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Uptake, accumulation and metabolization of the antidepressant fluoxetine by *Mytilus galloprovincialis*☆

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ABSTRACT

Fluoxetine, a selective serotonin re-uptake inhibitor (SSRI) antidepressant, is among the most prescribed pharmaceutical active substances worldwide. This study aimed to assess its accumulation and metabolization in the mussel *Mytillus galloprovincialis*, considered an excellent sentinel species for traditional and emerging pollutants. Mussels were collected from Ria Formosa Lagoon, Portugal, and exposed to a nominal concentration of fluoxetine (75 ng L^{-1}) for 15 days. Approximately 1 g of whole mussel soft tissues was extracted with acetonitrile:formic acid, loaded into an Oasis MCX cartridge, and fluoxetine analysed by liquid chromatography with tandem mass spectrometry (LC-MS*n*). After 3 days of exposure, fluoxetine was accumulated in 70% of the samples, with a mean of 2.53 ng g^{-1} dry weight (d.w.) and norfluoxetine was only detected in one sample (10%), at 3.06 ng g^{-1} d.w. After 7 days of exposure, the accumulation of fluoxetine and norfluoxetine increased up to 80 and 50% respectively, and their mean accumulated levels in mussel tissues were up to 4.43 and 2.85 ng g^{-1} d.w., respectively. By the end of the exposure period (15 days), both compounds were detected in 100% of the samples (mean of 9.31 and 11.65 ng g^{-1} d.w., respectively). Statistical analysis revealed significant accumulation differences between the 3rd and 15th day of exposure for fluoxetine, and between the 3rd and 7th against the 15th day of exposure for norfluoxetine. These results suggest that the fluoxetine accumulated in mussel tissues is likely to be metabolised into norfluoxetine with the increase of the time of exposure, giving evidence that at these realistic environmental concentrations, toxic effects of fluoxetine in mussel tissues may occur.

1. Introduction

According to the latest report of 2011 of the Organisation for Economic Co-Operation and Development (OECD), the consumption of antidepressants increased more than 60% over the past decade (Silva et al., 2012). Selective serotonin re-uptake inhibitors (SSRIs), widely marketed since the mid-1980s (Schultz and Furlong, 2008), include fluoxetine that is among the most prescribed pharmaceutical active substance at national level and worldwide. Fluoxetine, as the other SSRIs, is primarily prescribed to patients diagnosed with clinical depression, obsessive-compulsive disorder, panic disorder, social phobia, and attention-deficit disorder (Schultz and Furlong, 2008). In mammals, following oral ingestion, fluoxetine is metabolized and the primary metabolite formed is N-desmethyl product (norfluoxetine) which is more potent than the parent compound (Silva et al., 2012).

One of the main inputs of pharmaceutical compounds to natural waters is from wastewater treatment plants (WWTPs) (Silva et al., 2012). The annual rate of discharge of pharmaceuticals from municipal WWTPs may reach kilogram levels (Paterson and Metcalfe, 2008). Furthermore, fluoxetine concentrations ranging from 0.41 to 141 ng L^{-1} were already reported in surface waters from Canada, Spain and USA (Silva et al., 2012). Under chronic exposure conditions, there is potential for pharmaceuticals to accumulate in fish and in other aquatic organisms (Paterson and

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Metcalfe, 2008) and a range of 79.1–9.8 ngg^{-1} wet weight (w.w.) of fluoxetine was reported to be accumulated in *Elliptio spp.* mussels from Crabtree Creek, USA, near a WWTP effluent channel and the downstream sites (Bringolf et al., 2010). Along with its main metabolite, norfluoxetine, fluoxetine is undoubtedly the SSRI most investigated, both in the aquatic compartments and in biota (Silva et al., 2012) and was reported as the most toxic (Brooks et al., 2003), at levels of at least one order of magnitude lower when compared with the other SSRIs (Silva et al., 2015).

The phylogenetically ancient and highly conserved neurotransmitter and neurohormone serotonin has been found in vertebrates and invertebrates, although its specific physiological role and mode of action is unknown for many species (Kreke and Dietrich, 2008). SSRIs affects a wide range of aquatic organisms, both vertebrate and invertebrate, but there is large variation in the sensitivity of organisms (Sumpter et al., 2014). By increasing the bioavailability of serotonin, fluoxetine has been described to disrupt the endocrine systems and many biological functions within invertebrates, such as reproduction, metabolism, moulting and behaviour (Bossus et al., 2014; Fong and Ford, 2014; Silva et al., 2015).

The first data on fluoxetine and norfluoxetine accumulation in brain, liver, and muscle tissues of different fish species of effluent-dominated ecosystems was reported by Brooks et al. (2005). Since then, some studies reported fluoxetine accumulation in fish tissues (Silva et al., 2015) and fewer on refer other aquatic organisms, such as mussels (Bringolf et al., 2010; Franzellitti et al., 2014; Maruya et al., 2014).

Fluoxetine, with basic characteristics, binds easily with particulate materials (Zenker et al., 2014). Thus, dietary routes of fluoxetine exposure and uptake may be particularly important for bivalves (Franzellitti et al., 2014). Bivalves are filter feeders sessile organisms which draw in water and particles from their surrounding environment (Dodder et al., 2014). There is still a paucity of data regarding accumulation and metabolization of pharmaceuticals, such as fluoxetine, by bivalves and these mechanisms deserve additional clarification (Boxall et al., 2012; Franzellitti et al., 2014; Du et al., 2015). The blue mussel *Mytilus galloprovincialis* is generally considered a good bioindicator of environmental quality and of accumulation of numerous contaminants (Bebianno et al., 2015). In addition, the exposure to fluoxetine has clearly triggered several biological responses in this species, namely endocrine disruption (Gonzalez-Rey and Bebianno, 2013). Therefore, the aim of this study was to evaluate the uptake, accumulation, and metabolization of fluoxetine in mussels, *M. galloprovincialis*, exposed to an environmental realistic concentration of fluoxetine (75 ng L^{-1}) to complement previous findings. To our knowledge, this is the first study reporting the metabolization of this pharmaceutical in bivalves (mussels).

2. Materials and methods

2.1. Standards and chemicals

Reference standards of fluoxetine and norfluoxetine hydrochloride, the labelled surrogates fluoxetine-d5 hydrochloride, and norfluoxetine-d6 oxalate, all with ≥98% purity, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Stock and intermediate solutions were prepared in methanol at 5 mg mL^{-1} and 250 μg mL^{-1}, respectively, and stored at −20 °C, for a maximum of 6 months. Mixed standard working solutions, renewed before each analytical run, were prepared at 2.5 and 50 ng mL^{-1} in methanol:water (10:90), and used for linearity, accuracy, and repeatability assays. The labelled surrogates were typically prepared to obtain a final concentration of 50 ng mL^{-1}.

HPLC-grade acetonitrile and methanol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Water was prepared from a Millipore Milli Q system (Bedford, MA, USA). Ammonium hydroxide and formic acid (98%) were obtained from Merck (Darmstadt, Germany); hydrochloric acid 37% was from Carlo Erba (Milan, Italy).

2.2. Sample collection and exposure assay

Mussels *M. galloprovincialis* were collected from a small fishing and tourist boats harbour in the Ria Formosa Lagoon, Portugal (37°06′58.5″N 7°37′44.0″W). Mussels were transported alive to the laboratory, the shell was cleaned, and they were acclimatized for one week in aerated natural seawater. Mussels were then separated and placed in several aquaria (n = 35, 1 mussel L^{-1}); four for controls (un-exposed) and three exposed to a nominal concentration of fluoxetine (75 ng L^{-1}). The aquaria were kept at constant temperature (18.6 °C ± 1), salinity (33 ± 0.4), pH (8.1 ± 0.2) and oxygen saturation (>98% ± 2). Mussels were not fed until the end of the experiment and no mortality occurred. Water was changed every 48 h and fluoxetine concentration re-established (Gonzalez-Rey and Bebianno, 2013).

At each sampling time (0, 3, 7, and 15 days), mussels (n = 10) were removed from control and exposed aquaria, freeze-dried and individually stored at −20 °C to determine fluoxetine accumulated in mussel tissues. Fifteen mussels were removed from each aquaria and individual shell biometric measured – average shell length size: 67.60 ± 5.08 mm, width: 37.31 ± 1.77 mm and height 25.48 ± 1.93 (Table S1, Supporting Information) and the Condition index (CI) quantified as the ratio shown in equation (1). Data is presented in Table S1.

$$\mathrm{CI}\ (\%) = \left[\text{whole soft tissue (w.w.)} \times \text{whole body tissue with shell (w.w.)}^{-1}\right] \times 100 \quad (1)$$

2.3. Experimental procedure

Each mussel whole soft tissues was grounded into powder, and ±1 g was homogenized and spiked with surrogate standards (fluoxetine-*d*5 and norfluoxetine-*d*6) and extracted twice with 10 mL acetonitrile with 0.1% formic acid (Schultz et al., 2010). After mixing for 5 min, ultrasonication for 15 min, and centrifugation for 10 min at 4 °C and 5400*g*, the supernatant was collected and the extraction repeated. The supernatants were finally pooled and centrifuged for 10 min at 4 °C and 20,000*g*. For solid phase extraction with Oasis MCX cartridge (150 mg, 6 mL, Waters, Milford, MA, USA), the extract was loaded into the cartridge previously conditioned with 5 mL methanol and 5 mL acidified water at pH 2.5. To rinse 2 mL 0.1 N HCl and 2 mL 5% methanol in water were used. Elution was done with 2 mL methanol following 6 mL 5% ammonium hydroxide in methanol. Finally, the eluate was evaporated to dryness under a gentle stream of nitrogen, at 40 °C, and the dried extracts were stored at −20 °C until analysis, that took place in 48 h maximum.

For liquid chromatography coupled to tandem mass spectrometry (LC-MS*n*) analysis, the dried eluate was reconstituted in 1 mL methanol:water (10:90) and microfiltered through durapore membrane filters 0.22 μm (Millipore, Milford, MA, USA). A 20 μL injection volume was used with a flow rate at 200 μL min^{-1} and a gradient of (A) water with 0.5% formic acid and (B) methanol with 0.5% formic acid, as presented in Table S2. A chromatographic column ZORBAX Eclipse XDB-Phenyl (150 × 3.0 mm; 3.5 μm), maintained at 45 °C, and guard-column of the same packing material were used. A hybrid Quadrupole Ion Trap Mass Spectrometer (LCQ