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Comparison of the sensitivity of seven marine and freshwater bioassays as regards antidepressant toxicity assessment

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Abstract The hazards linked to pharmaceutical residues like antidepressants are currently a major concern of ecotoxicology because they may have adverse effects on non-target aquatic organisms. Our study assesses the ecotoxicity of three antidepressants (fluoxetine, sertraline and clomipramine) using a battery of marine and freshwater species representing different trophic levels, and compares the bioassay sensitivity levels. We selected the following bioassays: the algal growth inhibition test (*Skeletonema marinoi* and *Pseudokirchneriella subcapitata*), the microcrustacean immobilization test (*Artemia salina* and *Daphnia magna*), development and adult survival tests on *Hydra attenuata*, embryotoxicity and metamorphosis tests on *Crassostrea gigas*, and in vitro assays on primary cultures of *Haliotis tuberculata* hemocytes. The results showed high inter-species variability in EC_{50}-values ranging from 43 to 15,600 µg/L for fluoxetine, from 67 to 4,400 µg/L for sertraline, and from 4.70 µg/L to more than 100,000 µg/L for clomipramine. Algae (*S. marinoi* and *P. subcapitata*) and the embryo–larval stages of the oyster *C. gigas* were the most sensitive taxa. This raises an issue due to their ecological and/or economic importance. The marine crustacean *A. salina* was the least sensitive species. This difference in sensitivity between bioassays highlights the importance of using a test battery.

Keywords Antidepressant · Test battery · Marine ecotoxicity · Freshwater ecotoxicity · Sensitivity comparison

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Introduction

Over the last two decades, the presence of pharmaceutical compounds in aquatic environments has been a well-known fact that has forced the scientific community to consider this type of contamination as a potential issue that deserves attention (Kümmerer 2001; Santos et al. 2010). However, these chemicals known as "emerging contaminants" do not have usually regulatory status. Yet, antidepressant class should draw particular attention. These drugs act by blocking the reuptake of mainly serotonin and norepinephrine in the nerve synapses; as a result, the effective concentration of these neuromediators increases in the intrasynaptic space and stimulates serotoninergic and noradrenergic neurons. This effect is used to treat clinical depression, but also other psychiatric disorders and anxiety (e.g. Pélissolo et al. 1996). Concern has been voiced due to the increasing prescription to treat mental disorders. For example, in France the prescription of antidepressants grew

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sevenfold between 1980 and 2001 (Amar and Balsan 2004). In 2001, 6 % of the French population was medicated with antidepressants (Gasquet et al. 2005) and the most consumed molecules were fluoxetine, paroxetine, amitriptyline, clomipramine, and venlafaxine (Olié et al. 2002; ANSM 2012). The increasing consumption of drugs is likely to result in the presence of higher concentrations in the environment, and to a more likely occurrence of toxic effects on aquatic organisms (reviewed in Fent et al. 2006; Ankley et al. 2007; Minagh et al. 2009). Some neurotransmitters on which antidepressants act and their transporters are common to different species (Caveney et al. 2006), so we can expect physiological effects on invertebrates. Some antidepressants have been detected at concentrations of a few ng/L in freshwater systems and also coastal waters. For example, amitriptyline concentrations ranged between 1.4 and 6.0 ng/L in the Hérault watershed (France) and reached approximately 10 ng/L in Mediterranean coastal waters (Togola and Budzinski 2008). In the Great Lakes, the concentrations of fluoxetine ranged between 3.5 and 62 ng/L in Lake Michigan (Blair et al. 2013) and venlafaxine between 0.14 and 15.8 ng/L in Lake Ontario (Li et al. 2010). Most of the toxicity studies on aquatic organisms reports adverse effects of antidepressants (e.g. Brooks et al. 2003b; Fent et al. 2006), even at realistic environmental concentrations (De Lange et al. 2006; Painter et al. 2009; Di Poi et al. 2013a). However, the interactions between antidepressants and non-target organisms are still poorly understood. This leads to a growing demand for toxicity data in aquatic ecosystems, especially in coastal environments.

Aquatic pollution has been traditionally documented in terms of chemical concentrations of contaminants; however, these measurements remain insufficient to evaluate the deleterious effects of emerging contaminants on organisms. These analyses should be complemented with biological criteria from bioassays to design a more comprehensive approach to aquatic pollution assessment (Chapman and Long 1983). Acute toxicity tests are routinely used to evaluate the quality of waters in coastal areas subjected to anthropogenic effects and to assess the toxicity of pollutants such as heavy metals, polyaromatic hydrocarbons or pesticides (e.g. Almeida et al. 2012; Mai et al. 2012; Bao et al. 2013; Mottier et al. 2013), and now pharmaceutical toxicity (e.g. reviewed in Fent et al. 2006; Di Poi et al. 2013b; Minguez et al. 2014a, b). These routine tests are simple, relatively inexpensive and rapid methods. They can be used to compare the sensitivity of various bioassays to chemical pollutants. More and more pharmaceutical toxicity assessment results are published in standardized freshwater organisms (e.g. Minagh et al. 2009; Minguez et al. 2014b) but data are globally missing for marine organisms. In addition, studies mainly focus on only one species but works assessing their toxicity on several organisms are scarce (Brooks et al. 2003b; Johnson et al. 2007; Minagh et al. 2009). However, multispecies approaches would be more environmentally relevant and thus lead to the current study which used a battery of seven freshwater and marine organisms to test the toxicity of three antidepressants.

A battery of bioassays using different species representative of the ecosystem under focus is necessary to obtain relevant information on potential ecological risks in polluted environments. Species selected for such a battery of tests should differ taxonomically and play different roles in ecosystems, have different routes of exposure, and be easily available and cultured in the laboratory. As a result, well-known lab-cultured organisms that are also used for standardized toxicity tests are usually selected. The test species used in our study were chosen to represent marine and freshwater ecosystems at different trophic levels. Up to now, no studies has compared the sensitivity of toxicity tests performed on freshwater and marine species. This kind of information can be useful for risk assessment in marine waters. Indeed, right now an additional assessment factor is added when no marine data are available, without really knowing if marine organisms are more or less sensitive than freshwater ones (Marchand and Tissier 2005). The representative species of the marine ecosystems were as follows: (1) the diatom *Skeletonema marinoi* is an important primary producer in the North Adriatic and Mediterranean seas and a valuable food source for filter organisms including zooplankton and bivalves (Godhe et al. 2006); (2) the crustacean *Artemia salina*, more often found in brackish waters, is of major importance in the aquaculture industry and has been proposed as an uniform world-wide test system for toxicity assessment of chemical substances (Persoone and Wells 1987); (3) the Pacific oyster *Crassostrea gigas* is a leading aquaculture species at the worldwide level. Owing to its filter-feeding mode of life and its high sensitivity to a large range of pollutants, it has been widely used as a sentinel organism in ecotoxicological studies, especially its early life stages (e.g. Di Poi et al. 2013b; Mottier et al. 2013). We also investigated cytotoxicity in (4) hemocytes of the marine gastropod *Haliotis tuberculata* (European abalone). Abalones are sensitive indicators of coastal pollution events (Gorski and Nugegoda 2006; Lin and Liao 1999), and in vitro primary cultures of hemocytes are considered as an useful screening tool for characterizing the risk and impact of pollutants such as pharmaceuticals (Minguez et al. 2014b). In addition, we also studied the responses of three freshwater organisms: (1) the green alga *Pseudokirchneriella subcapitata* is an important primary producer frequently used in ecotoxicological tests (Blaise and Vasseur 2005); (2) the crustacean *Daphnia magna* commonly known as the water

flea, can be found in almost any permanent body of freshwater (Tatarazako and Oda 2007). It is commonly used as a test organism in aquatic toxicology (acute and chronic toxicity assays). It occupies a key position in the aquatic food web by linking primary and secondary productions (a highly abundant grazer of phytoplankton and a major dietary component of fish and invertebrate predators) (Dodson and Hanazato 1995; Tatarazako and Oda 2007; Miner et al. 2012). *Daphnia magna* is suitable as a zooplankton model organism (Dodson and Hanazato 1995) like its marine equivalent *A. salina*; finally, (3) the cnidarian *Hydra attenuata* is ubiquitous in freshwater environments and can regenerate itself. *Hydra attenuata* has already been used in the assessment of the teratogenic potential of several chemical substances (reviewed by Pascoe et al. 2003).

This work reports about the toxicity of three antidepressants common to all partners of the Pharm@ecotox project. We tested these compounds separately by using several acute bioassays and test species representing different trophic levels in aquatic ecosystems. The objectives were (1) to compare the sensitivity levels of seven bioassays and to identify the most sensitive ones to be used in a battery for preliminary toxicity screening of antidepressants; and (2) to compare the toxicity of three antidepressants that belong to two main families of antidepressants: fluoxetine and sertraline are selective serotonin re-uptake inhibitors (SSRIs), and clomipramine is a serotonin-norepinephrine reuptake inhibitor (SNRI).

Materials and methods

The antidepressants we used for the bioassays were fluoxetine HCl, sertraline HCl and clomipramine HCl; all three were supplied in analytical grade by Kemprotec Limited® (Maltby, Middlsbrough, U.K.).

Algal growth inhibition assays

Freshwater algal tests were conducted following the NF EN ISO 8692 guideline (2012) using the microalga *P. subcapitata* AC152 obtained from Algobank (Caen, France). Marine algal tests were performed following the NF EN ISO 10253 guideline (2006) using the diatom *S. marinoi* AC174 provided by Algobank (Caen, France). All algal growth inhibition tests were conducted at 20 ± 1 °C with continuous shaking at 100 rpm and continuous white light (4,000 lux). The toxicity tests were performed in 96-well cell culture plates. Each substance, the medium and the algal inoculums were mixed to obtain an initial algal concentration of 10^4 cells/mL in 0.21 mL of bioassay volume. At least three replicates were used per concentration. Cell density was measured after 72 h of exposure. The results were quantified as average growth rates calculated from cell numbers based on chlorophyll fluorescence measurements (680 nm, TECAN Infinite® M200 microplate reader). The percentages of inhibition of average specific growth relative to controls were calculated for each concentration. All the controls met the acceptability criteria, i.e. an algal growth factor >32 after 72 h from the initial concentration and potassium dichromate toxicity between 0.24 and 1.03 mg/L.

Microcrustacean immobilization assays

Daphnia magna acute immobilization test

Acute tests were conducted following the NF EN ISO 6341 guideline (1996) using the water flea *D. magna* Straus (cladocera, crustacea) obtained from the Institut National de l'Environnement Industriel et des Risques (INERIS, Verneuil-en Halatte, France). Neonates less than 24 h old were used for the tests.

Artemia salina acute immobilization test

Acute tests were performed using the brine shrimp *A. salina*. Brine shrimp eggs were supplied by JBL GmbH & Co.KG (Neuhofen, Germany). Nauplii were used within 48 h of hatching.

Both *Daphnia* and *Artemia* experiments were run in 10 mL-culture volume at 20 ± 1 °C under a 16:8 h light:dark regime. Twenty-five individuals were used for the controls and each treatment was subdivided into five replicates containing five individuals each. Immobility (i.e. no movement following shaking of the test pill bottles) was observed after 24 and 48 h with the latter being the endpoint for effect calculation. All the conditions for test validation were met (e.g. no more than 10 % mortality in the controls and no more than 20 % loss of compounds).

Adult survival and developmental toxicity on the cnidarian *Hydra attenuata*

Hydra attenuata, obtained from Environnement Canada (Centre Saint-Laurent, Montréal), were cultured in *Hydra* medium at 20 ± 1 °C under 16:8 h light:dark regime (800 lux) using glass dishes and following the procedure adapted from Trottier et al. (1997). Tests were conducted on *Hydra* sampled within 48 h after feeding and selected at random from stock cultures. Adult survival tests were carried out on non-budding *Hydra* polyps of similar size and calculated as an endpoint.

Developmental toxicity levels were assessed on gastric sections of *Hydra* by studying their capacity to regenerate

an entire organism. The gastric part is composed of undifferentiated cells. It was isolated from the differentiated tissues of the head and foot by dissecting the section above a bud and below the head (Pachura-Bouchet et al. 2006). Adults or gastric sections were then placed into each well of a 12-well plate containing 3 mL of exposure medium. All conditions were conducted in triplicate. The polyps were not fed throughout the test (96 h); they were exposed continuously to each chemical and monitored every 24 h. The morphological changes or the degree of regeneration were recorded and regeneration was scored using Wilby's classification (1988). A score of five or less corresponds to lethality (Pachura-Bouchet et al. 2006).

Embryotoxicity and metamorphosis assays on the Pacific oyster *Crassostrea gigas*

Embryotoxicity bioassay

Acute tests were conducted following the standardized AFNOR XP-T-90-382 procedure (2009). Spawning was induced in conditioned oyster genitors provided by a commercial hatchery (Guernsey Sea Farm Ltd., Guernsey, UK), and the eggs were fertilized with sperm at a 1:6 (egg:sperm) ratio. Twenty minutes post-fertilization, the embryos were distributed into glass pillboxes containing 25 mL of filtered, sterilized natural seawater (FSNS) (0.22 µm). The oysters were exposed to the three antidepressants for 36 h at 22 ± 1 °C. After observation under an inverted binocular microscope, the percentage of normal development (i.e. number of normal D-shaped larvae vs. number of embryos and abnormal larvae) was calculated.

Metamorphosis bioassay

Twenty-one day-old pediveliger larvae were provided by SATMAR (Société ATlantique de MARiculture) hatchery (Barfleur, France) and a minimum of 50 individuals were exposed in 12-well plates in a final volume of 1.5 mL of FSNS. Epinephrine at 10^{-4} M (Sigma Aldrich®, St Louis, MO, USA) was added to promote larval settlement and metamorphosis (Coon and Bonar, 1987). Exposure to the antidepressants was conducted for 24 h at 22 °C. The metamorphosis rate (%) was assessed by counting the number of metamorphosed larvae versus the total number of dead larvae and non-metamorphosed larvae.

Cytotoxicity on primary cultures of abalone hemocytes, *Haliotis tuberculata* (gastropoda)

Adult abalones were supplied by Ormasub® (Cherbourg, France) and maintained in natural seawater at the Centre de Recherche en Environnement Côtier (C.R.E.C, Luc-sur-Mer, Basse-Normandie, France) until use for ecotoxicity tests on their hemocytes. Hemocytes were cultured as previously described by Lebel et al. (1996). The cells were exposed for 48 h and the medium was changed every day. Each concentration was tested in quadruplicate (i.e. four wells per concentration) and cell exposure was repeated at least three times using at least three abalones (experiment replicates).

MTT assay

Cellular viability was estimated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The test was adapted to molluscan cell cultures by Domart-Coulon et al. (2000). A total of 5×10^5 cells per well was used. Briefly, 10 % (v/v) of the MTT stock solution (5 mg MTT/mL PBS 1X) was added to the culture dishes. After 24 h of incubation at 17 °C, an equal volume of isopropanol containing 0.04 N HCl was added to each culture. The absorbance was measured at a wavelength of 570 nm with a 630 nm reference.

Lysosomal membrane stability (LMS)

After exposure, lysosomal membrane stability was assessed using the neutral red (NR) uptake assay following the method of Coles et al. (1995) and adapted to microplate cultures. A total of 3×10^5 cells per well was used for the assay. Briefly, 10 % (v/v) of the NR stock solution (0.5 % NR in PBS 1X) was added to each well. After 1 h of incubation at 17 °C, the medium was removed, and the wells were washed first with 3 % formaldehyde in artificial sterile seawater and then twice with PBS 1X. NR was extracted from lysosomes using 1 % glacial acetic acid in 50 % ethanol. After 30 min at room temperature, the plates were transferred to a TECAN Infinite® M200 microplate reader, and absorbance was measured at a wavelength of 540 nm with a 650 nm reference.

Statistical analyses

Dose–response modeling

We used non-linear regressions using the Hill equation to calculate Effective Concentrations (EC_{10}, EC_{20}, EC_{50}) based on each endpoint sub-cited. These regressions were obtained using Excel® macro REGTOX (Vindimian 2012). We took regression-based point estimates EC_{10} and EC_{20} as equivalent to No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC), respectively, as suggested in OECD guidelines (OECD 201 2002). The use of NOEC and LOEC data in ecotoxicology has been severely criticized because these

values are highly dependent on test design (e.g. the selected concentrations of chemicals used in toxicity tests and data variability) (Warne and van Dam 2008). On the contrary, EC_x are more consistent, more reliable, and less variable estimates (Chapman et al. 1996).

Relationship between exposure time and observed toxicity

We analyzed the relationship between exposure time and EC_{50} in each bioassay using Spearman correlation test.

Species sensitivity distribution modeling

SSD curves were fitted with Excel® macro "SSD generator V1" (US EPA 2005) using the acute EC_{50}-values derived from the dose–response curves. Using the SSD model, one can demonstrate variations in species sensitivity using data based on single-species bioassays. From a regulatory point of view, this model is used to calculate a chemical concentration protective of most species in the environment. In the present study, we used SSD to illustrate differences in antidepressant toxicity levels and differences between bioassay sensitivity levels, so all the organisms were used to build the models. We used the geometric mean of EC_{50}-values not to over-represent the organisms for which two endpoints were assessed, i.e. *C. gigas*, *H. tuberculata* and *H. attenuata.* One SSD curve was drawn for each antidepressant. From each SSD curve, slope and the Hazardous Concentration HC_{50} were numerically derived. HC_{50}-values correspond to the antidepressant concentrations that affect 50 % of the species in an assemblage. The SSD curve of each antidepressant was fitted using log-logistic.

Mean rank sensitivity

To compare the mean sensitivity to antidepressants of the seven selected bioassays, we ranked the marine and freshwater organisms from the most sensitive (rank 1) to the least sensitive one (rank 7) to each antidepressant based on EC_{50}-values. We then calculated the mean of the three ranks (expressed as mean ± SD). Finally, species were clustered using the Ward method (Ward 1963) on the basis of their sensitivity to antidepressants, after the transformation of toxicity data in a Euclidean distance matrix. These statistical analyses were performed with R software version 2.15.1 (R Development Core Team 2012).

Results

The mean sensitivity ranking of the seven bioassays is presented in Fig. 1. It reflects which bioassay was the most sensitive to all three compounds. Marine and freshwater bioassay sensitivity levels are also compared. The tests exhibited different sensitivity levels. Bioassays using algae *S. marinoi* and *P. subcapitata*, and the early life-stages of the oyster *C. gigas* were particularly sensitive to antidepressants. In vivo tests using *D. magna*, *H. attenuata* and the in vitro test with *H. tuberculata* hemocytes displayed intermediate sensitivity. In contrast, the bioassay using *A. salina* was far less sensitive in the assessment of antidepressant toxicity.

The toxicity of three antidepressants was assessed and presented in Table 1. Even if the exposure time of the different bioassays was different, toxicity values were not correlated with exposure time ($R = 0.216, 0.028, 0.028$ for fluoxetine, sertraline and clomipramine, respectively). The classification of the Commission of the European Communities (1996) considered compounds as very toxic when $EC_{50} < 1$ mg/L, toxic when $1 \text{ mg/L} < EC_{50} < 10$ mg/L, harmful when $10 \text{ mg/L} < EC_{50} < 100$ mg/L, and harmless for aquatic organisms when $EC_{50} > 100$ mg/L. Based on this classification, the three antidepressants were very toxic to the algae *S. marinoi* and *P. subcapitata* and to early stages of *C. gigas*; toxic to the gastropod *H. tuberculata*, the crustacean *D. magna* and the cnidarian *H. attenuata*. For the marine crustacean *A. salina*, the two SSRIs were toxic whereas the SNRI was harmless. The toxicity (EC_{50}) range of fluoxetine was 43–15,600 µg/L (marine organisms) and 200–8,680 µg/L (freshwater organisms). For sertraline it was 67–4,400 µg/L (marine organisms) and 150–1,790 µg/L (freshwater organisms), and for clomipramine: 4.70–4,770 µg/L (marine organisms) and 460–2,950 µg/L (freshwater organisms).

The SSD models for each antidepressant are represented in Fig. 2. The three curves displayed different shapes that can be defined by the Hazardous Concentration HC_{50}, and the slopes. According to the calculated HC_{50}-values, the toxicity ranking of the antidepressants was (from the most to the least toxic compound): sertraline (688.69 µg/L) > clomipramine (1230.61 µg/L) > fluoxetine (1550.46 µg/L). Based on the slopes, sertraline appeared as a more potent toxic than the other two compounds (slope: sertraline = 1.451 vs. clomipramine = 0.714 and fluoxetine = 0.899). Whatever the antidepressant, the most sensitive bioassay was the assessment of growth inhibition of the marine alga *S. marinoi*, and the least sensitive one was the immobilization test of the marine crustacean *A. salina*. The sensitivity rank of the other bioassays depended of the assessed antidepressant.

Discussion

Like other drugs, antidepressants are designed for specific effects in humans, but they also potentially bring about

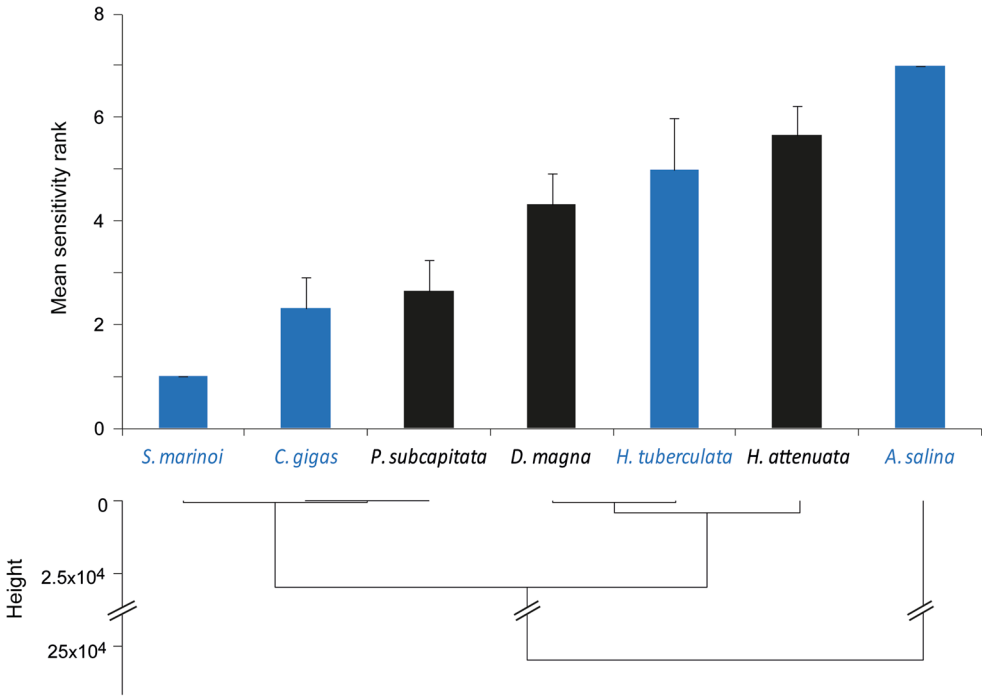

Fig. 1 Mean sensitivity ranks for the 7 bioassays (mean ± SD). Species were clustered on the basis of their mean sensitivity to the three antidepressants (Euclidean distances, Ward method). The height of each node in the cluster dendrogram is proportional to the value of the intergroup similarity. Marine species are represented in *blue* and freshwater species in *black* (Color figure online)

changes in non-target organisms likely to be exposed to those compounds. These compounds are now traced and found in aquatic environments thanks to the improvement of analytical tools and techniques (Daughton and Ternes 1999; Fent et al. 2006). It is important to estimate the concentrations of these compounds that can produce a toxic response in non-target aquatic organisms. Acute toxicity tests are the first step in the risk assessment procedure, and require a battery of taxonomically-different organisms.

Bioassay sensitivity levels in the assessment of antidepressant toxicity

The comparison of the mean sensitivity to antidepressants of the seven bioassays evidenced a sensitivity gradient. The most sensitive tests were those performed on microalgae and *C. gigas* early life-stages. The bioassays using algae are required for all risk assessments of chemical compounds because algae are major players in the functioning of ecosystems due to their role in nutrient cycling and oxygen production. They form the first trophic level of many aquatic food chains; therefore, a toxic effect at their level may cause cascading effects at higher levels. To our knowledge, antidepressant toxicity is not documented for *S. marinoi*, so that the comparison with other studies is not feasible. Toxicity data on *P. subcapitata* are more numerous but quite different from one study to another due to different exposure times. For fluoxetine, we obtained a 72 h-EC_{50} of 200 µg/L. A 96 h-EC_{50} of 44.99 µg/L and 120 h-EC_{50}-values of 24 and 39 µg/L were reported by Johnson et al. (2007) and Brooks et al. (2003a, b), respectively. For sertraline, our result (72 h-EC_{50} = 0.15 mg/L) is identical to those of Minagh et al. (2009) (72 h-EC_{50} = 0.14 mg/L). Toxicity mechanisms could be related to interactions with efflux pumps as suggested by Munoz-Bellido et al. (2000). The early life-stages of the oyster *C. gigas* also appeared very sensitive to antidepressants. Hardege et al. (1997) have shown that a short exposure of zebra mussels during spawning to 10^{-6} M imipramine or desipramine, two tricyclic antidepressants, leads to an abnormal embryonic development. A comparison of the mean EC_{50}-values obtained from available data about other types of pollutants shows that antidepressants are more toxic on embryo–larval development than some herbicide substances (Mottier et al. 2013), but less than some metals (e.g. His et al. 1999; Mai et al. 2012). Metamorphosis is a relevant endpoint to be considered in ecotoxicological studies because bivalves experience essential morphological and anatomical changes during that stage (Baker and Mann, 1994). However this endpoint has rarely been used (His et al. 1997; Di Poi et al. 2013a, b; Mottier et al. 2013). Overall, our three antidepressants had greater effects on metamorphosis than several herbicides in the same bivalve species and using the same bioassay (Mottier et al. 2013).

Among the bioassays displaying intermediate sensitivity to antidepressants was the *D. magna* immobilization test. *Daphnia* sp. plays a significant ecological role in trophic chains as a primary consumer of phytoplankton and as a food source for secondary consumers (Miner et al. 2012).

Table 1 Ecotoxicity data for fluoxetine, sertraline and clomipramine on selected aquatic species and endpoints

Test species	Endpoint	Fluoxetine			Sertraline			Clomipramine		
		EC_{50} (μg/L)	EC_{10} (μg/L)	EC_{20} (μg/L)	EC_{50} (μg/L)	EC_{10} (μg/L)	EC_{20} (μg/L)	EC_{50} (μg/L)	EC_{10} (μg/L)	EC_{20} (μg/L)
Marine species:										
Skeletonema marinoi	72 h growth inhibition	43 [21–71]	5	12	67 [59–86]	35	44	4.70 [2.30–4.80]	4.40	4.60
Artemia salina	48 h immobilization	13,810 [8,510–18,650]	5,730	7,930	4,080 [3,480–4,410]	2.61	3,070	>100,000	nd	nd
Crassostrea gigas	36 h embryotoxicity	192 ± 41	146	161	67 ± 66	44	50	157 ± 41	119	131
Crassostrea gigas	24 h metamorphosis	188 ± 65	108	132	1,099 ± 203	818	910	515 ± 115	233	311
Haliotis tuberculata	48 h MTT	15,600 ± 3,000	4,432	7,504	4,400 ± 1,890	1,806	2,539	4,765 ± 170	695	1,352
Haliotis tuberculata	48 h LMS	2,388 ± 299	1,085	1,451	737 ± 118	287	406	1,318 ± 182	840	992
Freshwater species:										
Pseudokirchneriella subcapitata	72 h growth inhibition	200 [180–210]	90	120	150 [140–160]	70	100	460 [410–490]	140	220
Daphnia magna	48 h immobilization	5,910 [4,420–6,500]	2,960	3,820	1,150 [950–1,280]	710	850	2,740 [1,790–2,910]	1,360	1,760
Hydra attenuata	96 h adult survival	7,940 [7,610–8,410]	6,960	7,300	1,710 [1,620–1,810]	1,460	1,540	2,950 [2,860–2,990]	2,850	2,890
Hydra attenuata	96 h polyp regeneration	8,680 [6,550–10,430]	5,620	6,570	1,790 [1,660–1,950]	1,480	1,590	2,950 [2,910–2,970]	2,850	2,890

EC_{50}: median effective concentration. EC_{10} and EC_{20} are considered as equivalent to no effect (NOEC) and low effect (LOEC) concentration values, respectively. EC_{50}-values are expressed as mean ± SD or with their 95 %-confidence interval between brackets

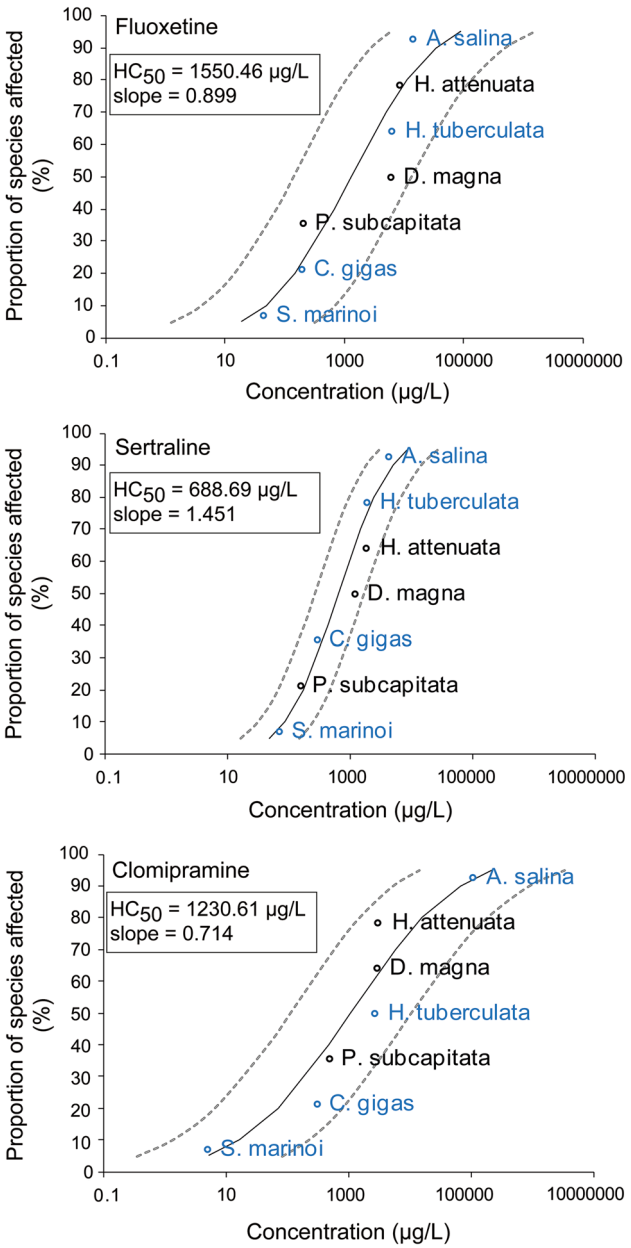

Fig. 2 Species sensitivity distribution curves of the 3 antidepressants and details of species sensitivity ranking. *Plain curves* (log-logistic regressions) are based on acute EC_{50}-values and *broken lines* represent the 95 %-confidence intervals. For *Crassostrea gigas*, *Haliotis tuberculata* and *Hydra attenuata* for which two endpoints were assessed, the geometric mean of the two EC_{50}-values was used to build SSD curves. Marine species are represented in *blue* and freshwater species in *black*. HC_{50}: hazardous concentration for 50 % of species (Color figure online)

Our EC_{50}-values were within the ranges reported by Christensen et al. (2007) and/or Minagh et al. (2009): sertraline EC_{50} = 0.92 or 1.3 mg/L, respectively and fluoxetine EC_{50} = 6.4 mg/L. The cnidarian *H. attenuata* was also moderately sensitive to antidepressants. *Hydra* spp. are known as one of the freshwater invertebrates that are the most sensitive to organic and inorganic compounds after *D. magna* (Beauregard and Ridal 2000). For example, pharmaceutical compounds like diazepam inhibit the polyp regeneration at 10 µg/L (Pascoe et al. 2003).

Artemia salina was weakly sensitive to antidepressants. Other studies have assessed the toxicity of some pharmaceutical compounds (e.g. antibacterial, narcotic, anticonvulsant, etc.) on *A. salina* larvae. They always found EC_{50}-values higher than 100 mg/L, i.e. in the upper limit of the classification of the Commission of the European Communities (1996) indicating harmless compounds (reviewed by Webb 2001). However, it is important to specify that we may not have used the most sensitive age of this organism. Sánchez-Fortún et al. (1997) showed that older shrimps (i.e. from 72 h-old) were more sensitive than younger ones.

In addition to all these in vivo assays, we assessed in vitro sensitivity of abalone hemocytes. Cell culture can offer several advantages over whole animal studies like reduced animal use. Short-term primary cultures from abalone target tissues help in the assessment of marine pollutants (Gaume et al. 2012; Mottin et al. 2010). Among suitable cell types, hemocytes represent an interesting model as they are one of the first lines of defense against foreign particles, like pollutants or pathogens (Auffret and Oubella 1994; Pipe and Coles 1995). In our study, we compared the sensitivity levels of the abalone hemocyte assays with results from in vivo EC_{50} data. The sensitivity of abalone hemocytes was similar to that of *D. magna* or *H. attenuata*. In a previous study we found correlations between in vivo data in *Daphnia* and in vitro data in abalone hemocytes, suggesting similar in vivo and in vitro toxicity mechanisms (Minguez et al. 2014b). Therefore, abalone hemocytes could also play an important role as screening tools as part of a test battery.

Toxicity assessment of three antidepressants

The analysis of our EC_{50}-values revealed that there was considerable variability in the toxicity range for a given substance depending on the bioassay; toxicity ranged from low µg/L to mg/L. The current risk assessment procedures for marine waters propose the use of freshwater ecotoxicity data when marine data are not available. However, to avoid a bias in the extrapolation from freshwater to marine ecosystems, freshwater and marine organisms should have equal sensitivity to the substances to be tested. However, most of the time marine bioassays display higher sensitivity (Sverdrup et al. 2002), as observed in the present study. The reasons why marine tests generally have higher sensitivity than freshwater tests are not known. Thus, risk assessment in marine waters should include marine data, or at least keep in mind that potentially strong differences exist between marine and freshwater results (Sverdrup et al. 2002).

In situ exposure levels are unlikely to produce drastic acute effects on our marine and freshwater organisms. Commonly reported environmental concentrations are in the low ng/L range (Kümmerer 2001; Fent et al. 2006). However, these compounds displayed high acute toxicities on algae or *C. gigas* larvae so that chronic effects due to exposure to more environmentally realistic concentrations should not be excluded. In addition to the use of concentrations higher than those found in the environment, these bioassays assess the effects of pollutants on only one species at a time whereas in the field organisms are in interaction. For example, *D. magna*, due to its trophic position, could also be indirectly affected by antidepressant exposure since algae are particularly sensitive to these compounds. Thus, studies are needed to assess the indirect effects of these compounds related to biotic interactions by assessing multi-trophic level responses in microcosms and mesocosms under more environmentally realistic conditions. Moreover, these compounds are also present in a complex mixture whose toxicity can be greater (e.g. Cleuvers 2003; Christensen et al. 2007). This issue of chemical mixtures should also be considered in future research and in the procedures for risk assessment.

Species sensitivity distribution (SSD) of ecotoxicological data is considered as one of the most effective approaches for ecological risk assessment because it aims at protecting biodiversity (review in Wheeler et al. 2002). Usually, the 95 % protection level is calculated (HC_5; Wheeler et al. 2002). However, for this model the more toxicity data there are the more reliable HC_5 will be. Wheeler et al. (2002) recommend the use of at least ten toxicity data. In our study, we used HC_{50}-values only to illustrate the toxicity patterns of the three antidepressants and sensitivity variations between bioassays, but we did not use them in a regulatory framework. Comparing the three models, sertraline emerged as a more potent toxic than the other two compounds whose SSD curves had gentler slopes. This higher toxicity was also confirmed by its HC_{50} that was half as much as the other two. Sertraline is known as the most acutely toxic antidepressant across all trophic groups (algae, invertebrates, fish and amphibians; reviewed by Brausch et al. 2012).

Conclusion

Our study reports about the ecotoxicity of fluoxetine, sertraline and clomipramine on a battery of bioassays using marine and freshwater organisms. The results show a wide range of variations in EC_{50}-values ranging from low µg/L to mg/L for the three antidepressants. This difference in sensitivity between bioassays highlights the need to use a test battery. In terms of environmental hazard classification (Commission of the European Communities 1996) based on acute toxicity levels, antidepressants can be very toxic to aquatic organisms. These high toxicity levels should be verified with chronic exposure experiments and more environmentally relevant concentrations, because concentrations measured in the field are orders of magnitude lower than those involving acute toxicity. Their impacts on algal species are of particular concern. As primary producers, algae constitute the first level of trophic chains; thus a toxic effect at their level may induce effects at higher levels. The higher sensitivity of the early life stages of the oyster *C. gigas* could also raise an issue because of potentially important ecological and economic consequences. When comparing mean sensitivity to antidepressants bioassay sensitivity levels ranked as follows (from the most sensitive to the least sensitive): *S. marinoi* > *C. gigas* > *P. subcapitata* > *D. magna* > *H. tuberculata* > *H. attenuata* > *A. salina*. Studies of chronic toxicity on a wider range of organisms would be useful to further study the ecotoxicity of antidepressant molecules.

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Conflict of interest The authors declare that they have no conflict of interest.

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