Insights on the metabolization of the antidepressant venlafaxine by meagre (*Argyrosomus regius*) using a combined target and suspect screening approach

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Abstract

Bioaccumulation of pharmaceuticals in fish exposed to contaminated water can be shaped by their capability to metabolize these xenobiotics, affecting their toxicity and animal welfare. In this study the *in vivo* metabolization of the antidepressant venlafaxine by the juvenile marine fish meagre (*Argyrosomus regius*) was evaluated using a combined target and suspect screening analytical approach. Thirteen venlafaxine metabolites were identified, namely N-desmethylvenlafaxine and N,N-didesmethylvenlafaxine, which were unequivocally identified using analytical standards, and 11 more tentatively identified by suspect screening analysis, including two Phase II metabolites formed by amino acid conjugation. All of them were detected in the liver, while in plasma and brain only 9 and 6 metabolites, respectively, were detected. Based on these findings, for the first time, a tentative metabolization pathway of venlafaxine by *A. regius* is proposed. Contrarily to what happen in humans, N-demethylation was identified as the main route of metabolization of venlafaxine by fish. Our findings highlight species-specificity in the metabolization of venlafaxine and allow a better understanding of venlafaxine's toxicokinetic in fish. These results emphasize the need to investigate the biotransformation of xenobiotics by non-target organisms to have an integrated overview of their environmental exposure and to improve future evaluations of environmental risk assessment.

Keywords: Pharmaceuticals, biotransformation, fish, water exposure, LC-HRMS

1. Introduction

Venlafaxine is an antidepressant belonging to the serotonin-norepinephrine reuptake inhibitors (SNRIs) family. It is used in the treatment of depression and anxiety disorders and it is among the most prescribed antidepressants worldwide (Magalhães et al., 2014). For instance, from 2005 to 2015, the consumption of venlafaxine in Germany has significantly increased from around 5 tonnes/year to 25 tonnes/year (Boulard et al., 2020). Venlafaxine is excreted in human urine as unchanged compound (4.7%) or transformed in metabolites, such as O-desmethylvenlafaxine (56%), N,O-didesmethylvenlafaxine (16%), N-desmethylvenlafaxine (1%) and N,O-didesmethyl-N-desmethylvenlafaxine (Magalhães et al., 2014). Although venlafaxine is mainly excreted in its metabolized form, it has been detected in raw wastewater, showing concentrations only three times lower than those of its main human metabolite O-desmethylvenlafaxine (Schlüsener et al., 2015). This may be justified by the weak statistically provable relation between the concentration of pharmaceuticals in urine and in raw wastewater described by Winker et al. (2008), due to environmental effects occurring during the passage from human excretion to wastewater treatment plants (WWTP) influents. Regardless all this, venlafaxine has been detected in different environmental compartments all over the world, such as in surface waters (Birch et al., 2015; Čelić et al., 2019; Paíga et al., 2016; Schlüsener et al., 2015), at levels up to 349 ng/L, in tap water (0.5-1.9 ng/L) (Giebułtowicz and Nałęcz-Jawecki, 2014), and also in sediments (Santos et al., 2016) and suspended particulate matter (Boulard et al., 2020), reaching concentrations up to 26.4 ng/g and around 12 ng/g, respectively.

The ubiquitous presence of venlafaxine in the environment implies a chronic exposure of non-target organisms to this contaminant, even at low concentrations. Venlafaxine acts by inhibiting the reuptake of serotonin and norepinephrine from the

presynaptic cleft, thus increasing the levels of these two neurotransmitters in the synapse. Since serotonin participates in different regulatory and endocrine functions in fish, changes in its levels may exert different toxic effects (Santos et al., 2010). Indeed, several studies have described that venlafaxine is able to affect fish metabolic response (Best et al., 2014; Maulvault et al., 2019), behaviour (Maulvault et al., 2018b; Painter et al., 2009), reproduction (Galus et al., 2013; Parrott and Metcalfe, 2017) and survival (Schultz et al., 2011).

Fish have shown potential to bioaccumulate venlafaxine (Arnnok et al., 2017; Grabicova et al., 2017; Huerta et al., 2018; Moreno-González et al., 2016), however bioaccumulation can be modified by metabolization. In fact, the metabolization of xenobiotics is a key factor to reduce their accumulation, body burden and toxicity (Connors et al., 2013). In humans, venlafaxine is extensively metabolized in liver by cytochrome P450 (CYP) isoenzymes. Different studies have revealed that CYP1A2, CYP2D6, CYP2C9, and CYP3A4 are the CYP isoforms involved in human metabolization of venlafaxine (Magalhães et al., 2014). Fish share some metabolic pathways with humans and thus are capable of metabolize some pharmaceuticals (e.g. fluoxetine (Smith et al., 2010); carbamazepine (Valdés et al., 2016), tramadol (Tanoue et al., 2017) and diclofenac (Lahti et al., 2011)). Like in humans, the primary metabolization organ in fish is the liver, and this process is mainly mediated by CYP450, but other pathways may also be involved (Burkina et al., 2015). CYP isoenzymes differ among species and some mammalian CYP isoforms (e.g. CYP2 family) possess just a few piscine orthologues (Connors et al., 2013). Thus, changes in pharmaceuticals metabolization are expected between humans and fish.

Despite their importance for the evaluation of risk assessment, the metabolization of pharmaceuticals by fish has barely been studied. In this context, the objective of this

study was to investigate the *in vivo* metabolization of the antidepressant venlafaxine by the juvenile meagre (*Argyrosomus regius*) using a combined target and suspect screening analytical approach. For the first time a tentative metabolization pathway of venlafaxine by fish is proposed. The distribution of metabolites in different tissues (liver, brain and plasma) is also evaluated.

2. Materials and Methods

2.1. Chemicals and Reagents

Venlafaxine was purchased from Sigma-Aldrich and venlafaxine human metabolites (N-desmethylvenlafaxine, O-desmethylvenlafaxine, N,N-didesmethylvenlafaxine, N,O-didesmethylvenlafaxine and N,N-didesmethyl-O-desmethylvenlafaxine) were purchased from Toronto Research Chemicals (Ontario, Canada). Venlafaxine-d6 (VLF-d6) was used as internal standard and was purchased from CDN Isotopes (Quebec, Canada). All standards were of high-grade purity (>98%). Methanol, acetonitrile and water were provided by Merck (Darmstadt, Germany). All solvents were MS grade. Formic acid 98-100% was purchased from Merck (Darmstadt, Germany) and ammonia 30% (as NH_3) was purchased from Panreac (Barcelona, Spain).

2.2. Experimental design – *In vivo* exposure experiments

2.2.1. Test organism, fish rearing and acclimation

Juvenile meagre (*A. regius*) was selected as model organism, because: i) it is a predator fish species that tends to accumulate organic chemical contaminants (Bodin et al., 2014); ii) its commercial interest for human consumption has been growing in the last years, especially in Mediterranean area, so the data obtained can contribute to the assessment of seafood safety (possible risk of human exposure) (FAO, 2019); and iii) the

early life stages of fish species (larvae and/or juvenile) are more susceptible to deleterious effects due to exposure to environmental chemical contamination.

Meagre specimens were raised at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) until reaching the juvenile stage. Then, fish with similar biometric characteristics (total length: 6.8 ± 0.4 cm; total weight: 2.6 ± 0.5 g) were transported to the aquaculture facilities of the Laboratório Marítimo da Guia (MARE-FCUL, Cascais, Portugal), where they were randomly distributed in 50 L glass tanks filled with natural UV-disinfected seawater for acclimation. Animal density was kept below 5g body weight/L in each tank in order to avoid physiological stress. Each tank had independent functioning and it was equipped with a protein skimmer (ReefSkimPro, TMC Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal) and biological filtration (model FSBF 1500, TMC Iberia, Portugal). Dead fish and faeces were daily removed, and seawater was partially daily renewed (25% of the total volume of the tank). Fish acclimation was done for a period of 30 days in the following abiotic conditions: i) dissolved oxygen (DO) > 5 mg/L; ii) temperature (19.0 ± 0.5 ºC); iii) pH = 8.0 ± 0.1; iv) salinity = 35 ± 1 ‰; and v) photoperiod = 12h light: 12h dark. Temperature, pH, salinity and DO were daily checked using a multi-parameter probe (Multi 3420 SET G, WTW, Germany). Fish were fed with a commercial fishmeal formulation for juvenile marine fish obtained from SPAROS Lda (Olhão, Portugal), that consisted of 48% crude protein and 18% crude fat. Detailed feed composition can be found elsewhere (Maulvault et al., 2018a). Seawater quality was daily checked by determining ammonia, nitrite and nitrate levels, and total alkalinity was weekly measured. More detailed information can be found elsewhere (Maulvault et al., 2018a).

149 **2.2.2. Venlafaxine exposure experiments**

150 Two treatments, namely control and exposure to venlafaxine at a nominal
151 concentration of 20 µg/L, were carried out using 10 individuals per tank. Each treatment
152 was performed in triplicate. For the exposure to venlafaxine, a stock solution of
153 venlafaxine (20 mg/L) was prepared on a weight basis in deionized water (total volume
154 = 500 mL). Then, seawater in the exposure tanks was spiked daily at a nominal
155 concentration of 20 µg/L of venlafaxine. The exposure experiments were performed
156 under the same abiotic conditions of the acclimation period (see section 2.2.1). The
157 exposure time was 28 days, followed by 7 additional days (until 35 days), during which
158 the spiking of venlafaxine was stopped. A schematic representation of the experimental
159 design is shown in Figure 1. During all the experiment, in both treatments (control and
160 venlafaxine exposure), fish were fed on a daily basis with the same amount of a
161 commercial fishmeal formulation, i.e., 2% of average body weight. Seawater abiotic
162 parameters were also monitored daily. No mortality was observed during the 35 days of
163 the experiment.

164

165 **2.2.3. Sampling**

166 A total of 10 fish were randomly sampled from each treatment (between the 3
167 replicate tanks) at days 28 (end of the exposure phase) and 35 (end of the experiment).
168 Then, fish were euthanized by immersion in an overdose MS222 solution (2,000 mg/L;
169 Sigma-Aldrich, USA) buffered with sodium bicarbonate (1 g of $NaHCO_3$ and 1 g of
170 MS222 per litre of seawater) for 10 min. Blood was collected by puncture of the caudal
171 vein and centrifuged (10,000 *g*, 15 min, 4 ºC). Then, plasma samples were collected,
172 pooled in two composite samples (5 individuals per pool, n = 2) and kept at -80 ºC until
173 further analysis. After blood collection, fish was dissected to collect muscle, liver and

brain tissues. Two composite samples were also prepared for each tissue, consisting in a pool of 5 individuals (n = 2). After that, composite samples were freeze-dried at -50 ºC, 10^{-1} atm of vacuum pressure for 48h (Power Dry LL3000, Heto, Czech Republic), homogenized and stored at -80 ºC until analysis.

Seawater samples from each replicate tank were collected at days 14, 28 and 35 for the quantification of venlafaxine and its metabolites. Water samples were collected in glass bottles and kept at -20 ºC until further analysis.

2.4. Sample preparation

For the extraction of venlafaxine and its metabolites from fish muscle, liver and brain, 50 mg (or 25 mg for brain) of freeze-dried tissue were extracted with 1 mL of a mixture methanol:water (75:25, *v/v*) using ultrasonic assisted extraction for 15 min in ice bath. Then, samples were centrifuged (7,500 rpm, 15 min, 4 °C) and the supernatant was collected in a glass tube. This protocol was repeated three times and all supernatants were combined and evaporated under a gentle stream of nitrogen to remove the organic solvent. Then, the evaporated extracts were reconstituted in 50 mL Milli-Q water with 0.1% formic acid and a clean-up step was performed by solid phase extraction (SPE) using Oasis® MCX (3cc, 60 mg) (Waters, Ireland). Briefly, SPE cartridges were conditioned with 3 mL of methanol and 3 mL of 0.1% formic acid in water. Then, the reconstituted extract was percolated through the SPE cartridge at a flow rate of 3.0-5.0 mL/min. After that, cartridges were washed with 5 mL of 2% formic acid in water and dried under vacuum for 5 min. Finally, compounds were eluted with 6 mL of 5% ammonia in methanol, evaporated until dryness under a gentle stream of nitrogen and reconstituted in 1 mL of a mixture of methanol:water (50:50, *v/v*). Before analysis, extracts were filtered by PVDF syringe filters (0.22 µm) (Merck Millipore, Ireland) and an aliquot of 150 µL

of extract was transferred to an insert. Finally, 1.5 µL of a 1 ng/µL VLF-d6 standard solution was added as internal standard.

For the analysis of fish plasma, 50 µL of methanol were added to 50 µL of fish plasma, centrifuged (5,000 rpm, 10 min, 4 ºC) and, then 60 µL of supernatant were transferred in an insert. Finally, 0.6 µL of a 1 ng/µL VLF-d6 standard solution was added before analysis.

Seawater samples were filtered by 0.22 µm PVDF syringe filters (Merck Millipore, Ireland). Then, 100 µL of methanol was added to 900 µL of filtered seawater and water samples were analyzed by direct injection. Previous to analysis, 10 µL of a 1 ng/µL VLF-d6 standard solution was added.

2.5. Combined target and suspect screening approach

The identification of venlafaxine metabolites generated by fish was done by combining two different analytical approaches (Figure S1), namely: i) target analysis that allowed the quantification of venlafaxine and its selected human metabolites in fish tissues and plasma, using analytical standards commercially available; and ii) suspect screening analysis that allowed the identification of other possible metabolites of venlafaxine generated by fish, for which there is not commercial analytical standards available, as well as the proposal of a tentative metabolization pathway by meagre.

2.5.1. Target analysis

Quantification of venlafaxine and its human metabolites in fish tissues and plasma as well as in the seawater of the tanks was performed by ultra-high performance liquid chromatography coupled with a quadrupole linear ion trap mass spectrometry detector (UPLC-QqLIT) from Waters-ABSciex using an adapted method from Gros et al. (2012).

The selected compounds were analysed in the positive ionization mode. Detailed information on the mass spectrometer parameters and on the quality parameters for the analysis of venlafaxine and its human metabolites in the different matrices is given in Supporting Information (Tables S1-S3). Relative recoveries were determined by comparing concentrations obtained after the whole extraction procedure, calculated using internal standard and a matrix-matched calibration curves, with the initial spiking levels. All the concentrations detected in the different fish tissues and plasma were corrected by the respective relative recoveries.

2.5.2. Suspect Screening analysis

Suspect screening analysis of fish tissues and plasma was performed using liquid-chromatography coupled to a high-resolution mass spectrometer (LC-HRMS) for elucidation of the tentative metabolites of venlafaxine generated by fish. Chromatographic analysis was carried out in an Aria TLX-1 chromatographic system from Thermo Fisher Scientific comprising a PAL autosampler and two mixing quaternary pumps. The chromatographic separation was performed on a Thermo Hypersil GOLD PFP column (100 x 2.1 mm; 1.9 µm) (Thermo Scientific, USA). For positive mode, the selected mobile phase consisted in 0.1% formic acid in water (A) and acetonitrile (B), using the following linear gradient: 0-1.0 min, 5% B; 1.0-8.0 min, 5-100% B; 8.0-10.0 min maintain 100% B; 10.0-12.0 min, return to initial conditions (5% B); 12.0-13.5 min, re-equilibration of the column. For negative mode, the mobile phase was water (A) and acetonitrile (B) and the linear gradient consisted in: 0-1.0 min, 5% B; 1.0-6.0 min, 5-100% B; 6.0-8.0 min maintain 100% B; 8.0-8.5 min, return to initial conditions (5% B); 8.5-11.0 min, re-equilibration of the column. A flow rate of 0.5 mL/min was used for both

ionization modes, the column was kept at room temperature and the autosampler was operated at 10 ºC. An injection volume of 10 µL was used.

The LC system was coupled to a high-resolution mass spectrometer LTQ-Orbitrap Velos™ (Thermo Fisher Scientific), equipped with a heated electrospray ionization source (HESI-II). Analysis were performed on both positive and negative ionization mode following a methodology adapted from Jaén-Gil et al. (2019). Chromatograms and mass spectra were acquired through two parallel scan events using Data Dependent Acquisition mode (DDA): 1) a full-scan mode from a mass-to-charge (*m/z*) range of 100 to700 at a resolving power of 60,000 FWHM, followed by 2) the MS/MS full-scan fragmentation from a *m/z* range of 50 to700 at 30,000 FWHM of the three most intense ion masses selected in each MS scan event. For positive ionization mode, mass spectrometry conditions were set up as follow: spray voltage at 3.5 kV, source heater temperature at 300 ºC, capillary temperature at 350 ºC, sheath gas flow at 40 and auxiliary gas flow at 20 (arbitrary units). For negative mode, spray voltage was kept at 3.0 kV, source heater temperature at 450 ºC, capillary temperature at 450 ºC, sheath gas flow at 35 and auxiliary gas flow at 10 (arbitrary units). MS/MS fragmentation scans were acquired in collision-induced dissociation (CID) at a normalized collision energy of 20 eV (activation Q of 0.250 and activation time of 30 ms) and an isolation width of 2 Da. The entire system was controlled via Aria Software under Xcalibur 2.1 software. An example of a chromatogram is presented in Supporting Information (Figure S2).

2.5.3. Suspect screening data processing

An automated data processing methodology adapted from Jaén-Gil et al. (2018) using Compound Discoverer 3.0 software (Thermo Scientific) was used for the identification of tentative metabolites of venlafaxine by fish. This methodology was based

on an automatic *in silico* prediction of a list of tentative venlafaxine metabolites to be found in the different fish tissues. For this purpose, venlafaxine chemical structure was firstly pinpointed as the parent compound into the software. Then, a list of 8 Phase I and 16 Phase II potential chemical transformations were defined to be applied to the parent compound structure, considering the most common Phase I and Phase II metabolic reactions involved in xenobiotics metabolization. A combination of a maximum of three consecutive chemical transformations was set. Finally, a list containing a total of 971 suspected exact masses was automatically predicted by the software (Table S4, Supporting Information) and were further used for compound detection and identification by searching the compounds list in fish samples from both treatments (control and exposure to venlafaxine at a nominal concentration of 20 µg/L).

Prior to automatic data processing, computational data files (chromatograms and mass spectra) were loaded into the software. Then, automatic data processing started with data filtering in the *m/z* range of 100-500 and by setting a peak intensity threshold at 1.5 signal-to-noise ratio. Chromatographic alignment was performed using a mass tolerance error of 5 ppm and a maximum retention time shift of 0.3 min. Then, the list of predicted compounds (including all the tentative metabolites of venlafaxine) was automatically compared to the accurate masses obtained in the experimental data, applying a mass tolerance error of 5 ppm and a minimum chromatographic peak intensity of 1000 counts. The compounds (venlafaxine and predicted metabolites) that were found in the samples were included into a list of detected compounds. For identification purposes, MS/MS *in-silico* elucidation was automatically carried out by comparing predicted fragment structures with those of the MS/MS spectra of the samples, using a mass tolerance error of 5 ppm and a signal-to-noise ratio of 3. After data processing, detected compounds with a FISh score ≥ 65% (MS/MS reliability percentage) with at least two characteristic *in-*

298 *silico* fragment structures were selected as potential venlafaxine metabolites. Finally, the
299 predicted structures of these compounds and their MS/MS spectra were manually checked
300 in order to avoid false positives. A combination of different criteria has to be fulfilled to
301 support the structural elucidation of tentatively identified venlafaxine metabolites,
302 namely MS/MS fragmentation spectra, mass accuracy (± 5 ppm), isotopic pattern, and
303 retention time (Gonzalez-Gil et al., 2019). In the case of the human venlafaxine
304 metabolites, tentative identified compounds were confirmed with analytical standards
305 commercial available.
306
307 **3. Results and Discussion**
308 Albeit it was shown that juvenile meagre (*A. regius*) was able to bioaccumulate
309 the antidepressant venlafaxine in its tissues and plasma (Maulvault et al., 2018a), no data
310 is available on its ability to metabolize this antidepressant. Thus, two complementary
311 analytical approaches (target and suspect screening analysis) were used for the
312 identification of potential venlafaxine metabolites by meagre and to assess their
313 distribution through different fish tissues.
314
315 **3.1. Target analysis**
316 Concentrations of venlafaxine in juvenile meagre exposed to contaminated
317 seawater by 28 days significantly varied according to the tissue, following the general
318 trend: liver > brain > plasma > muscle (Table 1). Concentrations of venlafaxine ranged
319 from 6,808 ± 1,177 ng/g, d.w. (liver) to 423 ± 159 ng/g, d.w. (muscle). The tissue-specific
320 bioconcentration factors (BCF) of venlafaxine were also calculated using its measured
321 concentration in water and the concentration in each tissue/plasma. BCF followed the
322 same trend of concentrations, i.e. liver (BCF = 354 ± 61) > brain (BCF = 130 ± 3) >

plasma (BCF = 67 ± 4) > muscle (BCF = 22 ± 8). For all tissues, BCF were lower than the threshold limit of 2,000 (ECHA, 2017).

Metabolization of venlafaxine by juvenile meagre was evaluated taking into account the pharmacokinetics and metabolization of this antidepressant by humans (Magalhães et al., 2014), as well as the commercial availability of analytical standards. Thus, five human metabolites of venlafaxine were selected for evaluation of their presence in fish samples (tissues and plasma) and in the water of the exposure experiment following a target analysis approach (Table 1).

After 28 days of exposure, besides venlafaxine, 2 out of 5 human metabolites of venlafaxine were detected in fish tissues (liver, brain and muscle) and plasma, namely N-desmethylvenlafaxine and N,N-didesmethylvenlafaxine. N-desmethylvenlafaxine was the metabolite attaining the highest concentration in all fish tissues, reaching levels up to 121 ± 44, 111 ± 9 and 30.5 ± 18.8 ng/g, d.w., in liver, brain and muscle, respectively, and 21.9 ± 4.6 ng/mL in fish plasma (Table 1). On the other hand, N,N-didesmethylvenlafaxine was only detected in fish brain and plasma, but in the latter at levels below the method quantification limit (<1.68 ng/mL) (Table 1). Due to analytical limitations, it was not possible to analyse N,N-didesmethylvenlafaxine in liver samples, because this metabolite presented a very low recovery (<10%) after sample preparation. Furthermore, at the end of the experiment (day 35), there was an increase in the concentration of N-desmethylvenlafaxine in the different fish tissues (199 ± 32, 120 ± 1 and 56.7 ± 1.7 ng/g, dw, in liver, brain and muscle, respectively), while in fish plasma its concentration decreased (11.1 ± 0.5 ng/mL) (Table 1). A small decrease in the concentration of N,N-didesmethylvenlafaxine in brain was also reported. As observed for unchanged venlafaxine (Maulvault et al., 2018a), liver was the organ that showed the highest concentration of venlafaxine metabolites, followed by brain, plasma and muscle.

Nevertheless, venlafaxine metabolites reached concentrations with 1-2 orders of magnitude less than the parent compound. These lower concentrations are expected, since metabolization of xenobiotics is a process of detoxification of organisms, which intends to produce more hydrophilic and less toxic metabolites and, in this way, easiest to be excreted. Therefore, it is not expected that they accumulate in fish tissues. Moreover, the raise in the concentration of metabolites at the end of the experiment is in agreement with this detoxification process, since after stopping the spiking of the contaminant, fish continued to metabolize venlafaxine present in their tissues as a reaction of depuration to the exposure to the contaminant. Additionally, the results of target analysis also showed that fish do not share the metabolization pathway of venlafaxine with humans. While O-demethylated metabolites are the main human metabolites of venlafaxine (Magalhães et al., 2014), our results indicate that in fish the N-demethylated metabolites are the predominant ones (Table 1).

The concentration of venlafaxine in seawater was monitored throughout the experiment. Venlafaxine was not detected in seawater of the control treatment whereas during the exposure phase it was kept around 20 µg/L (20.9 ± 1.8 µg/L at day 14, and 19.2 ± 1.6 µg/L at day 28), decreasing to 17.6 ± 1.4 µg/L at the end of the experiment (day 35) (Table S4). As there was only a 25% of seawater renewal per day, at the end of the experiment venlafaxine was still in the water of the exposure treatments. Besides, venlafaxine could also be excreted by fish as unchanged compound, contributing for its detection in water. Venlafaxine metabolites showed the same trend than the parent compound and they were only detected in seawater from the exposure treatment. Among the 5 selected human metabolites of venlafaxine N-desmethylvenlafaxine and N,N-didesmethylvenlafaxine showed the highest concentrations (up to 3.93 ± 0.23 and 4.39 ± 0.35 µg/L, respectively, at day 28), which is in agreement with the excretion of these

373 metabolites by fish (Table S5). On the other hand, O-desmethylvelafaxine and N,O-
374 didesmethylvenlafaxine, two metabolites that were not detected in fish tissues, were
375 quantified in seawater at a residual level during all the experiment (up to 1.24 ± 0.07 and
376 0.33 ± 0.02 µg/L, respectively), whereas N,N-didesmethyl-O-desmethylvenlafaxine was
377 not detected in seawater of the exposure tanks. Since O-demethylated metabolites were
378 not detected in fish tissues or plasma, their presence in seawater might be associated to
379 degradation of venlafaxine during the exposure experiment. In fact, O-
380 desmethylvenlafaxine was identified as the main transformation product of venlafaxine
381 under both aerobic and anaerobic conditions, while N,O-didesmethylvenlafaxine could
382 also be formed at residual levels (Gasser et al., 2012).
383
384 **3.2. Suspect screening analysis**
385 A suspect screening approach was used for an in-depth evaluation of the
386 metabolization of venlafaxine by fish. This analytical approach allowed the tentatively
387 identification of venlafaxine metabolites generated by juvenile meagre (*A. regius*),
388 including those metabolites that are not common with humans and for which there is not
389 commercial analytical standards available, as well as to propose a possible metabolization
390 pathway.
391 Fish liver, brain and plasma were considered for suspect screening analysis, since
392 they showed higher levels of venlafaxine and its metabolites by target analysis (Table 1).
393 Moreover, these are also tissues that can actively contribute to the metabolization of
394 venlafaxine by fish, given that liver is the main metabolization organ of xenobiotics, but
395 plasma may also contribute to this process together with brain, due to the presence of
396 enzymes that are involved in metabolic reactions (Burkina et al., 2015).

Potential venlafaxine metabolites generated by fish were investigated applying a suspect screening methodology that compared the obtained accurate masses after compound detection with a list of 971 predicted venlafaxine metabolites generated by *in-silico* tools. Automated software data processing together with manual data reviewing allowed the detection of 14 compounds in samples, including the parent compound (venlafaxine) and 13 metabolites (11 Phase I and 2 Phase II). All tentatively identified metabolites were detected in ESI positive mode. Retention time and chemical structure elucidation based on the MS/MS *in-silico* fragmentation were used for identification purposes, given that different peaks could share the same exact mass. For venlafaxine and its human metabolites N-desmethylvenlafaxine and N,N-didesmethylvenlafaxine, structures were confirmed with reference standards commercial available by comparison of retention time, accurate masses and MS/MS fragmentation. A summary of the 13 tentatively identified venlafaxine metabolites, including their retention time, accurate mass, elemental composition and suggested chemical structure, is present in Table S6.

Tentatively identified Phase I metabolites were mainly obtained by oxidative reactions (dealkylation, hydroxylation and oxidation) and Phase II metabolites by amino acid conjugation processes. A tentative metabolization pathway of venlafaxine by meagre (*A. regius*) is proposed in Figure 2. As it can be seen, suspect screening results confirmed N-demethylation as the main route of metabolization of venlafaxine by fish. Besides the confirmation of the presence of the two metabolites identified by target analysis (N-desmethylvenlafaxine and N,N-didesmethylvenlafaxine), suspect screening analysis allowed the tentative identification of 9 more Phase I metabolites of venlafaxine in fish. One of these metabolites (M294A and M294B), which results from the hydroxylation of the molecule of venlafaxine, has been previously identified in the crustacean *Gammarus pulex* exposed to venlafaxine (Jeon et al., 2013). The hydroxylation of venlafaxine can

occur in two different positions (at the amine group or in the cyclic C-H bond) (Jeon et al., 2013), despite the exact position of this modification could not be elucidated with the acquired MS/MS data. Most of the tentative venlafaxine metabolites identified in this work resulted from demethylation and hydroxylation reactions, however dehydrogenation reactions could also occur, forming double bonds in the cyclohexane ring (e.g. M294C, M276, M280, M262 and M244). A combination of dehydrogenation and hydroxylation reactions led to the formation of double bonds in the cyclohexane ring, due to the introduction of an oxygen in the molecule followed by a loss of water. These reactions were previously described for the transformation of venlafaxine during chemical processes (Lambropoulou et al., 2017).

Most Phase I reactions are mediated by cytochrome P450 (CYP) isoenzymes, which have a crucial role in detoxification processes (Burkina et al., 2015). In humans, venlafaxine is extensively metabolized in liver by CYP isoenzymes CYP1A2, CYP2D6, CYP2C9, CYP2C19, and CYP3A4. From these, O-demethylation reactions are predominantly associated to CYP2D6, while N-demethylation is partially mediated by CYP3A4 (Magalhães et al., 2014). Fish do not have piscine orthologues of mammalian CYP2B, 2C and 2D enzymes, though they have CYP1A and CYP3A-like enzymes (Burkina et al., 2018). Thus, these interspecies variations are in agreement with the observed predominance of N-demethylated metabolites in the metabolization of venlafaxine by fish (Figure 2). Nevertheless, fish may have other unknown enzymes capable of catalysing reactions associated with a specific human CYP even if there is any piscine orthologues of human enzymes (Connors et al., 2013). This could justify the presence of two tentatively identified metabolites (M235 and M251), generated by both O- and N-demethylations together with other oxidation reactions (Figure 2).

Two Phase II metabolites of venlafaxine formed by amino acid conjugation were tentatively identified in fish liver and brain. One resulted from the conjugation of venlafaxine with glutamine (M424) and the other one from the conjugation of the Phase I metabolite N,N-didesmethylvenlafaxine with glycine (M325) (Figure 2). Fish form conjugated metabolites to enhance the hydrophilicity of metabolites that are rapidly excreted by the organism, since they can be recognized by efflux transport proteins present in secretory epithelial tissues (Tierney et al., 2014).

In general, venlafaxine was not extensively metabolized by meagre, showing a high bioaccumulation (Table 1; Figure 3). This could be justified by the lack in fish of CYP2 family enzymes orthologues to the human ones that are directly involved in venlafaxine metabolization (Burkina et al., 2018; Connors et al., 2013). Therefore, an accumulation of this pharmaceutical in fish body might be favoured. A low capacity of fish to metabolize other antidepressants, like fluoxetine, was also described for rainbow trout (Connors et al., 2013). Nevertheless, a total of 13, 9 and 6 metabolites were detected in fish liver, plasma and brain, respectively (Figure 3).

All tentatively identified metabolites were detected in liver and M262 was the metabolite showing a higher area (of the chromatographic peak) at the end of the exposure phase (day 28), while after 35 days Phase II metabolites were the predominant ones, followed by M235 and M251 (Figure 3b; Table S7). This high prevalence of metabolites in liver was expected, since this organ is the major site of xenobiotics metabolization. In the case of the Phase II metabolite M424, its peak area showed an increment of 14 times from day 28 to day 35, showing a peak area higher than venlafaxine at the end of the experiment (Figure 3b; Table S7). This could be related with the effort of the organism in eliminating venlafaxine by converting it in a more water soluble metabolite that could

be easily excreted, given that fish conjugate amino acids with xenobiotics to enhance their excretion (Tierney et al., 2014).

In fish plasma, 9 Phase I metabolites were tentatively identified and from these M294A showed the highest peak area, followed by M276 and M262. Higher peak areas of venlafaxine metabolites were detected at day 28 than at day 35 (Figure 3a; Table S7). The same trend was seen for venlafaxine (Table 1).

Only 6 metabolites were detected in fish brain and M262, M276, M244 and M280 were the predominant ones. At the end of the experiment (day 35), in general, an increase in the abundance of the venlafaxine metabolites in fish brain was observed, especially for M276 and M262 that reached peak areas similar to venlafaxine. At day 35, also the Phase II metabolite M424 was detected, though at residual levels compared to the other metabolites (Figure 3c; Table S7).

The suspect screening analysis results shown herein are indicative of the trend of metabolization of venlafaxine by fish. However, it is not possible to compare the relative abundance of metabolites between the different tissues, since the MS signal response is affected by the high matrix effect usually associated to biological matrices. A lower MS signal response for venlafaxine and its metabolites was observed in fish liver and brain, which could be attributed to their high content of lipids and proteins (Tanoue et al., 2014; Valdés et al., 2016). Additionally, it should be noted that venlafaxine metabolites may also have differences in the response in mass spectrometry detection, thus some precaution should be taken on the evaluation of the metabolization profile of venlafaxine in the different tissues based only on chromatographic peak areas. Differences in mass spectrometry response to different compounds can vary, even if they share a similar structure, and the presence of metabolites can be overestimated or underestimated (Rubirola et al., 2014). For an accurate quantification of venlafaxine metabolites the

corresponding analytical standards need to be used. In the case of the tentatively identified venlafaxine metabolites, these standards are not commercially available yet.

3.3. Comparison of venlafaxine metabolization between aquatic organisms

The metabolization and the presence of venlafaxine metabolites in aquatic organisms has only been investigated in a limited number of studies. Notwithstanding, as these studies followed a target analysis methodology, they just evaluated the presence of venlafaxine human metabolites, which are the ones that have commercial analytical standards available. Table 2 compiles data from literature on the presence of venlafaxine and its metabolites in different aquatic organisms. Independently of the aquatic organism considered, in general, there is a more pronounced presence of venlafaxine than of its metabolites. This could be observed either in exposure experiments (Santos et al., 2019; Serra-Compte et al., 2018) or in field samples (Álvarez-Muñoz et al., 2015; Koba et al., 2018). Contrarily to what was described for fish in the present study, river biofilms, the freshwater crustacean *G. pulex* and mussels were able to metabolize venlafaxine in both demethylated forms (O- and N-desmethylvenlafaxine) (Jeon et al., 2013; Santos et al., 2019; Serra-Compte et al., 2018). Nevertheless, N-desmethylvenlafaxine was the most abundantly detected metabolite in all organisms. N,O-didesmethylvenlafaxine was also found in mussels exposed to near 10 µg/L of venlafaxine, reaching a maximum concentration of 7.8 ng/g dw (Serra-Compte et al., 2018).

Besides the experiments under laboratory controlled conditions, the presence of venlafaxine metabolites in wild aquatic organisms collected in field studies has also been reported. However, most studies only focused on the occurrence of O-desmethylvenlafaxine, the main human venlafaxine metabolite. Low levels of O-desmethylvenlafaxine (<0.08-4.8 ng/g dw.) were detected in different species of bivalves

collected in Portugal, Spain and Italy, while venlafaxine concentration varied from 2.1 to 36.1 ng/g dw (Álvarez-Muñoz et al., 2015). On the other hand, besides O-desmethylvenlafaxine, Martinez Bueno et al. (2014) also found other three human venlafaxine metabolites in mussels collected in the Mediterranean Sea, though their levels never exceeded 3.8 ng/g dw, whereas venlafaxine was detected at concentrations up to 2.7 ng/g dw. No data on the occurrence of venlafaxine and its metabolites in surface water was available. In the case of other studies on fish, O-desmethylvenlafaxine was found in liver (0.12-5.2 ng/g ww) (Koba et al., 2018), and in homogenates (< 0.05 ng/g ww) (Metcalfe et al., 2010) of fish collected in Czech Republic and Canada, respectively. Its presence could be due to the bioaccumulation of this metabolite from the environment rather than be generated by metabolization of venlafaxine, since the present study showed the incapability of fish to form O-desmethylvenlafaxine. In fact, O-desmethylvenlafaxine was detected in river water, where the fish were collected, at higher levels than those of venlafaxine (Czech Republic: max conc. 870 *vs* 580 ng/L; Canada: mean conc. 979 *vs* 507 ng/L) (Koba et al., 2018; Metcalfe et al., 2010). On the other hand, Metcalfe et al. (2010) found a higher concentration of N-desmethylvenlafaxine in fish homogenate (up to 1.24 ng/g ww) than O-desmethylvenlafaxine (<0.05 ng/g ww), despite the mean concentration of N-desmethylvenlafaxine in river water was one order of magnitude lower (96 ng/L) than those of O-desmethylvenlafaxine (979 ng/L). In addition, a more hydrophilic character of N-desemethylvenlafaxine at physiological pH (logD7.4 = 0.03) comparatively to O-desmethylvenlafaxine (logD7.4 = 1.07) reinforces the idea that, besides its bioaccumulation, fish preferably metabolize venlafaxine to N-desmethylvenlafaxine, contributing to its high levels in fish homogenates.

4. Conclusions

The present study highlights the importance of assessing the metabolization of pharmaceuticals by aquatic organisms like fish for the evaluation of environmental risk assessment of chemical contaminants, given that metabolization of pharmaceuticals may modify the body burden and toxicity of xenobiotics. By combining target and suspect screening analytical approaches, it was possible to tentatively identify 13 possible venlafaxine metabolites, including two Phase II metabolites, in the marine juvenile fish meagre. For the first time, a metabolization pathway of this antidepressant in fish is proposed. Our results evidenced that fish do not share the metabolization pathway of venlafaxine in humans, and N-demethylation appeared as the main route of metabolization in meagre. Species-specificity regarding xenobiotics metabolization are highlighted in this study and conduct to a better understanding of venlafaxine's toxicokinetic in fish. Metabolites identified may also be used as biomarkers of exposure to environmental contaminants.

Ethical Statement

Fish trials were conducted according to legal regulations (EU Directive 2010/63), and approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University, overseen by the Portuguese National Competent Authority (Direção-Geral de Alimentação e Veterinária, DGAV). All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences, by the Federation of European Laboratory Animal Science Associations (FELASA).

Author's contribution

Lúcia H.M.L.M. Santos: Conceptualization, Methodology, Investigation, Formal Analysis, Software, Validation, Writing – Original Draft; **Ana Luísa Maulvault:** Investigation, Writing – Review & Editing; **Adrián Jaén-Gil:** Software, Writing – Review & Editing; **António Marques:** Resources, Writing – Review & Editing; **Damià Barceló:** Resources, Supervision, Writing – Review & Editing; **Sara Rodríguez-Mozaz:** Conceptualization, Resources, Supervision, Writing – Review & Editing.

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Figure Captions

Figure 1 – Experimental design. VLF – Venlafaxine

Figure 2 - Proposal of the metabolization pathway of venlafaxine by juvenile meagre fish. VLF -Venlafaxine; N-DVLF – N-desmethylvenlafaxine; NN-DDVLF – N,N-didesmethylvenlafaxine. Note: the underlined compounds were unequivocally identified using analytical standards.

Figure 3 – Relative distribution of the tentatively identified venlafaxine metabolites, in terms of chromatographic peak area per mg or mL of sample, at days 28 and 35 on: a) fish plasma; b) liver; and c) brain. VLF – venlafaxine. Please note the scale for the y-axis (Area/mg or Area/mL) change between boxes.

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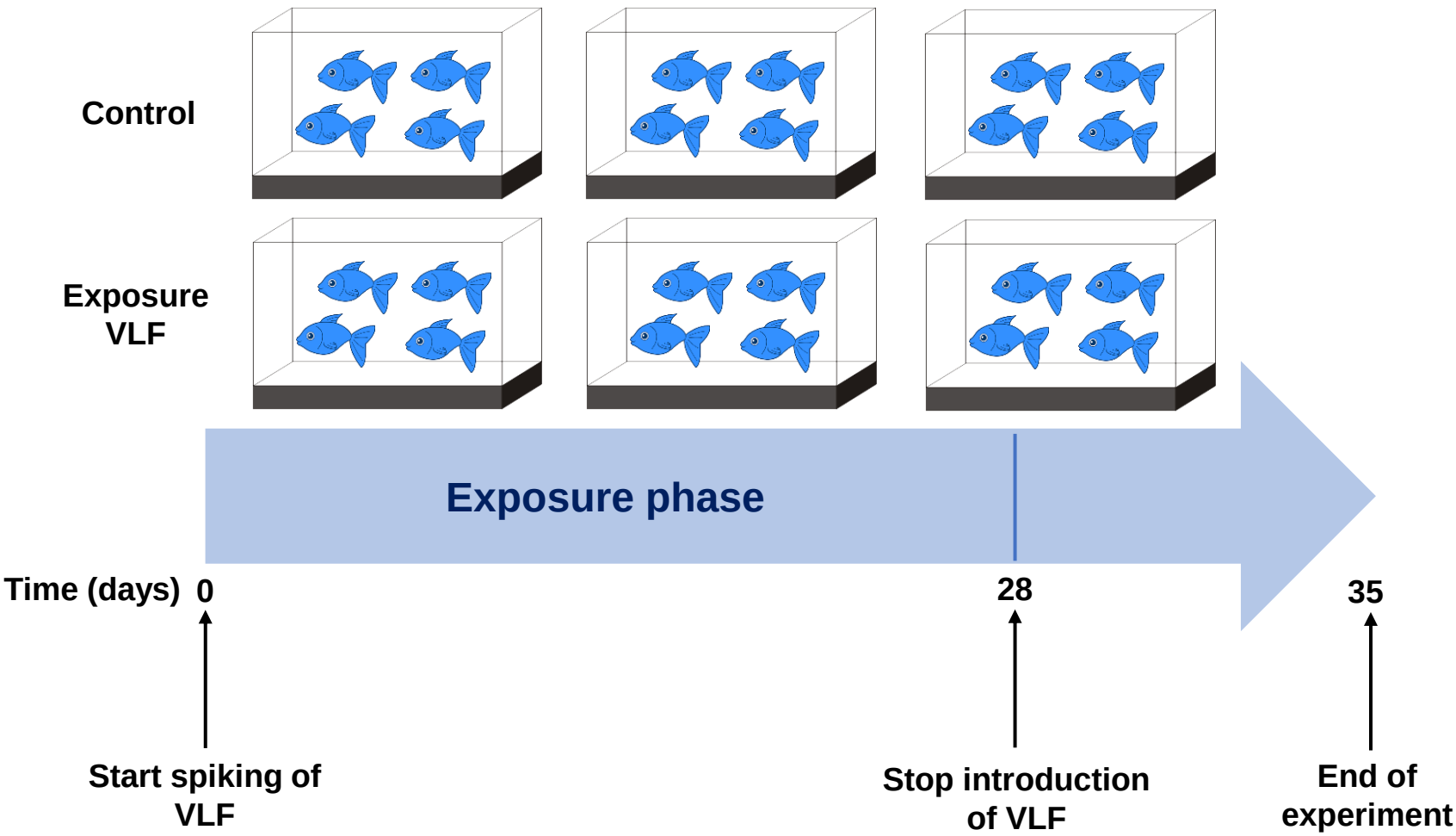
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Exposure
VLF
Exposure phase
Time (days)
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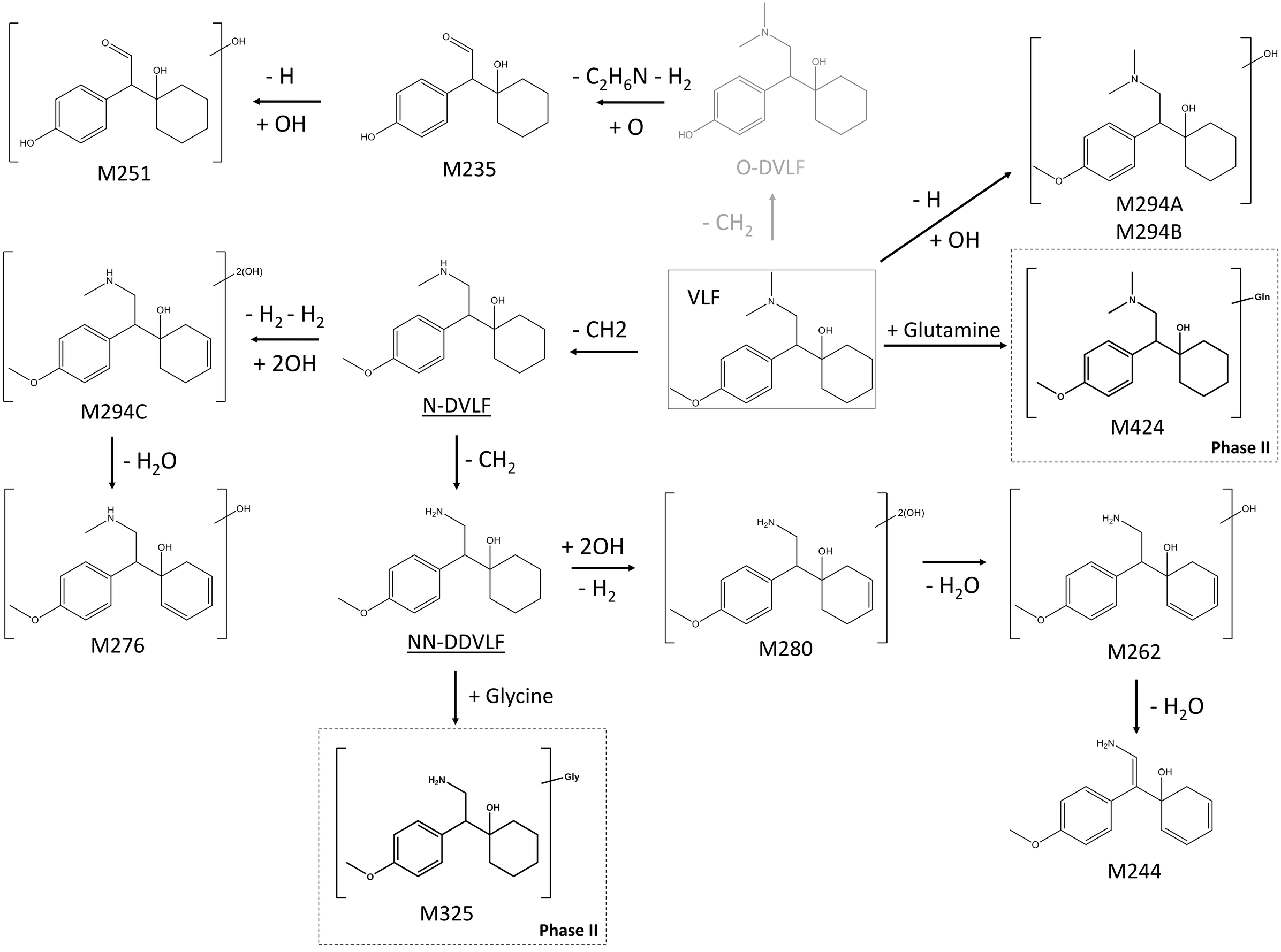
28
35
Start spiking of
VLF
Stop introduction
of VLF
End of
experiment


M251
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+ OH
M235
- C2H6N - H2
+ O
O-DVLF
- CH2
M294A
M294B
- H
+ OH
M294C
- H2 - H2
+ 2OH
N-DVLF
- CH2
VLF
+ Glutamine
M424
Phase II
- H2O
M276
- CH2
NN-DDVLF
+ 2OH
- H2
M280
- H2O
M262
- H2O
M244
+ Glycine
M325
Phase II

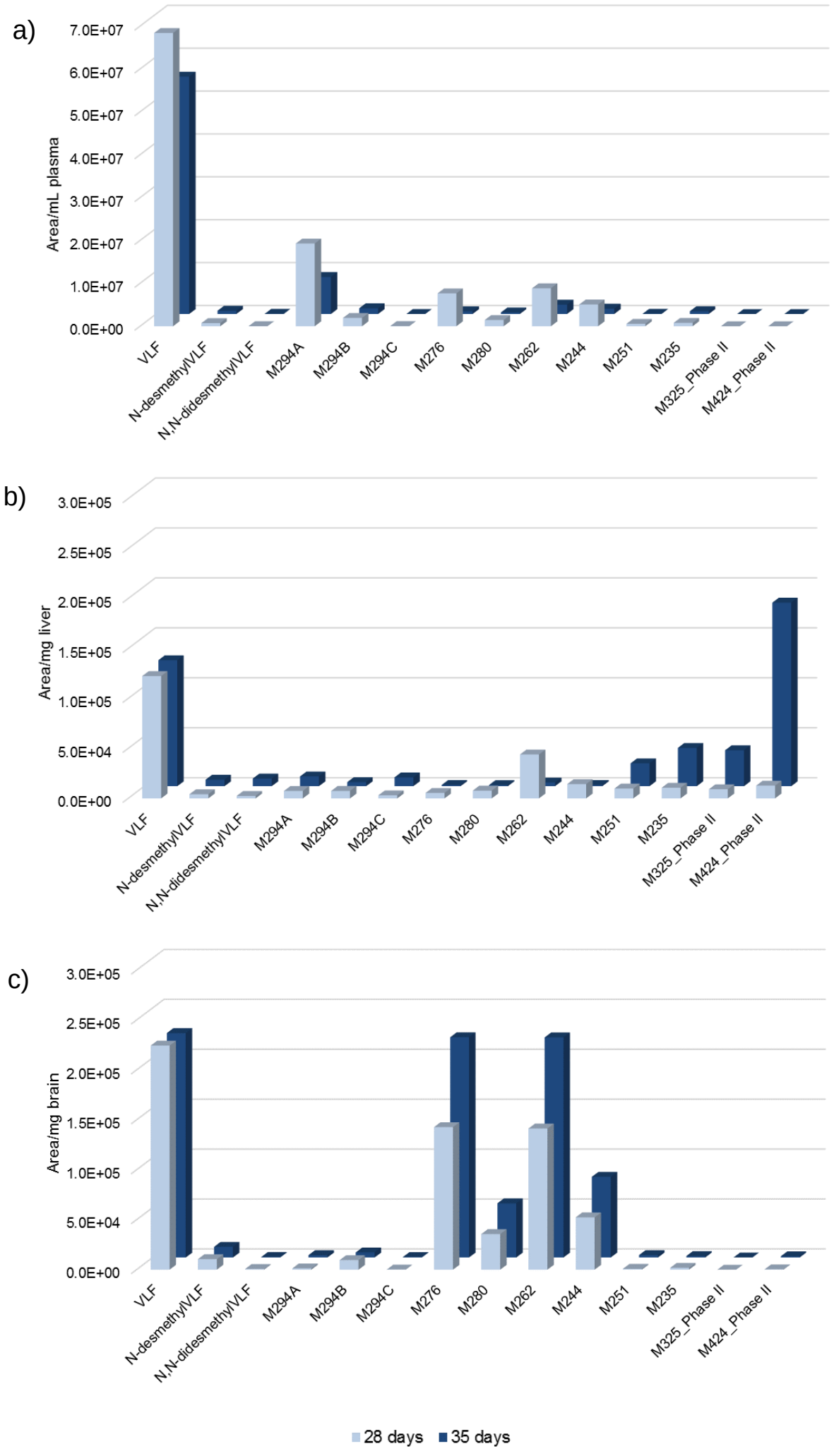
a)
Area/mL plasma
7.0E+07
6.0E+07
5.0E+07
4.0E+07
3.0E+07
2.0E+07
1.0E+07
0.0E+00
b)
Area/mg liver
3.0E+05
2.5E+05
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
c)
Area/mg brain
3.0E+05
2.5E+05
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
VLF
N-desmethylVLF
N,N-didesmethylVLF
M294A
M294B
M294C
M276
M280
M262
M244
M251
M235
M325_Phase II
M424_Phase II
28 days
35 days

Table 1 – Concentration (±SD) of venlafaxine and its metabolites in fish tissues, expressed in ng/g, dry weight (d.w.), and in fish plasma, expressed in ng/mL

Fish tissue		Venlafaxine*	O-desmethylVLF	N-desmethylVLF	N,N-didesmethylVLF	N,O-didesmethylVLF	N,O-didesmethyl-N-desmethylvenlafaxine
Brain	Control	2.55 ± 2.14	n.d.	13.3 ± 6.5	n.d.	n.d.	n.d.
	28 days	2,507 ± 64	n.d.	111 ± 9	27.7 ± 2.0	n.d.	n.d.
	35 days	2,406 ± 691	n.d.	120 ± 1	22.4 ± 0.7	n.d.	n.d.
Liver	Control	21.7 ± 1.0	n.d.	n.d.	—†	n.d.	n.d.
	28 days	6,808 ± 1,177	n.d.	121 ± 44	—†	n.d.	n.d.
	35 days	6,371 ± 210	n.d.	199 ± 32	—†	n.d.	n.d.
Muscle	Control	<MDL	n.d.	n.d.	<MDL	n.d.	n.d.
	28 days	423 ± 159	n.d.	30.5 ± 18.8	n.d.	n.d.	n.d.
	35 days	535 ± 15	n.d.	56.7 ± 1.7	n.d.	n.d.	n.d.
Plasma	Control	<MDL	n.d.	n.d.	n.d.	n.d.	n.d.
	28 days	1,292 ± 80	n.d.	21.9 ± 4.6	<MQL	n.d.	n.d.
	35 days	673 ± 194	n.d.	11.1 ± 0.5	<MQL	n.d.	n.d.

*Data from Maulvault et al. (2018b); †It was not quantified due to its low extraction recovery. n.d. – not detected; <MDL – below method detection limit; <MQL – below method quantification limit

Table 2 – Overview of the presence and metabolization of venlafaxine and its metabolites by different organisms. Results expressed in ng/g (dry weight).

Organism		Compound	Concentration	Field/Exposure experiment	Analytical approach	Reference
Biofilm		Venlafaxine O-desmethylVLF N-desmethylVLF	48,100-48,900 190-208 227-268	Exposure experiment	Target	Santos et al. (2019)
Mussels (*Mytilus galloprovincialis*)		Venlafaxine O-desmethylVLF N-desmethylVLF N,O-didesmethylVLF	2,256-3,917 15.2-55.7 13.3-276.8 <0.30-7.8	Exposure experiment	Target	Serra-Compte et al. (2018)
Fish (*Argyrosomus regius*)	Muscle	Venlafaxine N-desmethylVLF	423-535 30.5-56.7	Exposure experiment	Target	Maulvault et al. (2018b) Present study
	Liver	Venlafaxine N-desmethylVLF	6,371-6,808 121-199			Maulvault et al. (2018b) Present study
	Brain	Venlafaxine N-desmethylVLF N,N-didesmethylVLF	2,406-2,507 111-120 22.4-27.7			Maulvault et al. (2018b) Present study
	Plasma	Venlafaxine N-desmethylVLF N,N-didesmethylVLF	673-1,292 µg/L 11.1-21.9 µg/L <1.68 µg/L			Maulvault et al. (2018b) Present study
Freshwater crustacean (*Gammarus pulex*)		Venlafaxine N-desmethylVLF (MVE264B) O-desmethylVLF (MVE264A) MVE250 (N,N-didesmethylVLF or N,O-didesmethylVLF) MVE280	MVE264B showed the highest intensity	Exposure experiment	Suspect screening	Jeon et al. (2013)

Organism	Compound	Concentration	Field/Exposure experiment	Analytical approach	Reference
	MVE294				
Mussels (*Mytilus galloprovincialis*)	Venlafaxine O-desmethylVLF N-desmethylVLF N,O-didesmethylVLF N,N-didesmethylVLF	<2.5-2.7 <1.1-3.7 <0.5-3.0 2.5-3.5 3.8	Field	Target	Martinez Bueno et al. (2014)
Mussels (*Mytilus spp.*)	Venlafaxine O-desmethylVLF	2.7-36.1 <0.08-4.8	Field	Target	Álvarez-Muñoz et al. (2015)
Clams (*Chamalea galina*)	Venlafaxine O-desmethylVLF	2.1 ± 1.3 <0.18	Field	Target	Álvarez-Muñoz et al. (2015)
Pacific oysters (*Crassostrea gigas*)	Venlafaxine O-desmethylVLF	2.3 ± 0.2 1.4 ± 0.01	Field	Target	Álvarez-Muñoz et al. (2015)
Fish (*Cyprinus carpio* L.) liver	Venlafaxine O-desmethylVLF	0.47-3.9* 0.12-5.2*	Field	Target	Koba et al. (2018)
Fish (*Pimephales promelas*) homogenate	Venlafaxine O-desmethylVLF N-desmethylVLF	n.d.-1.20* n.d.-<0.05* n.d.-1.24*	Field (caged fish)	Target	Metcalfe et al. (2010)

*Results expressed in wet weight