The Biological Effects of Pharmaceuticals in the Marine Environment

Biological effects of pharmaceuticals in the marine environment

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Abstract

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

37 **1. Introduction on pharmaceuticals in the marine environment**

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

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

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

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

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

Common classes of pharmaceuticals occurring in marine ecosystems and accumulated by non-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 17β-
93 estradiol (E2), 17α-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 lormetazepam (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, metprolol, 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_{50} 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_2). 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 G2 (PGG2), before its reduction
190 to prostaglandin H2 (PGH2), 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 ABCC2 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-κB 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- kB pathway in both *Mytilus spp.* and clam *R. philippinarum,* increasing the mRNA
266 level of a genuine NF-kB gene (Maria et al. 2016, Milan et al. 2013). NF-kB 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

269 cytoplasm, NF-kB is translocated to the nucleus, binds to specific DNA sequences (NF-kB responsive
270 elements) and regulates the transcriptional induction of iNOS, as well as a number of genes mediating
271 inflammatory responses and apoptosis (Regoli & Giuliani, 2014). Also genes involved in the PPAR
272 signalling pathway (i.e. acetyl-coenzyme A acyltransferase 1, phosphoenolpyruvate carboxykinase,
273 and stearoyl-CoA desaturase) were transcriptionally up-regulated in bivalves exposed to IBU,
274 confirming the effects of NSAIDs on lipid and fatty acid metabolism of non-target organisms
275 (Mezzelani et al. 2018b, Milan et al. 2013). Similar evidence was obtained for the effects of these
276 drugs on p53 pathway activated by stimuli including ROS, trace metals, DNA damage and hypoxia.
277 The p53 tumor suppressor protein is a transcription factor which binds to specific DNA regions, the
278 p53 responsive elements (Figure 1) enhancing transcription of stress response genes involved in a
279 variety of anti-proliferative processes, cell cycle checkpoints and arrest, DNA damage and repair,
280 apoptosis (Regoli & Giuliani, 2014). Among these, both mussels and clams exposed to NSAIDs
281 revealed an up-regulation of several related genes including a putative baculoviral IAP repeat-
282 containing 2 (BIRC2), X-linked inhibitor of apoptosis (XIAP), BCL2-associated athanogene
283 4(BAG4), B-cell CLL/lymphoma 2 (BCL2), suggesting increased anti-apoptotic activity in contrast
284 to effects measured in target species (Mezzelani et al. 2018a,b, Milan et al. 2013).

285 The effects of NSAIDs in promoting the generation of ROS and the related pro-oxidant
286 mechanisms in marine organisms were confirmed at the cellular, functional level by the significant
287 modulation of antioxidant enzymes (catalase CAT, superoxide dismutase SOD, glutathione reductase
288 GR, glutathione S-transferases GST), the enhancement of peroxidation products like
289 malondialdehyde and lipofuscin, the increment in micronuclei frequency and DNA damages observed
290 in *Mytilus spp.*, *R. philippinarum*, *Gibbula umbillicalis* and *Hediste diversicolor* (Bebianno &
291 Gonzalez-Rey, 2015, Mezzelani et al. 2018a,b, Milan et al. 2013). The decrement of neutral lipids
292 and the inhibition of Acyl-CoA oxidase activity (ACOX) in *Mytilus spp.* further supported COX-
293 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 serotoninergic 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 µgL) 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-κB 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-α, 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-kB
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-α.

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 (0.03-5µg/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 + -K+ pump; Na+/Ca2+ exchanger), mussels (*M.*
429 *galloprovincialis*) revealed a significant down-regulation of sodium-dependent serotonin transporter
430 (SC6A4) at environmental levels of CBZ (1µg/L, 28 days) (Mezzelani et al. 2021). Similarly, the sea
431 bream (*Sparus aurata*) exposed to low levels of this drug (6.95µg/L, 28 days) highlighted the
432 transcriptional modulation of genes involved in transmembrane transport of Ca^{2+} 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 β-adrenergic receptors (β-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 β-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). β-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. β-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 β-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 β-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 β-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). β-adrenergic receptors were transcriptionally regulated in zebrafish embryos exposed
516 to propranolol (10 μg/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 (Rybaczyk 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, ERα1, ERβ1 and ERβ2,
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, 17β-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^{2+-} 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 (Välitalo
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

656 clarithromycin, with EC50 values similar to realistic seawater concentrations (reviewed by Välitalo
657 et al.2017). Reported effects spanned from protein synthesis and inhibition or interference with DNA
658 replication, to the modulation of photosynthesis-mediated calcification and enhancement of
659 cyanobacteria biofilms formation; the latter results highlight how antibiotic residues in marine
660 environment may also influence the biofilm-associated ecological functions of cyanobacteria, i.e.,
661 promoting precipitation of carbonate and the increment of atmospheric carbon dioxide concentration
662 (Kovalakova et al. 2020, Välitalo et al.2017). At a lower extent compared to cyanobacteria, also green
663 algae exhibit a certain sensitivity toward antibiotics: several species, such as *Pseudokirchneriella*
664 *subcapitata, Desmodesmus subspicatus, Chlorella vulgaris, Scenedesmus vacuolatus,* and
665 *Tetraselmis suecica*, were affected by the macrolides clarithromycin and erythromycin with EC50
666 values below 1 mg/L: mechanisms of toxicity could be related to the inhibition of pathways involved
667 in chloroplast and photosynthetic metabolism, leading to final impairment of cell growth (Välitalo et
668 al. 2017). Long-term exposure to environmental levels of sulfamethoxazole and norfloxacin inhibited
669 the green microalgae *Chlorella sp.*, while stimulated the growth of *Prorocentrum lima,* indicating a
670 role of antibiotics in the bloom of red tides (Niu et al. 2019). The bactericide and bacteriostatic effects
671 of these compounds can also cause disappearance of some microbial subpopulations with consequent
672 effects on their ecological functions like modulation of biogeochemical cycles, changes in nitrogen
673 transformation, methanogenesis, sulfate reduction, nutrient cycling, and organic matter degradation
674 (Kokalokova et al. 2020): the inhibition of denitrification by sulfonamides was shown to stimulate
675 the release of nitrous oxide (N_2O) with consequent enhancement of eutrophication processes and
676 greenhouse effects (Mezzelani et al. 2018a).

677 Beside the indirect effects modulated by ARGs, knowledge is actually limited on direct,
678 chronic toxicity of environmental antibiotics in non-target species. In this respect, the clam, *R.*
679 *philippinarum* exposed to realistic concentrations of trimethoprim, highlighted the alteration of
680 haemocytes parameters, with a significant decrement in lysosomal membrane stability, while
681 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, Välitalo 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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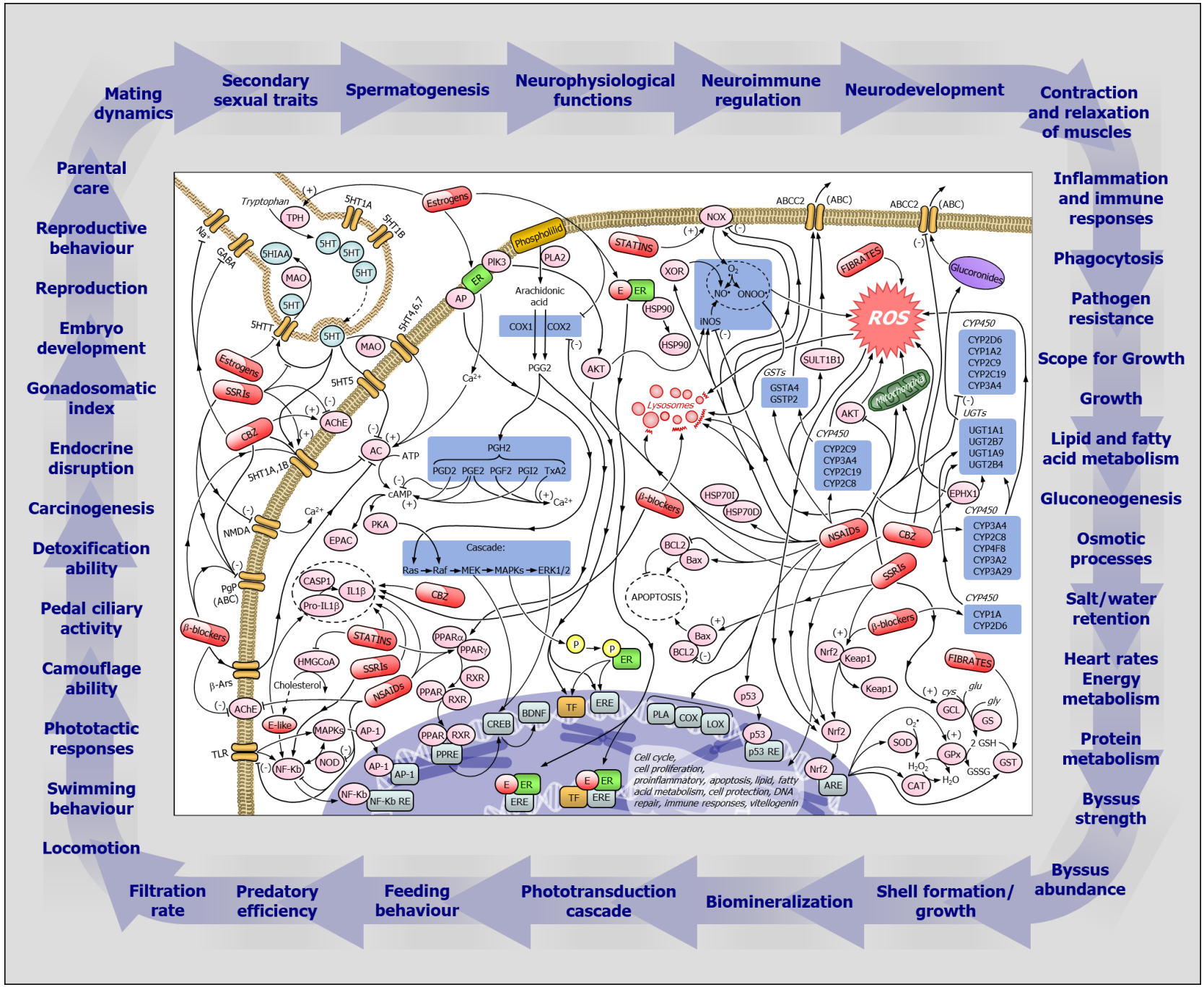
1006 **CAPTION OF FIGURE 1**

1007 **Figure 1:** Pathways of action, metabolism and toxicity of the most frequently detected therapeutic
1008 classes in marine environment: non-steroidal anti-inflammatory drugs (NSAIDs), psychiatric drugs
1009 (Selective Serotonin Reuptake Inhibitors SSRIs and carbamazepine CBZ), cardiovascular drugs (β-
1010 blockers), lipid regulators (statins and fibrates), steroidal hormones (estrogens). Acronyms arranged
1011 in alphabetical order: ABCC2: ATP Binding Cassette Subfamily C Member 2; ABC: ATP-binding
1012 cassette transporters; AC: adenylyl cyclase; AChE: acetylcholinesterase; AKT: protein kinase B; AP:
1013 activator protein; AP-1: activator protein 1; ARE: antioxidant responsive element; ATP: adenosine
1014 triphosphate; BAX: Bcl-2-associated X protein; BCL2: B-cell lymphoma 2 protein; BDNF: brain-
1015 derived neurotrophic factor; cAMP: cyclic adenosine monophosphate; CASP1: caspase 1 CAT:
1016 catalase; CBZ: carbamazepine; COX: cyclooxygenase; COX1: cyclooxygenase 1; COX2:
1017 cyclooxygenase 2; CREB: cAMP response element-binding protein; CYP450: cytochrome P450
1018 enzymes *Cys:* cysteine; E: estrogen; E-like: estrogen like compounds; EPAC: exchange protein
1019 activated by cyclic AMP; EPHX1: epoxide Hydrolase 1; ER: estrogen receptor; ERE: estrogen
1020 response element; ERK1/2: extracellular signal-regulated kinases 1 and 2; GABA: γ amino-butyric
1021 acid receptor; GCL: glutamate-cysteine ligase; *Glu:* glutamate; *Gly:* glycine; GPx: glutathione
1022 peroxidases; GS: glutathione synthetase; GSH: reduced glutathione; GSSG: oxidized glutathione;
1023 GST: glutathione S-transferase; HMGCoA: β-Hydroxy β-methylglutaryl-CoA; HSP70 I/D: heat
1024 shock protein 70 I and 70D; HSP90: heat shock protein 90; IL1β: interleukin 1 beta; iNOS: inducible
1025 nitric oxide synthetase; Keap1: Kelch-like ECH-associated protein 1; LOX: leukotrienes; MAO:
1026 monoamine oxidase; MAPKs: mitogen-activated protein kinases; NF-kB RE: nuclear factor-kB
1027 responsive element; NF-kB: nuclear factor-kB; NMDA: N-methyl-D-aspartate receptor; NOD:
1028 nucleotide-binding oligomerization domain proteins; NOX: NAPH oxidases; Nrf 2: nuclear factor
1029 (erythroid-derived 2)-like 2; NSAIDs: non-steroidal anti-inflammatory drugs; P: phosphate; p53 RE:
1030 protein 53 responsive element; p53: protein 53; PGD2: prostaglandin D2; PGE2: prostaglandin E2;
1031 PGF2: prostaglandin F2; PGG2: prostaglandin G2; PGH2: prostaglandin H2; PGI2: prostaglandin I2;
1032 PgP: P-glycoprotein; PKA: protein kinase A; PLA: phospholipase; PLA2: phospholipase A2; PlK3:
1033 Polo like kinase 3; PPAR: peroxisomal proliferator activated receptor; PPARα: peroxisomal
1034 proliferator activated receptor α; PPAR β: peroxisomal proliferator activated receptor β; PPRE:
1035 peroxisomal proliferator responsive element; Pro- IL1β: Pro-inflammatory interleukin 1 beta;
1036 Ras/Raf/MEK: Ras-signalling cascade; RXR: retinoid X receptor; SOD: superoxide dismutase;
1037 SSRIs: selective serotonin re-uptake inhibitors; SULT1B1: sulfotransferase Family 1B Member 1;
1038 TF: transcription factor; TLR: Toll like receptors; TPH: tryptophan hydroxylase; *Trp:* tryptophan;
1039 TxA2: thromboxane A2; UGTs: UDP-glucuronosyltransferases; XOR: xanthine oxido-reductase; β-

1040 Ars: β-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.

1043


Mating dynamics
Secondary sexual traits
Spermatogenesis
Neurophysiological functions
Neuroimmune regulation
Neurodevelopment
Contraction and relaxation of muscles
Inflammation and immune responses
Phagocytosis
Pathogen resistance
Scope for Growth
Growth
Lipid and fatty acid metabolism
Gluconeogenesis
Osmotic processes
Salt/water retention
Heart rates Energy metabolism
Protein metabolism
Byssus strength
Byssus abundance
Shell formation/ growth
Biomineralization
Phototransduction cascade
Feeding behaviour
Predatory efficiency
Filtration rate
Locomotion
Swimming behaviour
Phototactic responses
Camouflage ability
Pedal ciliary activity
Detoxification ability
Carcinogenesis
Endocrine disruption
Gonadosomatic index
Embryo development
Reproduction
Reproductive behaviour
Parental care
ROS
Lysosomes
Mitochondria
Cascade:
Ras → Raf → MEK → MAPKs → ERK1/2
PGH2
PGD2 PGE2 PGF2 PGI2 TxA2
Arachidonic acid
COX1 COX2
PGG2
Cell cycle, cell proliferation, proinflammatory, apoptosis, lipid, fatty acid metabolism, cell protection, DNA repair, immune responses, vitellogenin
CYP450
CYP2D6
CYP1A2
CYP2C9
CYP2C19
CYP3A4
UGTs
UGT1A1
UGT2B7
UGT1A9
UGT2B4
GSTs
GSTA4
GSTP2
CYP450
CYP2C9
CYP3A4
CYP2C19
CYP2C8
CYP450
CYP3A4
CYP2C8
CYP4F8
CYP3A2
CYP3A29
CYP450
CYP1A
CYP2D6
Estrogens
SSRIs
CBZ
β-blockers
STATINS
FIBRATES
NSAIDs
Glucoronides
Tryptophan
TPH
5HT
5HIAA
MAO
AChE
AC
ATP
cAMP
PKA
EPAC
NMDA
PgP (ABC)
β-Ars
TLR
NF-Kb
NOD
MAPKs
AP-1
E-like
HMGCoA
Cholesterol
CASP1
IL1β
Pro-IL1β
PPARα
PPARγ
RXR
PPAR
PPRE
CREB
BDNF
TF
ERE
NF-Kb RE
PIK3
PLA2
Phospholilid
AP
ER
AKT
XOR
HSP90
iNOS
NOX
ABCC2
(ABC)
SULT1B1
EPHX1
HSP70I
HSP70D
BCL2
Bax
APOPTOSIS
p53
p53 RE
PLA
COX
LOX
Nrf2
Keap1
ARE
GCL
GS
GPx
GST
SOD
CAT
GSSG
2 GSH
cys
glu
gly