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REVIEW

Effects of pharmaceuticals on immune parameters of aquatic invertebrates

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Abstract

Pharmaceuticals are a large group of chemicals used either by humans for personal health or by agribusiness to enhance the growth and health of livestock. Pharmaceuticals are considered to be emerging environmental contaminants. Indeed, several studies have shown that these compounds continuously enter aquatic ecosystems. Both pharmaceutical consumption and erroneous discharge of unused or expired medications make notable contributions to the introduction of pharmaceuticals into the environment. Additionally, pharmaceuticals consumed by humans and livestock are not entirely absorbed by organisms and are excreted and passed into wastewater and surface water. Although most pharmaceuticals are designed for human consumption, they can affect non-target organisms that share certain homologous receptors with humans. This review intends to summarise the most recent information concerning the effects of some classes of pharmaceuticals on the immune parameters of aquatic invertebrates.

Key Words: pharmaceuticals; hemocytes; immune parameters; aquatic invertebrates

Introduction

Among emerging environmental contaminants, pharmaceuticals are a large group of chemicals used either by humans or by agribusiness to enhance the growth or health of livestock (Heberer, 2002). Although information concerning the total annual production and use of pharmaceuticals is generally fragmentary, the US Environmental Protection Agency (EPA) reported that pharmaceuticals, including prescription drugs, veterinary drugs and diagnostic agents, are produced in large quantities - thousands of tons per year - worldwide (http://www.epa.gov/ppcp/basic2.html). In this context, Cleuvers (2003) reported that several kilotons of non-steroidal anti-inflammatory drugs (NSAIDs), a group of substances widely used to treat pain and inflammation, are produced yearly. In Italy, the annual consumption of prescribed drugs was estimated at 209.58 tonnes of amoxicillin, 22 tonnes of β-blockers, 7.6 tonnes of antilipidaemics, 1.9 tonnes of ibuprofen, 26.67 tonnes of antacids and 6.4 tonnes of diuretics (Calamari *et al.,* 2003). In the United Kingdom, 2.56 tonnes of fluoxetine (an antidepressant) were consumed in 2000 (Sebastine and Wakeman, 2003), whereas 22,266 million prescriptions were issued in 2007 in the United States (Modern Medicine Pharmacy, 2010). Zheng *et al*. (2012) reported that the annual consumption of antibiotics in China was approximately 180,000 tons.

Regarding veterinary medicine, Sarmah *et al*. (2006) provided an exhaustive review on the use and environmental occurrence of veterinary pharmaceuticals worldwide. In aquaculture in particular, the intensive farming that has been developed throughout the world (mainly in Asia) requires the application of many pharmaceuticals, mostly antibiotics. Sapkota *et al*. (2008) reported that the type and the total amount of antibiotics used per year vary markedly on a country-by-country basis for the top 15 aquaculture-producing countries (China, Indonesia, Taiwan, India, Philippines, and Norway, in particular). At the same time, the authors observed that the absence of data for some countries was not necessarily indicative of a lack in antibiotic usage but, rather, a lack of information available in these countries (Sapkota *et al*., 2008). In any case, of the 26 antibiotics examined from the FAO list, oxytetracycline, chloramphenicol and oxolinic acid were the most commonly used antibiotics (Sapkota *et al*., 2008). Additionally, it has been estimated that approximately 75% of most of the antibiotics incorporated in feed used in aquaculture systems enter aquatic environments

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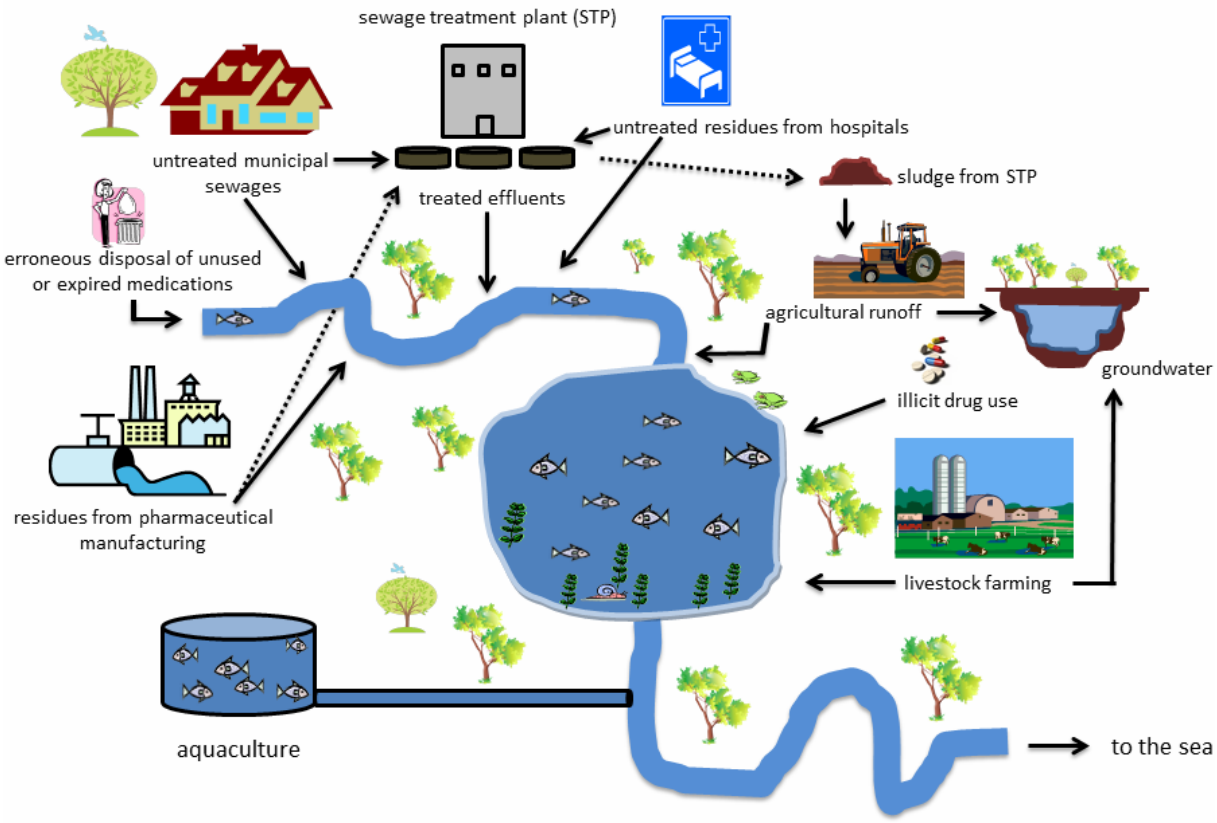

Fig. 1 A scheme summarising the origins and fate of human and veterinary pharmaceuticals in the environment.

directly and accumulate in sediments (Richardson and Bowron, 1985; Halling-Sørensen *et al*., 1998).

Overall, the main sources of pharmaceuticals in the environment are human and veterinary drug use, residues from pharmaceutical manufacturing and hospitals, and illicit drug use. Humans, in particular, contribute to the presence of these substances in the environment when pharmaceuticals are used and when unused or expired medications are erroneously disposed of. Exhaustive schemes of possible sources and pathways of pharmaceuticals in aquatic environments were reported by Heberer (2002) and on the EPA website (http://www.epa.gov/ppcp/pdf/drawing.pdf) and are summarised in Figure 1. Some pharmaceuticals are metabolised and converted by organisms into more easily excreted metabolites, others are converted into more soluble forms by the formation of conjugates, and other substances are excreted in an unaltered form (Daughton and Ternes, 1999). The excreted metabolites or the conjugated and unaltered parent compounds can then be subjected to further transformations in sewage treatment plants (STPs), where the elimination rates vary markedly according to the construction and treatment technology used, the hydraulic retention time, the time of year and the performance of the STP (Fent *et al*., 2006).

Although many pharmaceuticals show low environmental persistence, the main concern is that low persistence can be compensated by continuous introduction of these substances into aquatic ecosystems, where many compounds occur in the ng/l - µg/l range (Daughton and Ternes, 1999; Fent *et al*., 2006; Sarmah *et al*., 2006; Kümmerer, 2009; Zheng *et al*., 2012). However, the levels of pharmaceuticals can be higher in untreated water. According to Fent *et al*. (2006), aquatic organisms are particularly vulnerable to pharmaceuticals. Indeed, due to the continuous introduction of pharmaceuticals into aquatic ecosystems, the exposure of aquatic organisms may be chronic and multi-generational (Fent *et al*., 2006). Therefore, a major concern is not necessarily the acute effects of pharmaceuticals on organisms but, rather, the manifestation of imperceptible effects that can accumulate over time to yield truly profound changes in the biochemical and physiological processes of organisms.

Effects of pharmaceuticals on immune parameters of aquatic invertebrates

Antibiotics

Antibiotics are largely used worldwide to treat disease and protect the health of animals. Regarding the effects of antibiotics on non-target species, Gust *et al*. (2013) recently evaluated the short-term effects (3 days) of environmentally relevant concentrations of antibiotics, as a mixture (ciprofloxacin, 100 ng/l; erythromycin, 50 ng/l;

novobiocin, 100 ng/l; oxytetracycline, 200 ng/l; sulfamethoxazole, 50 ng/l; and trimethoprim, 50 ng/l), on the immune responses of the pond snail *Lymnaea stagnalis*. No significant effects were observed on haemocyte viability and count after 3 days of exposure, whereas intracellular levels of thiols were significantly decreased in snail haemocytes. Additionally, phagocytic activity was significantly decreased by 28 % in the haemocytes of snails exposed to the antibiotic mixture compared to the control. At the level of immune-related gene expression, the antibiotic mixture increased Toll-like receptor 4 (TLR4) mRNA expression and reduced glutathione reductase (GR) mRNA expression (Table 1).

Hemocytes from the freshwater bivalve *Dreissena polymorpha* were exposed *in vitro* to different concentrations (0.2, 1 and 5 µM) of the antibiotic trimethoprim (TMP, 5-[3,4,5-trimethoxybenzyl]pyrimidine-2,4-diamine), and the potential genotoxicity and cytotoxicity were evaluated by the SCGE (single-cell gel electrophoresis) assay, apoptosis frequency and the lysosomal membrane stability test (NRRA, Neutral Red Retention Assay) (Binelli *et al*., 2009a). The results demonstrated that TMP markedly affected mussel haemocytes, even if cytotoxic and genotoxic effects were mostly observed at the highest TMP concentrations tested (Table 1).

In the same freshwater mussel species, different hemocyte parameters were also measured after *in vivo* exposure to three concentrations (1, 3 and 10 nM) of TMP for 96 h. The SCGE assay, the micronucleus (MN) test, apoptotic frequency measurements and the NRRA assay were performed in mussel hemocytes (Binelli *et al*., 2009b). The study demonstrated a moderate cyto- and genotoxicity of TMP on mussel hemocytes. Indeed, only a slight increase in DNA damage was recorded by apoptosis induction and MN frequency, while significant differences in lysosomal membrane stability from baseline levels were measured with 3 and 10 nM at the end of exposure only (Binelli *et al*., 2009b) (Table 1).

In the freshwater mussel *Elliptio complanata*, the separate and combined effects of the antibiotics ciprofloxacin, erythromycin, novobiocin, oxytetracycline, sulfamethazole and TMP (commonly found in urban wastewater effluents) on mussel immune parameters were evaluated *in vitro* (Gust *et al*., 2012). Most of the tested antibiotics, individually or as mixtures, caused marked alterations in hemocyte viability, phagocytosis, lysozyme and cyclooxygenase (COX) activities (Table 1). Overall, the authors observed that antibiotics, alone and as mixtures, modulate the immune parameters of *E. complanata* at environmentally relevant concentrations. Of the antibiotics tested, erythromycin, TMP and sulfamethazole each caused effects similar to those of the mixture, and no additive effects of the antibiotics were observed.

Gagné *et al*. (2006) evaluated the immunotoxic effects of antibiotics in the freshwater mussel *E. complanata*. Hemolymph was collected and treated *in vitro* for 24 h with increasing concentrations of TMP, novobiocin, oxytetracycline, and sulfamethazole. While novobiocin and sulfamethazole decreased phagocytic activity, TMP and oxytetracycline increased it (Table 1). The authors observed that phagocytic activity was negatively correlated with the number of polar functional groups of the compounds, suggesting that the potential of drugs to decrease phagocytosis was related to their polarity.

These studies suggest that immunomarker responses can vary markedly, depending on drug type, animal species and methodological approach (*in vitro* or *in vivo* exposure). In this context, it is important to highlight that *in vivo* exposures can cause variations in the hemolymph levels of some endogenous factors, such as oestrogens, neuro-immune modulators and cytokine-like proteins, which have been shown to influence immune responses (Canesi *et al*., 2007a).

Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are the sixth top-selling class of drug worldwide, and some of them are sold over the counter (Langman, 1999). Among NSAIDs, ibuprofen (IBU) is a propanoic acid derivative (2-[4-(2-methylpropyl)phenyl]propanoic acid) widely used as an analgesic, antirheumatic and antipyretic (Fent *et al*., 2006; Praveen Rao and Knaus, 2008). IBU is a nonselective inhibitor of both cyclooxygenase (COX)-1 and -2 isozymes.

The effects of IBU (0, 0 + ethanol, 100, 500, and 1,000 µg/l) on hemocyte parameters of the clam *Ruditapes philippinarum* (=*Venerupis philippinarum*) were investigated after a 7-day exposure (Matozzo *et al*., 2012). The exposure of clams to the highest IBU concentration significantly reduced their total hemocyte count (THC), whereas no significant changes were observed in both the diameter and volume of hemocytes. Significant increases in hemocyte proliferation were recorded in clams that were exposed to the two highest tested concentrations of IBU. Exposure of clams to 1,000 µg IBU/l significantly reduced uptake of the vital dye Neutral Red (NR) and increased hemolymph lactate dehydrogenase (LDH) activity, which is indicative of cytotoxicity. Conversely, IBU did not induce DNA fragmentation in hemocytes (Table 1). Overall, the results obtained demonstrated that IBU caused marked alterations in the immune parameters of clams and indicated several mechanisms of action of IBU, mostly at the cell membrane level (Matozzo *et al*., 2012).

IBU-mediated lysosomal membrane destabilisation was also demonstrated in hemocytes from *R. philippinarum* exposed to various concentrations of IBU for 35 days (Aguirre-Martínez *et al*., 2013). The authors stated that the level of toxicity calculated for IBU suggested that environmental concentrations in the µg/l range can be extremely toxic for the lysosomal membrane stability of clams (Table 1).

A series of studies has been performed to investigate the effects of NSAIDs, namely diclofenac (DCF), paracetamol (PCM) and IBU, on the hemocytes of the zebra mussel *D. polymorpha*. The first study demonstrated that environmentally relevant concentrations of DCF (2-[(2,6-

dichlorophenyl)amino]phenylacetic acid) induced negligible cellular and genetic damage because a slight decrease in lysosomal membrane stability was observed at the end of exposure at the highest concentration tested (Parolini *et al*. 2011a) (Table 1). One of the most used analgesic and antipyretic agents in human medicine is PCM (N-(4-hydroxyphenyl)acetamide). To evaluate the effects of PCM on *D. polymorpha*, mussels were exposed to PCM environmental concentrations (1, 5 and 10 nM) for 96 h, and cyto-genotoxicity was determined in hemocytes by the lysosomal membrane stability test (NRRA), the SCGE assay, the MN test and apoptotic frequency assessment (DNA diffusion assay) (Parolini *et al*. 2010). The results revealed moderate cyto-genotoxicity in mussel hemocytes because no primary DNA fragmentation was measured by the SCGE assay and only a slight increase in fixed DNA damage was recorded by apoptotic and MN frequencies. A significant reduction in lysosomal membrane stability was observed at 5 and 10 nM at the end of the exposures (Table 1). Lastly, mussels were exposed to environmentally relevant IBU concentrations (0.2, 2 and 8 mg/l) for 96 h, and cyto-genotoxicity was evaluated as reported in the studies above (Parolini *et al*. 2011b). Additionally, in this case, a slight cyto-genotoxicity was found at the IBU concentration of 0.2 mg/l, and higher IBU concentrations were able to significantly increase both genetic and cellular damage (Table 1).

Considering that organisms are most likely exposed to a mixture of substances in the environment, Parolini and Binelli (2012) investigated the effects of a mixture of the three NSAIDs mentioned above (DCF, IBU and PCM) on hemocytes from *D. polymorpha*. The mussels were exposed to different environmental concentrations of the mixture, and the cyto-genotoxic effects were evaluated by means of the Neutral Red Retention (NRR) assay, the SCGE assay, the DNA diffusion assay and the micronucleus test. Exposure to the mixture induced significant cellular stress in bivalves, most likely due to increased oxidative stress, and this significantly increased DNA fragmentation and the frequency of apoptotic and micronucleated cells (Table 1).

Likewise, Luna-Acosta *et al*. (2012) evaluated the toxic effects of a mixture of two herbicides (diuron and isoproturon, each at 5 µg/l) and one pharmaceutical (IBU, at 5 µg/l) on the immune parameters of the oyster *Crassostrea gigas*. No cell mortality was recorded, and phagocytosis was significantly inhibited by almost 50% after 6 h of exposure (Table 1). Additionally, exposure to the mixture significantly decreased catecholase-type phenoloxidase activity (by 20%), highlighting once again that a mixture of contaminants can exert more pronounced effects than a single substance.

In a recent study, the *in vitro* effects of IBU on the immune parameters of the colonial ascidian *Botryllus schlosseri* were evaluated (Matozzo *et al*., 2014). Hemocytes were exposed for 1 h to 100 and 1000 µg IBU/l, and the effects on hemocyte viability and morphology (shape factor), lysosomal membrane stability (NRRA), phagocytic activity, apoptosis (TUNEL reaction), and hydrolytic (acid phosphatase) and oxidative (phenoloxidase and peroxidase) enzyme activities were evaluated. The exposure of hemocytes to IBU did not significantly affect cell viability but did increase the percentage of round cells. IBU significantly reduced both phagocytic activity and lysosomal membrane stability but significantly increased the percentage of hemocytes positive for TUNEL reaction (indicative of DNA fragmentation). A significant decrease in the percentage of hemocytes positive for acid phosphatase was recorded at 1,000 µg/l, while no significant variations were recorded in the percentage of hemocytes positive for phenoloxidase and peroxidase (Table 1). The results obtained indicated that exposure of ascidian hemocytes to IBU induces marked alterations in cell function.

Anticancer agents

In aquatic environments, the occurrence of chemotherapeutic and immunosuppressive agents used to treat cancers is of increasing concern. Among such chemicals, cyclophosphamide (CP) acts as a neurotoxicant (Rzeski *et al*., 2004; Xiao *et al*., 2007). Intracellular enzymes transform CP into active alkylating metabolites, which crosslink with DNA strands (Anderson *et al*., 1995). A non-negligible percentage of CP (up to 10 – 20 %) can be excreted unchanged (Anderson *et al*., 1995; Johnson *et al*., 2008). In aquatic environments, active compounds and metabolites show poor degradability and high persistence. Consequently, CP and metabolites have been detected in waste and surface waters (Buerge *et al*., 2006; Johnson *et al*., 2008).

At present, only one study has investigated the negative effects of anticancer agents in aquatic animals. Canty *et al*. (2009) evaluated the cytotoxicity and genotoxicity of CP on hemocytes from the mussel *Mytilus edulis* and celomocytes from the sea star *Asterias rubens* following 7 days of exposure (18 to 180 mg/l). In mussels, no significant effects on NRR were recorded, whereas a significant increase in the induction of micronuclei and DNA strand breaks was observed, with a strong correlation between micronuclei induction and DNA strand breaks. In sea stars, no significant differences in NRR were observed between CP-exposed animals and seawater controls. Conversely, significant increases in micronuclei induction and DNA strand breaks were detected after 5 and 7 days of exposure to 32 and 56 mg CP/l (Table 1).

Lipid regulators

Blood lipid regulators are a class of pharmaceuticals that can be detected in the ng/l - µg/l range in wastewaters and surface waters (Fent *et al*., 2006). Gust *et al*. (2013) evaluated the effects of a hypolipaemic mixture containing atorvastatin (50 ng/l), gemfibrozil (100 ng/l) and bezafibrate (100 ng/l) on the immune responses of *L. stagnalis.* The mixture increased intracellular reactive oxygen species (ROS) levels (2.9-fold) and decreased thiol levels but did not affect the phagocytic capability of hemocytes. Additionally, the hypolipaemic mixture increased (2.9-fold) nitric oxide synthetase isoform 1 (NOS-1) mRNA expression and decreased (0.4-fold)

TLR4 mRNA expression (Table 1). Reduced thiol levels in hemocytes, associated with increased ROS levels and NOS expression suggested that the oxidative burst can have detrimental effects (*i.e.*, inflammation) on snail hemocytes.

The effects of bezafibrate and gemfibrozil on immunocytes of *Mytilus* spp were investigated both *in vitro* and *in vivo* (Canesi *et al*., 2007a). *In vitro* exposure to both compounds induced rapid lysosomal membrane destabilisation, extracellular lysozyme release, NO production and decreased phagocytic activity. The effect of fibrates were partly mediated by the activation of ERK and p38 MAPKs (Mitogen Activated Protein Kinases) (Table 1). In the *in vivo* experiment, mussels were injected with 0.01, 0.1 and 1 nmol/animal (corresponding to 3.61, 36.18 and 361.8 ng/g dry weight for bezafibrate and to 2.50, 25.03 and 250.35 ng/g dry weight for gemfibrozil), and hemocytes were collected after 24 h. Both compounds caused a concentration-dependent lysosomal destabilisation and extracellular lysozyme release, with a 50 % effect at 0.1 nmol. Conversely, phagocytic activity increased (+24 %) at the highest concentration tested (Table 1). The results obtained indicated that environmental concentrations of hypolipaemic drugs can affect mussel immune function (Canesi *et al*., 2007a).

In an *in vitro* study, Gagne *et al*. (2006) observed an induction of phagocytosis after the exposure of hemocytes from *E. complanata* to both bezafibrate and gemfibrozil (Table 1).

Antihypertensive drugs

At present, only two studies have evaluated the effects of antihypertensive drugs on the immune parameters of aquatic invertebrates. In the first study, snails (*L. stagnalis*) were exposed to a mixture of antihypertensive drugs, including atenolol (500 ng/l), furosemide (300 ng/l), hydrochlorothiazide (300 ng/l) and lisinopril (50 ng/l), for 3 days (Gust *et al*. 2013). The mixture caused a decrease in phagocytosis and upregulated TLR4, NOS-1, NOS-2 and superoxide dismutase (SOD) expression compared to the controls (Table 1). The authors suggested that the decrease in phagocytic activity was a consequence of increased NO production.

In the second study, hemocytes from *D. polymorpha* were exposed *in vitro* to five increasing concentrations (from 0.001 to 10 mg/l) of atenolol for 96 h (Parolini *et al*., 2011c). Hemocyte viability was significantly reduced after 48 h of exposure to 0.01 mg/l of atenolol, and cell viability decreased markedly after 48 and 96 h of exposure to 10 mg/l of atenolol (Table 1).

Antidepressant and anticonvulsant agents

One of the most frequently detected substance in surface waters is fluoxetine, the active ingredient of Prozac® (Metcalfe *et al*., 2010; Bringolf *et al*., 2010). Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) that is prescribed as an antidepressant in large amounts worldwide to treat depression and other psychological disorders (Brooks *et al*., 2003; Nentwig, 2007).

In a recent study, cAMP/PKA regulation and ABCB mRNA expression were assessed in hemocytes from the mussel *Mytilus galloprovincialis* exposed *in vivo* to 0.3 ng/l fluoxetine for 1 week (Franzellitti and Fabbri, 2013). There is evidence that mammalian transcriptional regulation of the ABCB1 gene encoding P-glycoprotein (Pgp) is mediated through the phosphorylation activity of the cAMP-dependent protein kinase (PKA). Although this regulatory pathway needs to be more fully investigated in molluscs, the aforementioned study demonstrated that fluoxetine significantly decreased cAMP levels and PKA activity and induced ABCB mRNA down-regulation. The authors stated that their study provides the first evidence for the cAMP/PKA-mediated regulation of ABCB mRNA expression in mussels (Table 1). Overall, these results demonstrated that the impairment of transduction pathways induced by fluoxetine may affect the ability of mussels to cope with stressful conditions in the environment (Franzellitti and Fabbri, 2013).

We have evaluated the effects of fluoxetine on the immune parameters of the clam *V. philippinarum*. Clams were exposed to various fluoxetine concentrations (0, 1, 5, 25, 125 and 625 µg/l) for 7 days, and the effects on the total hemocyte count (THC), the diameter and volume of hemocytes, hemocyte proliferation, Neutral Red uptake (NRU), and lysozyme activity in cell-free hemolymph (CFH) were evaluated (Munari *et al*., 2014). A significant increase in THC values was observed in clams exposed to 25 µg/l compared with controls, whereas no significant variations were recorded in either the diameter or the volume of hemocytes. Hemocyte proliferation increased significantly in animals exposed to 25, 125 and 625 µg/l compared with controls, whereas NRU decreased significantly in the hemocytes of clams exposed to 1 and 5 µg/l (Table 1).

Gust *et al*. (2013) observed that a mixture containing psychiatric drugs, including venlafaxine (200 ng/l), carbamazepine (200 ng/l) and diazepam (10 ng/l), did not affect immunocompetence (defined by hemocyte density, viability, phagocytosis, ROS and thiol levels) in *L. stagnalis.* However, the mixture induced significant changes in the expression of immune-related genes. TLR4, heat-shock protein 70 (HSP70) and Selenium-dependent glutathione peroxidase (SeGPx) gene expression was upregulated, while allograft inflammatory factor-1 (AIF-1), catalase (CAT) and GR gene expression was downregulated (Table 1). The gene expression induction suggested that the psychoactive substances led to glutathione-dependent peroxidase activity (SeGPx) and the protection response against protein denaturation (HSP70). The reduced CAT and GR gene expression in hemocytes suggested decreased ROS handling and inflammation, whereas the increased TLR4 expression in snail hemocytes was most likely indicative of either a strong inflammation signal or a compensation mechanism against the loss of Toll-like receptor signalling.

Gagné *et al*. (2006) demonstrated that an high concentration of carbamazepine (14 mg/l) is

Table 1 Effects of pharmaceuticals on immune parameters of aquatic invertebrates

Pharmaceuticals	Species/exposure	Immune parameters	References
Antibiotics			
Mixture (ciprofloxacine, erythromycine, novobiocin, oxytetracycline, sulfamethoxazole, trimethoprim)	*Lymnaea stagnalis* *in vivo* exposure	Haemocyte viability = Haemocyte count = Phagocytic activity ↓ Thiol levels ↓ Gene expression ↓↑	Gust *et al*., 2013
Trimethoprim	*Dreissena polymorpha* *in vitro* exposure	DNA damage ↑ Apoptosis ↑ Lysosomal membrane stability ↓	Binelli *et al*., 2009a
Trimethoprim	*D. polymorpha* *in vivo* exposure	DNA damage ≈ Apoptosis ↑ Micronuclei ↑ Lysosomal membrane stability ↓	Binelli *et al*., 2009a
Ciprofloxacin, erythromycin, novobiocin, oxytetracycline, sulfamethazole, trimethoprim (alone and as mixture)	*Elliptio complanata* *in vitro* exposure	Haemocyte viability ↕ ROS levels ↕ Thiol levels ↕ Phagocytosis ↕ Lysozyme activity ↕ NO production ↕ COX activity ↕	Gust *et al*., 2012
Trimethoprim, novobiocin, oxytetracycline, sulfamethazole	*E. complanata* *in vivo* exposure	Phagocytic activity ∩	Gagné *et al*., 2006
NSAIDs			
Ibuprofen	*Ruditapes philippinarum* *in vivo* exposure	THC ↓ Haemocyte diameter = Haemocyte volume = Cell proliferation ↑ NR uptake ↓ LDH activity↑ DNA fragmentation =	Matozzo *et al*., 2012
Ibuprofen	*R. philippinarum* *in vivo* exposure	Lysosomal membrane stability ↓	Aguirre-Martínez *et al*., 2013
Diclofenac	*D. polymorpha* *in vivo* exposure	DNA damage = Apoptosis = Micronucleui = Lysosomal membrane stability ≈	Parolini *et al*., 2011a
Paracetamol	*D. polymorpha* *in vivo* exposure	DNA damage ≈ Apoptosis ≈ Micronuclei ↑ Lysosomal membrane stability ↓	Parolini *et al*., 2010
Ibuprofen	*D. polymorpha* *in vivo* exposure	DNA damage = Apoptosis ≈ Micronuclei ≈ Lysosomal membrane stability ↓	Parolini *et al*., 2011b
Diclofenac + paracetamol + ibuprofen	*D. polymorpha* *in vivo* exposure	DNA damage ↑ Apoptosis ↑ Micronuclei ↑ Lysosomal membrane stability ↓	Parolini and Binelli, 2010

Ibuprofen + diuron + isoturon	*Crassostrea gigas* *in vivo* exposure	Cell mortality = Phagocytosis ↓ Catecholase-type phenoloxidase activity ↓	Luna-Acosta *et al*., 2012
Ibuprofen	*Botryllus schlosseri* *in vitro* exposure	Haemocyte viability = % of round cells ↑ Phagocytosis ↓ Apoptosis ↑ Acid phosphatase ↓ Phenoloxidase = Peroxidase = Lysosomal membrane stability ↓	Matozzo *et al*., 2014
Anticancer agents			
Cyclophosphamide	*Mytilus edulis* *Asterias rubens* *in vivo* exposure	Lysosomal membrane stability (*M.e.*) = Micronuclei (*M.e.*) ↑ DNA damage (*M.e.*) ↑ Lysosomal membrane stability (*A.r.*) = Micronuclei (*A.r.*) ↑ DNA damage (*A.r.*) ↑	Canty *et al*., 2009
Lipid regulators			
Atorvastatin + gemfibrozil + bezafibrate	*L. stagnalis* *in vivo* exposure	Thiol levels ↓ ROS levels ↑ Phagocytosis = NOS1 expression ↑ TLR4 expression ↓	Gust *et al*., 2013
Bezafibrate Gemfibrozil	*Mytilus* spp. *in vitro* exposure	Lysozyme release ↑ NO levels ↑ Phagocytosis ↓ Lysosomal membrane stability ↓	Canesi *et al*., 2007a
Bezafibrate Gemfibrozil	*Mytilus* spp. injection	Lysosomal membrane stability ↓ Lysozyme release ↑ Phagocytosis ↑	Canesi *et al*., 2007a
Bezafibrate Gemfibrozil	*E. complanata* *in vitro* exposure	Phagocytosis ↑	Gagné *et al*., 2006
Antihypertensive drugs			
Atenolol + furosemide + hydrochlorothiazide + lisinopril	*L. stagnalis* *in vivo* exposure	Phagocytosis ↓ TLR4 ↑ NOS-1 ↑ NOS-2 ↑ SOD ↑	Gust *et al*., 2013
Atenolol	*D. polymorpha* *in vitro* exposure	Haemocyte viability ↓	Parolini *et al*., 2011c
Antidepressant agents			
Fluoxetine	*Mytilus galloprovincialis* *in vivo* exposure	cAMP ↓ PKA activity ↓ ABCB mRNA ↓	Franzellitti and Fabbri, 2013
Fluoxetine	*V. philippinarum* *in vivo* exposure	THC ↑ Haemocyte diameter = Haemocyte volume = Cell proliferation ↑ NR uptake ↓	Munari *et al*., 2014

Venlafaxine + carbamazepine + diazepam	*L. stagnalis* *in vivo* exposure	Haemocyte density = Haemocyte viability = Phagocytosis = ROS levels = Thiol levels = TLR4 ↑ HSP70 ↑ SeGPx↑ AIF-1 ↓ CAT ↓ GR ↓	Gust *et al*., 2013
Carbamazepine	*E. complanata* *n vitro* exposure	Phagocytosis ↑ Cell adherence ↓ Esterase activity ↑ Lipid peroxidation =	Gagné *et al*., 2006
Carbamazepine	*M. galloprovincialis* *in vivo* exposure	Lysosomal membrane permeability ↓	Martin-Diaz *et al*., 2009
Carbamazepine	*D. polymorpha* *in vitro* exposure	Hemocyte viability ↓	Parolini *et al*., 2011c
Estrogens			
17β-estradiol	*M. galloprovincialis* *in vitro* exposure	ROS production ↑ DNA damage ↑ Protein carbonylation ↑ Lipid peroxidation ↑ CAT mRNA ↑ SOD mRNA ↑ GST mRNA ↑	Koutsogiannaki *et al*., 2014
17β-estradiol	*M. galloprovincialis* *in vitro* exposure	Hemocyte adhesion ↑	Koutsogiannaki and Kaloyianni, 2011
17β-estradiol (i), 17α-ethinylestradiol (i), EDC mixture (ii)	*M. galloprovincialis* *in vitro* (i) and *in vivo* (ii) exposure	(i) Lysosomal membrane stability ↓ (i) Phagocytosis ↕ (i) Lysozyme release ↑ (ii) Lysosomal membrane stability ↓ (ii) Phagocytosis ↑ (ii) Lysozyme release ↑	Canesi *et al*., 2007b
17β-estradiol	*M. galloprovincialis* *in vitro* (i) and *in vivo* (ii) exposure	(i) Phagocytosis ↕ (i) Oxyradical production ↑ (ii) Lysosomal membrane stability ↓ (ii) Phagocytosis ↕ (ii) Lysozyme release ↑	Canesi *et al*., 2006
17β-estradiol	*Mya arenaria* injection	Hemocyte viability = Phagocytosis ↓	Gauthier-Clerc *et al*., 2006

Symbols:
=: no significant variations
≈: moderate effects
↓: decrease
↑: increase
↕: effects depending on experimental plan, namely exposure to a single substance or to a mixture, or to various concentrations of pharmaceuticals (see Text for details)
∩: effects depending on drug type (see Text for details)

necessary to increase phagocytic activity and to reduce cell adherence in *E. complanata* hemocytes exposed *in vitro* for 24 h. In that study, esterase activity was significantly increased to a threshold concentration of 0.7 mg/l, whereas lipid peroxidation was not affected (Gagné *et al*., 2006) (Table 1).

In *D. polymorpha* (*in vitro* study), hemocyte viability was significantly compromised after 48 h of exposure to 0.01 mg/l of carbamazepine (Table 1); however, exposure to 0.1 mg/l was able to cause a significant increase in cell mortality already after 24 h (Parolini *et al*., 2011c).

In mussels, a significant decrease in haemocyte lysosomal membrane permeability was observed after exposure to 0.1 - 10 µg/l of carbamazepine for 7 days (Martin-Diaz *et al*., 2009) (Table 1).

Estrogens

In the last decades, increasing attention has been given to evaluating negative effects of estrogens in aquatic organisms. One of the most documented effects of estrogens is the induction of vitellogenins, precursors of the egg-yolk proteins, vitellins, which provide energy reserves for embryo development (Matozzo *et al*., 2008).

However, it has been demonstrated that estrogens can also affect hemocyte parameters in aquatic invertebrates. Koutsogiannaki *et al*. (2014) recently evaluated the effects of 17β-estradiol (E_2) on oxidative parameters of *M. galloprovincialis* hemocytes. Results demonstrated that exposure of hemocytes to 25 nM of E_2 for 30 min caused a significant increase in ROS production and, consequently, a significant increase of DNA damage, protein carbonylation and lipid peroxidation. Increases in mRNA levels of the antioxidant enzymes CAT, SOD and glutathione S-transferase were also recorded (Table 1).

In the same mussel species, incubation of hemocytes with E_2 (5, 25 and 50 nM) caused a significant increase in adhesion of cells to extracellular matrix proteins, mostly to laminin-1, collagen IV and oxidized collagen IV (Koutsogiannaki and Kaloyianni, 2011) (Table 1).

The immunomodulatory role of E_2 in *Mytilus* hemocytes was investigated both *in vitro* and *in vivo* (Canesi *et al*., 2006). *In vitro* exposure of hemocytes to E_2 (5-25 nM) rapidly stimulated phagocytosis and oxyradical production; however, higher concentrations of E_2 (50 nM) inhibited phagocytosis. *In vivo* (= injection) exposure of mussels to 5, 25 and 100 pmol of E_2 for 6 and 24 h significantly affected hemocyte lysosomal membrane stability, phagocytosis, and extracellular release of hydrolytic enzymes (Table 1).

In addition, Canesi *et al*. (2007b) demonstrated that both natural (E_2) and synthetic (17α-ethinylestradiol, EE) estrogens can influence markedly hemocyte parameters in *M. galloprovincialis*. *In vitro* exposure of hemocytes, affected lysosomal membrane stability (decrease), phagocytosis (it generally increased at lower concentrations and decreased at higher concentrations) and lysozyme release (after E_2 exposure only). *In vivo* exposure (= injection) of mussels to a mixture of endocrine disrupting compounds (EDCs), including E_2 and EE, induced a clear dose-dependent lysosomal membrane destabilization, a significant stimulation of the phagocytic activity and a significant increase in lysozyme release (Table 1).

Specimens of the soft-shell clam *Mya arenaria* were injected with 10, 20 or 40 nmol of E_2, and the effects on hemocyte parameters were evaluated (Gauthier-Clerc *et al*., 2006). Cell viability did not change during the exposure, whereas significant decreases in phagocytic capacity of hemocytes were observed in clams treated with 10 and 20 nmol E_2 (Table 1).

Overall, results of the studies above indicate that hemocytes of aquatic invertebrates are potential targets of EDCs.

Concluding remarks

Although the impact of pharmaceuticals on aquatic environments needs to be more fully investigated, the data reported in the present review (summarised in Table 1) indicate that a variety of drugs can markedly influence immune parameters of non-target species. In this context, further studies are needed to better understand the relationship between pharmaceutical-mediated immunomodulation and the capability of animals to respond to pathogens. Nevertheless, efforts should be directed at evaluating the effects of drug mixtures because animals are more realistically exposed to complex drug mixtures in their environments.

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