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Assessment of cytotoxic and immunomodulatory properties of four antidepressants on primary cultures of abalone hemocytes (*Haliotis tuberculata*)

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ABSTRACT

Pharmaceutical compounds like antidepressants found in surface waters raise concerns due to their potential toxicity on non-target aquatic organisms. This study aimed at investigating the *in vitro* cytotoxicity and immunomodulatory properties of four common antidepressants, namely Amitriptyline, Clomipramine, Citalopram and Paroxetine, on primary cultures of abalone hemocytes (*Haliotis tuberculata*), after 48 h-exposure. Effects on immunocompetence (phagocytosis, levels of reactive oxygen species, esterase activity and lysosomal membrane destabilization) were assessed. Results obtained by MTT assays revealed that acute toxicity is unlikely to occur in the environment since the LC_{50}s of the four antidepressants are at the mg/L level. The different immunological endpoints displayed a biphasic response, with an increase at the lowest concentration (*i.e.* 1 μg/L) followed by a decrease at higher concentrations. Overall, Amitriptyline and Clomipramine, the two tricyclic antidepressants, had higher immunomodulatory capacities than the two selective serotonin reuptake inhibitors Citalopram and Paroxetine. Amitriptyline was the most potent and Citalopram the least potent drug in altering immune function in *H. tuberculata*.

1. Introduction

Pharmaceutical compounds are a class of emerging contaminants for which increasing demands are made from both scientific community and the mainstream media for information on their potential toxicities in water (Fent et al., 2006; Pascoe et al., 2003; Santos et al., 2010). The environmental risk associated with these compounds is linked with their specific mode of action since they are designed to highly interact with biological systems in order to provide beneficial effects. Although they are mainly used for medical and veterinary purposes, pharmaceutical compounds may be a potential threat to non-target aquatic organisms. Environmental issues may arise when excreted drugs enter water *via* sewage effluent from domestic dwellings and hospitals or from landfill leachates (Fent et al., 2006; Pascoe et al., 2003; Santos et al., 2010). Many recent studies have revealed the presence of measurable concentrations of pharmaceutical compounds in aquatic environments from nanogram to low microgram-per-liter range (Fent et al., 2006; Santos et al., 2010). Aquatic organisms are particularly important targets as they are exposed over their whole life cycle.

Among the most prescribed compounds, the antidepressants raise concern since their presence in aquatic environments has been confirmed in previous studies (review in Minagh et al., 2009). Antidepressants are used to treat clinical depression in humans but also other psychiatric disorders. There are many different families of antidepressants available today. The two most common groups are TriCyclic Antidepressants (TCAs) and Selective Serotonin Reuptake Inhibitors (SSRIs). They act by inhibiting the reuptake of monoamines (*i.e.* serotonin, norepinephrine and dopamine) from the pre-synaptic nerve cleft and increasing extracellular levels of these neurotransmitters (Brooks et al., 2003; Shelton, 2003). TCAs are the oldest class of antidepressants and are still used extensively. However, for the treatment of depressive disorders, SSRIs have replaced TCAs because they are better tolerated and safer (Anderson et al., 2000). Most of the studies about physiological effects of antidepressants have focused on fluoxetine (review in Nentwing, 2007 and Santos et al., 2010; Di Poi et al., 2013) and few have examined the aquatic toxicity of other antidepressants (Fong, 1998; Fong and Hoy, 2012; Fong and Molnar, 2013; Getz et al., 2011; Painter et al., 2009). All these studies reported adverse effects, sometimes even at environmental concentrations, on immunity (Gagné et al., 2006), reproduction, development and

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growth (*e.g.* Brooks et al., 2003; Campos et al., 2012; Fong, 1998; Henry and Black, 2008; Péry et al., 2008; Sánchez-Argüello et al., 2009), as well as various behaviors like prey capture of hybrid striped bass (Gaworecki and Klaine, 2008), predator avoidance behavior of larval fathead minnows (Painter et al., 2009), amphipod geotaxis/phototaxis (Guler and Ford, 2010), foot detachment in marine snails (Fong and Molnar, 2013), and memory processing in cuttlefish (Di Poi et al., 2013).

Acute and chronic toxicities of antidepressants have been assessed mainly by *in vivo* approaches. However, a number of ethical, scientific and economic reasons have led to the development and application of *in vitro* techniques. *In vitro* methods to assess the toxicity of contaminants may be a first step to justify more intensive *in vivo* studies with whole organisms or mesocosms (Gura, 2008; Parolini et al., 2011). The major application of these techniques is the understanding of mechanisms involved in the cellular response to pollutants. Moreover, cells represent a key level of biological organization.

The European abalone *Haliotis tuberculata* is a marine benthic gastropod living in coastal areas along the eastern Atlantic to the west coast of Africa. Abalones are considered sensitive indicator species for coastal pollution (Gorski and Nugegoda, 2006; Lin and Liao, 1999). As shown in marine bivalves (Binelli et al., 2009; Canesi et al., 2007; Matozzo et al., 2011), short-term primary cultures from abalone target tissues would help in the assessment of the biological impact of marine pollutants (Gaume et al., 2012; Mottin et al., 2010). Among suitable cell types, hemocytes represent an interesting model since they are one of the first lines of defense against stressors, like pollutants or pathogens (Auffret et al., 2006; Pipe and Coles, 1995). The immune response mainly involves phagocytosis and is complemented by an array of defense mechanisms which may include lysosomal activity and generation of highly reactive oxygen metabolites (ROS) (Anderson et al., 1995; Wootton and Pipe, 2003). Chemically induced immunological disorders have been well documented in an increasing number of species (reviewed in Auffret et al., 2006; Gust et al., 2010; Latire et al., 2012; Mottin et al., 2010). Such effects are particularly important since they may involve higher susceptibility to infection and associated diseases.

The aim of the present study was to investigate the potential immunotoxicity of four common antidepressant compounds on abalone cultivated hemocytes. These pharmaceutical compounds belong to the two most common antidepressant families, namely Amitriptyline (Ami, Elavil®) and Clomipramine (Clomi, Anafranil®) as TCAs, and Citalopram (Citalo, Celexa®) and Paroxetine (Parox, Paxil®) as SSRIs. They are detected worldwide in water at varied concentrations. For example, Amitriptyline was found at 207 ng/L in effluents of a wastewater treatment plant in South Wales, UK (Kasprzyk-Hordern et al., 2008); Clomipramine at few ng/L in Galicia, Spain (Esteban et al., 2012); Citalopram at 223 ng/L in Canada and up to 840 μg/L in Indian effluent treatment plant near drug manufactures (Lajeunesse et al., 2012; Larsson et al., 2007); Paroxetine from a few ng/L (effluent in Canada) (Lajeunesse et al., 2012) to 2 μg/L (sewage effluent of a Norwegian university hospital) (Kallenborn et al., 2008). The toxicity of these different compounds was assessed on the hemocyte viability, the phagocytosis activity, the production of ROS, the stability of lysosomal membranes and the esterase activity.

2. Materials and methods

2.1. Study organisms

Adult European abalones with shell length between 9 cm and 11 cm were sampled by Ormasub® on the Northern Cotentin peninsula (France). Organisms were maintained in natural and continuously aerated seawater at 17 °C and fed with a mixed algal diet (*Laminaria* sp. and *Palmaria* sp.) at the Centre de Recherche en Environnement Côtier (C.R.E.C., Luc-sur-Mer, Basse-Normandie, France). Prior to their use in our study, abalones were acclimated for at least 2 weeks.

2.2. Primary cell cultures

Hemocytes were cultured as previously described by Lebel et al. (1996). Briefly, after a medio-lateral incision in the abalone foot, the hemolymph was withdrawn using a 20 mL syringe with a 25 G needle. Hemolymph was transferred into a sterile tube and diluted 1:4 in cooled sterile anticoagulant modified Alsever's solution (115 mM glucose; 27 mM sodium citrate; 11.5 mM EDTA; 382 mM NaCl) (Bachère et al., 1988). Cells were rapidly placed into 12-well plates at a density of 0.5×10^6 cells per well with the addition of three volumes of artificial sterile seawater (ASSW), and maintained at 17 °C for 90 min to allow cells to adhere onto the bottom of the culture well. Then, the ASSW was replaced by Hank's sterile 199 medium modified by the addition of 250 mM NaCl, 10 mM KCl, 25 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 10 mM Hepes (final pH 7.4), and supplemented with 2 mM L-glutamine, 100 μg/mL streptomycin, 60 μg/mL penicillin G and 2 mM concanavalin A. Cell cultures were incubated at 17 °C overnight.

2.3. Exposure design

Cells were exposed for 48 h to antidepressant compounds: Paroxetine, Citalopram, Clomipramine or Amitryptiline. These compounds will be referred thereafter as Parox, Citalo, Clomi and Ami, respectively. Experiments were performed in two steps: (1) assessment of antidepressant cytotoxicities and calculation of lethal concentrations (LCs), and (2) study of the immunomodulatory properties of the four compounds on cells exposed to different LCs. Exposure concentrations used during the immunomodulatory property assessment were: LC 10, 20 and 50. Cells were also exposed to a more environmentally relevant concentration, *i.e.* 0.001 mg/L. Each concentration was tested in quadruplicate (*i.e.* four wells per concentration). The medium was changed every day. The cell exposure was repeated at least three times, *i.e.* using at least three abalones (experiment replicates).

2.4. MTT assay

Cellular viability was estimated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay, which is a sensitive and quantitative colorimetric assay measuring the capacity of mitochondrial succinyl dehydrogenase in living cells to convert a yellow substrate (MTT) into a dark blue formazan product (Mosmann, 1983). This test was adapted to molluscan cell cultures by Domart-Coulon et al. (2000). Briefly, 10% (v/v) of the MTT stock solution (5 mg MTT/mL PBS 1X) was added to the culture dishes. After 24 h incubation at 17 °C, an equal volume of isopropanol containing 0.04 N HCl was added to each culture to dissolve the converted formazan. The absorbance was measured at a wavelength of 570 nm with a 630 nm reference.

2.5. Lysosomal membrane stability

After treatments, the lysosomal membrane stability was assessed using neutral red uptake assay (NRU) following the method employed by Coles et al. (1995) and adapted to microplate cultures. Neutral red (NR) dye accumulates in the lysosome compartment of cells, (Lowe and Pipe, 1994). It spreads into cells by membrane diffusion or pinocytosis and an alteration in its uptake

may reflect damage to the plasma membrane and consequent changes in the volume of the lysosome (Lowe and Pipe, 1994). Dead cells or cells with membrane damage cannot accumulate the dye, so during washing steps the dyestuff will not be retained. Wells with dead or damaged cells will be less colored. A total of 3×10^5 cells were used for the assay and was conducted in triplicate. Briefly, 10% (v/v) of the neutral red stock solution (0.5% neutral red in PBS 1X) was added to each well. After 1 h incubation at 17 °C the medium was removed, and wells were washed first with 3% formaldehyde in ASSW to fix cells 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 the intensity of coloration was measured at a wavelength of 540 nm with a 650 nm reference.

2.6. Other immunological endpoints

Three other immunological parameters, *i.e.* phagocytosis, reactive oxygen species (ROS) production and esterase activity were determined using a flow cytometer (Gallios, Beckman Coulter®), as previously described by Mottin et al. (2010) and Latire et al. (2012) on abalone hemocytes, and 10,000 events were counted for each sample. For phagocytosis assays, 7 μL of bead solution (carboxylate-modified FluoSpheres®, yellow-green fluorescence, 1 μm diameter, Molecular Probes) was added to each well. Cells were incubated for 60 min at 17 °C in the dark. The percentage of phagocytic cells was evaluated as the percentage of hemocytes that had engulfed at least three beads (*i.e.* immunoefficiency) (Delaporte et al., 2003). ROS production was measured using 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma, final concentration = 100 μM) (Lambert et al., 2003). Cells were incubated in the dark at 17 °C for 20 min. Then, 6 μL of phorbol 12-myristate 13-acetate (PMA, final concentration = 10 μg/mL) was added to each well, and the cells were incubated for 60 min at 17 °C in the dark. Esterase activity was measured using the non-specific liposoluble substrate fluorescein diacetate (FDA, Invitrogen). The FDA probe was added to each well at a final concentration of 5 μM and cells were incubated for 60 min at 17 °C in the dark. Both ROS production and esterase activity results were expressed as the percentage of cells exhibiting fluorescence.

Following the incubation, the wells were scraped gently, and the samples were centrifuged at 300 × *g* for 10 min at 4 °C. The resulting pellet was mixed with 300 μL of 3% formaldehyde in ASSW. Samples were stored at 4 °C until analyses.

2.7. Data analysis

Non-linear regressions using the Hill equation on cytotoxicity results (MTT tests) allowed us to calculate lethal concentrations (LC_{10}, LC_{20}, LC_{50}). These regressions were obtained using the Excel® macro REGTOX (Vindimian, 2012). Results are expressed as means + standard deviations (S.D.). Statistical analyses were undertaken with R software version 2.15.1 (R Core Team, 2012), and performed on values resulting from quadruplicate wells containing cells repeated at least three times. Data normality and homoscedasticity were checked but were not assumed even with data transformation. Moreover, as we used pseudoreplication (*i.e.* the same organism was used to test all the concentrations), the significance of the differences between mean values was estimated using Wilcoxon tests. To assess an integrated impact of the four antidepressants on the abalone hemocytes activity, we applied the "Integrated Biomarker Response", which combined all the measured immunological parameters into one general stress index (IBR, Beliaeff and Burgeot, 2002). Since the IBR value is directly dependent on the number of biomarkers in the data set, the obtained IBR value must be divided by the number of biomarkers used (IBR/*n*; Broeg and Lehtonen, 2006). Results of data standardization procedure needed for IBR calculation were presented in star plots for each tested antidepressant.

Table 1
Lethal concentrations of the two TriCyclic Antidepressants, Amitriptyline and Clomipramine and the two selective serotonin reuptake inhibitors, Citalopram and Paroxetine, at 10, 20 and 50% determined through MTT tests on abalone hemocytes.

	LC_{10} (mg/L)	LC_{20} (mg/L)	LC_{50} (mg/L)
Amitriptyline	17.03	24.42	45.24
Clomipramine	0.70	1.35	4.76
Citalopram	7.50	12.91	32.94
Paroxetine	0.70	1.33	4.16

3. Results

Two compounds of the same family of antidepressant displayed different levels of toxicity on abalone hemocytes, assessed by MTT tests (Table 1). In the family of TCAs, Ami was less cytotoxic than Clomi, and in the family of SSRIs it was Citalo which was less cytotoxic than Parox. For example, looking at LC_{50}, a concentration ten-fold higher is needed for Ami to obtain the same effect than Clomi (*i.e.* 50% of the cell population affected by the tested compounds).

As these antidepressants displayed different cytotoxicities, we aimed at determining whether they also had different effects on four parameters of the immune response. Fig. 1 shows the phagocytic activity of hemocytes exposed to each compound. Ami showed a different phagocytosis effect than the other compounds since it was the only antidepressant inducing a significant dose-dependent stimulation of the hemocyte immunoefficiency. Clomi and Citalo displayed the same effect profile on phagocytosis with a significant increase at the lowest exposure concentration (1 μg/L) and then a decrease at higher concentrations. For Parox this effect profile is shifted to higher concentrations with a significant increase of the immunoefficiency at LC_{10} followed by a decrease at higher concentrations. The ROS production assessed in PMA-stimulated hemocytes exposed to the four antidepressant compounds is shown in Fig. 2. The environmental concentration induced a significant increase of ROS production for Ami, Clomi and Parox. Other tested concentrations induced rather a decrease in ROS production. The same profile is observed for Citalo but a significant ROS-production increase was observed at LC_{10} and was followed by a decrease at higher concentrations. The stability of lysosomal membranes was also impaired by the four compounds (Fig. 3). The lowest concentration of the four antidepressants had no effect on lysosomes. Ami involved a significant permeabilization of lysosomes in 80% of the cells from the LC_{10} and reached 100% of destabilized cells at LC_{50} ($p < 0.001$). The other three compounds displayed almost the same lysosome destabilization profile. Citalo and Parox began to affect the lysosomal membrane stability from LC_{20} in 20% of cells. At LC_{50}, all the three compounds destabilized lysosomal membranes in almost 100% of the hemocytes. Esterase activity was significantly reduced whatever the tested antidepressant compound at all the effective concentrations (Fig. 4). Fig. 5 combines the effects observed on the immune response of abalone hemocytes exposed to antidepressants. TCAs seemed to affect the immune response more strongly (IBR/*n* = 1.15 and 0.62 for Ami and Clomi, respectively) than SSRIs (IBR/*n* = 0.10 and 0.49 for Citalo and Parox, respectively). The main action of Ami was on lysosomal membrane stability and Clomi on phagocytosis. Among SSRIs, Citalo was found to be the less potent immunomodulator.

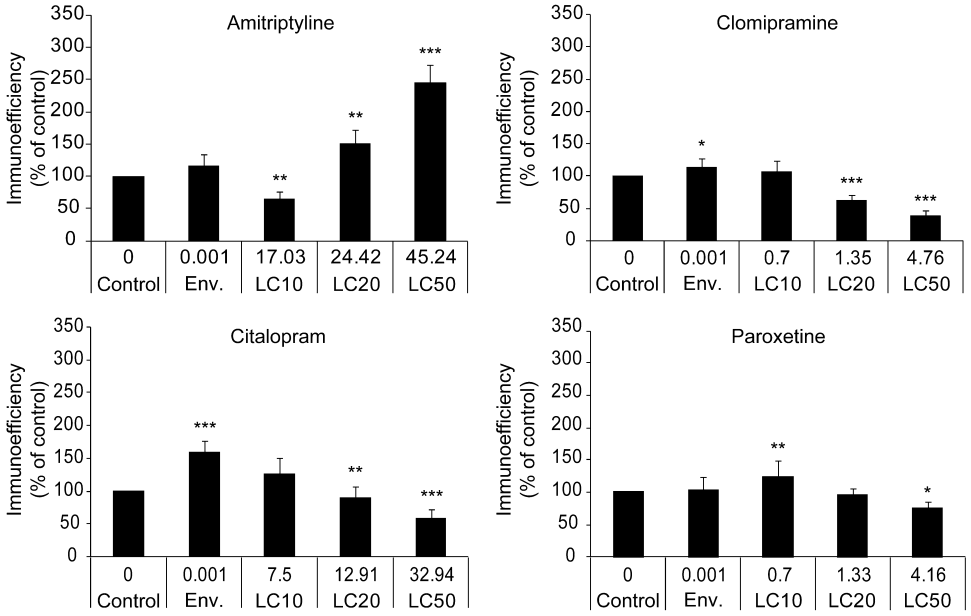

Fig. 1. Phagocytic activity in abalone hemocytes exposed to antidepressants. Relative phagocytic activities (hemocytes having engulfed at least three beads) of cells exposed to different concentrations of Amitriptyline, Clomipramine, Citalopram or Paroxetine were given compared to the 100% control (concentrations in mg/L, LC: lethal concentration). Each data point represents the mean percentage + S.D. of triplicate cultures. Significant differences from control cells were indicated by stars: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

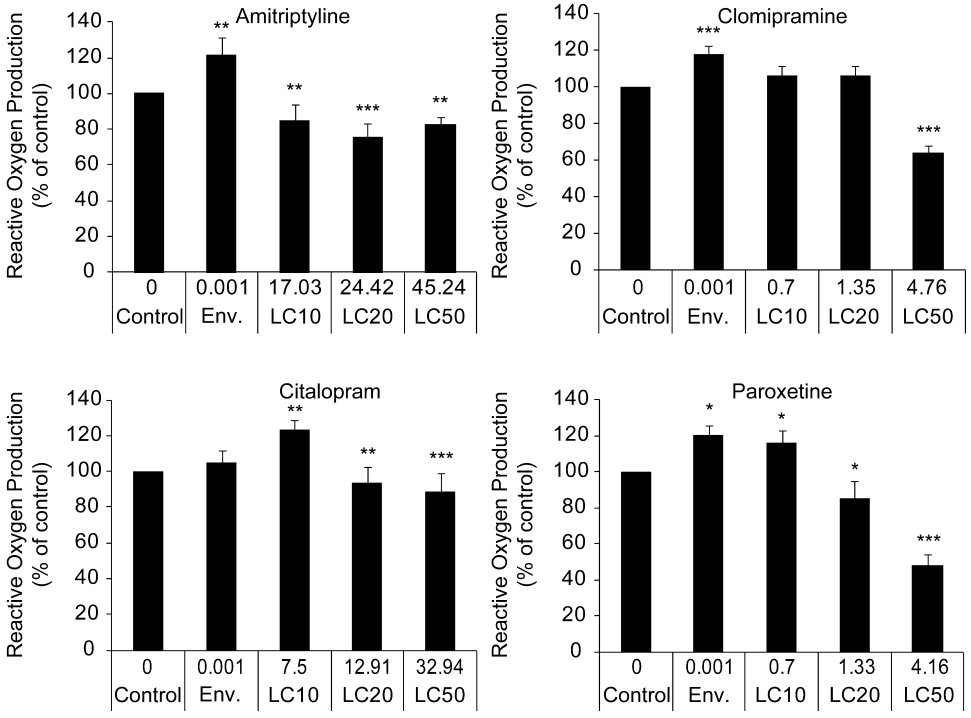

Fig. 2. ROS production in abalone hemocytes exposed to antidepressants. ROS production in cells exposed to different concentrations of Amitriptyline, Clomipramine, Citalopram or Paroxetine were given compared to the 100% control (concentrations in mg/L, LC: lethal concentration). Each data point represents the mean percentage + S.D. of triplicate cultures. Significant differences from control cells were indicated by stars: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

4. Discussion

In comparison to other pollutants present in aquatic environments, pharmaceuticals differ both by their high interaction with biological systems since they are specifically designed to modify physiological/biochemical functions, and by their continuous introduction into surface waters causing life cycle exposures of aquatic organisms (Pascoe et al., 2003). The protection of aquatic life from potential impacts of pharmaceuticals has become an important element within recent environmental assessment regulation. The development and application of *in vitro* methods in the ecotoxicity assessment of pollutants contribute also to the protection of organisms. The *in vitro* assays have the advantages of being easy to use and reproducible. The assays also allow the screening of a large number of compounds in a short period of time (Caminada et al., 2006). However, their major disadvantage is the difficulty of extrapolating the results to the *in vivo* situation. Even if the absolute sensitivity of *in vitro* assays is generally lower than that of

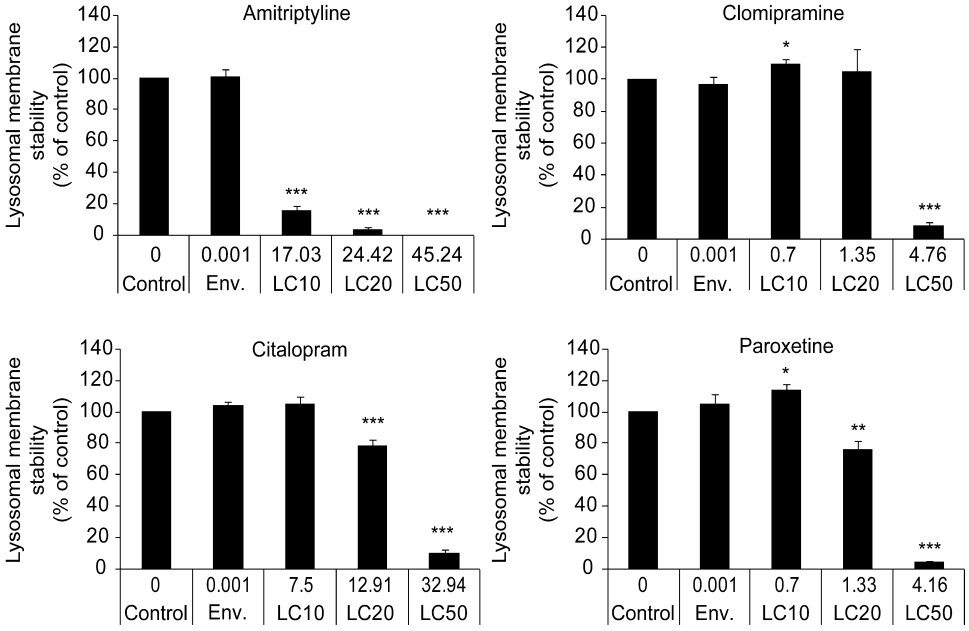

Fig. 3. Lysosomal membrane stability in abalone hemocytes exposed to antidepressants. Lysosomal membrane stability in cells exposed to different concentrations of Amitriptyline, Clomipramine, Citalopram or Paroxetine were given compared to the 100% control (concentrations in mg/L, LC: lethal concentration). Each data point represents the mean percentage + S.D. of triplicate cultures. Significant differences from control cells were indicated by stars: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

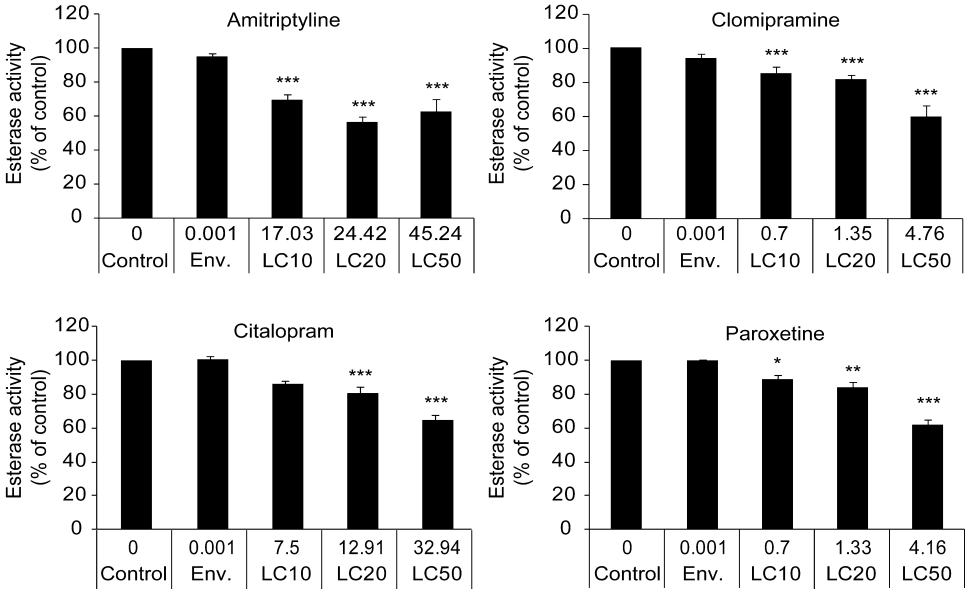

Fig. 4. Esterase activity in abalone hemocytes exposed to antidepressants. The esterase activities in cells exposed to different concentrations of Amitriptyline, Clomipramine, Citalopram or Paroxetine were given compared to the 100% control (concentrations in mg/L, LC: lethal concentration). Each data point represents the mean percentage + S.D. of triplicate cultures. Significant differences from control cells were indicated by stars: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

in vivo tests, positive correlations between *in vitro* endpoints and the LC_{50} *in vivo* data have been highlighted on fishes (reviewed in Kilemade and Quinn, 2003). Indeed, abalone hemocytes seem to be less sensitive to antidepressants (LC_{50} at the mg/L range) than classic normalized *in vivo* tests (LC_{50} at the μg/L range) (*e.g.* Henry et al., 2004; Christensen et al., 2007). However, we highlighted a significant positive correlation between NRU assay on abalone hemocytes and acute toxicity data from *Daphnia magna* (unpublished data). Caminada et al. (2006) have found a similar correlation between toxicity data on *D. magna* and fish cell lines, suggesting that *in vivo toxicity* may be estimated from *in vitro* experiments. The exposure to drug concentrations causing acute lethal toxicity is unlikely to occur since most of the LC_{50} reported in the literature are at the mg/L levels. Nevertheless, it is important to confirm that acute toxic effects do not occur before progressing to chronic studies.

Herein, we described for the first time the effects of four antidepressants, *i.e.* Ami, Clomi, Citalo and Parox, at a cellular level on the European abalone *H. tuberculata*, a marine benthic gastropod inhabiting coastal water areas exposed to treated wastewater disposal. Using primary cultures, we demonstrated that short-term exposure to these antidepressant compounds may affect functional parameters of abalone hemocytes at an environmental concentration of 1 μg/L. Our results showed that this lower concentration involved an immunostimulation with significant increases of phagocytosis and/or ROS production. Immunostimulation at low doses has been observed in bivalves exposed to different kinds of pollutants

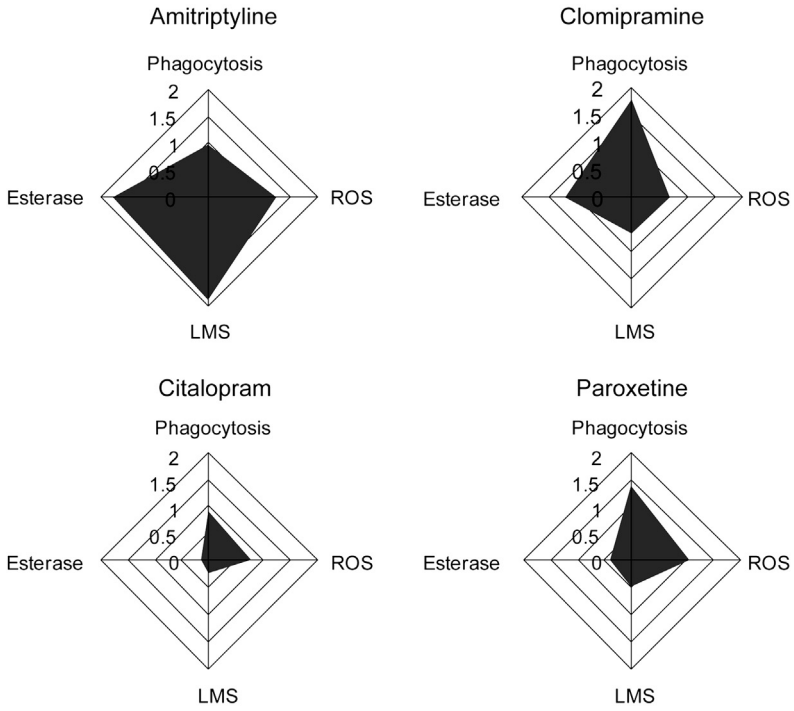

Fig. 5. Star plots representing the four immunological parameters, *i.e.* phagocytosis, ROS production, lysosomal membrane stability (LMS) and esterase activity for each antidepressant. Each of the four axes of the star plots represents the relative degree of response of one biomarker.

(*e.g.* metals, 17β-estradiol) (Brousseau et al., 1999; Canesi et al., 2006; Sauvé et al., 2002). This stimulation may be explained by a hormetic effect. However, for the entire organism, these stimulatory responses of the immune system observed at low levels of chemical stress should not be considered as beneficial because there may be an energetic cost of the hormetic response. All organisms have a finite energy content for growth, reproduction and maintenance/survival (Cody, 1966). By trying to protect themselves against chemical stressors, organisms would have less energy for their growth or reproduction suggesting potential threat to populations.

Hemocytes are involved in digestion, metabolite transport, shell repair and are the main immune effector cells (Cheng, 1981). After 48 h of exposure, cell metabolic activity was reduced, with an LC_{50} estimated at 45.24 ± 2.81 and 4.76 ± 0.09 mg/L for the two TCAs (Ami and Clomi, respectively), and 32.94 ± 6.64 and 4.16 ± 0.12 mg/L for the two SSRIs (Citalo and Parox, respectively). In one family of drugs, two compounds may have different toxicities and thus may be either nocive or toxic. Antidepressants are classified either on their molecular structure and/on the way they interfere with the serotonergic and norepinephrinic neurotransmitter systems. Derijks et al. (2008) underlined that this classification can be quite confusing from a pharmacological point of view, and cited the example of Clomipramine classified as TCA but pharmacologically showing very much similarity with SSRIs. Our results followed this observation since Clomi was found to have similar toxicity on cell viability as Parox, an SSRI compound. These two compounds displayed the same effective concentrations. Citalopram was found less toxic than the other SSRI compounds. This was often explained by its higher selectivity to serotonergic receptors (Brucculeri et al., 2005; Hiemke and Härtter, 2000).

Hemocyte-mediated phagocytosis is the core of the molluscan innate immune system (Pipe and Coles, 1995) although the exposure to a wide spectrum of toxicants generally impaired this mechanism (review in Auffret et al., 2006). In the present study, abalone hemocytes were also exposed to three lethal concentrations of each antidepressant assessed with MTT assays. The exposure to Ami induced a dose-related increase of phagocytosis whereas Clomi, Citalo and Parox induced a decrease. In vertebrates, Ami is a pleiotropic tricyclic antidepressant that can interact with a variety of receptors (*e.g.* histaminic, cholinergic, serotonin and adrenergic receptors) (Hajhashemi et al., 2010). Thus, even if all of these receptors have not been highlighted yet in abalone hemocytes, we can suppose that the non-specificity of Ami on receptors could explain its different effect on hemocytes. Baines et al. (1992) reported that serotonin increased phagocytosis *in vitro* in the cockroach *Periplaneta americana* hemocytes. As antagonist of serotonergic receptors, SSRI compounds could block the stimulating action of serotonin. To determine the specific pathway of the different antidepressants, known antagonists and agonists of the different receptors could be used in future studies on abalone hemocytes.

Phagocytosis is then followed by the production of reactive oxygen species (ROS) by hemocytes to destroy ingested foreign particles (Anderson et al., 1995; Wootton and Pipe, 2003). For this step of the immune response, different effect patterns were observed. For TCA compounds, Ami induced a significant reduction of ROS production non-dependent on the exposure concentration whereas Clomi induced a significant reduction only at higher concentrations. The two SSRI compounds showed the same effect pattern with a significant stimulation of ROS production at LC_{10} followed by a decrease at the other concentrations. Recent studies revealed that mitochondria play an important role in ROS production in vertebrates and invertebrates (Abele et al., 2007; Donaghy et al., 2010, 2012; Murphy, 2009; Sena and Chandel, 2012). Mitochondria release ROS during electron transfer for oxidative phosphorylation and the formation of proton gradient for ATP formation (Gagné et al., 2006). In the present study, concentrations of exposure were determined following MTT assays providing an indication on the mitochondrial integrity. So, the impairment of the mitochondrial activity could here explain the decrease of ROS production. However, we observed that the LC_{10} of the two SSRIs, Citalo and

Parox, stimulated rather than inhibited ROS production. Gagné et al. (2006) showed that pharmaceuticals and personal care products have the ability to increase the rate of electron transport in mitochondria. Thus, the two LC_{10} would not be sufficient to completely disrupt the mitochondrial activity and the production of reactive oxygen species.

The degradation of ingested foreign particles also passed through lysosomes. Lysosomes are involved in numerous functions, including nutrition, tissue repair, cellular defense, and turnover of membranes, organelles and proteins (Auffret and Oubella, 1994). Pollutants are known to cause in several organisms not only membrane destabilization but also profound structural and functional alterations of lysosomes (Lowe et al., 1981; Moore, 1988; Regoli, 1992). The four tested antidepressants greatly affected the stability of lysosomal membranes in abalone hemocytes. Antidepressants as basic lipophilic compounds are known to permeate through membranes and accumulate in the acidic interior of lysosomes where they are protonated and thus become unable to diffuse back into the cytosol (Daniel et al., 2001). Thus, if the accumulation capacity is exceeded, permeability of the lysosomal membrane would be impaired initiating increased autophagy and also hydrolytic enzyme release (Nicholson, 2003). Daniel and Wójcikowski (1997) found that Ami was the most accumulated drug in lysosomes. A reduction in lysosome membrane stability has been reported in mussels and oysters exposed to heavy metals, and has been proposed as an indicator of cell damage (Moore et al., 2006).

Exposure to the different antidepressants induced a significant decrease in the non-specific esterase activity. These enzymes are involved in the intracellular degradation of foreign particles. We have previously evidenced that all tested antidepressants affected ROS production and lysosomes. Thus, this observed decrease in esterase activity is in agreement with a general disturbance of killing mechanisms. Such disturbances, *i.e.* a decrease of the non-specific esterase activity associated with changes in lysosome contents and/or ROS production have been observed on *Crassostrea gigas* hemocytes exposed to organic compounds, *e.g.* insecticide and PAHs (Gagnaire et al., 2006).

The use of an integrative index combining several biomarkers has been reported from a number of ecotoxicological studies (*e.g.* Broeg and Lehtonen, 2006; Damiens et al., 2007; Tsangaris et al., 2011). We decided here to apply this index to summarize the effect of the four antidepressants on the four immunological responses assessed on abalone hemocytes. The application of the IBR allowed the identification of the antidepressant with the strongest immunomodulatory ability. Amitriptyline would be the most immunomodulatory compound (IBR/n = 1.15) followed by Clomi (0.62), Parox (0.49) and Citalo (0.10). Results highlighted a strong effect of Ami on abalone hemocytes with a main action on lysosome (membrane stability and esterase activity). However this impairment of lysosomes did not affect the phagocytosis since Ami was the compound inducing a concentration-dependent increase of phagocytosis. Thus, cells would be able to ingest foreign particles but not to degrade them. Nonetheless, these foreign particles have to be eliminated. Cells can thus trigger apoptosis. TCAs are known to be potent inducers of apoptosis in lymphocytes *in vitro* (Karlsonn et al., 1998), but this apoptosis induction remains to be confirmed in invertebrate hemocytes. The other three compounds have less effect on immune response. Clomi, the second tested TCA displayed immunomodulatory properties similar to those of the SSRI Parox, with an action mainly on phagocytosis. As previously said, Clomi would show great pharmacologically similarity with SSRIs, but its chemical structure (*i.e.* three aromatic cycles) could explain its higher impact on abalone immune response compared with Parox. Citalo was the antidepressant showing less effects on the abalone immune response.

5. Conclusion

This study showed for the first time an acute *in vitro* immunotoxicity of four antidepressants on abalone. After 48 h of exposure, concentrations affecting 50% of hemocyte populations were higher than environmental concentrations. However, the more ecological relevant concentration tested here induced notable effects (*i.e.* increases of phagocytosis and/or ROS production), suggesting a potential risk for abalones. The observed phagocytosis and ROS production responses were biphasic, enhancing both parameters at the environmental concentration (*i.e.* 1 μg/L) and decreasing it at high concentrations. The IBR showed that the two TCAs, Ami and Clomi had higher immunomodulatory capacities than the two SSRIs Citalo and Parox, Ami being the most potent and Citalo the least potent in altering immune function in *H. tuberculata*.

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