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Ibuprofen and diclofenac in the marine environment - a critical review of their occurrence and potential risk for invertebrate species

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Abstract

The presence of the two main non-steroidal anti-inflammatory drugs (NSAIDs), diclofenac (DCF) and ibuprofen (IBU), in marine and estuarine systems has been reported. Although the available information about the toxicity of these compounds in aquatic organisms has increased in recent years, the database about marine organisms, specifically invertebrates, is limited. Consequently, the assessment of potential risks posed by these compounds to aquatic species should be improved, given their relevance for aquatic life and the trophic chain. To fill this gap, we analyze the potential risk of IBU and DCF in marine invertebrates. To assess the risk, the database was built with available information from the scientific literature about the occurrence, bioaccumulation, and toxicity of both compounds in the estuaries and marine environments. Risk assessment of both compounds in these environments is scarce and based essentially on their acute toxicity. Data compiled in this review, including environmental concentrations and toxicity thresholds, were used to calculate risk quotients and classify the risk for invertebrate communities in coastal areas with different contamination levels. The results indicated a higher risk for DCF than IBU. Additionally, the simultaneous presence of the two NSAIDs in the aquatic environment increases the risk for exposed organisms. The use of classical methods (e.g., biochemical markers, gene expression), new approaches

(e.g., adverse outcome pathway, AOP), and omic techniques has contributed to understanding the underlying toxicity mechanisms and improving the risk assessment. However, in a global change scenario involving multiple drivers and pressures such as ocean acidification, heat waves, climate change, *etc.*, the risk assessment for these emerging pollutants needs improvement. This can be achieved by increasing our knowledge about the metabolic pathway and biotransformation routes of these compounds in marine species and understanding how these changes can affect the bioaccumulation, toxicity, fate, and behavior of both NSAIDs.

Keywords: Non-steroidal anti-inflammatory drugs, saltwater ecosystems, invertebrates, bioaccumulation, adverse effects, risk assessment

INTRODUCTION

Marine pollution is a global environmental problem that requires joint action at the international level to minimize the impact on marine ecosystems and biodiversity. It arises as a consequence of anthropogenic activities on both land and sea. The chemicals released into the sea correspond to regulated and unregulated products. Many of them are known as emerging pollutants. This term includes many chemical compounds such as fire retardants, musks, industrial solvents, pesticides, personal care products, nanomaterials, microplastics, and pharmaceuticals, among others.

The most relevant legislations for the protection and regulation of the aquatic environment at the European level concerning the presence of contaminants are the Water Framework Directive (WFD, EC 2000) and the Marine Strategy Framework Directive (MSFD, 2008/56/EC and amendment EU 2017/845). The first one establishes a list of substances (the priority list) to assess the chemical status of water bodies (up to 12 nautical miles from the coastal line). In contrast, the MSFD is based on Descriptor 8, which states, "Concentrations of contaminants are at levels not giving rise to pollution effects." Other initiatives to control pollution, including seafood, are related to regulatory agencies (ECHA, EFSA) and regional or international programs or conventions. Tornero and Hanke[1] summarized the potential chemical contaminants in the marine environment to monitor and assess the chemical pollution in European marine waters. Many substances are considered emerging pollutants and are not included in the routine monitoring program yet. Among the emerging pollutants, pharmaceuticals are a relevant group.

Pharmaceutical drug consumption from human or veterinary use can reach several thousands of tons in some countries[2-4]. Their presence has been reported in more than 70 countries representing all continents, and more than 600 substances have been detected in the aquatic environment[5]. Pharmaceutical compounds can be released into aquatic systems from different sources. After human or veterinary administration, drugs are metabolized and excreted as unaltered parental forms or metabolites (or metabolite mixtures). For this reason, wastewater treatment plants (WWTPs), hospital discharges, aquaculture facilities, and animal farming are considered the major pathways of drug entry into the aquatic environment[6-11]. Conventional WWTPs are typically designed for solids and organic loadings removal by primary and secondary treatments. However, these processes only partially remove the majority of pharmaceuticals from influents, and effluents discharge a complex mixture of unchanged compounds and metabolites/transformation products into the aquatic compartments, which can exhibit higher toxicity than the parental compounds[12,13]. Additional sources of drugs are industrial (pharmaceutical manufacturing) discharges, incorrect disposal of expired or unused medicines, and runoff of soils where sewage sludge or animal manures are applied as fertilizers[10]. The continuous input of these substances into the aquatic environment, coupled with their design to be biologically active also at low doses, can provoke negative effects for aquatic fauna, ecosystems, and in the end, even for human health.

Among pharmaceuticals, non-steroidal anti-inflammatory drugs (NSAIDs) are one of the main groups detected in aquatic ecosystems. This fact is related to their extensive prescription and over-the-counter commercialization worldwide, as well as their specific properties, which influence the inflow of these compounds to WWTPs and/or aquatic systems, their removal efficiency in WWTPs, mobility through WWTPs and natural waters, degradation rates, and persistence into the aquatic media[14,15]. They are employed to reduce pain or inpatient recovery from a medical condition or ailment. Two of the more prescribed NSAIDs are diclofenac (DCF) and ibuprofen (IBU) (chemical properties for both compounds are shown in Supplementary Table 1), and they have been detected in surface water, groundwater, estuaries, and coastal waters[16-18]. Most pharmaceuticals are traditionally considered pseudo-persistent pollutants due to the continuous release from various sources that exceed the natural degradation rate (half-life time for DCF and IBU are reported in Supplementary Table 1). Hernández-Tenorio and coauthors[19] reviewed the occurrence of DCF and IBU in different aqueous matrices (surface water, WWTPs influents, hospital wastewater, groundwater, drinking water, and raw water) worldwide, reporting a wide range of concentrations of both compounds: 0.08-19,300 ng/L for DCF (0.08-44 ng/L drinking water, 0.1-19,300 ng/L for surface water, 6-2,770 ng/L groundwater, 12-15,087 raw water, 26-10,340 ng/L WWTPs influents, and 40-3,040 ng/L hospital wastewater) and 3.7-141,000 ng/L for IBU (3.7-6,297 ng/L for surface water, 10.2-97 ng/L drinking water, 46.1-106,490 ng/L WWTPs influents, 48.7-988 ng/L groundwater, 88-141,000 ng/L hospital wastewater, and 325-117,460 raw water). For both compounds, the highest levels and variation of concentrations were recorded in China, Brazil, and Pakistan, primarily due to factors such as high population density, extensive pharmaceutical production and consumption, and low or lack of removal in WWTPs. Accumulation of both compounds and the induction of various ecotoxicological effects are documented in aquatic organisms, particularly from freshwater environments[11]. It should be pointed out that the European Union included DCF in the first Watch List published in 2015, a list of prioritized chemicals potentially harmful to aquatic organisms subject to continuous monitoring in the aquatic environment for up to four years[20]. However, it was later excluded[21].

The available information about the IBU and DCF occurrence in aquatic environments and their effects on organisms is mainly focused on freshwater environments. At the same time, the database about seawater systems and species is scarce[22,23]. Invertebrates represent the overwhelming majority of animals living in marine systems, both in terms of biomass and number of species, with worldwide distribution and extraordinary ecological and biological diversity. They have a key role in the ecosystem functioning, participating in nutrient recycling and the transfer of energy from primary producers and higher levels of the food web[24,25]. Additionally, many species are economically and commercially relevant, being widely used for human consumption. The ability of these organisms to accumulate a wide variety of contaminants present in the aquatic environments means that they play an important role in the transfer of contaminants along the food web, posing a risk to both animal and human health. All these characteristics, coupled with high sensitivity to environmental stresses, small size, short life cycle, relatively easy to culture and manipulate in the laboratory or collection in the field, and the existence of standardized protocols, make invertebrates widely used in field monitoring and laboratory studies of contaminant toxicity[26-28].

This work summarizes and analyses the information available in the literature about DCF and IBU in terms of occurrence and effects. First, we analyzed the environmental occurrence in water and sediments from coastal and estuarine areas, as well as open seawater, to identify the range of concentrations in which the two compounds occur and the areas of particular interest, especially those with high levels of DCF and IBU. The occurrence in the marine invertebrate was also evaluated to assess the bioconcentration potential of DCF and IBU, considering laboratory and field studies. Finally, we examined the possible effects induced by the two pharmaceuticals in marine invertebrates at different organization levels, encompassing acute effects

at the organism level to sublethal alterations at the sub-cellular level (biochemical, genetic, protein expression, *etc.*). These data are essential for understanding the toxicity of selected compounds, determining the concentrations at which the different effects occur, elucidating the possible mechanisms of action, and the risks associated with environmental exposure. A systematic literature search was conducted from September 2022 to December 2022 using the Google Scholar, Scopus and ScienceDirect databases, focusing on scientific publications from 2004 to 2023. A total of 107 studies were included in the database used for this review. For each chemical, we used different combinations of keywords, including ibuprofen, diclofenac, occurrence, marine, aquatic, invertebrates, effects, bioaccumulation, toxicity, and risk assessment. The risk associated with the presence of DCF and IBU in the coastal and estuarine ecosystems was studied using data from the literature, along with risk quotients calculated based on the data compiled in this review. Individual and mixture potential risks have been considered, along with exploring how new approaches (e.g., adverse outcome pathway, AOP) can improve the risk assessment.

OCCURRENCE

The presence of pharmaceuticals and their metabolites has been reported in aquatic ecosystems worldwide, with a wide range of concentrations (ranging from μg/L to ng/L). WWTPs are the most relevant sources of these contaminants. Adeleye and coauthors[5] reviewed the occurrence of pharmaceutical compounds in wastewaters around the world, observing that analgesics, particularly acetaminophen, ibuprofen, diclofenac, aspirin, and ketoprofen are commonly detected in wastewaters and occur at very high concentrations. The highest concentrations have been reported to be 1,090,000 ng/L for acetaminophen in Kenya, 603,000 and 8,560 ng/L, respectively, for ibuprofen and ketoprofen in Spain, 115,100 ng/L for diclofenac in South Africa, and 1,407,000 for aspirin in South Korea. As indicated above, WWTPs are designed to remove nutrients, pathogens, and particulate matter from influents, and typically, specific treatments for pharmaceutical removal were not available. In addition to the type and conditions of removal treatment, numerous factors influence the removal percentage and the environmental presence of drugs. These factors include the physicochemical properties of compounds, climatic conditions (precipitation and temperature), population density, pharmaceutical consumption rate, and the presence of hospitals and drug manufacturers. Hence, the variation in these factors results in drug presence to vary according to the compound, country, and season[29-31]. Adeleye and coauthors[5] indicated that the concentrations of DCF and IBU in WWTP effluents vary between 0.1-15,000 ng/L and 1-21,000 ng/L, respectively, with the highest concentrations detected in South Africa and Korea for both compounds.

Pharmaceuticals and their metabolites enter surface water and are transported by rivers, reaching estuaries and, finally, the sea, where the concentrations decrease significantly due to dilution effects, sorption to sediments, and transformation processes[32-34]. In recent years, several reviews on pharmaceutical levels in marine environments have been published[5,27,35,36]. The concentrations in seawater (estuary, bays, coastal, and offshore) and sediment are summarized in Table 1.

DCF and IBU presence in saltwater has been reported worldwide, although most available data correspond to the North Hemisphere and the European coasts. Very little information has been found on the occurrence and distribution of DCF and IBU in South America, Africa, and Asia. Waleng and Nomngongo[83] highlighted the growing concern about pharmaceutical contamination in Africa and Asia due to the increasing pharmaceutical consumption rate, improper wastewater treatment resources, reckless use and disposal of pharmaceuticals, poor sanitation, and lack of drug return programs. Similarly, very little is known about the presence of DCF and IBU in more remote areas such as the Antarctic and Arctic regions. Only four research works can be found in the bibliography on this subject[44,45,76,85].

Table 1. Occurrence of DCF and IBU in the marine environment (seawater and sediment)

Drug	Country	Study site	Matrix	Study period	Concentration (aqueous matrix: ng/L, sediment: ng/g dw)	Analytical method	LOQ (ng/L) (aqueous matrix: ng/L, sediment: ng/g dw)	LOD (ng/L) (aqueous matrix: ng/L, sediment: ng/g dw)	Refs
DCF	Germany	Baltic Sea	Nr	2009 (May)	9.2	LC-MS/MS	2		[37]
	Poland	Baltic Sea (Gulf of Gdańsk)	Estuary seawater	2012	92.6	LC-MS/MS	0.5	0.2	[38]
	Poland	Baltic Sea (Gulf of Gdańsk)	Coastal seawater	2012 (October and December)	79-102	GC-MS	9	3	[39]
	Poland	Baltic Sea (Bay of Puck)	Coastal seawater	2017-2018	196	LC-MS/MS	1	0.3	[40]
	UK	North Sea (Humber estuary)	Estuary seawater	2016-2017	32.35-205.8	UHPLC-MS/MS	5.91	1.77	[41]
	UK	North Sea (North Scotland)	Nr	2005 (November)	< 0.12	LC-MS/MS	0.41	0.12	[42]
	Belgium	North Sea	Coastal seawater	2017 (February and April)	10	UHPLC-Q-Orbitrap-HRMS		0.4	[43]
			Harbor seawater		12.3-69				
	Norway	Arctic regions	Seawater	2007	1-48	nr	nr	nr	[44]
	Antarctica	Antarctic Peninsula	Coastal seawater	2012-2013	77-15,087	LC-MS/MS	14.3	4.3	[45]
	Ireland	East and West coast of Ireland	Coastal seawater	2011-2012	0.06-0.55	LC-MS/MS	22		[46]
	France	Seine estuary	Estuary seawater	2002 (from March to November)	7.7-63.4	GC-MS		0.5	[46]
	France	Mediterranean Sea	Coastal seawater	Nr	< 1500	GC-MS		2.6	[47]
	Portugal	Arade river estuary	Estuary seawater	2010 (from August to November)	4-31	LC-MS/MS	nr	nr	[48]
	Portugal	Atlantic Ocean	Coastal seawater	2013 (from June to September)	1.14-241	UHPLC-MS/MS		0.02	[49]
	Portugal	Atlantic Ocean (Oporto)	Coastal seawater	2013 (July)	3-33	UHPLC-MS/MS	0.02	0.06	[50]
	Spain	Atlantic Ocean (Cadiz Bay and Huelva estuary)	Coastal seawater	2011(October)	3.1-27.6	UHPLC-MS/MS	0.2	0.1	[51]
			Estuary seawater		0.3-9.2				
	Spain	Atlantic Ocean (Bay of Cadiz)	Sediment	2011 (March and September)	< 0.10-1.5	UHPLC-MS/MS		< 0.1	[52]
	Spain	Atlantic Ocean (Gran Canaria Island)	Coastal seawater	2011-2012	23.7-343.6	LC-MS/MS	4.6	1.4	[53]
	Spain	Atlantic Ocean	Sediment	2014 (summer)	10	GC-MS		0.7	[54]
	Spain	Atlantic Ocean (Bay of Cadiz)	Coastal seawater	2015 (summer)	1.5-24.3	UHPLC-QqQ-MS/MS	0.2	0.1	[55]
			Ocean seawater		0.3-2.5				
	Spain	Mediterranean Sea	Coastal seawater	Nr	4	UHPLC_MS/MS	2.4	0.7	[56]
	Israel	Mediterranean Sea		July 2009 and January 2011	6.1	LC-MS/MS	2.0		[38]
	Tunisia	Mediterranean Sea	Coastal seawater	2017-2018	3-23	UHPLC-MS/MS	nr	nr	[57]
	Italy	Mediterranean Sea	Open	2014	0.02	UHPLC-MS/MS		0.02	[58]

			seawaters	(November)					
	Italy	Ionian Sea (Augusta Bay)	Coastal seawater	October 2017 and April 2018	0.5-441	LC-MS/MS	0.38		[59]
			Sediment		1.1		0.56		
	Greece and Turkey	Aegen Sea and Dardanelles		2010, 2011	9.7	LC-MS/MS	2.0		[38]
	Greece	Aegean Sea	Offshore and coastal seawater	2013 (December)	< 4.1-16.3	UHPLC-MS/MS	4.1	1.4	[60]
	Greece	Aegean Sea (Lesvol Island)	Coastal seawater	2014 (April and May)	2.2-14	LC-MS/MS	2.0	0.7	[61]
	Turkey	Sea of Marmara	Coastal seawater	2019 (April and October)	< 27-1,300	LC	93	28	[62]
	Turkey	Sea of Marmara (Golden Horn Estuary)	Estuary seawater	2019-2020	110-1,120	LC	92	27	[63]
			Sediment		< 30.6		102	30.6	
	Saudi Arabia	Red Sea	Coastal waters	2016 (March and May)	1.6-10,221.1	LC-MS/MS		1.60	[4]
	Taiwan	China Sea	Coastal seawater	2009 (June and July)	2.42-57.10	LC-MS/MS		2.5	[64]
	China	China Sea (Jiulong River)	Estuary seawater	2014 (from March to December)	0.819-11.0	LC-MS/MS		0.1	[65]
	China	Pearl River Delta	Sediment	October 2014 and March 2015	nd-0.03	UHPLC-MS/MS	0.1	0.03	[66]
	Malaysia	China Sea (PulauKukup)	Sediment	Nr	< 0.093-0.228	LC-MS/MS	0.062	0.093	[67]
	Singapore	Indian Ocean	Nr	Nr	4-38	UHPLC-MS/MS		0.93	[68]
	Singapore	Pacific Ocean	Coastal seawater	2011 (June and July)	< 1.5-11.6	LC-MS/MS		1.5	[69]
	Singapore		Estuary seawater	Nr	10	LC-MS/MS		0.01	[70]
	Singapore	Pacific Ocean	Mangrove waters	2012-2013	< 0.04-1.2	LC-MS/MS			[71]
	USA	Pacific Ocean (Southern California)	Coastal seawater	2006- 2007	0.6	LC-MS/MS		2.5 (Reporting Limit)	[72]
	Canada	Atlantic Ocean (Nova Scotia)	Marine watershed	2005 (from May to October)	4-6	GC-MS		3	[73]
	Brazil	Atlantic Ocean (Santos Bay)	Coastal seawater	2014 (March)	< 7.4-19.4	LC-MS/MS	7.4	1.0	[74]
	Brazil	(Santos Bay)	Coastal seawater	2016 (December)	4.01-4.78	LC-MS/MS	3.0	0.81	[75]
	Brazil	Atlantic Ocean (Santos Bay and North Coast of Salvador)	Sediment estuary	Nr	< 0.10-1.06	LC-MS/MS		0.1	[18]
IBU	Germany	Baltic Sea	Nr	2009 (May)	109	LC-MS/MS	3.6		[37]
	Poland	Baltic Sea (Gulf of Gdańsk)	Estuary seawater	2012	34.9	LC-MS/MS	10.0	3.3	[38]
	Poland	Baltic Sea (Gulf of Gdańsk)	Coastal seawater	2012 (October and December)	48	GC-MS	5	2	[39]
	UK	North Sea (North Scotland)	Nr	2005 (November)	< 0.52	LC-MS/MS	1.72	0.52	[42]
	UK	North Sea (Humber estuary)	Estuary seawater	2016-2017	24.93-6,297.14	UHPLC-MS/MS	4.83	1.45	[41]
	Norway	Arctic Ocean	Seawater	2002	0.7	GC-MS	0.09		[76]
	Norway	Arctic regions	Seawater	2007	0.4-52	nr	nr	nr	[44]
	Norway,	North Sea	Coastal and	2014 (October)	0.57-22	LC-MS/MS		0.28	[77]

Germany and Belgium		offshore seawater						
Antarctica	Antarctic Peninsula	Coastal seawater	2012-2013	37-10,053	LC-MS/MS	24	7.2	[45]
France	Seine estuary	Estuary seawater	2002 (from March to November)	< 2.0-26.1	GC-MS		0.4	[78]
France	Mediterranean Sea	Coastal seawater	Nr	< 1,600	GC-MS		1.7	[47]
Portugal	Atlantic Ocean	Coastal seawater	2013 (from June to September)	1.25-222	UHPLC-MS/MS		0.08	[49]
Portugal	Atlantic Ocean (Oporto)	Coastal seawater	2013 (July)	5-110	UHPLC-MS/MS	0.26	0.08	[50]
Portugal	Arade river estuary	Estuary seawater	2010 (from August to November)	9-28	LC-MS/MS	nr	nr	[48]
Spain	Atlantic Ocean (Cadiz Bay and Huelva estuary)	Coastal seawater	2011 (October)	2.8-18.3	UHPLC-MS/MS	3.5	1	[51]
		Estuary seawater		4.3-195.0				
Spain	Atlantic Ocean (Bay of Cadiz)	Sediment	2014 (summer)	97.1-101.3	GC-MS	0.1		[54]
		Coastal seawater		10		11		
Spain	Atlantic Ocean (Bay of Cadiz)	Coastal seawater	2015 (summer)	3.5-1,219.7	UHPLC-QqQ-MS/MS	3.5	1	[55]
		Ocean seawater		< 3.5-32.3				
Spain	Mediterranean Sea	Coastal seawater	Nr	16	UHPLC-MS/MS	3.3	1	[56]
Israel	Mediterranean Sea	Coastal seawater	July 2009 and January 2011	7.5	LC-MS/MS	3.6		[37]
Italy	Mediterranean	Open seawater	2014 (November)	0.063-1.08	UHPLC-MS/MS		0.056	[58]
Italy	North Adriatic Sea (Venice)	Coastal seawater	2010 (May)	70	LC-MS/MS	3.6		[37]
Italy	Adriatic Sea (Venice)	Coastal seawater	2011(February and March)	< 0.049-1.146	UHPLC-QTRAP-MS/MS	0.049		[79]
Italy	Ionian Sea (Augusta Bay)	Coastal seawater	October 2017 and April 2018	2.7-2,550	LC-MS/MS	2.15		[59]
Greece and Turkey	Aegean Sea and Dardanelles	Coastal seawater	2010, 2011	35	LC-MS/MS	3.6		[37]
Greece	Aegean Sea (Lesvol Island)	Coastal seawater	2014 (April and May)	9.4-16	LC-MS/MS	3.6	1.2	[61]
Turkey	Sea of Marmara	Coastal seawater	2019 (April and October)	< 15-2,130	LC	47	15	[62]
Turkey	Sea of Marmara (Golden Horn Estuary)	Estuary seawater	2019-2020	60-2,460	LC	50	15	[63]
		Sediment		< 91-215		91	27.2	
Saudi Arabia	Red Sea	Coastal seawater	2016 (March and May)	46.2-508.7	LC-MS/MS		26.7	[4]
		Estuary seawater		0.261				
Taiwan	China Sea	Coastal seawater	2009 (June and July)	< 2.50-53.60	LC-MS/MS		2.5	[64]
Taiwan	China Sea	Coastal seawater	2010 (October)	12.1	LC-MS/MS		2.5	[80]
Taiwan	China Sea (Xiamen Bay)	Coastal seawater	2019 (August)	9.4	LC-MS/MS	3.1	0.92	[81]

		(bay and estuary)						
China	Jiulong River	Estuary seawater	2014 (from March to December)	< 0.1-20.7	LC-MS/MS		0.1	[65]
Malaysia	China Sea (Kota Kinabalu)	Coastal seawater	2018- 2019	10.2-42.7	LC-MS/MS	3.1	0.92	[82]
Singapore	Pacific Ocean	Coastal seawater	2011 (June and July)	< 2.2-9.1	LC-MS/MS		2.2	[69]
Singapore	Indian Ocean	Nr	Nr	41-121	LC-MS/MS		1	[68]
South Africa	Indian Ocean (Durban coast)	Coastal seawater	2018 (September)	0.166	LC	35	11	[83]
USA	Pacific Ocean (San Francisco Bay)	Coastal seawater	2010 (February)	12	LC-MS/MS		3.6	[37]
USA	Salish Sea (Bellingham Bay)	Sediment	2010 (April and June)	21.70	LC-MS/MS		21.7	[84]
USA	Pacific Ocean (Southern California)	Coastal seawater	2006-2007	30	LC-MS/MS		50 (Reporting limit)	[72]
Canada	Atlantic Ocean (Nova Scotia)	Marine watershed	2005 (from May to October)	3-230	GC-MS		6	[73]
Brazil	Atlantic Ocean (Santos Bay)	Coastal seawater	2014 (March)	326.1-2,094.4	LC-MS/MS	35	10	[74]
Brazil	Atlantic Ocean (Santos Bay and North Coast of Salvador)	Sediment estuary	Nr	0.77-18.8	LC-MS/MS		0.1	[18]

DCF: diclofenac; dw: Dry weight; GC-MS: gas chromatography coupled to MS; IBU: ibuprofen; LC: traditional liquid chromatography with diode array detection and ultraviolet-visible spectroscopy; LC-MS/MS: liquid chromatography coupled to tandem MS; LOD: limit of detection; LOQ: limit of quantification; MS: mass spectrometry; nr: not reported; UHPLC: ultra-high performance liquid chromatography; UHPLC-MS/MS: UHPLC coupled to tandem MS; UHPLC-Q-Orbitrap-HRMS: UHPLC coupled to hybrid Q-Orbitrap high-resolution full-scan mass spectrometry; UHPLC-QqQ-MS/MS: UHPLC triple quadrupole coupled to tandem MS; UHPLC-QTRAP-MS/MS: UHPLC coupled to hybrid triple quadrupole linear ion trap MS.

Estuaries are transition areas between freshwater and seawater environments with high ecological and economic relevance, as they provide habitat for many species and recreation activities. They receive water from one or more rivers, acting as a confluence for contaminants, particularly pharmaceuticals, which pose a potential risk to these systems[86].

In European estuaries, the concentrations of DCF and IBU are between 0.3-1,120 ng/L and < 0.2-6,297.16 ng/L, respectively. The maximum levels of DCF and IBU are recorded to be 205.8 ng/L and 6,297 ng/L, respectively, in the Humber Estuary on the East Coast of England[41]. In the Golden Horn Estuary located northeast of the Sea of Marmara, the maximum levels of both compounds are found to be 1,120 ng/L and 2,460 ng/L, respectively[63]. In both sites, the high drug concentrations are related to the proximity of highly urbanized and industrial areas where WWTP effluents are present. Only three research works have been found on DCF and IBU levels in estuaries from the Asian continent, reporting very low concentrations ranges of 0.819-11 ng/L and 0.1-20.7 ng/L, respectively[65,70,81].

Also, in coastal seawater, wide variations have been reported for both compounds. The concentrations ranged between 0.06-10,221.1 ng/L for DCF and 0.049-2,550 ng/L for IBU. The highest values recorded in this matrix [Table 1] correspond to the Red Sea (10,221.1 ng/L, Saudi Arabia) for DCF and to the Augusta Bay (2,550 ng/L, Italy, Ionic Sea), the Santos Bay (2,094.4 ng/L, Brazil, Atlantic Ocean), and the Sea of Marmara (2,130 ng/L, Turkey) for IBU[4,59,62,74]. As for estuaries, the high levels recorded for both drugs in

these areas have a common cause: the discharge of untreated or insufficiently treated wastewater into the coastal water. A direct discharge of municipal raw sewages occurs in Augusta Bay[59], while both Santos Bay and Red Sea wastewaters are insufficiently treated. This is due to the absence of primary and secondary treatments in the Brazilian WWTPs[74] and to the overload of the WWTPs treatment capacity. The overload is a consequence of the development and the demographic increase that the eastern coast of the Red Sea has suffered in the last decades[4]. In the case of the Sea of Marmara, in addition to the presence of deep-sea discharge points of municipal WWTPs, hydrodynamic factors, such as a low rate of mixing and renewal of water masses, play an important role in the accumulation of selected compounds in the coastal waters[62].

Both pharmaceuticals were also detected in coastal waters of remote areas, such as the Antarctic Peninsula, isolated from human influence. González-Alonso *et al.* reported concentrations of 15,087 and 10,053 ng/L for DCF and IBU, respectively, in seawater samples collected from Hope Bay[45]. It is important to note that the samples were taken at a discharge point for urban wastewater into the sea, which explains the high recorded values.

The variability observed for both coastal and estuary concentrations of DCF and IBU could also be related to the seasonal fluctuation of drug levels. Studies reported in Table 1 refer to both punctual sampling and series of sampling over a period of time (one or more months, one year, *etc.*). It is known that variations in pharmaceutical concentrations in aquatic environments are often related to changes in usage (higher usage in winter to treat seasonal illnesses) and local environmental conditions (lower concentrations in the wet season due to dilution by precipitation)[41,49,87].

Data on DCF and IBU presence in open seawaters are scarce. As expected, the concentration ranges of both compounds reported in Table 1 (0.02 to 16.3 ng/L for DCF and 0.063 to 32.3 ng/L for IBU) are smaller compared to those observed in coastal and estuarine waters when considering sorption, degradation, and dilution[55].

In aquatic systems, pharmaceuticals are subjected to sorption processes on sediment that depend on the properties of the sediments (e.g., organic matter content and pH values) and the physicochemical properties of pharmaceuticals (e.g., dissociation constant pK_a and octanol-water partition coefficient, log K_{ow}). According to the classification reported by Rogers[88], an octanol-water partition coefficient (log K_{ow}) below 2.5 denotes low sorption, while the range of $2 < \log K_{ow} < 4$ signifies medium sorption, and log K_{ow} above 4 indicates high sorption. The octanol-water partition coefficients of DCF and IBU are 4.51 and 3.97, respectively (https://pubchem.ncbi.nlm.nih.gov). Hence, DCF and IBU sorptions on sediment are high and medium, respectively, which allows them to be detected in both water and sediment[63]. Similarly, the pKa values, ranging from 3.99 to 4.3 for DCF and from 4.45 to 5.3 for IBU, indicate that both compounds in seawater (pH close to 8.2) could be negatively ionized, showing low sorption on the surface sediments[59,89]. Very few studies have been conducted to determine the concentration of DCF and IBU in marine sediments. The highest values for both compounds were found in sediments from the Golden Horn estuary in the Sea of Marmara (< 30.6 ng DCF/g dw and < 91-215 ng IBU/g dw, as reported in Table 1), which can be related to high levels of DCF and IBU present in seawater, as previously reported[63]. Other studies were carried out in bays from different world areas, including the Atlantic Ocean (Bay of Cádiz in Spain and Santos Bay in Brazil), the Ionic Sea (Augusta Bay, Italy), the Pacific Ocean (Bellingham Bay in the Salish Sea, USA), and the South China Sea [Table 1]. DCF concentrations measured at a depth between 0 and 10 cm are similar in all study areas, ranging from < 0.093 to 1.5 ng/g dw, except Cadiz Bay, where a value of 10 ng/g dw was recorded. IBU showed a wide range of variation compared to DCF, reaching the maximum concentration (101.3 ng/g dw) in the same Bay of Cadiz. Xie *et al.* investigated the occurrence of 34

pharmaceuticals in sediments from the Pearl River Delta[66]. They observed that DCF and IBU were only present in one of the 13 sampling sites and at very low concentrations of 0.005 and 0.002 ng/L, respectively, compared with other compounds such as ketoprofen, spectinomycin, or norfloxacin, which were present at concentrations of 11, 7.2, and 2.2 ng/g dw, respectively. A review of the results of studies reported in Table 1 shows that the concentrations of DCF and IBU in marine sediments are generally lower compared to other anti-inflammatory/analgesics, such as fenoprofen and acetaminophen, or other drug classes (antibiotics, antihypertensives, or estrogens). This indicates that the presence of DCF and IBU in the sediments is determined by factors other than drugs and environmental properties (e.g., consumption rate). Sediments can act both as natural repositories and sources for pharmaceuticals, first adsorbing them and subsequently releasing them back into the water column during tidal changes, storm events, or bioturbation. This makes sediments an important pathway for entry into filter-feeding organisms living near the bottom.

BIOACCUMULATION

The DCF and IBU concentrations vary widely in water and sediments depending on the sampling area [Table 1]. One must consider the exposure, physicochemical conditions, and lifestyles involved to assess the hazardous effects of both compounds in their respective environmental compartments. While avoidance is an option for pelagic species to reduce exposure, it is a limited option for benthic species. Consequently, establishing a general pattern regarding the preferential exposure of pelagic or benthic marine invertebrates to NSAIDs is not an easy task, as it depends on several variables that need to be considered. In Tables 2 and 3, we have summarized data on DCF and IBU concentrations in various marine invertebrates from laboratory studies [Table 2] and field studies [Table 3].

The analytical method employed for the bioaccumulation assessment of both compounds has mainly been LC-MS, as shown in Tables 2 and 3. However, some results have been obtained using LC and GC-MS. The reported differences for LOD or LOQ vary based on the analytical technique employed. Thus, lower detection limits have been reported for both compounds when using LC-MS or LC-MS/MS.

Laboratory assays evaluate the pharmaceutical accumulation capacity in target organisms under controlled conditions. The knowledge generated with this type of assay can help us to understand the mechanisms of drug accumulation (e.g., toxicokinetic and toxicodynamic properties), identify hazards associated with exposure, and provide a basis for understanding the accumulation in the field.

According to the EU regulation 253/2011[109], the bioconcentration factor (BCF) represents the compound accumulation in the whole body solely from the water. Its value can be used to identify and classify bioaccumulative substances. A chemical is considered bioaccumulative when its BCF is greater than 2000 and classified as very bioaccumulative when its BCF exceeds 5000. According to the BCF data reported in Table 2, DCF and IBU can be considered substances with low bioconcentration potential in mollusks. The same conclusion can be reached by evaluating the $\log K_{ow}$. The $\log K_{ow}$ values for DCF and IBU are 4.26 and 3.97, respectively, which are below the threshold limit ($\log K_{ow} \geq 4.5$) indicated by the European Medicines Agency for the identification of bioaccumulative substances in human pharmaceuticals[110].

Bivalves, particularly mussels, were widely used to test pharmaceutical bioaccumulation[98]. The DCF accumulation in bivalve tissues has been studied in *Mytilus* and *Ruditapes* species, highlighting their differences [Table 2]. The resulting BCF values were 6.84-10.1, 180, 24.3, and 60.3 L/kg ww for *Mytilus galloprovincialis*, *Mytilus trossulus*, *Ruditapes decussatus,* and *Ruditapes philippinarum,* respectively, suggesting the existence of different mechanisms of drug uptake, detoxification, and excretion mechanisms in different species. Most studies have focused on *Mytilus galloprovincialis,* and they have tested different

Table 2. Bioconcentration factors for DCF and IBU in invertebrate marine species (all species included in the table belong to the Mollusca phylum) under laboratory-controlled exposure (*in vivo* assays)

Drug	Species	Exposure condition	Drug concentrations in organisms	Analytical method	LOQ (ng/g)	LOD (ng/g)	BCF	Refs
DCF	*Mytilus galloprovincialis*	Exposure: 0.5 µg/L, 14 d	1.19 ng/g ww	LC		1	2.4 L/kg ww	[90]
		Exposure: 1 and 100 µg/L, 3 d	6.5 and 520 ng/g ww (1.0 and 100 µg/L respectively)	LC-MS		10	6.84 and 5.0 L/kg ww (1.0 and 100 µg/L respectively)	[91]
		Exposure: 1 µg/L, 28 d	7.1 ng/g ww	LC	15	5	7.4 L/kg ww	[92]
		Exposure: 1 µg/L, 28 d	9.7 ng/g ww	LC		5	10.1 L/kg ww	[93]
		Exposure: 2.5 µg/L, 60 d	0.41, 0.91, 0.56 ng/g ww (respectively at 14, 30 and 60 d)	LC		1	0.16, 0.36, 0.22 L/kg ww (respectively at 14, 30 and 60 d)	[94]
		Exposure: 10.0 ± 1.6 µg/L, 58 d	132.6 ± 16.6 ng/g ww (visceral mass)	LC-MS/MS	29		13 L/kg ww	[95]
		Exposure: 25 µg/L, 14 d	14.90 ± 7.89 ng/g ww	LC		1	0.28-0.912 L/kg ww*	[26]
		Exposure: 100 and 600 µg/L, 7 d	502 and 1,836 ng/g ww (100 and 600 µg/L respectively)	LC-MS		1	16.3 and 11.03 L/kg ww (100 and 600 µg/L respectively)	[96]
	Mytilus trossulus	Exposure: 1 and 10,000 µg/L, 8 d	180 and 82,000 ng/g ww (respectively at 1 and 10,000 µg/L)	LC-MS/MS	nr	nr	180 and 10 L/kg ww (respectively at 1 and 10,000 µg/L)	[97]
		Exposure: 133.3 µg/L, 5 d	2.22 ng/g dw	GC-MS	20	10	57.43 L/kg dw	[98]
	Ruditapesdecussatus	Exposure: 1 µg/L, 7 d	24.3 ng/g dw	LC		5	24.3 L/kg dw	[99]
	Ruditapesphilippinarum		60.3 ng·g^{-1} dw				60.3 L/kg dw	[99]
IBU	*Crassostrea gigas*	Exposure: 1 and 100 µg/L, 7 d	0.2 and 29.4 ng/g ww (respectively at 1 and 100 µg/L)	LC	nr	nr	0.2 and 0.294 L/kg ww (respectively at 1 and 100 µg/L)*	[100]
	Mytilus galloprovincialis	Exposure: 2.5 µg/L, 60 d	6.4, 6.1, 11.3 ng/g ww (respectively at 14, 30 and 60 d)	LC		0.5	2.6, 2.5, 4.5 L/kg ww (respectively at 14, 30 and 60 d)	[94]
		Exposure: 25 µg/L, 14 d	1.63 ± 1.00 ng/g ww	LC			0.025-0.105 L/kg ww*	[26]

BCF: Bioconcentration factor; DCF: diclofenac; dw: dry weight; GC-MS: gas chromatography coupled to MS; IBU: ibuprofen; LC: traditional liquid chromatography with diode array detection and ultraviolet-visible spectroscopy; LC-MS/MS: liquid chromatography coupled to tandem MS; LOD: limit of detection; LOQ: limit of quantification; MS: mass spectrometry; nd: not detected; nr: not reported; ww: wet weight; *values calculated using concentrations reported by authors and the nominal concentration used in the assays.

concentrations and exposure times. The trend in the DCF accumulation seems to be directly proportional to the exposure concentration. The BCF values varied from 2.4 after 14 days of exposure at 0.5 µg/L of DCF[90] to 16.3 L/kg ww after 7 days of exposure at 100 µg/L[96]. However, some authors observed a decrease in BCF values at higher exposure concentrations. Mezzelani *et al.*[26] obtained BCF values ranging between 0.28 and 0.912 L/kg ww for mussels exposed at 25 µg/L of DCF for 14 days, and Bonnefille *et al.*[96] observed that exposure to 600 µg/L of DCF during 8 days resulted in a BCF of 10 L/kg ww, a lower value compared to 16.3 L/kg ww obtained after exposure at 100 µg/L. A similar result was also obtained by Ericson and coauthors[97], who exposed *Mytilus trossulus* specimens to DCF at 1 and 10,000 µg/L for 8 days and obtained BCF values of 180 and 10 L/kg ww, respectively. However, the same authors indicate that these results must be used cautiously due to the uncertainty of the underlying mechanisms for high accumulation. In the presence of high concentrations of contaminants, exposed organisms may respond by isolating themselves from the aquatic medium by closing the shell and switching to anaerobic metabolism, slowing down the

Table 3. The concentration of DCF and IBU in invertebrate marine species (all species included in the table belong to the Mollusca phylum) from field studies

Drug	Species	Study location	Study period	Detected concentration	Analytical method	LOQ (ng/g)	LOD (ng/g)	Refs
DCF	*Anomalocardiabrasiliana*	Northeast of Brazil (estuary and coastal areas)	July-August 2019	nd-2.1 ng/g ww	LC-MS	0.81	0.24	[101]
	Mytilus edulis	Northeast of Brazil (estuary and coastal areas)	July-August 2019	nd-3.0 ng/g ww	LC-MS	0.81	0.24	[101]
	Mytilus galloprovincialis	Atlantic Ocean (Portugal, coastal areas)	January-October 2015	0.5-4.5 ng/g dw	LC-MS/MS	0.5	0.2	[102]
		Central Adriatic Sea (Portonovo Bay, coastal areas)	July, August and September 2014	16.11 ± 14.72 ng/g dw	LC		1	[26]
		Adriatic Sea (Italy, coastal areas)	2014-2017	< LOQ-231 ± 67.2 ng/g dw	LC	1.37		[103]
		Tyrrhenian Sea (Italy, coastal areas)	2014-2017	< LOQ-280.1 ± 161.8 ng/g dw	LC	1.37		[103]
		Northwest Adriatic Sea (Italy, coastal lagoon)[a]		2.1-4.6 ng/g ww	GS-MS	1		[104]
	Mytilus trossulus	Gulf of Gdansk (southern Baltic Sea)		560 ± 130 ng/g dw	GC-MS	5	2	[105]
	Neritealineata	Klang River (Malaysia, estuary)		0.93-7.41 ng/g ww	LC-MS		0.35	[106]
	Ruditapesphilippinarum	Ria Formosa lagoon (Portugal, coastal lagoon)	June to July 2016, 2017, and 2018	1.5-1.6 ng/g ww	LC-MS/MS	0.02	0.006	[107]
IBU	*Anomalocardia brasiliana*	Northeast of Brazil (estuary and coastal areas)	July-August 2019	nd-8.2 ng/g ww	LC-MS	0.72	0.22	[101]
	Mytilus edulis	Northeast of Brazil (estuary and coastal areas)	July-August 2019	nd-5.4 ng/g ww				[101]
	Mytilus galloprovincialis	Central Adriatic Sea (coastal areas)	July, August and September 2014	9.39 ± 0.59 ng/g dw	LC		0.5	[26]
		Adriatic (Italy, coastal areas)	2014-2017	< LOQ-143.7 ± 242 ng/g dw	LC	8	2.4	[103]
		Tyrrhenian Sea (Italy, coastal areas)	2014-2017	< LOQ-22.99 ± 26.01 ng/g dw	LC	8	2.4	[103]
	Mytilus trossulus	Gulf of Gdansk (southern Baltic Sea)		730 ± 290 ng/g dw	GC-MS	3	1	[105]
	Ostrea lurida	Coos and Netarts Bays (Oregon coastal estuaries)	Summer 2013, fall 2013, spring 2014	nd-10.5 ng/g ww	LC-MS/MS	nr	nr	[108]
	Ruditapesphilippinarum	Ria Formosa lagoon (Portugal, coastal lagoon)	June to July 2016, 2017, and 2018	0.9-1 ng/g ww	LC-MS/MS	1.05	0.32	[107]

(a) Mussel transplanted for 28 d; (b) Clams transplanted for one month along a dilution gradient of WWTP effluent discharge and in a "clean site" under no direct influence of WWTP discharge (concentrations detected in seawater: DCF 390-800 ng/L, IBU 10-40 ng/L). DCF: Diclofenac; dw: dry weight; GC-MS: gas chromatography coupled to MS; IBU: ibuprofen; LC: traditional liquid chromatography with diode array detection and ultraviolet-visible spectroscopy; LC-MS/MS: liquid chromatography coupled to tandem MS; LOD: limit of detection; LOQ: limit of quantification; MS: mass spectrometry; nd: not detected; nr: not reported; ww: wet weight.

filtration rate or increasing metabolization, and excretion processes, significantly reducing the accumulation of contaminants[98,111,112]. Studies realized with IBU in the mussel *Mytilus galloprovincialis* showed similar results to those observed with DCF. Mezzelani[26,94] reported higher BCF values at lower exposure doses, for example, 2.5-4.5 L/kg ww at 2.5 μg/L compared to 0.025-0.105 L/kg ww at 25 μg/L. On the other hand,

nearly similar BCF values of 0.2 and 0.294 L/kg ww have been found in the oyster *Crassostrea gigas* exposed to low and high IBU concentrations of 1 and 100 μg/L, respectively[100]. Unfortunately, the available information about DCF accumulation under laboratory conditions is scarce, and even more so for IBU (only three papers were found in our review). This scarcity of data makes it difficult to draw conclusions about the relationship between DCF and IBU accumulation, exposure time, exposure concentrations, and species.

In general, information about bioaccumulation in invertebrates in the field is scarce and primarily focused on mollusk species. This focus is likely due to their sedentary lifestyle, which facilitates their sampling[113] and allows researchers to obtain data representative of the area where they have been collected.

In freshwater, the residues of NSAIDs detected in invertebrates are relatively higher than those in other pharmaceutical classes. The most frequent NSAIDs detected are DCF and IBU[114]. Swiacka and coauthors[28] reviewed the occurrence of pharmaceuticals in biota from freshwater and seawater environments. They concluded that antibiotics and NSAIDs are the most relevant classes detected in marine biota, reaching concentrations above 500 ng/g. In our bibliographic review, we found concentrations in this range in *Mytilus trossulus* from the Gulf of Gdansk in the Baltic Sea for both DCF and IBU (560 ± 130 ng/g dw and 730 ± 290 ng/g dw, respectively, as shown in Table 3). These levels reflect the high concentrations detected in seawater, as discussed in Section 2. Swiacka and coauthors[28] indicated that according to UNESCO and HELCOM[115], the Baltic Sea is one of the most contaminated seas in the world, with anti-inflammatories and analgesics being the substances of greatest concern. Within these groups, DCF, IBU, and paracetamol are the most frequently detected compounds in all seawater compartments.

High DCF and IBU concentrations of 109-280.1 ng/g dw and 143.7 ng/g dw, respectively, have also been detected by Mezzelani and coauthors[26,103] in *Mytilus galloprovincialis* from the Adriatic and Tyrrhenian coastal areas during 2014-2017. They sampled different sites along the coast, including natural parks, mussel farms, touristic areas, *etc.*, and detected both compounds in all sites, with a higher frequency of detection for DCF compared to IBU (31.15% *vs.* 9.35%). These authors detected the most elevated values in tourist areas with recreational beaches, mussel farms, and canal harbors. Additionally, they recorded seasonal changes with higher levels in spring and summer, which they related to the greater anthropogenic pressure in tourist areas.

Both compounds were also detected in mollusk species from estuaries and coastal areas in Portugal[102,107], Brazil[101], the USA[108], and Malaysia[106], with concentrations in the low nanogram per gram range (under 8 ng/g ww and 4.5 ng/g dw for DCF and 11 ng/g ww for IBU, as shown in Table 3). Sampling points from all cited works are characterized by their proximity to environmental contamination sources, such as farming and industrial areas, cities with high population density, tourist areas, the presence of WWTPs, harbors, *etc.* Most of these studies reported significant spatial and temporal variability in the bioaccumulation of DCF and IBU. Bioaccumulation is conditioned by numerous factors besides pharmaceutical physicochemical properties in the field. These factors include environmental variables, such as temperature, salinity, pH, dissolved oxygen, organic matter content, *etc.*, as well as biotic factors, like body size, growth, life stage, lipid content, behavior, respiration and feeding strategy, disease status, *etc.* These factors contribute to significant variability in the data obtained from field studies[114]. All of these, together with the scarcity of available data for DCF and IBU, highlight the need for more studies and more complex approaches to better understand the bioaccumulation of both compounds in marine invertebrates.

EFFECTS

As previously indicated, aquatic invertebrates are continuously exposed to active substances released into the environment, which accumulate in their tissues, producing a wide range of effects. These effects include acute effects, which are less likely and usually occur during accidental discharge of drugs, and chronic effects, which are related to prolonged exposure to different concentrations (generally low) of contaminants over an extended period of time.

Studies published between 2010 and 2023 on the toxic effects of DCF and IBU on marine invertebrates under laboratory-controlled exposure are summarized in Tables 4 and 5, respectively. The toxicities of both compounds have been assessed on diverse invertebrate species, including crustaceans, echinoderms, mollusks, and polychaeta.

Acute toxicity data are very scarce for both NSAIDs. Survival under drug exposure was evaluated in the early life stages of the shrimp *Siriella armata* and the copepod *Tisbe battagliai,* showing LC_{50} values ranging from 0.01 to 15.8 mg/L for DCF, depending on the species and the exposure time, and an LC_{50} value of 49.7 mg/L for *Tisbe battagliai* exposed to IBU, as reported in Tables 4 and 5[118-120]. Increased mortality was also observed by Ericson *et al.* in adult specimens of *Mytilus trossulus* after exposure to DCF at concentrations higher than 1,000 μg/L[97]. According to Directive 93/67/ECC (CEC)[148], EC_{50} can be used to classify the risk associated with chemical compounds. Accordingly, an EC_{50} less than 1 mg/L is considered very toxic to aquatic organisms, while an EC_{50} in the range of 1 to 10 mg/L is regarded as toxic, and an EC_{50} in the range of 11 to 100 mg/L is considered harmful. Data reported above indicate that DCF presents higher toxicity than IBU and that the toxic effect level varies between species. However, acute effects observed in laboratory studies occur at concentrations higher than those typically found in marine environments (in the range of ng/L, as shown in Table 1). This suggests that mortality from DCF and IBU may not be observed in a natural population except in areas with very high levels of contamination (e.g., proximity to WWTP effluent discharge and accidental spills). In coastal and estuarine waters, sublethal effects are more likely to occur. These effects include a wide range of alterations, such as changes in behavior, development, and reproduction, as well as metabolic alterations, tissue/organ abnormalities, and biochemical and genetic changes/damage.

Some studies have been conducted to evaluate the DCF and IBU effects on the larval development of sea urchins (*Arbacia lixula*, *Lytechinus variegatus*, and *Paracentrotus lividus*), marine bivalves (*Mytilus galloprovincialis* and *Perna perna*), and shrimp (*Palaemon serratus*), as reported in Tables 4 and 5. DCF negatively impacts the embryo development of all studied organisms, showing different sensitivity levels between species. *Mytilus galloprovincialis* showed increased shell malformation and reduced larval development at the lowest exposure concentration, closest to environmentally relevant concentrations (LOEC: 0.01 μg/L, 48 h), followed by *Parocentrotus lividus* (LOEC: 12.5 μg/L, 48 h), *Palaemon serratus* (LOEC: 900 μg/L, 50 days), *Arbacia lixula* (LOEC: 50,000 μg/L, 3 h), and finally *Perna perna*, which exhibited growth alterations at exposure concentrations 10^7 times higher compared to *Mytilus galloprovincialis* (LOEC: 100,000 μg/L, 48 h) [Table 4][75,117,121,123,126,127]. IBU was also shown to induce embryotoxicity in different species. It was classified as highly toxic for embryo-larval development by Pusceddu[138] and Aguirre- Martínez[139], who studied its effects on sea urchins and mussels. Species-specific differences were also observed in invertebrates exposed to IBU. Sea urchins (*Parocentrotus lividus*) showed decreased embryo development at lower exposure concentrations compared to *Mytilus galloprovincialis* (LOEC: 0.01 and 100 μg/L, respectively)[127,139]. On the other hand, the sea urchin *Lytechinus variegatus* and the mussel *Perna perna* exposed to IBU-spiked sediments showed similar inhibition of larval development from an exposure concentration of 15 ng/g dw[138]. The different responses observed in marine invertebrates

Table 4. Ecotoxicological effects of DCF in marine invertebrate species under laboratory conditions

Taxonomic	Species	Exposure concentrations (μg/L)	Exposure time	Tissue	Endpoints	Effects	Refs
Crustacean	*Carcinus maenas*	0.01, 0.1	7 d	Hemolymph	Physiology	↑ hemolymph osmolality and osmoregulation capacity (both concentrations)	[116]
Crustacean	*Palaemon serratus*	36, 900	50 d	Larvae	Embriotoxicity	↑ specific growth rate (LOEC: 900 μg/L)	[117]
Crustacean	*Siriella armata*	250, 20,000	2, 4 d	Neonates	Mortality	NOEC: 8,000 (2 d) and 250 (4 d) μg/L LOEC: 10,000 (2 d) and 500 (4 d) μg/L LC_{50}: 10.43 μg/L (2 d) and 2,919 μg/L (4 d)	[118]
Crustacean	*Tisbe battagliai*	0-120,000	2 d	Nauplii	Mortality	LC_{50}: 9,500 μg/L (2 d)	[119]
			2 d			LC_{50}: 15,800 μg/L (2 d)	[120]
Echinoderm	*Arbacia lixula*	50, 500, 5,000, 50,000	180 min	Embryos	Embryotoxicity	↓ number of correctly developed embryos (50,000 μg/L)	[121]
				Gametes	Reproduction	↑ number of degenerated eggs (5,000 and 50,000 μg/L)	
Echinoderm	*Asterias rubens*	0.01, 0.1, 1, 10, 100, 1,000	120 min	Gametes	Reproduction	↓ sperm motility (≥ 1 from 20 min) and swimming speed (≥ 0.1 from 20 min), EC_{50} (60 min): 2,335.8 μg/L ↓ % fertilization, EC_{50} (60 min): 616.48-2610 μg/L	[122]
Echinoderm	*Parocentrotus lividus*	12.5, 125, 1,250, 12,500	2 d	Embryos	Embryotoxicity	↓ larval length (all exposure concentrations) ↑ abnormal development (all exposure concentrations)	[123]
Echinoderm	*Psammechinus miliaris*	0.01, 0.1, 1, 10, 100, 1,000	120 min	Gametes	Reproduction	↓ sperm motility (exposure concentrations ≥ 1, after 20 min), EC_{50} (60 min): 378.22 μg/L ↓ % fertilization, EC_{50} (60 min): 247.31-429.37 μg/L	[122]
Mollusc	*Mytilus edulis*	1, 1,000	1, 4 d	Digestive gland	Oxidative stress	↑ GST, (4 d, 1 and 1,000 μg/L), LPO (↓ 1 d and ↓ 4 d, 1 and 1,000 μg/L)	[120]
					Genotoxicity	↑ DNA damage (4 d, 1,000 μg/L)	
		1, 1,000	14 d	Digestive gland	Oxidative stress	↓ GST (1,000 μg/L)	[124]
						down-regulation of GADPH (1 and 1,000μg/L, 14 d) upregulation of BRAFLDRAFT_282392 (1 and 1,000 μg/L, 7 and 14 d), class 1 ADH (1,000 μg/L, 7 d)	
		200, 1,000	7 d	Digestive gland	Oxidative stress	↓ GR, CAT (1,000 μg/L)	[125]
				gills	oxidative stress	↑ GR (1,000 μg/L)	
					oxidative stress, alteration of energy metabolism, cytoskeleton, protein modification and transport	oxidation and changes in protein abundance (caspase, HSP, SOD, *etc.*)	
Mollusc	*Mytilus galloprovincialis*	1, 10	2 d	Larvae	Embryotoxicity	shell deformation and disturbance in shell growth (both exposure concentrations)	[126]

						alteration in gene expression (genes involved in shell formation and biotransformation) (both exposure concentrations)	
		0.01, 0.1, 1, 10, 100, 1,000	2 d	Larvae	Embryotoxicity	↑ shell malformations and ↓ embryo development (LOEC: 0.01 μg/L)	[127]
		1	28 d	Whole body	Oxidative stress and detoxification	↑ SOD, CAT, GPx and GST, ↓ LPO and GSH/GSSG ratio	[92]
		0.250	15 d	Digestive gland	Oxidative stress	↑ SOD (7 d), ↑ GST (7 d), CAT (3d and ↓ 15 d), and LPO (↑ 3 d and ↓ 7 d)	[128]
				Gills	Oxidative stress	↑ SOD (3 d), ↑ GR (3 d), ↓ LPO (7 d)	
					Neurotoxicity	AChE (↑ 3 and 7 d)	
				Gonads	Vitellogenesis/endocrine disruption	female: ↑ ALP (3 and 7 d) male: ↑ ALP (3 d)	
		0.5	14 d	Digestive gland	Lysosomal responses	↑ lipofuscin, ↓ neutral lipids	[90]
		1, 100	3 d	Digestive gland, gills	DCF mechanism of action	↓ PG biosynthesis (both exposure concentrations)	[91]
		100	7 d	Digestive gland	Metabolism alteration	tyrosine and tryptophan metabolism modulation, steroid hormone biosynthesis modulation	[129]
		0.005, 0.01, 0.1	60 min	Hemocytes	Oxidative stress	↑ LPO (allexposureconcentrations)	[130]
					Immunotoxicity	↓ LMS (all exposure concentrations)	
					Genotoxicity	↑ DNA damage (0.005 and 0.01 μg/L)	
					Cytotoxicity	↑ O_2^- and NO (all exposure concentrations)	
		2.5	14, 30 and 60 d	Hemocytes	Immunotoxicity	↓ granulocytes/hyalinocytes ratio (from 30 d)	[11]
					Genotoxicity	↑ DNA fragmentation (14 and 60 d) and MN frequency (30 d)	
		25	14 d	Hemocytes	Immunotoxicity	↓ LMS	[26]
					Genotoxicity	↑ DNA fragmentation, ↑ MN frequency	
Mollusc	*Mytilus trossulus*	1, 100, 1,000, 5,000, 10,000	21 d		Physiology	↓ byssus strength and threads abundance (LOEC: 10,000 μg/L)	[97]
						↓ scope for growth (LOEC: 100 μg/L)	
						↑ mortality (LOEC: 10,000 μg/L)	
						energy balance alteration (LOEC: 100 μg/L)	
		4, 40	25 d (12 d exposure + 12 d depuration)	Various tissues	Tissue abnormality	accumulation of lipofuscin in gills, mantle and digestive gland (4 and 40 μg/L) necrosis (4 and 40 μg/L) gonadal artresia (4 and 40 μg/L) hepatopancreas atrophy (40 μg/L) hepatopancreas vacuolization (4 and 40 μg/L) gill deformations (4 and 40 μg/L)	[131]
		68.22	7 d	Digestive	Tissue abnormality	necrosis, local inflammation, digestive tubules	[132]

				gland			
				Gills	Oxidative stress	↓ GR ↑ cytosolic protein content	
					Tissue abnormality	gill deformations (edema, enlarged gills, inflammation and necrosis)	
				Gonads	Tissue abnormality	atresia, lesions (granulocitoma), inflammation and necrosis	
Mollusc	*Perna perna*	10, 100, 1,000, 10,000, 100,000	2 d	Larvae	Embryotoxicity	↑ inhibition of larval development (100000 μg/L) NOEC: 10,000μg/L LOEC: 100,000μg/L IC_{50}: 18,000μg/L	[75]
		0.02, 0.2, 2	2, 4 d	Digestive gland	Oxidative stress	↓ LPO (0.2 μg/L, 4 d)	[75]
					Genotoxicity	↑ DNA damage (2 μg/L, 2 d)	
					DCF mechanism of action	↑ COX (0.2 μg/L, 4 d)	
				Gills	Oxidative stress	↓ GST (0.02, 0.2 and 2 μg/L after 2 d, 2 μg/L after 4 d), ↓ GPx (0.2 μg/L after 2 d, 2 μg/L after 4 d), LPO (↑ 0.2 and 2 μg/L after 2 d, ↓ 0.02 and 0.2 μg/L after 4 d)	
					Neurotoxicity	↑ AChE (2 μg/L, 2 d)	
					Xenobiotic biotransformation	↓ EROD (0.2 μg/L, 4 d)	
					Genotoxicity	↓ DNA damage (0.2 and 2 μg/L, 2 d)	
					DCF mechanism of action	↓ COX (0.02 and 0.2 μg/L after 2 d, 0.2 and 2 μg/L after 4 d)	
		0.02, 0.2, 2	2, 4 d	Hemocytes	Immunotoxicity	↓ LMS (all exposure concentrations and times)	[75]
		10, 1,000, 31,250, 100,000, 250,000, 1,000,000	1 d	Sperm	Reproduction	↑ inhibition of fertilization LOEC: 31,250 μg/L EC_{50}: 389,000μg/L	[75]
Mollusc	*Ruditapes decussatus*	1	30 d	Whole body	Oxidative stress	↑ SOD, CAT and GPx ↓ GSH/GSSG ratio	[99]
Mollusc	*Ruditapes philippunarum*	1	30 d	Whole body	Physiology	↓ respiration rate	[99]
					Metabolic capacity and energy-related metabolism	↓ protein content ↑ glycogen content	
					Oxidative stress	↑ GSH/GSSG ratio	
		15	14 d	Digestive gland	Oxidative stress and detoxification	↑ SOD (1d), CAT (1 d), GR (1 and 7 d), GST (1 and 7 d), LPO (1 d) and MT (7 d) ↓ T-GPx (3 h)	[133]
					Neurotoxicity	↑ AChE (1, 7 and 21 d)	
				Gills	Oxidative stress	↑ SOD (7d) and CAT (7 d) ↓ LPO (14 d) and MT (14 d)	
					Neurotoxicity	↑ AChE (7 d)	

Polychaete	*Arenicola marina*	0.01, 0.1, 1, 10, 100, 1,000	120 min		Reproduction	↓ sperm motility and swimming speed (exposure concentrations higher than 1 μg/L, after 90 min), EC_{50} (120 min): 106.77 μg/L ↓ % fertilization, EC_{50} (120 min): 112.61-565.53 μg/L	[122]
Polychaete	*Hediste diversicolor*	0.5, 1, 2	28 d	Whole body	Oxidative stress and detoxification	↑ GST (1 and 2 μg/L)	[134]

AChE: Acetylcholinesterase; ADH: alcohol dehydrogenase; ALP: alkaline-labile-phosphate; CAT: catalase; COX: cyclooxygenase; DCF: diclofenac; EC_{50}: half maximal effective concentration; EROD: ethoxyresorufin-O-deethylase; ETS: electron transport system; GADPH: glyceraldehyde-3-phosphatase; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione; GST: glutathione S-transferase; HSP: heat shock protein; IC_{50}: half maximal inhibitory concentration; LC_{50}: lethal concentration 50; LMS: lysosomal membrane stability; LOEC: low observed effect concentration; LPO: lipid peroxidation; MN: micronuclei; MT: metallothionein; NO: nitric oxides; NOEC: no observed effect concentration; O_2^-: superoxide anions; PG: prostaglandins; T-GPx: total glutathione peroxidase; SOD: superoxide dismutase.

exposed to DCF and IBU can be attributed to differences in species-specific sensitivity and distinct mechanisms of action and bioaccumulation for each compound[149].

Studies on adult organisms revealed that DCF and IBU affect reproduction and physiology. Mohd Zanuri *et al.* studied the effects of exposure to concentration gradients of DCF and IBU (0.01-1,000 μg/L, 120 min) on the reproductive success of the polychaete *Arenicola marina*, the sea star *Asteria rubens*, and the sea urchin *Psammechinus miliaris,* as reported in Tables 4 and 5[122]. Under DCF exposure, a common effect on the three species was the reduction of swimming speed and sperm motility at low exposure concentrations (0.1 and 1 μg/L), with a faster response observed in *Asterias rubens* and *Psammechinus miliaris* (20 min) compared to *Arenicola marina* (90 min). Additionally, a reduction in the fertilization percentage was observed after incubation of oocytes and sperm at DCF concentrations ≥ 1 μg/L in *Asterias rubens* and ≥ 0.01 μg/L in *Psammechinus miliaris* and *Arenicola marina*. According to their results, the authors classified DCF as toxic to gametes for *Asterias rubens* and very toxic for *Psammechinus miliaris* and *Arenicola marina*[122]. IBU was toxic for reproduction in a concentration range similar to or slightly higher than DCF (≥ 1 and 10 μg/L). However, unlike DCF, its effects were more pronounced in *Psammechinus miliaris* than in other tested organisms, demonstrating the species-specific effects of the two NSAIDs. The study of Zanuri and coauthors[122] showed that the reproductive success of different species can be altered at concentrations close to those detected in more polluted coastal environments. Broadcast spawning invertebrates, such as mollusks and sea urchins, release their gametes into the water column[150] for external fertilization, and the presence of pollutants could affect sperm function, leading to DNA damage and disruption of sperm swimming ability, which may reduce fertilization success[122]. Other studies reported in this review showed the induction of negative effects at the highest exposure levels, which are far from environmentally relevant concentrations. These negative effects include a reduction in fertilization success in the sea urchin *Parocentrotus lividus* and the mussel *Perna perna* at 10,000 mg IBU/L (60 min) and 31.25 mg DCF/L (24 h), respectively. Additionally, DCF was found to accumulate in both the eggs and sperm of the sea urchin *Arbacia lixula*, leading to eggs degeneration and decreased development in embryos at concentrations of 5 and 50 mg/L, as reported in Tables 4 and 5[75,121,139].

Table 5. Ecotoxicological effects of IBU in marine invertebrate species under laboratory conditions

Taxonomic	Species	Exposure concentrations (μg/L)	Exposure time	Tissue	Endpoints	Effects	Refs
Crustacean	*Amphelisca brevicornis*	0.05, 0.5, 5, 50, 500 ng/g*	10 d	Whole body	Oxidative stress	↑ DBF (0.05, 0.5 ng/g), GST (0.05, 0.5, 50 ng/g) and GPx (0.05 ng/g) ↓ LPO (0.05 ng/g)	[135]
					Genotoxicity	↓ DNA damage (0.5, 50, 500 ng/g)	
Crustacean	*Carcinus maenas*	0.1, 5, 10, 50	28 d	Hemolymph		↓ LMS (exposure concentrations > 5 μg/L), LOEC: 5 μg/L	[136]
		0.1, 5, 10, 50	28 d	Hepatopancreas	Oxidative stress and detoxification	↑ DBF (5, 10 and 50 μg/L), GST (5, 10 and 50 μg/L), GPx (5, 10 and 50 μg/L) and LPO (all exposure concentrations)	[137]
					Genotoxicity	↑ DNA damage (50 μg/L)	
				Gills	Oxidative stress and detoxification	↑ EROD (50 μg/L), DBF (5,10 and 50 μg/L), GST (5, 10 and 50 μg/L), GPx (10 and 50 μg/L) and LPO (all exposure concentrations)	
					Genotoxicity	↑ DNA damage (5, 10 and 50 μg/L)	
				Muscle	Oxidative stress and detoxification	↑ DBF (10 μg/L), GST (10 and 50 μg/L), GPx (5, 10 and 50 μg/L) and LPO (0.1, 5 μg/L)	
					Genotoxicity	↑ DNA damage (0.1, 5 and 50 μg/L)	
				Gonads	Oxidative stress and detoxification	↑ GST (5, 10 and 50 μg/L), GPx (0.1, 5, 10 and 50 μg/L), LPO (0.1, 5 and 50 μg/L)	
Crustacean	*Tisbe battagliai*	0-120,000	2 d	Nauplii	Mortality	LC_{50}: 49,700 μg/L (2 d)	[119]
Echinoderm	*Lytechinus variegatus*	1.5, 15, 150.8, 1,508 ng/g dry weight*	2 d	Larvae	Embryotoxicity	↓ embrio-larval development (exposure concentrations ≥ 15 ng/g dw)	[138]
Echinoderm	*Parocentrotus lividus*	0.01, 0.1, 1, 5, 10	2 d	Larvae	Embryotoxicity	↓ larval development (0.01, 0.1, 5, 10 μg/L), EC_{50}: 0.01 μg/L (2 d), LOEC: 0.01 μg/L	[139]
Echinoderm	*Parocentrotus lividus*	0.1, 1, 10, 100,000, 500,000, 1,000,000, 100,000,000	60 min	Gametes	Reproduction	↓ % fertilization (10,000,000μg L^{-1}), EC_{50}: 2,065,000 μg/L (60 min)	[139]
Echinoderm	*Psammechinus miliaris*	0.01, 0.1, 1, 10, 100, 1,000	120 min		Reproduction	↓ sperm motility (exposure concentrations ≥ 1 μg·L^{-1} afterfrom 20 min) and swimming speed exposure concentrations ≥ 1 μg·L^{-1} after (≥ 1 from 20 min), EC_{50} (60 min): 845.98 μg/L ↓ % fertilization, EC_{50} (60 min): 437.03-4.56 × 10^6 μg/L	[122]
Mollusc	*Crassostrea gigas*	1, 100	7 d	Gills	Oxidative stress and detoxification	↑ CAT (1μg/L, 4 d), and GST (100 μg/L, 4 d) ↓ GR (1 and 100 μg/L, 4 and 7 d)	[100]
					Gene transcription	↑ CYP2AU2 (1 μg/L, 7 d), CYP356A1 (1μg/L, 7 d), CYP3071A1 (1 μg/L, 7 d), GST-ω (100 μg/L at 1 d and 1 μg/L at 7 d), GST-π (100 μg/L at 4 d and 1 μg/L at 7 d), COX-like (100 μg/L, 7 d), FABP-like (1μg/L, 1 and 7 d)	
Mollusc	*Mytella charruana*	0.02, 0.15, 1.51 1.5, 15, 150.8, 1,508 ng/g dry weight*	24 h 2 d	Hemolymph	Immunocytotoxicity	↓ LMS (0.15150.8 and 1.51508 ng/g dry weight)	[138]

Mollusc	*Mytilus galloprovincialis*	0.01, 0.1, 1, 10, 100, 1,000	2 d	Larvae	Embryotoxicity	alteration of embryo development (↑ shell malformations) (LOEC: 100 μg/L)	[127]
Mollusc	*Mytilus galloprovincialis*	0.250	15 d	Digestive gland	Oxidative stress	↑ SOD (7d), GR (3 and 7 d), CAT (3 and 7 d) and LPO (3 and 7 d)	[140]
				Gonads	Vitellogenesis/endocrine disruption	Female: ↑ ALP (7 and 15 d) Male: ↑ ALP (3, 7 and 15 d)	
		0.250	15 d	Gills	Oxidative stress	↓ GR (3, 7 and 15 d), CAT (3, 7 and 15 d), and GST (3 d) ↑ LPO (7 d)	[140]
		0.5	14 d	Digestive gland	Lysosomal responses	↓ neutral lipids	[90]
				Hemocytes	Immunotoxicity	↓ LMS	
		2.5	14, 30 and 60 d	Digestive gland	Lysosomal responses	↑ neutral lipids (30 d)	[11]
				Hemocytes	Immunotoxicity	↓ LMS (from 14 d)	
				Hemocytes	Immunotoxicity	↓ granulocytes/hyalinocytes ratio (from 1,430 d)	
						↑ phagocytosis (from 30 d)	
					Genotoxicity	↑ MN frequency (14 and 30 d)	
		25	14 d	Digestive gland	Oxidative stress and detoxification	↓ GST	[26]
					Genotoxicity	↑ DNA fragmentation and MN frequency	
				Hemocytes	Immunotoxicity	↓ LMS	
		250,000	15 d	Digestive gland		modulation of NF-kB pathway (up or down-expression of various genes)	[141]
						modulation of chitinase and GM2 activator protein genes	
Mollusc	*Mytilus trossulus*	1, 100, 1,000, 5,000, 10,000	21 d (14 d of exposure+7 d of depuration)		Physiology	↓ scope for growth (100 and 1,000 μg/L after 14 d of exposure) ↑ mortality (1,000, 5,000 and 10,000 μg/L) energy balance alteration (LOEC: 1,000 μg/L)	[97]
Mollusc	*Perna perna*	1.5, 15, 150.8, 1,508 ng/g dry weight	Larvae	Larvae	Embryotoxicity	↓ embryo-larval development (≥ 15 ng/g dw)	[138]
Mollusc	*Ruditapes philippinarum*	0.1, 5,10, 50	14 d	Hemocytes	Immunotoxicity	↓ LMS (all exposure concentrations)	[142]
				Digestive gland	Oxidative stress	↑ GPx (0.1, 5μg·L^{-1}), ↑ LPO (5, 10, 50 μg/L), GST (↑ 0.1, 10, 50 μg/L, ↓ 5 μg/L)	
					Genotoxicity	↓ DNA damage (10, 50 μg/L)	
					Neurotoxicity	↑ AChE (5, 50 μg/L)	
		0.1, 5, 10, 50	35 d	Hemocytes	Immunotoxicity	↓ LMS (all exposure concentrations from 14 d)	[143]
		15	14 d	Digestive gland	Oxidative stress and detoxification	↑ T-GPx (7 d), GR (1 d), GST (1 d), LPO (1 d) and, MT (1 d) ↓ SOD and GR (both after 14 d)	[133]
					Neurotoxicity	↑ AChE (1 d)	
				Gills	Oxidative stress and	↑ SOD, GR, LPO, and MT (all after 7 d)	

					detoxification	↓GST (3 h)	
					Neurotoxicity	↓ AChE (7 d)	
		100, 1,000	7 d	Digestive gland	Oxidative stress	↓ SOD (: 100 and 1,000 μg/L after 5 and 7 d)	[144]
				Gills	Neurotoxicity	↓ AChE (after 1d at 100 μg/L and after 7 d at all concentrations), ↑ AChE (1,000 μg/L after 3 d)	
		100, 1,000	7 d	Hemocytes	Immunotoxicity	↓ total hemocytes count (1,000 μg/L)	[145]
						↓ pinocytosis (1,000 μg/L)	
						↑ haemocyte proliferation	
					Cytotoxicity	↑ lactate dehydrogenase activity (1,000 μg/L)	
Polychaete	*Arenicola marina*	0.01, 0.1, 1, 10, 100, 1,000	120 min		Reproduction	↑ sperm swimming (exposure concentrations ≥ 10 μg/L after 30 min), EC_{50} not applicable	[122]
Polychaete	*Hediste diversicolor*	0.05, 0.5, 5, 50, 500 ng/g*	14 d	Whole body	Oxidative stress	↑ LPO (5 ng/g)	[146]
					Neurotoxicity	↑ AChE (500 ng/g)	
					Cellular energy status	↓ mitochondrial electron transport (5 ng/g)	[147]
					Metabolism of monoamines	↓ monoamine oxidase activity (0.5 ng/g)	
					Inflammation	↓ COX activity (all exposure concentrations)	

*Toxicity assay with sediment. AChE: Acetylcholinesterase; ALP: alkaline-labile-phosphate; CAT: catalase; COX: cyclooxygenase; CYP: cytochrome P450; DBF: dibenzylfluorescein; EC_{50}: half maximal effective concentration; EROD: ethoxyresorufin-O-deethylase; FABP: fatty acid binding protein; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione S-transferase; GST-ω and GST-π: glutathione S-transferase isoforms; HSP: heat shock protein; IBU: ibuprofen; LC_{50}: lethal concentration 50; LMS: lysosomal membrane stability; LOEC: low observed effect concentration; LPO: lipid peroxidation; MN: micronuclei; MT: metallothionein; NF-kB: nuclear factor k-light-chain-enhanced of activated B cells; T-GPx: total glutathione peroxidase; SOD: superoxide dismutase.

DCF and IBU also alter physiological parameters in *Mytilus trossulus*[97]. The exposure negatively affected feeding and respiration rates (LOEC: 100 μg/L for both IBU and DCF), as well as the byssus strength and thread abundance ($LOEC_{DCF}$: 10,000 μg/L), with significant consequences on organism survival.

Adverse effects of DCF and IBU on the physiology, larval development, fertility, and reproductive success of invertebrates can lead to significant ecological impacts on populations and ecosystems. These impacts may include reduced survival, decline of populations, and effects on higher trophic levels. Furthermore, species like *Ruditapes philippinarum* and *Mytilus galloprovincialis* represent important economic resources in many coastal areas worldwide. Therefore, negative impacts at the population level could also have economic consequences.

Most of the studies found in our review focus on DCF and IBU responses at the sub-organism level, including tissue alterations, immune, geno-, and neurotoxicity, as well as modulation of the activity of various metabolic and protective enzymes and the expression of different genes. These endpoints are particularly relevant because most offer greater sensitivity than growth and reproduction effects, making them useful in determining the contribution of pollutants toward sub-lethal toxicity in real scenarios.

The only data available on tissue alterations in marine invertebrates are for *Mytilus trossulus* exposed to DFC, which show that this compound is able to induce damage to the mantle, gills, gonads, and digestive gland (e.g., inflammation, necrosis, deformations, *etc.*) at a relatively low concentration of 4 μg/L and an exposure time of 12 days, with possible consequences on respiratory, digestive, and reproductive systems[131,132].

Immuno- and genotoxic effects and antioxidant and detoxification responses associated with DCF and IBU exposure are the most investigated aspects in marine invertebrates, particularly in mollusk species.

The immunotoxicity studies in marine invertebrates are focused on the adverse effects of drugs on hemocytes, cells that play a key role in internal defense. The most studied marker is lysosomal membrane stability (LMS). Destabilization of the lysosomal membrane may result in activation and liberation of hydrolytic enzymes to the cytosol with possible alteration of immunological functions, the integrity of cells, and higher levels of biological organization (e.g., embryogenesis and larval development)[15,151]. The minimum effective concentrations on LMS were 0.02 μg/L for DCF (*Perna perna*, 24 h) and 0.1 μg/L for IBU (*Ruditapes philippinarum*, 14 days), indicating that immunotoxic effects can be produced in coastal and estuarine environments on resident invertebrates in the short and medium term[80,142]. Studies on immunotoxicity of DCF and IBU were conducted on various organisms, exposing them to different concentrations and durations. The results revealed that: (i) both DCF and IBU can modulate immunological parameters in marine bivalves (*Mytilus galloprovincialis*, *Perna perna*, *Mytella charruana,* and *Ruditapes philippinarum*) and crabs (*Carcinus maenas*); (ii) the alteration of immunotoxic parameters (LMS, granulocytes/hyalinocytes ratio, phagocytosis capacity, neutral lipid level, capability to perform pynocitosis, hemocytes proliferation, and lactate dehydrogenase activity) is species-, dose-, and time-dependent; (iii) some effects persist for an extended period up to 30 and 60 days, as reported in Tables 4 and 5[11,26,75,90,130,136,142,143].

In humans, NSAIDs act by inhibiting the cyclooxygenase enzymes COX-1 and/or COX-2, thereby blocking the formation of prostaglandin. The mechanism of NSAIDs action is presumed to be similar also in marine bivalves[152]. The modulation of COX activity and prostaglandin production was observed in *Mytilus galloprovincialis* (1 and 100 μg/L, 3 days) and *Perna perna* (LOEC: 0.2 μg/L for digestive gland and 0.02 μg/L for gills, 4 days) exposed to DCF, as reported in Table 4[75,96,140]. A possible effect of IBU on prostaglandin and COX was suggested by Milan *et al.* in *Ruditapes philippinarum*[144]. The decrease of COX activity was also observed by Maranho *et al.* in the polychaete *Hediste diversicolor* exposed to IBU spiked sediments (LOEC: 0.05 ng/g, 14 days exposure), as reported in Table 5[147]. Even though the role of prostaglandins in invertebrates has not yet been fully elucidated, they apparently participate in numerous biological functions, including development and reproduction, homeostatic cellular balance, ion transport, oogenesis, spermatogenesis, and immune defense. The participation in the noted biological functions could be disrupted under exposure to DCF and IBU[120,130,149,153]. Gonzalez-Rey and Bebianno[140] hypothesized a possible increase in H_2O_2 production as a consequence of a decrease in COX activity and accumulation of arachidonic acid that leads to the alteration of the oxidative state of cells. It is well recognized that NSAIDs can promote the formation of oxygen-reactive species (ROS) and, consequently, the induction of oxidative,

cytotoxic, and genotoxic damages[154].

The presence of ROS in cells implies the activation of antioxidant defense, which involves a group of enzymes that work to inactivate ROS and are widely used as biomarkers of oxidative stress. These enzymes include catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and peroxidase (GPx), as well as reduced (GSH) and oxidized (GSSG) glutathione and ethoxyresorufin-O-deethylase (EROD). Glutathione S-transferase (GST) is also included in this group since it reduces the ROS lipid hydroperoxides to alcohol and H_2O and participates in detoxification processes by conjugation with xenobiotic[154]. Finally, the measure of lipid peroxidation (LPO) is used as a biomarker of cellular oxidative damage. The alteration of enzyme activities and LPO levels after DCF exposure was observed in the mussels *Mytilus edulis*, *Mytilus galloprovincialis*, *Mytilus trossulus*, *Perna perna*, the clams *Ruditapes philippinarum* and *Ruditapes decussatus*, the crab *Carcinus maenas*, and the ragworm *Hediste diversicolor,* as reported in Table 4. In Table 5, we can observe that exposure to IBU also led to changes in antioxidant enzyme activity and lipid peroxidation (LPO) levels in various bivalve mollusk species (*Mytilus galloprovincialis*, *Ruditapes philippinarum*, and *Crassostrea gigas*), as well as in the amphipod *Amphelisca brevicornis* and the worm *Hediste diversicolor*. When the defense system is functioning correctly, we can observe the activation of antioxidant and detoxification enzyme, leading to a consequent reduction of LPO levels (or early LPO induction followed by reduction). This was observed particularly in the digestive gland of various *Mytilus* species exposed to different DCF concentrations (from 0.250 to 1,000 μg/L) and in the gills of *Ruditapes philippinarum* (15 μg/L), as reported in Table 4. These observations imply that the antioxidant defenses effectively counteract the toxic effects induced by DCF. When the antioxidant defenses become overwhelmed, cellular oxidative stress is established, which can lead to the inactivation of enzymes (decreased activity) and cellular damage (indicated by increased LPO levels)[155]. A reduction of enzyme activity was observed particularly in the gills of organisms exposed to DCF (*Mytilus trossulus* and *Perna perna*). However, in these organisms, the decrease in enzyme activity is not followed by oxidative damage. A beneficial action of DCF cannot be excluded, as its anti-inflammatory properties could counteract the inflammation produced in cells by the increase in reactive oxygen species (ROS)[156,157]. The most frequent responses observed in IBU-exposed organisms, regardless of species, tissue, time, and concentration of exposure, were the increase of both enzyme activity and LPO levels. As previously indicated, the persistence of high LPO levels may be a sign of the failure of antioxidant defenses to detoxify the excess ROS production and the production of significant oxidative damage that is usually accompanied by other effects such as DNA damage or protein degradation and cell death. Indeed, DCF and IBU showed the ability to induce genotoxic effects in various invertebrate species, as reported in Tables 4 and 5. An increase in DNA fragmentation was observed in hemocytes of *Mytilus galloprovincialis* exposed in vitro to low DCF concentration (0.005 μg/L). However, in vivo exposure resulted in a more pronounced effect of concentration, depending on species and tissue, with 2.5 μg/L in hemocytes of *Mytilus galloprovincialis*, 2 μg/L in the digestive gland of *Perna perna*, and 1,000 μg/L in the digestive gland of *Mytilus edulis*. IBU also induces enhancement of DNA fragmentation and/or frequency of apoptotic and necrotic cells (micronuclei) at low exposure concentrations in *Mytilus galloprovincialis* (2.5 μg/L in hemocytes) and *Carcinus maenas* (5 μg/L in hepatopancreas and gills and 0.1 μg/L in muscle).

Another biomarker affected by the presence of DCF and IBU in the aquatic medium is acetylcholinesterase (AChE), which is commonly studied as a marker of neurotoxic effects. The most common effect observed in the studies reported in this review is the enhancement of its activity, which can be associated with cell apoptosis, where AChE is released from cells after membrane disruption. This was reported in *Mytilus galloprovincialis*, *Perna perna*, and *Ruditapes philippinarum* after DCF exposure at concentrations of 0.250, 2, and 15 μg DCF/L, respectively, as well as after IBU exposure in *Ruditapes philippinarum* in a

concentration range of 10-1,000 μg IBU/L and *Hediste diversicolor* at a concentration of 500 ng IBU/g, as reported in Tables 4 and 5. The alterations in the structure and functions of the nervous system can have important consequences at different levels of biological organization, including cell development, inflammation, apoptosis, sensory alteration, neuromuscular dysfunctions, paralysis, and death of organisms. However, the neurotoxic effects of DCF and IBU on marine invertebrates, as well as the role of AChE in this group of organisms, are still poorly understood.

Finally, few studies focused on gene expression, protein, and metabolite modulation. Serrano *et al.* observed increased transcription in genes encoding antioxidant and auxiliary enzymes (GST, COX, CAT, heat shock protein, and cytochrome P450) in the oyster *Crassostrea gigas* exposed to IBU (1 and 100 μg/L) for 7 days[100]. IBU exposure at a concentration of 250,000 μg/L for 15 days also induced the modulation of chitinase and GM2 activator genes, which are supposed to have a role in general stress responses, as well as many genes related to the NF-kB pathway, a group of transcription factors involved in cellular responses to stress and the regulation of immune response and inflammation in *Mytilus galloprovincialis*[141]. Proteomic analyses were used to evaluate the effect of DCF on *Mytilus edulis*, revealing possible oxidation and changes in the abundance of proteins belonging to the family of caspases and heat shock proteins, well-known for their involvement in responses to stress conditions[125]. Additionally, changes were observed in proteins GADPH and BRAFLADRAFT-282392[124]. To date, Bonnefille and coauthors[129] are the only ones who have tested the potentially toxic effects of DCF on the metabolic pathways of the Mediterranean mussel (*Mytilus galloprovincialis*). They observed that this compound modulates the metabolism of tyrosine, tryptophan, and prostaglandins at a concentration of 100 μg/L for 7 days. They related these alterations to the potential impairment of biological functions such as byssus formation, osmoregulation, and reproduction. Results from omic analyses are very useful in understanding the mode of action of drugs and the biological and metabolic processes affected by exposure.

The review of available data on DCF and IBU effects on marine invertebrates, particularly at concentrations close to the ranges detected in coastal/estuarine areas and open seawaters, reveals a wide range of responses that depend on factors such as the target organism and tissue, exposure time, and concentration. This produces variable patterns that are sometimes difficult to interpret. In addition, gaps in our knowledge regarding both DCF and IBU mechanisms of action and the biology of marine invertebrates (including biochemical and molecular mechanisms and metabolic pathways) are evident.

RISK ASSESSMENT

The environmental risk assessment of a substance involves different steps to determine whether its occurrence at a certain level can negatively affect the environment. These steps include (i) identifying substances capable of producing adverse effects (hazard identification); (ii) establishing the relationship between dose-response assessment; and (iii) determining the exposure conditions. It is essential to know the exposure concentrations and routes to identify the potential risk.

The Technical Guidance Document on Risk Assessment of the European Commission (EU TGD) describes the evaluation procedure[158]. In summary, the procedure is based on comparing the predicted environmental concentration (PEC) or measured environmental concentration (MEC) with the concentration that does not represent any hazard for exposed organisms (predicted no effect concentration, PNEC). The ratio between these parameters allows to establish a risk quotient (RQ), as given by:

$$RQ = \frac{PEC \vee MEC}{PNEC}$$

The PEC or MEC can be modified depending on the site, season, and physicochemical characteristics of aquatic ecosystems. In summary, these changes produce variations in the PEC or MEC and, consequently, in the RQ.

The PNEC calculation is carried out by using single-test toxicity data. In this laboratory assay, parameters such as EC_{50}, LC_{50} or NOEC are determined using an assessment factor (AF), as given by:

$$PNEC = \frac{NOEC \vee LC_{50} \vee EC_{50}}{AF}$$

If the RQ value exceeds 1, it implies a potential risk. On the contrary, there is no apparent risk if RQ is less than 1.

The critical point is to establish the assessment factor (AF). Different criteria are employed depending on the nature of the toxicity data. Thus, the AF lies in the range of 10-1,000 and its application accounts for the degree of uncertainty when extrapolating from lab toxicity test data for a limited number of species to a "real environment". However, the selection of the AF is very inconsistent among studies, showing marked differences even when a similar toxicity database is used[159]. Durán and Beiras[160] recommended a test battery of five taxonomic groups, including algae, mollusks, crustaceans, echinoderms, and chordates, using toxicity thresholds (LOEC and NOEC) rather than EC_{50} values. They also emphasized prioritizing information originating from sublethal endpoints and sensitive early life stages. Although the most generalized protocol to establish the risk assessment is the TGD of the EU[148]. Other methods, such as the OECD[161], propose similar AF under similar database availability. Hansen[162] proposed using a bioanalytical system as an additional tool to conventional bioassays.

Table 6 shows the RQs collected from several scientific publications for DCF and IBU. The quotients were calculated for specific areas with the highest measured concentration in marine waters from sampled areas (MEC or MMC, except for two studies that used PEC) to represent the "worst case scenario", following the TGD 2003 of the EU[158]. Data for the occurrence of these compounds were measured in seawater ecosystems. However, all PNEC values from the three trophic levels (algae, crustacean, and fish) used for RQ calculations are from freshwater systems. The lack of data on toxicity for marine species has supported the use of freshwater data.

The RQs in coastal seawater generally show insignificant to moderate risk for both NSAIDs, as shown in Table 6. Most of these quotients are calculated for relatively low environmental concentrations (4.3-441 ng/L for DCF and 9.4-53.60 ng/L for IBU), except for the Sea of Marmara and the Cadiz Bay, where higher DCF and IBU concentrations in seawater were detected. These higher concentrations correspond to the areas with the highest risk for aquatic invertebrates[55,62]. It should be noted that an elevated risk is also

Table 6. Summary of RQs calculated for DCF and IBU in coastal seawater and sediment

Drug	Matrix (sampling site)	Target organisms for PNEC data	RQ	AF	Drug concentration used for RQ calculation	Risk classification	Refs
DCF	Seawater (Baltic Sea, Bay of Puck)	*Vibrio fisheri*, *Pseudokirchneriella subcapitata*, *Ceridaphnia dubia*, *Salmo trutta*	3.51	100-1,000	MEC (196 ng/L)	High risk	[40]
	Seawater (Baltic Sea, Germany)	*Synechococcus leopoliensis*	0.0048	10,000	MEC (4.3 ng/L)	Insignificant risk	[163]
	Seawater (China Sea, Taiwan)	*Pseudokirchneriella subcapitata*, *Daphnia magna*, *Danio rerio*	0.004	NR	MMC (53.6 ng/L)	Insignificant risk	[64]
			0.165	NR	PEC (2,230 ng/L)	Moderate risk	
	Seawater (Aegean Sea, Greece)	*Pseudokirchneriella subcapitata*, *Daphnia magna*, *Danio rerio*	0	1,000	MMC (16.3 ng/L)	Insignificant risk	[60]
	Seawater (Atlantic Ocean, Bay of Cadiz)	*Synechococcus leopoliensis*, *Daphnia magna*, *Pimephales promelas*	< 0.01	1,000	MEC (24.3 ng/L)	Insignificant risk	[55]
	Seawater (Atlantic Ocean, Portugal)	Algae, crustacean, and fish (species not reported)	0.0001-10	1,000	MEC (1.14-241)	Insignificant-high risk	[49]
	Seawater (Mediterranean Sea, Tunisia)	*Pseudokirchneriella subcapitata*, *Daphnia magna*,*Pimephales promelas*	< 0.01	1,000	MEC (23 ng/L)	Insignificant risk	[57]
	Seawater (Sea of Marmara, Turkey)	*Pseudokirchneriella subcapitata* , *Daphnia magna*, *Salmo trutta*	0.31-26	10	MEC (1,120 ng/L)	Moderate-high Risk	[62]
	Seawater (Ionian Sea, Italy)	*Synechococcus leopoliensis*, Daphnia *magna, Danio rerio*	< 0.01	1,000	MEC (441 ng/L)	Insignificant risk	[59]
	Sediment (Ionian Sea, Italy)		< 0.01*		MEC (1.1 ng/g dw)	Insignificant risk	
IBU	Seawater (China Sea, Taiwan)	*Vibrio fisheri*, *Daphnia magna*	0.006	NR	MMC (57.1 ng/L)	Insignificant risk	[64]
			2.165	NR	PEC (19,700 ng/L)	High risk	
	Seawater (Atlantic Ocean, Bay of Cadiz)	*Desmodesmus subspicatus*, *Daphnia magna*, *Pimephales promelas*	0.01-0.3	1,000	MEC (1,219.7 ng/L)	Insignificant-moderate risk	[55]
	Seawater (Atlantic Ocean, Portugal)	Algae, crustacean, and fish (species not reported)	< 1	1,000	MEC (1.25-222)	Insignificant-high risk	[49]
	Seawater (Sea of Marmara, Turkey)	*Pseudokirchneriella subcapitata* , *Daphnia magna*, *Pimephales promelas*	0.05-0.43	1,000	MEC (2,130 ng/L)	Low-moderate risk	[62]
	Seawater (China Sea, Xiamen Bay)	NR	0.00143	NR	MEC (9.4 ng/L)	Low risk	[81]

The risk was classified according to the following: RQ > 1 "high risk", 0.1< RQ < 1 "moderate risk", 0.01< RQ <0.1, "low risk", and RQ < 0.01 "insignificant risk"[61]. *RQ for sediment was calculated using $PNEC_{sed}$ derived from $PNEC_{water}$, using the partitioning equilibrium method, according to the equation given by: $PNEC_{sed} = [(PNEC_{water} \times K_p)/d] \times 1000$ where K_p is the sediment-water partition coefficient and *d* is the sediment density, assumed as an average value of 1.70 g/cm^3[59]. DCF: diclofenac; dw: Dry weight; IBU: ibuprofen; MEC: measured environmental concentration; MMC: maximum measured concentration; NR: not reported; PEC: predicted environmental concentration; RQ: risk quotient.

observed at lower concentrations in the Bay of Puck (196 ng/L for DCF) and on the Portuguese coast (222 ng/L for IBU)[40,49]. This is because the RQ values are strongly influenced by the selection of AF, target species (different sensitivity between species), and endpoints (EC_{50}, LC_{50}, and NOEC).

Table 7 compiles the DCF and IBU toxicity thresholds for marine species from different taxonomic groups. We utilized these thresholds to calculate PNEC and corresponding RQ values for the risk assessment of coastal and estuarine environments based on data reported in this review.

As recommended by Beiras[159] and Durán and Beiras[160], we used five taxonomic levels (algae, crustaceans, and fish plus two additional marine taxonomic groups) to enhance the sensitivity and protective values of RQs. Since data for marine fish are unavailable, we combined data sets of marine and freshwater species to derive environmental quality standards for transitional and coastal waters, following the EU TGD[158].

Table 7. Ecotoxicity data for DCF and IBU towards selected marine and freshwater species representing different trophic levels

Drug	Taxon	Species	Endpoint	Toxicity threshold	mg/L	Refs
DCF	Algae	*Dunaliella tertiolecta*	Population growth (96 h)	$EC_{50}/3$	61.9	[164]
		Skeletonema costatum	Population growth (72 h)	$EC_{50}/3$	1.6	[120]
		Isochrysis galbana	Population growth (72 h)	NOEC	> 5	[159]
	Mollusc	*Mytilus trossulus*	Byssus strength (7 d)	LOEC	10	[97]
		Perna perna	Larval development	NOEC	10	[75]
	Crustacean	*Artemia salina*		$EC_{50}/3$	33.3	[165]
		Siriella armata	Neonate mortality (48 h)	$EC_{50}/3$	3.5	[118]
		Tisbe battagliai	Neonate mortality	$EC_{50}/3$	5.3	[120]
			Neonate mortality (48 h)	$EC_{50}/3$	3.2	[119]
		Palaemon serratus	Larval growth (50 d)	LOEC	0.9	[117]
	Echinoderm	*Parocentrotus lividus*	Larval length (48 h)	LOEC	0.0125	[123]
			Early larval growth (48 h)	LOEC	0.0125	[123]
	Fish	*Pimephales promelas*		$EC_{50}/3$	177.3	[166]
		Oryzias latipes	Adult mortality (96 h)	$EC_{50}/3$	3.36	http://cfpub.epa.gov/ecotox
		Danio rerio	Larval abnormalities (80 h)	LOEC	12.5	[159]
IBU	Algae	*Isochrysis galbana*	Population growth (72 h)	EC_{10}	22.6	[159]
		Skeletonema costatum	Population growth (96 h)	$EC_{50}/3$	2.7	[159]
	Mollusc	*Mytilus galloprovincialis*	Larval morphology (48 h)	LOEC	0.1	[127]
		Mytilus trossulus	SFG reduction (14 d)	LOEC	0.1	[98]
	Crustacean	*Tisbe battagliai*	Neonate mortality (48 h)	$EC_{50}/3$	16.6	[119]
	Fish	*Pimephales promelas*		$EC_{50}/3$	1.6	[166]
			Embryo/larval development (33 d)	NOEC	3	[159]
		Oryzias latipes	Larval mortality (96 h)	$EC_{50}/3$	> 33.3	[159]
		Leomis macrochinus	Survival (96 h)	$EC_{50}/3$	57.7	[159]

DCF: diclofenac; EC_{50}: half maximal effective concentration; IBU: ibuprofen; LOEC: low observed effect concentration; NOEC: no observed effect concentration.

Threshold values reported in the bibliography for freshwater fish were used in these cases. Additionally, sublethal endpoints (NOEC and LOEC) were used for calculation. In cases where endpoints were unavailable, $EC_{50}/3$ was used, following the approach suggested by Durán and Beiras[160]. According to the Manual on the Methodological Framework to Derive Environmental Quality Standards (EQS Manual)[167], we used an AF of 10 for PNEC calculation for both DCF and IBU. Regarding MEC, we used data from Table 1. The reported data cover a wide range of concentrations, spanning from a few ng/L to a few μg/L. To cover various contamination levels, we selected a range of values representative of low, medium, and high contamination levels. The RQ values and their corresponding risk classifications are reported in Table 8. We established a safe level for DCF below 100 ng/L. Concentrations of DCF higher than 102 μg/L and 1,500 μg/L present moderate and high risks, respectively, for organisms living in coastal areas, as indicated in Table 8. On the other hand, IBU presents a low to moderate risk at higher environmental concentrations than DCF (ranging from 109 μg/L to 6,297 μg/L, as shown in Table 8), suggesting lower IBU toxicity for organisms living in coastal and estuarine areas.

The highest risk for both NSAIDs is detected in areas previously identified as subject to strong anthropogenic pressure, such as high pharmaceutical consumption, the presence of industrial activities and hospitals, the discharge of effluents from WWTPs, and low removal efficiency from WWTPs. In addition, a higher risk is also observed in the Red Sea, the Humber estuary, Cadiz Bay, and the Antarctic Peninsula.

Table 8. RQ calculation and risk classification for coastal and estuarine environments

Drug	MEC (ng/L)	RQ	Risk classification	Reference for environmental concentrations
DCF	0.02	0.000016	Insignificant	[58]
	102	0.0816	Low	[39]
	205	0.164	Moderate	[41]
	441	0.3528	Moderate	[59]
	1,120	0.896	Moderate	[63]
	1,500	1.2	High	[47]
	10,221.1	81.77	High	[4]
	15,087	12.07	High	[45]
IBU	0.049	0.0000049	Insignificant	[79]
	109	0.0109	Low	[37]
	222	0.0222	Low	[49]
	508	0.0508	Low	[4]
	1,219.7	0.122	Moderate	[55]
	1,600	0.16	Moderate	[47]
	2,550	0.255	Moderate	[59]
	6,297	0.6297	Moderate	[41]
	10,053	1.005	High	[45]

Environmental concentrations (ng/L) from Table 1 were used as MEC. PNEC values used for RQ calculation were 12.5 and 100 μg/L for DCF and IBU, respectively, and they are derived from data in Table 7 using an AF of 10. The risk was classified according to the following: RQ >1 "high risk", 0.1< RQ <1 "moderate risk", 0.01 < RQ < 0.1, "low risk", RQ < 0.01 "insignificant risk"[61]. AF: Assessment factor; DCF: diclofenac; IBU: ibuprofen; MEC: measured environmental concentration; PNEC: predicted no effect concentration; RQ: risk quotient.

The risk classification from our study is consistent with the results from the literature data reported in Table 6, particularly at the highest MEC (e.g., moderate risk for IBU at Cadiz Bay and the Sea of Marmara for DCF). However, differences at the lowest MEC can be related to the AF and endpoint considered for calculation.

The risk for the sampling sites reported in this review ranged between low and high. Specifically, 80% of sites showed low risk for DCF and 83% for IBU, 10% of sites showed moderate risk for DCF and 15% for IBU, and 10% of sites showed high risk for DCF and 2% IBU.

Besides the single-toxicity test, another way to obtain information about safe levels for risk assessment is to use species sensitivity distribution (SSD). The basic premise is to assume that sensitive species can be described using a parametric distribution (e.g., logistic)[168]. This approach was employed by Trombini *et al.* for DCF and IBU using acute toxicity data (LC_{50} or EC_{50} values) from aquatic species, mainly freshwater species[23]. The hazard effect concentration (HEC5) calculated for DCF and IBU were 4.5 and 4.4 mg/L, respectively. Using AF of 5, we calculated the PNEC, which is close to 0.8 mg/L for both compounds. However, this value is higher than some chronic toxicity data for these compounds[168], which fall in the order of g/L, indicating that some precautions should be considered to derive safe levels for aquatic species as they may not be fully protective for the environment. Posthuma and coauthors[168] also highlighted the limitations of this approach.

Yanagihara *et al.* pointed out that the acute SSDs for saltwater species can be estimated using those of freshwater species[169]. However, the number of species considered will be a crucial factor in obtaining more results. The authors also mentioned that the establishment of relationships for chronic toxicity SSD data was not feasible due to the insufficient size of the database available for such an analysis.

Most pollutant-associated risk classifications are based on acute-type responses. However, as discussed in the previous section, at environmentally relevant concentrations, DCF and IBU trigger a wide range of responses at the sub-organism level that can have important consequences on the health status of organisms. These responses are not typically considered by classical environmental risk assessment models, highlighting the need for a more comprehensive approach to assessing the potential ecological impacts of these substances. Mezzelani *et al.* employed a multidisciplinary approach integrating the measurement of drug bioaccumulation with many biomarker responses to assess the perturbation of cellular districts and molecular pathways[26,90]. The observed variations were analyzed using a quantitative model and weighted criteria (Sediqualsoft), which generates a cellular hazard index known as the Hazard Quotient for biomarkers (HQ_{BM})[170,171]. The HQBM takes into account the relevance and magnitude of the measured biomarkers. To calculate this index, each biomarker is assigned a specific "weight" based on its toxicological relevance, and a "threshold" is defined to establish the minimum percentage considered biologically relevant, as given by:

$$HQ_{BM} = \frac{\sum_{j=1}^{N} Effect(j)_{1<Effect(j)\leq 2}}{numbiomark_{1<Effect(j)\leq 2}} + \sum_{k=1}^{M} Effect_W(k)_{Effect(j)>2}$$

The exposure of *Mytilus galloprovincialis* to both NSAIDs showed slight to moderate hazard depending on the exposure concentration. Specifically, a slight hazard for both compounds after 14 days of exposure at 0.5 μg/L, a slight hazard for DCF and a moderate hazard for IBU after 60 days of exposure at 2.5 μg/L, and a moderate hazard for both chemicals have been identified after exposure to 25 μg/L for 14 days[26,90,94].

MIXTURE RISK ASSESSMENT

One of the main challenges of ecotoxicology is assessing the impact of pollutants in the real world, where the substances are not present in individual forms but as complex mixtures. The joint action of a species can be different from the sum of the individual effects, as the interaction between components can produce diverse effects. The main toxicological interactions include synergism, antagonism, potentiation, inhibition, and masking[172,173].

Models have been used to analyze the combined effect of species using data from individual compounds. The classical approach is based on concentration addition (CA) and independent action (IA). The CA model assumes that all mixtures of the component have a similar mode of action and act on the same biochemical pathways and target sites. The mixture toxicity is expressed as:

$$ECx_{mix} = \left(\sum_{i}^{n} \frac{p_i}{EC_{xi}} \right)^{-1}$$

where n is the number of compounds, ECx_{mix} is the effect of concentration of the mixture provoking an x% effect and EC_{xi} is the concentration of component i provoking the same effect (x%) as the mixture when

applied individually, and p_i is the fraction of component i in the mixture.

The IA model assumes that the chemical model of action shows dissimilarity in the interaction with molecules and target sites. As a consequence, the relative effect of a compound in the mixture can be unaffected by the occurrence of other compounds. In this model, the global toxicity can be related only to specific compounds in the mixture. The equation applies for the mixture toxicity is expressed as:

$$E(C_{mix}) = 1 - \prod_{i=1}^{n} \left(1 - E(C_i)\right)$$

where $E(C_{mix})$ is the effect of the total concentration in the mixture and $E(C_i)$ is the effect generated by component i at the concentration C_i.

The CA model has been employed for the mixture of different chemical compounds[174-179]. Although the classical approaches have been employed widely due to their simplicity and usefulness for regulators, the results are subjected to some bias. Thus, the CA model overestimates the toxicity, and the IA model underestimates it[119]. Nonetheless, in the real world, the predictive power of both models will decrease due to the complexity of biological systems and the specificity of the pathways of chemical mixtures. Other alternative methods have been developed to overcome the limitations of CA and IA models. Among alternative methods, two-stage prediction (TSP), integrated fuzzy concentration addition-independent action model (INFCIM), toxic equivalency factors (TEF), mixture toxicity indices (MTI), median effect/combination index (CI-isobologram equation), and quantitative structure-activity relationship (QSAR) can be cited.

The risk associated with the mixtures can be assessed using the RQ. According to Backaus and Faust[180], the RQs of mixtures can be calculated assuming the individual ratios PEC/PNEC ratios as follows:

$$RQ_{PEC/PNEC} = \sum_{i=1}^{n} \frac{PEC_i \vee MEC_i}{PNEC_i}$$

The risk can be calculated for a specific organism using data from different experiments or, in a more general way, using data from different trophic levels according to the following equation:

$$RQ = \sum_{i=1}^{n} \frac{PEC_i \vee MEC_i}{min \frac{(EC_{50} differenttropiclevels)*1}{AF}}$$

Information about the mixture toxicity of pharmaceuticals is very scarce. This is especially remarkable in the marine environment. However, the mixture toxicity for DCF and IBU was assessed in the marine copepod *Tisbebattagliai* by Trombini *et al.*[119]. The study was carried out on neonate nauplii (< 24 h old) exposed for 48 h to both pharmaceutical compounds, following the UK Environment Agency protocol[181]. The toxicity of both compounds expressed as LC_{50} has been reported to be 9.5 and 49.7 mg/L, respectively, indicating higher toxicity for DCF. According to the EU Directive 93/67/EEC[148], individual compounds can be identified as toxic and harmful. Nevertheless, the occurrence of the compounds in the environment is a mixture of components, and the estimation based on individual components can underestimate the risk. The species *Tisbe battagliai* showed a low risk of exposure to individual or mixture compounds[119]. However, the mixture risk was higher than individual compounds. This observation agrees with the CA model that assumes that compounds with similar modes of action increase the overall toxicity of the mixture since DCF and IBU act on the same biochemical pathways and target molecules. However, these authors questioned the accuracy of the classical models (CA and IA) in assessing the risk of the occurrence of these drugs in real scenarios at low concentrations.

Similar to the risk assessment of individual compounds, the models used for assessing the risk of contaminant mixtures are primarily based on acute toxicity data. However, there is a lack of studies focusing on the sublethal effects of mixtures, especially involving DCF and IBU, on marine invertebrates. In a study conducted by Gonzalez-Rey and coauthors[182], they investigated the effects of two mixtures, DCF and IBU (250 ng/L each), in combination with fluoxetine (75 ng/L) and copper (5 µg/L), on the mussel species *Mytilus galloprovincialis*. The researchers observed notable changes in biochemical responses and gene expression related to oxidative stress, neurotoxicity, and endocrine disruption. Importantly, these alterations differed from the effects observed in a single compound exposure. Biochemical alterations (SOD, CAT, LPO, MT, and AChE) were also observed in the clam *Scrobicularia plana* exposed to a mixture of IBU, ciprofloxacin, and flumequine during a 21-day period, as reported by Trombini[183]. Similarly, Ericson *et al.* reported a decrease in the scope for growth and in the ability to attach to the substrate in the mussel species *Mytilus edulis* when exposed to a combination of DCF, IBU, and propanolol at concentrations ranging from 1-10,000 µg/L[97]. Fabbri *et al.* conducted a study on the effects of a complex mixture, which included IBU and DCF at concentrations ranging from 0.01 to 1 µg/L, with a 48-hour exposure on the embryos of *Mytilus galloprovincialis*[184]. The researchers observed a retarded development and shell malformations in the exposed embryos. Additionally, Luna-Acosta *et al.* reported that a mixture of IBU and the herbicides diuron and isoproturon (at a concentration of 5 µg/L) induced early effects at both the biochemical level (resulting in a decrease of catecholase-type phenoloxidase activity in the plasma) and the cellular level (leading to a decrease of phagocytosis activity in hemolymph) in the oyster species *Crassostrea gigas* after a 6-hour exposure[185].

In toxicological studies, replicating a completely realistic scenario is challenging due to the complexity and variability of contaminant mixtures in the marine environment, which may include pharmaceuticals, metals, micro and nanoplastics, pesticides, and more. Additionally, the toxicity of these mixtures can be

influenced by changes in physicochemical variables. Therefore, some authors have investigated the combined effects of DCF and changes in water pH, temperature, and salinity, which are alterations typically associated with climate change. Munari *et al.* showed that seawater acidification increases the sensitivity of the larvae of *Ruditapes philippinarum* to DCF, altering survival (increased mortality) and growth (increased morphological abnormalities)[153]. Similarly, González-Ortegón *et al.* reported an increased DCF toxicity in larvae of the marine shrimp *Palaemon serratus* when exposed to DCF in combination with changes in temperature and salinity[117]. However, other studies carried out with bivalve mollusks (*Mytilus* and *Ruditapes* species) did not show clear synergistic effects between DCF and changes in temperature, pH, or salinity[92,93,99,186].

NEW APPROACHES (AOPS)

The development of a new approach for assessing toxicity, which takes into account the underlying mechanisms of the toxicity process, has received great attention. This is because the classical approach relies on assessment factors that often lack ecological relevance and uncertainty. Additionally, there are economic and practical difficulties in applying it to the current chemical products in the market and the new products generated by the chemical industry. The World Health Organization (WHO), through the International Program on Chemical Safety (IPCS), is taking steps to develop a scientific foundation for chemical management that reduces the reliance on animal testing. They aim to generate risk identification tools by utilizing new technologies capable of providing high-throughput biological data at lower organizational levels (ecotoxicogenomic) while harnessing the increasing computational power capacity for data processing and prediction using Big Data and Artificial Intelligence (AI).

The Adverse Outcome Pathway (AOP) is a conceptual framework[187] that depicts the sequence of logical events or processes in a biological system. It considers understanding adverse effects and refining current risk assessment. The final objective is to develop predictive models for human and environmental toxicology.

The core principle of AOP is that exposure to chemical substances at a certain dose can trigger a molecular initiating event (MIE) (e.g., receptor binding), leading to a cascade of key events (KE), which can ultimately result in a pathological effect that is considered an adverse outcome. AOP represents the sequence of events from the exposure of an individual to a chemical, leading to an understanding of the adverse effect at the population level. The link between chemicals and MIE represents a "small jump" concerning toxicological endpoints. This approach establishes stronger connections to identify the mechanisms that underlie the toxic effect and can generate a more generalized and predictive model for aquatic species. AOPwiki (https://aopwiki.org) is a database whose objective is to be the repository of all AOPs developed as a part of the OECD AOP Development Effort, supported by the Extended Advisory Group on Molecular Screening and Toxicogenomics. Currently, there is no complete view of the MIEs and KEs and the concentration that triggers these events.

The use of omic approaches to assess the effect of pollutants on marine organisms has shown a high potential in understanding the response mechanisms without the need for any "a priori" hypothesis. Many pollutants reach the marine environment through the discharge of WWTPs, carrying a complex mixture of pollutants, posing a potential threat to the health of marine ecosystems. Environmental metabolomics has been shown to be an efficient tool for assessing the effect of multi-contamination in marine organisms[188]. It has been reported that this exposure led to alterations in a large number of metabolites, including those belonging to amino acid metabolism, neurohormones, purine and pyridine metabolism, as well as citric acid cycle intermediates and oxidative stress defenses[188]. These changes reflect the alteration of biological

processes. Nevertheless, the use of a metabolomics approach to assess the exposure of marine organisms to NSAIDs is very scarce. Bonnefille *et al.* identified that the DCF effects on the Mediterranean mussel (*Mytilus galloprovincialis*) were related to two main metabolomics pathways: tyrosine, mostly down-regulated and tryptophan metabolism, mostly up-regulated[129]. Furthermore, the study suggested a potential impairment of mussel osmoregulation and reproduction due to exposure to DCF. Through a transcriptomic approach, a similar mode of action of IBU was revealed in mussels (*Mytilus galloprovincialis*) and humans. The study found similarities in the expression of genes associated with the Nuclear Factor kappa B (NF-kB) pathway, which is involved in immune and inflammation responses. Almeida *et al.* reported that the effects of the selected NSAIDs revealed a non-consistent oxidative challenge, supporting that the prooxidant mechanisms do not represent the primary mode of action of these pharmaceuticals[149].

The metabolomics approach has been shown to be useful in defining adverse outcome pathways for pharmaceuticals.

CONCLUSIONS

The risk assessment of individual compounds for aquatic species in the estuaries and marine environment involves analyzing the ratio between ecotoxicology information and the environmental concentrations. Currently, the presence of pharmaceutical compounds, specifically non-steroidal anti-inflammatory drugs (IBU and DCF), has increased in the marine environment. To evaluate the risk linked to these compounds, we have compiled data from the scientific literature, encompassing a diverse range of environmental concentrations and biological responses (such as bioaccumulation rates and effects of various levels of biological organization). These variations are contingent on location, environmental conditions, and the species under consideration. This suggests that the risk associated with the presence of these two drugs can vary depending on the species, site, and exposure concentrations being considered. Diclofenac showed a higher risk than ibuprofen for aquatic species. The risk for both compounds was generally low, encompassing approximately 80% of the reported sites, while a reduced number of sites showed high risk, ranging from 2% to 10%. Due to the concurrent presence of both compounds in the marine environment, with similar primary sources (e.g., wastewater), the risk of the mixture of DCF and IBU exhibited a higher risk compared to individual compounds. In addition to the conventional methods for risk assessment, alternative approaches have been considered, such as the use of adverse outcome pathways and toxicogenomic tools (including omics techniques such as transcriptomic, proteomic, and metabolomics analyses) to gain insights into the underlying mechanisms of toxicity. However, taking the research a step further will necessitate expanding the knowledge base on various species and employing tools such as Big Data and artificial intelligence. Additionally, the marine ecosystem is a dynamic environment influenced by numerous drivers and pressures (e.g., ocean acidification, heat waves, climate change, *etc.*) that can impact the fate, behavior, and physiological responses of organisms to these compounds. As a result, evaluating the risk of NSAIDs in the marine environment presents challenges, as it will require addressing the information gaps through further research and data collection.

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Authors' contributions

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