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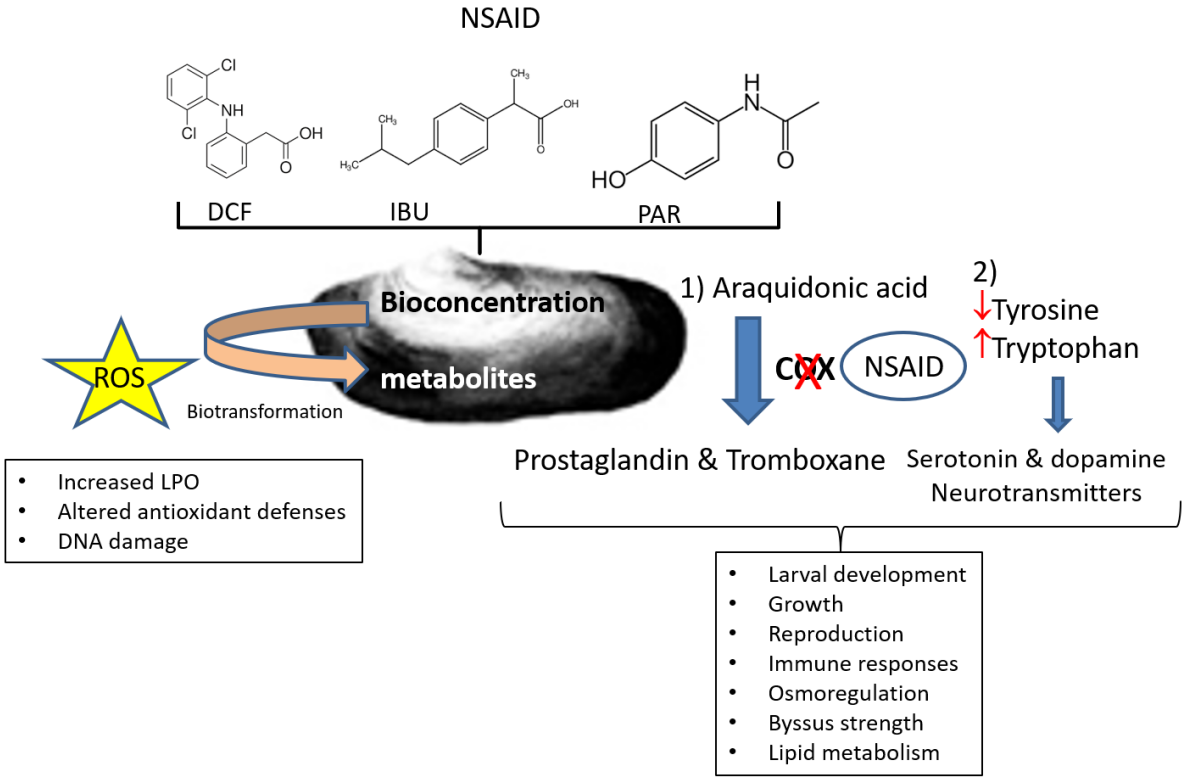
NSAID
DCF
IBU
PAR
Bioconcentration
metabolites
ROS
Biotransformation
1) Araquidonic acid
COX
NSAID
2)
↓Tyrosine
↑Tryptophan
Prostaglandin & Tromboxane
Serotonin & dopamine
Neurotransmitters
• Increased LPO
• Altered antioxidant defenses
• DNA damage
• Larval development
• Growth
• Reproduction
• Immune responses
• Osmoregulation
• Byssus strength
• Lipid metabolism

# Anti-inflammatory drugs in the marine environment: bioconcentration, metabolism and sub-lethal effects in marine bivalves

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**Abstract**

Pharmaceuticals such as non-steroidal anti-inflammatory drugs (NSAIDs) have been found in the marine environment. Although there is a large body of evidence that pharmaceutical drugs exert negative impacts on aquatic organisms, especially in the freshwater compartment, only limited studies are available on bioconcentration and the effects of NSAIDs on marine organisms. Bivalves have a high ecologic and socio-economic value and are considered good bioindicator species in ecotoxicology and risk assessment programs. Therefore, this review summarizes current knowledge on the bioconcentration and the effects of three widely used NSAIDs, diclofenac, ibuprofen and paracetamol, in marine bivalves exposed under laboratory conditions. These pharmaceutical drugs were chosen based on their environmental occurrence both in frequency and concentration that may warrant their inclusion in the European Union Watch List. It has been highlighted that ambient concentrations may result in negative effects on wild bivalves after long-term exposure. Also, due to food-chain transfer, higher trophic level organisms may be impacted due to food-chain transfer (e.g., humans are shellfish consumers). Overall, the three selected NSAIDs were reported to bioconcentrate in marine bivalves, with recognized effects at different life-stages, immune responses being the main target of a long-term exposure. The studies selected support the inclusion of diclofenac on the European Union Watch List and highlight the importance of conducting research for ibuprofen and paracetamol due to their demonstrated negative effects, under laboratory conditions, on marine bivalves exposed to environmental realistic concentrations.

**Keywords:** Pharmaceuticals, marine environment, bivalves, metabolism, sub-lethal effects.

# 1. ECOTOXICOLOGICAL IMPLICATIONS OF THE OCCURRENCE OF PHARMACEUTICAL DRUGS IN THE MARINE ENVIRONMENT

Pharmaceuticals are compounds used in human and veterinary medicine which, because of their manufacture, application and inappropriate disposal, combined with low degradation rates for some of them in Wastewater Treatment Plants (WWTPs), are present in the aquatic environment (Arpin-Pont et al., 2016; Mezzelani et al., 2018a; Monteiro and Boxall, 2010; Pazdro et al., 2016). The contamination of water with pharmaceutical residues is considered an emerging environmental concern in the field of water policy (Directives 2013, 2008, 2000), suggesting a careful monitoring for some of these compounds to determine the risk that they may pose to wildlife.

In the aquatic milieu, these compounds are present in freshwater and marine water bodies, in surface waters, wastewater effluents, groundwater and even in drinking water, at concentrations ranging between a few ng/L and up to several µg/L (Brausch et al., 2012; Mezzelani et al., 2018a; Runnalls et al., 2010). Although pharmaceuticals have been found in seawater from marine/coastal environments, usually within the same wide range of concentrations as the ones measured in freshwater environments, fewer studies are available on the occurrence of pharmaceuticals in the marine system with regard to freshwater ecosystems (Álvarez-Muñoz et al., 2015; Alygizakis et al., 2016; Arpin-Pont et al., 2016; Ebele et al., 2017; Gaw et al., 2014; Mezzelani et al., 2018a). Furthermore, pharmaceutical drugs have also been detected in aquatic organisms belonging to different levels of the ecological hierarchy from algae, bivalves, crustaceans to fish (Álvarez-Muñoz et al., 2015; Ebele et al., 2017; Mezzelani et al., 2018a). Among these groups, bivalves are of interest due to their socio-economic value, as they are highly consumed shellfish (Voultsiadou et al., 2010). Thus, the ingestion of bivalves contaminated with drugs may pose a health risk to the human population (e.g., potential antibiotic resistance) (Rodrigues et al., 2019). Moreover, bivalves have been commonly used as bioindicator species as they

exhibit adequate characteristics such as a wide distribution, bioaccumulation potential, well-known life-cycles, a sedentary lifestyle and filter feeding activity that suit their use in ecotoxicology and monitoring programs (McEneff et al., 2014; Powell and Cummins, 1985; Viarengo and Canesi, 1991). It has already been demonstrated that several drugs induce negative impacts on bivalves, due to their biological activity, and high liposolubility for some of them, with most of the literature focusing on effects on subcellular and cellular processes (Fabbri and Franzellitti, 2016; Mezzelani et al., 2018a; Prichard and Granek, 2016). Alterations in immunological, antioxidant, detoxification, neurological and metabolic parameters (either as enzymatic activity and/or gene expression/transcription), as well as DNA and other biomolecules are frequently considered endpoints (Gonzalez-Rey and Bebianno, 2014; Lacaze et al., 2015; Martin-Diaz et al., 2009). However, at the individual level, changes in growth, reproduction and behavior (e.g., filter-feeding and respiratory rates) were reported (Honkoop et al., 1999; Solé et al., 2010).

Among the pharmaceutical drugs found in the aquatic environment, the non-steroidal anti-inflammatory drugs (NSAIDs): ibuprofen (IBU) and diclofenac (DCF), as well as the analgesic drug paracetamol (PAR) have been detected with high frequency in the water bodies of different countries, as a consequence of their high consumption (reviewed in Mezzelani et al., 2018a). PAR, although it is structurally not a NSAID, shares its analgesic and antipyretic properties with this class of drugs (Jóźwiak-Bebenista and Nowak 2014) and for this reason is included in the present study. DCF was already included in the first surface water EU Watch List, published in 2015, together with other drugs such as three hormones (17-β-estradiol, 17-α-ethinylestradiol and estrone), and three antibiotics of the macrolide group (erythromycin, clarithromycin and azithromycin) (EC, 2015). NSAIDs are applied to humans to alleviate pain and inflammation, through the inhibition of the reaction catalyzed by the cyclo-oxygenase enzymes (COX-1 and COX-2), decreasing biosynthesis of prostaglandins and thromboxane from arachidonic acid (Bacchi et al., 2012). In bivalves, the exposure to

NSAIDs has also been responsible for the modulation of COX enzymes (Bebianno et al., 2017; Courant et al., 2018; Milan et al., 2013).

DCF, one of the most popular "pain-killers", has an estimated annual global consumption of 1443 tons (Acuña et al., 2015). The removal rate of DCF in WWTPs varies between studies; however, maximal removals of 75% have been reported (Bueno et al., 2012; Gómez et al., 2007; Zhang et al., 2008). In the marine environment, this drug has been found at concentrations up to 15 µg/L (Mezzelani et al., 2018a). In marine bivalves, DCF was found at concentrations between 0.5 and 4.5 ng/g dry weight (DW) (2 and 18 ng/g WW, conversion factor of 4, according to Álvarez-Muñoz et al., 2018, 2019) in mussels *Mytilus galloprovincialis* and *M. edulis* collected along the Portuguese Atlantic coast (Cunha et al., 2017). Capolupo et al. (2017) reported DCF concentrations between 2.1 and 4.6 ng/g wet weight (WW) in mussels *M. galloprovincialis* transplanted to a costal lagoon in Ravenna (Italy), which is in the same range as concentrations determined by Cunha et al. (2017) in Portugal.

IBU has a consumption rate of about 163 tons per year in the European Union (Sebastine and Wakeman, 2003). Although this drug was found to be efficiently removed (>70%) in WWTPs (Bueno et al., 2012), it has been detected in the marine environment at concentrations up to 2 µg/L (Mezzelani et al., 2018a). Limited data is available on the detection of IBU in wild populations of marine bivalves, however, Mezzelani et al. (2016a) detected this drug at approximately 9 ng/g DW (36 ng/g WW) in mussels, *M. galloprovincialis,* collected in the Portonovo bay in the Italian Central Adriatic Sea.

PAR is one of the leading substances in terms of use, with a consumption reaching thousands of tons per year. Sebastine and Wakeman (2003) determined a total consumption of PAR in the United Kingdom in 2000 which was 1136 tons. This drug has a high removal rate in WWTPs (> 90%) (Moreno-González et al., 2015), but has still been detected in seawater near the discharge of wastewater effluents with concentrations up to 200 µg/L, as reported by Togola and Budzinski (2008). However,

typically, the concentrations of PAR found in marine waters are in the high ng/L concentration range (Célic et al., 2019; Mijangos et al., 2018; Vidal-Dorsch et al., 2012). Regarding the occurrence of PAR in natural populations of marine bivalves, concentrations up to 115 ng/g DW (60 ng/g WW) were reported in mussels, *M. edulis,* collected on the Belgian coast (Wille et al., 2011).

Considering worldwide distribution, concentrations, the high frequency of DCF, IBU and PAR occurrence and the inclusion of DCF in the Watch List, the aim of the present review is to gather published laboratory evidence on bioconcentration and the effects of these drugs on marine bivalves. Google Scholar, Scopus, ScienceDirect and Web of Science were selected as databases for the literature search. The methodological approach selected articles by using, for each pharmaceutical, various combinations of keywords dealing with their effects on marine bivalves, these were: pharmaceutical drugs, non-steroidal anti-inflammatory drugs, marine environment, effects, toxicity, bioconcentration, metabolism, biomarkers and marine bivalves. As articles on this review topic are available only since 2010, the published literature selected was from 2010-2020.

# 2. DCF, IBU AND PAR BIOCONCENTRATION, METABOLISM AND EFFECTS ON LABORATORY EXPOSED MARINE BIVALVES

Bivalves are exposed to contaminants discharged into the marine system near coastal sites where they naturally occur and where they can also be harvested. Due to their filter-feeding habits, they can concentrate pharmaceuticals drugs present in the water column in their tissues (e.g., gills, digestive gland) (McEneff et al., 2014; Viarengo and Canesi, 1991). Thus, they provide valuable information not only on the exposure but also on the effects of the drugs under study (DCF, IBU, PAR), contributing to the monitoring of the risk they represent to aquatic organisms and also to humans as shellfish consumers.

## 2.1 BIOCONCENTRATION AND METABOLISM OF DCF, IBU AND PAR IN LABORATORY EXPOSED BIVALVES

### 2.1.1 DCF, IBU and PAR bioconcentration in marine bivalves

The tissue concentrations and bioconcentration factors (BCFs) of the NSAIDs DCF, IBU and PAR reported in marine bivalves, are summarized in Table 1. Ericson et al. (2010) reported that DCF was accumulated in *M. trossulus* exposed for 8 days to 1 µg/L (180 ng/g WW) and 10,000 µg/L (82,000 ng/g WW). Despite the expected higher accumulation at higher exposures, the BCF was reduced from 180 to 10 L/kg WW at the highest concentration. In the same species (*M. trossulus*), Świacka et al. (2019) related the concentration of DCF to the length of exposure: DCF concentration in mussels increased from 0 to ~8 µg/g DW (~2,000 ng/g WW) over 3 days and decreased by a factor of 9 at the end of the exposure period (day 5). Despite the nominal concentration of 133.3 µg/L, the authors measured the concentration of DCF in the exposure medium, finding that at a concentration of 102 (day 1) and 15 µg/L (day 5), the corresponding BCF in mussels increased along the 5-day exposure by a factor

of 3; and after 5 days depuration DCF was approximately 10 fold lower compared to day 1. Ericson et al. (2010) described a similar BCF trend, with higher values at lower exposure concentrations. In *M. galloprovincialis* mussels exposed for 3 days to DCF 1 and 100 µg/L, this drug was bioconcentrated in their tissues, in a dose dependent manner (1 µg/L: 0.026 mg/kg DW (6.5 ng/g WW); 100 µg/L: 2.08 mg/kg DW (520 ng/g WW)) (Courant et al., 2018). The previous results were coincident with values reported by Świacka et al. (2019) within a comparable time frame. In the same mussel species, Bonnefille et al. (2017) showed DCF levels of 2,008 µg/kg DW (502 ng/g WW) at 100 µg/L and 7,343 µg/kg DW (1,836 ng/g WW) at 600 µg/L. However, only a slight difference of 1.4-fold in BCF was obtained, with higher BCF values at the lowest exposure concentration. In a comprehensive study considering the three targeted NSAIDs, *M. galloprovincialis* were exposed to 25 µg/L of each drug for 14 days (Mezzelani et al., 2016a). After this period, mussels showed a DCF tissue concentration of 14.9 ng/g DW (3.7 ng/g WW) and 9-fold lower IBU concentration, while PAR was not detectable. In another parallel study by the same authors, carried out with the same length of exposure but with 50-fold lower drug concentrations, only DCF was quantified in the mussel tissue, while the other drugs were not detected. Comparing both studies, the 50-fold lower water DCF levels resulted in a 3-fold lower tissue concentration but 16-fold higher BCF value in mussels under the lowest dose (0.5 µg/L). More recently, Mezzelani et al. (2018b), using the same mussel species, demonstrated that a longer exposure (60 days) to DCF and IBU at the environmentally relevant water concentration of 2.5 µg/L resulted in different bioaccumulation patterns for both drugs: IBU was accumulated 16-, 7- and 20-fold more than DCF after 14, 30 and 60 days, respectively. These results differ from the trend described at 25 µg/L by Mezzelani et al. (2016a) for a comparable exposure period (14 days), in which bioaccumulation of IBU was 9-fold lower than DCF. With regard to the time trend in drug uptake over the 60-day exposure period, Mezzelani et al. (2018b) found an increase in DCF tissue concentrations and its associated BCF until the mid-term of the

exposure (14 days) and then a decrease towards the end (60 days). Higher fluctuations on BCFs were associated with drug presence (16-fold, at 0.5 and 25 µg/L) rather than length of exposure (1.4-fold at 14 with 60 days, at DCF of 2.5 µg/L) (Mezzelani et al., 2018b, 2016b). Overall, Mezzelani et al. (2018b, 2016a, 2016b) studies evidenced that a 50-fold (0.5 and 25 µg/L) and 10-fold (2.5 and 25 µg/L) difference in DCF concentration was reflected only in slight changes in BCF values. In the case of IBU, the same authors (Mezzelani et al., 2018b, 2016a) revealed that tissue concentrations and associated BCF in exposed mussels decreased by a 159-fold factor with increasing doses (2.5 to 25 µg/L) after 14 days but only a slight increase (1.7-fold) was revealed over time (from 14 to 60 days). Recent studies conducted by Freitas et al. (2019a, 2019b) reported a BCF for DCF between 7.4 and 10.1 L/kg WW in *M. galloprovincialis* exposed for 28 days to 1 µg/L. These results together with those by Bonnefille et al. (2017), Ericson et al. (2010) and Mezzelani et al. (2018b, 2016b) coincide on higher BCF values at lower DCF concentrations. The decrease of BCF values at higher and less realistic DCF exposure concentrations may be possibly related with a reduction in the filtration rate, a response already reported to occur in bivalves exposed to pharmaceutical drugs (Solé et al., 2010). At lower exposure concentrations, possibly, the pharmaceutical drug is not considered “stressful” and thus, the rate of accumulation is maintained similar to control conditions, resulting in higher BCFs. In Table 1, differences in BCF values between mussel species are also evidenced. That is, at a comparable DCF dose, 18-fold higher BCF was measured in *M. trossulus* in comparison with *M. galloprovincialis*, possibly revealing different mechanisms of drug uptake, detoxification and excretion in the two species (Ericson et al., 2010; Freitas et al., 2019b), but also depending on the organisms’ condition (e.g., age, size, nutritional status). However, more studies with different mussel species exposed to similar conditions are necessary to properly assess this issue. In a recent study (Costa et al., 2019), DCF was accumulated in the tissues of the clams *Ruditapes philippinarum* and *R. decussatus* exposed to 1 µg/L for 7 days. Comparing both

species, *R. philippinarum* accumulated 2-fold more DCF than *R. decussatus.* When comparing BCFs between clams and mussels, values reported for clams after 7-day exposure were comparable to those found in mussels for the same DCF concentration but when exposed for 28 days (Freitas et al., 2019a, 2019b). Another study, with oysters *Crassostrea gigas,* exposed to IBU for 7 days revealed tissue bioconcentration was dependent on the exposure concentration (133 times higher at 100 than at 1 µg/L) but with similar BCFs (Serrano et al., 2015). The differences in DCF and IBU tissue concentrations in the former studies were related to the ability of bivalves to assimilate the drug through filtration up to a certain period of time, decreasing afterwards. The decrease in NSAIDs concentrations and the lack of detection of PAR in the studies by Mezzelani et al. (2016a, 2016b) was associated with drug metabolism, as will be discussed in the next section.

#### 2.1.2 DCF, IBU and PAR metabolism and metabolite bioconcentration

The metabolism of DCF in marine bivalves, compared with other organisms, is poorly investigated, as reviewed in Sathishkumar et al. (2020). In mammals, metabolism of DCF partitions between acyl glucuronidation and phenyl hydroxylation, with the former reaction catalyzed primarily by uridine 5'-diphosphoglucuronosyl transferase 2B7 while the latter is catalyzed by cytochrome P450 (CYP) 2C9 and 3A4 (Tang, 2003). The main metabolites of phase I biotransformation are 4′-hydroxy-diclofenac (4'-OH) and 5-hydroxy-diclofenac (5-OH). In the second phase of the detoxification, in mammals, both DCF and its hydroxylated metabolites are conjugated with glucuronide and/or sulfate. Both, hydroxylated phase I metabolites and conjugates are rapidly excreted from the body. However, in mammals, DCF and the hydroxylated DCF metabolites can undergo further oxidation, yielding a reactive and toxic benzoquinone imine metabolite (Boelsterli, 2003). To prevent toxicity, quinine imines undergo glutathione conjugation, resulting in the non-toxic 4'-OH and 5-OH DCF glutathione conjugates. Likewise, the formation of reactive metabolites in acyl

glucuronidation of DCF, another metabolic pathway, leads to glutathione conjugates (Boelsterli, 2003). With regard to marine bivalves, only two studies are available on the identification of DCF metabolites in marine bivalves exposed to laboratory conditions (Bonnefille et al., 2017; Świacka et al., 2019). The study by Świacka et al. (2019) determined the concentrations of two DCF metabolites (4'-OH and 5-OH) in *M. trossulus*. The previously reported decrease in the concentration of DCF in mussels' tissues after 3 days was related with the metabolism of the drug, being confirmed by the concentration of 4'-OH and 5-OH metabolites. A maximum tissue concentration of 0.73 µg/g DW (183 ng/g WW) for 4'-OH on day 4 and of 1.182 µg/g DW (295 ng/g WW) for 5-OH on day 3 were reached in the exposed mussels. Comparing the metabolite tissue concentrations with the maximum levels of the parent compound (2,000 ng/g WW), 4'-OH and 5-OH levels were, 10-fold and 6.7-fold lower, respectively. The authors hypothesized the presence of DCF metabolites as resulting from the action CYP3 enzymes, as they are present in mussels and resemble human CYP3A4 (Zanette et al., 2013), also involved in the metabolism of DCF in humans. Bonnefille et al. (2017) also determined 13 DCF metabolites in mussels exposed to the drug. Three of them were phase I metabolites (including 4'-OH and 5-OH) and the remaining 10 were phase II metabolites, mainly sulfate and amino acids conjugates. These 13 described metabolites allowed the authors to propose a partial metabolic pathway for DCF metabolism in mussels. The authors also revealed that, despite the fact that the generation of reactive DCF metabolites (glucuronide conjugates, arene oxide or quinone imines) was not observed in their study, it does not exclude that they were formed.

In the case of IBU, there is no information on metabolite identification in marine invertebrates. However, the IBU biotransformation pathway has been hypothesized to occur as for vertebrate fish species (Aceña et al., 2017; Lahti et al., 2011). In mammals, IBU is almost completely metabolized, with little to no unchanged drug found in the urine. IBU is mainly metabolized by CYP 450 2C9 and 2C8 (phase I)

biotransformation pathways followed by glucuronidation (phase II), resulting in metabolites like carboxy-ibuprofen, hydroxy-ibuprofen and acyl glucuronides (Mazaleuskaya et al., 2015). It has been suggested that, as for other drugs, as bivalves contain CYP 450 isoforms and conjugation enzymes (e.g., GST) they are likely to transform IBU into its oxidized or conjugated metabolites (Parolini et al., 2011).

With regard to PAR, previous studies have suggested that similar detoxification mechanisms may exist between mammals and non-target organisms that are environmentally exposed to the drug (Nunes et al., 2014; Parolini et al., 2010). In mammals, under therapeutic dosages, over 90% of PAR is conjugated with co-factors (namely with sulphate and glucuronic acid) forming non-toxic conjugated metabolites that are promptly excreted (Xu et al., 2008). Under these conditions, less than 10% of PAR is metabolized by the CYP 450 enzymes (primarily CYP 2E1, 1A2, and 3A4) to produce a toxic intermediate metabolite called N-acetyl-p-benzoquinoemine (NAPQI) that is further detoxified by intracellular glutathione (Xu et al., 2008). However, a different scenario can occur at higher amounts of PAR administration and/or deficiency in intracellular glutathione. Under these conditions NAPQI can accumulate and exert toxic effects on endogenous proteins, lipids and nucleic acids (Hinson et al., 2004, Jaeschke and Bajt, 2006). Thus, the toxicity of PAR exposure in non-target organisms was suggested to be caused by saturation or exhaustion of co-factors involved in detoxification (Almeida and Nunes, 2019; Nunes et al., 2014). Parolini et al. (2010) hypothesized a similar mechanism for PAR action in a freshwater mussel, where NAPQI was likely to interact with proteins and nucleic acids and promote the increase of reactive oxygen and nitrogen species, together injuring cell macromolecules. More recently, Bebianno et al. (2017) showed that PAR elicited an inhibition of CYP30C1, CYP3071A1 and FABP-like transcripts highlighting their role in drug metabolism, and transport in oyster gills. Despite the association between PAR toxicity and the formation of the reactive NAPQI metabolite, there are no studies on the identification of PAR metabolites in marine organisms.

Overall, former bivalve studies confirmed a low bioconcentration potential at higher and less environmentally realistic doses, possibly due to a reduction in the filtration rate under more stressing conditions. At environmentally realistic concentrations, most drugs were detected in their tissues, with no clear time- and concentration-dependent trends but with some marked species particularities, probably due to differences in biotransformation and/or excretion capacity. However, despite the low tissue levels detected for some NSAIDs, an ecotoxicological concern cannot be excluded. Also, more studies on drug metabolite identification are needed to properly assess their negative impacts, as reactive metabolites and/or reactive oxygen species could be formed during drug biotransformation and damage essential cell macromolecules.

## 2.2 BIOLOGICAL RESPONSES INDUCED BY DCF, IBU AND PAR IN LABORATORY EXPOSED BIVALVES

The effects of the selected drugs on marine bivalves (from early-life stages to adulthood) were studied at concentrations of environment and non-environment relevance, at the individual and sub-individual level using several physiological, subcellular and cellular endpoints.

### 2.2.1 Sub-lethal effects induced by DCF, IBU and PAR on mussels

In Table 2 studies concerning the sub-lethal effects of the selected NSAIDs on marine bivalves are summarized.

#### 2.2.1.1 Early life stages

*NSAIDs effects on larval development*

The early life stages of mussels have also been addressed with NSAIDs exposures and impacts reflected mostly as development impairment. DCF and IBU (0.01- 1,000 µg/L) affected larval development and shell formation of *M. galloprovincialis* when exposed for 48 h at concentrations as low as 0.01 µg/L of DCF, showing in an embryotoxicity assay an inverted U-shaped dose response curve, with no effects at the highest concentration tested (Fabbri et al., 2014). This U-shapped response, commonly observed in drug toxicity evaluation, is defined as "hormesis" and it is characterized by stimulation at low doses and inhibition at higher ones (Calabrese, 2008). In order to properly assess the "hormesis" effect, a temporal component should be incorporated into the examination/equation; however, most of the studies deal with the effects of multiple doses at a single time-point scale. In the same Fabbri et al. (2014) study, contrary to DCF observations, embryo development was only compromised by IBU at higher concentrations (100 and 1,000 µg/L) in a dose dependent manner. The different responses to DCF and IBU in the early life stages of mussels are probably attributable to their distinct mechanisms of action, bioaccumulation and biotransformation rates, resulting in higher sensitivity to DCF. Shell malformations in *M. galloprovincialis* embryos after a 48-h post fertilization embryotoxicity test were also seen after DCF (1 and 10 µg/L) exposures with the genes involved in shell formation and biotransformation seen as the most affected (Balbi et al., 2018). Also, Fontes et al. (2018) found that an ample range of DCF exposures (20 ng/L – 1,000 mg/L) impaired embryo-larval development in *Perna perna* mussels. IBU exposures through contaminated sediments (0.15 – 1508 ng/g DW) were also responsible for slowing down the embryo-larval development of the same mussel species *P. perna* after a 2-day exposure (Pusceddu et al., 2018).

2.2.1.2 Adults

*NSAIDs effects on reproduction*

Besides the impacts on early life stages, Fontes et al. (2018) also considered the effects of DCF exposures (20 ng/L – 1,000 mg/L) in adult mussels *P. perna*, revealing that the impairment on reproduction occurred only at the non-environmental concentrations.

*NSAIDs' effects on physiological parameters*

The effects on physiological parameters due to DCF and IBU exposures were assessed in *M. trossulus,* both in environmental (1 ug/L) and non-environmental (10,000 μg/L) concentrations for up to 21 days (Ericson et al., 2010). For both drugs, it was shown that the responses in mussels exposed to lower concentrations (1 - 100 μg/L) did not differ from those not exposed. However, higher concentrations (1,000 and 10,000 μg/L) and long-term exposures resulted in lower scope for growth (SFG) and byssus strength. The reduced SFG in contaminated mussels was related to fewer energy resources available for biologic processes. Indeed, at higher exposure concentrations, mortality (16%) was also associated to less energy available for homeostasis and maintenance. According to these authors, under stress conditions, mussels can satisfy higher energy demands by increasing their filtration rate, which is also an energy demanding process that can lead to decreased SFG. The reduced byssus strength decreases the capacity of the mussels to attach to surfaces, possibly leading to reduced survival and impairment on recruitment and with detrimental population consequences. PAR exposure (23 and 403 μg/L) for 10 days affected mussels' (*M. galloprovincialis)* physiology by, in comparison with the control condition, increasing their feeding rate in a non-concentration dependent manner (Solé et al., 2010). However, comparing the PAR exposure levels (23 and 403 μg/L), a decrease in the feeding rate was observed at the highest exposure concentration. Also, Piedade et al. (2020) showed that PAR (0.5 – 500 μg/L, 96 h exposure) increased the ingestion of food (algal cells) by *Mytilus* spp. although in a dose-dependent pattern, which is partly in agreement with the previously published data. The increased food ingestion was

399 followed by a significant reduction of the gill's glycogen levels and a slight (not
400 significant) reduction in the digestive gland (Piedade et al., 2020). The previous authors
401 hypothesized that PAR exposure stimulated an energetically demanding response in
402 mussels, reflected in an increase absorption of nutrients from the media and a
403 depletion of cellular energetic reserves.
404
405 *NSAIDs effects on immunological parameters*
406 In addition to physiological consequences, the most consistence response to
407 NSAIDs in bivalves at all life stages is the modulation of immunological parameters. In
408 the previously referred Pusceddu et al. (2018) study but with the mussel *Mytella*
409 *charruana* exposed to IBU contaminated sediment, a decrease of haemocytes
410 lysosomal membrane stability (LMS) was related to consequences at higher levels of
411 biological organization since the embryo-larval development depends on the energy
412 released from the yolk by lysosomal action. Studies by Mezzelani et al. (2018b, 2016a,
413 2016b) also described a decrease in the LMS in *M. galloprovincialis* at DCF and IBU
414 concentrations of 25 µg/L, and IBU at a much lower one (0.5 µg/L). A decrease in LMS
415 was also confirmed after PAR exposure in the same species (Mezzelani et al., 2016b).
416 More recently, Munari et al. (2019) reported that DCF (0.05 and 0.50 µg/L) affected the
417 overall immune haemocyte response of *M. galloprovincialis*, compromising their
418 defense capacity against pathogens.
419
420 *NSAIDs effects on oxidative stress related parameters*
421 Another consequence of exposure to NSAID in marine bivalves is the production
422 of reactive oxygen species (ROS) responsible for oxidative stress, cellular damage,
423 DNA strand break formation and inactivation of enzymes when the antioxidant
424 defenses become overwhelmed (Regoli and Giuliani, 2014). In the previously referred
425 work by Fontes et al. (2018) with *P. perna*, lower concentrations (0.02 – 2 µg/L) of DCF
426 were also responsible for DNA damage, cellular damage (increased lipid peroxidation;

LPO) and decrease in antioxidant defenses (glutathione peroxidase; GPX) and the cytochrome P450 related ethoxyresorufin O-deethylase (EROD) activity. A study with *M. galloprovincialis* hemocytes treated and non-treated with ultrasounds and exposed for only 1 h to DCF (5 ng/L- 20 µg/L) showed that at higher concentrations (>100 ng/L) the drug induced cell mortality, while at lower concentrations (<100 ng/L) it significantly increased the levels of superoxide anions, nitric oxides (NO) and LPO products (Toufexi et al., 2016). The induction of ROS in DCF exposed mussel hemocytes was considered an unspecific mode of action related to the activation of NADPH oxidase and NO synthase in the hemocytes alleviated by the partial DCF degradation after ultrasound treatment. However, no clear consensus has been reached on the effects of DCF on the antioxidant defense system in marine mussels, since responses have been shown to be tissue-specific and transient. That is, Gonzalez-Rey and Bebianno (2014) found transient and tissue-specific antioxidant responses and LPO levels in *M. galloprovincialis* exposed for 15 days to 250 ng/L of DCF. An increase in LPO at the third day of exposure was counteracted by the effectiveness of antioxidant enzymes in the elimination of ROS, leading to reduction of cellular damage; however, estrogenic disturbances in DCF exposed females were suggested as altered alkali labile phosphate levels. Studies by Freitas et al. (2019a, 2019b) with whole tissue of mussel *M. galloprovincialis* exposed to DCF (1 µg/L) for 28 days showed a decrease in the energy metabolism (reduction in the electron transport system activity-ETS), an increase of the activity of the antioxidant and detoxification enzymes (namely, catalase-CAT, superoxide dismutase-SOD and glutathione S-transferases-GSTs) and no LPO occurrence confirming effectiveness of the antioxidant defenses, as pointed out by Gonzalez-Rey and Bebianno (2014). Other pathways such as the detoxification and defense system (enhanced GSTs) and oxidative stress (DNA damage and LPO occurrence) were reported in blue mussels (*Mytilus* spp.) after 96h injection with non-environmental concentrations of DCF (Schmidt et al., 2011). In a more realistic approach, prolonged water exposure (14 days) of the same mussel species to DCF (1

and 1,000 µg/L) resulted in DNA damage, induced oxidative stress and decreased GSTs activity (only at 1,000 µg/L at day 14) (Schmidt et al., 2014). In the same study, after an additional 7 days of depuration period, a recovery of LPO to control values was reached although a certain oxidative stress status was still evident through the presence of oxidized aconitase, an enzyme involved in the mitochondrial tricarboxylic acid cycle, and considered as a marker of cellular stress. Mezzelani et al. (2018b) also addressed the effects of 2.5 µg/L DCF and IBU for up to 60 days, showing that neither acetylcholinesterase (AChE; indicator of neurotoxicity), nor antioxidant defenses (e.g., CAT, glutathione reductase-GR, glutathione peroxidase-GPX) showed significant variations with the two drugs and exposure times. Also, their results on total oxyradical scavenging capacity supported the lack of prooxidant effects induced by DCF and IBU. The previously referred study, Gonzalez-Rey and Bebianno (2012, 2011) also investigated the impacts of IBU on the antioxidant and endocrine system of the mussels *M. galloprovincialis* exposed for 14 days to the same environmental concentrations of the drug (250 ng/L). IBU caused significant fluctuations of several oxidative stress biomarkers in the mussels: increased SOD activity, reduced CAT, GR and GSTs activities (antioxidant and detoxification enzymes) and increased LPO levels. The oxidative effects of short-term exposure (96 h) to increasing concentrations of PAR (0.5 – 500 µg/L) of *Mytilus* spp. were assessed by Piedade et al. (2020). As no significant alterations were observed in their oxidative stress parameters, the authors hypothesized that the activation of the defensive adaptive mechanisms limited the action of the drug on antioxidant defenses and oxidative damage.

*NSAIDs effects on multiple general stress responses*

In addition to the biochemical responses previously reported, Omics analyses were performed to complement and unravel the mechanistic actions of NSAIDs in mussels. Jaafar et al. (2015) applied a proteomic approach using a two gel

electrophoresis analysis that revealed changes in the presence and expression of 4 oxidized proteins (caspase 3/7-4; cytosolic heat-shock cognate protein 70; a predicted enolase-like protein and arginine kinase) indicative of key structural, metabolic and stress-response protein alterations. Also, Mezzelani et al. (2018b) applied several biological endpoints including transcriptomic analysis and observed that biological effects measured at both molecular and cellular level confirmed the immune system, lipid metabolism and cellular turnover as the main biological targets of NSAIDs in mussels. Koagouw and Ciocan (2019) showed changes in mRNA expression of signaling pathways involved in apoptosis (e.g., heat shock protein-70, caspase-8, B-cell lymphoma-2, Fas cell surface) that were down-regulated in *M. edulis* gonads after 7 days of exposure to PAR (40 ng/L, 250 ng/L and 100 µg/L). Former studies in mussels by Mezzelani et al. (2016a, 2016b) and more recently by Munari et al. (2019, 2018) showed similar molecular and cellular responses after exposure to DCF, IBU and PAR (0.05 - 25 µg/L) after the same exposure period. The coincident changes revealed by the previously mentioned authors were alterations in the immune system, lipid metabolism and genotoxicity as the main targets of the referred NSAIDs with an early onset of responsiveness at low drug levels and increased effects at higher doses. The association between DCF and IBU and lipid metabolism was due to the high affinity of these drugs for peroxisome proliferator-activated receptors (PPARs), a response already long reported in mussels by Lehmann et al. (1997), suggesting the reduction of β-oxidation pathways and a concomitant consumption of energy reserves. However, even though previous studies using cellular parameters showed that pro-oxidant effects are not the main consequences of these drug exposures, the previously referred study by Schmidt et al. (2014), reported changes in protein expression dealing with oxidative stress (e.g., down-regulation of glyceraldehyde-3-phosphate dehydrogenase, GADPH; down-regulation of aconitase; up-regulation of class 1 alcohol dehydrogenase, ADH), and involved molecular chaperones in mussels exposed to DCF.

509

510 *NSAIDs mode of action on COX and other metabolic pathways*

511 As indicated before, the main mode of action of NSAIDs in humans is the
512 inhibition of cyclooxygenases (COX-1 and COX-2), thus preventing the formation of
513 prostaglandins and thromboxane from arachidonic acid (Gierse et al., 1995). This
514 mode of action was also confirmed in bivalves, where prostaglandins were reported to
515 be involved in several physiological functions including immunity and reproduction
516 (Deridovich and Reunova, 1993; Stanley-Samuelson, 1987). To evaluate the NSAIDs'
517 mode of action on bivalves, biochemical and Omics analyses were performed.
518 Evidence of COX modulation by the inhibition in the synthesis of prostaglandin-$E_2$
519 (PGE2) was revealed in *M. galloprovincialis* exposed for 72 h, more significantly at 100
520 than at 1 µg/L DCF (Courant et al., 2018). Another NSAID mode of action, different
521 from COX inhibition, was proposed in *M. galloprovincialis* exposed to DCF (Bonnefille
522 et al., 2018). Through a metabolomic approach, the authors indicated two altered main
523 metabolic pathways: (1) a down-regulation of the tyrosine (e.g., catecholamines)
524 metabolism and (2) an up-regulation of the tryptophan (e.g., serotonin) metabolism,
525 which were, in turn, related with the impairment of byssus formation, osmoregulation
526 and reproduction in mussels. The down-modulation of catecholamines itself was also
527 related to the impairment of byssus formation, giving further support to former
528 observations on byssus strength reduction after NSAIDs exposures by Ericson et al.
529 (2010). Through a transcriptomic approach, a similar mode of action of IBU was
530 revealed in mussels, *M. galloprovincialis,* and humans in genes associated with the
531 Nuclear Factor kappa B (NF- κB) pathway (involved in immune and inflammation
532 responses) in the study of Maria et al. (2016).

533

534 ***2.2.2*** **Sub-lethal effects induced by DCF, IBU and PAR on clams**

535

536 2.2.2.1. Early life stages

537 *NSAIDs effects on larval development*

538 In clams *R. philippinarum*, larval development was affected by DCF (0.5 µg/L)
539 after 96 h exposure (Munari et al., 2016), which is in accordance with the previous
540 study by Fabbri et al. (2014) on mussels at which DCF impacted their larval
541 development at concentrations as low as 0.01 µg/L (50-fold lower).

542

543 2.2.2.2. Adults

544

545 *NSAIDs effects on immunological parameters*

546 As reported for mussels, the influence of NSAIDs in the modulation of immunity
547 related parameters was also consistently demonstrated in the clams *R. philippinarum*
548 (Aguirre-Martínez et al., 2013; Matozzo et al., 2012; Munari et al., 2019, 2018). That is:
549 DCF (0.05 and 0.50 µg/L) affected their overall haemocyte response with a reduced
550 immune surveillance against pathogens (Munari et al., 2019, 2018); while in the study
551 by Aguirre-Martínez et al., (2013) IBU (0.1-50 µg/L) was responsible for the alteration
552 of clams' immune parameters already at environmental concentrations, as revealed by
553 the decrease in LMS in a concentration- and time-dependent manner. However, in the
554 same clam species *R. philippinarum*, significant effects on immunotoxicity (evaluated
555 as total haemocyte count and pinocytic activity decreases and haemocyte proliferation
556 and lactate dehydrogenase activity increases) were only significant at non-
557 environmental concentrations (>500 µg/L) (Matozzo et al., 2012).

558

559 *NSAIDs effects on oxidative stress related parameters*

560 In *R. philippinarum* DCF and IBU (15 µg/L) independent exposures were followed
561 up (from 3 h up to 21 days and including 7 days of depuration) in the Trombini et al.
562 (2019) study. The authors evidenced an induction of oxidative stress and neurotoxicity
563 in the exposed clams already in the first 24 h. Another recent study by Costa et al.

(2019), with *R. philippinarum* and *R. decussatus* adult clams exposed to DCF (1 µg/L) for 7 days showed greater variation in the antioxidant defenses than in the energetic pathways that resulted in no oxidative damage in contaminated clams. Exposing also, *R. philippinarum,* for 14 days to IBU (0.1-50 µg/L), showed that despite a clear induction of the detoxification enzymes (EROD and GSTs activities) and increased antioxidant defenses (GPX activity), LPO still occurred while DNA damage and neurotoxicity signs were not detected (Aguirre-Martínez et al., 2016). Furthermore, the previously referred authors described the occurrence of the "hormesis" effect due to the increase of the responses at low concentrations of the drug followed by a decrease at higher ones, as previously described in mussels (Fabbri et al., 2014). A neurotoxicity marker (decreased AChE activity) and antioxidant defense capacity (decreased SOD activity) was described in *R. philippinarum* exposed to IBU (100 and 1,000 µg/L) for up to 7 days in the study of Milan et al. (2013). PAR (0.05-5.0 mg/L) exposure for 96 h did not cause increased LPO levels in the clams *R. decussatus* and *R. philippinarum,* thanks to the efficiency of the antioxidant GR and GSTs enzymes (Antunes et al., 2013). Neither LPO occurred under a prolonged exposure (28 days) to these same drug concentrations (0.05-5.0 mg/L) in the Manila clam *R. philippinarum*, with an increase in the antioxidant enzymes SOD and CAT already observed at 0.05 and 0.5 mg/L (Correia et al., 2016). In the study by Nunes et al. (2017) with *R. philippinarum* clams, an exposure of 96 h of PAR (0.25-25 µg/L) was performed and clams were allowed a further 10 days in non-contaminated water for recovery. These authors observed that, after the PAR contamination, the antioxidant defenses, metabolism and energy reserves were compromised, however, after the depuration period, they recovered from the stress to pre-pulse values.

*NSAIDs effects on multiple general stress responses*

The previously mentioned study by Milan et al. (2013) also reported changes in the transcription of multiple genes involved in biotransformation, eicosanoid

metabolism (e.g., phospholipase A2, prostaglandin D synthase), apoptosis, peroxisomal proliferator-activated receptor signaling pathway, inflammatory and immune responses that were justified by a similar mode of action of IBU in vertebrates and bivalves.

### 2.2.3. Sub-lethal effects induced by DCF, IBU and PAR on oysters

#### 2.2.3.1 Adults

*Effects on oxidative stress related parameters*

With regard to antioxidant defenses, Serrano et al. (2015) observed a decrease in GR activity in IBU exposed oysters, possibly affecting the recycling of the antioxidant GSH. However, as no impacts were seen in GPX activity (which requires GSH as co-factor), it was suggested that GSH might be supplied by other mechanisms such as GSH biosynthesis. The previously mentioned Bebianno et al. (2017) study, also assessed biochemical parameters related to oxidative stress in *C. gigas* exposed to PAR (1 and 100 µg/L) for 1, 4 and 7 days showing that no oxidative stress occurred in gills, which was in accordance with their former studies that showed that pro-oxidant mechanism do not seem to be the primary toxicity mode of action of PAR and other NSAIDs in bivalves as previously pointed out by Gonzalez-Rey and Bebianno (2014, 2012, 2011) for DCF and IBU in mussels.

*NSAIDs effects on multiple general stress responses*

Changes in the transcription of genes related to the cytochrome P450 system have been studied on the oyster, *C. gigas* exposed to IBU (1 and 100 µg/L) for 7 d (Serrano et al., 2015). The changes in the transcription of fatty acid binding protein-like (FABP-like), CYP isoforms (e.g., CYP365A1), GSTs (-ω and -π) proteins were

associated with IBU transport and biotransformation in the cells affecting the clearance of IBU, especially at lower concentrations. Once more, this response was related to the "hormesis" effect, characterized by a low-dose stimulation and high dose-inhibition as described previously for the effects of NSAIDs in marine mussels (Fabbri et al., 2014) and clams (Aguirre-Martínez et al., 2016). In the same oyster species, *C. gigas*, Bebianno et al. (2017) assessed gene transcription responses (related to oxidative stress and biotransformation) as a consequence of PAR (1 and 100 µg/L) exposures for 1, 4 and 7 days revealing that transcriptional changes were also concentration and tissue dependent.

Overall, the effects of the NSAIDs: DCF, IBU and PAR were studied in the marine mussels: *M. galloprovincialis, M. trossulus*, *M. edulis*, *P. perna* and *M. charruana,* clams: *R. philippinarum* and *R. decussatus*, and the oyster: *C. gigas* at a wide range of concentrations (DCF: 0.005 – 10,000 µg/L; IBU: 0.25 – 10,000 µg/L; PAR: 0.25 – 5,000 µg/L). The larger number of reports on DCF is likely related to its proven acute toxicity and controversial inclusion in the Watch List of Chemicals of environmental concern. Although some of the tested concentrations (e.g., 10,000 µg/L) do not have environmental relevance, they were selected as tools to unveil specific responses to drugs, with more recent reports covering scenarios that are more realistic. As far as time of exposure refers, most of the studies are chronic (over 7 days) with some of the most recent ones including a depuration phase to evaluate recovery to pre-exposure conditions. Among marine bivalves, the most frequently investigated organisms were by far mussels, followed by a few species of clams and oysters. However, as for all the tested NSAIDs, even at low concentrations, negative impacts were recorded in all bivalve species. Therefore, it is difficult to predict which will be the most affected species and by which drug. Possibly, the limited number of studies performed with clams and oysters is related with ease of capture and maintenance of mussels; however, since all are important shellfish species which are highly appreciated for

human consumption, their study deserves consideration. In general, the tested NSAIDs showed impact in several endpoints such as larval development, reproduction, osmoregulation, immune parameters, genotoxicity and oxidative stress in addition to the changes associated with their particular mode of action.

The ecotoxicological risk of pharmaceutical drugs for marine bivalves will be linked to the uptake, bioaccumulation, metabolism and detoxification of the parental drug; however, their toxicity also depends on the drugs particular mode of action and the biotransformation processes of drugs. The latter leading to the generation of reactive metabolites and/or reactive oxygen species and probably leading to changes in the species' metabolism that can ultimately result in unpredicted consequences. Moreover, since shellfish are an important food resource, the toxicity related to parental NSAIDs and their metabolites may also have consequences for human health.

## 3. CONCLUSIONS AND FUTURE PERSPECTIVES

The presence of pharmaceuticals in aquatic systems is a well-recognized and growing problem worldwide, since the release of these contaminants can lead to alterations from molecular to population level in their aquatic inhabitants. However, due to heterogeneous and fragmented data it is difficult to define a list of environmental priority substances. In this review, the NSAIDs DCF and IBU and the analgesic PAR were selected taking into account both the controversial inclusion of DCF in the EU Watch List, for its demonstrated acute toxicity, as well as the high consumption and/or frequent occurrence in the aquatic environment in the case of all of these. Marine bivalves were selected because of their suitability as bioindicators that provide valuable information not only on the presence but also on the harm pharmaceutical drugs can cause. NSAIDs bioaccumulation potential is of great relevance although some of the studies assessing the effects of these drugs in marine bivalves still lack this information. Even though tissue concentrations may not be well correlate with exposure

time or concentration, due to the biotransformation/excretion in bivalves, still it provides useful information on the levels accumulated and highlights the need to include the identification of metabolites. The investigation of the effects of the selected pharmaceutical drugs in marine bivalves (from early life stages to adulthood) was assessed acutely and chronically mainly by using sub-individual (biochemical and Omics parameters) and developmental endpoints indicative of larval development in early life stages. Parameters related to cytotoxicity, oxidative stress, immunological status and genotoxicity, were among the most investigated in adults. In general, the studies on the effects of the selected NSAIDs showed a non-consistent oxidative challenge, supporting that the prooxidant mechanisms do not represent the primary mode of action of these pharmaceuticals. However, immune responses appeared to be the main target of the long-term of these exposures. Despite an evident effort in recent studies to apply environmentally realistic scenarios (in the low µg/L range and longer exposure times, up to 28 days), some studies were still performed with non-environmentally concentrations (e.g., 1 mg/L), short time exposures (1h) and non-relevant pathways (intraperitoneal injection), not properly estimating the effects of these drugs on bivalves. Moreover, even in studies applying realistic approaches, most of them lack the evaluation of the effects within a time-frame that would provide key information on how the organisms react to the drugs and the consideration of the "hormesis" effect. The inclusion of metabolite determinations and the toxic evaluation of drug mixtures under combined climatic scenarios also deserve further consideration.

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Table 1: Tissue concentration and bioconcentration factor (BCF) of the NSAIDs DCF, IBU and PAR in marine bivalves exposed under laboratory conditions.

| Drug | Exposure concentration (µg/L) | Days of exposure (d) and depuration (D) | Species | Tissue concentration (ng/g WW)* | BCF (L/kg WW)* | Reference |
|---|---|---|---|---|---|---|
| **DCF**<br>**IBU** | 1, 1000, 10000 | 21 d | *M. trossulus* | 180 (1.0 µg/L DCF, 8 d)<br>82000 (10000 µg/L DCF, 8 d), NQ for IBU | 180 (1.0 µg/L DCF, 8 d)<br>10 (10000 µg/L DCF, 8 d), NQ for IBU | Ericson et al. (2010) |
| **DCF** | 133 | 5 d + 5 D | *M. trossulus* | 555 (1 d), 1948 (3 d), 215 (5 d), 52.5 (5 D) | 3.05 (1 d), 9.57 (5 d) | Świacka et al. (2019) |
| **DCF** | 1; 100 | 3 d | *M. galloprovincialis* | 6.5 (1.0 µg/L)<br>520 (100 µg/L) | 6.84 (1.0 µg/L)**<br>5.4 (100 µg/L)** | Courant et al. (2018) |
| **DCF** | 100; 600 | 7 d | *M. galloprovincialis* | 502 (100 µg/L)<br>1836 (600 µg/L) | 16.3 (100 µg/L)<br>11.3 (600 µg/L) | Bonnefille et al. (2017) |
| **DCF**<br>**IBU**<br>**PAR** | 25 | 14 d | *M. galloprovincialis* | 3.7 (DCF)<br>0.4 (IBU)<br>ND (PAR) | 0.149 (DCF)**<br>0.0163 (IBU)** | Mezzelani et al. (2016a) |
| **DCF**<br>**IBU**<br>**PAR** | 0.5 | 14 d | *M. galloprovincialis* | 1.19 (DCF)<br>ND (IBU)<br>ND (PAR) | 2.4 (DCF)** | Mezzelani et al. (2016b) |
| **DCF**<br>**IBU** | 2.5 | 60 d | *M. galloprovincialis* | DCF: 0.41 (14 d), 0.91 (30 d), 0.56 (60 d)<br>IBU: 6.4 (14 d), 6.1 (30 d), 11.3 (60 d) | DCF: 0.16 (14 d), 0.36 (30 d), 0.22 (60 d)**<br>IBU: 2.6 (14 d), 2.5 (30 d), 4.5 (60 d)** | Mezzelani et al. (2018b) |
| **DCF** | 1 | 28 d | *M. galloprovincialis* | 7.1 | 7.4** | Freitas et al. (2019a) |
| **DCF** | 1 | 28 d | *M. galloprovincialis* | 9.7 | 10.1** | Freitas et al. (2019b) |
| **DCF** | 1 | 7 d | *R. philippinarum*, *R. decussatus* | 22 (*R. philippinarum*)<br>10 (*R. decussatus*) | 22 (*R. philippinarum*)**<br>10 (*R. decussatus*)** | Costa et al. (2019) |
| **IBU** | 1; 100 | 7 d | *C. gigas* | 0.22 (1.0 µg/L)<br>29.4 (100 µg/L) | 0.22 (1.0 µg/L)**<br>0.29 (100 µg/L)** | Serrano et al. (2015) |

DCF, diclofenac; IBU, ibuprofen; PAR, paracetamol; WW, wet weight, NQ, not quantified, ND, not determined; DW, dry weight

Note: The tissue concentrations levels and the bioconcentration factors (BCFs) (*) were converted to equal units (µg/g WW for concentrations and L/kg for BCFs). In the absence of reported BCFs, the values indicated (**) were calculated based on the information provided by the studies (tissue and water concentrations), applying a conversion factor of 4. The experimental BCFs were reported, whenever possible.

Table 2: Sub-lethal effects of the NSAIDs (DCF, IBU and PAR) in marine bivalves exposed under laboratory conditions.

| Drug | Exposure concentration (µg/L) | Days of exposure (d) and depuration (D) | Species | Endpoints | Reference |
|---|---|---|---|---|---|
| **DCF** | 133 | 5 d + 5 D | *M. trossulus* | Bioconcentration, metabolites identification | Świacka et al. (2019) |
| **DCF** | 100; 600 | 7 d | *M. galloprovincialis* | Bioconcentration, metabolites identification | Bonnefille et al. (2017) |
| **DCF IBU** | 0.01-1000 | 2 d | *M. galloprovincialis* | Embryotoxicity (developmental effects) | Fabbri et al. (2014) |
| **DCF** | 1; 10 | 2 d | *M. galloprovincialis* | Embryotoxicity (developmental effects), mRNA transcription of genes involved in shell formation and biotransformation | Balbi et al. (2018) |
| **DCF** | 0.02-1× $10^6$ | 1 d | *P. perna* | Embryo-larval development effects, oxidative stress biomarkers, xenobiotic metabolism | Fontes et al. (2018) |
| **IBU** | 0.15-1508 ng/g DW (sediment) | 2 d (*P. perna*)<br>1 d (*M. charruana*) | *P. perna*<br>*M. charruana* | Embryo-larval development effects, lysosomal membrane stability | Pusceddu et al. (2018) |
| **DCF IBU** | 1, 1000, 10000 | 21 d | *M. trossulus* | Bioconcentration, physiological indicators | Ericson et al. (2010) |
| **PAR** | 23; 403 | 10 d | *M. galloprovincialis* | Behavior (feeding rate), drug metabolism, oxidative stress related parameters | Solé et al. (2010) |
| **PAR** | 0.5-500 µg/L | 3 d | *Mytilus spp*. | Food uptake and nutritional reserve status, oxidative stress related parameters | Piedade et al. (2020) |
| **DCF IBU PAR** | 25 | 14 d | *M. galloprovincialis* | Bioconcentration, immunotoxicity, neurotoxic responses, oxidative stress biomarkers, genotoxic effects | Mezzelani et al. (2016a) |
| **DCF IBU PAR** | 0.5 | 14 d | *M. galloprovincialis* | Bioconcentration, immunotoxicity, oxidative stress biomarkers, genotoxicity, fatty acid metabolism, neurotoxic effects, transcriptomic analysis | Mezzelani et al. (2016b) |
| **DCF IBU** | 2.5 | 60 d | *M. galloprovincialis* | Bioconcentration, immunotoxicity, neurotoxic effects, oxidative stress biomarkers, genotoxicity, transcriptomic analysis | Mezzelani et al. (2018b) |
| **DCF** | 0.05; 0.50 | 14 d | *M. galloprovincialis* | Immunotoxicity, oxidative stress biomarkers, genotoxicity | Munari et al. (2019, 2018) |
| **DCF** | 0.005-20 | 1 h | *M. galloprovincialis* | Cytotoxicity, oxidative stress biomarkers, genotoxicity | Touxefi et al. (2016) |
| **DCF** | 0.25 | 15 d | *M. galloprovincialis* | Oxidative stress biomarkers | Gonzalez-Rey and Bebianno (2014) |
| **DCF** | 1 | 28 d | *M. galloprovincialis* | Bioconcentration, energy metabolism, oxidative stress biomarkers | Freitas et al. (2019a, 2019b) |
| **DCF** | 1; 1000 | 4 d | Mytilus spp. | Oxidative stress and reproduction | Schmidt et al. |

| | | | | | |
|---|---|---|---|---|---|
| | | | | biomarkers, genotoxicity | (2011) |
| **DCF** | 1; 1000 | 14 d + 7 D | Mytilus spp | Oxidative stress biomarkers, genotoxicity, proteomic analysis | Schmidt et al. (2014) |
| **IBU** | 0.25 | 15 d | *M. galloprovincialis* | Oxidative stress biomarkers, endocrine disruption | Gonzalez-Rey and Bebianno (2012, 2011) |
| **DCF** | 200; 1000 | 7 d | *M. edulis* | Oxidative stress biomarkers, proteomic analysis | Jaafar et al. (2015) |
| **PAR** | 0.04; 0.25, 100 | 7 d | *M. edulis* | Histology, gene expression | Koagouw and Ciocan (2019) |
| **DCF** | 1; 100 | 3 d | *M. galloprovincialis* | Bioconcentration, modulation of prostaglandin biosynthesis | Courant et al. (2018) |
| **DCF** | 100 | 7 d | *M. galloprovincialis* | Metabolite identification, metabolism pathways identification | Bonnefille et al. (2018) |
| **IBU** | 0.25 | 15 d | *M. galloprovincialis* | Transcriptomic analysis related with metabolic processes and inflammation/immunity | Maria et al. (2016) |
| **DCF** | 0.5 | 4 d | *R. philippinarum* | Embryotoxicity (developmental effects), oxidative stress biomarkers | Munari et al. (2016) |
| **IBU** | 0.1-50 | 35 d | *R. philippinarum* | Immunotoxicity | Aguirre-Martinez et al. (2013) |
| **IBU** | 100-1000 | 7 d | *R. philippinarum* | Immunotoxicity, cyto-genotoxicity | Matozzo et al. (2012) |
| **DCF IBU** | 15 | 14 d + 7 D | *R. philippinarum* | Oxidative stress biomarkers, neurotoxicity | Trombini et al. (2019) |
| **DCF** | 1 | 7 d | *R. philippinarum, R. decussatus* | Bioconcentration, physiological parameters (respiration rate), energy metabolism, oxidative stress biomarkers, neurotoxicity | Costa et al. (2019) |
| **IBU** | 0.1-50 | 14 d | *R. philippinarum* | Oxidative stress biomarkers, genotoxicity, neurotoxicity | Aguirre-Martínez et al. (2016) |
| **IBU** | 100; 1000 | 7 d | *R. philippinarum* | Oxidative stress biomarkers, neurotoxicity, gene transcription | Milan et al. (2013) |
| **PAR** | 50; 500; 5000 | 4 d | *R. decussatus, R. philippinarum* | Oxidative stress biomarkers | Antunes et al. (2013) |
| **PAR** | 50; 500; 5000 | 28 d | *R. philippinarum* | Oxidative stress biomarkers | Correia et al. (2016) |
| **PAR** | 0.25-25 | 4 d + 10 D | *R. philippinarum* | Oxidative stress biomarkers, energy metabolism | Nunes et al. (2017) |
| **IBU** | 1; 100 | 7 d | *C. gigas* | Bioconcentrations, gene transcription, oxidative stress biomarkers, genotoxicity | Serrano et al. (2015) |
| **PAR** | 1; 100 | 7 d | *C. gigas* | Cyto-genotoxicity, oxidative stress biomarkers, gene transcription | Bebianno et al. (2017) |

DCF, diclofenac; IBU, ibuprofen; PAR, paracetamol; DW, dry weight

**Highlights**

- NSAIDs, some included in the Watch List, are of concern in the marine environment.
- Studies on bioconcentration and effects of NSAIDs on marine organisms are limited.
- NSAIDs accumulated by marine bivalves, showed mainly sub-lethal effects.
- Immune responses are the main target of long-term exposure to NSAIDs.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: