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Review

Toxicity of the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) acetylsalicylic acid, paracetamol, diclofenac, ibuprofen and naproxen towards freshwater invertebrates: A review

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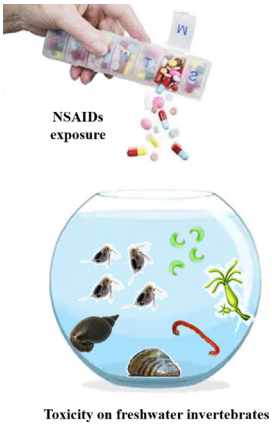
HIGHLIGHTS

- NSAIDs are one of the main pharmaceutical categories found in freshwater worldwide.
- Their peculiar chemico-physical feature can cause adverse effects to organisms.
- Freshwater invertebrates are excellent model organisms to assess NSAIDs toxicity.
- Acute effects of NSAIDs occur only at high, unrealistic concentrations.
- NSAIDs cause chronic effects at low, environmentally relevant concentrations.

GRAPHICAL ABSTRACT



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ABSTRACT

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) represent one of the main therapeutic classes of molecules contaminating aquatic ecosystems worldwide. NSAIDs are commonly and extensively used for their analgesic, antipyretic and anti-inflammatory properties to cure pain and inflammation in human and veterinary therapy. After use, NSAIDs are excreted in their native form or as metabolites, entering the aquatic ecosystems. A number of monitoring surveys has detected the presence of different NSAIDs in freshwater ecosystems in the ng/L - μg/L concentration range. Although the concentrations of NSAIDs in surface waters are low, the high biological activity of these molecules may confer them a potential toxicity towards non-target aquatic organisms. The present review aims at summarizing toxicity, in terms of both acute and chronic toxicity, induced by the main NSAIDs detected in surface waters worldwide, namely acetylsalicylic acid (ASA), paracetamol (PCM), diclofenac (DCF), ibuprofen (IBU) and naproxen (NPX), both singularly and in mixture, towards freshwater invertebrates. Invertebrates play a crucial role in ecosystem functioning so that NSAIDs-induced effects may result in hazardous consequences to the whole freshwater trophic chain. Acute toxicity of NSAIDs occurs only at high, unrealistic concentrations, while sub-lethal effects arise also at low, environmentally relevant concentrations of all these drugs. Thus, further studies represent a priority in order to improve the knowledge on NSAID toxicity and mechanism(s) of action in freshwater organisms and to shed light on their real ecological hazard towards freshwater communities.

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Contents

1. Pharmaceuticals as emerging contaminants of freshwaters

In the last two decades, pharmaceutical compounds have been identified as emerging contaminants for aquatic ecosystems. Pharmaceutical compounds are extensively and increasingly used both in human and veterinary therapy, including agriculture and aquaculture (Boxall et al., 2012). Pharmaceuticals have been designed to have a specific mode of action, targeting specific organs, metabolic pathways or receptors to modulate physiological functions, to treat a disease and to restore the health of the organism. For these reasons, pharmaceuticals play a pivotal role in our society, which commonly uses, and often abuses, a number of these molecules. For instance, in the European Union (EU) alone, it has been estimated that about 3000 different substances are used in human therapy, including anti-inflammatory drugs, contraceptives, antibiotics, β-blockers, lipid regulators, neuroactive drugs and many others (Fent et al., 2006). After their use, pharmaceuticals are excreted unchanged or as metabolites entering the sewage. As wastewater treatment plants (WWTPs) own a limited removal efficiency for several drugs, they are discharged in WWTP effluents contributing to the contamination of surface waters and, rarely, of groundwater and drinking water (Santos et al., 2010). According to the trend of production and use, as well as their pharmacokinetic and chemico-physical properties, different pharmaceuticals are detected in aquatic ecosystems in the ng/L to mg/L concentration range worldwide (Santos et al., 2010; Al Aukidy et al., 2014; Bagnis et al., 2018; Fekadu et al., 2019). In fact, a recent comprehensive review of measured environmental concentrations (MECs) for both human and veterinary pharmaceuticals on a global scale showed that 631 different substances were found water samples worldwide (aus der Beek et al., 2016). Thus, the presence of pharmaceuticals in aquatic ecosystems represents one of the main concerns that ecotoxicology has to face (Fent et al., 2006; Santos et al., 2010; Boxall et al., 2012).

2. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in freshwater ecosystems

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) represent one of the most relevant therapeutic class found in aquatic ecosystems worldwide (aus der Beek et al., 2016). NSAIDs are administered for their analgesic, antipyretic and anti-inflammatory properties to cure pain and inflammation in both human and veterinary therapy. NSAIDs inhibit the synthesis and the release of prostaglandins from arachidonic acid, acting as non-selective inhibitors of cyclooxygenase (COX) enzymes, namely cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoforms (Gierse et al., 1995). Different NSAIDs have been prescribed extensively or are commercialized over-the-counter worldwide. For instance, more than 70 million prescriptions are written each year in the United States, while considering the over-the-counter sale, more than 30 billion NSAID doses are consumed annually in the United States alone (Wiegand and Vernetti, 2017). Because of their huge and increasing use, coupled with their specific pharmacokinetic properties, NSAIDs reach detectable concentrations in both sewage and surface water, accounting for 15% of pharmaceuticals measured in aquatic ecosystems worldwide (Santos et al., 2010). NSAIDs and analgesics are the most frequently detected class of pharmaceuticals in the environment (Fekadu et al., 2019), as a number of monitoring surveys have reported levels of NSAIDs exceeding 1 μg/L in influent and effluents of WWTPs, while lower concentrations (in the ng/L range) have been found in surface waters (Santos et al., 2010; aus der Beek et al., 2016; Bagnis et al., 2018; Fekadu et al., 2019). Diclofenac is the most frequently detected pharmaceutical in environmental samples, while also ibuprofen and naproxen were detected nearly as often as diclofenac globally (aus der Beek et al., 2016). Moreover, five NSAIDs, including acetylsalicylic acid (ASA), paracetamol (PCM), diclofenac (DCF), ibuprofen (IBU) and naproxen (NPX) are included in the list of sixteen substances that were detected in surface, drinking, and groundwater of all the five United Nations (UN) regional groups (i.e., Africa Group, Asia-Pacific Group, Eastern Europe Group, Latin American and Caribbean States Group, and Western Europe and Others Group, which also includes North America, Australia and New Zealand), with global average concentrations ranging between 0.032 and 0.922 μg/L (aus der Beek et al., 2016). Although the concentrations of NSAIDs in freshwaters can be considered as relatively low, their high biological activity may pose a serious risk towards non-target species at different levels of the ecological hierarchy, leading to dissimilar toxic effects. To date, some previous reviews or meta-analyses have summarized the occurrence, toxicity and/or environmental risk of diverse pharmaceuticals (e.g., aus der Beek et al., 2016; Bagnis et al., 2018; Fekadu et al., 2019; Ohoro et al., 2019) or a specific compound (e.g., diclofenac; Acuña et al., 2015; Lonappan et al., 2016) in aquatic ecosystems, but none has specifically focused on the toxicity of drugs belonging to a specific class of pharmaceuticals towards freshwater invertebrates. Thus, the present review aimed at summarizing the toxicity induced by the exposure to ASA, PCM, DCF, IBU and NPX towards freshwater invertebrates. Freshwater invertebrate species globally account for approximately 2% (150,000 estimated species grouped in 17 *phyla*; Strayer et al., 2006) of an estimated 6.7 million invertebrate species (Collen et al., 2012). Although individually small and inconspicuous, aquatic invertebrates play a pivotal role in ecosystem functioning, including the transfer of energy from autotrophs to higher levels of the food web and the recycling of nutrients

(e.g., Pingram et al., 2014; Macadam and Stockan, 2015). Moreover, many invertebrate species are easy to be cultured and maintained under laboratory conditions, and are very sensitive to exogenous stresses, including the exposure to environmental contaminants, making them excellent model organisms in ecotoxicological surveys. A systematic literature research was performed in Google Scholar, Scopus and Web of Science databases. Literature research was focused on papers published in the 2000–2019 period of time, using for each single pharmaceutical compound different combinations of keywords dealing with their effects on freshwater invertebrates, including pharmaceutical drugs, Non-Steroidal Anti-Inflammatory Drugs, freshwater, invertebrates, effects, toxicity.

3. Features of focal Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Acetylsalicylic acid, diclofenac, naproxen, ibuprofen and paracetamol are the most common NSAIDs detected in aquatic environments (Fekadu et al., 2019). The main physico-chemical properties of the investigated NSAIDs are reported in Table 1. Acetylsalicylic acid (ASA; 2-(acetyloxy)benzoic acid) has remained for over 90 years as one of the most prescribed analgesics in human medical care worldwide (Katzung and Trevor, 2015). ASA is commonly used to reduce pain, fever, or inflammation and after oral administration it overwhelms hepatic metabolic reactions that transform it into conjugates (e.g., glucoronides) ease to be excreted. Although as much as 80% of a single therapeutic doses of ASA is metabolized in the liver, the remaining part is excreted as unchanged parent compound, entering the sewage. ASA was detected in sewage effluents and surface waters at maximum levels of 1.5 and 3.1 μg/L (e.g., Ternes, 1998; Schulman et al., 2002) respectively, even if concentrations up to 13 μg/L (Santos et al., 2010 and references therein) and even 59.6 μg/L were detected in wastewater treatment plants from Spain (Metcalfe et al., 2003). ASA was detected in surface waters, groundwater and/or tap or drinking waters from 15 Countries worldwide, with global average and maximum measured environmental concentrations of 0.922 μg/L and 20.96 μg/L, respectively (aus der Beek et al., 2016).

Paracetamol (PCM; N-(4-hydroxyphenyl)acetamide), also known as acetaminophen, is an analgesic and antipyretic drug. Although PCM does not own a proper anti-inflammatory action, it is usually included in the NSAID therapeutic group by a toxicological point of view because its mechanism of action is similar to that of NSAIDs (Misra et al., 1990). PCM can be purchased as an over-the-counter sale drug in most Countries worldwide and represents one of the most frequently detected pharmaceuticals in surface waters, wastewaters and drinking water. A recent review showed that PCM was detected in surface waters, groundwater and/or tap or drinking waters from 29 Countries worldwide, with global average and maximum measured environmental concentrations of 0.161 μg/L and 230 μg/L, respectively (aus der Beek et al., 2016).

Diclofenac (DCF; 2-[(2,6-dichlorophenyl)amino] phenylacetic acid) is a phenylacetic acid used to reduce inflammation and pain associated with arthritis, osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis (Todd and Sorkin, 1988). As DCF can be sold both as an over-the-counter sale drug and under medical prescriptions, it is one of the main drugs used worldwide and, consequently, one of the main pharmaceuticals contaminating the aquatic ecosystems. As WWTPs have a limited efficiency of removal, DCF is commonly detected at low μg/L range in WWTP effluents of Europe and North and South America (Roberts and Thomas, 2006; Gómez et al., 2007). Accordingly, DCF was commonly detected also in surface waters, in concentrations ranging between low ng/L up to low μg/L (Metcalfe et al., 2003; Bound and Voulvoulis, 2006; Gros et al., 2006). Because of its occurrence in aquatic ecosystems and potential toxicity, the European Union has included DCF to the list of the Water Framework Directive (2013/39/EU) as priority molecules to be monitored in aquatic ecosystems. However, as sufficient high-quality monitoring data were obtained for DCF, the EU commission decided that this substance should be removed from the watch list (commission implementing decision 2018/840/EU). DCF was detected in surface waters, groundwater and/or tap or drinking waters from 50 Countries worldwide, with global average and maximum measured environmental concentrations of 0.032 μg/L and 18.74 μg/L, respectively (aus der Beek et al., 2016).

Ibuprofen (IBU; ((+/−)-2-(*p*-isobutylphenyl) propionic acid with R and S isomers) is used to relieve the symptoms of arthritis, rheumatic disorders, pain and fever (Hayashi et al., 2008). IBU represents one of the core pharmaceuticals included in the "Essential Drug List" of the World Health Organization (WHO), and it is produced in large amounts worldwide (Heckmann et al., 2007). Because of its over-the-counter sale, large prescription volume and high excretion rate (~70–80% of the therapeutic dose), IBU has been identified as one of the main pharmaceuticals in aquatic ecosystems. Moreover, IBU has relatively high mobility into aquatic environments, but a lower persistence in comparison with other pharmaceuticals (Buser et al., 1999). IBU was detected in moderate to high concentrations both in the effluents of WWTPs and in surface waters during surveys carried out in both Europe and North America (Metcalfe et al., 2004; Santos et al., 2010). IBU was detected in surface waters, groundwater and/or tap or drinking waters from 47 Countries worldwide, with global average and maximum measured environmental concentrations of 0.108 μg/L and 303 μg/L, respectively (aus der Beek et al., 2016).

Naproxen (NPX; (S) 6-methoxy-α-methyl-2-naphthalene acetic acid) is a prototypical member of NSAIDs, commonly used in the treatment of migraine, rheumatoid arthritis, and osteoarthritis. Naproxen is metabolized in the liver and eliminated in its unchanged form (10% of the dose) or as metabolites (60% of the dose) through the urine and feces. NPX can undergo diverse biotransformation pathways, the most important being conjugation with glucuronic acid to form naproxen-β-1-O-acyl glucuronide, as well as O-dealkylation made by CYP2C9 and CYP1A2 enzymes, leading the production of 6-Odesmethylnaproxen (Huq, 2006). NXP was commonly detected in surface waters at concentrations up to 32 μg/L in Pakistan, 4.5 mg/L in Canada, 0.328 mg/L in China, and 0.24 mg/L in Japan (Brun et al., 2006; Komori et al., 2013; Zhao et al., 2010). A recent review showed that NPX was detected in surface waters, groundwater and/or tap or drinking waters from 45 Countries worldwide, with global average and maximum measured environmental concentrations of 0.050 μg/L and 32 μg/L, respectively (aus der Beek et al., 2016).

Table 1
Main physico-chemical properties of the investigated Non-Steroidal Anti-Inflammatory Drugs (NSAIDs).

	Acetylsalicylic acid	Paracetamol	Diclofenac	Ibuprofen	Naproxen
Formula	$C_9H_8O_4$	$C_8H_9NO_2$	$C_{14}H_{11}Cl_2NO_2$	$C_{13}H_{18}O_2$	$C_{14}H_{14}O_3$
Molar mass (g/mol)[b]	180.158	151.163	318.1	206.3	230.3
Solubility in water (g/L at 25 °C)[a]	1.46	4.15	0.00237	0.021	0.0159 mg/L
pKa[b]	3.49	9.38	4.15	4.91	4.15
logP[b]	1.20	0.46	4.40	3.70	3.18

[a] Drugbank (https://www.drugbank.ca), accessed 05/2020.
[b] Puckowski et al., 2016.

4. Accumulation and biotransformation of NSAIDs in freshwater invertebrates

The studies on accumulation of NSAIDs in freshwater invertebrates were performed on different species belonging to different *taxa*. NSAID residues in invertebrates were relatively higher compared with other classes of pharmaceuticals and the most frequent NSAIDs accumulated and measured in invertebrates were DCF and IBU (Miller et al., 2018). Focusing on freshwater invertebrates, some studies investigated the accumulation of NSAIDs in invertebrates from different ecosystems worldwide. Measureable concentrations of DCF (12.4 ng/g dry weight) and IBU (183 ng/g dry weight) were found in *Hydropsyche* spp. individuals from the River Segre (Spain; Huerta et al., 2015). An in-situ study aimed at developing an analytical method to determine emerging contaminants in benthic invertebrates, namely *Gammarus fossarum*, *Potamopyrgus antipodarum* and *Chironomus riparius*, organisms were exposed upstream and downstream of a WWTP located on a French river that drains dense urban and industrial areas in the Southeast of France (Berlioz-Barbier et al., 2014). IBU was measured in *G. fossarum* specimens (concentration range 60.6–105.4 ng/g wet weight) but not in *P. antipodarum* specimens (<limit of quantification), while DCF was measured in *C. riparius* specimens (concentration range 26–51.5 ng/g wet weight; Berlioz-Barbier et al., 2014). Diclofenac was detected in measurable concentrations in *Planorbis* spp. (13 ng/g wet weight), *Hyalella azteca* (20 ng/g wet weight), *Utterbackia imbecillis* (15 ng/g wet weight) and *Corbicula fluminea* (23 ng/g wet weight) from the North Bosque River (Texas, USA; Du et al., 2015). Diclofenac was accumulated in diverse mussel species from the Taihu Lake (China), including *Anodonta* spp. (2.45 ng/g dry weight), *Bellamya* spp. (3.14 ng/g dry weight), *Corbiculidae* (2.59 ng/g dry weight), and in Siberian prawn *Exopalaemon modestus* (6.7 ng/g dry weight), while IBU was measured at higher mean concentrations than DCF in tissues of *Bellamya* spp. (71.23 ng/g dry weight), *Corbiculidae* (41.6 ng/g dry weight), and in Siberian prawn (24.96 ng/g dry weight) (Xie et al., 2015). Grabicova et al. (2014) measured DCF in *Erpobdella octoculata* specimens (19.66 ng/g wet weight) from the Zivny Stream (Czech Republic), while Ruhí et al. (2016) found DCF in *Hydropsyche* (9 ng/g dry weight) and IBU in *Hydropsyche* (182.7 dry weight) and *Phagocata vitta* (30.9 dry weight) from the Segre River (Spain). A study by Ikkere et al., (2018) investigated the presence of different NSAIDs (i.e., tolfenamic acid, meloxicam, carprofen, flunixin, diclofenac, ibuprofen, phenylbutazone, ketoprofen and mefenamic acid) in soft tissues of four freshwater mussels, namely *Unio tumidus*, *Anodonta anatina*, *Anodonta cygnea* and *Dreissena polymorpha*, from Latvian ecosystems. Only IBU was detected in half of analyzed samples in concentrations ranging between 0.52 and 109 ng/g wet weight. A recent study by Yang et al. (2020) investigated the levels, bioaccumulation, and trophic transfer of 45 pharmaceuticals and personal care products, including some DCF and IBU, in highly urbanized rivers, namely the New Qinhuai River, the Qinhuai River and a section of the Yangtze River (China). DCF and IBU were detected in measureable concentrations in phytoplankton (DCF concentration range = 1.3–8.4 ng/g wet weight; IBU concentration range = 14.5–35.8 ng/g wet weight), zooplankton (concentration range = 2.1–12.4 wet weight; IBU concentration range = 20.9–48.9 ng/g wet weight) and three invertebrate species (i.e., freshwater shrimps, mussels and snails; concentration range = 1.1–5.9 wet weight; IBU concentration range = 4.8–11.6 ng/g wet weight) from the three rivers (Yang et al., 2020).

After uptake, NSAIDs can undergo biotransformation processes. Cytochrome P450 mixed function oxidase (MFO) systems play a crucial role in oxidation of drugs and xenobiotics in humans and in a number of species, including bacteria, plants, fish and aquatic invertebrates (Snyder, 2000; Rewitz et al., 2006; Gottardi et al., 2016). Different P450 gene families (CYP) have been characterized in fish and invertebrates (Stegeman and Livingstone, 1998). The CYP2 family, particularly the subfamily CYP2C9, has been identified as the responsible for NSAID biotransformation (Blanco et al., 2005; Zanger et al., 2008). Another pathway of biotransformation of carboxylate NSAIDs, such as ASA, DCF, NPX and IBU) involves the glucuronic acid conjugation catalyzed by the uridine diphosphoglucuronosyl transferase superfamily of enzymes, resulting in acyl glucuronides (Pritchard, 1993). These compounds are reactive intermediates that can undergo acyl migration and hydrolysis and can also form adducts with nucleophilic amino acid residues (Pritchard, 1993). Many NSAID-derived acyl glucuronides, including those obtained from DCF and IBU, have been shown to form covalent bonds with intra and extracellular proteins, with toxicological consequences (Boelsterli, 2007). Information on biotransformation products is currently limited to surface waters and biota. In detail, they have been scarcely determined across invertebrates, with only 12 reported concentrations (Miller et al., 2018). However, to date none of such studies included NSAIDs. Thus, developing new methods to measure biotransformation products in invertebrates represents a priority in NSAID ecotoxicology. The measurement of accumulated levels of NSAIDs and their biotransformation products in organisms should allow to perform a more reliable risk assessment for these compounds in the environment and to address prioritization of hazardous compounds, as well as to study potential pharmacological or toxicological effects for the understanding of the risk. In fact, the quantification of NSAIDs associated with effect-based studies should allow to shed light on the cause-effect relationship and threshold associated with the onset of the effect, avoiding extrapolation of exposure concentrations to observed effects (Miller et al., 2018).

5. Toxicity of NSAIDs towards freshwater invertebrates

Toxic effects induced by the exposure to the main NSAIDs measured in freshwater ecosystems, namely acetylsalicylic acid, paracetamol, diclofenac, ibuprofen and naproxen, in freshwater invertebrates were performed on different model species belonging to different *taxa* (Fig. 1). The number of studies investigating NSAID chronic toxicity was higher than those focused of acute toxicity. Crustaceans, mainly the Cladoceran *Daphnia magna*, were the main model organisms used to explore both acute and chronic toxicity of NSAIDs, followed by Mollusca. In Tables 2–7 are summarized the studies investigating the acute or chronic toxicity of selected NSAIDs and mixtures towards freshwater invertebrates. Acute toxicity describes the effects induced by either a single exposure or multiple exposures in a short time period and appears as lethal endpoints (e.g., mortality or immobilization). Chronic toxicity describes the onset of adverse effects resulting from prolonged and repeated exposure to stressors, which appears as sub-lethal endpoints (e.g., growth inhibition, molecular or biochemical alterations, behavioral changes).

5.1. Toxic effects induced by acetylsalicylic acid (ASA)

The toxicity of acetylsalicylic acid (ASA) was investigated on two crustacean species (*Daphnia magna* and *Daphnia longispina*) and a planarian species (*Dugesia japonica*) (Table 1). Acute and chronic toxicity of ASA was investigated on *Daphnia magna* and *Daphnia longispina* through the assessment of survival (i.e., immobilization or mortality) or reproduction and growth (Marques et al., 2004a). After 48 h of exposure, the 50% Effect Concentration (EC_{50}) of ASA for *D. longispina* was 647.31 mg/L and it was about half compared to that calculated for *D. magna* (1293.05 mg/L). These results were different from those found by other studies of *D. magna*, showing that the 50% Lethal Concentration (LC_{50}) at 48 h of ASA was 88.33 mg/L (Gómez-Oliván et al., 2014) and the EC_{50} at 48 h was 88.1 mg/L (Cleuvers, 2004). Increasing concentrations of ASA significantly affected the fecundity of *D. magna* and *D. longispina*. Similar No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) for both the Cladoceran species (i.e., NOEC = 1.00 mg/L and LOEC = 1.80 mg/L) were

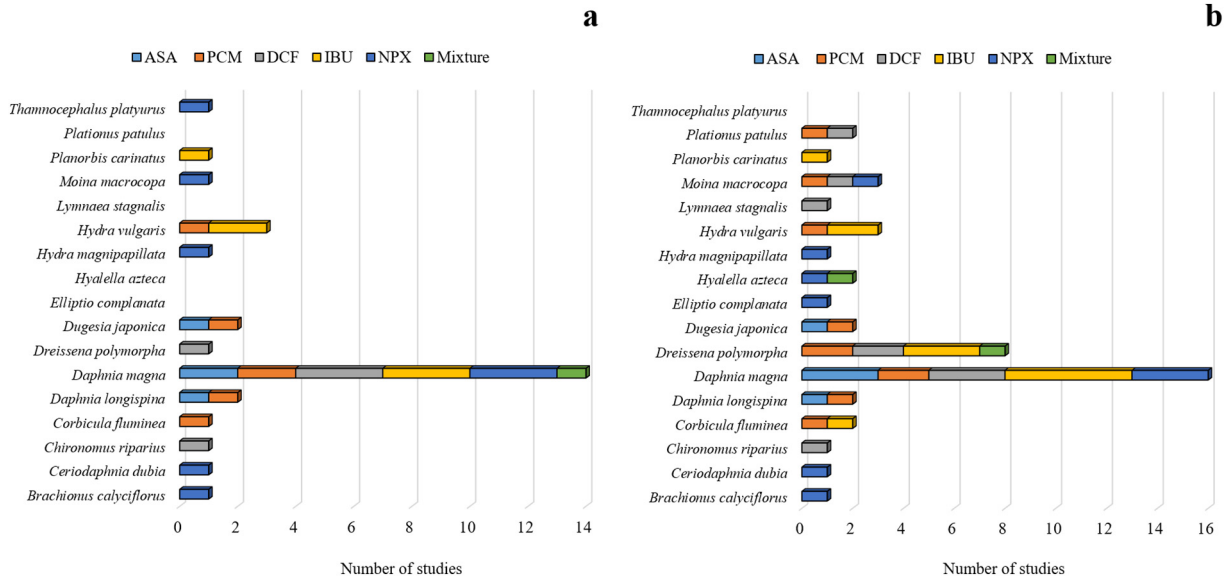

Fig. 1. Studies investigating acute (panel a) or chronic (panel b) toxicity of acetylsalicylic acid (ASA), paracetamol (PCM), diclofenac (DCF), ibuprofen (IBU), naproxen (NPX) and NSAID mixture (Mixture) towards different freshwater invertebrates.

found (Marques et al., 2004a). Considering the population intrinsic growth rate (r), ASA boosted the population growth in *D. longispina*, whereas an opposite trend was observed for *D. magna* (Marques et al., 2004a). Sub-lethal effects were induced by the exposure to 17.9 mg/L of ASA in terms of defects in eye regeneration, unstimulated behavior, and scrunching in regenerating tails, but not in full worms, in the freshwater planarian *D. japonica*, suggesting developmental injuries (Zhang et al., 2019).

Chronic toxicity of ASA was also investigated at biochemical level by the application of a battery of different biomarkers of oxidative stress and genotoxicity, namely lipid peroxidation, protein carbonyl content, activity of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase and DNA fragmentation (Gómez-Oliván et al., 2014). The exposure to a single concentration (8.8 mg/L) of ASA, corresponding to equal to the Lowest Observed Adverse Effect Level (LOAEL) obtained from a previous acute assay, induced the modulation of the activity of antioxidant enzymes, as well as the increase of lipid peroxidation and DNA fragmentation in treated specimens compared to controls (Gómez-Oliván et al., 2014).

5.2. Toxic effects induced by paracetamol (PCM)

The toxicity of paracetamol (PCM) was assessed on diverse freshwater invertebrate species, including a cnidarian (*Hydra vulgaris*) and a rotifer (*Plationus patulus*) species, different crustacean (*Daphnia magna, Daphnia longispina* and *Moina macrocopa*) and bivalve (*Dreissena polymorpha* and *Corbicula fluminea*) species, as well as a planarian species (*Dugesia japonica*) (Table 2).

PCM median lethal concentrations (LC_{50}) for *D. magna* was 224, 40.0, 8.06 and 5.32 mg/L at 24, 48, 96 h and 21 days, respectively (Du et al., 2016). In the same species, the PCM median effect concentrations (EC_{50}) for body length, number of carapaces per adult, number of broods per female and egg production per female was 4.78, 4.21, 2.38 and 1.12 mg/L, respectively (Du et al., 2016). A study performed by Nunes et al. (2014) investigated the toxicity of PCM towards different freshwater species, including *D. magna* and *D. longispina*. PCM toxicity was widely variable among species, even among species that were phylogenetically related. Considering acute toxicity in terms of EC_{50} for invertebrates, *D. magna* (4.7 mg/L) was more sensitive than *D. longispina*

Table 2
List of studies investigating the adverse effects induced by acetylsalicylic acid (ASA) exposure towards freshwater invertebrates. Test species, range of ASA concentrations used, duration of the exposure and investigated endpoints are reported.

	Taxonomic	Concentration range	Duration/endpoint	Test type	References
Daphnia magna	Arthropoda	77.5–101.5 mg/L	48 h/immobility	Acute	Gómez-Oliván et al., 2014
Daphnia magna	Arthropoda	1–320 mg/L	24 and 48 h/immobility	Chronic	Cleuvers, 2004
Daphnia longispina	Arthropoda	347–900 mg/L	48 h/immobility	Acute	Marques et al., 2004a
Daphnia magna	Arthropoda	900–2350 mg/L	48 h/immobility	Acute	Marques et al., 2004a
Daphnia longispina	Arthropoda	1–10 mg/L	21 days/reproduction	Chronic	Marques et al., 2004a
Daphnia magna	Arthropoda	1–10 mg/L	21 days/reproduction	Chronic	Marques et al., 2004a
Dugesia japonica	Platyhelminthes	0.0015–15 mg/L	12 days/lethality, eye regeneration assay, unstimulated behavioral assay, phototaxis, thermotaxis, scrunching	Acute/chronic	Zhang et al., 2019
Daphnia magna	Arthropoda	8.8 mg/L	48 h/superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), Lipid peroxidation, protein carbonyl content, Single cell gel electrophoresis, detection of oxidized bases	Chronic	Gómez-Oliván et al., 2014

Table 3
List of studies investigating the adverse effects induced by paracetamol (PCM) exposure towards freshwater invertebrates. Test species, range of PCM concentrations used, duration of the exposure and investigated endpoints are reported.

Species	Taxonomic	Concentration range	Duration/endpoint	Test type	References
Daphnia magna	Arthropoda	4–972 mg/L	48 h/immobility	Acute	Du et al., 2016
Daphnia magna	Arthropoda	0.08–6.48 mg/L	21 days/reproduction	Chronic	Du et al., 2016
Daphnia magna	Arthropoda	4.0–8.9 mg/L	48 h/immobility	Acute	Nunes et al., 2014
Daphnia magna	Arthropoda	0.53–4.0 mg/L	21 days/reproduction	Chronic	Nunes et al., 2014
Daphnia longispina	Arthropoda	48.6–85 mg/L	48 h/immobility	Acute	Nunes et al., 2014
Daphnia longispina	Arthropoda	7.9–60.0 mg/L	21 days/reproduction	Chronic	Nunes et al., 2014
Plationus patulus	Rotifera	2–32 mg/L	25 days/population growth	Chronic	Sarma et al., 2014
Moina macrocopa	Arthropoda	2–32 mg/L	10 days/population growth	Chronic	Sarma et al., 2014
Hydra vulgaris	Cnidaria	0.01–10 mg/L	7 days/polyp structure	Acute	Pascoe et al., 2003
Hydra vulgaris	Cnidaria	0.01–10 mg/L	10 days/feeding and bud production, polyp regeneration	Chronic	Pascoe et al., 2003
Dugesia japonica	Platyhelminthes	0.0018–18 mg/L	12 days/lethality, eye regeneration assay, unstimulated behavioral assay, phototaxis, thermotaxis, scrunching	Acute/chronic	Zhang et al., 2019
Corbicula fluminea	Mollusca	0.05–532.78 mg/L	96 h/survival	Acute	Brandão et al., 2014
Corbicula fluminea	Mollusca	3.88–61.95 μg/L	28 days/catalase (CAT), glutathione S-transferases (GSTs), and glutathione reductase (GRed), lipid peroxidation	Chronic	Brandão et al., 2014
Dreissena polymorpha	Mollusca	30–450 μg/L	1 h/Neutral Red Retention Assay (NRRA), single cell gel electrophoresis assay, DNA Diffusion assay	Chronic	Parolini et al., 2009
Dreissena polymorpha	Mollusca	0.154–1.51 μg/L	96 h/Neutral Red Retention Assay (NRRA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), single cell gel electrophoresis assay, DNA Diffusion assay, micronucleus test	Chronic	Parolini et al., 2010

(67.9 mg/L). Moreover, PCM caused mortality during a chronic toxicity reproduction test with *D. magna* at the highest tested concentrations (between 1.2 and 1.7 mg/L). Although treated specimens generated offspring, they did not survive over the whole duration of the experiment.

A different response was observed for *D. longispina*, which showed a significant delay in the first reproductive event and a reduction in the fecundity, but no mortality. A study by Sarma et al. (2014) exposed the rotifer *Plationus patulus* and the cladoceran *Moina macrocopa* to

Table 4
List of studies investigating the adverse effects induced by diclofenac (DCF) exposure towards freshwater invertebrates. Test species, range of DCF concentrations used, duration of the exposure and investigated endpoints are reported.

Species	Taxonomic	Concentration range	Duration/endpoint	Test type	References
Daphnia magna	Arthropoda	2–486 mg/L	48 h/immobility	Acute	Du et al., 2016
Daphnia magna	Arthropoda	0.5–40.5 mg/L	21 days/reproduction	Chronic	Du et al., 2016
Daphnia magna	Crustacea	29.5–75 mg/L	48 h/immobility	Acute	de Oliveira et al., 2016
Daphnia magna	Crustacea	5–5000 μg/L	21 days/reproduction; 48 h/expression of the genes related to the detoxification metabolism, growth, development and reproduction	Chronic	Liu et al., 2017
Plationus patulus	Rotifera	2–32 mg/L	25 days/population growth	Chronic	Sarma et al., 2014
Moina macrocopa	Crustacea	2–32 mg/L	10 days/population growth	Chronic	Sarma et al., 2014
Daphnia magna	Crustacea	5–50 mg/L	48 h/immobility and hsp70-induction	Acute and chronic	Haap et al., 2008
Dreissena polymorpha	Mollusca	60–250 μg/L	1 h/Neutral Red Retention Assay (NRRA), single cell gel electrophoresis assay, DNA Diffusion assay	Chronic	Parolini et al., 2009
Dreissena polymorpha	Mollusca	0.001–10 mg/L	96 h/trypan blue exclusion test, MTT reduction assay	Acute	Parolini et al., 2011a
Dreissena polymorpha	Mollusca	95–637 ng/L	96 h/Neutral Red Retention Assay (NRRA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), single cell gel electrophoresis assay, DNA Diffusion assay, micronucleus test	Chronic	Parolini et al., 2011b
Lymnaea stagnalis	Mollusca	100–1000 μg/L	3 days/hemocyte density and viability, hemocyte phagocytosis capacity and hemocyte-related oxidative activities	Chronic	Boisseaux et al., 2017
Chironomus riparius	Arthropoda	34.0 μg/g	10 days/survival, growth and developmental stage	Acute and chronic	Nieto et al., 2017

Table 5
List of studies investigating the adverse effects induced by ibuprofen (IBU) exposure towards freshwater invertebrates. Test species, range of IBU concentrations used, duration of the exposure and investigated endpoints are reported.

Species	Taxonomic	Concentration range	Duration/endpoint	Test type	References
Daphnia magna	Arthropoda	1–200 mg/L	48 h/immobility	Acute	Du et al., 2016
Daphnia magna	Arthropoda	0.4–32.4 mg/L	21 days/reproduction	Chronic	Du et al., 2016
Daphnia magna	Arthropoda	10–160 mg/L	48 h/immobility	Acute	Heckmann et al., 2005
Daphnia magna	Arthropoda	10–160 mg/L	12 days/reproduction and survival	Chronic	Heckmann et al., 2005
Daphnia magna	Arthropoda	20–80 mg/L	48 h/immobility	Acute	Heckmann et al., 2007
Daphnia magna	Arthropoda	20–80 mg/L	14 days/growth rate (PGR), reproduction, survival, somatic growth and population size structure	Chronic	Heckmann et al., 2007
Daphnia magna	Arthropoda	20–80 mg/L	10 days/reproduction, survival, growth rate	Chronic	Hayashi et al., 2008
Daphnia magna	Arthropoda	0.5–50 μg/L	6 h and 48 h/enzyme activity and gene expression	Chronic	Wang et al., 2016
Daphnia magna	Arthropoda	0.5–50 μg/L	21 days/reproduction	Chronic	Wang et al., 2016
Hydra vulgaris	Cnidaria	0.01–10 mg/L	7 days/polyp structure	Acute	Pascoe et al., 2003
Hydra vulgaris	Cnidaria	0.01–10 mg/L	10 days/feeding and bud production, polyp regeneration	Chronic	Pascoe et al., 2003
Hydra vulgaris	Cnidaria	0.1–100 mg/L	96 h/survival and polyp regeneration	Acute and chronic	Quinn et al., 2008
Dreissena polymorpha	Mollusca	45–909 μg/L	1 h/Neutral Red Retention Assay (NRRA), single cell gel electrophoresis assay, DNA Diffusion assay	Chronic	Parolini et al., 2009
Dreissena polymorpha	Mollusca	0.2–8 μg/L	96 h/Neutral Red Retention Assay (NRRA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), single cell gel electrophoresis assay, DNA Diffusion assay, micronucleus test	Chronic	Parolini et al., 2011c
Dreissena polymorpha	Mollusca	0.206–206 μg/L	7 days/mRNA changes of enzymes and other proteins involved in the prevention from protein damage and oxidative stress, biotransformation, elimination and reversible protein posttranslational modification	Chronic	Contardo-Jara et al., 2011
Corbicula fluminea	Mollusca	0.1–50 μg/L	21 days/lysosomal membrane stability, etoxyresorufin O-deethylase, dibenzylfluorescein dealkylase, gluthathione-S-transferase (GST), gluthathione reductase (GR), gluthathione peroxidase (GPX), lipid peroxidation, DNA damage	Chronic	Aguirre-Martínez et al., 2015
Planorbis carinatus	Mollusca	0.1–100 mg/L	21 days/survival and reproduction	Acute/chronic	Pounds et al., 2008

Table 6
List of studies investigating the adverse effects induced by Naproxen (NPX) exposure towards freshwater invertebrates. Test species, range of NAP concentrations used, duration of the exposure and investigated endpoints are reported.

Species	Taxonomic	Concentration range	Duration/endpoint	Test type	References
Brachionus calyciflorus	Rotifera	1–100 mg/L	24 h/mortality 48 h/reproduction	Acute/chronic	Isidori et al., 2005
Thamnocephalus platyurus	Arthropoda	1–100 mg/L	24 h/mortality	Acute	Isidori et al., 2005
Ceriodaphnia dubia	Arthropoda	1–100 mg/L	48 h/immobility 7 days/reproduction	Acute/chronic	Isidori et al., 2005
Hyalella azteca	Arthropoda	76.6 and 339.2 mg/kg	48 h/lipid peroxidation level, protein carbonyl content, activity of the antioxidant enzymes, and DNA damage	Chronic	García-Medina et al., 2015
Elliptio complanata	Mollusca	0.57–23 mg/L	24 h/phagocytic activity, intracellular esterase activity, cell adherence and lipid peroxidation	Chronic	Gagné et al., 2006
Daphnia magna	Arthropoda	1–320 mg/L	24 and 48 h/immobility	Chronic	Cleuvers, 2004
Moina macrocopa	Arthropoda	0.33–30 mg/L	48 h/immobility 21 days/reproduction	Acute and chronic	Kwak et al., 2008
Daphnia magna	Arthropoda	0.33–30 mg/L	48 h/immobility 21 days/reproduction	Acute and chronic	Kwak et al., 2018
Daphnia magna	Arthropoda	0.01–100 mg/L	24 and 48 h/immobility	Acute	Gheorghe et al., 2016
Hydra magnipapillata	Cnidarian	35–70 mg/L	24, 48 and 72 h/survival	Acute	Yamindago et al., 2019
Hydra magnipapillata	Cnidarian	40 mg/L	6, 12, 24 h/morphological changes in polyps, transcriptomic analyses	Chronic	Yamindago et al., 2019
Daphnia magna	Arthropoda	20.4–51.3 mg/L	48 h/immobility	Acute	Gómez-Oliván et al., 2014
Daphnia magna	Arthropoda	2.9 mg/L	48 h/superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), lipid peroxidation, protein carbonyl content, single cell gel electrophoresis	Chronic	Gómez-Oliván et al., 2014

Table 7
List of studies investigating the adverse effects induced by the exposure to complex mixtures of NSAIDs towards freshwater invertebrates. Test species, range of concentrations of NSAIDs included in the mixture, duration of the exposure and investigated endpoints are reported.

Species	Taxonomic	Concentration range	Duration/endpoint	Test type	References
Daphnia magna	Arthropoda	DCF (10.0–181.3 mg/L) IBU (58.4–134.1 mg/L) NAP (26.2–426.6 mg/L) ASA (38.1–135.2 mg/L)	24 and 48 h/immobility	Acute	Cleuvers, 2004
Hyalella azteca	Arthropoda	DCF (4.2–27.0 mg/L) PCM (2.4–15.1 mg/L) IBU (1.2–2.7 mg/L) NAP (2.5–9.8 mg/L) ASA (2.5–3.2 mg/L)	72 h/superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), lipid peroxidation, protein carbonyl content	Chronic	Gómez-Oliván et al., 2014
Dreissena polymorpha	Mollusca	DCF (0.1–1.5 μg/L) PCM (0.5–13 μg/L) IBU (0.1–9 μg/L)	96 h/Neutral Red Retention Assay (NRRA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), single cell gel electrophoresis assay, DNA Diffusion assay, micronucleus test	Chronic	Parolini and Binelli, 2012

increasing concentrations of PCM (concentration range 2–32 mg/L) in order to assess changes in population growth. Population growth curves of both the species were affected by PCM concentrations, showing a decrease in organism density with increasing levels of the drug. Moreover, the daily rate of population growth was negatively affected by PCM exposure in both the zooplanktonic species. A 7-day exposure to increasing PCM concentrations (concentration range 0.01–10 mg/L) did not affect the survival of *Hydra vulgaris* specimens at concentrations up to 1.0 mg/L, while no adverse effects on feeding and bud formation were induced after 17 days of exposure to the same concentration range. Moreover, the ability of dissected polyps to regenerate hypostome, tentacles and foot was not altered (Pascoe et al., 2003). All the studies mentioned above showed that acute effects of PCM occur only at mg/L concentrations, while sub-lethal effects arise also at lower, often environmentally relevant, concentrations. A decreased unstimulated speed in day 12 regenerating tails was observed in *D. japonica* specimens exposed to 15.5 mg/L of PCM (Zhang et al., 2019).

Chronic toxicity of PCM was also investigated at low levels of the biological organization. Biochemical effects of PCM exposure were investigated in the freshwater clam *Corbicula fluminea* following short- (96 h) and long-term (28 days) exposures to increasing PCM concentrations (Brandão et al., 2014). No mortality was observed in clams over short- or long-term exposures. PCM did not modulate catalase activity but induced a significant decrease of glutathione S-transferase (GST) and glutathione reductase (GR) activity over both short- and long-term exposures. A significant increase of lipid peroxidation was noted at the end of short- and long-term exposure to the highest PCM tested concentrations. These results indicated that the exposure to increasing PCM concentration caused notable changes in the cellular redox status of *C. fluminea* (Brandão et al., 2014). The in-vitro cytogenotoxicity of PCM was investigated through the application of a battery of four biomarkers (i.e., the comet test to investigate DNA fragmentation and frequency of apoptotic and necrotic cells, and the neutral red retention assay - NRRA) on hemocytes collected from the zebra mussel *Dreissena polymorpha* exposed for 1 h to 30, 150 and 450 μg/L (Parolini et al., 2009). Dose-dependent decrease in the stability of lysosome membranes (NRRA), coupled with a significant increase in both primary (DNA fragmentation) and fixed (frequency of apoptotic and necrotic cells) genetic damage was induced by PCM. In-vivo 96-h exposures of *D. polymorpha* specimens to three, environmentally relevant PCM concentrations (0.154; 0.75 and 1.51 μg/L) showed that this drug can alter the oxidative status of this bivalve species (Parolini et al., 2010). Low PCM concentrations did not cause neither mortality of zebra mussel over the duration of the experiment nor changes in hemocyte viability. Although PCM did not induce primary genetic injuries in zebra mussel hemocytes at all the tested concentration, a significant increase of fixed genetic damage, in terms of both micronuclei and apoptotic frequency, was noted at the end of the exposure to the highest tested concentrations. Moreover, a significant destabilization of lysosomal membranes and significant modulation of catalase, glutathione peroxidase (GPx) and GST activity was induced by the exposure to 0.75 and 1.51 μg/L of PCM.

5.3. Toxic effects induced by diclofenac (DCF)

Acute and chronic toxicity of diclofenac (DCF) towards freshwater invertebrates was explored on a rotifer (*Plationus patulus*), two crustaceans (*Daphnia magna* and *Moina macrocopa*), a chironomid (*Chironomus riparius*), a bivalve (*Dreissena polymorpha*) and a gastropod (*Lymnea stagnalis*) species (Table 3).

Mortality of *D. magna* specimens arose after only 24 h of exposure to a high DCF concentration (486 mg/L). DFC exposure caused 50% mortality (EC_{50}) in *D. magna* after 21 days of exposure to 2.00 mg/L and a significant reduction of egg production at the lowest exposure concentrations of 0.50 mg/L (Du et al., 2016). A study by de Oliveira et al. (2016) calculated that the EC_{50} for *D. magna* was 123.3 mg/L, but no effects on population growth was noted after the exposure to a range of increasing DCF concentrations (concentration range 29.5–75 mg/L). A 21-days exposure to four increasing DCF concentrations (concentration range 5–5000 μg/L) did not cause significant changes in molting frequency, number of eggs produced in the first brood, total number of eggs per individual, total number of broods per individual, body length and growth rate in *D. magna* specimens (Liu et al., 2017). In the same study, 96-h exposure to 50 μg/L of DCF induced significant changes in the expression of some genes related to detoxification, growth, development and reproduction, which were inhibited after 24 h and overexpressed after 48 h of exposure (Liu et al., 2017). In contrast, the exposure to increasing concentrations of DCF (concentration range 2–32 mg/L) affected the population growth curves of the rotifer *Plationus patulus* and the cladoceran *Moina macrocopa,* leading to a decrease in organism density

with increasing levels of drug and negative effects on the daily rate of population increase (Sarma et al., 2014).

Chronic toxicity of DCF was investigated also at molecular and biochemical level in different invertebrate species. A research by Haap et al. (2008) investigated the toxicity of DCF at biochemical level in *D. magna* by assessing the modulation of heat shock protein 70 (hsp70) level as a biomarker for proteotoxicity, showing that the modulation of such protein occurred only at concentrations of DCF higher than 40 mg/L. The cyto-genotoxicity of DCF was investigated through an in-vitro approach by exposing for 1 h hemocytes collected from the zebra mussel *D. polymorpha* to 60, 126 and 250 μg/L (Parolini et al., 2009). A significant cytotoxic effect, in terms of destabilization of the lysosomal membranes, was noted only after the exposure to 250 μg/L of DCF, while both primary genetic lesions (i.e., DNA fragmentation) and fixed damage to DNA (i.e., frequency of apoptotic and necrotic cells) occurred after the exposures to all the tested concentrations. A further in-vitro experiment (Parolini et al., 2011a) investigated the toxicity of increasing DCF concentrations (0.001, 0.01, 0.1, 1 and 10 mg/L) on three different cell typologies of the zebra mussel, namely hemocytes, gill and digestive gland cells. After 96 h of exposure, the viability of DCF treated gill cells was significantly reduced already at the lowest tested concentration. Moreover, the viability of DCF-treated digestive gland cells was significantly reduced already after 48 h exposure to 0.01 mg/L, while hemocyte viability was reduced already at the lowest concentration (0.001 mg/L). An in-vivo 96-h exposure of the zebra mussels to three increasing concentrations (95, 318 and 637 ng/L) of DCF showed a negligible cyto-genotoxicity. In fact, only a slight decrease of lysosomal membrane stability was observed at the end of exposure to the highest tested concentration (637 ng/L), while no other effects arose (Parolini et al., 2011b). DCF sub-lethal toxicity in terms of immunotoxicity was assessed on the gastropod *Lymnaea stagnalis* exposing specimens for 3 days to environmentally relevant (concentration range 1–10 μg/L) and therapeutic concentrations (concentration range 100–1000 μg/L) of DCF (Boisseaux et al., 2017). Diclofenac induced immune responses, while no immunosuppression was observed. DCF significantly affected the immunocapacity and the immunoefficiency of the snails' hemocytes. This effect is typical of an inflammatory response, confirmed by the increase of the NADPH-oxidase activity, mainly at 1000 μg/L. A 10-days chronic toxicity test with *C. riparius* was performed to assess effects on survival, growth and developmental stage, in terms of biomass, as well as emergence rates and sex ratio after 21 days of exposure to DCF-spiked sediments. No effects on survival and no change in the sex-ratio were induced by DCF exposure. In contrast, DCF decreased the emergence ratio in organisms exposed at concentrations of 34.0 μg/g of DCF (Nieto et al., 2017).

5.4. Toxic effects induced by ibuprofen (IBU)

Acute and chronic toxicity of ibuprofen (IBU) towards freshwater invertebrates was investigated on a crustacean (*Daphnia magna*), a cnidarian (*Hydra vulgaris*), two bivalve (*Dreissena polymorpha* and *Corbicula fluminea*) and a gastropod (*Planorbis carinatus*) species (Table 4).

Mortality of all *D. magna* specimens was caused after 24 h of exposure to high levels of IBU (200 mg/L), while EC_{50} was calculated as 3.97 (Du et al., 2016). The 48 h EC_{50} for immobilization was estimated as 108 mg/L, while the reproduction was reduced above 10 mg/L and that survival was unaffected at concentrations up to 40 mg/L, with no survival above 80 mg/L (Heckmann et al., 2005). A further study revealed that a 14-days exposure of *D. magna* to increasing IBU concentrations affected life-history traits and population performance of the species (Heckmann et al., 2007). Population growth rate significantly decreased at all the IBU tested concentrations, while survival was affected only at the end of the exposure to 80 mg/L of IBU. In contrast, reproductive effort was affected also by the exposure to the lowest IBU tested concentrations, whereby the 14-days EC_{50} was calculated as 13.4 mg/L, while it was completely inhibited at 80 mg/L. Similar results were obtained by Hayashi et al. (2008), who exposed 5-days old *D. magna* specimens to the same range of IBU concentrations tested by Heckmann et al. (2007) for 10 days. Specimens exposed to 40 mg/L of IBU generated significantly fewer offspring than controls, while no reproduction occurred at 80 mg/L. Moreover, a significant delay of the first reproductive event occurred at all the tested IBU concentrations. *Daphnia magna* survival was affected after the exposure to 80 mg/L during the 10-day exposure, while the population grew after the exposure to control, 20 and 40 mg/L of IBU. In contrast, a significant decrease of population growth was noted at the end of the exposure to 80 mg/L of IBU (Hayashi et al., 2008). A 7-days exposure to increasing IBU concentration did not affect the survival of the cnidarian *H. vulgaris* at concentrations up to 1 mg/L, while after 17 days of IBU administration neither feeding nor bud formation nor ability of dissected polyps to regenerate a hypostome, tentacles and foot were affected (Pascoe et al., 2003). However, another study showed that the exposure to 5 mg/L of IBU inhibited the regeneration of the cnidarian, with a 96-h IC_{50} (i.e., the concentration that inhibits 50% of the embryos to develop) was calculated as 3.84 mg/L (Quinn et al., 2008). Acute and sub-lethal effects of IBU were also investigated on the freshwater Keeled rams horn snails (*Planorbis carinatus*) exposed for 72 h or 21 days to different IBU concentrations. The 48- and 72-h LC_{50} were 17.1 mg/L at both the time points, while the 21-days LOEC and NOEC based on the survival of specimens were calculated as 45.36 and 5.36 mg/L, respectively. In addition, the 21-days LOEC and NOEC calculated for snail reproduction (i.e., hatching success) were 5.36 and 2.43 mg/L, respectively, while for growth were 2.43 and 1.02 mg/L, respectively (Pounds et al., 2008).

Chronic toxicity of IBU was investigated also at molecular and biochemical level through the application of different techniques. A study by Wang et al. (2016) investigated the modulation of the expression of CYP360A, CYP314, and GST genes involved in the detoxification process and the responses of their associated enzymes activity, as well as in some physiological parameters (e.g., growth and reproduction) in *D. magna* specimens exposed to three environmentally relevant concentrations of IBU. The exposure to IBU did not affect the reproduction of cladocerans, in terms of total amount of eggs produced and total number of clutches per female, as well as of body length of treated specimens. The same experiment showed that the treatment with 0.5 μg/L of IBU inhibited the expression of CYP360A gene, while at 50 μg/L an overexpression of such gene occurred. A similar trend was also induced by the exposure to at 0.5 μg/L of IBU towards the GST gene, while CYP314 expression was inhibited after a short time exposure (6 h), while was overexpressed after prolonged exposure time (48 h). Similarly, erythromycin *N*-demethylase and aminopyrine *N*-demethylase were both inhibited after 6-h exposure but overexpressed after 48-h exposure to 0.5 μg/L. Moreover, an induction of glutathione S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT) activity was observed in short-term exposure to IBU, while a dose-dependent increase of EROD activity and methane dicarboxylic aldehyde (MDA) content occurred (Wang et al., 2016). The cyto-genotoxicity of IBU was investigated through an in-vitro approach by 1-h exposure of zebra mussel hemocytes to different IBU concentrations (Parolini et al., 2009). The stability of lysosomal membranes was reduced at the endo of the exposure to 450 and 909 μg/L of IBU, while genotoxicity, in terms of DNA fragmentation and frequency of apoptotic cells, occurred in response to all the tested concentrations. A further 96-h in-vivo exposure of the zebra mussel showed that IBU induced a slight cyto-genotoxicity on hemocytes at 0.2 μg/L of IBU, while higher concentrations (2 and 8 μg/L) significantly affected lysosomal membrane stability and arose both primary and fixed genetic damage. In addition, IBU modulated the activity of antioxidant and detoxifying enzymes at all the tested concentrations, suggesting that this drug can imbalance the oxidative status of mussels and provoke the onset of oxidative stress also at low, environmental concentrations (Parolini et al., 2011c). Similar results were obtained on the clam *Corbicula fluminea* (Aguirre-Martínez et al., 2015). IBU

induced a destabilization of lysosomal membrane at all the tested concentrations, as well as the increase of the activity of phase I and II enzymes, including the activation of GR and GPx at the highest tested concentration (50 mg/L). Moreover, an increase of lipid peroxidation, but not of DNA damage was observed at the end of the exposure to 50 μg/L (Aguirre-Martínez et al., 2015). Another study performed on the zebra mussel investigated the effects of 7-days exposure to IBU at molecular level, exploring the changes in the expression of mRNA of enzymes and other proteins involved in the prevention of protein damage (hsp70) and oxidative stress (SOD, CAT and metallothionein), in the biotransformation (GST, aryl hydrocarbon receptor) and elimination (P-glycoprotein) of xenobiotics, as well as in reversible protein post-translational modification (protein phosphatase 2A). Zebra mussel specimens exposed to the lowest tested concentration of IBU experienced an oxidative stress situation as pointed out by the induction of mRNA levels observed for SOD, CAT and metallothionein in in the digestive gland after 1 or 4 days of treatment. At the higher concentrations, an increase in levels of transcripts for GST occurred, suggesting the activation of biotransformation processes of IBU or by-products deriving from oxidative stress (Contardo-Jara et al., 2011).

5.5. Toxic effects induced by naproxen (NPX)

Acute and chronic toxicity induced by naproxen (NPX) towards freshwater invertebrates was investigated on a rotifer (*Brachionus calyciflorus*), four crustacean (*Thamnocephalus platyurus*, *Ceriodaphnia dubia*, *Daphnia magna, Hyalella azteca*), a cnidarian (*Hydra magnipapillata*) and a bivalve (*Elliptio complanata*) species (Table 5). Acute and chronic toxicity of NPX was assessed through standard bioassays using rotifers (*Brachionus calyciflorus*) and crustaceans (*Thamnocephalus platyurus*, *Ceriodaphnia dubia*). Naproxen acute toxicity for *T. platyurus* and *B. calyciflorus* (LC_{50}; mortality) was calculated as 62.48 mg/L and 84.09 mg/L, respectively, while for *C. dubia* (EC_{50}; immobilization) was 66.37 mg/L (Isidori et al., 2005). Other studies showed that acute toxicity of NPX, in terms of immobilization EC_{50} to crustaceans, were in the same order of magnitude (Cleuvers, 2004; Gheorghe et al., 2016; Kwak et al., 2018). The study by Isidori et al. (2005) showed that chronic tests were responsive at lower concentrations compared to acute ones. Chronic toxicity in terms of inhibition or population growth for rotifers and crustaceans (EC_{50}) was 0.56 (0.40–0.62) mg/L for *T. platyurus* and 0.33 (0.11–0.63) mg/L for *C. dubia*, respectively. Similar results were obtained by Kwak et al. (2018) on *D. magna* and *M. macrocopa*.

Sub-lethal effects of NPX were also focused on effects at molecular and biochemical levels. The exposure to NPX and its photoproducts din not induce genotoxicity neither in *E. coli* nor in *S. typhimurium* (Isidori et al., 2005). Immunotoxic effects of NPX, in terms of phagocytosis, intracellular esterase activity, adherence to microplate wells and lipid peroxidation, was tested on hemolymph collected from the freshwater mussel *Elliptio complanata* exposed in vitro to 2.5–100 μM (concentration range 0.57–23 mg/L) of NPX. Threshold effect level for phagocytosis, intracellular esterase activity and adherence to microplate wells was 35, 152 and 4 μM of NPX, respectively, while a significant decrease in lipid peroxidation occurred (Gagné et al., 2006). A study of the amphipod *Hyalella azteca* investigating the onset of oxidative stress and the subsequent oxidative damage to genetic material induced by NPX sodium enriched sediments showed that 48 h of exposure to sediments enriched with 76.6 and 339.2 mg/kg of NPX induced a dose-dependent increase of genotoxicity, lipid peroxidation and protein carbonylation, as well as an increase of SOD and CAT activity coupled with a decrease of GPx activity (García-Medina et al., 2015). Similar effects were found in *D. magna* exposed to 0.017 mg/L of NAP that showed a significant increase of SOD and CAT activity and lipid peroxidation, while a decrease was noted for GPx. Moreover, a significant increase of genetic damage (i.e., DNA fragmentation) was noted after 48 and 96 h of exposure to NAP (Gómez-Oliván et al., 2014). Lastly, a recent work by Yamindago et al. (2019) showed that NPX median lethal concentrations (LC_{50}) in *H. magnipapillata* was 51.99 mg/L, 44.93 mg/L, and 42.50 mg/L after 24, 48, and 72 h of exposure, respectively. Morphological observation of the exposed cnidarian showed that 40 mg/L of NPX stimulated the contraction of body column and tentacles after 24 h. A KEGG pathway analysis of the genes differentially expressed in *H. magnipapillata* after NPX exposure for 6, 24, or 48 h pointed out various cellular and metabolic effects, including protein processing in the endoplasmic reticulum, Wnt signaling, and tryptophan metabolism (Yamindago et al., 2019).

5.6. Toxic effects induced by mixtures of NSAIDs

Acute and chronic toxicity induced by mixtures of NSAIDs towards freshwater invertebrates was investigated on two crustaceans (*Daphnia magna* and *Hyalella azteca*) and a bivalve species (*Dreissena polymorpha*) only (Table 6). A study of Cleuvers (2004) investigated the ecotoxicity of four NSAIDs, namely DCF, IBU, NPX and ASA, on *D. magna*. Toxicities of single NSAIDs were relatively low, with half-maximal EC_{50} values in *Daphnia magna* ranging from 68 to 166 mg/L. The toxicity of the mixture composed by the four NSAIDs at different concentrations ($EC_5/4$, $EC_{10}/4$, $EC_{20}/4$, $EC_{50}/4$, and $EC_{80}/4$) was considerable, even at concentrations that caused no or slight effects when the organism were exposed to the single substances. Parolini and Binelli (2012) investigated the sub-lethal effects induced by a mixture of three common NSAIDs, namely diclofenac, ibuprofen and paracetamol, on the freshwater bivalve zebra mussel (*Dreissena polymorpha*). Zebra mussels were exposed to three mixtures in which each single drug was included at the median value of surface waters (Low), wastewater effluents (Mid) and predicted environmental concentration (PEC; High), similar to those previously tested in experiments assessing the toxicity of each single molecule independently (Parolini et al., 2010, 2011b, 2011c, 2013). The exposure to the three mixtures induced a significant cellular stress in bivalves, in terms of destabilization of the lysosome membranes (i.e., NRRA) probably caused by the alteration of the oxidative status of the bivalves, as indicated by the modulation of the antioxidant enzyme activity. Moreover, the mixtures induced significant increase of both primary (i.e., DNA fragmentation) and fixed (i.e., apoptosis and micronuclei) genetic damage. The effects induced by the mixture resulted higher than those observed in previous studies when the single drugs were tested individually at concentrations similar to those included in the mixtures (Parolini et al., 2010, 2011b, 2011c). Another study investigating the toxicity of binary mixtures of DCF with PCM, IBU, NPX, and ASA compared to that of the same NSAIDs in their isolated form, on the amphipod *Hyalella azteca* showed that NSAIDs induced oxidative stress both in isolated form and in binary mixtures (Gómez-Oliván et al., 2014). In detail, modulation of SOD, CAT, and GPx activity, as well as increase of lipid peroxidation and protein carbonylation occurred with NSAIDs in isolated form and at higher extend when NSAIDs where in binary mixtures.

6. Conclusions and future needs

The exposure to the most common NSAIDs found in the aquatic ecosystems, namely acetylsalicylic acid, diclofenac, ibuprofen, naproxen, paracetamol, as well as to their mixtures, might represent a risk for non-target, freshwater invertebrates. Although data from standardized tests, in terms of EC_{50} or LC_{50} from acute or chronic toxicity tests, are comparable, it is not simple to draw a scale of toxicity of NSAIDs because of some contrasting results among studies testing the toxicity of the same drug towards the same model species or the lack of information for specific drugs. However, according to EC_{50} values obtained on *D. magna*, which was the model species on which all the NSAIDs were tested, DCF and IBU can be identified as the more toxic drugs, followed by PCM, NPX and ASA. Simultaneously, although different responses occurred within phylogenetic-related species, cladocerans can be indicated as the more sensitive organisms to NSAID exposure. Although acute toxicity of NSAIDs occurs only at high, unrealistic concentrations,

much higher than those currently measured in freshwaters worldwide, sub-lethal effects due to long-term exposures cannot be neglected. A growing number of studies performed on diverse invertebrate species belonging to different levels of the ecological hierarchy showed that the exposure to low, environmentally relevant concentrations of NSAIDs, both independently and in mixture, can cause a variety of adverse effects at molecular, biochemical and cellular level, while effects at individual level (e.g., growth, survival, reproduction) seem to be less probable, although they cannot be underestimated considering that are used for risk assessment. For instance, concentrations of PCM similar to those measured in environments worldwide induced ecologically relevant effects on two species belonging to Cnidaria and Mollusca *taxa* (i.e., *Hydra vulgaris* and *Corbicula fluminea*, respectively). Despite these alarming findings, it is important to bearing in mind that chronic toxicity data, in term of sub-lethal effects at individual or sub-individual levels, come from two different approaches, the first one performed on whole animals exposed in vivo and the second one on single cells isolated or collected by animals and then exposed in vitro to NSAIDs. These approaches result in different outcomes, which can lead to different considerations concerning the toxicity of NSAIDs towards aquatic organisms. For instance, EC_{50} or IC_{50} from whole animal experiments support the conclusion that NSAIDs in the environment are at much lower concentrations than those causing toxicity, while biochemical or molecular investigations from both in vitro and in vivo studies pointed out a potential hazard of these drugs also at environmentally relevant concentrations. However, some caveats need to be considered, mainly related to the uncertainty of the real amount of xenobiotic reaching the cells, the realism of exposures and the lack of cells mechanisms owned by cells to mitigate the effects of xenobiotics, as instead the whole organism have, that might result in a potential overestimation of the effect. In addition, although NSAIDs-induced molecular or biochemical effects were induced in whole body or tissues/organs isolated after in vivo exposures, they are early, and often specific, responses that the organism activate as a consequence of an exposure to these drugs. To date, there is a dearth of information concerning the effects at cellular and tissue levels induced by NSAIDs in freshwater species, including invertebrates, as well as on the propagation of the effects from the lowest levels of the biological organization to the highest ones. Moreover, sublethal effects highlighted by short- and mid-term exposures might be also more worrisome considering that in natural ecosystems, invertebrates are exposed to measurable NSAID concentrations for their whole lifespan. For all the reasons mentioned above, the assessment of the risk of NSAIDs towards aquatic species comparing acute or sublethal effects only could not be accurate. Thus, an approach using measured environmental concentrations (MECs) combined with predicted no effect concentrations (PNECs) was proposed by European commission (2003) to screen compounds with potential environmental risks and it was recently refined to properly identify the priority pollutants that should be regularly monitored in surface waters (Zhou et al., 2019). According to this approach, DCF and IBU were prioritized as high risk pharmaceuticals, PCM as moderate risk one (Zhou et al., 2019; Palma et al., 2020), while ASA as a negligible risk compound (Gómez-Canela et al., 2019). To date, the environmental risk of NPX and NSAID mixture was not assessed. This approach is crucial considering that the increasing production and use of NSAIDs worldwide might result in a notable increase in their environmental levels occurring in aquatic ecosystems, with a consequent enhancement of the risk related to the exposure to these pharmaceuticals towards non-target, freshwater invertebrates. For these reasons, further studies should be needed to enlarge the knowledge on NSAID toxicity towards aquatic organisms, not only invertebrates but also vertebrates, considering long-term exposures and the use of alternative and innovative assays to shed light on the mechanism(s) of action of these pharmaceutical compounds. Moreover, as the most of the studies on NSAID toxicity were performed on native, parental compounds, exploring the toxicity of NSAID metabolites, which might results also more toxic than the parental ones (e.g., Marques et al., 2004b; Isidori et al., 2005), should contribute to understand the hazard of these drugs. Lastly, considering that NSAIDs occur in aquatic ecosystems in complex 'cocktails' and few previous studies demonstrated that NSAID mixtures induced higher effects than the single compounds, further studies aimed at investigating the toxicity of binary or complex NSAID mixtures should be a priority to shed light on adverse effects, mechanism(s) of action and ecological risk of these therapeutics towards freshwater communities.

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