above MDLs).

Total lipid content was determined for 1.5–2 g d.w. of pooled samples by the A.O.A.C. method (A.O.A.C., 1990).

Bioaccumulation factors (BAFs, in L·kg^{-1}) of CBZ found in fish were estimated as the ratio between CBZ concentration in whole fish (μg kg^{-1} w.w, corresponding to values in d.w. divided by 4, the ratio w.w./d.w. for both species), divided by the freely dissolved CBZ concentration in water samples (μg L^{-1}). Concentrations of CBZ in river water correspond to a previous study, Valdés et al. (2014), only for campaigns where fish were captured, and are stated in Table S3 (Supporting information). These water samples were discrete, while fish had been exposed to varying concentrations of CBZ for a longer period. Therefore, estimated BAFs should only be considered as possible tendency of CBZ bioaccumulation (Huerta et al., 2016). Nevertheless, CBZ water concentrations are within the range of levels found in other sampling campaigns at the same sampling sites and seasons (period October 2011–July 2012, Valdés et al., 2014).

2.3. Laboratory study

2.3.1. Exposure conditions

Female adults of the native fish species *J. multidentata* (Cyprinodontiformes, Anablepidae) (total weight: 0.5 ± 0.2 g; standard length: 29 ± 2 mm) were selected for laboratory bioassays, considering their usefulness as a bioindicator of water pollution (Ballesteros et al., 2007, 2011; Cazenave et al., 2008; Guyón et al., 2012; Hued et al., 2012; Maggioni et al., 2012; Roggio et al., 2014). Fish were collected by fish net at a reference site (Yuspe River, 64°32'W; 31°17'S, Córdoba, Argentina) (Hued et al., 2012), and transported to the laboratory in aerated 20 L water tanks. Fish were acclimatized in a temperature controlled room at 21 ± 1 °C, 12:12 h light:dark photoperiod in 15 L fresh water aerated glass aquarium, for 2 weeks prior to the experiments and fed ad libitum twice a day with commercial fish pellets (TetraMin®, USA). Fish were starved 24 h prior to the experiment to avoid prandial effects, preventing deposition of feces in the course of the assay.

The bioconcentration assay was conducted at 100 μg L^{-1} of CBZ for 48 h as a first approach to measure CBZ accumulation and possible biotransformation in organs of *J. multidentata*. According to Wang and Gardinali (2013), uptake of CBZ from reclaimed water (1.229 μg L^{-1} CBZ) by mosquito fish (*Gambusia holbrooki*) reaches almost the maximal concentration after 48 h exposure.

The bioconcentration of CBZ in *J. multidentata*, experimentally exposed to CBZ, was assessed as follows: 60 fish were randomly distributed in six 15 L aerated glass aquariums, containing 10 fish/aquarium (1 L per fish). There were 3 control aquariums supplemented with 0.002% methanol, and 3 exposure aquariums supplemented with 100 μg L^{-1} CBZ. After 48 h of exposure, individuals were weighed and their size was measured. They were immediately anesthetized with ice, sacrificed by transecting the spinal cord and dissected to remove gills (12 ± 4 mg), brain (6 ± 2 mg), liver (7 ± 3 mg), intestine (11 ± 3 mg) and muscle (62 ± 27 mg). The organs were weighed and stored in pools of 10 organs in plastic tubes at −20 °C until analysis (n = 3 for each condition).

2.3.2. Analysis of exposure water and exposed biota

Water samples from each treatment were taken before starting the exposure and after 48 h. Aquarium water was analyzed within 2 days to verify the amount of CBZ and metabolites, following the methodology reported by Valdés et al. (2014).

A methodology was optimized for the analysis of pharmaceuticals in small quantities (mg) of fish tissues. Pools of each organ (n = 10 per pool) were freeze-dried and triturated. Twenty-five milligrams of gill, intestine and muscle, and 10 mg of liver and brain were weighed in 1.5 mL plastic tubes. One milliliter of methanol was added to each sample tube, achieving the extraction by ultrasonication for 15 min in an ice-bath. Supernatants were separated by ultracentrifugation (8000 × g, 10 min, 4 °C) into glass tubes. This procedure was repeated twice. Combined supernatants were dried under a gentle stream of nitrogen at 30 °C. Different clean-up methods were evaluated previously and SPE (Oasis HLB) was the one reaching acceptable recoveries for most compounds and lower standard deviations (Figure S2). Therefore, dried extracts were dissolved in 50 mL of ultrapure water and purified by solid phase extraction (SPE) according to the protocol of Gros et al. (2012). SPE eluates were dried and transferred to vials where they were reconstituted in 1 mL methanol/water 10:90 (*V*/*V*), adding 50 μL of 1 mgL^{-1} internal standard mixture solution. The same HPLC-MS/MS method developed by Gros et al. (2012) and Huerta et al. (2013) and used for analysis of fish homogenates was applied. Matrix-matched calibration curves (range 0.1–100 μg L^{-1}, $R^2 > 0.999$) were prepared using *Odontesthes bonariensis* tissue extracts as an alternative fish species (same superorder, *Acanthopterygii*), which were collected in areas where pharmaceuticals were not detected (San Roque reservoir, Córdoba, Argentina) (Valdés et al., 2014). Recoveries and matrix effects were calculated as described in Section 2.2.2. Recovery percentages (n = 3) were evaluated at 100 ng g^{-1} d.w. of gill, liver, intestine and brain, and 40 ng g^{-1} d.w. of muscle. For matrix effects calculation, the spiked concentration was 10 μg L^{-1} for all fish tissues (equivalent to 400 or 1000 ng g^{-1} d.w., depending on the weight of the organ analyzed). Limits of quantification (LOQ) were calculated as the lowest point in the matrix calibration curve that can be accurately quantified (S/N ≥ 10), while limits of detection (LOD) were calculated as LOQ divided by 3.3 (LOD = LOQ/3.3).

Bioconcentration factors (BCFs, in L·kg^{-1}) of CBZ in *J. multidentata* organs were estimated as the ratio between the concentration of the pharmaceutical in fish organ (μg kg^{-1} w.w.) divided by the concentration of the same pharmaceutical in water (μg L^{-1}), assuming stationary state. According to the OECD Guideline N° 305 (OECD, 2012), the estimated time to reach stationary state for CBZ, with a log K_{OW} = 2.45, would be 1–2 days.

2.4. Statistical analysis

Statistical analyses were carried out using the Infostat Software Package 2013 (Di Rienzo et al., 2013). Since concentrations of CBZ in tissues are not independent, statistical differences among brain, gills, intestine, liver and muscle were tested using Friedman ANOVA analysis for multiple dependent samples. This last test was also used to compare CBZ metabolites levels within organs. Significance level was set at α = 0.05, for all analysis. Linear mixed models were applied to assess differences in the bioaccumulation of pharmaceuticals between fish species (*G. affinis* and *J. multidentata*), followed by LSD Fisher comparison test.

3. Results and discussion

3.1. Field study

3.1.1. Pharmaceuticals in fish of Suquía River

Pharmaceuticals concentration in homogenates of *G. affinis* and *J. multidentata* collected at S1 and S2 of the Suquía River are presented

in Table 1. During the dry season, neither *G. affinis* nor *J. multidentata* could be captured in S1 (closer to the WWTP, Fig. 1). The 20 pharmaceuticals analyzed were detected at least once in wild fish samples, with 7 out of 20 being detected in all samples (100% frequency): atenolol, nadolol, diazepam, lorazepam, clopidogrel, salbutamol and hydrochlorothiazide, ranging from 1 to 67 ng g^{-1} d.w. The analgesic codeine showed the highest concentration found in fish (163 ng g^{-1} d.w.). These results agree, or are higher than previous reported values for pharmaceuticals in fish homogenates, captured at WWTP impacted surface waters worldwide, including freshwater (rivers and lakes), estuaries and marine water resources (Table 1). Huerta et al. (2013) reported similar values of clopidogrel, carazolol, sotalol, salbutamol and diclofenac in homogenates of fish collected at 4 Mediterranean rivers. However, propranolol, venlafaxine and citalopram values found in the present work were in general higher in *G. affinis* and *J. multidentata* compared to that study. CBZ levels in fish from the Suquía River were similar to the ones reported in homogenates of *G. holbrooki*, exposed for 7 days to reclaimed water of Florida International University (Wang and Gardinali, 2013), in muscle of common carp (*Cyprinus carpio*) of Taihu lake (Xie et al., 2015), in marine mussels and clams from USA and Europe estuaries and coastal zones (Wille et al., 2011; Klosterhaus et al., 2013; Martínez Bueno et al., 2013; McEneff et al., 2014; Álvarez-Muñoz et al., 2015). Moreover, Álvarez-Muñoz et al. (2015) found lower levels among 10 out of the 20 pharmaceuticals analyzed in this study in mullet (*Liza aurata*) and flounder (*Platichthys flesus*), collected from Tagus (Portugal) and Scheldt (Netherlands) estuaries. Recently, Moreno-González et al. (2016) found 18 out of these 20 pharmaceuticals in golden gray mullet (*Liza aurata*) and black goby (*Gobius niger*) of the Mar Menor lagoon (Spain), reaching similar or lower levels than the ones reported in fish from the Suquía River. In their work, authors reported that both CBZ metabolites were not found simultaneously in the same fish sample, their concentrations were always lower than those of the parent compound and that concentrations of 2-OH-CBZ were higher than CBZ-EP concentrations. In comparison, the same conclusions were not drawn from this study, probably because of high variability among fish pooled samples of the same species (Table S2), besides possible differences between species of both studies. There is agreement, however, in the relative concentrations of metabolites, since higher 2-OH-CBZ concentrations with respect to CBZ-EP levels were also found in fish of this study.

In general, the comparison of pharmaceuticals in *G. affinis* (wet season) shows a trend to higher levels in S1 with respect to S2, even though the differences are not statistically significant ($P > 0.05$).

Table 1
Pharmaceuticals concentration in homogenates of *G. affinis* and *J. multidentata*, reported as mean ± standard deviation (in ng g^{-1} dry weight). S1: Capilla de los Remedios, S2: Río Primero.

Compound	Therapeutic group	Season	S1	S2		Freq. (%)	Log P[1]	Reported values	References
			G. affinis	*G. affinis*	*J. multid.*				
Atenolol	β-Blockers	Wet	32 ± 49	17 ± 4	15 ± 14	100	0.43	<0.17–0.41	Álvarez-Muñoz et al. (2015)
		Dry	–	57 ± 23[a]	11 ± 10[b]				
Carazolol	β-Blockers	Wet	9 ± 14	2 ± 1	nd (0.04)	80	2.71	<0.15–3.8	Huerta et al. (2013)
		Dry	–	13 ± 1[a]	4 ± 5[b]			<0.10–0.31	Álvarez-Muñoz et al. (2015)
Metoprolol	β-Blockers	Wet	nd	<MQL (0.67)	nd (0.2)	60	1.76	<0.60–1.19	Álvarez-Muñoz et al. (2015)
		Dry	–	<MQL (0.67)	nd (0.2)				
Nadolol	β-Blockers	Wet	14 ± 24	<MQL (0.03)	1 (2)	100	0.87		
		Dry	–	23 (11)	5 (8)				
Propranolol	β-Blockers	Wet	11 ± 20	nd (0.09)	85 ± 148	80	2.58	4.2	Huerta et al. (2013)
		Dry	–	17.78 ± 0.04[a]	<MQL[b] (0.29)			<0.29–1.21	Álvarez-Muñoz et al. (2015)
Sotalol	β-Blockers	Wet	<MQL (0.88)	nd (0.26)	<MQL (0.88)	80	0.05	<0.88	Huerta et al. (2013)
		Dry	–	<MQL (0.88)	nd (0.26)			<0.24–0.88	Álvarez-Muñoz et al. (2015)
Carbamazepine	Psychiatrics	Wet	33 ± 54	8 ± 14	nd (0.04)	60	2.45	1.0–1.8	Du et al. (2014)
		Dry	–	8 ± 11	nd (0.04)			<0.04–0.25	Álvarez-Muñoz et al. (2015)
CBZ-EP	Psychiatrics (metabolite)	Wet	9 ± 15	nd (0.09)	nd (0.09)	40	2.31		
		Dry	–	5 ± 7	nd (0.09)				
2-OH-CBZ	Psychiatrics (metabolite)	Wet	17 ± 30	nd (0.03)	2 ± 4	60	2.46		
		Dry	–	13 ± 9	nd (0.03)				
Citalopram	Psychiatrics	Wet	<MQL (0.41)	51 ± 25	nd (0.12)	60	3.74	0.8	Huerta et al. (2013)
		Dry	–	45 ± 6	nd (0.12)			<0.16–0.41	Álvarez-Muñoz et al. (2015)
Diazepam	Psychiatrics	Wet	21 ± 32	28 ± 9	38 ± 34	100	3.08		
		Dry	–	41 ± 9[a]	6 ± 7[b]				
Lorazepam	Psychiatrics	Wet	30 ± 42	<MQL (1.62)	<MQL (1.62)	100	2.53		
		Dry	–	56 ± 28	31 ± 41				
Sertraline	Psychiatrics	Wet	24 ± 37	nd (0.32)	nd (0.32)	60	5.15	6.9–14	Du et al. (2014)
		Dry	–	58 ± 9[a]	14 ± 18[b]				
Venlafaxine	Psychiatrics	Wet	9 ± 16	18 ± 12	nd (0.04)	80	2.74	0.6	Huerta et al. (2013)
		Dry	–	35 ± 10[a]	4 ± 7[b]			<0.15–1.33	Álvarez-Muñoz et al. (2015)
Clopidogrel	Antiplatelet agent	Wet	5 ± 8	<MQL (0.13)	nd (0.04)	100	4.03	<0.13	Huerta et al. (2013)
		Dry	–	14 ± 1	9 ± 9				
Codeine	Analgesics/ anti-inflammatories	Wet	75 ± 130	nd (0.06)	nd (0.06)	60	1.34		
		Dry	–	163 ± 82	46 ± 50				
Diclofenac	Analgesics/ anti-inflammatories	Wet	<MQL (1.66)	nd (0.5)	nd (0.5)	40	4.15	4.1–8.8	Huerta et al. (2013)
		Dry	–	<MQL (1.66)	nd (0.5)			<0.62–2.16	Álvarez-Muñoz et al. (2015)
Hydrochlorothiazide	Diuretic	Wet	30 ± 53	13 ± 5	5 ± 8	100	−0.58	<0.17–0.57	Álvarez-Muñoz et al. (2015)
		Dry	–	67 ± 6[a]	21 ± 24[b]				
Levamisole	Antihelmintic	Wet	nd (0.04)	22 ± 11	11 ± 11	80	2.36		
		Dry	–	22 ± 31	11 ± 12				
Salbutamol	Asthma treatment	Wet	26 ± 38	18 ± 5	22 ± 17	100	0.88	2.6	Huerta et al. (2013)
		Dry	–	47 ± 9[a]	6 ± 6[b]				
Total		Wet	347	178	187				
		Dry	–	685	168				

The significant of "a" and "b" on the upper right corner of mean values correspond to statistical significant differences between values.
Mean values with different letters indicate significant differences between species, within the same season ($P < 0.05$).
[1] Data from Huerta et al. (2013). nd: not detected, MDLs in ng g^{-1} d.w. <MQL: below method quantification limit, in ng g^{-1} d.w. (Huerta et al., 2013).

Concerning seasonal variation, except from CBZ, a trend to higher pharmaceutical accumulation was observed in *G. affinis* during the dry season (considering the total sum of pharmaceuticals). Since the river flow is much lower at this time of the year, higher total pharmaceuticals concentration in the water can be expected during the dry season and, therefore, higher bioaccumulation in fish. However, the same pattern is not observed for *J. multidentata*, which shows greater variability during both seasons, but a similar total load (sum of all compounds detected at each season).

There are noticeable differences in the accumulation patterns of both studied fish species. Thus, *G. affinis* accumulates a wider variety of compounds, affording a higher total load of pharmaceuticals than *J. multidentata* during the dry season, not in the wet season, where the total load was similar between both species. The 20 studied pharmaceuticals were always quantified in *G. affinis*, at least during the dry season, while 5 out of the 20 compounds were not detected in *J. multidentata*. *G. affinis* is an introduced species that shares the same ecological habitat than *J. multidentata*, with same trophic level and similar diets (Haro and Bistoni, 2007). The total lipid content was different between both species ($P < 0.05$): 4.6 ± 0.2% in *G. affinis* (both seasons and stations), and 3.5 ± 0.5% in *J. multidentata* (both seasons). This difference in lipids content could explain the higher accumulation of hydrophobic pharmaceuticals in *G. affinis* (e.g. sertraline). However, it does not account for differences in the accumulation of all pharmaceuticals, since some compounds with low log K_{OW} were also accumulated in higher amounts by *G. affinis* (e.g. atenolol and hydrochlorothiazide). Therefore, it is likely to think that other mechanisms (rather than lipophilicity), such as receptor-binding interactions or differences in the biotransformation rates, may be involved in the accumulation of polar pharmaceuticals in fish (Ramirez et al., 2009; Tanoue et al., 2014). Nichols et al. (2015) studied the effect of pH on waterborne diphenhydramine (a weak base) bioconcentration by fathead minnows. Authors found that the ionized fraction of the pharmaceutical contributes substantially to the observed distribution of total pharmaceutical between blood and tissues and that some factor other than lipid content controls its distribution, suggesting it could be its binding to tissue proteins (exceeding its binding to plasma proteins) as well as considering effects at the gill surface (acidification). In the present study, protein content of each fish species was not analyzed, but it could be another factor influencing the different accumulation observed. Tanoue et al. (2015) studied the intra-species variability of 20 pharmaceuticals pharmacokinetics, including CBZ, in wild cyprinoid fish and found that brain/plasma ratios for psychotropic agents varied up to 28-fold among individual fish, attributing this differences to plasma pharmaceuticals levels, plasma protein content and differences in influx and efflux mechanisms at the blood-brain barrier. This might all be possible reasons of differences observed in the present study among individuals (pooled samples) and between fish species.

Accumulation of CBZ as well as their metabolites (CBZ-EP, 2-OH-CBZ) occurred in wild *G. affinis* from Capilla de los Remedios (S1) and Río Primero (S2). The estimated CBZ BAFs for *G. affinis* were 208 L kg^{-1} in S1 (wet season), 43 and 50 L kg^{-1} in S2 during dry and wet seasons, respectively (Table S3). These BAFs are within the range of already reported values in fish of freshwater resources impacted by WWTPs, ranging from 2.5 to 264 L kg^{-1} (Garcia et al., 2012; Zenker et al., 2014; Xie et al., 2015). In our current work, we did not observe either CBZ or its metabolites (except for 2-OH-CBZ during wet season) in wild *J. multidentata*, reinforcing the concept of different uptake and/or biotransformation pathways for different fish species.

Whenever fish bioaccumulated CBZ, the corresponding BAF was higher than 1, meaning that the total fish concentration was higher than the concentration in river water. However, it should be noticed that the presence of CBZ in wild fish could be caused by other sources, e.g. feed, particulate matter, sediments, etc.

To elucidate if the occurrence of CBZ metabolites in fish was the result of bioconcentration from water or biotransformation of CBZ by fish, we carried out controlled exposures of fish to CBZ in the laboratory.

3.2. Laboratory exposure experiment

3.2.1. Carbamazepine water concentration

At the beginning of the experiment, measured CBZ concentrations in the exposure water were: 0.62 ± 0.04, 11 ± 3 and 76 ± 3 μg L^{-1} CBZ, for nominal concentrations of: 0.5, 10 and 100 μg L^{-1} CBZ, respectively. After 48 h exposure, measured concentrations were: 0.54 ± 0.02, 13.24 ± 0.03 and 78 ± 14 μg L^{-1} CBZ, respectively. So far, non-appreciable drop of CBZ concentration in the exposure water was observed, meaning that the uptake of CBZ by fish is minimum compared to its amount in water. CBZ metabolites (CBZ-EP and 2-OH-CBZ) were not detected in any of the exposure solutions (LOD: 3 ng L^{-1}). Neither CBZ (LOD: 0.2 ng L^{-1}) nor CBZ metabolites were detected in control samples.

3.2.2. Carbamazepine tissue distribution

The optimized methodology, as well as the validation criteria for measuring not only CBZ and its two metabolites, but also 16 further pharmaceuticals in small sample amounts (10–25 mg) of fish gills, intestine, liver, brain and muscle, is presented in Table S3. Recoveries ranged from 40 to 128%, while matrix effects varied between −96% (ion suppression) to 164% (ion enhancement). CBZ and metabolites recoveries ranged from 59 to 105%, with matrix effects between −78% (ion suppression) and 27% (ion enhancement). The major problem faced with matrix interference was ion suppression (causing higher detection limits). To this respect, we observed that both liver and intestine were the most complicated matrices for the analysis of pharmaceuticals. The use of internal isotopically labeled standards, in addition to calibration curves prepared from fish tissues (matrix-matched calibration curve), were of considerable importance for the accurate quantification of pharmaceuticals in fish organs. Higher matrix effects and LODs are commonly reported in the literature for the analysis of pharmaceuticals in fish liver, which could be explained by the higher lipid and protein contents in this tissue (Ramirez et al., 2009; Huerta et al., 2013; Tanoue et al., 2014). Dilution is an option to diminish this matrix interference, as it was applied in this work (dilution 1/50–1/100); however, this is possible only if high sensitive MS equipment is used for measurements. Therefore we recommend a more exhaustive clean-up method to analyze pharmaceuticals in liver and intestine. In brain, gills and muscle, the optimized method was suitable for all pharmaceuticals, except for carazolol, sertraline and diclofenac. LODs ranged from 0.1 to 30.3 ng g^{-1} d.w., while LOQs ranged from 0.4 to 100 ng g^{-1} d.w. in the five studied organs. Despite of the low amount of biomass considered (10–50 mg), limits of quantification are in agreement with reports from the literature for pharmaceuticals in fish tissues (Du et al., 2012; Ramirez et al., 2007; Subedi et al., 2011; Zhao et al., 2015); although they were higher than some reports from other authors (Huerta et al., 2013; Tanoue et al., 2014; Liu et al., 2015). Thus, the optimized method can be considered satisfactory for analyzing pharmaceuticals in small amount of fish tissues, as it was the case in this work, using a simple and relatively fast procedure.

This method was successfully applied to quantify CBZ and their metabolites, CBZ-EP and 2-OH-CBZ, in the five studied organs of *J. multidentata* exposed to 100 μg L^{-1} CBZ for 48 h (Table 2). Concentrations of CBZ and its metabolites were below LODs in control fish. Conversely, in exposed fish, CBZ was accumulated in all studied organs, denoting that the detoxification and elimination systems were exceeded. Even though differences between organs were not statistically significant ($P > 0.05$), the highest concentrations of CBZ occurred in brain (701 ± 206 ng g^{-1} w.w.) and liver (688 ± 83 ng g^{-1} w.w.). Brain accumulation indicates that CBZ crosses the blood–brain barrier of *J. multidentata*, which would be explained by its mechanism of action, since brain is the target organ in psychiatric human treatments (Rogawski and Löscher, 2004). CBZ accumulation in liver could be associated to the organ's main function of detoxification in vertebrates

Table 2
Carbamazepine (CBZ), carbamazepine-10, 11-epoxide (CBZ-EP) and 2-hydroxycarbamazepine (2-OH-CBZ) concentration (ng g^{-1} dry weight-d.w and wet weight-w.w.), sum of the three compounds ($\sum$ (CBZ + Met.), in ng g^{-1} d.w. and w.w.), ratio between metabolite and CBZ concentration (2-OH-CBZ:CBZ and CBZ-EP:CBZ) and bioconcentration factor of CBZ (BCF, in L kg^{-1} w.w.).

	Gill		Intestine		Liver		Brain		Muscle	
	d.w.	w.w.	d.w.	w.w.	d.w.	w.w.	d.w.	w.w.	d.w.	w.w.
CBZ (ng g^{-1})	1731 ± 348	407 ± 82	1655 ± 345	372 ± 77	2154 ± 259	688 ± 83	3182 ± 936	701 ± 206	1775 ± 60	430 ± 14
2-OH-CBZ (ng g^{-1})	243 ± 34	57 ± 8	221 ± 127	50 ± 28	335 ± 63	107 ± 20	218 ± 99	48 ± 22	283 ± 97	69 ± 24
CBZ-EP (ng g^{-1})	175 ± 37	41 ± 9	nd	nd	nd	nd	nd	nd	246 ± 122	60 ± 30
Σ (CBZ + Met.) (ng g^{-1})	2150	505	1876	421	2490	796	3400	749	2304	559
2-OH-CBZ:CBZ	0.15 ± 0.04		0.13 ± 0.06		0.16 ± 0.05		0.07 ± 0.03		0.16 ± 0.05	
CBZ-EP:CBZ	0.11 ± 0.04		–		–		–		0.14 ± 0.07	
BCF CBZ (L kg^{-1})		5		5		9		9		6

(van der Oost et al., 2003), in agreement with its usually higher pollutants accumulation (Brooks et al., 2005; Ramirez et al., 2009).

CBZ levels in muscle (430 ± 14 ng g^{-1} w.w.) and liver (688 ± 83 ng g^{-1} w.w.) are in agreement with values reported by Garcia et al. (2012). In this study, bluntnose minnows (*Pimephales notatus*), exposed to 298 μg L^{-1} CBZ at different days in an uptake and depuration 42 d test, accumulated 324–414 and 892–1503 ng g^{-1} w.w. CBZ in muscle and liver, respectively. Tanoue et al. (2015) recently reported distribution of pharmaceuticals, including CBZ, in different tissues of wild crucian carp (*Carassius carassius*) and common carp (*Cyprinus carpio*) of 2 WWTP impacted rivers in Japan, with CBZ concentration in the range 0.043–0.12 μg L^{-1}. Even though being a field study, CBZ concentrations reached higher levels in liver (0.0526–0.204 ng g^{-1} w.w.) and brain (0.0212–0.165 ng g^{-1} w.w.) compared to gills (0.0454–0.117 ng g^{-1} w.w.) and muscle (0.0150–0.05 ng g^{-1} w.w.), as it was found in this study.

The biotransformation of CBZ by fish is not fully understood to date. In humans, CBZ is metabolized by a number of pathways, including 10,11-oxidation via the epoxide-diol pathway, hydroxylation and formation of iminostilbene (Fig.S1) (Breton et al., 2005). The first pathway includes the formation of carbamazepine-10, 11-epoxide (CBZ-EP), the principal metabolite of CBZ with anticonvulsant properties, which is almost completely hydrolyzed to 10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine (diOH-CBZ) before excretion. This pathway is mainly mediated by the cytochrome P450 (CYP) CYP3A4 isozyme, while a minor role is attributed to CYP2C8 (Kalapos, 2002). Another pathway is the hydroxylation of the six-membered aromatic ring, leading to the formation of 2-hydroxycarbamazepine (2-OH-CBZ), 3-OH-CBZ, 1-OH-CBZ, etc. (Cunningham et al., 2010). In fish, there is one report where the biotransformation of 12 pharmaceuticals known to be substrates for specific human CYPs, including CBZ, was quantified in vitro using rainbow trout liver S9 fractions (Connors et al., 2013). Authors mentioned that some CYP3A-like isoforms have been identified in several fish species. However they did not find measurable metabolism of CBZ (Connors et al., 2013). Since CBZ metabolites were not added at the beginning of the exposure experiment in the present study, and were also not detected in the exposure media throughout the studied period, its occurrence in organs of experimentally exposed fish can be mostly attributed to the biotransformation of the parental compound by *J. multidentata*. Namely, the metabolite 2-OH-CBZ was found in the five organs analyzed, ranging from 48 to 107 ng g^{-1} w.w. This could imply that CBZ hydroxylation metabolic pathway would be present in these organs. On the other hand, CBZ-EP, the main active human CBZ metabolite, was detected in fish muscle and gills (60 and 41 ng g^{-1} w.w. respectively).

These results are in contrast with the findings of Connors et al. (2013) in rainbow trout liver S9 fraction. As possible reasons for absence of metabolism in that study, authors stated other organs, e.g. fish gills, may also be capable of metabolizing pharmaceuticals. As it was found in the present study, not only gills, but also liver, intestine, brain and muscle showed CBZ metabolism (Table 2). Different exposure conditions (*in vitro* vs. *in vivo* tests, time of exposure, temperature of media, CBZ concentration, etc.) as well as individual species metabolic abilities could be reasons for differences found in CBZ fish metabolism between both studies.

In clinical practice, the ratio CBZ-EP/CBZ is widely used to characterize CBZ metabolism. In the study of Liu and Delgado (1995), this ratio was presented for epileptic children, reporting an average value of 0.137 ± 0.052 for total CBZ-EP/CBZ measured in serum of children. This value is in good agreement with the ratios obtained in muscle and gills of *J. multidentata* during this work (Table 2), suggesting that a similar epoxide metabolic pathway could be involved in fish, compared to humans. As for 2-OH-CBZ/CBZ ratio, similar values were obtained for all the organs of *J. multidentata* (range 0.07–0.16) (Table 2).

Moreno-González et al. (2016) have reported concentrations of 2-OH-CBZ and CBZ-EP in wild fish collected at the Mar Menor lagoon. These authors detected 2-OH-CBZ (0.07–0.3 ng g^{-1} d.w.) and CBZ-EP (n.d.—0.2 ng g^{-1} d.w.) in muscle of golden gray mullet (*Liza aurata*), but not in liver, mentioning that the uptake through gill, rather than dietary uptake of CBZ, could be the reason for such concentrations in muscle. Such idea is in agreement with our current results for CBZ-EP in *J. multidentata*, since CBZ-EP was only detected in gills and muscle. However, 2-OH-CBZ was detected in all the organs analyzed in *J. multidentata*, with higher levels in liver. These differences could be attributed not only to different exposure scenarios (different CBZ exposure concentrations in water, marine vs. laboratory exposure), but also to metabolic pathways of each fish species. Moreover, invertebrates might show differences in CBZ biodegradation compared to vertebrates. In an experiment with marine mussels, Boillot et al. (2015) detected CBZ-EP and acridine in digestive glands (with functions equivalent to vertebrates liver), gills and mantels of mussels (*Mytilus galloprovincialis*), after 3 days exposure to 100 μg L^{-1} CBZ. However, these authors did not detect 2-OH-CBZ in mussel organs. Nevertheless, Álvarez-Muñoz et al. (2015) reported CBZ-EP and 2-OH-CBZ (<MQL—1.3 ng g^{-1} d.w.) in other species of mussels from the Ebro delta (*Chamelea gallina* and *Crassostrea gigas*). These contradictory results reinforce the concept of species-specific metabolic pathway for CBZ, negating the simplification of a single mechanism for similar species.

The net amount of bioconcentrated CBZ, including the sum of the parental compound plus both metabolites, follows the order liver ≈ brain > muscle ≈ gill ≈ intestine. A similar trend was found for the analysis of CBZ in different wild fish species (Ramirez et al., 2009; Du et al., 2012; Tanoue et al., 2015), and also for the analysis of antidepressants and other pharmaceuticals (Brooks et al., 2005; Lajeunesse et al., 2009; Liu et al., 2015).

CBZ bioconcentration factors (BCF) in *J. multidentata* organs ranged from 5 to 9 L kg^{-1} w.w. It is worth to mention that BCFs < 10 L kg^{-1} w.w. have been previously reported for CBZ and other relatively polar pharmaceuticals (Meredith-Williams et al., 2012; Almeida et al., 2014; Boillot et al., 2015). These BCFs are lower than those usually measured for traditional persistent organic pollutants, with log K_{OW} > 3. Our

current results are in agreement with the ones reported by Garcia et al. (2012), with BCFs ranging 2–7 L kg^{-1} in plasma, liver, brain and muscle of *Pimephales notatus* and *Ictalurus punctatus*, using experimental exposures. When the bioaccumulation of CBZ was studied using an aquatic trophic chain, Vernouillet et al. (2010) found a 24-h BCF of 2.2 for the algae *Pseudokirchneriella subcapitata* exposed to 150 mg L^{-1} CBZ, a BAF of 12.6 for the crustacean *Thamnocephalus platyurus* fed with the CBZ-treated algae, and only traces of CBZ in the cnidarians *Hydra attenuate* fed with the contaminated crustacean. Even though differences in CBZ levels, and different organisms used for these experiments, BCF and BAFs are within the same order of magnitude than the ones obtained in this work for fish organs. Moreover, when mussels (*Dreissena polymorpha*) were exposed to low CBZ concentration (0.236 μg L^{-1}), they accumulated CBZ at 1.1, 4 and 6-fold after 1, 4 and 7 days of exposure, respectively (Contardo-Jara et al., 2011). In other experiment with juvenile rainbow trout, exposed for 10 d to low and high levels of a mixture of 5 pharmaceuticals, where CBZ was present at 1.6 and 43 μg L^{-1}, Lahti et al. (2011) reported BCFs of 0.4-0.3 in plasma of fish. More recently, Almeida et al. (2014) reported BCFs between 0.11 and 1.2 in two species of clams (*Venerupis decussate* and *Venerupis philippinarum*), exposed to 0.3–9 μg L^{-1} CBZ for 96 h. Lastly, Tanoue et al. (2015) reported CBZ BAFs for wild common and crucian carp exposed to 0.049–0.077 μg L^{-1} CBZ in river water, in the range 0.42–1.5 for plasma, brain, liver, kidney, muscle and gills. All in all, although differences between wild organisms and laboratory exposures exist, it is clear that CBZ is differentially accumulated among diverse organs of the exposed biota. This accumulation seems to occur via the uptake of CBZ from water, with BCFs < 10 L kg^{-1}. Such low BCFs would usually be taken as a criterion of "no bioaccumulation potential for a given substance". However, it has been stated that certain chemicals with log K_{OW} values < 5 have greater BAFs than BCFs, by approximately 1–2 orders of magnitude and, therefore, BCF should not be used as a sole predictor of bioaccumulation potential, but rather to estimate metabolic biotransformation rates that can then be incorporated into BAF models (Arnot and Gobas, 2006).

4. Conclusions

Twenty pharmaceuticals, including CBZ and two CBZ-metabolites, were accumulated in wild *G. affinis*, captured in polluted areas of the Suquía River, while only fifteen compounds were observed in wild *J. multidentata*. Thus, wild *G. affinis* accumulated a wider variety of compounds, resulting in a higher total load of pharmaceuticals than wild *J. multidentata* during the dry season, but not during the wet season. Concerning seasonal variation (wet – dry season), a higher pharmaceuticals accumulation trend was observed in wild *G. affinis* during the dry season, in agreement with the worse water quality of the basin during this period. Conversely, this was not the case for wild *J. multidentata*, which shows a greater variability in the accumulation of pharmaceuticals during both seasons. So far, our current results trigger the need of analyzing multiple species to account for the ecological impact of pollutants, negating the simplification of a single, "representative model" during ecotoxicological tests.

An optimized analytical method for the analysis of pharmaceuticals in small amounts of fish tissues was applied to evaluate the uptake and tissue distribution of CBZ and its metabolites in different tissues of *J. multidentata*, experimentally exposed to CBZ for 48 h at 100 μg L^{-1}. CBZ was preferentially accumulated in brain and liver, but also in gills, intestine and muscle of experimentally exposed *J. multidentata*. The metabolite 2-OH-CBZ was also present in the five organs, while CBZ-EP was only observed in muscle and gills. Considering that 2-OH-CBZ and CBZ-EP were not added during the experiment, nor detected in the exposure media, it is likely to think that CBZ is being biotransformed by exposed fish, probably using a metabolic pathway similar to that used by humans. To our knowledge, this is the first report evidencing the biotransformation of CBZ by fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.03.045.

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