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# Biological effects of pharmaceuticals in the marine environment

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Short title: Pharmaceuticals in the marine environment

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**Keywords:** Contaminants of Emerging Concern; Environmental Pharmaceuticals; Mechanism Of  
Action; Multiple Stressors; Interactive Effects; Non-target species.

23    **Abstract**

24    Environmental pharmaceuticals represent a threat of emerging concern for marine ecosystems.  
25    Widely distributed and bioaccumulated, these contaminants could provoke adverse consequences on  
26    aquatic organisms, acting with modes of action like those reported for target species. Compared to a  
27    pharmacological use, organisms in field conditions are exposed to complex mixtures of compounds  
28    with either similar, different, or even contrasting therapeutical effects. This review summarizes  
29    current knowledge on main cellular pathways modulated by the most common classes of  
30    environmental pharmaceuticals occurring in marine ecosystems and accumulated by non-target  
31    species, including non-steroidal anti-inflammatory drugs (NSAIDs), psychiatric drugs,  
32    cardiovascular and lipid regulator agents, steroidal hormones and antibiotics. An intricate network of  
33    possible interactions is shown with both synergistic and antagonistic effects on the same cellular  
34    targets and metabolic pathways. This complexity reveals the intrinsic limit of the single-chemical  
35    approach to predict long-term consequences and future impact of pharmaceuticals at organismal,  
36    population and community levels.

## 1. Introduction on pharmaceuticals in the marine environment

The progress of medical science and continuous formulation of more efficient pharmaceutical drugs has allowed to reduce the impact of several pathologies, determining an increased life expectancy, better prevention of diseases, and general improvement of health quality (Mezzelani et al. 2018a). Consequently, global pharmaceutical market has quickly grown both in terms of total sales and number of synthesized active pharmaceutical ingredients, with a further increase expected in the future due to development and aging of human population (IQVIA, 2019, COM//2019/128 final).

Beside their beneficial role to human society, the huge consumption of pharmaceuticals has become an environmental concern for the widespread occurrence of these compounds in aquatic ecosystems and their potential adverse effects on non-target species. Specifically designed for being biologically reactive at very low concentrations, active ingredients have the potential to interfere on biochemical and physiological processes of non-target species, with virtually unknown long-term effects on marine ecosystems. The urgent need to elucidate such aspects is reflected in international actions, such as the development of guidelines for the Ecopharmacovigilance and regulatory approaches in Europe, USA, Japan and Australia (Jose et al. 2020). The European Strategic Approach to Pharmaceuticals in the Environment encourages efforts to enlighten long-term consequences of these compounds through innovative and multidisciplinary ecosystems-oriented approaches, ensuring that such actions do not limit the access to safe and effective treatments for humans and animals (COM//2019/128 final).

Pharmaceutical residues can enter the aquatic environment by a number of pathways including primarily wastewater treatment plants (WWTPs), but also industrial and hospital discharges, aquaculture facilities, animal farming, runoff of soils and direct discharge of untreated wastewater (Bagnis et al. 2019; Couto et al. 2019, de Oliveira et al. 2020, Mezzelani et al. 2018a). WWTPs are often of limited efficiency toward pharmaceuticals which have biorefractory behaviour and are minimally removed by primary and secondary wastewater treatment processes (Peake et al. 2016).

62 Tertiary treatments based on finer filtration techniques (sand filter, microfiltration, ultrafiltration,  
63 nanofiltration, reverse osmosis) and absorption by activated carbon, represent more advanced  
64 technologies with higher removal capacity, but of still limited application due to the elevated costs.  
65 Transfer of pharmaceuticals from WWTPs to surface waters has thus been extensively documented  
66 precluding the entry of these compounds in the marine environment (Couto et al. 2019, Sathishkumar  
67 et al. 2020).

68 The risk of pharmaceuticals in coastal areas has been ignored for a long time, hypothesizing  
69 an illimited dilution capacity of seawater. Contrasting with this misleading view, the presence of  
70 pharmaceuticals in marine ecosystems has been demonstrated worldwide, at concentrations ranging  
71 from a few ng/L up to hundreds of µg/L (Biel-Maeso et al. 2018, González-Alonso et al. 2017,  
72 Kümmerer, 2010, Mezzelani et al. 2018a). From the initial detection of a few anticancer drugs and  
73 synthetic steroids in early '90, more than 160 active principles were measured in 2010, and almost  
74 300 compounds have been actually reported in aquatic environments for various therapeutical classes  
75 like antibiotics, non-steroidal anti-inflammatory drugs, antidepressants, antihypertensives and  
76 antiepileptics (Aherne et al. 1990, Kümmerer, 2010, Mezzelani et al. 2018a). Despite the  
77 environmental persistence of pharmaceuticals is influenced by several variables including physical-  
78 chemical characteristic of the molecules, temperature, pH and photolysis by solar irradiation (Couto  
79 et al. 2019), the huge consumption and release might confer to these drugs the behaviour of pseudo-  
80 persistent pollutants. The urgency to prioritize the environmental sustainability of more than 4000  
81 active principles potentially reaching natural ecosystems, has been identified as a major research need  
82 but, at this time, comprehensive monitoring programs for such emerging pollutants remain neither  
83 regulated nor mandatory. A basic prerequisite to achieve this goal is to integrate a better knowledge  
84 on released loads, with the assessment of bioavailability, uptake and biological effects of  
85 pharmaceuticals in non-target organisms.

86 Common classes of pharmaceuticals occurring in marine ecosystems and accumulated by non-  
87 target species include non-steroidal anti-inflammatory drugs (NSAIDs), psychiatric and

88 cardiovascular drugs, steroids and antibiotics. The detection of steroids hormones in sewage effluents  
89 in early '70 provided the first impetus to the study of pharmaceuticals in aquatic environments.  
90 Natural and synthetic steroids are largely used in human and veterinary medicine and the amounts of  
91 estrogens released by livestock and aquaculture are similar or even higher than the contribution  
92 related to human population (Aris et al. 2014, Beardmore et al. 2001, Liu et al. 2015). The 17 $\beta$ -  
93 estradiol (E2), 17 $\alpha$ -ethynylestradiol (EE2) and Estrone (E1) are the more frequently detected  
94 hormones in aquatic environments, and their accumulation has been shown in bivalves and in fish  
95 collected from natural environments (Guedes-Alonso et al. 2017). Other active principles measured  
96 in tissues of marine organisms include: diethylstilbestrol, estriol, norgestrel, norethisterone, megestrol  
97 acetate, progesterone, testosterone, boldenone, nandrolone, cortisone, prednisone and prednisolone.  
98 Among 15 steroids detected in molluscs, crabs, shrimps and fish from the southern coast of China,  
99 norgestrel and progesterone were the most frequently detected and the occurrence of six synthetic  
100 steroids in the feed demonstrated the illegal use of these drugs in local aquaculture farms (Liu et al.  
101 2015).

102       Antibiotics have received scientific attention since the '90, given the direct correlation between  
103 their growing consumption, occurrence in aquatic ecosystems and rapid expansion of antibiotic  
104 resistance in microorganisms. Similarly to steroid hormones, antibiotics in human medicine are  
105 coupled with their massive use in livestock and aquaculture. There are actually 5 antibiotics included  
106 as priority active principles in the watch-list of the EU Water Framework Directive 2018/840/EU  
107 (erythromycin, clarithromycin, azithromycin, amoxicillin and ciprofloxacin) but a recent study  
108 suggested that also trimethoprim, sulfamethoxazole, tetracycline, oxytetracycline and ofloxacin are  
109 of great environmental concern based on their common detection in surface waters and adverse effects  
110 on microbial communities (Kovalakova et al. 2020). Bioaccumulation of antibiotics has been reported  
111 in several marine vertebrates and invertebrates. As an example, sulfamethoxazole was dominant in  
112 fish sampled from south Africa, in fish and shellfish from the Red Sea and in bivalves from the  
113 Mediterranean and South Atlantic coastal areas (Ali et al. 2018, Álvarez-Muñoz et al. 2015, Ojemaye

114 & Petrik, 2019). Several antibiotics residues were detected in both organisms and fish feeds from the  
115 mariculture areas of the Pearl River Delta highlighting again that, despite the use of antibiotics as  
116 growth promoters in livestock and aquaculture has been forbidden in EU since 2006, they are still  
117 used in other parts of the world (Kovalakova et al. 2020, Xie et al. 2019).

118 NSAIDs have been shown as common pollutants in marine organisms, from benthic  
119 invertebrates up to top predators, with a certain biomagnification behaviour highlighted by the  
120 increased concentrations at higher trophic levels (Sathishkumar et al 2020). Diclofenac (DIC) has  
121 been detected in mussels from Belgian coasts, Mediterranean, South-East Atlantic Ocean, San  
122 Francisco Bay (Capolupo et al. 2017, Cunha et al. 2017, Mezzelani et al. 2020), and in fish species  
123 from Spanish and South Africa coasts (Ojemaye & Petrik, 2019). Occasionally, other active principles  
124 like acetaminophen, salicylic acid, nimesulide (NIM), naprossen and ibuprofen (IBU) have also been  
125 measured in tissues of marine vertebrates and invertebrates (Wolecki et al. 2019).

126 Possibly surprisingly for some readers, psychiatric drugs are highly represented in marine  
127 biota. The antiepileptic carbamazepine (CBZ) was measured in more than 90% of mussels collected  
128 from the Tyrrhenian Sea, Adriatic Sea, and Belgian coasts, in oysters from Ebro delta, and in several  
129 fish and shellfish species sampled in the Red Sea and Pearl River Delta (Ali et al. 2018, Mezzelani et  
130 al. 2020, Xie et al. 2019). The elevated distribution of this antiepileptic drug in marine organisms is  
131 partly related to its refractory properties which confer to CBZ an average half-life >200 days in  
132 aquatic ecosystems (Bu et al. 2016, Zhu et al. 2019). Marine organisms can also contain residues of  
133 antidepressants such as paroxetine (PAR), sertraline (SER), venlafaxine (VEN), citalopram (CIT),  
134 lorazepam (LOR), fluoxetine (FLU) and its metabolites. LOR and PAR were detected in more  
135 than 40% of wild mussels from the Mediterranean, while 64% of bivalves collected along the  
136 California west coast contained SER; bioaccumulation of VEN, CIT and FLU has been further  
137 documented in organisms from the Mediterranean, Red Sea and Atlantic Ocean (Ali et al. 2018,  
138 Martínez-Morcillo et al. 2020, Mezzelani et al. 2020).

139 A few data are also available on bioaccumulation of cardiovascular drugs (CVDs) which  
140 include various therapeutic classes, like beta-blockers (atenolol, metoprolol, propranolol), angiotensin-  
141 converting-enzyme inhibitors, angiotensin II receptor antagonists (valsartan) and calcium channel  
142 blockers (diltiazem). Such active principles have been detected in fish and invertebrates from the  
143 Portuguese and Spanish coasts, and from the Red Sea (Ali et al. 2018, Álvarez-Muñoz et al. 2015,  
144 Moreno-González et al. 2016): considering the massive worldwide consumption of these drugs for  
145 the treatment of the first cause of human death (World Health Organization, 2016), the limited number  
146 of studies on their environmental occurrence and biological consequences represent an important gap  
147 of knowledge.

148

## 149 **2. Biological effects of pharmaceuticals on marine organisms**

150 Elucidating biological effects and impacts of pharmaceuticals in marine ecosystems is a  
151 faceted and complex challenge. Organisms are typically exposed to complex chemical mixtures  
152 where individual compounds can interact through several mechanisms, causing either synergistic or  
153 antagonistic feedback, cascade effects which in turn modulate cellular and physiological  
154 responsiveness to other stressors, potentially up to population dynamics and ecosystem functioning.

155 Pioneering studies on deleterious effects and ecological risk of environmental pharmaceuticals  
156 were based on the application of acute toxicity tests on bacteria, algae, invertebrates and vertebrates.  
157 This approach revealed an apparent lack of risk with EC<sub>50</sub> values for selected endpoints at  
158 concentrations more than one order of magnitude higher than those typically found in marine  
159 ecosystems. However, these data more realistically demonstrate that the onset of acute toxicity is  
160 highly improbable for such compounds, and that the exclusive use of ecotoxicological bioassays may  
161 underestimate potential adverse outcomes in marine organisms exposed to low doses but in long-  
162 term, chronic conditions (Brausch & Rand, 2011, Mezzelani et al. 2018a).

163 Pharmaceuticals exert their biological activity targeting metabolic, enzymatic, or cell-  
164 signalling processes through well characterized Mechanisms of Action (MoA): since molecular



165 targets are often evolutionarily conserved in many aquatic species, specific alterations at molecular  
166 and cellular levels may represent sensitive early warning signals for these drugs before the onset of  
167 long-term toxicological effects or changes at higher levels of biological organization. Available data  
168 on the effects of pharmaceuticals in non-target species are still too fragmented for delineating the  
169 overall impact of such active principles on marine organisms.

170 The aim of the next paragraphs is to summarize the common pathways of action, metabolism  
171 and toxicity of the most frequently detected therapeutic classes in marine environment, trying to  
172 elucidate parallelisms between target and non-target organisms. Compared to target “patients” who  
173 assume specific drugs in response to specific diseases, in field conditions non-target marine  
174 organisms are typically exposed to multiple stressors with combinations of several pharmaceuticals  
175 and compounds with either similar, completely different, or even contrasting therapeutical effects. In  
176 this respect, to provide a more ecologically relevant synthesis of responsiveness to such complexity,  
177 individual pathways of various pharmaceutical classes will be combined in a single picture (Figure  
178 1) to highlight the intricate network of metabolic connections which can not be individually  
179 disentangled and in turn modulate potential adverse outcomes at biological and environmental levels.

180

### 181 *3.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)*

182 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are widely consumed worldwide to treat  
183 a variety of symptoms including fever, pain, inflammation, rheumatoid arthritis, muscular and  
184 skeletal disorders. Mechanism of action and modulated pathways in target and non-target species are  
185 summarized in Figure 1. Irritation or injury conditions provoke increased levels of arachidonic acid  
186 (AA), a polyunsaturated fatty acid synthesized from cell membranes by cytosolic phospholipase A2  
187 (PLA<sub>2</sub>). The AA is transformed by the cyclo-oxygenase pathway (COX) with the formation of  
188 prostaglandins (PGs), an important group of lipid inflammatory mediators, collectively known as  
189 eicosanoids. The first COX reaction converts AA to prostaglandin G<sub>2</sub> (PGG<sub>2</sub>), before its reduction  
190 to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), and the final conversion to five biologically active primary PGs:

191 prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), prostaglandin F2 (PGF2), prostacyclin (PGI2)  
192 and thromboxane A2 (TxA2). These eicosanoids differently modulate, either increasing or  
193 decreasing, the intracellular levels of cyclic adenosine monophosphate (cAMP) and calcium, exerting  
194 their effects on important neurophysiological functions, modulation of inflammation and immune  
195 responses, protein metabolism, contraction and relaxation of muscles or blood vessels (Burian, 2007).  
196 NSAIDs prevent the conversion of AA to prostaglandins and thromboxanes by inhibiting one or both  
197 the isoforms of the COX enzyme (Figure 1): COX-1, constitutively expressed in several tissues and  
198 involved in homeostatic, cytoprotective functions, while the inducible COX-2 is mainly responsible  
199 for pain perception and inflammation (Bacchi et al. 2012, Ghosh et al. 2015). According to their  
200 MoA, available NSAIDs can be classified as “Non-Selective” (i.e. IBU, ketoprofen KET) acting on  
201 both COX-1 and COX-2, “Selective“ (i.e. celecoxib and rofecoxib) which target only the COX-2,  
202 and “Semi-selective“ (i.e. DIC, indomethacin, meloxicam) with a higher affinity for COX-2 but also  
203 able to inhibit COX-1 pathways. Given the role of prostaglandins in the activation of both innate and  
204 adaptive immune responses, their inhibition by NSAIDs leads to the modulation of immune system  
205 which, in worst conditions, can result in excessive inflammatory cascades, accumulation of activated  
206 macrophages and ulceration.

207 COX metabolism of AA, prostaglandins (PGs) and other biologically active derivatives of  
208 polyunsaturated fatty acids have been detected in several marine invertebrates, such as sponges,  
209 corals, and molluscs. These compounds include PGI2, PGE2, PGD2, PGF2, with similar functions as  
210 in mammals (Di Costanzo et al. 2019). Evidence that AA metabolism and COX pathways are  
211 modulated in aquatic organisms by environmental NSAIDs have been documented in a large number  
212 of laboratory studies. Exposure to DIC (1 to 100 µg/L) caused the inhibition of PGE2 synthesis in *M.*  
213 *galloprovincialis*, and changes of COX activity in the digestive gland of the brown mussel *Perna*  
214 *perna* (0.5 µg/L) (reviewed by Sathishkumar et al. 2020). Similarities in MoA of NSAIDs between  
215 vertebrates and invertebrates were further confirmed in clams *Ruditapes philippinarum* and *Mytilus*  
216 *spp.*: following exposure to IBU (10-100 µg/L), KET and NIM (0.5-2.5 µg/L) transcriptional profile

217 revealed the differential expression of genes involved in AA metabolism, including up-regulation of  
218 PLA2, down-regulation of Thromboxane A Synthase 1(TBXAS1), Progesterone receptor (PGR1),  
219 Prostaglandin E2 receptor EP4 subtype (PTGER4) (Almeida et al. 2020a, Mezzelani et al. 2018b,  
220 Milan et al. 2013).

221 Despite invertebrates lack an adaptive immunity, they possess a potent and complex innate  
222 immune system similar to that of vertebrates. Free circulating haemocytes (i.e. granulocytes and  
223 hyalinocytes) are responsible for cell-mediated immunity and, when activated, are responsible of  
224 phagocytosis, reactive oxygen species production, release of hydrolytic enzymes and antimicrobial  
225 peptides. Early activation of immune system was observed in marine species in response to NSAIDs.  
226 Transcriptional profiles of marine invertebrates exposed to DIC, IBU, KET and NIM highlighted the  
227 modulation of genes regulating lysosomal enzymes, chitinases, Toll-like and NOD-like receptors  
228 (TLRs, NLRs), lectins (rhamnose-binding lectin RBL, hepatic lectin HLEC) and scavenger receptors  
229 (Almeida et al. 2020a, Mezzelani et al. 2018b, Milan et al. 2013). NLRs are intracellular sensors of  
230 pathogen-associated molecular patterns that enter the cell via phagocytosis, interacting with TLRs to  
231 regulate the immune response: the activation of immune system in response to environmental  
232 NSAIDs (0.05-25µg/L), progressed from transcriptional up to cellular level in *M. galloprovincialis*  
233 and *R. philippinarum*, with a significant impairment of haemocytes responsiveness, compromised  
234 lysosomal membrane stability, inhibition of phagocytosis activity and granulocytes-hyalinocytes  
235 ratio, and an overall reduction of immune surveillance against pathogens (Almeida et al. 2020a).

236 NSAIDs are also known to be substrates for the cytochrome P450 (CYP) biotransformation  
237 pathway (Figure 1). In mammals, CYP2C9 is the most important oxidase primarily responsible for  
238 metabolism of several NSAIDs (i.e. celecoxib, IBU, and naproxen), but also cytosolic phase I  
239 enzymes (i.e. CYP3A4, CYP2C19, CYP2C8) catalyse oxidative transformations; phase II reactions  
240 by UDP-glucuronosyltransferases (i.e. UGT1A1, UGT2B7, UGT1A9 and UGT2B4) produce  
241 glucuronides and reactive metabolites which are finally excreted through bile and ABC2 efflux  
242 transporters (Bindu et al. 2020). The role of P450 in NSAIDs metabolism has been considered a key

243 factor in overcoming the adverse effect of these drugs and the elevated DIC toxicity has been  
244 associated to its poor metabolization by cytochrome P450. Despite the P450 biotransformation  
245 pathway in marine invertebrates still need to be fully elucidated, transcriptional responsiveness of  
246 phase I and phase II-related genes was observed in response to environmental levels of NSAIDs  
247 (Mezzelani et al. 2018a,b, Milan et al. 2013). Bivalves (*M. galloprovincialis* and *R. philippinarum*)  
248 exposed to DIC, IBU, KET and NIM revealed the modulation of drug metabolism genes including,  
249 among others, CYP1A, phase II-related genes (GSTA4, SULT1B1, GSTP2) and heat shock proteins  
250 (HSP70L, HSP70I) (Figure 1).

251 In addition to COX inhibition and metabolism through CYP450, NSAIDs exert cytotoxic side-  
252 effects which mostly derive from their capability to enhance the generation of reactive oxygen  
253 species, ROS (Ghosh et al. 2015). Well known mechanisms include a direct targeted toxicity on  
254 mitochondria, the potent induction (i.e. by DIC, naprossen, aspirin) of NADPH-oxidases (NOX) and  
255 xanthine oxidoreductase (XOR), all typical sources of oxyradicals and lipid peroxidation (Figure 1);  
256 NSAIDs contribute to prooxidant mechanisms also through their capability to uncouple inducible  
257 nitric oxide synthase (iNOS), resulting in the generation of NO· instead of nitric oxide (NO); further,  
258 NSAIDs modulate the ROS-mediated induction of apoptosis through the inhibition of Akt signalling  
259 pathways, the downregulation of the transcription factor NF- $\kappa$ B and the alteration of p53 pathway  
260 (Figure 1). Additional effects of NSAIDs depend on their high affinity for peroxisome proliferator  
261 activated receptors (PPARs), with consequences on lipid metabolism, while their reactivity with  
262 peroxidases enzymes is responsible for the inhibition of the acetylcholinesterase activity and the  
263 consequent increase of cholinergic transmission (Muraoka & Miura, 2009).

264 In agreement with prooxidant effects described for target species, NSAIDs were shown to  
265 modulate NF- $\kappa$ B pathway in both *Mytilus spp.* and clam *R. philippinarum*, increasing the mRNA  
266 level of a genuine NF- $\kappa$ B gene (Maria et al. 2016, Milan et al. 2013). NF- $\kappa$ B family is a group of  
267 heterodimeric transcription factors found in almost all animal cell types and involved in cellular  
268 responses to stress, cytokines, ROS, ultraviolet radiation and trace metals; after its activation in the

cytoplasm, NF- $\kappa$ B is translocated to the nucleus, binds to specific DNA sequences (NF- $\kappa$ B responsive elements) and regulates the transcriptional induction of iNOS, as well as a number of genes mediating inflammatory responses and apoptosis (Regoli & Giuliani, 2014). Also genes involved in the PPAR signalling pathway (i.e. acetyl-coenzyme A acyltransferase 1, phosphoenolpyruvate carboxykinase, and stearoyl-CoA desaturase) were transcriptionally up-regulated in bivalves exposed to IBU, confirming the effects of NSAIDs on lipid and fatty acid metabolism of non-target organisms (Mezzelani et al. 2018b, Milan et al. 2013). Similar evidence was obtained for the effects of these drugs on p53 pathway activated by stimuli including ROS, trace metals, DNA damage and hypoxia. The p53 tumor suppressor protein is a transcription factor which binds to specific DNA regions, the p53 responsive elements (Figure 1) enhancing transcription of stress response genes involved in a variety of anti-proliferative processes, cell cycle checkpoints and arrest, DNA damage and repair, apoptosis (Regoli & Giuliani, 2014). Among these, both mussels and clams exposed to NSAIDs revealed an up-regulation of several related genes including a putative baculoviral IAP repeat-containing 2 (BIRC2), X-linked inhibitor of apoptosis (XIAP), BCL2-associated athanogene 4 (BAG4), B-cell CLL/lymphoma 2 (BCL2), suggesting increased anti-apoptotic activity in contrast to effects measured in target species (Mezzelani et al. 2018a,b, Milan et al. 2013).

The effects of NSAIDs in promoting the generation of ROS and the related pro-oxidant mechanisms in marine organisms were confirmed at the cellular, functional level by the significant modulation of antioxidant enzymes (catalase CAT, superoxide dismutase SOD, glutathione reductase GR, glutathione S-transferases GST), the enhancement of peroxidation products like malondialdehyde and lipofuscin, the increment in micronuclei frequency and DNA damages observed in *Mytilus spp.*, *R. philippinarum*, *Gibbula umbilicalis* and *Hediste diversicolor* (Bebianno & Gonzalez-Rey, 2015, Mezzelani et al. 2018a,b, Milan et al. 2013). The decrement of neutral lipids and the inhibition of Acyl-CoA oxidase activity (ACOX) in *Mytilus spp.* further supported COX-mediated effects on AA and lipid metabolisms, while the inhibition of acetylcholinesterase activity

294 in IBU and DIC exposed organisms confirmed effects on cholinergic transmission (Mezzelani et al.  
295 2018b, Sathishkumar et al. 2020).

296 Despite clear mechanistic evidence support molecular and cellular effects of NSAIDs in  
297 marine organisms, long term consequences on organismal, population, community or ecosystem  
298 functioning are still difficult to be predicted. Nonetheless, the effects of DIC in *Mytilus spp.* revealed  
299 the disturbance of tyrosine and tryptophan/serotonin pathways allowing the authors to hypothesize  
300 adverse consequences for osmotic and reproduction processes (Swiacka et al. 2019). In addition,  
301 NSAIDs were shown to modulate genes involved in shell formation and biomineralization of early  
302 mussel embryos, such as chitin synthase-CS, carbonic anhydrase-CA and extrapallial protein-EP  
303 (Balbi et al. 2018), while in adult mussels these drugs impacted various physiological traits reducing  
304 the byssus abundance and strength, along with a significant decrease of the scope for growth; the  
305 reduced energy available for growth is considered as an overall index of performance which, if not  
306 compensated, might anticipate future adverse consequences at the population level.

307

### 308 3.2 Central Nervous System/Psychiatric Drugs

309 Pharmaceuticals targeting the central nervous system also represent environmental  
310 contaminants of emerging concern which include antidepressants acting as Selective Serotonin  
311 Reuptake Inhibitors SSRIs (fluoxetine FLU, paroxetine PAR, fluvoxamine FVX, venlafaxine VEN,  
312 sertraline SER, citalopram CIT and escitalopram ESC), and the antiepileptic carbamazepine CBZ  
313 (Hillhouse & Porter 2015).

314 The SSRIs exert their function at the synaptic cleft by increasing the extracellular level of  
315 serotonin (or 5-hydroxytryptamine 5-HT), a small molecule that functions both as a neurotransmitter  
316 in the central nervous system and as a hormone in the periphery. Serotonin is synthesized from  
317 tryptophan (Figure 1) and stored in synaptic vesicles which prevent its degradation by the enzyme  
318 monoamine oxidase (MAO). Following the action potential, vesicles fuse with the presynaptic  
319 membrane and release their content into the synaptic cleft where serotonin can bind 5-HT receptors,

320 modulating the associated signalling pathways. Some of the 5-HT receptors (as 5-HT1A and 5-HT1B)  
321 act as inhibitory autoreceptors, indicating whether high levels of 5-HT are present in the synapse, and  
322 thus inhibiting the further release of additional serotonin (Nichols & Sanders-Bush 2003). The excess  
323 of 5-HT in the synapse is either degraded by MAO or taken back into the presynaptic terminal by  
324 active serotonin transporter, 5-HTT (Figure 1). SSRIs are designed to inhibit the serotonin transporter  
325 (5-HTT) which determine the accumulation of this neurotransmitter in the synaptic cleft, enhancing  
326 the interaction with 5-HT receptors. These receptors are characterized by seven subtypes of  
327 transmembrane peptides which modulate different downstream effects and multiple cascades of  
328 biochemical reactions (Figure 1); the activation of 5-HT1 and 5-HT5 receptors results in the inhibition  
329 of adenylyl cyclase activity (AC), decreased cellular levels of cAMP and of cAMP-dependent protein  
330 kinases (PKA); conversely, the other receptors (5-HT4, 6, 7) produce an opposite, excitatory response  
331 increasing cellular levels of cAMP and the activation of downstream signalling pathways including  
332 MPAK, ERK 1/2 and the CREB cascade resulting in the induction of BDNF gene involved in  
333 neuroimmune regulation, fatty acid oxidation and gluconeogenesis (Faure et al. 2006).

334 Despite simpler than in mammals, serotonergic signalling system is evolutionary conserved  
335 and active in marine organisms, including fish and molluscs, where serotonin is involved in many  
336 neurophysiological processes, including sensitization and facilitation of withdrawal reflexes, feeding  
337 behaviour, locomotion and reproduction. Exposure of *M. galloprovincialis* to environmental  
338 concentrations of FLU (0.03-300 ng/L) significantly decreased cAMP levels and PKA activity: these  
339 effects were consistent with the inhibition of serotonin reuptake by FLU, its accumulation in post-  
340 synaptic cleft, more elevated interaction with 5-HT1 and consequent inhibition of AC activity (Fabbri,  
341 2015, Franzellitti et al. 2014).

342 Interestingly, the 5-HT1 receptor, coupled to the inhibition of AC, is the unique serotonin receptor  
343 pharmacologically demonstrated in invertebrates. Mussels exposed to FLU exhibited a significant  
344 upregulation of 5-HT1 mRNA and down-regulation of the ATP-binding cassette (ABC) transporter  
345 P-glycoprotein (Pgp), a key component of the multixenobiotic resistance (MXR) mechanism: the

346 modulation of Pgp by SSRIs supports its role in mussels as a general response toward a wide range  
347 of chemical stressors (Fabbri, 2015, Franzellitti et al. 2014).

348 The increased serotonergic neurotransmission in response to SSRIs has been postulated to  
349 represent a key process in marine species, influencing many physiological responses (Fabbri, 2015).  
350 In this respect, neurotoxic effects of low levels of FLU (0.03-5 µg/L) were reported for various  
351 bivalves, including *Mytilus.spp* and *R. philippinarum* with a significant inhibition of  
352 acetylcholinesterase activity (Figure 1), leading to impairment of numerous cholinergic pathways,  
353 hyperstimulation of nicotinic and muscarinic receptors, and disrupted neurotransmission (Cortez et  
354 al. 2018, Franzellitti et al. 2014, Gonzalez-Rey & Bebianno, 2013, Munari et al. 2014). In response  
355 to FLU and SER the amphipod *Echinogammarus marinus* showed a significant downregulation of  
356 *rhodopsin* and *arrestin*, neurological genes related to behaviour and phototransduction cascade, while  
357 juvenile oysters, *C. gigas*, chronically exposed to FLU (28-days, 0.1 and 10µg/L), highlighted a  
358 transient stimulation of shell growth suggesting a role of serotonin in the regulation of feeding and  
359 metabolism in bivalves (Bossus et al. 2014, Gonzalez-Rey & Bebianno, 2013). Additional studies  
360 further demonstrated serotonergic effects on locomotion in various marine invertebrates, regulating  
361 pedal ciliary activity in marine snails (*Tritonea diomedea*), pedal muscle contractions and swimming  
362 in nudibranchs (*Melibe leonine*), gill ciliary activity in mussels and oysters, increased locomotion in  
363 crabs (*C. maenas*) and in amphipods (*E. marinus*), inhibited swimming behaviour in early life stages  
364 of barnacles (*Amphibalanus Amphitrite*), rotifers (*Brachionus plicatilis*) and mussels (*M.*  
365 *galloprovincialis*) (Estévez-Calvar et al. 2017, Fong & Ford, 2014). Low levels of FLU (1-10 ng/L)  
366 caused phototactic responses in amphipods (*Gammarus pulex*) and inhibited striking prey efficiency  
367 in newborn cuttlefish (*Sepia officinalis*). Similarly, VEN (5 ng/L) acted as a neurodevelopmental  
368 toxicant for early life stages of *S. officinalis*, affecting the architecture of the vertical lobe, a key brain  
369 structure for cognitive processing: a decrease in norepinephrine and in the relative number of NMDA-  
370 like receptor binding sites was observed after 20 days of exposure, supporting a neurological



371 mechanism of action (Bidel et al. 2016). In the same study, a higher exposure concentration (100  
372 ng/L) impaired the camouflage ability of the cuttlefish, a critical behaviour for its survival.

373         Beside their effects on serotonergic pathway, SSRIs have also either antioxidant or pro-  
374 oxidant activities depending on tissues, dose and presence of a pre-existing oxidative insult (Stefan  
375 et al. 2020). In this respect, mechanisms of action include modulation of intracellular ROS formation,  
376 antioxidants levels and interactions with key redox signalling pathways, such as the Keap1–Nrf2, NO  
377 and NF- $\kappa$ B pathways (Figure 1). Among the antioxidant effects, SSRIs can decrease the ROS levels  
378 by suppressing immune cells and secretion of interleukins typically associated to an over-production  
379 of oxyradicals; the inhibition of 5-HT reuptake and related metabolism (Kumar & Kumar 2009), as  
380 well as the reduced activity of CYP450 (i.e. CYP2D6, CYP1A2, CYP2C9, CYP2C19, CYP3A4)  
381 further lower the intracellular ROS release (Figure 1), thus contributing to enhance stability of  
382 antioxidant enzymes less subjected to protein carbonylation (Eren et al. 2007, Kumar & Kumar 2009,  
383 Rebai et al. 2017). Antioxidant mechanisms of SSRIs have been experimentally demonstrated also in  
384 terms of inhibition of NADPH oxidase (NOX) and consequent decrease of superoxide anion,  
385 inhibition of iNOS activity and NO levels, modulation of mitochondrial activity and apoptosis  
386 induction via the mitochondrial pathway (de Oliveira, 2016). In such less prooxidant cellular  
387 conditions, FLU increased cysteine availability, mRNA expression of glutamate cysteine ligase and  
388 glutathione synthesis (Eren et al. 2007, Moretti et al, 2012), while chronic exposure to SSRIs has  
389 been associated to enhanced levels of antioxidants through the up regulation of Nrf2 and expression  
390 of dependent genes (Bouvier et al. 2017) (Figure 1).

391         In contrast to the above antioxidant effects which typically occur in the presence of an already  
392 existing oxidative unbalance, SSRIs can act as prooxidants in non-stressed organisms, further  
393 complicating the prediction of their ecological impact in natural, field conditions. Prooxidant effects  
394 of FLU have been reported in terms of lipid peroxidation, disruption of cytosolic and mitochondrial  
395 membranes, increased mRNA expression of TNF- $\alpha$ , inflammation and apoptosis in hepatic tissues;  
396 FLU was shown to activate apoptotic pathway by increasing levels of proapoptotic Bax protein, while

397 lowering the expression of the antiapoptotic Bcl2 (Djordjevic et al. 2011). Modulation of the NF- $\kappa$ B  
398 pathway by SSRIs can further induce both antioxidant and prooxidant effects (Figure 1): the latter  
399 derive from the inhibition of Nrf2 and contemporary induction of NADPH oxidase while antioxidant  
400 effects are modulated through the increased transcription of several antioxidant genes, and by  
401 inhibiting ROS production via TNF- $\alpha$ .

402 Overall, data on oxidative effects of SSRIs agree with findings measured in several marine  
403 species. Induction of glutathione S-transferase activity was measured in *M. galloprovincialis*, *Perna*  
404 *perna* and *R. philippinarum* in response to FLU exposure (0.03-5 $\mu$ g/L), suggesting the activation of  
405 xenobiotic metabolism and antioxidant response: the concomitant impairment of immune parameters,  
406 decreased lysosomal stability, increment of MDA and a biphasic variation of antioxidant enzymes  
407 (CAT, GR, glutathione peroxidases, SOD) revealed a transient onset of oxidative insult (Franzellitti  
408 et al. 2014, Munari et al. 2014). FLU caused a significant down regulation of alkali-labile phosphates  
409 (ALP) in mussels gonads (*M. galloprovincialis*) suggesting potential endocrine disruptive effect of  
410 SSRIs, given the positive correlation between ALP and vitellogenin-like proteins levels (Franzellitti  
411 et al. 2014, Gonzalez-Rey & Bebianno, 2013, Munari et al. 2014). At physiological level, mussels  
412 (*M. californianus*) exposed for 107 days to environmental levels of FLU (0.3, 3, 30, and 300 ng/L)  
413 revealed adverse effects on algal clearance rates, growth and gonadosomatic index.

414 Among pharmaceuticals targeting the Central Nervous System, a particular environmental  
415 relevance should be given to carbamazepine (CBZ), an anticonvulsant drug used in human medicine  
416 to treat epilepsy, neuropathic pain and maniac disorders. In mammals, CBZ was shown to block the  
417 voltage-gated sodium channels preventing the generation of action potential and depolarization of  
418 cell, thus reducing the frequency of impulses during epileptic crisis (Ambrosio et al. 2002, Siebel et  
419 al. 2010); CBZ provoke the same effect also acting as an agonist at the gamma-aminobutyric acid  
420 (GABA) receptor with inhibition of glutamate release, and entry of chloride into the cell (Figure 1).

421 As mentioned above, biological targets of pharmaceuticals are evolutionarily conserved  
422 among vertebrates and invertebrates and many studies highlighted a certain similarity for the MoA

423 of CBZ in non-target marine species (Figure 1). The reduction of ion-channel opening by CBZ is at  
424 least partially mediated by a decrease of cAMP levels through direct inhibition of AC and consequent  
425 reduction of neuronal excitability (Fabbri, 2015). CBZ has been shown to inhibit AC and PKA  
426 activities in *Mytilus spp.*, and to down-regulate the expression of ABCB mRNA, potentially lowering  
427 the detoxification ability of mussels (Fabbri, 2015). Concerning the effects of CBZ on voltage-,  
428 ligand-gated channels and ion transporters (Na<sup>+</sup> + K<sup>+</sup> pump; Na<sup>+</sup>/Ca<sup>2+</sup> exchanger), mussels (*M.*  
429 *galloprovincialis*) revealed a significant down-regulation of sodium-dependent serotonin transporter  
430 (SC6A4) at environmental levels of CBZ (1 µg/L, 28 days) (Mezzelani et al. 2021). Similarly, the sea  
431 bream (*Sparus aurata*) exposed to low levels of this drug (6.95 µg/L, 28 days) highlighted the  
432 transcriptional modulation of genes involved in transmembrane transport of Ca<sup>2+</sup> such as calcium  
433 binding protein v2-like, calcium calmodulin-dependent serine protein kinase (CASK), and potassium  
434 intermediate/small conductance calcium-activated channel (KCNN4), representing an additional  
435 proof of similar mechanisms of action of CBZ across species (Hampel et al. 2017).

436 In mammals, CBZ undergoes to hepatic biotransformation resulting in epoxidation, aromatic  
437 hydroxylation, and conjugation reactions (Figure 1). The major drug-metabolizing enzymes involved  
438 in CBZ biotransformation include CYP3A4, CYP3A5, CYP2C8, EPHX1 and UGT2B7, with  
439 carbamazepine metabolites being excreted through the ABCC2 drug transporter. The cytochrome  
440 P450 biotransformation pathway and the enhanced production of ROS are partly responsible the  
441 major long-term side effects of CBZ, and similar metabolic pathways have been reported in non-  
442 target marine species exposed to low environmental levels of this drug. In *M. galloprovincialis*, CBZ  
443 (1 µg/L, 28 days) caused the up-regulation of genes involved in drug metabolism such as cytochrome  
444 P450 (CYP4F8, CYP3A2, CYP3A29) and sulfotransferases (SULT1B1), as well as the differential  
445 expression of genes involved in cell protection, cell cycle and DNA repair. Among these, a significant  
446 upregulation was observed for the stress induced protein Sestrin-3 (SEST3) that has a role in  
447 protection against oxidative and genotoxic stress, for genes belonging to GIMAP family (GIMAP4,  
448 GIMAP7) with a role in defence, cell differentiation and apoptosis, and for genes coding for

449 Baculoviral IAP repeat-containing protein (BIRC1, BIRC7), which modulate apoptotic processes. It  
450 should be also highlighted the significant up-regulation of several genes modulating immune response  
451 and inflammation (MYNA, LYZ1, DEFI, MRC1, IRG1, LITAF and PLA2) (Mezzelani et al. 2021).

452 Consistent with molecular evidences, CBZ (0.3-9µg/L) was shown to provoke cellular effects  
453 potentially impairing the health status of marine organisms (Figure 1). Onset of oxidative stress,  
454 activation of immune responses and neurotoxicity were observed in the bivalves *M. galloprovincialis*,  
455 *Venerupis decussata*, *R. philippinarum*, *Scrobicularia plana* and in the polychaete *Diopatra*  
456 *neapolitana*, revealing the induction of CYP450 3A4, modulation of antioxidant enzymes (CAT,  
457 SOD, GR and GST), accumulation of lipid peroxidation products (malondialdehyde, lipofuscin,  
458 neutral lipids), decrease of lysosomal membrane stability, inhibition of phagocytosis capacity and  
459 increase of AChE activity (Almeida et al. 2020a, Freitas et al. 2016, Hampel et al. 2017, Mezzelani  
460 et al. 2021).

461 CBZ was also documented to cause effects possibly leading to adverse consequences at higher  
462 levels of biological organization. In early life stages of *M. galloprovincialis*, environmental  
463 concentrations of CBZ affected embryo development with appearance of shell malformations in D-  
464 veligers 48 h post fertilization even at 0.01µg/L. Transcriptional analyses revealed the down-  
465 regulation of genes related to homeostasis of carbonate chemistry at the site of calcification (EP) and  
466 in organic matrix synthesis (CS); these genes control calcification rates and morphology of the shell,  
467 suggesting a mechanistic explanation for the negative impact of CBZ on regulation of shell  
468 biogenesis. The role of CBZ as endocrine disruptor, interfering with synthesis, bioavailability or  
469 breakdown of juvenile hormones or ecdysteroids, was highlighted by the significant modulation of  
470 androgen and estrogen metabolism (Hampel et al. 2017). At organismal level, this drug altered  
471 physiological condition (CI) and gonadosomatic index (GSI) in mussels, leading to hypothesize a  
472 potential impairment of reproductive capacity while lower values of CI in chronically exposed  
473 organisms were probably related to the reduced feeding capacity (Oliveira et al. 2017). Overall, these  
474 responses could trigger a slow-down in the metabolism, resulting in a decrease of the reproductive

475 performance and energy reserves, with long-term adverse consequences on population sustainability  
476 (Oliveira et al. 2017).

477

### 478 *3.3 Cardiovascular drugs and lipid regulating agents*

479 Cardiovascular drugs (CVDs) are largely used in human medicine for treatment of  
480 hypertension, myocardial infarction, heart failure and coronary artery disease. CVDs include an array  
481 of compounds that directly regulate the function of the heart and blood vessels, like beta-blockers,  
482 angiotensin converting-enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs) and  
483 calcium channel blockers. These drugs are commonly prescribed with lipid regulating agents (LRs),  
484 such as statins and fibrates, which indirectly reduce the risk of severe cardiovascular diseases by  
485 treating dyslipidaemias. Compared to other therapeutical classes described so far, ecotoxicological  
486 impacts of CVDs and LRs are quite unexplored, especially in marine species, with a few data  
487 available mostly for beta-blockers, angiotensin II receptor blockers and lipid regulators.

488 Beta-blockers target  $\beta$ -adrenergic receptors ( $\beta$ -Ars) which, after stimulation from endogenous  
489 catecholamines, activate the upregulation of adenylyl cyclase converting ATP to cAMP; the latter is  
490 used by cAMP-dependent protein kinase A (PKA) to phosphorylate calcium channels, thus increasing  
491 cellular calcium influx available for muscle contraction. By antagonizing  $\beta$ -Ars, beta-blockers (i.e.  
492 propranolol PRP, atenolol ATE, metoprolol MET) prevent the binding of norepinephrine and  
493 epinephrine, decreasing levels of cyclic AMP and PKA (Figure 1).  $\beta$ -blockers can also induce  
494 behavioural side-effects and depressive moods, indicating that they affect some central nervous  
495 mechanisms due to their high affinity towards serotonin receptors (Alhayek & Preuss 2021). ACE  
496 inhibitors and angiotensin II receptors blockers (ARBs) limit the action of the hormone angiotensin  
497 II which has constricting effects on blood vessels, as well as on salt and water retention: while ACE  
498 inhibitors reduce the levels of this compound by targeting the enzyme catalysing its formation (renin),  
499 ARBs (like Valsartan VAL and Lorsartan LRS) selectively bind to angiotensin type 1 (AT1)  
500 receptors.  $\beta$ -blockers are metabolized by P450 (CYP1A2 and CYP2D6) and phase II glucuronidation

501 reactions (Figure 1), while the majority of ARBs are poorly influenced by CYP enzymes and directly  
502 undergo to de-ethylation and glucuronidation processes. Among lipid regulators, statins decrease the  
503 levels of cholesterol competitively inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A reductase  
504 (HMG-CoA reductase), the main enzyme responsible for its biosynthesis, while fibrates lower  
505 triglycerides and low-density lipoproteins by stimulating the peroxisomal  $\beta$ -oxidation of fatty acids  
506 (Pahan, 2006) (Figure 1).

507         Despite investigations on the effects of CVDs and LRs in non-target organisms are still rather  
508 limited (Zhang et al. 2020), adrenoceptors of the  $\beta$ -type were reported in marine species with relevant  
509 pharmacological properties similar to those of their mammalian counterparts (Fabbri, 2015). A wide  
510 range of environmentally realistic concentrations of PRP (0.3–300 ng/L) significantly decreased  
511 cAMP levels and protein kinase A (PKA) activities in digestive gland of *M. galloprovincialis*,  
512 consistent with the antagonistic effect of this drug on  $\beta$ -Ars and serotonin receptors: these  
513 biochemical observations were paralleled by a similar pattern of mRNA expression for the ABCB1  
514 gene, encoding for the membrane transporter Pgp which is also modulated by cAMP and PKA  
515 (Fabbri, 2015).  $\beta$ -adrenergic receptors were transcriptionally regulated in zebrafish embryos exposed  
516 to propranolol (10  $\mu$ g/L and above for 96 h) and additional molecular effects of beta-blockers were  
517 observed on antioxidant responses (CAT) and apoptosis pathway (p53, PUMA) (Sun et al. 2014).  
518 The involvement of oxidative stress in metabolism of beta-blockers by marine organisms was  
519 confirmed in mussels exposed to PRP showing decreased lysosomal membrane stability, significant  
520 variations of antioxidant enzymes (CAT, GST), induction of carboxylesterase in digestive gland and  
521 inhibition of AChE in gills (Fabbri, 2015, Solé et al.2010) (Figure 1).

522         The comparison of pharmaceuticals effects between target and non-target species is difficult  
523 when considering angiotensin II receptor blockers (ARBs), given the paucity of investigations  
524 performed in marine species. In this respect, LRS (300-3000 ng/L), caused the induction of CYP450  
525 like activity, glutathione S-transferases and glutathione peroxidases in mussel *Perna perna* along with  
526 the onset of oxidative and cyto-genotoxic effects (Zhang et al. 2020).

527 A few studies carried out on lipid regulators indicated a significant reduction of triglycerides  
528 and an increase of fatty acids in of zebra mussels exposed to low levels of clofibrate (0.2 µg/L,  
529 Lazzara et al. 2012), while gemfibrozil (1 µg/L-1000 µg/L) caused oxidative stress conditions with  
530 enhanced lipid peroxidation and glutathione transferases (GSTs) (Quinn et al. 2011). Variations of  
531 plasma triglycerides and cholesterol levels in fish exposed to gemfibrozil were reflected in changes  
532 of representative hepatic genes involved in lipid metabolism, including FABP, APOA1, APOEB,  
533 FASN, SREBP2, HMGCR1, PPARA and SREBP1 (Al-Habsi et al. 2016, Skolness et al. 2012).

534 At physiological level 19-days of PRP exposure (100-1000µg/L) significantly decreased  
535 byssus strength and energy available for growth and reproduction in *M. edulis*, suggesting possible  
536 consequences at the population level. PRP affected the embryonic development of sea urchin  
537 *Paracentrotus lividus* at 5µg/L and led to fecundity decrease, growth inhibition and alterations of  
538 heart and metabolic rates in *Daphnia magna*, with reported multi-generational effects (Zhang et al.  
539 2020).

540

#### 541 3.4 Steroidal Hormones/estrogens

542 An increasing body of evidence has demonstrated the high environmental concern for  
543 steroidal hormones, provoking deleterious effects in non-target marine species at concentrations one  
544 order of magnitude lower than those of other active principles (Almeida et al. 2020b, Aris et al. 2014,  
545 Capoluopo et al. 2018, Roark, 2020). The main target of steroids hormones is the endocrine system,  
546 responsible for maintaining organisms homeostasis, development, behaviour and reproduction (Aris  
547 et al. 2014). Although various hormones have been measured in marine matrices, the majority of  
548 studies focussed on estrogens and particularly on 17α-ethinylestradiol (EE2), widely used as a major  
549 component in oral contraceptives, and considered the synthetic drug with the highest endocrine  
550 disrupting potency (Almeida et al. 2020b).

551 Physiological estrogens are produced from cholesterol and a detailed discussion on their  
552 effects is outside the aim of this paper since excellent reviews are already available both for mammals

553 and marine organisms (Fuentes & Silveyra, 2019, Milla et al. 2011). Very briefly, the classical  
554 mechanism of direct genomic signalling is modulated by the nuclear estrogen receptors (ERs) which  
555 act as ligand-activated transcription factors (Marino et al. 2006, O'Malley, 2005). Upon binding in  
556 the cytoplasm with estrogens (or estrogen-like xenobiotics), a conformational change of ER occurs,  
557 inducing receptor dimerization, the complex is then translocated to the nucleus, and its binding to  
558 chromatin at ERE sequences, enhances transcription of target genes (Figure 1). Estrogens can also  
559 regulate transcription of other genes by indirect genomic signalling when the estrogen receptor  
560 complexes do not bind directly to ERE sequences, but rather interact with other transcription factors  
561 (TFs) and response elements, thus activating or suppressing target gene expression (Figure 1). Along  
562 with the genomic signalling, estrogens are able to exert rapid cellular effects through nongenomic  
563 mechanisms. The interaction with membrane bound ER directly activates signalling cascades, such  
564 as the Ras/Raf/MAPK cascade, the phosphatidyl inositol 3 kinase (PI3K)/Akt kinase cascade, and the  
565 cAMP/ PKA signalling pathways which modulate intracellular levels of  $Ca^{2+}$  and NO, as well as the  
566 expression of ERE and TF regulated genes (Figure 1).

567 In a variety of tissues, ERs coexist with serotonin receptors, and many studies highlighted  
568 effects of estrogens signalling on serotonergic pathway. Both natural and pharmacologically induced  
569 changes in estradiol (E2) levels alter the concentration of serotonin, i.e. enhancing the activity of  
570 tryptophan hydroxylase (TPH), the rate-limiting step in synthesis of this neurotransmitter from  
571 tryptophan (Figure 1). In addition, E2 inhibits the gene expression of the serotonin reuptake  
572 transporter (5-HTT) and acts as an antagonist at the 5-HTT, thus promoting the permanence of  
573 serotonin in synapses and interstitial spaces (Rybaczynk et al. 2005). The estrogens signalling pathway  
574 is also closely related with prostaglandins and arachidonic acid metabolism (Figure 1), and the  
575 modulation of PG receptors by sex steroids has been shown as fundamental for key reproductive  
576 processes (Blesson et al. 2012).

577 In teleost fishes, three estrogen receptors have been characterized,  $ER\alpha1$ ,  $ER\beta1$  and  $ER\beta2$ ,  
578 and agonistic activity of EE2 revealed astonishing similarities in molecular pathways of this



579 signalling system between mammals and fishes (Amenyogbe et al. 2020). One of the most commonly  
580 measured effects in fish is the increment of vitellogenin (VTG), an estrogen-inducible yolk precursor  
581 protein normally produced only by mature females but detected also in juveniles and males of fish  
582 exposed to estrogen-like compounds. The synthesis of VTG is initiated with activation of ERs  
583 signalling pathway by estrogens, the enhancement of VTG gene expression, translation and  
584 maturation of the protein in the endoplasmic reticulum and Golgi apparatus, before the final  
585 packaging into secretory vesicles and release into the circulatory system. Feminization of males is a  
586 more severe effect reported in fish exposed to EE2, with development of ovotestes in oviparous  
587 species, presence of oocytes in male gonads, impaired spermatogenesis, decrement of sperm motility  
588 and sperm counts (Almeida et al. 2020b, Aris et al. 2014, Notch et al. 2007). Additional detrimental  
589 effects include an increased frequency of cancers allowing to hypothesize EE2 as a promoter of  
590 hepatic tumour formation, by reducing the capability to repair DNA adducts by nucleotide excision  
591 repair (NER) processes (Aris et al. 2014, Notch et al. 2007). Behavioural studies provided further  
592 support to adverse physiological effects of estrogens with predictable consequences at the population  
593 level. Among the several reported evidences, the sand goby *Pomatoschistus minutus* exposed to EE2  
594 (41ng/L, 31 days) revealed altered reproductive behaviour of males which normally exhibit specific  
595 movements to attract the females' attention and to provide parental care to developing eggs (Saaristo  
596 et al. 2010). Exposure to low levels of EE2 (1 ng/L, 10 days) impacted secondary sexual trait  
597 expression and mating dynamics of the Gulf pipefish *Syngnathus scovelli*, a species in which males  
598 receive and fertilize eggs into a specialized brood pouch where developing embryos are carried for  
599 approximately two weeks: the appearance in adult males of female-like secondary sexual traits, while  
600 not directly affecting their reproductive capability, induced females to discriminate in mate choice  
601 trials, an effect which would reduce male mating opportunities and long-term reproductive success  
602 in natural populations (Partridge et al. 2010). Similarly, in the sex-role reversal seahorse  
603 *Hippocampus erectus*, environmentally relevant concentrations of EE2 and progesterone (5 ng/L, 50

604 ng/L, 10 ng/L, 100 ng/L, 60 days), significantly inhibited male brood pouch development, impaired  
605 the expression of spermatogenesis genes in the testes and caused male feminization (Qin et al. 2020).

606 Knowledge on the effects and mechanisms of action of estrogens is more limited for  
607 invertebrates (Almeida et al. 2020b) but the presence of sex-dependent steroids and steroidogenic  
608 pathways has been described in different invertebrate groups, particularly in molluscs, where their  
609 role has been elucidated in the control of gametogenesis (Janer & Porte, 2007, Porte et al. 2006).  
610 Estrogen-like receptors have been characterized in gastropods and cephalopods and corresponding  
611 sequences have been identified in bivalves (Canesi et al. 2010). Although phylogenetically clustered  
612 with other steroid receptors, molluscan ERs appear to be functionally different, with a constitutive  
613 transcriptional activity, not further activated by estrogens. Nonetheless, 17 $\beta$ -estradiol (E2) was shown  
614 to activate 'alternative' modes of action in ganglia and immune cells of *Mytilus* spp, i.e. through  
615 modulation of Ca<sup>2+</sup> and kinase-mediated cascades (Canesi et al. 2004, 2006). Environmentally  
616 realistic concentration of EE2 (5 and 50 ng/L) determined significant increase in the expression of  
617 VTG and estrogen receptor 2 (ER2) in both female and male mussels (*M. edulis*), along with the  
618 decrease in serotonin receptors and COX mRNA levels (Almeida et al. 2020b). In *M.*  
619 *galloprovincialis* E2 affected different functional parameters and increased the expression of  
620 antioxidant genes in hepatopancreas (Canesi et al. 2010).

621 Available studies on marine invertebrates (polychaete worms, molluscs, and crustaceans)  
622 indicate that EE2 can cause developmental delays and female-biased sex ratios (Roark, 2020). In  
623 bivalves, changes in the reproductive function and energy metabolism were frequently observed. A  
624 lowered percentage of fertilized eggs and of normal larvae were caused in *M. galloprovincialis* by  
625 exposure to EE2 (5–500 ng/L, 48 h), probably due to the strong energy depletion on spermatozoa,  
626 resulting in a decreased viability when finally exposed to eggs (Almeida et al, 2020b). Gonadal atresia  
627 and a delayed gonadal development were caused by EE2 (50 and 500 ng/L, 10 days) in *M. trossulus*,  
628 while the rock oysters *Saccostrea glomerata* (at EE2 50 ng/L, 56 days) exhibited VTG induction and  
629 intersex in males and females, highlighting the role of estrogens in modulating steroidogenesis and

630 sexual reversion in molluscs (Almeida et al, 2020b). Overall, these data support the hypothesis that,  
631 although the invertebrate ERs do not mediate genomic estrogen signalling, conserved nongenomic  
632 pathways are likely candidates for similar mechanisms of action.

633

### 634 *3.5 Antibiotics*

635 A final comment on environmental pharmaceuticals should be related to antibiotics.  
636 Compared to other therapeutical classes, these drugs represent an emerging environmental hazard for  
637 the development of antibiotic resistant bacteria (ARB) and transfer of antibiotic resistance genes  
638 (ARGs), more than for the potential ecotoxicity of such molecules toward non-target species (Väitalo  
639 et al. 2017).

640 Antibiotic resistance derives from selective pressure on sensitive bacteria resulting in  
641 enrichment of ARGs, which are normally present in microbial communities at background levels.  
642 Further exacerbated by environmental factors and co-occurring contaminants, the presence of ARGs  
643 confers resistance to antibiotics mostly through transmembrane efflux pumps, enzymatic  
644 deactivation, and cellular protection (Carvalho & Santos, 2016, Zheng et al. 2021).

645 In the marine environment, ARGs may be subjected to horizontal gene transfer (HGT)  
646 representing a possible risk for organisms and humans: in this respect, the World Health Organization  
647 recognized the occurrence of ARB and ARGs as one of the most important public health concerns of  
648 this century (Ben et al. 2019, Kovalakova et al. 2020). ARGs can be transferred by direct contact with  
649 seawater or indirectly through food webs and seafood consumption. Shaping typology and resistance  
650 of gut bacteria, the transfer of ARGs can have adverse effects on important functions like digestive  
651 processes, immune responses and vulnerability to infectious diseases (Zheng et al. 2021).

652 The environmental impact of antibiotics and ARGs has been shown particularly on microbial  
653 communities, affecting richness and diversity of primary producers and decomposers, which are  
654 essential for the microbial ecosystem functioning (Zheng et al. 2021). Cyanobacteria and ammonium  
655 oxidizing bacteria appeared sensitive to antibiotics such as amoxicillin, ampicillin, ciprofloxacin and

clarithromycin, with EC50 values similar to realistic seawater concentrations (reviewed by Väitalo et al. 2017). Reported effects spanned from protein synthesis and inhibition or interference with DNA replication, to the modulation of photosynthesis-mediated calcification and enhancement of cyanobacteria biofilms formation; the latter results highlight how antibiotic residues in marine environment may also influence the biofilm-associated ecological functions of cyanobacteria, i.e., promoting precipitation of carbonate and the increment of atmospheric carbon dioxide concentration (Kovalakova et al. 2020, Väitalo et al. 2017). At a lower extent compared to cyanobacteria, also green algae exhibit a certain sensitivity toward antibiotics: several species, such as *Pseudokirchneriella subcapitata*, *Desmodesmus subspicatus*, *Chlorella vulgaris*, *Scenedesmus vacuolatus*, and *Tetraselmis suecica*, were affected by the macrolides clarithromycin and erythromycin with EC50 values below 1 mg/L: mechanisms of toxicity could be related to the inhibition of pathways involved in chloroplast and photosynthetic metabolism, leading to final impairment of cell growth (Väitalo et al. 2017). Long-term exposure to environmental levels of sulfamethoxazole and norfloxacin inhibited the green microalgae *Chlorella sp.*, while stimulated the growth of *Prorocentrum lima*, indicating a role of antibiotics in the bloom of red tides (Niu et al. 2019). The bactericide and bacteriostatic effects of these compounds can also cause disappearance of some microbial subpopulations with consequent effects on their ecological functions like modulation of biogeochemical cycles, changes in nitrogen transformation, methanogenesis, sulfate reduction, nutrient cycling, and organic matter degradation (Kokalokova et al. 2020): the inhibition of denitrification by sulfonamides was shown to stimulate the release of nitrous oxide (N<sub>2</sub>O) with consequent enhancement of eutrophication processes and greenhouse effects (Mezzelani et al. 2018a).

Beside the indirect effects modulated by ARGs, knowledge is actually limited on direct, chronic toxicity of environmental antibiotics in non-target species. In this respect, the clam, *R. philippinarum* exposed to realistic concentrations of trimethoprim, highlighted the alteration of haemocytes parameters, with a significant decrement in lysosomal membrane stability, while oxidative stress responses were only slightly affected (Binelli et al. 2009, Matozzo et al. 2015).

682 Similarly, low doses of sulfamethoxazole affected the metabolomic profile in *M. galloprovincialis*,  
683 with alterations in amino acids levels (aspartate, phenylalanine, valine, and tryptophan) pinpointing  
684 disturbances in osmotic regulation and energy metabolism (Serra-Compte et al. 2019). Mechanisms  
685 of action of antibiotics in non-target species are still to be clarified, and further effort is thus needed  
686 to fill this gap of knowledge (Kovalakova et al. 2020, Vålitalo et al. 2017).

687

## 688 **5. Final Thoughts**

689 Environmental consequences of pharmaceutical residues have recently emerged as a major  
690 research area in marine science. Fragmented information is still available for non-target species, with  
691 prevalence of studies focussing on NSAIDs and psychiatric drugs compared to cardiovascular,  
692 synthetic steroidal hormones and antibiotics. Well-documented effects of single classes of  
693 pharmaceuticals, dosed at low and environmentally realistic concentrations, evidenced marked  
694 similarities in modes of action between target and non-target species, showing the same cellular  
695 pathways involved in metabolism or onset of adverse consequences.

696 However, a key feature to consider when assessing the impact of pharmaceuticals on marine  
697 species is the typology and conditions of exposure. Target organisms typically assume specific  
698 compounds intended to alleviate a particular disturbance at defined posology and time of treatment:  
699 conversely, in field conditions non-target species are exposed, potentially for the entire duration of  
700 their life cycle, to low doses of several classes of co-occurring drugs that, at the same time, will  
701 modulate a variety of pathways and metabolic processes. This review highlighted the complexity of  
702 such interactions, revealing either synergistic or antagonistic effects on the same cellular targets.  
703 Noteworthy, the intricate network of mechanisms regulating organism responsiveness to  
704 pharmaceuticals might be further challenged by the simultaneous presence of other typologies of  
705 chemical pollutants (trace metals, polycyclic or halogenated hydrocarbons, microplastics, biotoxins,  
706 etc.) or environmental stressors (such as ocean acidification and temperature increase) which target  
707 the same cellular pathways.

708           The reviewed results need to be interpreted with a certain caution, given the high variability  
709 in reported biological endpoints, doses and mode of exposures, typologies of investigated drugs, life  
710 stage and characteristics of non-target species. Nonetheless, the intrinsic limit of a single-chemical  
711 approach appears evident, and predictions obtained evaluating potential risk of individual classes of  
712 pharmaceuticals, might not necessarily be confirmed in natural, environmental conditions.

713           Future research and field monitoring studies need to consider more comprehensive strategies  
714 for assessing the impact of multiple stressors, particularly considering that the continuous  
715 development of new drugs makes risk assessment of pharmaceuticals not affordable on a chemical  
716 characterization alone. Due to the lack of acute effects, it is imperative to move toward evaluation of  
717 chronic effects for which, however, the contribution of various stressors is more difficult to  
718 disentangle. Despite convincing mechanistic hypotheses can explain molecular or cellular effects of  
719 pharmaceuticals, only a few studies documented a progression of adverse consequences on  
720 physiological performances as a function of dose and time of exposure. In this respect, links with  
721 organismal, population, community or ecosystem functioning are still hard to be forecasted, but not  
722 less important for this difficulty.

723

#### 724 **Disclosure Statement**

725           The authors, Marica Mezzelani and Francesco Regoli, declare that they have no known competing  
726 financial interests or personal relationships with other people or organizations that could have  
727 inappropriately influenced the work reported in this paper.

728

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## CAPTION OF FIGURE 1

**Figure 1:** Pathways of action, metabolism and toxicity of the most frequently detected therapeutic classes in marine environment: non-steroidal anti-inflammatory drugs (NSAIDs), psychiatric drugs (Selective Serotonin Reuptake Inhibitors SSRIs and carbamazepine CBZ), cardiovascular drugs ( $\beta$ -blockers), lipid regulators (statins and fibrates), steroidal hormones (estrogens). Acronyms arranged in alphabetical order: ABCC2: ATP Binding Cassette Subfamily C Member 2; ABC: ATP-binding cassette transporters; AC: adenylyl cyclase; AChE: acetylcholinesterase; AKT: protein kinase B; AP: activator protein; AP-1: activator protein 1; ARE: antioxidant responsive element; ATP: adenosine triphosphate; BAX: Bcl-2-associated X protein; BCL2: B-cell lymphoma 2 protein; BDNF: brain-derived neurotrophic factor; cAMP: cyclic adenosine monophosphate; CASP1: caspase 1; CAT: catalase; CBZ: carbamazepine; COX: cyclooxygenase; COX1: cyclooxygenase 1; COX2: cyclooxygenase 2; CREB: cAMP response element-binding protein; CYP450: cytochrome P450 enzymes; Cys: cysteine; E: estrogen; E-like: estrogen like compounds; EPAC: exchange protein activated by cyclic AMP; EPHX1: epoxide Hydrolase 1; ER: estrogen receptor; ERE: estrogen response element; ERK1/2: extracellular signal-regulated kinases 1 and 2; GABA:  $\gamma$  amino-butyric acid receptor; GCL: glutamate-cysteine ligase; *Glu*: glutamate; *Gly*: glycine; GPx: glutathione peroxidases; GS: glutathione synthetase; GSH: reduced glutathione; GSSG: oxidized glutathione; GST: glutathione S-transferase; HMGCoA:  $\beta$ -Hydroxy  $\beta$ -methylglutaryl-CoA; HSP70 I/D: heat shock protein 70 I and 70D; HSP90: heat shock protein 90; IL1 $\beta$ : interleukin 1 beta; iNOS: inducible nitric oxide synthetase; Keap1: Kelch-like ECH-associated protein 1; LOX: leukotrienes; MAO: monoamine oxidase; MAPKs: mitogen-activated protein kinases; NF-kB RE: nuclear factor-kB responsive element; NF-kB: nuclear factor-kB; NMDA: N-methyl-D-aspartate receptor; NOD: nucleotide-binding oligomerization domain proteins; NOX: NAPH oxidases; Nrf 2: nuclear factor (erythroid-derived 2)-like 2; NSAIDs: non-steroidal anti-inflammatory drugs; P: phosphate; p53 RE: protein 53 responsive element; p53: protein 53; PGD2: prostaglandin D2; PGE2: prostaglandin E2; PGF2: prostaglandin F2; PGG2: prostaglandin G2; PGH2: prostaglandin H2; PGI2: prostaglandin I2; PgP: P-glycoprotein; PKA: protein kinase A; PLA: phospholipase; PLA2: phospholipase A2; PIK3: Polo like kinase 3; PPAR: peroxisomal proliferator activated receptor; PPAR $\alpha$ : peroxisomal proliferator activated receptor  $\alpha$ ; PPAR  $\beta$ : peroxisomal proliferator activated receptor  $\beta$ ; PPRE: peroxisomal proliferator responsive element; Pro- IL1 $\beta$ : Pro-inflammatory interleukin 1 beta; Ras/Raf/MEK: Ras-signalling cascade; RXR: retinoid X receptor; SOD: superoxide dismutase; SSRIs: selective serotonin re-uptake inhibitors; SULT1B1: sulfotransferase Family 1B Member 1; TF: transcription factor; TLR: Toll like receptors; TPH: tryptophan hydroxylase; *Trp*: tryptophan; TxA2: thromboxane A2; UGTs: UDP-glucuronosyltransferases; XOR: xanthine oxido-reductase;  $\beta$ -

1040    Ars:  $\beta$ -Adrenergic receptors; 5HIAA: 5-Hydroxyindoleacetic acid; 5HT 1,4,5,6,7: 5-  
1041    hydroxytryptamine receptors; 5HT: 5-hydroxytryptamine (Serotonin); 5HT1A: 5-hydroxytryptamine  
1042    1A receptor; 5HT1B: 5-hydroxytryptamine 1B receptor; 5HTT: 5-hydroxytryptamine transporter.

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