

# Elucidating venlafaxine metabolism in the Mediterranean mussel (Mytilus galloprovincialis) through combined targeted and non-targeted approaches

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] Title

2	Elucidating Venlafaxine Metabolism in the Mediterranean Mussel (Mytilus
3	galloprovincialis) through Combined Targeted and Non-Targeted Approaches
4	
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9	
10	

Abstract

12

13 Exposure of aquatic organisms to antidepressants is currently well documented, while little information is 14 available on how wild organisms cope with exposure to these pharmaceutical products. Studies on 15 antidepressant metabolism in exposed organisms could generate information on their detoxification 16 pathways and pharmacokinetics. The goal of this study was to enhance knowledge on the metabolism of 17 venlafaxine (VEN)—an antidepressant frequently found in aquatic ecosystems—in Mytillus galloprovincialis, 18 a bivalve that is present worldwide. An original tissue extraction technique based on the cationic properties 19 of VEN was developed for further analysis of VEN and its metabolites using targeted and non-targeted 20 approaches. This extraction method was assessed in terms of recovery and matrix effects for VEN 21 metabolites. Commercial analytical standards were applied to characterize metabolites found in mussels 22 exposed to 10 µg/L VEN for 3 and 7 days. Targeted and non-targeted approaches using liquid 23 chromatography (LC) combined with high-resolution mass spectrometry (HRMS) were implemented to 24 screen for expected metabolites based on the literature on aquatic species, and for metabolites not 25 previously documented. Four venlafaxine metabolites were identified, namely N-desmethylvenlafaxine and 26 O-desmethylvenlafaxine, which were clearly identified using analytical standards, and two other metabolites 27 revealed by non-target analysis. According to the signal intensity, hydroxy-venlafaxine (OH-VEN) was the 28 predominant metabolite detected in mussels exposed for 3 and 7 days.

29

# 30 Keywords

31 Bioaccumulation, pharmaceuticals, metabolites, mass spectrometry, bivalves

32

# 33 Highlights

- An extraction method based on the cationic properties of VEN was successfully developed.
   The non-targeted approach allowed the identification of new VEN metabolites in mussels.
   OH-VEN was the predominant metabolite detected in VEN-exposed mussels.
- 38

# 39 1 Introduction

40 Venlafaxine (VEN), an antidepressant prescribed for the treatment of human clinical and anxiety 41 disorders, is one of the most commonly detected pharmaceutically active compounds (PhACs) in the 42 aquatic environment (Brieudes et al., 2017; Moreno-González et al., 2016; Paíga et al., 2019). This 43 contamination affects the coastal marine environment, where recent studies have revealed the 44 bioaccumulation of VEN, among other PhACs, in different species (Alvarez-Muñoz et al., 2015a,b; 45 McEneff et al., 2013). Particularly, bivalves have shown the highest VEN accumulation (up to 36.0 46 ng/g dw) in relation to other aquatic organisms such as microalgae and fish (Alvarez-Muñoz et al., 47 2015b). The Mediterranean mussel (Mytilus galloprovincialis) is a species commonly used in 48 monitoring environmental contaminants in the marine environment thanks to its wide geographical 49 distribution, its filter-feeding behavior and ability to bioaccumulate contaminants (Gomez et al., 2012; 50 51 exposed Mediterranean mussels, Serra-Compte et al. (2018) detected three phase I metabolites, i.e. 52 O-desmethyl venlafaxine (ODV), N-desmethyl venlafaxine (NDV) and N,O-didesmethyl venlafaxine 53 (NODDV), while Gomez et al. (submitted) detected four phase I metabolites, i.e. ODV, NDV, NODDV 54 and N,N-didesmethyl venlafaxine (NNDDV). The two previous studies were conducted on the basis of 55 a targeted analysis geared towards monitoring known phase I metabolites, already reported in the 56 literature focused in humans. However, non-targeted approaches are promising to achieve a more 57 complete characterization of VEN metabolism, including previously unreported metabolites in 58 Mediterranean mussels. Non-targeted approaches are based on the generation of chemical profiles in

59 exposed and non-exposed organisms. Their comparison allow the detection of appearing signals in 60 exposed organisms, those signals corresponding to potential previously unreported metabolites. This 61 strategy helps elucidate biotransformation pathways in mussels, as already demonstrated for 62 diclofenac (Bonnefille et al., 2017). Meanwhile, Jeon et al. (2013) conducted the first experiment to 63 screen for VEN metabolites in freshwater crustaceans with a non-targeted approach using LC-HMRS, 64 and the findings suggested the involvement of biotransformation pathways. These authors 65 documented the structures of five VEN metabolites: three dealkylation products (ODV, NDV and 66 NODDV, confirmed with reference standards) and two hydroxylation products (both with tentatively 67 identified structures). In this context, to our knowledge no studies have focused on the VEN 68 metabolism in mussels using an untargeted approach, thereby highlighting the question as to whether 69 known biotransformation processes in humans are maintained in invertebrates. The goal of the 70 present study was therefore to investigate this strategy for identifying VEN metabolites (already known 71 and non-documented ones) produced in Mediterranean mussels (M. galloprovincialis) after exposure. 72 A tissue treatment method based on the cationic properties of VEN and its metabolites (known in 73 humans) was developed to increase the probability of metabolite detection and it was assessed in 74 terms of recovery and matrix effect.

#### 75 2 Materials and Methods

#### 76 2.1 Chemicals and materials

77 VEN ( $\geq$  98.5%), venlafaxine-d6 (VEN-d6, > 98%) and O-demethylvenlafaxine-d6 (ODV-d6, > 99.9%) 78 were purchased from Sigma-Aldrich (Steinheim, Germany). N,N-didesmethyl- O-desmethyl 79 venlafaxine (NNDDODV, ≥98%), ODV-glucuronide (ODV-glucu, > 98%) and NODDV-glucuronide 80 (NODDV-gluco,  $\geq$  95%) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while 81 ODV, NDV, NNDDV and NODDV were obtained at analytical grade (purity  $\geq$  98%) from Toronto 82 Research Chemicals Inc. (Toronto, ON, Canada). Stock standard solutions of individual compounds 83 were prepared at 100 mg/L concentration in methanol. Subsequent stock standard dilutions were 84 prepared with methanol. All standard solutions were stored at -20°C.

Ultrapure water was generated by the Millipore Simplicity UV system (Bedford, MA, USA) with a specific resistance of 18.2 MΩ.cm at 25°C. Dichloromethane (DCM) as pesticide analytical grade solvent, ammonia (NH<sub>3</sub>, 28%) as ACS reagent grade solution and HPLC/MS-grade solvents (methanol-MeOH and acetonitrile - ACN) were purchased from Carlo Erba (Val de Reuil, France).

89 Formic acid (98% purity) was obtained from Fisher Labosi (Elancourt, France) and ammonium acetate 90  $( \geq 98\%)$  was purchased from Sigma-Aldrich (Steinheim, Germany). Dispersive SPE tubes containing 91 Z-Sep-Plus (500 mg/12 mL) was obtained from Supelco (Bellefonte, PA, USA). Oasis® MCX 6 cc (150 92 mg, 30 µm, 6 cc) cartridges, obtained from Waters Corporation (Mildford, USA), and a Visiprep<sup>™</sup> DL 93 Solid Phase Extraction (SPE) Vacuum Manifold from Supelco (Bellefonte, USA) were used for solid 94 phase extraction. All chromatographic solvents were filtered through a 0.22 µm nylon membrane filter 95 (Nylaflo<sup>™</sup>, Michigan, USA). Glass-fiber filters (0.7 µm pore size) purchased from Whatman 96 (Maidstone, UK) were used to filter seawater.

# 97 2.2 Experimental design and sample preparation

98 M. galloprovincialis mussels were purchased in February 2017 from Mediterranean mussel suppliers 99 (Bouzigues, France) and immediately transported to the laboratory. After cleaning, the mussels were 100 acclimatized in aerated (9.9  $\pm$  0.4 mg/L) filtered natural seawater (salinity = 35  $\pm$  3 g/L, pH = 8.2  $\pm$  0.1 101 units) for 7 days before the experiment. During the acclimation and exposure periods, the seawater 102 was renewed every day (static renewal), the room temperature was regulated at  $15 \pm 2^{\circ}$ C, and the 103 mussels were fed Tetraselmis suecica phytoplankton (Greensea, Mèze, France) at constant density 104 (10,000 cells/mL). 100 mussels (shell length 6.0-7.2 cm) were randomly distributed in 20 glass 105 aquaria at a density of 5 mussels per 2 L water. Two groups were constituted: a solvent control group 106 (with the vehicle for VEN: MeOH) and a group exposed at a nominal VEN concentration of 10 µg/L. 107 Each group consisted of 10 aquaria of 5 mussels each. During the exposure period, the VEN 108 concentration was reestablished after daily water renewal. Seawater samples were taken daily to 109 quantify VEN and its known metabolites. Mussels were sampled at days 3 and 7 of exposure (five 110 aquaria per condition). All mussels were dissected and the digestive gland was frozen at -80°C for 111 further analysis, and the remaining soft tissues (the tissue of interest in our study) were frozen at 112 -80°C before freeze-drying (Heto Power dry LL 3000, Thermo). After lyophilisation, these tissues were 113 homogenized and ground into powder using an MM-2 vibrational mill (Retsch, Haan, Germany). Each 114 sample was then placed inside a clean glass bottle and stored at room temperature in the dark until 115 extraction and analysis. The mussel soft tissue analysis involved five replicates per group (solvent 116 control and exposed) for each day of exposure (3 and 7 days).

## 117 2.3 Sample extraction and LC-MS analysis

### 118 2.3.1 Tissue extraction method

119 In order to increase the probability of detection of phase I and phase II (known and unknown) VEN 120 metabolites in mussels, a tissue treatment method was developed using the physicochemical 121 properties of these compounds, especially the positive charge on the amine functional group. 500 mg 122 (± 1 mg) dry weight (dw) of tissue samples obtained by pooling two individuals was weighed in a 50 123 mL polypropylene centrifuge tube and spiked with VEN-d6 (500 µg/kg) and ODV-d6 (20 µg/kg). The 124 tissues were then extracted by sonication in 5 mL of extraction solvent (5% formic acid in MeOH) for 5 125 min. The samples were centrifuged at 23°C (3400 x g, 5 min) and the supernatant was decanted in 126 another 50 mL polypropylene centrifuge tube. Re-extraction was carried out for each sample and the 127 resulting supernatants were combined, and then 0.5 g of lipid removal sorbent Z-Sep Plus and 5 mL of 128 5 mM ammonium acetate were added to the combined supernatant. The sample was then vortexed 129 for 5 min and centrifuged at 23°C (3400 x g, 5 min). The extract obtained was transferred to a glass 130 bottle (250 mL capacity) and diluted with milliQ water to a 150 mL final volume. After dilution, the 131 sample pH was adjusted to  $1.4 \pm 0.1$ . Then water-diluted samples were loaded onto the SPE Oasis 132 MCX cartridges pre-conditioned with MeOH (6 mL), milliQ water (6 mL) and 2% formic acid in water (6 133 mL, pH: 1.40), respectively. After all samples were loaded at a flow rate of 1 mL/min, the cartridges 134 were dried (until there were no longer any drops coming out of them) and then washed with 2% formic 135 acid in water (6 mL, pH: 1.40), MeOH (2 mL), DCM (6 mL) and MeOH (3 mL). The analytes were 136 eluted with 6 mL of 5% ammonium hydroxide in MeOH (5:95, v/v), and the solvent was evaporated to 137 dryness under a gentle N<sub>2</sub> stream at 35°C. The residues were reconstituted with 200 µL of ACN:water 138 (10:90, v/v). Finally, the sample was centrifuged at  $13.4 \times 10^3$  rpm for 10 min to separate the residual 139 lipids. The clear solution was transferred to a vial for LC-MS analysis.

# 140 **2.3.2 Seawater extraction method**

A tailored version of the tissue extraction and cleaning method was applied to determine the real VEN concentration in the seawater sampled during the mussel exposure period. 18 mL of seawater was spiked with surrogate standard (VEN-d6 at 1  $\mu$ g/L) and ajusted to pH 1.4 ± 0.1. Solid-phase extraction was conducted with the same method used for the tissues, except for the washing step which was performed without DCM (6 mL). The analyte was eluted with fresh prepared ammonium hydroxide in MeOH and the solvent was evaporated to dryness under an N<sub>2</sub> stream. The residue was reconstituted in 200  $\mu$ L of ACN:water (10:90, v/v) and transferred to a vial for LC-MS analysis.

# 148 **2.3.3 Liquid chromatography and mass spectrometry conditions**

149 Extracts were analyzed on an HPLC Accela 600 pump coupled to a Q-Orbitrap HRMS mass 150 spectrometer (Thermo Fischer Scientific) equipped with a heated electrospray ionization probe (HESI) 151 source for detection. Chromatographic separation involved a reverse phase PFPP analytical column 152 (Discovery HS F5 100 mm x 2.1 mm; 3 µm particle size). The chromatography assays involved a 10 153 µL injection volume, a 0.35 mL/min flow rate and a binary gradient of ACN (A) and water (B), both 154 containing 0.1% formic acid, as follows: 10% A at 0 min, 50% A at 4-6 min, 60% A at 8-10 min, 70% A 155 at 14-16 min, 80% A at 17-19 min, 10% A at 21 min and a stop time at 27 min. Over a 27 min run, 156 data acquisition was performed simultaneously in positive and negative ionisation mode and the HESI 157 parameters were as follows: 55 arbitrary units (AU) sheath gas; 10 AU auxiliary gas; 275°C capillary 158 temperature; 200°C heater temperature, and the electrospray voltage was set at 4.0/-4 kV. The S-lens 159 radio frequency (RF) level was set at 100 AU. Full scan data in polarity switching mode were acquired 160 at a resolution of 35,000 full width at half maximum (FWHM) with an automatic gain control (AGC) of 161 10<sup>6</sup> and 250 ms of the maximum ion injection time. Moreover, MS<sup>2</sup> was achieved using a mass 162 inclusion list composed of the mass of the precursor ion of the compounds of interest. A 10 eV 163 absolute collision energy and 17,500 FWHM resolution of were used. The m/z scan range was set 164 between 50-750.

#### 165 2.4 Non-targeted metabolite analysis strategy

166 Chemical profiles from five SC samples and five exposed samples at a nominal VEN concentration of 167 10 µg/L were generated by LC-HRMS for each exposure day (3 and 7 days). The data processing and 168 signal identification strategies were from Bonnefille et al. (2017). Briefly, the data obtained in positive 169 and negative ionization mode were converted with the free MS Convert software package (from \*.raw 170 to \*mzxml) and extracted using an XCMS script (open source) on the basis of the R software. The 171 XCMS parameters were tailored to fit our data: m/z interval for peak picking of 0.01, signal-to-noise 172 ratio threshold of 5, group band-width of 20, with a 0.4 minimum fraction. After data processing, a 2-173 dimensional table containing information on each peak was generated (Courant et al., 2014). The 174 peaks detected only in the exposed samples (absent in the control samples) and found in all five 175 replicates were considered as potential VEN metabolites. The resulting signals were confirmed on the 176 basis of the fold change and extracted ion chromatogram. The fold change was calculated as the ratio 177 of the mean signal intensity in exposed samples to the mean signal intensity in the control samples. 178 Only signals with a fold change higher than 10 in negative or positive ionization mode were kept and 179 further processed to determine the structures associated with the signals. Moreover, extracted ion

180 chromatograms (EIC) were checked manually for all potential signals associated with metabolites so 181 as to confirm the absence of any signal in the controls. Peaks associated with the same molecule (e.g. 182 isotopes and/or dimers) were identified by checking the retention times and shapes of peaks obtained 183 in the chromatograms generated by the Xcalibur QualAnalytical Browser (Xcalibur 3.0, Thermo Fisher 184 Scientific Inc.). The same software was used to determine the elemental composition of unknown 185 metabolites and those with the best fits, along with C, H, N and O compositions that could be related 186 to VEN, are reported in the present study. Chromatographic retention time prediction of unknown 187 metabolites was conducted using the multiple linear regression model developed by Jeon et al. 188 (2013). With this model, an equation was obtained by plotting the retention time versus the molecular 189 weight (MW) and the distribution coefficient (log  $D_{ow}$ ) of the parent compound and its known 190 metabolites. The MW and log Dow values were generated by ACD/ChemSketch (version 12.01).

# 191 2.5 Targeted metabolite analysis

#### 192 **2.5.1 LC-MS analysis**

In the targeted analysis, only data acquired in positive electrospray ionization mode were considered, as this was the most sensitive mode for the molecules included in this section of the study (VEN and its known metabolites). Each compound was confirmed on the basis of: (1) the retention time, and (2) the mass accuracy (max 5 ppm deviation) of protonated molecules [M+H]<sup>+</sup> in full scan MS mode compared to values obtained for the analytical standards under the same analytical conditions.

# 198 2.5.2 Quantification of VEN and its known human metabolites in tissues

In order to quantify VEN and its known metabolites in mussel soft tissues, calibration curves were plotted for blank mussel tissues by adding a fixed amount of the internal standards: VEN-d6 (500 µg/kg dw) and ODV-d6 (20 µg/kg dw) and increasing quantities of the target analytes from 0 to 3000 µg/kg dw for VEN, and from 0 to 80 µg/kg dw for its known phase I (ODV, NDV, NNDDV, NODDV and NNDDODV) and phase II (ODV-glucu and NODDV-glucu) metabolites before extraction.

#### 204 **2.5.3 VEN quantification in seawater**

205 In addition, a seawater calibration curve was plotted by adding a fixed amount of internal standard 206 VEN-d6 (at 1 $\mu$ g/L) and increasing quantities (0–15  $\mu$ g/L) of the targeted VEN analyte in blank 207 seawater.

#### 208 2.5.3 Method performances

209 The method performances of both matrices were evaluated by taking into account aspects such as the

210 linearity, limit of detection (LOD) of the methods, absolute recovery, matrix effects and repeatability

#### Tissues

211 per analyte. Linearity was estimated with blank tests and calibration curves in tissues (five levels) at 212 the 250–3000  $\mu$ g/kg dw concentration range for VEN and 5–80  $\mu$ g/kg dw for its known metabolites, 213 and in seawater (seven levels) at 0.1–15  $\mu$ g/L concentration range for VEN. LODs of the tissues and 214 water methods were determined as the concentration resulting in a signal-to-noise ratio of 3 for each 215 analyte. Absolute recoveries were studied by comparison of the signal area of each analyte in blank 216 samples spiked before and after the extraction/purification process, both injected under the same 217 analytical conditions. Matrix effects were evaluated by comparison of the signal area of each analyte 218 in blank samples spiked after extraction/purification with the signal area of each analyte measured in a 219 pure standard injected under the same analytical conditions. Absolute recovery, matrix effects and 220 repeatability of the methods were evaluated by spiking tissues with 20 µg/kg (dw) and seawater with 221 1µg/L of all compounds in triplicate.

### 222 2.6 VEN bioconcentration factor and the mann Whitney non-parametric statistical test

An apparent bionconcentration factor (BCFa) was calculated for VEN as the ratio of the measured concentration in the tissues to the measured concentration in seawater. The mann Whitney nonparametric statistical test was performed to compare the data obtained at 3 days and 7 days. OriginPro 2018 statistical software was used for this test.

# 227 3. Results

#### 228 **3.1 Analytical performance**

The method performances are shown in Table 1. Both tissue and water methods demonstrated good linearity ( $r^2>0.98$ ) in the concentration range defined in both matrices for the 8 compounds included in the study. Absolute recoveries of phase I metabolites were higher than 91% in the tissues. Phase II metabolites had a lower absolute recovery (approximately 50%) in this matrix. A medium matrix effect was observed in tissues for all evaluated analytes (suppression between 32% and 59%). The average variability for all analytes evaluated never exceeded 21% in both matrices. The sensitivity of the methods in terms of LOD ranged from <1.0 to 8.0  $\mu$ g/kg dw in tissues and was 1 ng/L in seawater.

Analy <b>T</b> F	<b>able 1</b> Physicochemical properties, retention time, linearity, absolute recovery, matrix effets, average repetability and								D <sup>Meth</sup> ∶g dw)	
n	method LODs and LOQs observed for each molecule in LC-MS Q-Orbitrap.									
NODD - glucu <sup>f</sup>	425.47274	426.2122	2,78; 10,24	-3.58	3.96	0.9923	50 ± 7	-48 ± 3	17	8.0
ODV-glucu	ı <sup>f</sup> 439.49932	440.2279	2,78; 9,26	-3.65	4.21	0.9993	49 ± 19	-51 ± 8	16	8.0
NNDDODV	<sup>e</sup> 235.32204	236.1645	9,72; 10,38	-1.79	6.96	0.9832	96 ± 4	-59 ± 6	21	3.0
NODDV <sup>e</sup>	249.34862	250.1801	9,83; 10,59	-1.29	7.91	0.9988	98 ± 6	-41 ± 9	4	2.0
ODV <sup>e</sup>	263.3752	264.1958	9,23; 10,04	-1.36	8.02	0.9886	100 ± 5	-32 ± 8	5	5.0
NNDDV <sup>e</sup>	249.34862	250.1801	9,85; 14,75	-1.06	11.64	0.9987	97 ± 6	-55 ± 8	17	0.5
NDV <sup>e</sup>	263.3752	264.1958	10,44; 14,83	-0.55	12.08	0.9945	108 ± 12	-32 ± 12	9	1.5
VEN	277.40178	278.2114	9,26; 14,84	-0.62	14.23	0.9998	105 ± 7	-32 ± 11	4	0.5
Seawater										
VEN	-	-	-		14.18	0.9992	105 ± 11	14 ± 3	18	1,0 <sup>(µg/L)</sup>
237										

240 <sup>a,b,c,d</sup> values predicted with ACD/ChemSketch software; <sup>e</sup>phase I metabolite and <sup>f</sup>phase II metabolite.

# **3.2 VEN quantification**

The VEN concentrations were measured in water to control mussel exposure in aquaria. The exposure concentrations measured in the exposed aquaria ranged from 9.5 to 12.5  $\mu$ g/L (Table 2). No signal of the parent compound (VEN) was detected in the control samples (SC) of both matrices (seawater and tissues). Mean VEN concentrations in exposed mussel tissues were around 1.9 mg/kg dw and 2.5 mg/kg dw at days 3 and 7, respectively. Moreover, when considering the VEN concentration measured in water and exposed mussel tissues, the apparent bioconcentration factors (BCFa) were 177  $\pm$  27 L/kg dw and 229  $\pm$  29 L/kg dw for days 3 and 7, respectively. Finally, with the Mann Whitney non-parametric statistical test, for VEN and its metabolites ODV and NDV, no significant differences in the concentrations were observed between day 3 and day 7.

258 **Table 2** Mean concentrations of VEN and its metabolites NDV and ODV measured in the different conditions and matrices (seawater and tissue) of the study.

259

		Analytes						
Matrix	Conditions	VEN (μg/L in water ou μg/kg dw in tissue) (n=5)	NDV (μg/L in water ou μg/kg dw in tissue) (n=5)	ODV ν (μg/L in water ou μg/kg dw in tissue) (n=5)				
Convetor	SC	< LOD	< LOD	< LOD				
Seawaler	Exposure	11.0 ± 1.5	< LOD	< LOD				
	SC	< LOD	< LOD	< LOD				
Tissues	3rd day of exposure	1948 ± 493	50 ± 13	28 ± 7				
	7th day of exposure	2517 ± 341	34 ± 13	24 ± 8				

- 260
- 261

#### 262 **3.3 Non-targeted analysis of VEN and its metabolites in tissues**

The non-targeted approach led to the detection of four metabolites. All of these molecules were detected in positive mode, while no signal in the negative mode could be identified as a potential metabolite. The metabolite and parent compound identification process is described below. Relevant information on the identification of these molecules is also presented in Table 3.

#### 267 3.3.1 Venlafaxine

268 Among the signals detected in exposed mussels, absent in the control samples and found in all 269 replicates, a peak with molecular mass [M+H]+ 278.2114 was identified at 14.23 min (i.e. M in Table 270 3). The selection of this signal was confirmed by its fold change value of 132 (value >10) and its 271 extracted ion chromatogram (Fig. 1). Xcalibur software helped determine the elemental composition of 272 the signal and the suggestion with the smallest delta atomic mass unit (amu) was considered. The 273 experimental mass [M+H]+ 278.2114 associated with the retention time (14.23 min) and elemental 274 composition proposed (C17H27NO2) coincided with the identification of VEN (Table 1). The 275 experimental mass of VEN was compared with its theoretical mass and no difference was found 276 (Table 3). The identification of the signal corresponding to the isotopic ion <sup>13</sup>C ([M+H]<sup>+</sup> 279.2148) at 277 14.26 min reconfirmed the presence of this molecule (VEN) (Fig. 2). HCD fragmentation (at 10 eV) 278 demonstrated the presence of a fragment at m/z 260.2007 (Fig. 2, (1). This fragment was associated 279 with the loss of 18.0107 amu compared to the measured VEN m/z 278.2114, corresponding to a 280 neutral loss of H<sub>2</sub>O in the VEN structure. In addition, the signal corresponding to the isotopic ion <sup>13</sup>C 281 ([M+H]+ 261.2039) of this fragment was identified. A second fragment was observed at m/z 215.1427 282 (Fig. 2, (2)). This fragment corresponded to a loss of 45.0580 amu (equivalent to a loss of  $C_2H_7N$ ) relative to the previously observed fragment in the VEN structure. A third fragment was found at m/z 121.0646 (Fig. 2, (3)). The elemental composition of this fragment corresponded to  $C_8H_9O$ . Note that the signals corresponding to the fragments and 13C isotopic ions had peak shapes and retention times very similar to those of VEN, thereby indicating that they were related. The VEN identification was confirmed by injection of the analytical standard under the same conditions reaching the maximum confidence level (level 1) in the identification of elucidated signals from a non-directed analysis, according to the scale proposed by Schymanski et al. (2014).



**Figure 1**.Examples of extracted ion chromatograms of metabolites (NDV and OH-VEN) and the parent compound (VEN). Black peaks represent the exposed samples and green peaks are the control samples. No peaks were identified in the control samples for these molecules.



Figure 2. Relevant ions from the high resolution mass spectrum of fragmented VEN at 10 eV

# 328 3.3.2 Phase I metabolites

# 329 3.3.2.1 Oxidative metabolism-demethylation

Two metabolites identified as M1 and M2 (Table 3) displayed a [M+H]<sup>+</sup> of 264.1958. The signals of both metabolites demonstrated a fold change higher than 10 and were detected only in the exposed samples (Fig. 1). They presented a mass decrease of 14.0156 amu (equivalent to a loss of CH<sub>2</sub>) compared to the VEN structure, corresponding to dealkylation in the parent compound structure. M1 and M2 exhibited different retention times, indicating that demethylation occurred at different positions. N-demethylation enabled greater interaction of nitrogen-free electron pairs with pentafluorophenyl propyl groups of the stationary phase in the chromatographic column used.







Peak ID	Tr (min)	Assignment	Elemental composition	Chemical Structure	Measured [M+H]+ (Δm)	Fold change	Relevant [M+H] <sup>+</sup> fragment m/z, HCD 10 eV (Δm)	log D <sub>ow</sub> at pH 2 <sup>c</sup>	Tr predicted	ΔTr  observed-predicted <2	Confidence level
М	14.23	VEN	C <sub>17</sub> H <sub>27</sub> NO <sub>2</sub>	H <sub>3</sub> C OH	278.2114 (0.0 mmu)	132	260.2007 (0.7 mmu) 215.1427 (0.9 mmu) 121.0646 (0.7 mmu)	-0.62	13.15	1.08	Level 1
M1	12.08	NDV	C <sub>16</sub> H <sub>25</sub> NO <sub>2</sub>	H3C OH	264.1958 (0.0 mmu)	24	246.1850 (0.8 mmu) 215.1426 (1.0 mmu) 121.0649 (0.4 mmu)	-0.55	13.02	0.94	Level 1
M2	8.02	ODV	C <sub>16</sub> H <sub>25</sub> NO <sub>2</sub>	HO HO	264.1958 (0.0 mmu)	43	246.1849 (0.9 mmu) 133.0647 (0.6 mmu) 107.0493 (0.4 mmu)	-1.36	9.20	1.18	Level 1
М3	14.11	OH-VEN	C <sub>17</sub> H <sub>27</sub> NO <sub>3</sub>	H <sub>3</sub> C OH OH OH	294.2064 (0.0 mmu)	1214	276.1952 (1.1 mmu) 233.1530 (1.1 mmu) 215.1425 (1.1 mmu) 178.1222 (1.0 mmu) 121.0644 (0.9 mmu)	-0.86	12.53	1.58	Level 2
M4	4.85	OH-VEN	C <sub>17</sub> H <sub>27</sub> NO <sub>3</sub>	сн <sub>3</sub> н <sub>3</sub> с <sup>-N</sup> он <sub>4</sub> он	294.2064 (0.0 mmu)	11	276.1955 (0.8 mmu) 121.0644 (0.9 mmu)	-2.05 (2) -2.06 (3) -1.98 (4)	6.93 (2) 6.88 (3) 7.26 (4)	2.1 (2) 2.0 (3) 2.4 (4)	Level 3

344 In these conditions, greater chromatographic retention was observed for the N-demethylated 345 metabolite (M1;Tr: 12.08 min) compared to the O-demethylated metabolite (M2;Tr: 8.02 min) (Table 346 3). For M1, three fragments were highlighted (Fig. 3), with the first one noted at m/z 246.1850, 347 corresponding to  $H_2O$  loss. The second one was detected at m/z 215.1426, corresponding to the 348 elemental composition C<sub>15</sub>H<sub>19</sub>O. This fragment resulted from a CH<sub>5</sub>N loss consecutive to water loss, 349 as observed with VEN but for a dealkylated metabolite, thus confirming N-dealkylation. The last 350 fragment was detected at m/z 121.0649, corresponding to the elemental composition fragment C<sub>8</sub>H<sub>9</sub>O 351 already observed for VEN (Fig. 3).

352 Concerning M2, a fragment was observed at m/z 246.1849 (equivalent to loss of H<sub>2</sub>O). 353 HCDfragmentation demonstrated the presence of a second fragment at m/z 133.0647. With the 354 assistance of Xcalibur software, the elemental composition proposed as the first option for this 355 fragment was C<sub>9</sub>H<sub>9</sub>O (with a delta mm of -0.091) (Fig. 3) . Finally, a fragment was detected at m/z 356 107.0493 and associated with the elemental composition fragment C<sub>7</sub>H<sub>7</sub>O, as observed with VEN but 357 for a dealkylated metabolite. The presence of this fragment confirmed that a methyl group was 358 detached from the oxygen of the VEN structure. The injection of analytical standards of NDV and ODV 359 confirmed the identity of these metabolites at a confidence level of 1 in mussel tissues.

#### 360 **3.3.2.1 Oxidative metabolism-hydroxylation**

361 Two metabolites, i.e. M3 and M4, revealed a [M+H]<sup>+</sup> of 294.2064. The M3 fold change value was 362 higher than reported for M4, however both were greater than 10 (Table 3) and were detected only in 363 the exposed samples (Fig. 1). The peak shapes and retention times of these signals (M3 and M4) 364 differed from those observed for VEN, indicating that they were potential metabolites (Fig.1, Fig. 4 and 365 Table 3). These metabolites presented a mass shift of +15.9950 compared to VEN. This mass shift 366 could be associated with the biotransformation of an RH structure into an ROH structure, leading to 367 the formation of potential hydroxylated metabolites. M3 and M4 exhibited shorter retention times than 368 VEN, thus supporting the VEN hydroxylation hypothesis. Previous studies have highlighted the 369 formation of such metabolites in organisms such as crustaceans (Jeon et al., 2013) and juvenile lean 370 marine fish (Santos et al., 2020).

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Figure 4. Chromatogram of signals associated with M3 (13C isotopic ion and dimer). Comparison with
 VEN (Tr= retention time and NL=signal strength).

The addition of an OH group increases the molecular polarity, leading to earlier elution of the molecule in the reverse phase liquid chromatography system used. M3 showed a retention time of 14.11 min, while lower chromatographic retention was obtained for M4 (Tr: 4.85 min). The difference in retention times between M3 and M4 indicated that hydroxylation occurred at different positions in the parent compound (VEN).

392 For M3, two signals corresponding to (1) the <sup>13</sup>C isotopic ion ([M+H]<sup>+</sup> 295.2094), and (2) the molecular 393 dimer ([M+H]<sup>+</sup> 587.4052) observed at 14.11 min and 14.09 min, respectively, reconfirmed the 394 presence of this potential metabolite (Fig. 4). HCD fragmentation of M3 showed a set of five fragments 395 that allowed us to determine the position of the OH group in its structure (Fig. 5). The fragment at m/z 396 276.1952 corresponded to H<sub>2</sub>O loss from the initial molecule. The fragments observed at m/z 397 215.1425 and 121.0644 in M1 were common to those of VEN. These coincidences between M3 and 398 VEN fragments were evidence of the absence of OH group on the aromatic ring and on the hexane 399 cycle of the initial molecule. The elementary compositions C<sub>15</sub>H<sub>21</sub>O<sub>2</sub> and C<sub>11</sub>H<sub>16</sub>ON associated with 400 fragments m/z 233.1530 and 178.1222, respectively, confirmed the position of the OH group in the 401 carbon adjacent to the VEN amino group.

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417 Figure 5. Relevant ions from the high resolution mass spectrum of fragmented M3 at 10 eV. The
418 asterisks indicate fragments in common with the VEN fragmented spectrum.

In addition, in the absence of an analytical standard to ensure the identity of M3 at the maximum confidence level (level 1), a predictive chromatographic retention time model was applied to support our structure proposal for M3. This Tr prediction methodology was from Jeon et al. (2013). The chromatographic retention time was predicted using a multiple regression equation (Eq.1) formulated by plotting the retention time versus the molecular weight and the predicted log D<sub>ow</sub> of each compound with the available analytical standard (Table 1).

426 Retention time (Tr) =  $4.7107x \log D_{ow} + 0.0324x M (g/mol) + 7.0762 (r^2: 0.92) (Eq.1)$ 

If the Tr predicted for the elucidated structure was within 2 min of the observed Tr, we considered that it was a viable structure. The proposed M3 structure demonstrated a predicted log D<sub>ow</sub> of -0.86 and a predicted Tr of 12.53 min, showing a difference of less than 2 min with respect to the observed Tr (Table 3). The proposed M3 structure was therefore feasible with a confidence level of 2.

The information available for M4 was limited. The signal intensity was very weak, which made it difficult to identify the relevant fragments. Only two fragments were identified. The experimental mass [M+H]<sup>+</sup> 276.1955 was associated with the H<sub>2</sub>O loss. A second fragment was observed at m/z 434 121.0644 and its elemental composition was C<sub>8</sub>H<sub>9</sub>O, as observed for VEN. The presence of this last 435 fragment suggests that the additional OH group was not positioned in the aromatic ring. Three 436 possible OH group positions were noted for M3 when applying the predictive chromatographic 437 retention time model (Table 3). The M3 structure with the OH group at positions 2 and 3 of the hexane 438 cycle showed predicted retention times at 6.93 min and 6.88 min, respectively (Table 3). Both 439 positions had a delta retention time (calculated considering the observed and predicted value) of 2 min 440 and might have fit a possible M4 structure. Due to the limited information available, the proposed M4 441 structure had a confidence level of 3.

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# 443 **3.4 Venlafaxine metabolite quantification**

The two phase I metabolites generated by demethylation of VEN previously highlighted in mussel tissues were quantified on each exposure day (days 3 and 7). Mean concentrations of NDV ( $50 \pm 13$ and  $34 \pm 13 \mu g/kg dw$ ) and ODV ( $28 \pm 7$  and  $24 \pm 8 \mu g/kg$ ) were measured in tissues exposed to VEN for 3 and 7 days (Table 2). Considering the intensity obtained for hydroxylated M3 (i.e.  $1.5*10^6$  versus  $5*10^5$  for M1), it seems (hypothesis put forward in the absence of absolute quantification) that it was the most abundant of all of the metabolites identified in this study. However, M3 quantification was not possible due to the lack of a commercially available analytical standard.

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#### 452 4. Discussion

453 PhACs for human care such as VEN are continuously disseminated in the environment (Čelić et al., 454 2019; Paíga et al., 2016, Santos et al., 2016), resulting in high exposure of non-targeted organisms to 455 these pollutants, even at low concentrations. VEN has been demonstrated to bioaccumulate and 456 interact at the molecular level with aquatic organisms, thereby impacting their tissue metabolic 457 capacities and jeopardizing their adaptive reponse to an acute stressor, as noted by Best et al. (2014) 458 and Ings et al. (2012). In addition, VEN has been shown to have an impact on the behavior (Malvault 459 et al., 2018), reproduction (Parrott and Metcalfe, 2017) and survival (Schultz et al., 2011) of these 460 organisms, but little information is available on the VEN metabolism process involved. In-depth 461 knowledge on the metabolism of this substance in wildlife is necessary to unravel key events in the 462 potential toxicological pathways. Metabolic studies in a broad range of organism are very challenging but could benefit from advances in chemical analysis and combined targeted and non-targeted approaches, the latter recently used to elucidate small molecules such as metabolites (Bonefille et al., 2017; Santos et al., 2020). The present study in a marine invertebrate illustrates the potential offered by these non-targeted approaches for biomonitoring and conducting surveys on the marine environment.

468 The sample treatment step is crucial in the determination of unknown metabolites. The tissue 469 treatment method was developed based on the physicochemical properties of known VEN metabolites 470 in the human body (five phase I and two phase II metabolites). These compounds showed medium to 471 high polarity, so the MeOH solvent was ideal for their extraction. VEN and its metabolites have a basic 472 character due to the amine functional group present in their structures. A pH at least two units lower 473 than the pKa value favors the presence of a positive charge on the nitrogen of the amine group of 474 these compounds. Since the compounds of interest hadthis property, the sorbent SPE chosen 475 combined two mechanisms to enhance their retention, i.e. strong cation exchange and hydrophobic 476 interactions. This double retention mechanism enabled the recovery of phase I metabolites, but also of 477 glucuronide metabolites, which are highly complex due to their elevated polarity and sensitivity to 478 hydrolysis during the extraction process (Karinen et al., 2009; Ketola and Hakala, 2010). The 479 analytical method performance evaluation showed that the combined tissue extraction and purification 480 conditions enabled high absolute recoveries of phase I metabolites and glucuronide metabolites, along 481 with moderate matrix effects.

482 Application of the tissue treatment method to the VEN-exposed samples generated data that helped 483 determine the extent of parent compound accumulation in mussel tissues. The BCF values obtained 484  $(177 \pm 27 \text{ L/kg dw and } 229 \pm 29 \text{ L/kg dw for VEN exposure at days 3 and 7, respectively) were in a$ 485 range similar to those already reported for VEN-exposed mussels. A first study conducted in mussels 486 (*M. galloprovincialis*) exposed for 7 days to 1, 10 and 100  $\mu$ g/L VEN reported BCF values of 178 ± 8.0 487 L/kg dw, 146 ± 35 L/kg dw and 189 ± 15 L/kg dw, respectively (Gomez et al, submitted). In a second 488 study, the BCF values reported for mussels (*M. galloprovincialis*) exposed for 20 days to  $10.7 \pm 1.6$ 489 µg/L of VEN were between 213.3 and 528.1 L/kg dw for the different treatments applied while also 490 taking the effects of water warming and acidification into account (Serra-Compte et al., 2018). The 491 authors suggested that the positive ionized form of VEN would facilitate its adhesion to the biological 492 matrix, which could explain the high BCF value obtained.

493 The high VEN bioconcentration combined with the presence of cytochrome P450 in mussels favours 494 enzymatic reactions involved in the metabolism of this xenobiotic compound in mussels (Snyder, 495 2000). The application of a non-targeted approach to samples collected in VEN-exposed mussels led 496 to the identification of four phase I metabolites. Two metabolites resulted from the demethylation 497 process at two different VEN positions: N-demethylation and O-demethylation. These metabolites 498 have already been previously identified in laboratory-exposed mussels (Serra-Compte et al., 2018; 499 Gomez et al., submitted) and in mussels collected in coastal areas of France (Martínez Bueno et al., 500 2014), Portugal, Italy and Spain (Álvarez-Muñoz et al., 2015b). Both metabolites presented 501 concentrations two orders of magnitude lower than the parent molecule. This behavior may be 502 expected in the mussel detoxification process, where it is sought to produce more hydrophilic and less 503 toxic metabolites, thereby facilitating their excretion. Quantification of these metabolites showed that, 504 for both exposure days, in the tested mussels there was a slight trend in favor of the detoxification 505 pathway that generates VEN dealkylation at the nitrogen position, whereas ODV is the major 506 metabolite in humans (Sangkuhl et al., 2014). A similar behavior was observed by Santos et al. 507 (2020), showing that N-demethylated metabolites are the predominant ones in fish. They suggest that 508 due to the higher hydrophilic character of NDV at physiological pH compared to ODV, fish prefer to 509 metabolize venlafaxine to NDV, generating higher concentrations of this metabolite in fish. The above 510 explanation could potentially be the justification for the higher presence of NDV evidenced in mussels. 511 Finally, NDV and ODV metabolites were both formed early during the detoxification process since they 512 both presented higher concentrations at day 3. This was already observed in a previous study (Gomez 513 et al., submitted). However, the findings of the non-parametric statistical Mann Whitney test comparing 514 data obtained on days 3 and 7 revealed no significant differences between these days.

515 The two other metabolites identified were hydroxylated VEN metabolites at two different positions: 1) 516 on the carbon adjacent to the amine group, and 2) on the hexane cycle. Hydroxylation is one of the 517 two known N-dealkylation mechanisms. This mechanism involves formal hydroxylation of a C-H bond 518 on the carbon adjacent to the heteroatom (HAT, H-atom transfer) (Meunier et al., 2004). The above 519 findings support the hypothesis of the proposed structure for the VEN hydroxylated metabolite (M3), 520 which to our knowledge has not been previously described. This metabolite showed the highest 521 intensities of all metabolites identified in the study for both exposure days. Hydroxylated metabolites 522 were also shown to be major diclofenac metabolites in mussels (Bonnefille et al., 2017; Świacka et al., 523 2019). This also highlights the differences in biotransformation processes in mussels compared to 524 humans (Sangkuhl et al., 2014). Due to the low intensity of the second hydroxylated venlafaxine 525 metabolite (M4), there was not a sufficient number of fragments to increase the confidence level for 526 the suggested structure. This hydroxylated metabolite has been previously identified in crustaceans 527 (Jeon et al., 2013). Both hydroxylated metabolites (M3 and M4) were formed later during the 528 detoxification process since they both showed higher intensities at day 7.

529 No glucuronide metabolites were detected despite the fact that the method was found to be able to 530 retain glucuronide metabolites. This could possibly be explained by two hypotheses: 1) mussels are 531 not capable of producing glucuronides, or 2) the concentration at which these compounds are formed 532 is below the detection limit of our method. A previous study revealed that mussels are capable of 533 generating glucuronide metabolites from xenobiotic benzo(a)pyrene (Michel et al., 1995). Mussels 534 may therefore possibly be able to generate this type of metabolite from pharmaceuticals. The above 535 suggests that the concentration of these metabolites in the analyzed tissues was lower than our 536 detection limit.

#### 537 **5. Conclusions**

538 The non-targeted approach was demonstrated to be a relevant method for characterizing and 539 identifying VEN metabolites produced in Mediterranean mussels, thereby generating insight into the 540 VEN metabolism in mussels. Two hydroxylated metabolites were identified for the first time in this 541 organism, thus confirming its ability to efficiently dealkylate and then hydroxylate amino 542 pharmaceuticals. However, glucuronidation did not appear to be a major detoxification pathway for 543 these compounds. Our results showed that mussels did not share the same VEN metabolism pathway 544 as in humans, and N-demethylation appeared to be the main metabolization pathway in 545 Mediterranean mussels. This hydroxylation ability thus facilitates further interpretation of metabolism in 546 mussels. The identified metabolites could also be used as biomarkers of exposure to environmental 547 pollutants. It should be noted that despite the active metabolism of mussels, venlafaxine 548 bioaccumulates 117 times in this organism. This warrants further studies on the effects of these 549 metabolites and venlafaxine in mussels.

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#### 560 **References**

Alvarez-Muñoz, D., Huerta, B., Fernandez-Tejedor, M., Rodríguez-Mozaz, S., Barceló, D., 2015a.
Multi-residue method for the analysis of pharmaceuticals and some of their metabolites in
bivalves. Talanta 136, 174–182.

- Álvarez-Muñoz, D., Rodríguez-Mozaz, S., Maulvault, A.L., Tediosi, A., Fernández-Tejedor, M., Van
  den Heuvel, F., Kotterman, M., Marques, A., Barceló, D., 2015b. Occurrence of pharmaceuticals
  and endocrine disrupting compounds in macroalgaes, bivalves, and fish from coastal areas in
  Europe. Environ. Res. 143, 56–64.
- Best, C., Melnyk-Lamont, N., Gesto, M., Vijayan, M.M., 2014. Environmental levels of the
  antidepressant venlafaxine impact the metabolic capacity of rainbow trout. Aquat. Toxicol. 155,
  190–198.
- 571 Bonnefille, B., Arpin-Pont, L., Gomez, E., Fenet, H., Courant, F., 2017. Metabolic profiling identification
  572 of metabolites formed in Mediterranean mussels (*Mytilus galloprovincialis*) after diclofenac
  573 exposure. Sci. Total Environ. 583, 257–268.
- Brieudes, V., Lardy-Fontan, S., Vaslin-Reimann, S., Budzinski, H., Lalere, B., 2017. Development of a
  multi-residue method for scrutinizing psychotropic compounds in natural waters. J. Chromatogr.
  B Anal. Technol. Biomed. Life Sci. 1047, 160–172.
- Čelić, M., Gros, M., Farré, M., Barceló, D., Petrović, M., 2019. Pharmaceuticals as chemical markers
  of wastewater contamination in the vulnerable area of the Ebro Delta (Spain). Sci. Total Environ.
  652, 952–963.

- Courant, F., Antignac, J.-P., Dervilly-Pinel, G., Le Bizec, B., 2014. Basics of mass spectrometry based
   metabolomics. Proteomics 14:2369–2388.
- Gomez, E., Bachelot, M., Boillot, C., Dominique, M., Chiron, S., Casellas, C., Fenet, H., 2012.
  Bioconcentration of pharmaceuticals (benzodiazepines) and two personal care products (UV
  filters) in marine mussels (*Mytilus galloprovincialis*) under controlled laboratory
  conditions).Environ. Sci. Pollut. Res. 19:2561-2569.
- 586 Gomez, E., Boillot, C., Martinez Bueno, MJ., Munaron, D., Mathieu, O., Courant, F., Fenet, H. In vivo 587 exposure of marine mussel to venlafaxine: bioconcentration and metabolization. Sumitted
- Ings, J.S., George, N., Peter, M.C.S., Servos, M.R., Vijayan, M.M.Venlafaxine and atenolol disrupt
  epinephrine-stimulated glucose production in rainbow trout hepatocytes. Aquat. Toxicol. 106107, 48-55.
- Jeon, J., Kurth, D., Hollender, J., 2013. Biotransformation pathways of biocides and pharmaceuticals
  in freshwater crustaceans based on structure elucidation of metabolites using high resolution
  mass spectrometry. Chem. Res. Toxicol. 26, 313–324.
- Karinen, R., Andersen, J.M., Ripel, Å., Hasvold, I., Hopen, A.B., Mørland, J., Christophersen, A.S.,
  2009. Determination of heroin and its main metabolites in small sample volumes of whole blood
  and brain tissue by reversed-phase liquid chromatography-tandem mass spectrometry. J. Anal.
  Toxicol. 33, 345–350.
- 598 Ketola, R., Hakala, K., 2010. Direct analysis of glucuronides with liquid chromatography-mass 599 spectrometric techniques and methods. Curr. Drug Metab. 11, 561–582.
- Martínez Bueno, M.J., Boillot, C., Munaron, D., Fenet, H., Casellas, C., Gómez, E., 2014. Occurrence
   of venlafaxine residues and its metabolites in marine mussels at trace levels: Development of
   analytical method and a monitoring program. Anal. Bioanal. Chem. 406, 601–610.
- Maulvault, A.L., Santos, L.H.M.L.M., Paula, J.R., Camacho, C., Pissarra, V., Fogaça, F., et al., 2018.
  Differential behavioural responses to venlafaxine exposure route, warming and acidification in
  juvenile fish (Argyrosomus regius). Sci. Total Environ. 634, 1136–1147
- McEneff, G., Barron, L., Kelleher, B., Paull, B., Quinn, B., 2013. The determination of pharmaceutical
   residues in cooked and uncooked marine bivalves using pressurised liquid extraction, solid-

- 608 phase extraction and liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem.609 405, 9509–9521.
- 610 Meunier, B., de Visser, S.P., Shaik, S., 2004. Mechanism of oxidation reactions catalyzed by 611 cytochrome P450 enzymes. Chem. Rev. 104, 3947–3980.
- Michel, X.R., Beasse, C., Narbonne, J., 1995. In vivo metabolism of benzo (a) pyrene in the mussel
   *Mytilus galloprovincialis*. Arch. Environ. Contam. Toxicol. 28, 215–222.
- Moreno-González, R., Rodríguez-Mozaz, S., Huerta, B., Barceló, D., León, V.M., 2016. Do
  pharmaceuticals bioaccumulate in marine molluscs and fish from a coastal lagoon? Environ.
  Res. 146, 282–298.
- Paíga, P., Santos, L.H.M.L.M., Ramos, S., Jorge, S., Silva, J.G., Delerue-Matos, C., 2016. Presence
  of pharmaceuticals in the Lis river (Portugal): sources, fate and seasonal variation. Sci. Total
  Environ. 573, 164–177.
- Paíga, P., Correia, M., Fernandes, M., Silva, A., Carvalho, M., Vieira, J., Jorge, S., Silva, J., Freire, C.,
  Delerue-Matos, C., 2019. Assessment of 83 pharmaceuticals in WWTP influent and effluent
  samples by UHPLC-MS/MS : Hourly variation. Sci. Total Environ. 648, 582–600.
- Parrott, J.L., Metcalfe, C.D., 2017. Assessing the effects of the antidepressant venlafaxine to fathead
  minnows exposed to environmentally relevant concentrations over a full life cycle. Environ.
  Pollut. 229, 403–411.
- Sangkuhl, K., Stingl, J., Turpeinen, M., Altman, R., Klein, T., 2014. PharmaGKB summary: venlafaxine
  pathway. Pharmacogenet Genom. 24, 62–72.
- Santos, L.H.M.L.M., Ramalhosa, M.J., Ferreira, M., Delerue-Matos, C., 2016. Development of a
  modified acetonitrile-based extraction procedure followed by ultra-high performance liquid
  chromatography-tandem mass spectrometry for the analysis of psychiatric drugs in sediments.
  J. Chromatogr. A 1437, 37–48.
- Santos, L.H.M.L.M., Maulvault, A.L., Jaén-Gil, A., Marques, A., Barceló, D., Rodríguez-Mozaz, S.,
  2020. Insights on the metabolization of the antidepressant venlafazine by meagre (Argyrosomus
  regius) using a combined target and suspect screening approach. Sci. Total Environ. 737,
  140226

- Schultz, M.M., Painter, M.M., Bartell, S.E., Logue, A., Furlong, E.T., Werner, S.L., et al., 2011.
  Selective uptake and biological consequences of environmentally relevant antidepressant
  pharmaceutical exposures on male fathead minnows. Aquat. Toxicol. 104, 38–47.
- 639 Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ru, M., Singer, H.P., Hollender, J., 2014. Identifying
- 640 Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. Environ.
- 641 Sci. Technol. 48, 2097–2098.
- Serra-Compte, A., Maulvault, A.L., Camacho, C., Álvarez-Muñoz, D., Barceló, D., Rodríguez-Mozaz,
  S., Marques, A., 2018. Effects of water warming and acidification on bioconcentration,
  metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in
  marine mussels (*Mytilus galloprovincialis*). Environ. Pollut. 236, 824–834.
- 646 Silva, L.J.G., Martins, M.C., Pereira, A.M.P.T., Meisel, L.M., Gonzalez-Rey, M., Bebianno, M.J., Lino,
- 647 C.M., Pena, A., 2016. Uptake, accumulation and metabolization of the antidepressant fluoxetine
  648 by *Mytilus galloprovincialis*. Environ. Pollut. 213, 432–437.
- Snyder, M.J., 2000. Cytochrome P450 enzymes in aquatic invertebrates: Recent advances and future
  directions. Aquat. Toxicol. 48, 529–547.
- Świacka, K., Szaniawska, A., Caban, M., 2019. Evaluation of bioconcentration and metabolism of
   diclofenac in mussels *Mytilus trossulus* laboratory study. Mar. Pollut. Bull.141, 249–255.
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