Journal of Hazardous Materials 283 (2015) 350–358

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Sertraline accumulation and effects in the estuarine decapod *Carcinus maenas*: Importance of the history of exposure to chemical stress

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HIGHLIGHTS

- Bioaccumulation and effects of sertraline in a key estuarine species.
- MOA-related endpoint exhibiting non-monotonic hormetic responses.
- Life-threatening alterations in biomarkers of cholinergic transmission and oxidative stress.
- Effects dependent on crabs' history of exposure to contamination.
- Site-specific risk assessment needed for accurate inference from the lab to the field.

ARTICLE INFO

Article history:
Received 9 April 2014
Received in revised form 8 August 2014
Accepted 11 August 2014
Available online 28 August 2014

Keywords:
Bioaccumulation
Cholinergic neurotransmission
Oxidative stress
Exposure history
Risk assessment
Invertebrates

ABSTRACT

Sertraline is widely prescribed worldwide and frequently detected in aquatic systems. There is, however, a remarkable gap of information on its potential impact on estuarine and coastal invertebrates. This study investigated sertraline accumulation and effects in *Carcinus maenas*. Crabs from a moderately contaminated (Lima) and a low-impacted (Minho) estuary were exposed to environmental and high levels of sertraline (0.05, 5, 500 $\mu g\,L^{-1}$). A battery of biomarkers related to sertraline mode of action was employed to assess neurotransmission, energy metabolism, biotransformation and oxidative stress pathways. After a seven-day exposure, sertraline accumulation in crabs' soft tissues was found in Lima (5 $\mu g\,L^{-1}$: 15.3 $ng\,L^{-1}$ ww; 500 $\mu g\,L^{-1}$: 1010 $ng\,L^{-1}$ ww) and Minho (500 $\mu g\,L^{-1}$: 605 $ng\,L^{-1}$ ww) animals. Lima crabs were also more sensitive to sertraline than those from Minho, exhibiting decreased acetylcholinesterase activity, indicative of ventilatory and locomotory dysfunction, inhibition of antioxidant enzymes and increased oxidative damage at $\geq$0.05 $\mu g\,L^{-1}$. The Integrated Biomarker Response (IBR) index indicated their low health status. In addition, Minho crabs showed non-monotonic responses of acetylcholinesterase suggestive of hormesis. The results pointed an influence of the exposure history on differential sensitivity to sertraline and the need to perform evaluations with site-specific ecological receptors to increase relevance of risk estimations when extrapolating from laboratory to field conditions.

1. Introduction

Implementation of specific legislation worldwide is acting to reduce and mitigate the presence and effects of priority contaminants in aquatic systems. However, emerging contaminants (ECs) are placing a major challenge to ecological risk assessment and mitigation programmes because they are excluded from standard chemical evaluations and their biological effects are still poorly known, despite their recognised importance. Among ECs, antidepressants and their metabolites have been raising a great deal of concern. They are widely prescribed and frequently detected in wastewaters and streams influenced by their discharge [1–4]. In the European Union, their consumption increased by >80% in the last decade [5] due to escalating trends in mental health problems and psychiatric disorders. Their occurrence in aquatic

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http://dx.doi.org/10.1016/j.jhazmat.2014.08.035
0304-3894/

systems is considered to be due to low removal rates of conventional wastewater treatments, resulting in the discharge of contaminated effluents into receiving waters, with major public health and environmental risks [1,6]. Conceived to trigger a specific biological response, several studies evidenced that antidepressants may induce effects on non-target species with unexpected consequences in behavioural and reproductive traits at concentrations within the ng L^{-1} range [7]. This is the case for selective serotonin reuptake inhibitors (SSRIs) used to treat depression, anxiety and personality disorders. In humans and other vertebrates, serotonin (5-HT) is involved in the neuromodulation of several hormone-dependent physiological processes. In invertebrates and lower taxa, 5-HT is involved in functions as diverse as bivalve reproduction [8] or cilia regeneration in protozoa [9]. Based on high homology between receptors, relevant risk for undesirable outcomes may be expected for SSRIs in non-target organisms [10,11]. In good agreement, sertraline (SERT), a potent SSRI with few side effects in humans, is considered the most acutely toxic SSRI towards freshwater species (*e.g.*, algae, daphnids, fish) [12–14]. SERT has also been detected at concentrations as high as 0.037 μg L^{-1} in surface waters (USA) [4] and 0.100 μg L^{-1} (Norway) [15] and 0.106 μg L^{-1} (China) [3] in hospital effluents. Moreover, predicted environmental concentrations (PECs) range from 0.14 μg L^{-1} to 17.1 μg L^{-1} for untreated wastewaters [16]. However, knowledge on its accumulation in and/or effects on coastal and estuarine key invertebrates is scarce [17–19]. Studies are mostly focused in freshwater species and effects of fluoxetine [4,19–22], despite high human pressure and risk of contamination in these areas. Hence, research addressing the relationship between exposure, tissue accumulation and adverse effects of sertraline in such species is urgently needed to provide empirical data for risk assessment and develop predictive approaches [20].

It has been noted earlier that effects of pharmaceuticals should be better addressed by focusing on sensitive biomarkers and adverse outcomes related to their mode of action (MOA) [10,23]. Moreover, sublethal endpoints, such as disruption of fish behaviour [11,22], stress effects [19] and metabolic pathways [24] seem to be more sensitive to evaluate SSRIs than conventional testing based on standard laboratory species and survival, growth and reproduction. Also species and/or populations of different backgrounds (*e.g.*, geographic regions, genetic make-up, previous exposure history) may exhibit differential sensitivity to contaminants [25–28]. However, this is seldom taken into consideration often limiting ecological relevance to field scenarios and accurate site-specific risk assessment.

Carcinus maenas is a key invertebrate of European estuarine and coastal systems regularly used in laboratory and field studies addressing effects of contaminants, including pharmaceuticals [29–31]. It is a good biological indicator reflecting the levels of environmental contamination [32]. Its voracious foraging behaviour is considered to be a structuring feature of marine and estuarine benthic communities [33]. *C. maenas* is also a common prey of several species of crustacean, fish, aquatic birds, minks, otters and seals [34]. Recently, it was observed that the history of chronic exposure to even moderate pollution may influence *C. maenas* sensitivity to chemical and natural stress. Crabs under such conditions showed higher sensitivity to salinity stress but increased tolerance to organophosphate exposure possibly resulting from acclimation processes [28,31].

This work therefore investigated the accumulation and sublethal effects of SERT exposure in *C. maenas* originating from sites with differing contamination histories. The working hypotheses were that: (i) exposure to low environmental levels of sertraline would cause tissue accumulation and alterations in sub-individual biomarkers, impairing the health status of *C. maenas*; (ii) these effects would be dependent on the history of exposure to moderate contamination of the crabs, providing relevant information to the ecotoxicological assessment involved in risk calculations. To test this, sub-acute exposures to SERT were performed with crabs from two NW Iberian estuaries, a low-impacted and a moderately polluted by metals and polycyclic aromatic hydrocarbons (PAHs) [28]. Ten biomarkers providing health status information were assessed for their potential involvement in SERT MOA [29,35,36] and widespread use in integrated chemical–biological effects monitoring. They were related to neurotransmission, energy metabolism, biotransformation and oxidative stress pathways: activity of acetylcholinesterase in the thoracic ganglion (AChEg) and AChEm, lactate dehydrogenase (LDH) and $NADP^+$-dependent isocitrate dehydrogenase (IDH) in the muscle; activity of glutathione *S*-transferases (GST), catalase (CAT), glutathione peroxidase (GPx) and reductase (GR), and levels of total glutathiones (TG) and lipid peroxidation (LPO) in the digestive gland. AChE is a serine hydrolase acting in the cleavage of acetylcholine in synapses and neuromuscular junctions terminating the transmission of nervous impulse to postsynaptic cells. Blockage of AChE is life-threatening leading to paralysis and respiratory arrest. SERT was previously shown to inhibit human AChE activity and 5-HT and serotonergic neurons have been implicated in the modulation of cholinergic transmission in *Caenorhabditis elegans* [35,37], pinpointing the interest of AChE to assess SERT effects. To clarify the results, a characterisation of cholinesterase (ChE) forms in the crabs' thoracic ganglion was performed. This ganglion is composed of interneurons and motoneurons that are important components of the ventilatory central pattern generator [38]. Catalytic properties of muscle AChE, involved in crabs locomotion [39], were reported elsewhere [28]. LDH and IDH are involved in anaerobic and aerobic pathways of energy production, respectively; their induction under chemical challenge provides additional energy to deal with toxicant exposure [40–42]. GST act in detoxification of xenobiotics, and defence against oxidative damage, by catalysing conjugation of phase I metabolites with reduced glutathione (GSH), facilitating excretion from the organism. GSH is also a direct scavenger of oxyradicals formed during detoxification and a cofactor of GPx in the conversion of hydrogen peroxide into oxygen and water [43]. Oxidised glutathione is subsequently recycled in reactions involving GR activity. CAT is another antioxidant enzyme facilitating the conversion of hydrogen peroxide into less reactive components. When the balance between the generation of oxyradicals and its elimination by antioxidants is disrupted, oxidative damage to macromolecules will occur [44]. An influence of SERT on LPO and hepatic tissue damage was reported previously [36] suggesting its putative usefulness here. All these parameters may be altered by xenobiotic exposure, hence their wide use as environmental biomarkers [29,30,42,43,45]. Finally, the Integrated Biomarker Response (IBR) index was used for interpretation of the multibiomarker responses and evaluation of health status.

2. Methods

2.1. Crab sampling and acclimation

Crabs were collected at the mouth of the Minho and Lima estuaries as described previously [28]. Minho shows low pollution levels while Lima is historically contaminated by moderate levels of metals and PAHs among others (supplementary data). Previously no relevant genetic structuring of *C. maenas* populations could be found along the Iberian Peninsula coast [46], suggesting the patterns of physiological variation of *C. maenas* from these estuaries likely reflect environmental differences. Intermoult male crabs were used in the experiments (Minho: 4.2 ± 0.03 cm carapace width, mean ± SD; Lima: 4.5 ± 0.04 cm). In the laboratory, crabs

were placed in tanks containing filtered seawater (14 psu), continuously aerated, for 45 d (temperature: 15 ± 0.6 °C; photoperiod: 14:10 h day/night). During this acclimation they were fed frozen mussels (supplementary data).

2.2. Biochemical properties of ganglion ChEs

Thoracic ganglion was chosen to assess cholinergic transmission in central nervous system due to its role in ventilation [38] and 5-fold higher enzymatic content compared to cerebral ganglion [47]. This ganglion contains interneurons and motoneurons both are important components of the ventilatory central pattern generator [38]. The motoneurons innervate the levator and depressor muscles controlling the ventilatory appendages. Measurement of ChE substrate preferences and responses to selective inhibitors allows for their classification either as AChE (used as biomarker of exposure and/or effect) or pseudocholinesterase (also known as butyrylcholinesterase, BChE). Some studies described marine invertebrate ChEs with atypical properties including overlapping substrate preferences and atypical behaviour towards selective inhibitors [48] raising the need to clarify this possibility in other marine species for its adequate use as a reliable biomarker. The characterisation was performed as described previously [28] and using the Ellman's method [49]. Selective inhibitors used were eserine sulphate, BW284C51 and iso-OMPA; substrates were acetylthiocholine (ATCh), butyrylthiocholine (BTCh) and propionylthiocholine (PTCh) (supplementary data). Determinations were done in three pools of five crabs each per sampling site.

2.3. SERT accumulation and effects in C. maenas

Crabs collected at each sampling site were exposed to environmental and high levels of SERT (0.05, 5, 500 $\mu g\,L^{-1}$) for 7 d. Four crabs were exposed per glass aquaria, each containing 4 L of test medium. Three aquaria were prepared per treatment. Preliminary experiments have shown that the loading rate used provided healthy conditions for crabs of the average size employed here. Exposure conditions were as described for the acclimation. Medium renewal was performed every 48 h. Salinity, temperature, pH and dissolved oxygen were measured at 0 h and 48 h (Table S1). Samples of freshly prepared (0 h) and old (48 h) test media from the highest SERT treatment were collected for confirmation of real exposure concentrations. At the end of the exposure period, sub-samples of ganglion, leg muscle and digestive gland were collected, snap frozen, and stored at −80 °C until biomarkers determination. The digestive gland was selected because of its main role on detoxification of xenobiotics and hence high levels of biotransformation and anti-oxidant enzymes [31]. Remaining whole-body soft tissues were pooled and frozen at −20 °C until chemical quantification of SERT and its major metabolite norsertraline (NORS).

2.3.1. Chemical analysis of SERT and NORS

Water samples were extracted by solid phase extraction (SPE) with Oasis MCX cartridges and analysed by ultra high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) (supplementary data). SERT and NORS concentrations in crabs tissues were determined using a QuEChERS-based extraction method adapted from [50] and [51] and UHPLC–MS/MS analysis (supplementary data). Fluoxetine-d5 (1 $mg\,L^{-1}$ in acetonitrile) was used as internal standard. A Nexera UHPLC system coupled with a triple-quadrupole mass spectrometer detector LCMS-8030 was used. Chromatographic separation was performed on a Kinetex C18 column. Method detection limits (MDL) were defined as the minimum detectable amount of analyte with a signal-to-noise ratio (S/N) of 3:1 divided by the pre-concentration factor obtained with the extraction procedure. MDL for SERT and NORS in tissues were 1.76 $ng\,g^{-1}$ wet weight (ww) and 1.80 $ng\,g^{-1}$ ww, respectively. MDL in water were 10 $ng\,L^{-1}$ (SERT) and 3 $ng\,L^{-1}$ (NORS). Detailed QA/QC data is available in the supplementary data.

2.3.2. Biomarkers determination

All biomarkers were determined as previously described for *C. maenas* [31,42] (supplementary data). Microplate determinations were performed in a Bio Tek Power Wave 340 reader; cuvette absorbance assays were carried out with a Jasco 6405 UV/VIS spectrophotometer.

2.4. Data analysis

The Michaelis–Menten equation was used to investigate ganglion ChE properties through estimation of maximal velocity (V_{max}), Michaelis–Menten constant (K_m) and their ratio (V_{max}/K_m). Differences in the kinetic parameters obtained for the two sampling sites were assessed by comparison of regression slopes and intercepts of kinetic curves. Data concerning specific inhibitors and substrates were compared with the non-parametric Friedman test. As to biomarkers, the data were first analysed using a two-way hierarchical analysis of variance (ANOVA) model. The concentration of sertraline and the estuary of origin of the crabs were included as fixed factors, and the aquarium as nested random factor. Given that no effect of the aquarium could be observed on any of the parameters analysed, the nested factor was subsequently removed from the model. Contrast analysis was used to investigate potential differences in biomarkers in relation to SERT exposure concentrations and the estuary of origin of the crabs. ANOVA assumptions were tested with Shapiro–Wilk and the Cochran's tests. The IBR was calculated, following previous description [52] and subsequent modifications [53], for each SERT concentration tested in the Minho and Lima crabs (supplementary data). Statistical analyses were carried out in GMAV 5.0 [54].

3. Results

3.1. Biochemical properties of ganglion ChEs

The preferential substrate to ganglion ChE was ATCh, followed by PTCh and BTCh (Table S2). Activity inhibition by excess substrate was never detected. The catalytic efficiency for ATCh was higher in Lima than in Minho crabs ($p < 0.001$). Nevertheless, eserine sulphate and BW284C51 strongly inhibited ChE activity in a similar manner in crabs from both estuaries indicating these were true ChE and that the main form was AChE, respectively (Fig. 1). Incubation with iso-OMPA caused no relevant change in ChE activity (Fig. 1) further confirming the above.

3.2. SERT accumulation and effects in C. maenas

No mortality was recorded during the exposure experiments. Salinity, temperature, pH and dissolved oxygen were stable along the assays, within the expected values (Table S1). SERT concentration in freshly prepared medium of the highest test concentration was 566 ± 49 $\mu g\,L^{-1}$ (mean ± SD). SERT exposure levels will, thus, be indicated as the nominal concentrations hereafter. In old test medium (48 h), the concentration was 49% lower than in the freshly prepared. A residual level of NORS (0.035% of the SERT concentration) was detected. After the seven-day exposure, accumulation of SERT in soft tissues was found in crabs from the Minho estuary exposed to 500 $\mu g\,L^{-1}$ and in those from the Lima exposed to 5 $\mu g\,L^{-1}$ and 500 $\mu g\,L^{-1}$ (Table 1). Tissue levels found in Lima crabs were considerably higher than those measured in Minho crabs. Accumulation of NORS in soft tissues was only observed

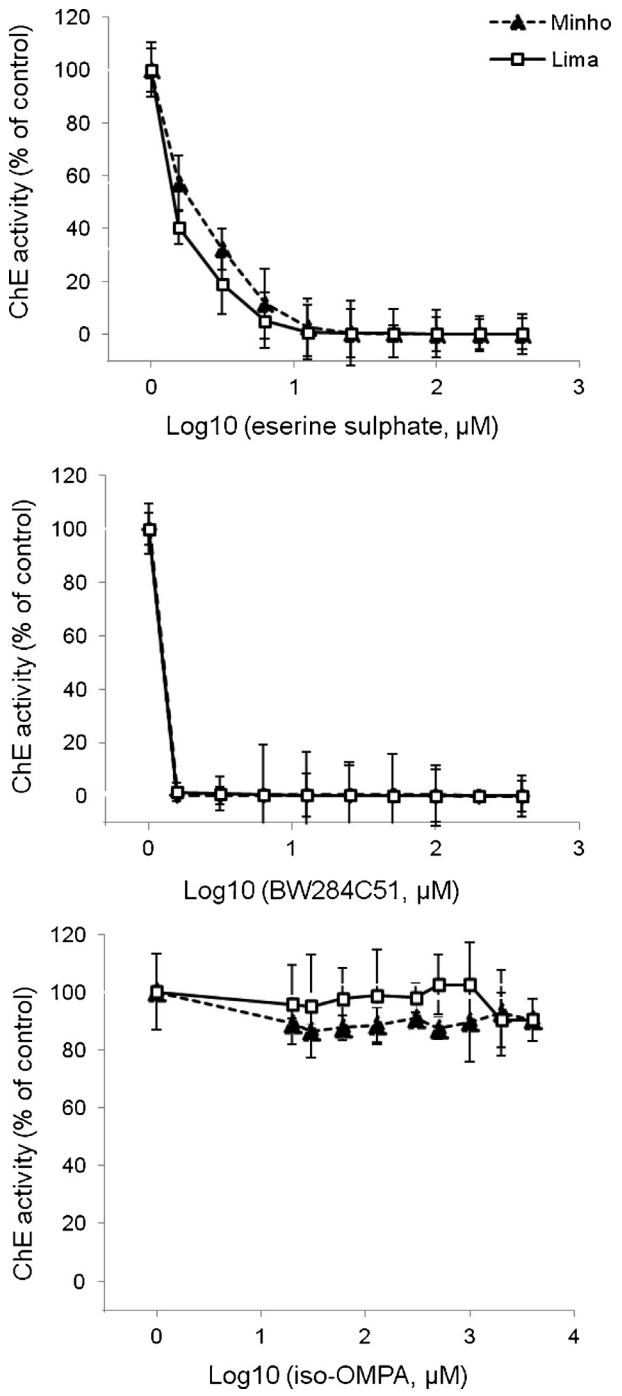

Fig. 1. Effects of specific inhibitors (eserine sulphate, BW284C51, iso-OMPA) on thoracic ganglion cholinesterase (ChE) activity (mean ± SE in % of control) of *C. maenas* from Minho and Lima estuaries. Acetylthiocholine was used as substrate.

in crabs exposed to 500 μg L^{-1} SERT. The levels detected were low (~100 ng g^{-1} ww) but similar between the two populations (Table 1).

Significant differences between control animals from the Minho and the Lima sites were found for CAT, GPx and GR activities (Fig. 2). For CAT, the activity levels were higher in the Minho than in the Lima crabs, whereas for GPx and GR the inverse pattern was found with lower activities measured in Minho crabs. The levels of all the other biomarkers evaluated were similar between the two control groups ($p > 0.05$) (Fig. 2). Two-way ANOVA indicated a significant main effect of SERT on the levels of TG and LDH activity

Table 1
Concentration (mean ± SD, ng g^{-1} ww; $n = 3$) of sertraline and norsertraline found in soft tissues of *C. maenas* collected in Minho and Lima estuaries, after seven-day exposure experiments.

Exposure	Sertraline	Norsertraline
Minho		
Control	n.d.	n.d.
0.05 μg L^{-1}	<1.76[a]	n.d.
5.0 μg L^{-1}	<1.76[a]	n.d.
500 μg L^{-1}	605 ± 18	115 ± 2
Lima		
Control	n.d.	n.d.
0.05 μg L^{-1}	<1.76[a]	n.d.
5.0 μg L^{-1}	15.3 ± 1.7	<1.80[a]
500 μg L^{-1}	1010 ± 10	111 ± 1

n.d., not detected.
[a] Limits of detection.

(Table 2). Significant *SERT* × *Sampling site* interactions were found for all the remaining biomarkers assessed (Table 3), indicating that SERT elicited different responses in Minho and Lima crabs.

In Minho crabs, differences among treatments were related to cholinergic neurotransmission (AChEg activity) and antioxidant defences (GR, GPx and CAT activity) (Fig. 2). A non-monotonic response was found for AChEg with a significant increase (+24%, $p < 0.05$) in activity observed at 5 μg L^{-1}, compared to the control treatment. This suggested a hormetic effect in Minho crabs. Exposure to SERT caused marked increases (1.5- to 4-fold) in the

Table 2
Results of full-factorial two-way ANOVAs performed to assess effects of sertraline exposure and the sampling site on the biomarkers investigated.

Parameter	Source of variation	*df*	*F*	*p*
Neurotransmission				
AChEg	Sertraline	3, 94	8.90	<0.001
	Sampling site	1, 94	33.62	<0.001
	Sertraline × sampling site	3, 94	12.89	<0.001
AChEm	Sertraline	3, 94	4.32	0.007
	Sampling site	1, 94	18.25	<0.001
	Sertraline × sampling site	3, 94	7.72	<0.001
Energy metabolism				
LDH	Sertraline	3, 94	3.62	0.016
	Sampling site	1, 94	0.72	0.789
	Sertraline × sampling site	3, 94	0.24	0.867
IDH	Sertraline	3, 94	8.18	<0.001
	Sampling site	1, 94	35.70	<0.001
	Sertraline × sampling site	3, 94	5.95	0.001
Biotransformation and anti-oxidant defences				
GST	Sertraline	3, 94	0.66	0.578
	Sampling site	1, 94	4.59	0.035
	Sertraline × sampling site	3, 94	3.00	0.035
GR	Sertraline	3, 94	41.73	<0.001
	Sampling site	1, 94	35.49	<0.001
	Sertraline × sampling site	3, 94	47.90	<0.001
GPx	Sertraline	3, 94	14.01	<0.001
	Sampling site	1, 94	14.65	<0.001
	Sertraline × sampling site	3, 94	4.78	0.004
CAT	Sertraline	3, 94	6.84	<0.001
	Sampling site	1, 94	20.32	<0.001
	Sertraline × sampling site	3, 94	2.86	0.042
TG	Sertraline	3, 94	4.14	0.009
	Sampling site	1, 94	0.23	0.635
	Sertraline × sampling site	3, 94	0.76	0.518
Oxidative damage				
LPO	Sertraline	3, 94	5.40	0.002
	Sampling site	1, 94	2.60	0.111
	Sertraline × sampling site	3, 94	2.81	0.044

AChEg, ganglion acetylcholinesterase, AChEm, muscle acetylcholinesterase, LDH, lactate dehydrogenase, IDH, $NADP^+$-dependent isocitrate dehydrogenase, GST, glutathione S-transferases, GR, glutathione reductase, GPx, glutathione peroxidase, CAT, catalase, TG, total glutathione, LPO, lipid peroxidation.

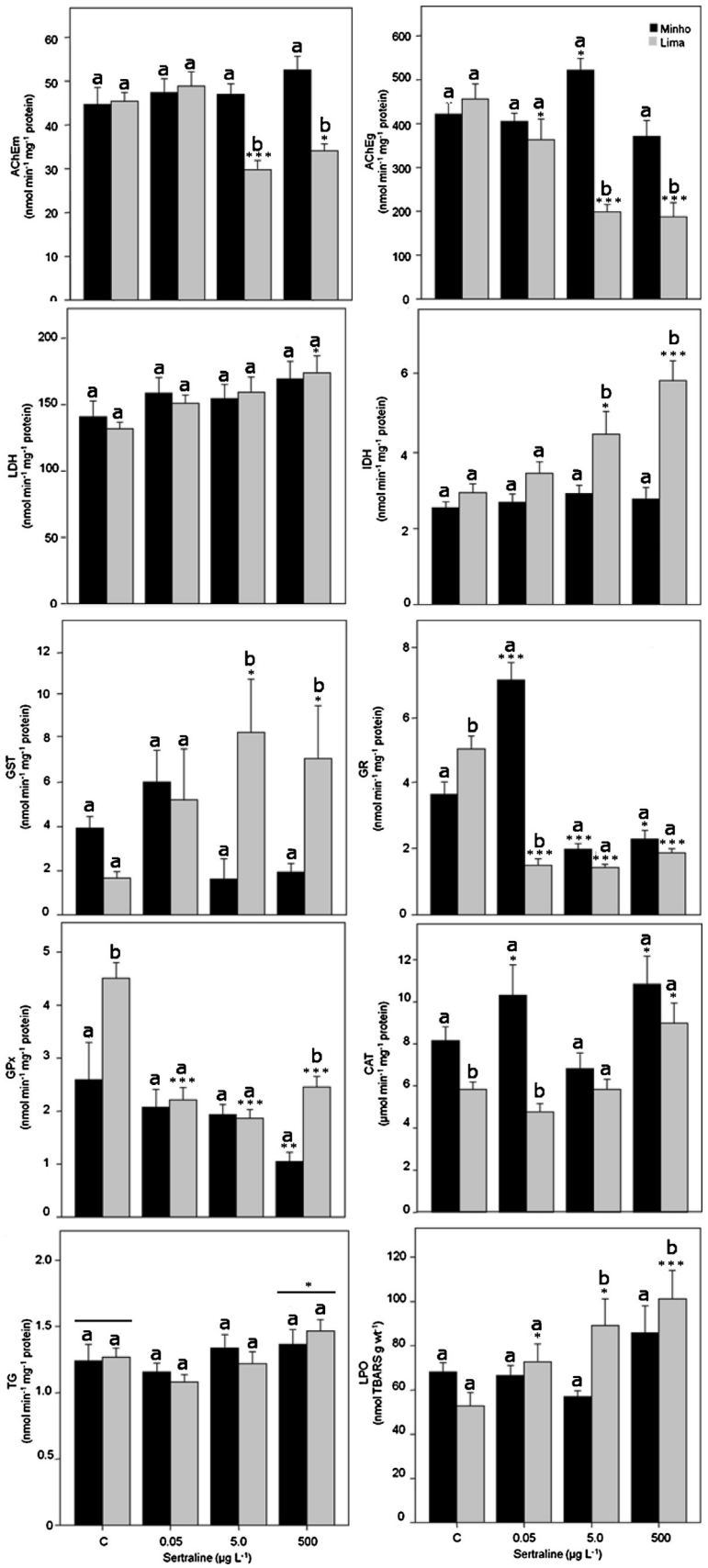

Fig. 2. Levels (mean ± SE) of biomarkers determined in *C. maenas* from the Minho and Lima estuaries exposed to sertraline. Activity of acetylcholinesterase in ganglion (*AChEg*) and muscle (*AChEm*), lactate dehydrogenase (LDH) and $NADP^+$-dependent isocitrate dehydrogenase (IDH) in muscle, glutathione *S*-transferases (GST), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) in digestive gland. Levels of total glutathiones (TG) and lipid peroxidation (LPO) in digestive gland. Differences between sampling sites within each treatment are identified by different letters; asterisks indicate differences within each estuary compared to the respective control group (two-way ANOVA followed by contrast analysis; $^{*}p < 0.05$, $^{***}p < 0.001$).

Table 3
Summary of the biomarker changes observed in crabs exposed to sertraline. The symbols denote increase (↑), decrease (↓) or no alteration (∼) relative to the respective control group.

Parameters	Sampling site					
	Minho			Lima		
	0.05 μg L^{-1}	5.0 μg L^{-1}	500 μg L^{-1}	0.05 μg L^{-1}	5.0 μg L^{-1}	500 μg L^{-1}
SERT in tissues	∼	∼	↑	∼	↑	↑
AChEg	∼	↑	∼	↓	↓	↓
AChEm	∼	∼	∼	∼	↓	↓
LDH	∼	∼	∼	∼	∼	↑
IDH	∼	∼	∼	∼	↑	↑
GST	∼	∼	∼	∼	↑	↑
GR	↑	↓	↓	↓	↓	↓
GPx	∼	∼	↓	↓	↓	↓
CAT	↑	∼	↑	∼	∼	↑
TG	∼	∼	∼	∼	∼	∼
LPO	∼	∼	∼	↑	↑	↑

SERT, sertraline, AChEg, ganglion acetylcholinesterase, AChEm, muscle acetylcholinesterase, LDH, lactate dehydrogenase, IDH, NADP$^+$-dependent isocitrate dehydrogenase, GST, glutathione S-transferases, GR, glutathione reductase, GPx, glutathione peroxidase, CAT, catalase, TG, total glutathione, LPO, lipid peroxidation.

coefficient of variation of GST, compared to the control group, a recognised consequence of exposure to detrimental compounds. GR activity was higher than the control (+95%) in crabs exposed to 0.05 μg L^{-1} and lower than the control in those exposed to 5 μg L^{-1} (−45%) and 500 μg L^{-1} (−37%) ($p < 0.001$). GPx was significantly decreased at 500 μg L^{-1}(−60%, $p < 0.001$), compared to the control group (Fig. 2). In contrast, CAT was significantly increased in animals treated with 0.05 μg L^{-1}(+26%) and 500 μg L^{-1} (+33%).

In Lima crabs, differences relative to the control treatment were found for all the biomarkers assessed, except TG levels (Fig. 2). Compared to controls, AChEg was decreased at all test concentrations (−20%, −56%, −59%, $p < 0.05$), and AChEm was decreased at 5 μg L^{-1} (−34%) and 500 μg L^{-1} (−25%) ($p < 0.05$). Energy metabolism enzymes showed a tendency to increase with the exposure concentration (Fig. 2). Significant differences relative to controls were found at 500 μg–L^{-1} for LDH (+32%, $p < 0.05$) and at 5 μg L^{-1} (+52%) and 500 μg L^{-1} (+100%) for IDH ($p < 0.05$). Increased GST activity, relative to controls, was found at 5 μg L^{-1} (∼5-fold) and 500 μg L^{-1} SERT (∼4-fold) ($p < 0.05$). An opposite trend was observed for GR and GPx activities, which were markedly inhibited in all SERT treatments (GR: −70%, −71%, −63%; GPx: −51%, −59%, −46%; $p < 0.001$). CAT activity was significantly increased but only in crabs exposed to 500 μg L^{-1} (+54%). LPO levels were increased in all SERT treatments (+38%, +69%, +92%, $p < 0.05$) compared to controls, indicating damage to cellular macromolecules.

Overall, after seven days, significant changes in biomarkers of cholinergic transmission in the central nervous tissue and muscle, and antioxidant defences and oxidative damage in the digestive gland were elicited by the lowest SERT exposure levels in crabs originating from the moderately polluted site but not in those from the low impacted site (Table 3). The IBR indicated distinct biological effects in crabs from these sites, relative to the respective control groups, providing a qualitative measure of the stress caused by SERT exposure (Fig. 3). No important differences among SERT treatments were noted in Minho crabs. But in Lima crabs all SERT treatments triggered high stress levels, although much stronger at 5 μg L^{-1} and 500 μg L^{-1}. Distinction among test treatments was provided essentially by the activities of AChEg, AChEm, IDH and GST.

4. Discussion

SERT is highly prescribed worldwide and the most acutely toxic SSRI to standard crustacean [12,13]. However, there is a lack of information on its effects in estuarine and coastal species. In this study, the seven-day exposure to waterborne SERT elicited tissue accumulation in *Carcinus maenas* from both study sites, but clearly higher (almost the double) in those from the moderately polluted Lima site. Low NORS/SERT ratios were found in crabs from both sites (0.19 in Minho and 0.11 in Lima crabs), supporting the hypothesis of low metabolisation and/or elimination by the green crab. Information related to the metabolisation pathway of SERT is limited. The available data resulted from studies with suspensions of mammalian liver microsomes, which demonstrated that SERT was N-demethylated to NORS by cytochrome P450 (CYP) enzymes [55]. In aquatic invertebrates the levels of total CYP are substantially lower than those found in vertebrates [56]. This suggests that low metabolisation of SERT is to be expected in invertebrates compared to vertebrate species, a hypothesis that appears to be supported by our findings. The very low NORS/SERT ratio (0.00035) found in the medium of the highest test concentration after 48 h exposure further supports this. Other studies detected concentrations of NORS in fish tissues greater than those of the parent compound [4,24], though in freshwater species. It is also of note that SERT is expected to undergo oxidative metabolism as its major elimination pathway [57]. Hence, dysfunction of the digestive gland could impair its elimination, which may also explain the higher tissue concentrations found in Lima crabs. Animals from this site exposed to levels causing bioaccumulation also showed significantly reduced antioxidant defences and high extent of oxidative damage to lipid macromolecules, both particularly cytotoxic, with multiple effects on enzyme activity and ATP production, as well as the initiation of apoptosis [58]. Because the potential of SERT to inhibit the reuptake of 5-HT is higher than that exhibited by NORS [57], these results suggest that its effects in decapods may be more severe than those induced in vertebrate species, which usually show much higher NORS/SERT ratios. Nevertheless, this data indicate potential for bioaccumulation in *C. maenas*.

SERT levels detected in *C. maenas* tissues were considerably higher than those reported for mussel *Geukensia demissa* (0.1 ng g^{-1} to 1.4 ng g^{-1} ww) in San Francisco Bay [17]. Such measurements were, however, obtained under conditions favouring extensive dilution, as the winter season and the relatively well-flushed system of San Francisco Bay [17]. Also, previous studies have shown that adverse effects of SSRIs such as sertraline and fluoxetine were more pronounced when the exposure took place at higher pH, in the 8.5–9 range [11,59], possibly due to a greater bioconcentration factor at increased pH [59]. Future studies addressing this issue would help to further clarify sertraline bioconcentration processes in this estuarine invertebrate and possible risk to higher trophic levels.

Different biomarker response patterns were observed in Minho and Lima crabs with non-monotonic responses elicited in Minho crabs. Globally, the pattern of variation of control groups is in good agreement with previous observations in crabs from these

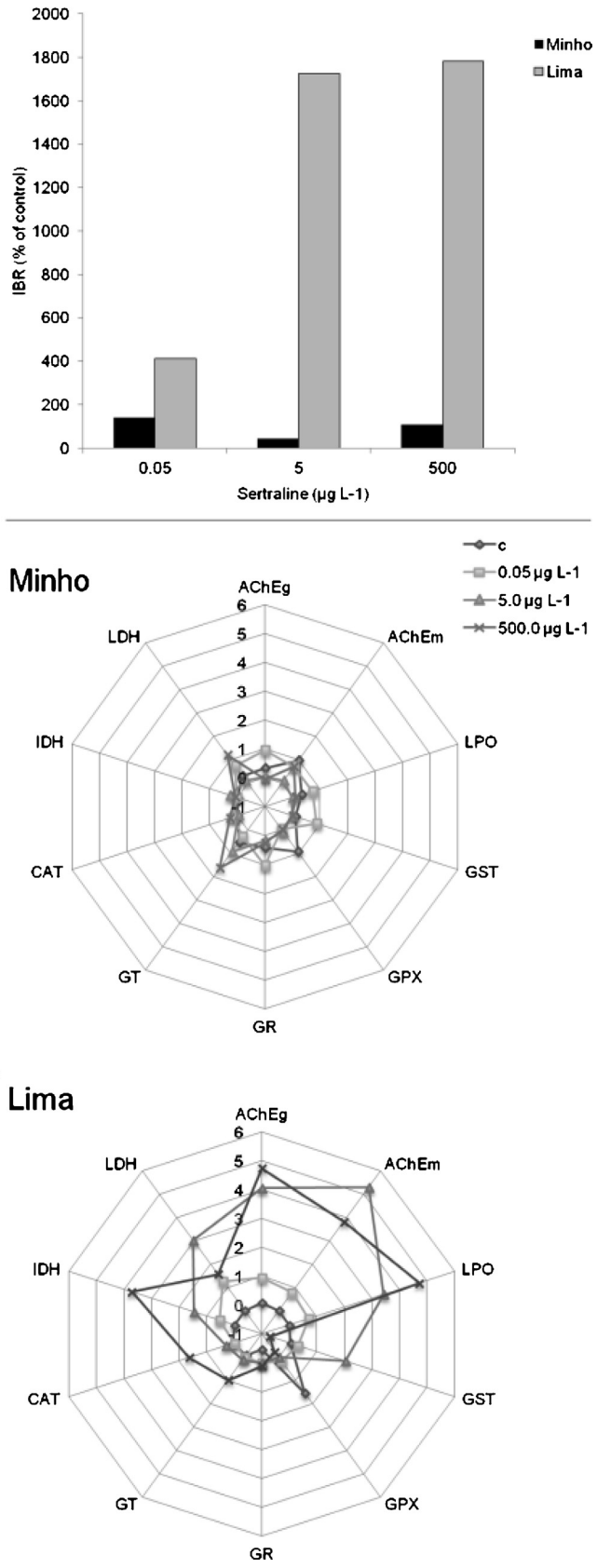

Fig. 3. Integrated Biomarker Response (IBR) index (in % of control) calculated with the biomarkers evaluated in *C. maenas* from the Minho and Lima estuaries exposed to sertraline (top). Contribution of each biomarker to the IBR value and the discrimination among treatments in each sampling site (star plots). Legend as provided in Fig. 2.

sites [31]. The biphasic U-shaped AChEg response of Minho crabs, contrasting with the enzymatic decrease observed in Lima organisms, raised the question of whether these differences could be attributed to different ChE forms in the thoracic ganglion of these populations. Previous investigations indicated the presence of true ChE and ATCh affinity in *C. maenas* ganglion, but possible relevant presence of BChE remained to elucidate [47]. Here, ganglion ChE characterisation confirmed AChE as the predominant form in both populations. Moreover, this form showed no response to iso-OMPA, a BChE inhibitor, further supporting our conclusion. Similar findings were reported for the central nervous system of other marine crustacean [60]. Given this, the consideration of a hormetic effect of SERT on the enzyme activity seems more plausible.

Hormetic dose–response curves, with typical occurrence of opposite effects at low and high doses, were described for a large number of chemicals and physical agents, including some pharmaceuticals and neurotransmitters; responses with a 10- to 100-fold stimulatory range were reported [61]. Moreover, hormetic responses were found also in high-risk or more sensitive individuals, as the Lima crabs appear to be. In such cases the biphasic concentration–response relationship becomes shifted to the left [62]. Considering this, AChEg responses of Lima crabs appear to be already on the inhibitory side of the U-shape concentration–response relationship, whereas those of Minho crabs are still on the stimulatory phase. The pattern of AChEm is also consistent with this, further supporting that Lima crabs were more sensitive to SERT than Minho ones. Increases in AChE activity, associated with altered locomotor behaviour, and biphasic responses in behavioural traits, were observed in other marine crustacean exposed to fluoxetine [29,63]. Interestingly, it was shown recently that serotonergic neurons, and 5-HT signalling, regulate cholinergic neurotransmission in *C. elegans* through both stimulatory and inhibitory inputs [35]. While no explanation is available for the paradoxical observations in crustacean, it may be hypothesised that SERT would affect both stimulatory and inhibitory responses through differential affinity to receptor subtypes, or differential activation of the same receptor in different neurons, that may lead to dual stimulatory or inhibitory inputs to cholinergic neurotransmission [35], consequently affecting AChE activity. Noteworthy is also the possibility of AChE inhibition by SERT [37], which could contribute to the decreased activity found. SSRIs were shown to inhibit AChE activity in human serum and erythrocyte membrane. This interaction is labile [37], suggesting that upon continuous exposure strong inhibition of AChE activity may occur but recovery may be possible following remediation measures. Nevertheless, both up- and down-regulations of AChE activity may have serious detrimental consequences to the crabs. Increased AChE expression and activity is a hallmark of cells undergoing apoptosis [64]. On the other hand, both lower cholinergic transmission and/or AChE inhibition will depress crabs ventilatory and locomotory functions [38,39], setting difficulty to find food and increasing risk of predation.

The increase in LDH (at 500 $\mu g\,L^{-1}$ SERT) observed in Lima crabs could provide additional energy to readily cope with the chemical stress induced by the exposure [65]. However, not only moderate changes were observed as the anaerobic pathway appears to be relevant only at very high concentrations, with concomitant SERT accumulation in tissues. The intense IDH increase (at 5 $\mu g\,L^{-1}$ and 500 $\mu g\,L^{-1}$) found in Lima crabs appears to be a more important mechanism to meet the energy requirements imposed by SERT exposure. IDH is considered as the most efficient pathway in ATP production. It is also active in the antioxidant defence system by supplying the NADPH necessary to GR-mediated regeneration of GSH [41]. Likewise, previous studies have found that Lima crabs seem to cope with toxicant challenge by increasing the aerobic pathway rather than the anaerobic route [28].

GST activity was another biomarker showing non-monotonic responses in the Minho crabs. Moreover, at 5 $\mu g\,L^{-1}$ and 500 $\mu g\,L^{-1}$ inverse effects were observed in Minho (decrease) and in Lima (induction) animals, again suggesting differential sensitivity to SERT. In Minho crabs, the increased levels of CAT and LPO indicate that SERT is able to cause the production of oxyradicals leading to oxidative stress. In animals with a history of exposure to moderate contamination this may not be compensated by the anti-oxidant system resulting in oxidative damage. Conversely, in animals originating from low impacted sites it is possible that alternative detoxification pathways may become active, preventing oxidative damage and possibly leading to reduced contribution of phase II biotransformation to SERT detoxification and elimination. Reports on the effects of SERT in detoxification and antioxidant defences of aquatic organisms are not available in the literature. Previous investigations on the effects of fluoxetine on *C. maenas* showed that, besides increasing locomotion, this SSRI could induce AChE, GST and GR activities and TG levels in Minho crabs at high exposure levels (120–750 $\mu g\,L^{-1}$) [29], contrasting with the lower exposure levels and effects found in the present study. Also in this case no oxidative damage was found in the exposed animals.

The results indicate that crabs from the moderately contaminated site are more susceptible to SERT, showing considerably lower health status than those from the low impacted site. Previous investigations supports that chronic exposure of *C. maenas* to moderate levels of contamination in this estuary may elicit differential sensitivity to further environmental contamination and natural stress [28,31]. The integration of biomarker responses (IBR) revealed that SERT caused low stress in Minho crabs with no clear differences among exposure levels. Lima crabs showed remarkable stress increases even at concentrations as low as 0.05 $\mu g\,L^{-1}$ SERT (over 200%), compared to controls. Biomarkers linked to ventilatory, locomotory and anti-oxidant functions, and oxidative damage, were the most affected by SERT. Also, reduced health status was observed at concentrations not causing relevant SERT accumulation in crab tissues. Biomarkers provide crucial early-warning measures of bioavailability and effects caused by environmental disturbance that may reflect at population levels [28]. These results highlight the suitability of biomarkers involved in cholinergic neurotransmission, detoxification, anti-oxidant defences and oxidative damage to assess contamination by SSRIs in *C. maenas*.

An important point here is that data employed to derive species sensitivity distributions or predicted no effect concentrations (PNECs) for hazard risk calculations are usually based on bioassays assessing conventional endpoints (*e.g.*, survival, growth and reproduction), performed with standard species (or clones) or organisms originating from pristine or low-impacted sites. The present results suggest that estimates derived from such procedures may not be sufficiently protective, as detrimental effects may go undetected using such an ecotoxicological approach. This study demonstrates the occurrence of changes with ecologically relevant repercussions in crabs from the moderately impacted estuary at SERT concentrations four orders of magnitude lower than those observed for crabs from the low impacted site. Such life threatening changes would be missed if regular testing approaches would be used. Moreover, impaired health status was found for crabs from the moderately polluted site at concentrations about three orders of magnitude lower than persistent changes found in the most sensitive freshwater species tested up-to-date using reproduction as endpoint [12]. Concerns are further deepened by the fact that additive effects to algae and aquatic invertebrates may be caused by mixtures of SERT with other SSRIs [13] frequently found in environmental samples (*e.g.*, fluoxetine, citalopram). This work supports that more sensitive MOA-related endpoints should be used to assess SSRIs toxicity [10,23], at broad concentration ranges to encompass the possibility of different responses at low and high exposure levels [61]. The results also stress the importance of tailored risk assessment, involving testing with site-specific criteria and ecological receptors, and accounting for dynamic natural and man-induced environmental change, to improve accuracy in extrapolation from laboratory testing to field conditions.

Acknowledgements

This work was supported by national funds, through FCT/MCTES (PIDDAC), and co-funded by the European Regional Development Fund (ERDF) through the COMPETE – Operational Competitiveness Programme, under the projects CRABTHEMES (PTDC/MAR/71143/2006 and FCOMP-01-0124-FEDER-007383), PSYCHOBASS (TDC/AAG MAA/2405/2012 and FCOMP 01 0124 FEDER 027808) and "PEst-C/MAR/LA0015/2013", "PEst-C/EQB/LA0006/2013". The work was partially funded by the Project ECORISK (reference NORTE-07-0124-FEDER-000054), co-financed by the North Portugal Regional Operational Programme (ON.2 – O Novo Norte), under the National Strategic Reference Framework (NSRF). A.P. Rodrigues was supported by a PhD training grant from FCT (SFRH/BD/65456/2009).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2014.08.035.

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