Electronic Supplementary Material (ESI) for Environmental Science: Processes & Impacts.

Supplemental Material:

Content:

2. Materials and methods

2.1. Materials and fish exposure

Eight PhACs were selected for this study based on their high usage rates and frequent occurrence in surface water in China.[1,2] The standards of roxithromycin (ROX), erythromycin (ERY), ketoconazole (KCZ), ibuprofen (IBU), diclofenac (DIC), propranolol (PRO), carbamazepine (CBZ) and 17α-ethinylestradiol (EE2) were purchased from the laboratory of Dr. Ehrenstorfer (Augsburg, Germany). Erythromycin-13C,d3, carbamazepine-d10, ibuprofen-d3 and estrone-d4 were obtained from Sigma-Aldrich (Flanders, New Jersey, USA). Crucian carp were purchased from the Nanjing Institute of Fishery Science (Nanjing, China).

Crucian carp (*Carassius auratus*) is an important economic species widely distributed in the freshwaters of China. It has been demonstrated to be a very sensitive species to study the toxic effects of pollutants on aquatic organisms.[3] In the laboratory, the fish were fed every day with commercial fish food (6% of body weight/day). According to OECD guideline 305,[4] the exposure water's quality was checked daily and maintained at conditions suitable for crucian carp (Water temperatures 9 ± 1 °C; pH 7.4 ± 0.3; DO 7.5 ± 0.5 and $CaCO_3$ 110.5 ± 3.5 mg L^{-1}). Feces and uneaten food were removed every day by suction. Natural light conditions were used throughout the experiment.

2.2. Biomarker assays, HSI, EROD and GST

Hepatosomatic index (HSI) was calculated as follow: $HSI = (W_L/W_B) \times 100$, where W_L is the weight of the liver in grams and W_B is the body weight in grams. Liver samples were homogenized in nine volumes of cold buffer (0.15 M KCl, 0.1 M Tris–HCl, pH 7.4) and centrifuged for 25 min (10,000×g) at 4 °C. The supernatants were used as the extract for enzymatic activity determination. The use of S9 fractions is convenient and relatively cheap compared with using cytosol and microsomes. The

EROD activity was determined at 572 nm using a microplate reader. Specifically, 10 μL of supernatant, 140 μL buffer (0.1 M Tris, 0.15 M KCl, pH 8.0) and 10 μL of 2 μM 7-ethoxyresorufin were added to a 96-well microplate. The reaction was then initiated at 25 °C for 30 min by adding 40 μL of 2.1 mg mL^{-1} NADPH. NADPH was replaced by H_2O in the control wells. The molar extinction coefficient of the resorufin was 73 L $mmol^{-1}$ cm^{-1}. The EROD activity was expressed as pmol mg pro^{-1} min^{-1}. GST activity was determined at 340 nm by adapting to a microplate reader, using 30 μL of homogenate and 150 μL of the reaction solution (100 μL of 0.1 mM potassium phosphate, 10 μL of 1.0 mM 1-chloro-2,4-dinitrobenzene, 10 μL of 1.0 mM GSH and 880 μL H_2O) in microplates, and this activity was measured for 3 min. GST activity was expressed as nmol mg pro^{-1} min^{-1}.

2.3. Sample extraction

Tissue samples were extracted by pressurized liquid extraction (PLE) using a Dionex ASE 350 system (Thermo Fisher, Germering, Germany). Each tissue sample (1 g wet weight (ww) for muscle, gill and brain and 0.5 g ww for liver) was thoroughly mixed with hydromatrix and the mixture was put into a 22-mL stainless steel extraction cell containing a glass-fiber filter (27-mm diameter, type D28, Dionex) in the cell inlet and outlet. The extracting solvent was 100% methanol, and the operating conditions were as follows: extraction temperature, 70 °C; extraction pressure, 1500 psi; preheating period, 5 min; static extraction, 5 min; nitrogen purge, 180 s; and three static cycles.

Each PLE extract was concentrated to approximately 1 mL in a Büchi R200 (Labortechnik, Flawail, Switzerland) rotary evaporator set at 45 °C and 19600 Pa in a 50-mL round-bottomed flask. Then, the extract was transferred to a 15-mL conical tube and the round-bottomed flask was rinsed twice with 0.5 mL of methanol and evaporated to dryness using a multi-sample N-EVAP with a nitrogen stream and water bath at 50 °C. After solvent evaporation the extract was redissolved in 1 mL of acetonitrile, which has a low solubility for lipids. A freezing-lipid technique was applied, by means of redissolving the extract in acetonitrile and storing the extract in the freezer at -80 °C for 4 h. Most of the lipids precipitated out of solution and the extract was immediately centrifuged at 0 °C and 12000×*g* for 15 min. The supernatants were decanted into a chromatography bottle and reconstituted with 1 mL of acetonitrile, and 50 µL of the 1 mg L^{-1} mixture containing the internal standards were added.

Water samples (500 mL) were passed through Oasis HLB 6cc SPE cartridges (500 mg, Waters, USA). The cartridges were preconditioned with 6 mL of methanol and 6 mL of water. The samples were eluted from the cartridges using 2 × 3 mL of methanol. The extracts were evaporated to dryness under a stream of nitrogen, reconstituted with 1 mL acetonitrile, and analyzed by UPLC/MS/MS.

2.4. Instrumental analysis

Liquid chromatography was performed on an Agilent 1290 ultra-high performance liquid chromatography (UPLC) system (Agilent Technologies) consisting of a binary pump, a micro-vacuum degasser, an autosampler equipped with

a 1200 bar injection valve and a column oven (set to 30 °C). Analytes were separated on an Eclipse Plus C18 (150 mm × 4.6 mm, 5 μm, Agilent Technologies) column preceded by a C18 guard column at a flow rate of 0.3 mL min^{-1}. The injection volume was 5 μL. A binary gradient consisting of 0.1% (v/v) formic acid/0.02 mM ammonium acetate in water and 100% acetonitrile was employed to achieve separation as shown in Table S1. Detection was performed with an Agilent 6460 triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI). The ROX, ERY, KCZ, PRO and CBZ in the tissue and water samples were analyzed in the positive mode, whereas the DIC, IBU and EE2 were analyzed in the negative mode. The capillary voltage was held at 4 KV and the gas flow was set at 6 L min^{-1}. The pressure of the nebulizing gas was 35 psi. The sheath gas temperature was held at 350 °C and the gas flow was 9 L min^{-1}. The triple quadrupole was used in the multiple-reaction monitoring (MRM) mode to identify and quantify the compounds. The collision energy, the fragmentor voltage, the precursor and the production ions optimized by the direct infusion of standard compounds are presented in Table S2.

Whole analytical procedures were monitored using strict quality assurance and control measures. The method sensitivity was calculated by determining the method detection limits (MDL), the limit of detection (LOD) and the limit of quantitation (LOQ) for the target compounds. The MDLs of the target compounds calculated using standard solutions were 0.05–0.12 ng L^{-1}. For the water samples, the LODs and LOQs of the target compounds were 0.07–0.15 and 0.27–0.65 ng L^{-1}, respectively. Similarly, the LODs and LOQs of the target compounds corresponding to fish tissues

were 0.1–0.3 and 0.4–1.2 ng g^{-1}, respectively. Satisfactory recoveries were obtained for the target compounds in the range of 83.7–105.2% for the water samples and 62.4–83.6% for the fish samples.

2.5. Environmental implications

An environmental risk assessment of individual pharmaceuticals was performed based on chronic data and acute data according to the EMA guideline and REACH guideline[5], respectively. Typically, the risk quotient (RQ) of individual pharmaceuticals is calculated via their measured environmental concentration (MEC) divided by the predicted no-effect concentration (PNEC), as shown in Equation (1):

$$RQ = \frac{MEC}{PNEC} \quad (1)$$

According to the REACH guidance document, to estimate a PNEC on the basis of toxicity data, when only the short-term/acute toxicity data EC50 or LC50 are available, the calculation of PNEC is obtained from the EC50 or LC50 divided by an assessment factor (AF) of 1000. Once the long-term/chronic NOEC values for one, two or three trophic levels are available, an AF of 100, 50 or 10 is used.[5] In this study, an AF of 1000, 100 or 10 was used in relation to the toxicity of PhACs. In this study, the acute or chronic toxicity data of the detected PhACs on non-target organisms were collected from the literature and are shown in Table S3.

The environmental risk assessment of pharmaceutical mixtures was evaluated by the approach recently proposed by Backhaus and Faust. The approach for calculating the mixture RQ is outlined by Equation (2): the calculation of $\Sigma RQ_{MEC/PNEC}$, based on

the sum of MEC/PNEC values. The toxicity data EC50, LC50 or NOEC are represented by EC50 in Equations (2).

$$\Sigma RQ_{MEC/PNEC} = \sum_{i=1}^{n} \frac{MEC_i}{PNEC_i} = \sum_{i}^{n} \frac{MEC_i}{\min\left(EC50_{aglse}, EC50_{daphnids,}\ EC50_{fish}\right)_i \times (1/AF_i)} \quad (2)$$

The biomarker response data for each point were standardized according to Eq.(3):

$$Yi = (Xi\text{-}m)/s \quad (3)$$

where *Yi* is the standardized value of the biomarker, *Xi* is the mean value of a biomarker at each point, and *m* and *s* are the mean value and the standard deviation of a biomarker with all of the sampling points considered, respectively.

Using standardized data, *Zi* was then calculated as *Zi* = *Yi* or *Zi* = –*Yi* in the case of a biomarker responding to contamination by induction or inhibition, respectively. The minimum value (min*Zi*) for each biomarker at all of the points was calculated from the standardized response value. The score of each biomarker response (*Si*) was calculated as:

$$Si = Zi + |minZi| \quad (4)$$

Finally, to obtain an integrated multi-biomarker response, the EIBR value was calculated as the sum of the weighting of the biomarker and the biomarker score as follows:

$$EIBR = \sum_{i}^{n} Si \times Wi / \sum_{i}^{n} Wi \quad (5)$$

where Wi is the weighting of each biomarker i, and molecular (EROD and GST) and physiological (HSI) biomarkers are weighted as 1 and 3, respectively, because it is assumed that an alteration at the physiological level would have a greater impact on the health of the organisms than changes at the molecular level.

Table S1.

Time (min)	Composition of the mobile phase (%)	
Positive mode	Eluent A1 (0.1% (v/v) Formic acid)	Eluent B1 (Acetonitrile)
0	80	20
0.5	80	20
4	10	90
5	10	90
5.5	80	20
Negative mode	Eluent A2 (0.02 Mm Ammonium acetate)	Eluent B2 (Acetonitrile)
0	80	20
0.5	80	20
6	5	95
6.5	80	20

Table S2.

Compound	Precursor ion (a)	Product ion (a)	Fragmentor voltage (v)	Collision energy (v)	polarity
ROX	837	679.6	140	10	Positive
ERY	734.2	576.4	140	20	Positive
KCZ	531.3	489.3	140	5	Positive
PRO	260.2	116.2	120	20	Positive
CBZ	237.2	194.2	140	20	Positive
EE2	295	145	160	44	Negative
DIC	294.2	250	80	6	Negative
IBU	205	161	60	0	Negative

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170 **Table S3.**

Compound	Non-target organism	Toxicity data (mg L^{-1})	Endpoint	Toxicity	AF	PNEC (ng L^{-1})	Reference
ROX	*Pseudokirchneriella subcapitata*	NOEC = 0.01	Growth	Chronic	100	100	6
	Selenastrum capricornutum	EC50 = 4.0	Growth	Acute	1000	4000	7
	Daphnia magna	EC50 = 7.1	Immobilization	Acute	1000	7100	8
	Oryzias latipes	LC50 = 288.3	Mortality	Acute	1000	288300	8
ERY	*Pseudokirchneriella subcapitata*	EC50 = 0.02	Growth	Chronic	1000	20	9
	Selenastrum capricornutum	EC50 = 0.037	Growth	Acute	1000	37	10
	Ceriodaphnia dubia	EC50 = 0.22	Growth	Chronic	1000	220	9
	Ceriodaphnia dubia	EC50 = 10.23	Immobilization	Acute	1000	10230	9
	Oryzias latipes	NOEC = 100	Growth	Chronic	100	1000000	11
	Oryzias latipes	LC50 > 100	Mortality	Acute	1000	100000	12
DIC	*Pseudokirchneriella subcapitata*	NOEC > 10	Growth	Chronic	100	100000	13
	Pseudokirchneriella subcapitata	EC50 = 16.3	Mobility	Acute	1000	16300	14
	Ceriodaphnia dubia	NOEC = 1	Reproduction	Chronic	100	10000	13
	Daphnia magna	EC50 = 22.43	Immobilization	Acute	1000	22430	14
	Oncorhynchus mykiss	NOEC = 0.001	Cytotoxicity	Chronic	100	10	15
	Oncorhynchus mykiss	LC50 = 5.6	Cytotoxicity	Acute	1000	5600	16
PRO	*Cyclotella meneghiniana*	NOEC = 0.094	Growth	Chronic	100	940	14
	Cyclotella meneghiniana	EC50 = 0.244	Growth	Acute	1000	244	14

	Ceriodaphnia dubia	NOEC = 0.009	cytotoxicity	Chronic	100	90	17
	Ceriodaphnia dubia	LC50 = 0.8	Reproduction	Acute	1000	800	18
	Oryzias latipes	NOEC = 0.0005	Reproduction	Chronic	10	50	18
	Oryzias latipes	LC50 = 11.4	Mortality	Acute	1000	11400	12
CBZ	*Desmodesmus subspicatus*	EC50 = 74.0	Growth	Chronic	1000	74000	17
	Cyclotella meneghiniana	EC50 = 31.6	Growth	Acute	1000	31600	14
	Ceriodaphnia dubia	NOEC = 0.025	Reproduction	Chronic	100	250	13
	Daphnia magna	EC50 = 13.8	Mobility	Acute	1000	13800	13
	Danio rerio	NOEC = 25	Embryos and larvae mortality	Chronic	100	250000	13
	Oryzias latipes	LC50 = 45.87	Mortality	Acute	1000	45870	12

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173 **Table S4.**

Compound	Non-target organism	Toxicity data (mg L^{-1})	Toxicity	AF	PNEC (ng L^{-1})	Reference
ROX	*Pseudokirchneriella subcapitata*	NOEC = 0.01	Chronic	100	100	6
ERY	*Pseudokirchneriella subcapitata*	EC50 = 0.02	Chronic	1000	20	9
PRO	*Oryzias latipes*	NOEC = 0.0005	Chronic	10	50	18
CBZ	*Ceriodaphnia dubia*	NOEC = 0.025	Chronic	100	250	13
DIC	*Oncorhynchus mykiss*	NOEC = 0.001	Chronic	100	10	15

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