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Fluoxetine accumulation and metabolism as exposure biomarker to better understand biological effects in gastropods

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Introduction

Fluoxetine is a widely used antidepressant frequently found in wastewater treatment plant effluent and in aquatic ecosystems at concentrations below $\mu\text{g/L}$.¹ Numerous studies have explored the sensitivity of invertebrates to waterborne fluoxetine showing marked differences of sensitivity. In a previous study, the New-Zealand mudsnail, *Potamopyrgus antipodarum*, and the European valve snail, *Valvata piscinalis*, were exposed to waterborne fluoxetine (3.7-100 $\mu\text{g/L}$) during 42 days. While effects were observed on the mudsnail reproduction and F1, the valve snail was not affected. Several assumptions were proposed to explain these results including dissimilar metabolic capacities or bioavailability of fluoxetine for the snails.² Indeed, recently, fluoxetine was measured in tissues of mussels *Eliptio complanata* exposed downstream of a municipal wastewater, assorted with reproductive effects under laboratory experiments.³ Both fluoxetine and norfluoxetine (the active metabolite) have been measured in tissues of fishes sampled or caged in streams downstream of urban and industrialized effluent discharges.⁴⁻⁷ Thus the aim of this study was to assess if differential sensitivity of gastropods snails to fluoxetine could be explained by its bioaccumulation and metabolism into norfluoxetine.

Materials and Methods

P. antipodarum and *V. piscinalis* were exposed in the same beakers to fluoxetine as previously described and concentrations were chosen in accordance with the biological effects observed in these species.² However, due to lack of abundance of *V. piscinalis* in our culture, exposure of the both species was not rigorously similar. The nominal exposure concentrations were 11, 33 and 100 $\mu\text{g/L}$ for

P. antipodarum plus a control, and 33, 100 $\mu\text{g/L}$ plus a control for *V. piscinalis*. Six replicates per concentration were prepared and exposed for 7 (*V. piscinalis*) and 14 days (*P. antipodarum*). Organisms were fed *ad libitum*. Adult snails were used at the start of the experiment. On days 7 and 14, 3 *P. antipodarum* were collected per condition for fluoxetine and norfluoxetine analysis and 3 *V. piscinalis* on day 7. Due to the lipophilic nature of fluoxetine,^{8,9} triacylglycerol (TG) content of snails was measured,¹⁰ as proxy of total lipid content, TG being the principal lipid class in mollusks.¹¹

Fluoxetine was measured in water on days 7 and 14 as published elsewhere,¹⁰ as well as fluoxetine and norfluoxetine in snails. Briefly, an easy and quick extraction similar to Quick Easy Cheap Effective Rugged Safe (QuEChERS) procedure¹² in a miniature version was developed to analyze both compounds in gastropods. The procedure involves an extraction of about 10 milligrams of matrix by 500 μL of a mixture of acetonitrile: water:hexane (50/20/30) and 100 mg of citrate buffer. Recoveries were 87% for fluoxetine and 86% for norfluoxetine. Nano-LC-nano-ESI MS/MS analysis was performed with a nano Ultimate3000 (Dionex[®]) coupled with a Qtrap3200 detector (AB Sciex[®]). Data were processed with Analyst 1.5. The chromatographic separation was performed in two steps: first a preconcentration step on a precolumn C18 (5 μm , 300 Å , LC Packings[®]) followed by a separation on a Pepmap C18 column (C18 3 μm x 75 μm x 100 Å , Dionex[®]). The column oven temperature was set to 60°C; injection volume was 1 μL in μL pickup mode; the flow rate was 300 nL/min. Samples were analyzed in positive mode with the mobile phase (A) CH₃CN/FA/H₂O (2/0.1/97.9) and (B) CH₃CN/FA/H₂O (80/0.08/19.92), with the following elution program: start at 60% (A), from 60 to 0% in 5 min and from 5 to 20 min, 100% (B). The column was re-equilibrated 20 min between runs. MS/MS detection was performed in the multiple reaction monitoring (MRM) mode using a NanoSpray[®] II source (AB Sciex[®]). Fluoxetine and norfluoxetine were identified by their retention times, two characteristic ion transitions (fluoxetine: 310>148 and 310>91, norfluoxetine: 296>134 and 296>30) and specific ratios of the responses of the transitions (deviation <20% with respect to analytical standards ratios). The quantification was carried out by double injection. The use of matrix-matched standards was selected. The matrix blank (snail) was prepared as for analysis of samples. Six-point calibration curves were built from the injection of these matrix prepared standards. This calibration intended to compensate for matrix effects. In these conditions, the limit of quantification for fluoxetine and norfluoxetine in snail samples was respectively 7.7 and 3.8 ng/g.

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Fluoxetine accumulation on days 7 and 14, was assessed by calculating bioaccumulation factor (BCF) estimates according to Nakamura *et al.*⁹ The estimate ($\text{BCF}_{\text{tot}}/\text{BCF}_{\text{tot}} = C_{\text{snail}}/C_{\text{water}}$) was calculated, using the fluoxetine concentrations measured in snails after 7 and 14 days (C_{snail}) for *P. antipodarum* and 7 days for *V. piscinalis*.

Results and Discussion

During the exposure, mean water temperature was $21.0 \pm 0.3^\circ\text{C}$, mean conductivity $332 \pm 25 \mu\text{S/cm}$ and mean pH 7.9 ± 0.3 . Chemical measurements showed a fluoxetine recovery in the exposure system depending on the tested concentration (from 25 to 98%), with mean measured exposure concentrations of 2.9, 24 and 79 μg fluoxetine/L (Table 1). Even if the gradient of exposure still was present, fluoxetine measured concentrations were lower than expected ones, with the lowest recovery rates for the lowest tested concentration. This is consistent with what was previously observed in similar exposure systems.² These lower exposure concentrations can certainly be explained by adsorption on the devices used for exposure system, as fluoxetine in a relatively stable compound.¹³

Fluoxetine was measured in both *P. antipodarum* and *V. piscinalis* (Table 1). No significant differences ($P > 0.05$) were noted between the fluoxetine concentrations in *P. antipodarum* after 7 or 14 days, suggesting that the bioaccumulation reached steady state after 7 days of exposure. In the Japanese medaka, bioaccumulation steady state was achieved after only 3 days of exposure.⁸ On the basis of these results which showed quick accumulation for fish and snail, we assumed that steady

state could also be reached in *V. piscinalis* after 7 days of exposure and we compared fluoxetine body burden in the two species on day 7. Concentrations of fluoxetine and estimate BCF_{tot} in *P. antipodarum* were significantly higher ($P < 0.05$) than in *V. piscinalis* (Tables 1 and 2),^{3,8,9,14} implying a higher bioaccumulation of the parent compound in *P. antipodarum* after 7 days of exposure. Exposure systems were rigorously similar for both species. As fluoxetine was waterborne, the contamination probably preferentially occurred across the gills. However, fluoxetine can adsorb on food as it does on sediment¹³ and thus oral route is not excluded and total exposure could be modified by various food consumption. Indeed, both snails have different feeding modes, *P. antipodarum* being a deposit feeder and *V. piscinalis* being a scraper.¹⁵

Many studies showed that fluoxetine concentrations were higher in lipid rich tissues, than in muscles (Table 2). Thus, it can be assumed that a difference in lipid content in

both snails could also explain the differences in BCF_{tot}. TG content in *P. antipodarum* was around seven times higher than in *V. piscinalis* (Figure 1), which probably explains the higher bioaccumulation of fluoxetine in the mudsnail compared to the valve snail. Therefore, the lesser sensitivity of *V. piscinalis* to waterborne fluoxetine compared to *P. antipodarum*² could

be at least partly explained by its lesser bioaccumulation in the whole body.

BCF estimates in both snails were far lower than in mussel, but higher than those calculated in fishes (Table 2). Fluoxetine bioaccumulation is strongly dependant of pH,⁹ and no data is available concerning the exposure pH of *E. complanata*,³ thus limiting the comparison

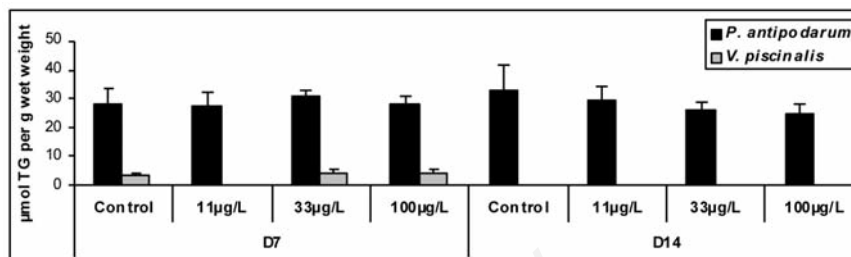


Figure 1. Levels of triglycerides in snails µmol/g (mean value and standard deviation).

Table 1. Measured concentrations of fluoxetine in water (µg/L), snails (µg/g), and of norfluoxetine in snails (µg/g).

Nominal fluoxetine concentration (µg/L)		Control	11 µg/L	33 µg/L	100 µg/L	
Measured concentration (µg/L)	D7	0.4	2.7	21.4	98.1	
	D14	<LD	3.1	26.7	60.3	
Fluoxetine (µg/g)	<i>P. antipodarum</i>	D0	<LOD	<LOD	<LOD	
		D7	<LOD	1.199±0.277	5.474±0.240	32.473±4.213
		D14	<LOD	1.755±0.309	6.272±0.503	33.478±4.312
	<i>V. piscinalis</i>	D0	<LOD	<LOD	<LOD	
D7	<LOD	-	2.827±1.136	13.862±3.226		
Norfluoxetine (µg/g)	<i>P. antipodarum</i>	D0	<LOD	<LOD	<LOD	
		D7	<LOD	<LOQ	0.009±0.002	0.099±0.014
		D14	<LOD	0.004	0.012±0.003	0.060±0.014
	<i>V. piscinalis</i>	D0	<LOD	<LOD	<LOD	
		D7	<LOD	-	0.008±0.003	0.058±0.026

D0, D7 and D14: days 0, 7 and 14; LOD, limit of detection; LOQ, limit of quantification.

Table 2. Bioaccumulation factor (BCF) and pseudo-BCF in aquatic organisms.

Species	Water concentration	Exposure duration	pH	Fluoxetine BCF	Norfluoxetine pseudo-BCF
<i>O. latipes</i> ⁹	10 µg/L	30 days	7.2	8.8 (B), 339 (L), 13 (B+L)	84 (B), 1500 (L), 100 (B+L)
			8.1	30 (B), 580 (L), 37 (B+L)	130 (B), 3300 (L), 170 (B+L)
			8.9	260 (B), 3100 (L), 330 (B+L)	650 (B), 3700 (L), 720 (B+L)
<i>O. latipes</i> ⁸	0.64 µg/L	7 days	7.4	74	117
<i>O. mykiss</i> ¹⁶	3 µg/L	8 days	-	59 (M), 143 (AT)	-
<i>P. antipodarum</i>	11 µg/L	7/14 days	7.9	400/585	1.3
	33 µg/L			228/261	0.5
	100 µg/L			411/424	0.8
<i>V. piscinalis</i>	33 µg/L	7 days	7.9	118	0.3
	100 µg/L			175	0.7
<i>E. complanata</i> ³	2.4 ng/L	14 days	-	125	-
	7 ng/L			1347	
	14 ng/L			1250	
	119 ng/L			665	

BCF, bioaccumulation factor; B, body; L, liver; B+L, body + liver; M, muscle; AT, adipose tissue.

between species. Dietary route of exposure and lower exposure concentrations might also be in cause.

As for fluoxetine, norfluoxetine was measured in both *P. antipodarum* and *V. piscinalis* (Table 1). To our knowledge, this is the first study to investigate *in vivo* metabolism of a pharmaceutical in mollusk. Contrary to parental compound, norfluoxetine concentrations measured in both species were not significantly different ($P > 0.05$), and norfluoxetine levels in snails represented less than 1% of total fluoxetine body burden. This suggests that *N*-demethylation is not the primary metabolite as in fish.¹⁶

Since norfluoxetine was measured in both snails, pseudo-BCFs estimates (pseudo-BCF₁₀₀) were calculated according to Nakamura *et al.*,⁹ the denominator being the concentration of fluoxetine in water and not the concentration of norfluoxetine. Pseudo-BCF estimates (Table 2) were much lower than those determined for fluoxetine. In fishes, higher norfluoxetine concentrations in comparison to fluoxetine are measured and pseudo-BCF estimates were far higher than in gastropods (Table 2). Thus *N*-demethylation capacity is higher in fishes than in gastropods. In snails, total accumulation of fluoxetine can be reduced to fluoxetine accumulation.

The biological effects in gastropods are supported by the higher bioaccumulation of fluoxetine in the mudsnail. In humans, the norfluoxetine is more active than the parent compound,¹⁷ as in a protozoan and a crustacean 24 h lethality-test.¹⁸ However, the presumed active metabolite is measured in similar very low quantities in both species, implying that metabolite activation into norfluoxetine does not explain the interspecific differences previously observed. However, other metabolites cannot be excluded. Thus further studies are needed in order to better assess these issues.

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