UNIWERSYTET GDAŃSKI
WYDZIAŁ OCEANOGRAFII I GEOGRAFII

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Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego

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Environmental fate and toxicity evaluation of selected pharmaceuticals and their metabolites using *Mytilus trossulus* as a model species

Rozprawa doktorska wykonana pod opieką

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PODZIĘKOWANIA

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SPIS TREŚCI

STRESZCZENIE

Liczne badania z ostatnich lat jednoznacznie wskazują, że pozostałości leków przedostające się do środowiska wodnego mogą stanowić poważne zagrożenie dla fauny i flory. Wśród farmaceutyków powszechnie wykrywanych w zbiornikach wodnych, wyróżnić należy zwłaszcza niesteroidowe leki przeciwzapalne (NLPZ), których głównym przedstawicielem jest diklofenak. Oprócz związków macierzystych, zagrożenie dla środowiska mogą stanowić także produkty transformacji farmaceutyków, jednak w tym przypadku skala problemu jest dopiero szacowana.

Głównym celem niniejszej pracy była ocena wpływu diklofenaku oraz jego głównego produktu transformacji, 4-OH diklofenaku, na bezkręgowce morskie, z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego. Aby tego dokonać, podczas badań wykorzystano markery z różnych poziomów organizacji biologicznej obrazujące fizjologiczne i biochemiczne efekty oddziaływania obu związków. Kolejnym celem było oszacowanie współczynnika biokoncentracji diklofenaku i jego metabolitu w tkankach *M. trossulus*. Oprócz wyznaczenia zdolności do biokoncentracji obu związków, podczas przeprowadzonych badań eksperymentalnych analizowano kinetykę absorpcji testowanych związków oraz wyznaczono bilans masy biorąc pod uwagę stężenia analitów zarówno w tkankach małży, wodzie, a także biofilmie. Dodatkowym celem niniejszej pracy było określenie stabilności obu testowanych związków w długoterminowym teście symulującym warunki w strefie przydennej Morza Bałtyckiego. Do celów niniejszej rozprawy należało także zebranie dostępnych danych literaturowych dotyczących zanieczyszczeń farmaceutycznych, w tym NLPZ, w środowisku wodnym, a w szczególności ich stężeń notowanych w poszczególnych komponentach ekosystemów oraz ich toksycznego oddziaływania na organizmy żywe.

Badania przeprowadzone w ramach niniejszej pracy potwierdziły, że zarówno diklofenak, jak i 4-OH diklofenak są pobierane przez małże *M. trossulus* i gromadzone w ich tkankach. Jednakże uzyskane wartości współczynnika biokoncentracji (BCF) wskazują, że proces ten zachodzi w niewielkim stopniu. Co więcej, dzięki uzyskanym wynikom potwierdzono, że związki te mogą być metabolizowane, a następnie wydalane przez małże. Wykazano również, że diklofenak i 4-OH diklofenak mają toksyczne działanie na małże *M. trossulus*, co w przypadku diklofenaku może być obserwowane już na poziomie stężeń regularnie notowanych w środowisku morskim. Przeprowadzone analizy potwierdziły, że markery histologiczne mogą stanowić przydatne narzędzie w ocenie wpływu farmaceutyków na organizmy wodne. Wykazano także, że diklofenak cechuje się znacznie wyższą stabilnością w przydennej wodzie morskiej i w obecności osadu niż jego metabolit, 4-OH diklofenak, oraz ulega biodegradacji w osadzie do 5-OH diklofenaku.

Słowa kluczowe:
Związki farmaceutyczne, NLPZ, diklofenak, metabolity, toksyczność, biokoncentracji, omułki

ABSTRACT

Numerous studies in recent years have clearly indicated that drug residues entering the aquatic environment can pose a serious threat to biota. Among the pharmaceuticals that are commonly found in water bodies, non-steroidal anti-inflammatory drugs (NSAIDs) are of particular note, with diclofenac being the most important representative. In addition to parent compounds, pharmaceutical transformation products may also pose a threat to the environment, but in this case the scale of the problem is yet to be estimated.

The main objective of the present study was to evaluate the effects of diclofenac and its main transformation product, 4-OH diclofenac, on marine invertebrates, using *Mytilus trossulus* as a model species. To accomplish this, markers from different levels of biological organization depicting the physiological and biochemical effects of the two compounds were used during the study. Another objective was to estimate the bioconcentration factor of diclofenac and its metabolite in *M. trossulus* tissues. In addition to determining the bioconcentration capacity of the two compounds, the absorption kinetics of the tested compounds were analyzed during the experimental studies and the mass balance was determined taking into account the concentrations of the analytes in both the mussel tissues, water, and biofilm. An additional objective of the present work was to determine the stability of both tested compounds in a long-term test simulating conditions present in the bottom zone of the Baltic Sea. The purpose of this thesis was also to collect available literature data on pharmaceutical contaminants, including NSAIDs, in the aquatic environment, in particular, their concentrations recorded in various components of ecosystems and their toxic effects on living organisms.

The studies conducted in this work confirmed that both diclofenac and 4-OH diclofenac are taken up by *M. trossulus* mussels and accumulated in their tissues. However, the bioconcentration factor (BCF) values obtained indicate that this process occurs to a small extent. Moreover, thanks to the results obtained, it was confirmed that these compounds can be metabolized and then excreted by the mussels. It was also shown that diclofenac and 4-OH diclofenac have toxic effects on *M. trossulus*, which in the case of diclofenac can be observed already at concentrations regularly recorded in the marine environment. The analyses conducted confirmed that histological markers can be a useful tool in assessing the effects of pharmaceuticals on aquatic organisms. Diclofenac was also shown to have significantly higher stability in bottom seawater in the presence of sediment than its metabolite, 4-OH diclofenac, and to biodegrade in sediment to 5-OH diclofenac.

Keywords:
Pharmaceutical compounds, NSAIDs, diclofenac, metabolites, toxicity, bioconcentration, mussels

1. WSTĘP

Farmaceutyki to związki aktywne biologicznie które znajdują zastosowanie m.in. w medycynie oraz weterynarii (Li, 2014). Dzięki swoim właściwościom są one wykorzystywane do leczenia szeregu dolegliwości zdrowotnych u ludzi oraz zwierząt (Fent i in., 2006; Boxall, 2004; Klatte i in., 2017). Środki farmaceutyczne są obecnie zaliczane do tak zwanych „nowo pojawiających się zanieczyszczeń" (eng. *new emerging pollutants*). Produkcja oraz konsumpcja leków wzrastają w niezwykle szybkim tempie, a trend ten jest obserwowany na całym świecie. Jest to związane m. in. z ciągłym wzrostem liczby ludności oraz łatwą dostępnością farmaceutyków, zwłaszcza tych sprzedawanych bez recepty, co z kolei często prowadzi do ich błędnego lub nadmiernego wykorzystywania (Caban i Stepnowski, 2021; Nikolaou i in., 2007; Li, 2014). Istotny problem stanowi także ograniczona świadomość społeczeństwa na temat właściwych sposobów utylizacji leków, co skutkuje ich powszechnym deponowaniem w kanalizacji (Gotz i Keil, 2007; Constantino i in., 2020; Zorpas i in., 2018). Ostatecznie znaczna ilość farmaceutyków stosowanych w gospodarstwach domowych przedostaje się do środowiska, ponieważ ich usuwanie w oczyszczalniach ścieków zachodzi w bardzo ograniczonym stopniu (Zhang i in., 2008; Nikolaou i in., 2007; Lonappan i in., 2016; Li, 2014). Znaczącym źródłem leków w środowisku pozostaje także masowa hodowla zwierząt, w ramach której wciąż powszechną praktykę stanowi nadużywanie środków przeciwdrobnoustrojowych, a także przeciwbólowych i przeciwzapalnych (Low i in., 2021; Kołodziejska i in., 2013; Wagner i in., 2021). Pozostałe źródła farmaceutyków to akwakultura, składowiska śmieci z przemysłu wytwórczego i farmaceutycznego oraz wysypiska śmieci (Nikolaou i in., 2007).

Farmaceutyki są dostarczane do środowiska wodnego w sposób ciągły (ang. *pseudo-persistent compounds*), a ich stężenie jest stale odnawiane. Biorąc pod uwagę ich szerokie spektrum aktywności biologicznej, mogą one negatywnie wpływać na organizmy wodne (Fent, 2007; Brausch i in., 2012; Li, 2014). Co więcej, związki te po przedostaniu się do środowiska wodnego ulegają procesom transformacji pod wpływem czynników biotycznych (bakterie i inne organizmy) oraz abiotycznych (fotoliza, hydroliza), w wyniku których powstają pochodne, które mogą w inny sposób oddziaływać na organizmy (Maculewicz i in., 2022; Nikolaou i in., 2007). Leki mogą także ulegać akumulacji w tkankach organizmów lub osadach (Nikolaou i in., 2007; Świacka i in., 2019; Li, 2014). Los farmaceutyków w środowisku wodnym jest jednak wciąż słabo poznany.

Jak wynika z danych literaturowych zebranych w pracach **przeglądowych numer 3 i 5**, do najczęściej stosowanych i tym samym wykrywanych w środowisku wodnym grup leków należą: antybiotyki, antydepresanty, beta-blokery, leki hormonalne, a także niesteroidowe leki przeciwzapalne (NLPZ) (Nikolaou i in., 2007; Fent i in., 2006). Według niektórych źródeł NLPZ mają największy udział spośród wszystkich grup farmaceutyków wykrywanych w środowisku (Nikolaou i in., 2007; Fent i in., 2006).

1.1. Obecność diklofenaku i jego metabolitów w środowisku wodnym oraz w tkankach organizmów

Spośród związków farmaceutycznych najczęściej wykrywanych w wodach powierzchniowych na całym świecie, a w związku z tym rozpatrywanych jako potencjalne zagrożenie dla środowiska należy wymienić diklofenak (Nikolaou i in., 2007; Fent i in., 2006; Lonappan i in., 2016). Związek ten, należący do NLPZ, jest stosowany do zwalczania bólu o umiarkowanym nasileniu oraz stanów zapalnych (Kołodziejska i Kołodziejczyk, 2018; Zhang i in., 2014; Lonappan i in., 2016). W wielu krajach jest on powszechnie dostępny bez recepty, co sprzyja jego znaczącej konsumpcji (Asukai i in., 2020).

Zgodnie z pracą Acuña i in. (2015) światowe zużycie diklofenaku w latach 2010-2013 wynosiło średnio 1443 tony rocznie. Należy jednak podkreślić, że dokładne liczby są trudne do oszacowania ze względu na mnogość wyrobów zawierających ten związek. Dodatkowo biorąc pod uwagę, że jest on wykorzystywany także w weterynarii, można założyć, że jego światowa konsumpcja jest obecnie jeszcze wyższa (Lonappan i in., 2016).

Ogromna konsumpcja diklofenaku ma bezpośrednie przełożenie na jego powszechną obecność w środowisku wodnym na całym świecie. Analiza 133 oryginalnych prac przeprowadzona przez Sathishkumar i in. (2020) wskazała, że związek ten należy do 5 najczęściej wykrywanych leków w różnych matrycach środowiskowych. Co szczególnie niepokojące, śladowe ilości diklofenaku oznaczane są nawet w próbkach wody pitnej, gdzie jego stężenie może wynosić do kilkudziesięciu ng/l (Carmona i in., 2014; Simazaki i in., 2015; Rodil i in., 2012).

Największa zawartość diklofenaku notowana jest jednak w wodach powierzchniowych, do których trafiają zanieczyszczenia zarówno z oczyszczalni ścieków, zakładów produkcyjnych, szpitali czy pól uprawnych (Reis-Santos i in., 2018; Ashfaq i in., 2017; Nikolau i in., 2007). Stężenie diklofenaku może osiągać od kilkuset ng/l do nawet kilkudziesięciu µg/l (Olaitan i in., 2014; Rivera-Jaimes i in., 2018). Ostatecznie związek ten przedostaje się do mórz i oceanów, gdzie jest regularnie notowany w wodach przybrzeżnych Bałtyku (Nödler i in., 2014; Borecka i in., 2015), Morza Śródziemnego (Togola i Budzinski, 2008; Nödler i in., 2014; Alygizakis i in., 2016; Brumovský i in., 2017), Morza Czerwonego (Ali i in., 2017) i wielu innych akwenów (Pereira i in., 2016; Kallenborn i in., 2018; Biel-Maeso i in., 2018; Gonzalez-Alonso i in., 2017). Diklofenak jest również coraz częściej oznaczany w osadzie rzecznym i morskim. W pracy Pintado-Herrera i in. (2013) stężenia diklofenaku w osadzie pobranym z Zatoki Kadyksu (Hiszpania) osiągały wartość do 10 ng/g suchej masy. W pracy Stewart i in. (2014) z kolei wykryto diklofenak o stężeniu do 2,5 ng/g suchej masy w osadzie pobranym z estuariów w pobliżu Auckland u wybrzeża Nowej Zelandii. Wciąż jednak niewiele wiadomo o losie tego związku w strefie przydennej środowiska morskiego, jaka część leku ulega akumulacji w osadach oraz czy może on ulegać biodegradacji w tej matrycy.

Rozpatrując obecność diklofenaku w środowisku, oprócz macierzystego związku należy mieć na uwadze także jego produkty transformacji. Do produktów transformacji zaliczane są zarówno metabolity, czyli pochodne powstające w wyniku procesów biochemicznych zachodzących w organizmach poddanych leczeniu, ale także produkty przemian biotycznych i abiotycznych mających miejsce bezpośrednio w środowisku (Maculewicz i in., 2022). Chociaż problem ten przez długi czas pozostawał w cieniu, obecnie wiadomo, że produkty transformacji leków mogą osiągać podobne, a w niektórych przypadkach nawet wyższe stężenia niż związki

macierzyste (Klein i in., 2021; Langford i Thomas, 2011; Martinez i in., 2016). Głównym produktem transformacji diklofenaku notowanym w środowisku jest 4-hydroksy (4-OH) diklofenak (Stülten i in., 2008; Scheurell i in., 2009; Osorio i in., 2014; Schmidt i in., 2018). Pochodna ta jest zarówno metabolitem wydalanym przez ludzi, jak i produktem powstającym w ramach procesów zachodzących już w środowisku (Li i in., 2014). Schmidt i in. (2018) oznaczyli 4-OH diklofenak w wodach jednego z Berlińskich kanałów na poziomie od 0,26 do 0,53 µg/l. Podobne stężenia tego związku wykryto także w rzekach oraz lagunach Karachi (Pakistan) (Scheurell i in., 2009). W pracy Hiba i in. (2021) z kolei wykryto 4-OH diklofenak w osadzie pochodzącym z francuskich rzek w pobliżu aglomeracji o średnim stężeniu 103 ± 107 ng/g. Co niepokojące, oznaczone średnie stężenie metabolitu w osadzie rzecznym było znacznie wyższe niż dla diklofenaku.

Doniesienia naukowe z ostatnich lat wielokrotnie potwierdzały obecność diklofenaku w tkankach organizmów, zarówno słodkowodnych, jak i morskich (Álvarez-Muñoz i in., 2015; Moreno-González i in., 2016). Diklofenak oznaczano w organizmach należących do różnych grup taksonomicznych oraz poziomów troficznych - zarówno autotroficznych mikro i makroglonach (Álvarez-Muñoz i in., 2015), skorupiakach (Wilkinson i in., 2018), mięczakach (Mezzelani i in., 2016; Cunha i in., 2017) i rybach (Omar i in., 2019). Zgodnie z badaniami Bean i in. (2017), stężenie diklofenaku w tkankach słodkowodnych ryb zamieszkujących najbardziej zanieczyszczone akweny znajdujące się w pobliżu dużych aglomeracji miejskich (rzeka i zbiornik Delaware, Stany Zjednoczone) może osiągać wartość powyżej 10 µg/g suchej masy. Z kolei w przypadku organizmów morskich, dotychczas maksymalne stężenie tego związku zostało zanotowane w tkankach małży i wyniosło 171,1 ng/g suchej masy (Mezzelani i in., 2020).

Obecność diklofenaku oraz innych leków w środowisku wodnym, ze szczególnym uwzględnieniem organizmów została omówiona w **publikacji 5**.

1.2. Wpływ diklofenaku i jego metabolitów na organizmy wodne

Rozpatrując toksyczny wpływ diklofenaku na organizmy wodne należy pamiętać, że obserwowane efekty mogą różnić się diametralnie pomiędzy poszczególnymi grupami taksonomicznymi. Oddziaływanie farmaceutyków na faunę i florę morską może być odzwierciedlone na różnych poziomach organizacji biologicznej, od poziomu molekularnego aż po zaburzenia homeostazy czy też zmiany o charakterze ogólnoustrojowym. Oprócz zależności od dawki i czasu ekspozycji, działanie diklofenaku w środowisku może być wzmacniane poprzez obecność innych zanieczyszczeń, w tym pozostałych farmaceutyków z grupy NLPZ o działaniu synergistycznym (Ericson i in., 2010).

Spośród organizmów wodnych dotychczas najlepiej poznano wpływ diklofenaku na ryby. Stężenia na poziomach regularnie notowanych w środowisku mogą powodować u tej grupy organizmów zaburzenia procesów metabolicznych, co wykazano poprzez analizy markerów stresu oksydacyjnego, takich jak glutation, transferaza glutationowa czy peroksydaza lipidowa (Nunez i in., 2020; Stepanova i in., 2013). W wielu pracach zaobserwowano także indukowane przez diklofenak zmiany związane z gospodarką hormonalną (Ji i in., 2013; Gröner i in., 2015). Ekspozycja na diklofenak spowodowała także powstanie zmian w skrzelach, wątrobie i nerkach u karpia i troci (Derakhsh i in., 2020; Schwarz i in., 2017). W kilku pracach z wykorzystaniem danio pręgowanego odnotowano także wystąpienie efektów teratogennych, takich jak

zaburzenia pracy serca (Zhang i in., 2020), a także wystąpienie deformacji u larw (Chen i in., 2014). Z kolei zaburzenia behawioralne powodowane przez diklofenak mogą obejmować wzrost agresji, ale również liczne zmiany w procesach lokomotorycznych u troci, danio pręgowanego i suma afrykańskiego (Schwarz i in., 2017; Xia i in., 2017; Ajima i in., 2015). Wiele podobnych efektów działania diklofenaku obserwowano także wśród małży (Parolini i in., 2011; Boisseaux i in., 2017; Fontes i in., 2018), skorupiaków (Lee i in., 2011; Nieto i in., 2013; Gomez-Olivan i in., 2014), wieloszczetów (Nunes i in., 2020; Zanuri i in., 2017) i innych organizmów wodnych (Zanuri i in., 2017; Sarma i in., 2014), dla których jednak dane są wciąż bardziej ograniczone i wybiórcze.

Bardzo słabo poznanym zagadnieniem pozostaje wpływ metabolitów diklofenaku na organizmy wodne. Badania związane z toksycznością produktów transformacji stanowią nowy, dopiero wyłaniający się trend w ekotoksykologii farmaceutyków (Maculewicz i in., 2022). Dotychczas toksyczny wpływ 4-OH diklofenaku na organizmy wodne zanotowano zaledwie w jednej pracy (Grabarczyk i in., 2020). Obserwacje dotyczyły jednak wyłącznie prostych wskaźników jak śmiertelność, inhibicja wzrostu i luminescencji związanych z toksycznością ostrą, która okazała się być niższa niż w przypadku związku macierzystego.

Zagadnienia związane z toksycznym wpływem diklofenaku, a także innych NLPZ i ich metabolitów na organizmy wodne zostały szczegółowo przedyskutowane w **publikacji 3**.

1.3. Małże z rodziny Mytilidae jako modelowe organizmy w ekotoksykologii farmaceutyków

Wśród modelowych organizmów wykorzystywanych w badaniach ekotoksykologicznych, małże posiadają szereg cech sprzyjających ich skutecznemu zastosowaniu zarówno w analizach środowiskowych, jak i laboratoryjnych (Boillot i in., 2015; Capolupo i in., 2017; Maranho i in., 2015; Morales-Caselles i in., 2008; Moschino i in., 2011; Orbea i in., 2006). Jako zwierzęta o osiadłym trybie życia, dają pewność, że ich stan fizjologiczny odzwierciedla stan środowiska w miejscu, z którego zostały pobrane. Odżywiają się głównie poprzez filtrację, co sprawia, że są zdolne do biokoncentracji, w wyniku czego stężenie zanieczyszczeń w ich tkankach może osiągać wielokrotnie wyższe wartości niż w otoczeniu. Według niektórych źródeł, małże wykazują niską zdolność do metabolizowania zanieczyszczeń organicznych w porównaniu z rybami i skorupiakami (Farrington i in., 2016; Dailianis, 2011). Poziomy aktywności enzymatycznej związanej z detoksykacją tych zanieczyszczeń, w tym związków farmaceutycznych, w tkankach omułków są znacznie niższe niż w przypadku innych organizmów, co sprzyja ich bioakumulacji (Farrington i in., 2016; Dailianis, 2011).

Spośród małży stosowanych w badaniach ekotoksykologicznych, szczególną uwagę należy zwrócić na organizmy należące do rodziny Mytilidae. Ich powszechna obecność w strefach przybrzeżnych mórz i oceanów całego świata umożliwia prowadzenie badań porównawczych z wykorzystaniem gatunków żyjących w różnych obszarach geograficznych (Beyer i in., 2017). Sprzyja temu także dobrze poznana fizjologia wielu przedstawicieli rodziny Mytilidae (Bao i in., 2018; Cappello i in., 2018; Hoher i in., 2015; Lacroix i in., 2017). Co więcej, organizmy te żyją zazwyczaj w dużych skupiskach, co umożliwia łatwy zbiór materiału do badań środowiskowych (Cuevas i in., 2015; Dailianis, 2011; Gosling, 2008). Jako łatwe w hodowli i stosunkowo odporne na zmianę warunków, dobrze sprawdzają się także w pracach laboratoryjnych (Mezellani i in., 2016).

Zastosowanie małży z rodziny Mytilidae w badaniach ekotoksykologicznych związanych z wpływem farmaceutyków na środowisko morskie zostało szczegółowo przedyskutowane w **publikacji 1**.

2. CELE PRACY

Do celów niniejszej rozprawy należało zebranie dostępnych danych literaturowych dotyczących zanieczyszczeń farmaceutycznych, w tym NLPZ, w środowisku wodnym, a w szczególności ich stężeń w biotycznych i abiotycznych elementach ekosystemów, oraz ich toksyczności na organizmy żywe. Głównym celem niniejszej pracy była ocena wpływu diklofenaku oraz jego głównego produktu transformacji, 4-OH diklofenaku, na bezkręgowce morskie, z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego.

Do szczegółowych celów pracy zaliczono:

1. Wyznaczenie gatunku modelowego do prowadzonych badań **(publikacja 1)**
2. Ocena toksyczności diklofenaku i jego metabolitu, 4-OH diklofenaku, na organizmy nietargetowe z wykorzystaniem *M. trossulus* jako gatunku modelowego **(publikacje 2 i 6)**
3. Zebranie danych literaturowych dotyczących toksyczności NLPZ, w tym diklofenaku, z perspektywy ekosystemów wodnych **(publikacja 3)**
4. Ocena stopnia biokoncentracji i metabolizmu diklofenaku i 4-OH diklofenaku w tkankach bezkręgowców morskich **(publikacja 4)**
5. Zebranie danych literaturowych dotyczących akumulacji zanieczyszczeń farmaceutycznych i ich metabolitów w dziko żyjących organizmach wodnych **(publikacja 5)**
6. Określenie stabilności diklofenaku i 4-OH diklofenaku w długoterminowym teście symulującym warunki w strefie przydennej Morza Bałtyckiego **(publikacja 7)**

PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY DOKTORSKIEJ

Publikacja 1

Świacka, K., Maculewicz, J., Smolarz, K., Szaniawska, A., & Caban, M. (2019). Mytilidae as model organisms in the marine ecotoxicology of pharmaceuticals-a review. Environmental Pollution, 254, 113082.

IF: 6,793; 5-letni IF: 6,939; Punktacja MEiN: 100

Publikacja 2

Świacka, K., Smolarz, K., Maculewicz, J., & Caban, M. (2020). Effects of environmentally relevant concentrations of diclofenac in Mytilus trossulus. Science of the Total Environment, 737, 139797.

IF: 7,963; 5-letni IF: 7,842; Punktacja MEiN: 200

Publikacja 3

Świacka, K., Michnowska, A., Maculewicz, J., Caban, M., & Smolarz, K. (2021). Toxic effects of NSAIDs in non-target species: a review from the perspective of the aquatic environment. Environmental Pollution, 273, 115891.

IF: 9,988; 5-letni IF: 10,366; Punktacja MEiN: 100

Publikacja 4

Świacka, K., Smolarz, K., Maculewicz, J., Michnowska, A., & Caban, M. (2021). Exposure of Mytilus trossulus to diclofenac and 4′-hydroxydiclofenac: Uptake, bioconcentration and mass balance for the evaluation of their environmental fate. Science of The Total Environment, 791, 148172.

IF: 10,753; 5-letni IF: 10,237; Punktacja MEiN: 200

Publikacja 5

Świacka, K., Maculewicz, J., Kowalska, D., Caban, M., Smolarz, K., & Świeżak, J. (2022). Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms-current state of knowledge. Journal of Hazardous Materials, 424, 127350.

IF: 14,224; 5-letni IF: 12,984; Punktacja MEiN: 200

Publikacja 6

Świacka, K., Maculewicz, J., Świeżak, J., Caban, M., & Smolarz, K. (2022). A multi-biomarker approach to assess toxicity of diclofenac and 4-OH diclofenac in Mytilus trossulus mussels-First evidence of diclofenac metabolite impact on molluscs. Environmental Pollution, 315, 120384.

IF: 9,988; 5-letni IF: 10,366; Punktacja MEiN: 100

Publikacja 7

Świacka, K., Maculewicz, J., Smolarz, K., & Caban, M. (2022). Long-term stability of diclofenac and 4-hydroxydiclofenac in the seawater and sediment microenvironments: Evaluation of biotic and abiotic factors. Environmental Pollution, 304, 119243.

IF: 9,988; 5-letni IF: 10,366; Punktacja MEiN: 100

Całkowity IF:69,70; Całkowity 5-letni IF:69,1; Suma punktów MEiN: 1000

3. MATERIAŁ I METODY

3.1. Zbiór materiału

Próbki do wszystkich przeprowadzonych eksperymentów zostały zebrane z Zatoki Gdańskiej - stacje Orłowo (**publikacja 2, 4 i 6**) i Mechelinki (**publikacja 7**). Materiał do badań: wodę, osad i małże zbierano na pokładzie statku *RV Oceanograf*. Wodę nadenną pobierano przy pomocy batometru. Od razu po zbiorze mierzono parametry takie jak temperatura i zasolenie przy użyciu sondy WTW, co pozwoliło na odzwierciedlenie warunków panujących w środowisku morskim podczas aklimatyzacji małży, jak i w trakcie eksperymentów. Kilka kilogramów wierzchniej warstwy osadu o miąższości do 5 cm zebrano za pomocą chwytaka Van Veena z głębokości 10 metrów. Osad wraz z wodą denną umieszczono w czterech 10 litrowych wiaderkach, które następnie przetransportowano do laboratorium i umieszczono w lodówce w temperaturze środowiskowej 5 °C (**publikacja 7**). Małże *M. trossulus* zbierano z głębokości 10-17 m przy użyciu dragi dennej o wymiarach 30×60 cm. Do poszczególnych eksperymentów zbierano od 300 do 1300 małży. Małże następnie transportowano do pomieszczeń hodowlanych, gdzie, w warunkach zbliżonych do naturalnych, były aklimatyzowane i poddawane ekspozycji.

3.2. Przebieg eksperymentów

Badania wykonane w ramach niniejszej pracy doktorskiej obejmują cztery niezależne eksperymenty wykonane w latach 2019-2022. Każdy z testów prowadzono z wykorzystaniem materiału zebranego bezpośrednio przed jego rozpoczęciem. Próbki wody i małży niezbędne do wykonania zamierzonych analiz biochemicznych i chemicznych (obecność leków) mrożono w temperaturze -80 °C.

3.2.1. Ekspozycja *M. trossulus* na diklofenak - publikacja 2

Po aklimatyzacji trwającej 3 tygodnie małże eksponowano przez 12 dni na dwa stężenia diklofenaku - 4 i 40 μg/l. W tym celu przygotowano dziewięć 15 l szklanych zbiorników ze sztuczną wodą morską o zasoleniu 7 PSU (dwa stężenia diklofenaku i kontrola, każdy wariant w trzech powtórzeniach). W każdym zbiorniku umieszczono 73 osobniki *M. trossulus*.

Eksperyment laboratoryjny podzielono na dwie części: 12-dniową ekspozycję omułków na diklofenak i 13-dniową fazę oczyszczania. Podczas ekspozycji wodę zmieniano co cztery dni i odnawiano stężenie diklofenaku. Przed każdą wymianą wody pobierano 0,5 l wody do analiz chemicznych. Na początku fazy oczyszczania, akwaria ponownie napełniano sztuczną wodą morską, ale już bez diklofenaku. Podczas fazy ekspozycji, co cztery dni przed wymianą wody ze wszystkich akwariów pobierano po cztery omułki do analiz chemicznych obecności diklofenaku i jego dwóch metabolitów: 4-OH diklofenaku i 5-OH diklofenaku. Dodatkowo w dniach 0 i 12, ze wszystkich akwariów pobrano po dwa osobniki do analiz histologicznych.

3.2.2. Ekspozycja *M. trossulus* na diklofenak i 4-OH diklofenak - publikacja 4 i 6

Eksperyment ekspozycyjny trwał siedem dni. W tym celu przygotowano 12 zbiorników wypełnionych 15 l sztucznej wody morskiej, w których umieszczono po 50 omułków. Do trzech zbiorników dodano diklofenak i 4-OH diklofenak o stężeniu nominalnym 100 μg/l. Zmierzone w wodzie 30 minut po dodaniu stężenie diklofenaku wyniosło 68,22 μg/l, a jego metabolitu

20,85 µg/l. Z tego względu wszystkie obliczenia wykonano na podstawie stężeń mierzalnych a nie nominalnych. Trzy zbiorniki posłużyły jako kontrola rozpuszczalnikowa. Każdego dnia eksperymentu pobierano 100 ml wody ze zbiorników z diklofenakiem i 4-OH diklofenakiem do analizy chemicznej. W ostatnim dniu eksperymentu, oprócz wody, zebrano 10 małży z każdego zbiornika i zamrożono w -80 °C w celu przeprowadzenia analiz chemicznych, natomiast pozostałe omułki pobrano i zamrożono w tej samej temperaturze do pomiaru wybranych biomarkerów (**publikacja 6**). Po zakończeniu eksperymentu woda ze zbiorników była delikatnie wylewana, aby nie naruszyć biofilmu. Następnie, używając czystej szczoteczki do zębów, zebrano biofilm ze ścian zbiorników oraz z muszli małży.

3.2.3. Stabilność diklofenaku i 4-OH diklofenaku - publikacja 7

Do testów stabilności diklofenaku i jego metabolitu wykorzystano reaktory symulujące warunki środowiskowe dla układów wodnych, jak i osadowo-wodnych. W tym celu przygotowano 30 szklanych butelek o objętości 1 l z nakrętkami, uprzednio przemytych metanolem. Sumarycznie przygotowano 15 bioreaktorów dla diklofenaku i jego metabolitu, po pięć wariantów eksperymentalnych w trzech powtórzeniach każdy. W celu sterylizacji wodę morską przefiltrowano dwukrotnie przy użyciu filtrów z włókna szklanego (Whatman, klasa GF/F), a następnie poddano autoklawowaniu w temperaturze 130 °C przez 2 godziny (Classic Prestige Medical Autoclave). Osad, podobnie jak wodę, sterylizowano dwukrotnie w autoklawie w tych samych warunkach. Następnie autoklawowany osad morski umieszczono w sześciu szklanych butelkach (trzy do badań z diklofenakiem i trzy z metabolitem), tak, by każda butelka zawierała około 200 cm^3 osadu. Butelki z autoklawowanym osadem wypełniono następnie sterylizowaną wodą morską o objętości 800 ml. Taką samą objętość osadu niepoddanego autoklawowaniu umieszczono w kolejnych sześciu szklanych butelkach, a następnie każdą butelkę wypełniono 800 ml sterylizowanej wody morskiej. Pozostałe 18 butelek bez osadu wypełniono niesterylizowaną wodą morską, sterylizowaną wodą morską i przefiltrowaną sztuczną wodą morską (objętość 800 ml). Dwa zestawy, tj., osad i osad autoklawowany, zostały przygotowane w celu sprawdzenia czy mikroorganizmy obecne w osadzie mogą biodegradować diklofenak i jego metabolit oraz określenia sorpcji tych związków w osadach.

Ponieważ stężenia metabolitów diklofenaku w środowisku morskim są znacznie niższe niż stężenia związku macierzystego, użyto 10-krotnie niższego stężenia 4-OH diklofenaku niż diklofenaku. Stężenie końcowe diklofenaku wynosiło 1000 µg/l, natomiast 4-OH diklofenaku 100 µg/l. Wszystkie zbiorniki były napowietrzane podczas eksperymentu, aby uniknąć warunków beztlenowych. Eksperyment prowadzono przez 29 dni, w temperaturze środowiskowej, w ciemności, aby uniknąć fotodegradacji badanych związków. Dodatkowo by sprawdzić stabilność warunków panujących w butlach, każdego dnia, w którym pobierano próbki do analiz chemicznych, mierzono pH i OD_{600} (gęstość optyczną).

3.3. Ekstrakcja farmaceutyków i analiza chromatograficzna

3.3.1. Ekstrakcja z tkanek *M. trossulus* i derywatyzacja

Tkanki omułków liofilizowano przez 72 h w temperaturze -80 °C i ciśnieniu 0,000547 Mbar. Następnie 0,5 g liofilizatu z każdej próbki umieszczano w celkach ekstrakcyjnych, które wypełniano ziemią okrzemkową. Przyspieszoną ekstrakcję rozpuszczalnikową (ASE) prowadzono przy użyciu aparatu Dionex ASE 350 w temperaturze 80 °C, pod ciśnieniem 1500 psi, stosując trzy cykle ekstrakcji za pomocą 1:1 (v/v) mieszaniny metanolu i wody dejonizowanej (0,05 mS). Następnie z każdej próbki pobierano ekstrakt, który filtrowano i rozpuszczono w wodzie dejonizowanej. Ekstrakcję do fazy stałej (SPE) uzyskanych próbek wykonano z wykorzystaniem kolumienek (Strata-X; Phenomenex; Torrance, USA). Kolumienki Strata-X kondycjonowano metanolem i wodą dejonizowaną. Następnie przez kolumienki przepuszczano próbki za pomocą podciśnienia generowanego przez pompkę wodną. Kolumienki przemywano za pomocą 5% wodnego roztworu metanolu, a następnie heksanu. Po osuszeniu sorbentu, próbki eluowano stosując metanol. Otrzymane ekstrakty zatężano, a następnie przenoszono do wialek chromatograficznych, w których odparowywano rozpuszczalnik pod delikatnym strumieniem azotu. Wysuszone próbki poddawano następnie derywatyzacji za pomocą mieszaniny -N,O-bistrifluoroacetamidu (BSTFA) +1% chlorotrimetylosilanu (TMCS) i pirydyny w temperaturze 60 °C przez 30 minut aby otrzymać trimetylosililowe pochodne analitów, które następnie analizowano za pomocą chromatografii gazowej ze spektrometrią mas (GC-MS).

3.3.2. Ekstrakcja z wody i derywatyzacja

Próbki wody pobrane ze środowiska naturalnego oraz uzyskane w trakcie eksperymentu laboratoryjnego filtrowano przy użyciu filtrów z włókna szklanego. Następnie wykonywano SPE przy użyciu specjalnych filtrów dyskowych (H2OPhilic DVB Speedisk®, Bakerbond). W celu kondycjonowania, sorbent przemywano metanolem, a następnie wodą destylowaną. W kolejnym etapie próbki przepuszczono przez dyski (za pomocą podciśnienia), a następnie dyski przemywano 5% metanolem. Następnie wkłady suszono przez 15 minut i przemywano heksanem. W ostatnim etapie zaabsorbowane anality wymywano z dysków za pomocą metanolu. Kolejne etapy tj. odparowywanie rozpuszczalnika pod azotem, suszenie, derywatyzacja i analiza GC-MS były takie same jak dla ekstraktów z tkanek małży.

3.3.3. Analiza ilościowa techniką GC-MS

Diklofenak i jego metabolity analizowano techniką GC-MS (GCMS-QP2010 SE, Shimadzu; Kyoto, Japonia) Fazą ruchomą był hel o początkowym natężeniu przepływu 2 ml/min i stabilnym ciśnieniu 100 kPa. Próbki o objętości 1 µl dozowano bez dzielenia strumienia przy temperaturze dozownika wynoszącej 300 °C. Temperatura początkowa gradientu wynosiła 100 °C i utrzymywano ją przez 1 minutę, a następnie stopniowo podnoszono do 300 °C (narost 8 °C/min) i utrzymywano przez 5 minut. Detekcję MS (EI, 70 eV) przeprowadzono w trybie pełnego skanowania lub monitorowania wybranych jonów (SIM, ang. *Selected Ion Monitoring*). Temperatura linii przesyłowej wynosiła 300 °C. Anality były identyfikowane na podstawie czasu retencji oraz obecności jonów ilościowych i jonów potwierdzających. Walidację analizy GC-MS przeprowadzono metodą wzorca zewnętrznego, stosując czysty wzorzec diklofenaku i jego metabolitów (Świacka i in., 2019). Główne

parametry metody, takie jak dokładność (80-120%) i precyzja (poniżej 11% RSD) były zgodne z analitycznymi standardami. Granice oznaczalności wyniosły od 0,001 µg/ml (instrumentalna granica oznaczalności, IQL) do 5 µg/ml dla każdego analitu (min. 8 punktów kalibracji), niezależnie od rodzaju próbki (woda lub tkanka) (Świacka i in., 2019).

3.4. Wskaźnik ogólnej kondycji (BMI)

Do określenia indeksu BMI zmierzono długość muszli małży oraz zważono mokrą masę tkanki. Indeks BMI został następnie obliczony zgodnie z poniższym wzorem:

$$BMI=W/L^3\ [mg/cm^3], \text{ gdzie}$$

L^3- długość muszli^3 [cm^3];
W- mokra masa tkanki [mg]

3.5. Analizy biochemiczne

Do określenia wpływu diklofenaku i jego metabolitu na małże *M. trossulus* wykorzystano markery stresu oksydacyjnego (aktywności następujących enzymów): transferaza glutationowa (GST), reduktaza glutationowa (GR), katalaza (CAT) i profenolooksydaza (proPO), a także marker neurotoksyczności: acetylocholinoesteraza (AChE). Aktywność enzymatyczną przeliczano na ilość białka, stąd też niezbędne było określenie zawartości białka w tkankach.

3.5.1. Aktywność proPO

Hemolimfę odwirowywano przy 400×g przez 5 minut w temperaturze 4 °C, a uzyskany supernatant, osocze, przenoszono do czystych probówek i wykorzystywano do dalszych analiz. 100 µl osocza z każdej próbki mieszano z 50 µl siarczanu dodecylosodowego (SDS, 5 mg/l) rozpuszczonego buforze Tris (pH 6,5) i inkubowano przez 15 minut w temperaturze 25 °C. Następnie 100 µl powstałej mieszaniny przenoszono do dołka na mikropłytce i dodawano do niej 1 ml 10 mM L-DOPA (3 mg/ml w 0,5 M HCl zawierającym 10 mM $CaCl_2$). Natychmiast po wymieszaniu próbki analizowano spektrofotometrycznie przy długości fali 480 nm przez 15 minut z 1-minutowymi przerwami na spektrofotometrze mikropłytkowym (Multiskan SkyHigh Microplate Spectrophotometer, Thermofisher). Czysty roztwór L-DOPA stanowił ślepą próbę (Muñoz i in., 2006). Aktywność ProPO obliczano w odniesieniu do ilość białka oznaczonego w hemolimfie (U/mg białka).

3.5.2. Aktywność GR, GST i CAT

Do oznaczenia aktywności GR i GST wykorzystano dostępne zestawy testowe (Gentaur). Analizy prowadzono zgodnie z instrukcją producenta dostępną w zestawie, z niewielkimi modyfikacjami. Około 0,1 g tkanki skrzeli homogenizowano w czterech objętościach buforu, używając ręcznego homogenizatora szklanego (Bionovo). Następnie uzyskany homogenat odwirowywano przy 10,000×g przez 15 minut w temperaturze 4 °C, a uzyskany supernatant przenoszono do probówek o objętości 1,5 ml. W zależności od aktywności enzymatycznej, w testach wykorzystywano od 10 do 100 µl roztworu, który

przenoszono do studzienek na mikropłytce, a następnie mieszano z odczynnikami zgodnie z instrukcją producenta. Aby ocenić aktywność GST, absorbancję monitorowano przez 10 minut z 1-minutowymi przerwami przy długości fali 340 nm. Dla GR wartość OD odczytywano co minutę przy 405 nm przez 30 minut. Aktywność obu enzymów obliczano w odniesieniu do ilości białka oznaczonego w skrzelach (GST: mmol/min/mg białka; GR: mU/mg białka).

CAT oznaczano zgodnie z metodą Cohena i in. (1996), wykorzystującą reakcję pomiędzy H_2O_2 i nadmiarem 2 mM $KMnO_4$, a następnie mierzono spektrofotometrycznie rozkład H_2O_2 po 3 minutach przy 480 nm. Reakcję prowadzono w łaźni wodnej z lodem. Aktywność CAT przeliczano na ilość białka cytozolowego, które oznaczono w skrzelach (U/mg białka).

3.5.3. Aktywność AChE

AChE mierzono zgodnie z metodą Ellmana i wsp. (1961). Około 0,1 g tkanki skrzeli homogenizowano w ośmiu objętościach zimnego buforu siarczanu Tris (pH 7,8) przy użyciu ręcznego homogenizatora szklanego (Bionovo). Następnie homogenat odwirowywano w temperaturze 4 °C przy 13 000×g przez 10 minut, a uzyskany supernatant przenoszono do czystych probówek. Dla aktywności AChE, 50 µl próbki rozpuszczano w 100 µl zimnego siarczanu Tris, a następnie 30 µl powstałej mieszaniny przenoszono do studzienki mikropłytki. Następnie dodawano 70 µl mieszaniny 0,6 mM odczynnika Ellmana (DTNB) i 1,2 mM jodku acetylocholiny. Absorbancję odczytywano co minutę przez 20 minut przy długości fali 415 nm.

3.5.4. Stężenie białka w tkankach

Stężenie białka w skrzelach i hemolimfie analizowano za pomocą metody fenolowej (Lowry i in., 1951) dostosowanej do analizy za pomocą czytnika mikropłytek. Stężenie białka w hemolimfie zostało wykorzystane tylko do obliczenia aktywności proPO, podczas gdy ilość białka w skrzelach została wykorzystana do obliczenia pozostałych biomarkerów. Krzywą wzorcową przygotowano z surowiczej albuminy bydlęcej (Sigma-Aldrich). Wzorzec albuminy rozcieńczono w wodzie destylowanej do uzyskania stężenia 2 mg/ml. Następnie przygotowano 6 roztworów o stężeniach 0, 1, 2, 5, 10, 20 i 40 µg/ml i objętości 400 µl. W przypadku próbek eksperymentalnych, 5-20 µl supernatantu z każdej badanej próbki przeniesiono do probówek i rozcieńczono wodą destylowaną do końcowej objętości 400 µl. Następnie do każdej probówki dodano odczynnik biuretowy o objętości 500 µl i inkubowano w temperaturze pokojowej przez 15 minut. Następnie zastosowano odczynnik Folina & Ciocalteu'a (rozcieńczony z wodą w stosunku objętościowym 1:2), którego 250 µl dodano do każdej próbki i inkubowano przez 30 minut do momentu wystąpienia reakcji barwnej. Ostatecznie 100 µl każdej próbki przeniesiono do studzienki na mikropłytce. Absorbancję mierzono przy długości fali 650 nm.

3.6. Analizy histologiczne

Tkanki miękkie małży zostały oddzielone od muszli i umieszczone w roztworze utrwalającym Davidsona (33% woda destylowana, 33% etanol [96%], 22% formaldehyd i 11% kwas octowy). Po dwóch dniach tkanki umieszczano w 10% roztworze formaldehydu buforowanym NaH_2PO_4 i Na_2HPO_4 na 10 dni. Następnie tkanki przeniesiono do bloczków histologicznych, odwodniono poprzez zanurzanie w roztworach o rosnącym stężeniu etanolu (od 70% do czystego etanolu) i ksylenie, impregnowano, zatopiono w parafinie i ostatecznie

przygotowano bloczki parafinowe z tkanką. Bloczki cięto z wykorzystaniem mikrotomu na skrawki o grubości 3 μm. Tak uzyskane preparaty pozostawiono na noc w temperaturze 60 °C, poddano deparafinacji w trzech kąpielach ksylenowych, a następnie uwodniono zanurzając w roztworach o malejącym stężeniu etanolu. Gotowe preparaty z tkankami barwiono hematoksyliną i eozyną (H&E). Przygotowane próbki były w dalszej kolejności analizowane pod mikroskopem świetlnym pod kątem zmian w gonadach, skrzelach i gruczołach trawiennych. Zastosowano binarną (0 dla braku i 1 dla obecności) ocenę występowania zmian.

4. WYNIKI I DYSKUSJA

4.1. Toksyczny wpływ diklofenaku i 4-OH diklofenaku na *M. trossulus*

4.1.1. Wpływ na kondycję małży

W dwóch przeprowadzonych eksperymentach analizowano wpływ diklofenaku na ogólną kondycję małży wyrażoną poprzez BMI (body mass index; **publikacje 2 i 4**). W niniejszych badaniach małże eksponowano na diklofenak o stężeniach 68 µg/l przez 7 dni (**publikacja 4**) lub 4 i 40 µg/l przez 12 dni (**publikacja 2**). W obu pracach nie zaobserwowano wpływu tego związku na kondycję mięczaków. Również w przypadku 4-OH diklofenaku nie zaobserwowano wpływu na kondycję małży (**publikacja 4**). Uzyskane wyniki potwierdziły, że potencjalne efekty diklofenaku i jego metabolitu mogą nie być odzwierciedlane poprzez analizy prostych, podstawowych wskaźników ogólnej kondycji organizmów, jak BMI. Również w pracy Gonzalez-Rey i Bebianno (2014) nie zaobserwowano wpływu diklofenaku na kondycję u małży *Mytilus galloprovinciallis*. Brak wpływu może wynikać z czasu ekspozycji, gdyż małże były eksponowane na diklofenak stosunkowo krótko. Przy dłuższej ekspozycji trwającej np. 2-3 miesiące wpływ testowanego leku na kondycję mógłby być widoczny.

4.1.2. Wpływ na markery biochemiczne

Spośród analizowanych biomarkerów enzymatycznych, wpływ testowanych związków zaobserwowano tylko w przypadku aktywności GR. Aktywność GR u małży eksponowanych na diklofenak i 4-OH diklofenak była niższa w porównaniu z małżami ze zbiorników kontrolnych, jednak różnice te były istotne statystycznie tylko dla diklofenaku. Funkcją GR w organizmie jest katalizowanie redukcji utlenionego glutationu (GSSG) do zredukowanego glutationu (GSH) przy użyciu dinukleotydu nikotynoamidoadeninowego (NADPH) jako koenzymu (Ramos-Martinez i in., 1983; Manduzio i in., 2004). Gonzalez-Rey i Bebianno (2014) zaobserwowali znaczącą indukcję aktywności GR u *M. galloprovincialis* eksponowanych na diklofenak o stężeniu 250 ng/l. W badaniach Jaafara (2015) aktywność GR była również zwiększona w skrzelach *M. edulis* eksponowanych na diklofenak w stężeniu 1 mg/l, podczas gdy w gruczołach trawiennych tych samych organizmów była ona zmniejszona. Z kolei Mezzelani i in. (2006a, b; 2018) nie zaobserwowali istotnej zmiany wartości GR u omułków narażonych na działanie diklofenaku w stężeniu 25 i 2,5 µg/l. Różne wyniki wspomnianych testów ekotoksykologicznych są skutkiem prowadzenia ich w odmiennych warunkach, ale podkreślają duże prawdopodobieństwo negatywnych skutków diklofenaku i jego metabolitu już na poziomie kluczowych szlaków metabolicznych / detoksykacyjnych.

Nie stwierdzono statystycznie istotnych zmian w aktywności pozostałych biomarkerów stresu oksydacyjnego: GST, CAT i Pro-PO (**publikacja 6, rys. 1**). Biorąc pod uwagę, że zarówno diklofenak, jak i 4-OH diklofenak zostały wykryte w tkankach omułków, brak widocznego wpływu na aktywność wymienionych biomarkerów może wskazywać na nieistotną rolę mechanizmów prooksydacyjnych w sposobie działania testowanych substancji chemicznych. Obserwacja ta jest zgodna z wynikami uzyskanymi przez Mezzelani i in. (2018b). W tym badaniu diklofenak w stężeniach środowiskowych (2,5 µg/l) podczas 60-dniowej ekspozycji nie powodował istotnych statystycznie zmian w aktywności biomarkerów stresu oksydacyjnego, w tym CAT, GST i innych enzymów zależnych od glutationu badanych u *M. galloprovincialis.* Pomimo, że zastosowałam 30-krotnie wyższe stężenie diklofenaku,

również nie zaobserwowaliśmy jego wpływu na markery stresu oksydacyjnego (z wyjątkiem GR) u małży z rodziny Mytilidae. Brak wpływu na markery stresu oksydacyjnego odnotowano także u *M. galloprovincialis* eksponowanego na diklofenak w stężeniach 25 µg/l i 0,5 µg/l przez 14 dni (Mezzelani i in., 2016a, b).

W niniejszej pracy analizowałam również wpływ diklofenaku i jego metabolitu na AChE, która jest enzymem zaangażowanym w neurotransmisję (Gonzalez-Rey i Bebianno, 2014). AChE jest powszechnym markerem wykorzystywanym do oceny efektów neurotoksycznych badanych związków (Gonzalez-Rey i Bebianno, 2014; Mezzelani i in., 2018b; Solé i in., 2010). Nie zaobserwowałam jednak wpływu diklofenaku i jego metabolitu na aktywność AChE. Wynik ten jest zgodny z ustaleniami Mezzelani i in. (2016a, b, 2018), którzy również nie zaobserwowali wpływu diklofenaku na aktywność neurotransmiterów u małży. Natomiast Gonzalez-Rey i Bebianno (2014) wykazali istotny wzrost aktywności AChE u *M. galloprovincialis* eksponowanego na diklofenak przy stężeniu 250 ng/L. Zgodnie z hipotezą założoną przez autorów wzrost aktywności AChE w grupie poddanej działaniu diklofenaku był związany raczej z poziomem białka witelogeniny w gonadach samic niż z zaburzeniami neurotransmisji przez lek (Gonzalez-Rey i Bebianno, 2014). Podobnie Fontes i in. (2018) zaobserwowali indukcję aktywności AChE w skrzelach *Perna perna* eksponowanych na diklofenak w stężeniu 2000 ng/L po 48 godzinach ekspozycji. Liczba doniesień opisujących modulację aktywności AChE w wyniku działania diklofenaku jest niewielka, a uzyskane wyniki niejednoznaczne, zatem nie ulega wątpliwości, że wpływ farmaceutyków na funkcjonowanie tego enzymu u bezkręgowców jest specyficzny dla organizmu i stężeń oraz wymaga dalszych badań.

Z kolei proPO jest enzymem pełniącym trzy funkcje u bezkręgowców, w tym małży: działa zarówno jako antyoksydant i detoksykant, ale jest również zaangażowana w mechanizmy obrony immunologicznej, takie jak melanizacja (Coles i Pipe, 1994; Luna-Acosta i in., 2017). Jej rola jest dość dobrze poznana u skorupiaków, natomiast wciąż niewiele wiadomo o jej funkcji u mięczaków (Coles i Pipe, 1994; Luna-Acosta i in., 2017). W jednej z prac wskazano na znaczący wpływ zanieczyszczeń na aktywność proPO u bezkręgowców (Laitano i Fernández-Gimenez, 2016). W naszych badaniach ujawniono akumulację lipofuscyny/melaniny w różnych tkankach omułków poddanych działaniu diklofenaku. Ze względu na tę obserwację postanowiono po raz pierwszy wykorzystać proPO do oceny wpływu diklofenaku i 4-OH diklofenaku na odporność omułków. Nie zaobserwowano jednak żadnego wpływu testowanych związków chemicznych na ten marker. Chociaż nie istnieją inne badania analizujące wpływ diklofenaku na aktywność proPO u małży, w nielicznych pracach odnotowano istotny wpływ ibuprofenu, również należącego do NLPZ, na aktywność tego enzymu (Luna-Acosta i in., 2012; Matozzo i in., 2012).

Aktywność biomarkerów enzymatycznych może być związana z wieloma czynnikami, takimi jak stężenie testowanego związku i czas ekspozycji, ale także cechy gatunku modelowego lub analizowanej tkanki. Co więcej, odpowiedź biomarkerów enzymatycznych jest zazwyczaj obserwowana w badaniach toksyczności ostrej, po krótkiej ekspozycji na wysokie stężenia.

4.1.3. Wpływ na markery histologiczne

Wpływ diklofenaku na markery histologiczne analizowano w dwóch eksperymentach przeprowadzonych w ramach niniejszej pracy. Zmiany histopatologiczne odnotowano już w trakcie wstępnych analiz (**publikacja 2**). Diklofenak w stężeniach występujących w środowisku (4 µg/l) powodował deformacje skrzeli, wakuolizację pęcherzyków trawiennych i nagromadzenie brązowego pigmentu (melaniny/lipofuscyny) w nabłonku różnych narządów. Dodatkowo, ekspozycja na diklofenak w stężeniu 40 µg/l powodowała atrofię i martwicę pęcherzyków trawiennych (**publikacja 2**). W związku z tym w kolejnym teście przeprowadzono bardziej wnikliwe obserwacje histopatologiczne, zarówno w przypadku diklofenaku, jak i 4-OH diklofenaku, uwzględniając także analizę statystyczną (**publikacja 6**).

Obserwowane efekty obejmowały zmiany regresywne występujące w układzie pokarmowym, oddechowym i rozrodczym. Do najczęściej stwierdzanych zmian należała atrezja gonad (**publikacja 6, rys. 2a**), zanik i martwica pęcherzyków trawiennych (**publikacja 6, rys. 2b)** oraz lokalnie występujące, rozproszone nacieki hemocytów (zapalenie), tworzące niekiedy granulocytomy. W skrzelach stwierdzono przerost nabłonka oddechowego (**publikacja 6, rys. 2c)**, obecność zmian zwyrodnieniowych, obrzęki (**publikacja 6, rys. 2d**), zrastanie się blaszek skrzelowych, częściową utratę blaszki i hiperplazję nabłonka. Obrzęk skrzeli występował z podobną częstotliwością do kontroli (**publikacja 6, rys. 3**). Z kolei martwicę skrzeli obserwowano tylko u omułków eksponowanych na leki i efekt ten wystąpił u ok. 17% małży (**publikacja 6, rys. 3**). Chociaż w skrzelach omułków eksponowanych na diklofenak i metabolit obserwowano także inne zmiany, to pojawiały się one sporadycznie i nie można ich przypisać wyłącznie efektom działania farmaceutyków (**publikacja 6, rys. 3**). Dodatkowo stwierdzono obecność brązowych komórek pigmentowych, zawierających melaninę i/lub lipofuscynę (**publikacja 6, rys. 2e i rys. 2f**). Komórki pigmentowe najczęściej występowały w nabłonku płaszcza, gdzie ich zwiększona produkcja była statystycznie istotna dla omułków poddanych działaniu 4-OH diklofenaku, ale także w nabłonku gruczołu trawiennego i skrzeli. W takich przypadkach brązowa pigmentacja występowała we wszystkich komórkach tworzących warstwę nabłonkową danego organu/struktury.

W gruczole trawiennym zmiany patologiczne były bardziej widoczne, jednak tylko w przypadku zmian martwiczych efekt ten był statystycznie istotny względem kontroli (**publikacja 6, rys. 5**). Zmiany obserwowane w układzie pokarmowym obejmowały atrofię pęcherzyków trawiennych, przerost lub martwicę nabłonka, lokalne stany zapalne oraz obecność komórek pigmentowych. Zmiany te często stwierdzano jako współwystępujące z martwicą komórek pęcherzyków (martwe lub rozpadające się komórki i/lub obszary tkanki) oraz zmianami degeneracyjnymi komórek układu pokarmowego. Obserwowano je u 20% małży ze zbiorników kontrolnych, 41% małży eksponowanych na 4-OH diklofenak i 38% małży eksponowanych na diklofenak. Miejscowe reakcje zapalne występujące w okolicy układu pokarmowego wystąpiły u 6,5% małży z kontroli, 13,7% małży narażonych na działanie 4-OH diklofenaku i u 21% małży narażonych na działanie diklofenaku.

Dotychczas nie powstały inne badania, w których przeprowadzono ocenę histologiczną małży narażonych na działanie diklofenaku. Ponadto, niniejsza praca jest pierwszą, która bada wpływ 4-OH diklofenaku na poziomie struktury poszczególnych organów i tkanek. W kilku innych pracach zaobserwowano jednak zmiany struktury w tkankach ryb narażonych na diklofenak. Podobnie do obserwacji uzyskanych podczas moich badań, farmaceutyk wywołał

zmiany martwicze w układach wydalniczym (nerki) i oddechowym (skrzela) pstrąga tęczowego i pstrąga potokowego (Schwaiger i in., 2004; Hoeger i in., 2005; Mehinto i in., 2010; Triebskorn i in., 2004). Efektem oddziaływania związku u ryb były także zmiany hiperplastyczne nerek u ciernika (Näslund i in., 2017). Z kolei Zhang i in. (2021) odnotowali uszkodzenia jelit (komórek nabłonka i błony śluzowej jelita) u raków słodkowodnych narażonych na diklofenak w stężeniu 1 i 10 mg/l. Efekty histopatologiczne działania NLPZ na organizmy wodne, w szczególności morskie, pozostają wciąż słabo poznane. Nasze badania potwierdzają jednak, że to narzędzie może okazać się bardzo przydatne w lepszym zrozumieniu toksyczności farmaceutyków.

4.2. Biokoncentracja diklofenaku i 4-OH diklofenaku w tkankach *M. trossulus* i biofilmie morskim

We wszystkich przeprowadzonych eksperymentach, niezależnie od testowanego stężenia oraz czasu ekspozycji zaobserwowano, że diklofenak jest pobierany przez małże *M. trossulus*. W dwóch testach, w których analizowano kinetykę tego procesu, stężenie diklofenaku w tkankach wzrastało w początkowych dniach ekspozycji, a następnie spadało (**publikacje 2 i 4**), co oznacza, że małże mogą metabolizować i wydalać ten związek. Dodatkowo w niniejszych badaniach w małżach eksponowanych na diklofenak zaobserwowano obecność hydroksypochodnych tego związku: 4-OH i 5-OH diklofenaku, co potwierdza doniesienia Bonnefille i in. (2017), że są to główne pochodne do których omułki metabolizują diklofenak.

Również w przypadku ekspozycji małży na 4-OH diklofenak zaobserwowano, że związek ten jest pobierany i gromadzony w ich tkankach. Na podstawie oznaczonych stężeń obliczono współczynniki BCF dla diklofenaku i 4-OH diklofenaku, które wyniosły odpowiednio 118,5 ± 17,5 oraz 69,7 ± 18,2 l/kg. Zgodnie z wytyczną OECD nr 305 wartości BCF uzyskane dla tych związków wskazują na ich niski potencjał do bioakumulacji w tkankach małży (OECD, 2016; OECD, 1992a, 1992b). Należy jednak podkreślić, że najbardziej wiarygodna ocena bioakumulacji wymaga osiągnięcia stanu równowagi pomiędzy stężeniem w tkankach organizmów i środowisku przy długotrwałej ekspozycji na badaną substancję. W naszych badaniach omułki były eksponowane na diklofenak i 4-OH diklofenak zbyt krótko, aby zaobserwować ich akumulację w stanie równowagi, co przedstawia kinetyczna zmiana stężenia w zbiornikach testowych oraz tkankach małży na przestrzeni eksperymentów (**publikacje 2 i 4**). Mimo to, w porównaniu z innymi badaniami uzyskane przez nas wartości BCF dla DIC są stosunkowo wysokie. W pracy Bonnefille et al. (2017), BCF dla *M. galloprovincialis* eksponowanego na diklofenak przy stężeniach 122,6 µg/l i 647,8 µg/l przez 7 dni wyniósł odpowiednio 16,5 i 11,3 l/kg. Z kolei w przypadku 4-OH diklofenaku nasza praca jest pierwszą, która analizowała i wykazała biokoncentrację tego związku w tkankach małży. Dodatkowo po raz pierwszy określono wpływ płci małży na biokocentrację diklofenaku i 4-OH diklofenaku. Zaobserwowano różnice w biokoncentracji metabolitu diklofenaku jednak nie były one istotne statystycznie, co wynikało najprawdopodobniej z małej próby. Są to pierwsze takie badania, które należałoby jednak powtórzyć z wykorzystaniem większej liczby osobników.

Dodatkowo zaobserwowano, że oba badane związki ulegają też w niewielkim stopniu, znacznie niższym niż w tkankach małży, nagromadzeniu w biofilmie morskim zebranym z muszli małży oraz ze ścian akwarium (**publikacja 4**).

4.3. Stabilność i dystrybucja diklofenaku i 4-OH diklofenaku w wodzie morskiej i osadzie

W kolejnej pracy przez 29 dni obserwowano stabilność diklofenaku oraz 4-OH diklofenaku w wodzie morskiej z osadem pobranym z okolicy ujścia oczyszczalni ścieków „Dębogórze". W zbiornikach z osadem zaobserwowano wyraźny, stopniowy spadek stężenia obu badanych związków (**publikacja 7, rys. 2**). Największy spadek stężenia diklofenaku zaobserwowano w zbiornikach z osadem, a największy spadek stężenia 4-OH diklofenaku w zbiornikach z osadem sterylizowanym. Stężenie diklofenaku zmniejszyło się o 31,5% w zbiornikach z osadem i o 20,4% w zbiornikach z osadem sterylizowanym podczas 29-dniowego doświadczenia. Stężenie 4-OH diklofenaku zmniejszyło się o 76,5% i 90,2% w zbiornikach z osadem i osadem sterylizowanym. Analiza statystyczna wykazała istotne różnice w stężeniu 4-OH diklofenaku pomiędzy zbiornikami z osadami (sterylizowanymi i niesterylizowanymi) a pozostałymi zbiornikami (woda środowiskowa, woda środowiskowa sterylizowana i sztuczna woda morska - **publikacja 7, rys. 2**) w dniach 1-29. W przeciwieństwie do metabolitu, w przypadku diklofenaku statystycznie istotne różnice pomiędzy zbiornikami z osadem a pozostałymi zbiornikami pojawiły się najwcześniej 7. dnia, a w niektórych kombinacjach (osad sterylizowany - woda środowiskowa) dopiero pod koniec eksperymentu (**publikacja 7, rys. 2**). Uzyskane wyniki wskazują więc, że w obecności osadów diklofenak i jego metabolit są łatwiej usuwane z wody. Co więcej, nasze obserwacje sugerują, że metabolit cechuje się dużo mniejszą stabilnością w wodzie z osadem niż związek pierwotny. (**publikacja 7, rys. 2**). Z kolei w żadnym ze zbiorników z diklofenakiem i 4-OH diklofenakiem bez osadu nie zaobserwowano statystycznie istotnego spadku stężenia leku po 29 dniach (**publikacja 7, rys. 2**). Może to świadczyć o znikomej roli mikroorganizmów w wodzie morskiej w biodegradacji diklofenaku i 4-OH diklofenaku, a także o niepodatności tych związków na abiotyczne procesy degradacji.

Również w pracy Gröning i in. (2007) zaobserwowano znaczący spadek stężenia diklofenaku w kolumnowym bioreaktorze ze złożem stałym wypełnionym osadami z potoku Münzbach (Niemcy). Osad i woda były pobierane z miejsca w pobliżu wylotu komunalnej oczyszczalni ścieków. Już po pięciu dniach od dodania diklofenaku zaobserwowano 93% spadek jego stężenia. Stężenie diklofenaku (1036 µg/l) zastosowane przez Gröninga i in. (2007) było zbliżone do zastosowanego w niniejszych badaniach (1000 µg/l). Pomimo tego, w niniejszej pracy po 29 dniach ubytek diklofenaku był znacznie mniejszy niż w badaniach Gröninga i in. (2007). Wśród potencjalnych przyczyn znaczących różnic w obserwacjach należy zwrócić uwagę na fakt, że w pracy Gröninga i in. (2007) eksperyment przeprowadzono w temperaturze 20 ºC, natomiast w naszych badaniach temperatura była czterokrotnie niższa (5 ºC). Zgodnie z licznymi pracami temperatura jest ważnym czynnikiem determinującym aktywność biodegradacyjną mikroorganizmów. Ponadto ważna jest statyka testu: w naszym przypadku kontakt woda-osad był mniejszy niż w teście kolumnowym.

W przeprowadzonym bilansie masy dla ostatniego dnia eksperymentu, największy ubytek 4-OH diklofenaku w matrycy wodnej zaobserwowano w zbiorniku z osadem sterylizowanym (82,75%), jednak tylko 1,5% masy metabolitu zostało oznaczone w sterylizowanym osadzie. Dodatkowo, zaobserwowano różnicę w masie metabolitu oznaczonej w osadzie między zbiornikami z osadem sterylizowanym i niesterylizowanym, jednak różnica ta nie była istotna statystycznie. Znaczny ubytek masy metabolitu w obu zbiornikach z osadem może więc być związany z jego degradacją biotyczną i abiotyczną (**publikacja 7, rys. 3**).

Tak jak w przypadku metabolitu, największe straty masy diklofenaku zaobserwowano w zbiornikach z osadami (~26,16%) (**publikacja 7, rys. 4**). Ubytek masy diklofenaku w matrycy wodnej w zbiornikach z osadem był jednak znacznie niższy niż jego metabolitu. Około 9% początkowej masy diklofenaku uległo adsorpcji na osadzie niesterylizowanym, a około 7% na osadzie sterylizowanym (**publikacja 7, rys. 4**). Nasze wyniki mogą wskazywać na większą tendencję diklofenaku do adsorpcji na osadzie bądź mniejszą stabilność 4-OH diklofenaku w obecności osadu. Dodatkowo w niesterylizowanym osadzie z każdego z trzech zbiorników z diklofenakiem wykryto 5-OH diklofenak w stężeniu 0,392 +/- 0,125 µg/kg suchej masy, podczas gdy w sterylizowanym osadzie nie wykryto metabolitów diklofenaku (5-OH diklofenak lub 4-OH diklofenak). Wyniki te wskazują na możliwość biotransformacji diklofenaku przez mikroorganizmy z osadów, jednak biorąc pod uwagę niewielką różnicę w oznaczonej masie diklofenaku pomiędzy osadem sterylizowanym i niesterylizowanym- proces ten jest nieznaczny. Zgodnie z licznymi badaniami 5-OH diklofenak jest metabolitem powszechnie wytwarzanym przez różne szczepy bakterii, w tym bakterie z osadów rzecznych (Gröning i in., 2007; Palyzová i in., 2019; Lu i in., 2019; Ivshina i in., 2019; Osorio-Lozada i in., 2008; Moreira i in., 2018). Nadal jednak niewiele wiadomo na temat zdolności biotransformacji farmaceutyków przez bakterie z osadów morskich. Utrata masy zarówno diklofenaku, jak i metabolitu w zbiornikach z osadami, niezwiązana z ich sorpcją, była wyraźnie wyższa w porównaniu ze zbiornikami z samą wodą morską (o 43-69% dla metabolitu i 4-17% dla diklofenaku). Ta ważna obserwacja może sugerować, że obecność osadów sprzyja usuwaniu obu związków chemicznych z matrycy wodnej. Należy podjąć badania, aby powiązać chemizm i właściwości morfologiczne osadu morskiego z usuwaniem leków ze środowiska.

5. WNIOSKI

Badania eksperymentalne przeprowadzone w ramach niniejszej pracy doktorskiej, opublikowane w cyklu czterech oryginalnych artykułów, a także dogłębna analiza danych literaturowych, składających się na trzy prace przeglądowe, pozwalają na wysunięcie następujących wniosków:

1. Małże z rodziny Mytilidae są dobrym gatunkiem modelowym w badaniach biokoncentracji i toksyczności farmaceutyków. Badania eksperymentalne wykonane w ramach niniejszej pracy doktorskiej potwierdzają, że bałtycki przedstawiciel rodziny Mytilidae, *M. trossulus*. może być z powodzeniem wykorzystywany w tego typu analizach (**publikacja 1, 2 i 6**)
2. Diklofenak i 4-OH diklofenak wykazują się toksycznym działaniem na małże *M. trossulus*. W przypadku diklofenaku negatywny wpływ na te organizmy może być obserwowany już na poziomie stężeń regularnie notowanych w środowisku (**publikacja 2, 3 i 6**).
3. Zmiany na poziomie tkankowym takie jak deformacja skrzeli, atrezja gonad, atrofia pęcherzyków trawiennych oraz akumulacja lipofuscyny/melaniny w nabłonku płaszcza mogą stanowić przydatne narzędzie w ocenie wpływu farmaceutyków na organizmy wodne. Zgodnie z uzyskanymi wynikami, wpływ ekspozycji na diklofenak i 4-OH diklofenak może być w większym stopniu odzwierciedlany na poziomie tkankowym niż na poziomie markerów biochemicznych (**publikacja 2, 3 i 6)**.
4. Zarówno diklofenak, jak i 4-OH diklofenak są pobierane przez małże *M. trossulus*, a w konsekwencji ulegają nagromadzaniu w ich tkankach. Biokoncentracja obu związków w tkankach *M. trossulus* różni się znacząco. Wyznaczone współczynniki BCF dla obu testowanych związków wskazują jednak, że proces ten zachodzi w umiarkowanym stopniu i zgodnie z powszechnie stosowanymi kryteriami oceny ich potencjał do biokoncentracji w środowisku wodnym należy uznać za niski (**publikacja 2 i 5)**.
5. Diklofenak jest metabolizowany, a następnie wydalany przez małże, a także ulega biotransformacji do 5-OH diklofenaku w osadzie morskim pobranym przy ujściu oczyszczalni ścieków (**publikacja 5 i 7**).
6. Obecność osadu morskiego przyczynia się do spadku stężenia diklofenaku i 4-OH diklofenaku w wodzie morskiej. Oba związki ulegają adsorpcji na osadzie morskim. Diklofenak cechuje się jednak znacznie wyższą stabilnością w wodzie morskiej w obecności osadu niż 4-OH diklofenak. Osad morski może pełnić ważną funkcję w eliminacji tych związków z wody **(publikacja 7)**.

6. LITERATURA

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7. DOROBEK NAUKOWY

OPUBLIKOWANE PRACE

1. **Świacka**, K., Maculewicz, J., Kowalska, D., & Grace, M. Do pharmaceuticals affect microbial communities in aquatic environments? A review. Frontiers in Environmental Science, 10, 2657 (IF: 5,411; 5-letni IF: 6,313 MEiN: 100)
2. **Świacka**, K., Maculewicz, J., Świeżak, J., Caban, M., & Smolarz, K. (2022). A multi-biomarker approach to assess toxicity of diclofenac and 4-OH diclofenac in Mytilus trossulus mussels-First evidence of diclofenac metabolite impact on molluscs. Environmental Pollution, 315, 120384 (IF: 9,988; 5-letni IF:10,366; MEiN: 100)
3. **Świacka, K**., Maculewicz, J., Smolarz, K., & Caban, M. Long-Term Stability of Diclofenac and 4-Hydroxydiclofenac in the Seawater and Sediment Microenvironments: Evaluation of Biotic and Abiotic Factors. Environmental Pollution, 304, 119243 (IF: 9,988; 5-letni IF:10,366; MEiN: 100)
4. Maculewicz, J., **Świacka**, K., Stepnowski, P., Dołżonek, J., & Białk-Bielińska, A. (2022). Ionic liquids as potentially hazardous pollutants: Evidences of their presence in the environment and recent analytical developments. Journal of Hazardous Materials, 129353 (IF: 14,224; 5-letni IF:12,984; MEiN: 200)
5. Maculewicz, J., Kowalska, D., **Świacka, K.,** Toński, M., Stepnowski, P., Białk-Bielińska, A., & Dołżonek, J. (2022). Transformation products of pharmaceuticals in the environment: their fate,(eco) toxicity and bioaccumulation potential. Science of the Total Environment, 802, 149916 (IF: 10,753; 5-letni IF:10,237; MEiN: 200)
6. **Świacka, K.,** Maculewicz, J., Kowalska, D., Caban, M., Smolarz, K., & Świeżak, J. (2022). Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms-Current state of knowledge. Journal of Hazardous Materials, 424, 127350 (IF: 14,224; 5-letni IF: 12,984; MEiN: 200)
7. **Świacka, K.,** Smolarz, K., Maculewicz, J., Michnowska, A., & Caban, M. (2021). Exposure of Mytilus trossulus to diclofenac and 4′-hydroxydiclofenac: Uptake, bioconcentration and mass balance for the evaluation of their environmental fate. Science of the Total Environment, 791, 148172 (IF: 10,753; 5-letni IF:10,237; MEiN: 200)
8. Mulkiewicz, E., Wolecki, D., **Świacka, K.,** Kumirska, J., Stepnowski, P., & Caban, M. (2021). Metabolism of non-steroidal anti-inflammatory drugs by non-target wild-living organisms. Science of the Total Environment, 791, 148251 (IF: 10,753; 5-letni IF:10,237; MEiN: 200)
9. **Świacka, K.,** Michnowska, A., Maculewicz, J., Caban, M., & Smolarz, K. (2021). Toxic effects of NSAIDs in non-target species: A review from the perspective of the aquatic environment. Environmental Pollution, 273, 115891 (IF: 9,988; 5-letni IF:10,366; MEiN: 100)
10. Maculewicz, J., **Świacka, K.,** Kowalska, D., Stepnowski, P., Stolte, S., & Dołżonek, J. (2020). In vitro methods for predicting the bioconcentration of xenobiotics in aquatic organisms. Science of The Total Environment, 739, 140261 (IF: 7,963; 5-letni IF: 7,842; MEiN: 200)
11. **Świacka, K**., Smolarz, K., Maculewicz, J., & Caban, M. (2020). Effects of environmentally relevant concentrations of diclofenac in Mytilus trossulus. Science of the Total Environment, 737, 139797 (IF: 7,963; 5-letni IF:7,842; MEiN: 200)
12. **Świacka, K.,** Maculewicz, J., Smolarz, K., Szaniawska, A., & Caban, M. (2019). Mytilidae as model organisms in the marine ecotoxicology of pharmaceuticals-a review. Environmental Pollution, 254, 113082 (IF: 6,793; 5-letni IF:6,939; MEiN: 100)

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Mytilidae as model organisms in the marine ecotoxicology of pharmaceuticals - A review☆

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Bioindicators

ABSTRACT

Growing production and consumption of pharmaceuticals is a global problem. Due to insufficient data on the concentration and distribution of pharmaceuticals in the marine environment, there are no appropriate legal regulations concerning their emission. In order to understand all aspects of the fate of pharmaceuticals in the marine environment and their effect on marine biota, it is necessary to find the most appropriate model organism for this purpose. This paper presents an overview of the ecotoxicological studies of pharmaceuticals, regarding the assessment of Mytilidae as suitable organisms for biomonitoring programs and toxicity tests. The use of mussels in the monitoring of pharmaceuticals allows the observation of changes in the concentration and distribution of these compounds. This in turn gives valuable information on the amount of pharmaceutical pollutants released into the environment in different areas. In this context, information necessary for the assessment of risks related to pharmaceuticals in the marine environment are provided based on what effective management procedures can be developed. However, the accumulation capacity of individual Mytilidae species, the bioavailability of pharmaceuticals and their biological effects should be further scrutinized.

1. Introduction

Pharmaceuticals are emerging contaminants that are detected in the aquatic environment, all over the world. Exhibiting a broad spectrum of biological activity, these chemicals pose a threat to the aquatic biota (Fent et al., 2006; Sanderson et al., 2004). Acute and chronic toxic effect of some medicines have already been proven (El-Bassat et al., 2011; Franzellitti et al., 2015; Mezzelani et al., 2018a). However, due to the difficulty in detecting pharmaceuticals in the aquatic environment and the conviction that their dilution prevents adverse effects on marine ecosystems, little attention has been paid to this problem so far (Fabbri and Franzellitti, 2016; Gaw et al., 2014). Global consumption data for pharmaceutical products are unclear and not detailed enough, resulting in insufficient information on their amount released into the environment (Brausch et al., 2012; Fabbri and Franzellitti, 2016; Fent et al., 2006; Kümmerer, 2008; Sanderson et al., 2004). Pharmaceuticals' consumption is estimated at 100,000–200,000 tonnes per year in Russia, China, South Africa, India and Brazil (Tijani et al., 2016; Van Boeckel et al., 2014). However, such estimates for other countries with high demand for pharmaceuticals, e.g. USA, UE and Japan, are lacking. Pharmaceuticals are not completely eliminated in Wastewater Treatment Plants (WWTPs), therefore they are easily released into the aquatic environment and eventually reach the marine ecosystems.

Pharmaceuticals enter the aquatic environment both in non-modified form and as metabolites (Fent et al., 2006; Mezzelani et al., 2016a; Ojemaye and Petrik, 2018). Knowledge about the fate of pharmaceuticals in the aquatic environment is still insufficient, mainly due to difficulties in their analysis. Once released into the environment, pharmaceuticals undergo complex processes related to a number of factors. In addition, these processes differ in marine and freshwater environments (Fabbri and Franzellitti, 2016; Ojemaye and Petrik, 2018). The number of studies conducted in the marine environments is relatively small compared to the freshwater studies (Brausch et al., 2012; Fabbri and Franzellitti, 2016;

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Mezzelani et al., 2016a; Ojemaye and Petrik, 2018). Some pharmaceuticals are readily biodegradable or photodegradable, while others, such as hormones, are highly persistent and accumulate in sediments and marine organisms (Gaw et al., 2014; Kümmerer, 2008). Environmental factors such as temperature and pH also affect the chemical and biological properties of pharmaceuticals, for example their metabolism and toxicity (Fabbri and Franzellitti, 2016; Fent et al., 2006; Gaw et al., 2014).

Pharmaceuticals undoubtedly pose a threat to the marine environment and some studies described their negative effects on marine organisms (Bonnefille et al., 2018; Capolupo et al., 2018; McRae et al., 2018; Munari et al., 2018). Moreover, they are constantly released into the environment (Desbiolles et al., 2018; Ericson et al., 2010; McRae et al., 2018; Schwaiger et al., 2004). Due to chronic exposure, even at a low concentrations, pharmaceuticals may affect marine organisms (Ferrari et al., 2003; Parolini and Binelli, 2012). The potential for bioaccumulation and their toxicity to the marine biota have not been sufficiently researched (Boxall et al., 2012; De Oliveira et al., 2016; Du et al., 2015; Franzellitti et al., 2014; Sanderson et al., 2004). The mode of action of pharmaceuticals is not well investigated, because one type of pharmaceutical may act at different levels, altering many physiological and biochemical pathways and molecular mechanisms. These substances may react with each other resulting in a synergistic or antagonistic effect (Comeau et al., 2008; Fent et al., 2006; Mezzelani et al., 2018b).

Therefore, it is important to find the most appropriate model organism that would enable a thorough investigation and better understanding of the effects of pharmaceutical products on marine fauna, as well as their fate in the marine ecosystem. Among the organisms that have the largest potential for this purpose, mussels seem to be the most promising. The aim of this review was to summarize the present state of knowledge and to discuss the potential and challenges arising from the use of Mytilidae mussels in the monitoring of pharmaceuticals and assessing their ecotoxicological effects.

1.1. Pharmaceuticals most frequently detected in the marine environment

The first group of pharmaceuticals most commonly detected in the environment are non-steroidal anti-inflammatory drugs (NSAIDs) (Kümmerer, 2008; Mezzelani et al., 2018b; Nikolaou et al., 2007). NSAIDs are antipyretic and analgesic drugs with rapidly growing consumption (Cleuvers, 2004; Comeau et al., 2008; McRae et al., 2018; Mezzelani et al., 2018b). NSAIDs concentrations detected in aquatic ecosystems worldwide range from few ng/L to several μg/L. The highest concentrations of these chemicals are recorded in places located close to WWTPs (Table 1) (Mezzelani et al., 2016a; Pal et al., 2010; Parolini and Binelli, 2012). Diclofenac, ibuprofen and ketoprofen are the most commonly identified NSAIDs in the aquatic environment (Alygizakis et al., 2016; Bort et al., 1999; Comeau et al., 2008; He et al., 2017; Kasprzyk-Hordern et al., 2008). Low concentrations of these pollutants are detected even in drinking water (Caban et al., 2016; Kasprzyk-Hordern et al., 2008).

Another equally important group of pharmaceuticals detected in marine waters are steroidal hormones, which belong to the group of endocrine disrupting compounds (EDCs) (Jean-Claude and Amiard-Triquet, 2015; Santos et al., 2018; Ting et al., 2017). EDCs are chemical compounds whose structure and mode of action is similar to natural hormones. EDCs interfere with the pathways of the endocrine system and disturb the synthesis, metabolism and functions of natural hormones. EDCs are frequently found in the aquatic environment, mainly in sediments, surface waters and sewage from WWTPs (Barreiros et al., 2016; Santos et al., 2018; Ting et al., 2017). The most dispersed EDCs in the marine ecosystems are: 17β-estradiol (E2, natural hormone) and 17α-ethinylestradiol (EE2, synthetic hormone) (Barreiros et al., 2016; Ricciardi et al., 2016; Smolarz et al., 2017; Ting et al., 2017; Zabrzańska et al., 2015).

Other important groups of pharmaceuticals detected in the marine environment worldwide are: antibiotics, lipid regulators, β-blockers, antiepileptic, anticancer and antidepressants (Białk-Bielińska et al., 2016; Desbiolles et al., 2018; Fent et al., 2006; Liu et al., 2018; Nikolaou et al., 2007; Ojemaye and Petrik, 2018). In Europe, pharmaceutical consumption is significant and three of the most frequently detected: E2, EE2 and diclofenac have been added to the European monitoring list of the surface water bodies (Barreiros et al., 2016; Nikolaou et al., 2007).

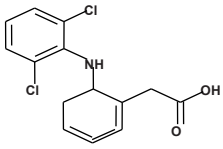
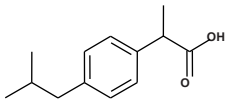
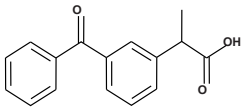
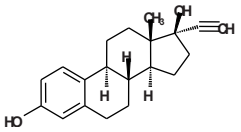
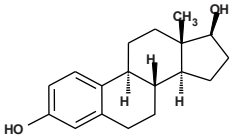
1.2. Lipophilicity of pharmaceuticals as a determinant of their bioaccumulation

Lipophilicity refers to the ability of a compound to dissolve in non-polar substances and lipids (Arnott and Planey, 2012; Waring, 2010). Lipophilic compounds have a high affinity to the hydrophobic phase and are poorly or not soluble in water. The lipophilicity may suggest whether the compound accumulates in sediment, water or biota (Arnott and Planey, 2012; Waring, 2010). For the determination of lipophilicity of the pharmaceutical, the octanol-water partition coefficient (log K_{ow}) is the most commonly used (Arnott and Planey, 2012; Waring, 2010). This coefficient measures the solubility ratio of a substance in a hydrophobic phase, such as octanol, to the hydrophilic (water) phase. Compounds with a high log K_{ow} coefficient easily accumulate in the cell membrane or penetrate into the cells and are harder to remove from the body (Arnott and Planey, 2012; Waring, 2010). According to the Organisation for Economic Co-operation and Development (OECD) guidelines 305 (OECD, 2016), compounds with log $K_{ow} > 3$ have a high potential to bioaccumulate. The lipophilicity of a compound is also determined by pH. If the pH is 8.0, non-ionized compounds like some pharmaceuticals are bioaccumulated by marine mussels due to their higher lipophilicity (Ojemaye and Petrik, 2018). Example of pharmaceuticals characterised by high lipophilicity are synthetic hormones such as ethynyloestradiol (EE2) (Fent et al., 2006). Log K_{ow} values of selected pharmaceuticals with potential for bioaccumulation are presented in Table 1.

2. Methods

To prepare this review a search for publications was conducted between June 2018 and March 2019 using publicly available databases of peer-reviewed scientific articles. The included databases were, among others, Science Direct, Google Scholar and Scopus. Various combinations of keywords directly relevant to the main topic were used to search these databases, including studied pharmaceuticals in combination with species names of Mytilidae family and terms such as 'bioconcentration', 'bioaccumulation' and toxicity effects of selected key pharmaceuticals. This resulted in formulation of three-level keywords based on the scheme "Search = species name + pharmaceutical type + concentration/accumulation/effect". The selection of articles for inclusion in this work was done by evaluating their relevance to the main topic. The use of at least one of the Mytilidae species was a key factor in the inclusion of data in this work. Moreover, only the main representatives of the most commonly consumed pharmaceutical groups: NSAIDs, EDCs, SSRIs, antihistamines, β-blockers and antibiotics were selected for this work. Particularly the pharmaceuticals that were studied in at least two publications were considered. The

Table 1
Concentrations of non-steroidal anti-inflammatory drugs and estrogenic hormones found in different countries.

Pharmaceutical		log K_{ow}	Water	Country	Range [ng/L]	References
NSAIDs						
Diclofenac		4.50[a]	Warta River	Poland	17–486	Kasprzyk-Hordern et al. (2008)
			Tyne River	UK	120–1036	Roberts and Thomas (2006)
			North Sea	Belgium	660	Wille et al. (2010)
			Mediterranean Sea	Greece	20	Alygizakis et al. (2016)
				Spain	4	Gros et al. (2012)
				France	1500	Togola and Budzinski (2008)
			Indian Ocean	Singapore	4–38	Wu et al. (2010)
				Taiwan	<2.5–53.6	Fang et al. (2012)
			Atlantic Ocean	Portugal	3–30	Paíga et al. (2015)
				Spain, Gran Canaria	23.7–343.6	Afonso-Olivares et al. (2013)
				Brazil (Santos Bay)	20	Pereira et al. (2016)
				Ireland	60–460	McEneff et al. (2014)
Ibuprofen		3.72[b]	Warta River	Poland	12–76	Kasprzyk-Hordern et al. (2008)
			Tyne River	UK	1979 –33,764	Roberts and Thomas (2006)
			Umgeni River	Southern Africa	229–278	Ngubane et al. (2019)
			Norwegian Sea	Norway (Tromsø)	0.7	Weigel et al. (2004)
			North Sea	Norway, Germany and Belgium	0.57–22	Brumovský et al. (2016)
			Indian Ocean	Southern Africa (Blue lagoon beach)	166	Ngubane et al. (2019)
				Singapore	41–121	Wu et al. (2010)
				Taiwan	<2.5–57.1	Fang et al. (2012)
			Atlantic Ocean	Portugal	6–110	Paíga et al. (2015)
				Brazil (Santos Bay)	2,090	Pereira et al. (2016)
Ketoprofen		2.81[b]	Warta River	Poland	6–47	Kasprzyk-Hordern et al. (2008)
			Mediterranean Sea	Spain	<8	Gros et al. (2012)
			Baltic Sea	Poland	34.9	Borecka et al. (2015)
			North Sea	Norway, Germany and Belgium	0.25–9.7	Brumovský et al. (2016)
			Atlantic Ocean	Portugal	10–17	Paíga et al. (2015)
					89.7	Lolić et al. (2015)
				Spain, Gran Canaria	41.6–106.3	Afonso-Olivares et al. (2013)
			Indian Ocean	Taiwan	>1.7–6.59	Fang et al. (2012)
Estrogenic hormones						
17α-ethinylestradiol (EE2)		3.70 –3.90[c]	River	Germany	0.1–8.9	Kuch and Ballschmiter (2001)
			Acushnet River	USA	3.01–4.67	Zuo et al. (2006)
			Mediterranean Sea	Italy	<0.8–28	Pojana et al. (2007)
			Atlantic Ocean	Brazil (Santos Bay)	<2.0	Lisboa et al. (2013)
			Pacific Ocean	Japan (Suruga Bay)	<3	Hashimoto et al. (2007)
17β-estradiol (E2)		3.10 –4.00[c]	River	Germany	0.15–5.2	Kuch and Ballschmiter (2001)
			Acushnet river	USA	0.56–0.83	Zuo et al. (2006)
			Baltic Sea	Germany	2.4–17.2	Beck et al. (2005)
			Mediterranean Sea	Italy	<1.0–175	Pojana et al. (2007)
			Atlantic Ocean	Brazil (Santos Bay)	<3.0–18.2	Lisboa et al. (2013)
				Canada (St. John's harbour)	1.8	Saravanabhavan et al. (2009)
			Pacific Ocean	Japan (Suruga Bay)	<1	Hashimoto et al. (2007)

[a] Sahar et al. (2011).
[b] Trinh et al. (2011).
[c] Yoon et al. (2003).

other groups of compounds were omitted due to insufficient number of reports, rare presence in the environment and doubts or lack of data in the context of the impact on mussels. In total, 81 articles on bioaccumulation and toxic effects of pharmaceuticals on Mytilidae mussels were selected for use in this study. The data were complemented by 53 articles on the presence of pharmaceuticals in the aquatic environment and 40 articles concerning general information presented. During the database research, studies were found including four species from the Mytilidae family: *M. edulis*, *M. trossulus*, *M. galloprovincialis* and *M. californianus.*

3. Mussels as bioindicators

3.1. Characteristic of mussels

Bivalves are a typical representatives of the aquatic fauna and belong to the Mollusca phylum. They are widespread, benthic and sedentary organisms characterised by burrowing lifestyle, but various species (those considered in the current review) live attached to the hard substrate by byssus threads (Bayne, 1976a,b; Dailianis, 2011; Gosling, 2008). Bivalves reproduce by ejection of gametes to the water column, where the random fertilization occurs. The dominant factors determining their distribution worldwide are salinity, temperature and type of substrate (Bayne, 1976a,b; Gosling, 2008). Bivalves constitute the main source of food for animals like fish, birds, crustaceans and starfish, thus they are an important link in the food chain (Bayne, 1976a,b; Dailianis, 2011; Gosling, 2008). Mussel clusters create a comfortable substrate for many organisms such as barnacles, algae, crustaceans, some larvae and eggs (Bayne, 1976a,b; Gosling, 2008). They are also important in terms of economy. In many countries, mussels are considered delicacies and are grown in maricultures (Barros et al., 2009; Gosling, 2008).

The use of bivalves in the ecotoxicological studies of pharmaceuticals has many advantages. Filter and/or facultative filter feeding and sedentary lifestyle favour the absorption and bioaccumulation of drugs and encourages bivalves' involvement in biomonitoring (Boillot et al., 2015; Capolupo et al., 2016; Maranho et al., 2015; Morales-Caselles et al., 2008; Moschino et al., 2011; Orbea et al., 2006). Mussels are resistant organisms and they can thrive even in the most unfavourable conditions. Their breeding is easy and thus they are often involved in the laboratory and field experiments (Bayne, 1976a,b; Dailianis, 2011; Mezzelani et al., 2016b). In addition, marine bivalves have a slower metabolic rate compared to vertebrates or crustaceans and are therefore better bioindicators (Farrington et al., 2016).

3.2. Mytilidae family

One of the bivalves families, which is well researched and involved in various ecotoxicological studies around the world, is Mytilidae (Capolupo et al., 2016; Marigómez et al., 2013; Viarengo et al., 2007). The main representatives of the family Mytilidae and their distribution are as follows: *Mytilus coruscus* (coasts of the Western Pacific Ocean), *Mytilus californianus* (coast of the North Eastern Pacific Ocean), *Mytilus edulis* (North Atlantic region), *Mytilus galloprovincialis* (Mediterranean) and *Mytilus trossulus* (northern Pacific and the Baltic Sea) (Beyer et al., 2017).

A number of field studies involving this family has already been performed. Representatives of Mytilidae such as *M. edulis* are widely used as indicators in numerous works on the effect of xenobiotics on stress biomarkers due to their well-known physiology (Bao et al., 2018; Cappello et al., 2018; Höher et al., 2015; Lacroix et al., 2017). For example, Boissel et al. (2017) used *M. edulis* to study the effects of cadmium on expression of genes involved in phagocytosis. De los Ríos et al. (2013) tested the immunological responses of *M. galloprovincialis* to the urban discharges. For the purpose of toxicity assessment, specific biomarkers were used. In addition, Mytilidae are often involved in numerous biomonitoring programs (Chiesa et al., 2018; Höher et al., 2015; Lacroix et al., 2017). Another advantage of Mytilidae is their wide geographical distribution. Species belonging to this family can be found in the coastal areas of almost every continent and their local populations are usually large and stable. As a result, studies involving this ubiquitous group can be carried out in different parts of the world and can be repetitive (Bayne, 1976a,b; Dailianis, 2011; Farrington et al., 2016). Furthermore, Mytilidae usually live in high concentrations and are therefore organisms suitable for effortless collection (Cuevas et al., 2015; Dailianis, 2011; Gosling, 2008).

4. Ecotoxicological studies involving Mytilidae

4.1. In-situ researches

4.1.1. Biomonitoring programs and studies

Biomonitoring is becoming one of the most common methods for analysing xenobiotics concentration, determination and effects in the aquatic environment. In many countries, e.g. France, USA, Spain, Great Britain and China, native mussels, mainly oysters and blue mussels, have been used as bioindicators for the detection of contaminants in the 1980s and 1990s (Bayne, 1976a,b; Farrington et al., 2016; Goldberg et al., 1978; Sericano et al., 1995). Professor Edward D. Goldberg was the first to propose the monitoring of contaminants using wild bivalves. He pursued first biomonitoring program “Mussel Watch” involving oysters and blue mussels. The program lasted for three years (1976, 1977 and 1978) and there are no previous biomonitoring studies with the use of mussels (Farrington et al., 2016). Within the biomonitoring framework, mussels were collected annually from about 100 stations located along the United States coast. In addition, in order to provide information on the pollution of the California coast in a shorter period of time, samples were taken monthly and bimonthly at Narragansett Bay and Bodega Head stations in California (Farrington et al., 2016). However, this program focused on contaminants such as trace metals, petroleum hydrocarbons and organochlorinated compounds, while monitoring of pharmaceuticals with the use of mussels has only recently started to develop (Bajt et al., 2019; Bayne, 1976a,b; Farrington et al., 2016; Maruya et al., 2014).

One of the largest local biomonitoring programs, which includes the monitoring of pharmaceuticals in mussels, is the Surface Water Ambient Monitoring Program (SWAMP) (Farrington et al., 2016; Goldberg et al., 1978; Sericano et al., 1995). The creation of this program was already proposed in 1999 and since then SWAMP has been constantly evolving. The main goal of this program is the assessment of the quality of California coastal waters. Mussel Watch Pilot Project on Emerging Contaminants is a part of SWAMP program using *M. edulis* and *M. californianus* for monitoring purposes (Farrington et al., 2016). Synthetic hormones and some analgesics are among contaminants monitored in mussel tissues. The main objectives pursued by the use of marine bivalves in water monitoring are the following:

a) Assessment of the presence of analyzed compounds in a given area and determination of their concentrations and extent. Monitoring how the range and concentration of pollutants change as a function of time (Bayne, 1976a,b; Beyer et al., 2017;

Farrington et al., 2016; Moschino et al., 2016; Richman et al., 2011).

b) Development of the effective monitoring methods using mussels and further chemical analysis of contaminants, which can be comparable between laboratories. Extension of these methods to the entire world (Bayne, 1976a,b; Beyer et al., 2017; Farrington et al., 2016; Moschino et al., 2016; Richman et al., 2011).

The involvement of mussels in pharmaceuticals' biomonitoring programs can bring many benefits, enabling a better diagnosis of environmental pollution (Bajt et al., 2019; Maruya et al., 2014). However, there are some weaknesses in the use of mussels in biomonitoring studies. In general, most of wild living mussels are not clinically healthy (Carella et al., 2018). They are affected by various biotic and abiotic stressors thus the presence of pathological changes of unknown nature is often inevitable. On the other hand, the absence of lesions in mussels may indicate the high environmental quality and low level of pollution. Environmental and anthropogenic factors affect the fitness of mussels, therefore it is difficult to determine the effect of the tested xenobiotics (Carella et al., 2018). To reduce environmental impact and to balance naturally occurring inter-individual variability, biomonitoring shall be performed using a large number of organisms.

Unfortunately, the number of studies dedicated to pharmaceuticals is still relatively low in comparison to those devoted to heavy metals (Kraak et al., 1991; Lafabrie et al., 2007; Rainbow et al., 2000; Richman et al., 2011), organic pollution monitoring (Lehtonen et al., 2016; Maisano et al., 2017; Okay et al., 2017) and plastic monitoring (Farrington et al., 2016; Li et al., 2018). For the purposes of pharmaceuticals monitoring, the most common method is the use of wild mussels, native to the monitored area (Caban et al., 2016; Marigómez et al., 2013). However, in places where mussels do not occur and which are potentially contaminated with pharmaceuticals, the cage placement method including representatives of Mytilidae may be employed (Bajt et al., 2019; Cappello et al., 2017b; Farrington et al., 2016; Freitas et al., 2015; Lehtonen et al., 2016; Maisano et al., 2017; Marigómez et al., 2013; Moschino et al., 2016; Serafim et al., 2011). The possibility to determine the pollution gradient in the defined area is yet another advantage (Lehtonen et al., 2016). The main limitations in the use of wild mussels in cause-and-effect and biomonitoring studies result from the impact of many factors, both environmental and biological, e.g. availability of food, reproductive status, presence of blooms and presence of other xenobiotics (Farrington et al., 2016). Therefore, it is not possible to investigate short-term temporal and spatial variability in bioconcentration of pharmaceuticals in the environment without the inclusion of laboratory tests (Farrington et al., 2016). Mussels are constantly accumulating pharmaceuticals in the area of their distribution. The concentrations of the analyzed pharmaceuticals, depending on their bioavailability and other properties, may be significantly higher in mussels than in the environment (Farrington et al., 2016). It is more likely that the compound will be detected in mussel tissues than in the water or sediments (Farrington et al., 2016; Moreno-González et al., 2016). However, the accumulative capacity of individual species of mussels to the selected pharmaceuticals should be more thoroughly studied. Depending on the predisposition of the species and chemical properties of tested pharmaceutical, mussels may completely metabolize and excrete the pharmaceutical, which in consequence may give false information about the absence of pharmaceutical in the area of interest (Almeida et al., 2014; Farrington et al., 2016; Moreno-González et al., 2016; Teixeira et al., 2017).

4.1.2. Examples of field experiments

Pharmaceuticals in WWTPs effluents reach relatively high concentrations, which increases the likelihood of their determination. Therefore, at the beginning most of the screening tests were carried out in the estuaries of WWTPs. Over time, however, mussels began to be used to detect pharmaceuticals in places away from WWTPs. The use of mussels for the detection of pharmaceuticals in the environment has therefore become popular and has enabled the detection of these chemicals in places other than the WWTPs outlets. In places where mussels do not occur, the cage method is used.

For example, Turja et al. (2014) investigated the effect of sewage from WWTP on physiological processes in *M. trossulus* in the Gulf of Finland. Mussels were transplanted and kept in cages for one month. Exposition groups were placed close to the discharge of WWTP. All cages were equipped with special samplers: Polar Organic Chemical Integrative Sampler (POCIS) measuring the concentration of dissolved pharmaceuticals. In order to assess the ecotoxicological impact of pollutants, a multibiomarker approach was used with selected biomarkers response, cellular energy allocation and morphometric condition index (CI) were performed. DNA damage and bioconcentration of organic compounds and trace metals were also investigated. The exposed mussels had damaged gills and the activity of antioxidant enzymes (superoxide dismutase and glutathione reductase) was significantly higher in the caged group. High level of DNA damage was also observed. This study also confirmed the occurrence of some pharmaceuticals from NSAIDs group in marine water. Data from POCIS suggest a higher number of pharmaceuticals at the stations closest to the WWTP. Nine out of nineteen analyzed pharmaceuticals were detected in water, mainly from the NSAIDs group. Unfortunately, the presence of these pharmaceuticals in the tissues of *M. trossulus* has not been analyzed. Lehtonen et al. (2016) also researched the impact of sewage effluents on mussels using the same method and species, *M. trossulus*. The research was carried out at the northern part of the Baltic Sea and the exposure time was four months. In order to investigate the toxicological impact of wastewater, selected biological endpoints including biochemical biomarkers and growth were performed. Unfortunately, the presence of pharmaceuticals in mussel tissues and in water has not been tested. Nevertheless, the results showed a negative effect of sewage on the condition of mussels and highlighted the occurrence of seasonal changes in the concentration of the tested compounds in mussel tissues. Moreover, the observed differences in enzymatic activity were more related to environmental factors than to the negative impact of the tested substances. Capolupo et al. (2017) investigated bioaccumulation and toxic effect of pharmaceuticals (EDCs, analgesics, anti-epileptics and β-blockers) in the sewage by employing *M. galloprovincialis*. Mussels were transplanted for 28 days in the coastal lagoon of Adriatic Sea, near the industrial and harbour complex. For the purpose of ecotoxicological evaluation, biomarker analysis was also incorporated. Diclofenac was the only drug detected in mussel tissues, and the concentrations of the remaining 6 pharmaceuticals were below the limit of detection (Table 2). Interestingly, some authors (D'Agata et al., 2014; Parrino et al., 2019) have highlighted the significant influence of anthropogenic loads on the hemocyte variation in Mytilidae mussels.

Three of presented field studies showed, among other things, the negative impact of sewage on marine organisms, which was confirmed by the disturbance of physiological processes of bivalves. However, this effect cannot be attributed only to pharmaceuticals, as the treated wastewater contains a mixture of different pollutants. Several studies have revealed that chronic exposure to treated wastewater affects the condition of mussels. For example, Zouiten et al. (2016) also investigated the ecotoxicological potential of

Table 2
Selected bioconcentration studies of pharmaceuticals involving Mytilidae.

Model organism	Number of mussels	Tested pharmaceuticals	Concentrations [ng/g]		References
			laboratory	field	
M. trossulus	n/a	Diclofenac	180 ± 0.02	–	Ericson et al. (2010)
		Ibuprofen	160 ± 0.02	–	
	10–151	Paracetamol	–	80	Caban et al. (2016)
		Flurbiprofen	–	210	
		EE2	–	310	
	10	Diclofenac	7780	–	Świacka et al. (2019)
M. edulis	n/a	Estrone	–	0.30 ± 0.10	Saravanabhavan et al. (2009)
	4	EE2	103	–	Ricciardi et al. (2016)
		4-nonylphenol	196.29	–	
	5	Oxolinic acid	0.5 ± 0.05	–	Le Bris and Pouliquen (2004)
		Oxytetracycline	1.83 ± 0.82	–	
Mytilus spp.	10	Carbamazepine	12,100 ± 0.4	–	McEneff et al. (2013)
		Diclofenac	13,000 ± 0.2	–	
		Gemfibrozil	4000 ± 0.0	–	
		Mefenamic acid	16,000 ± 0.5	–	
		Trimethoprim	21,700 ± 1.4	–	
	50	Tetracycline	–	0.55	Chiesa et al. (2018)
M. galloprovincialis	5	Diclofenac	–	2.1–4.6	Capolupo et al. (2017)
	15	4′OH-Diclofenac	89.0 ± 42.7	–	Bonnefille et al. (2017)
		5′OH-Diclofenac	10.7 ± 4.7	–	
	50	Azithromycin	–	13.3	Álvarez-Muñoz et al. (2015)
	8	Carbamazepine	0.53–13	–	Oliveira et al. (2017)
	5	Cetirizine	0.2 ± 0.1	–	Teixeira et al. (2017)
			12.2 ± 1.8		
	10	Fluoxetine	–	4.83	Silva et al. (2016)
		Norfluoxetine	–	13.52	
		Citalopram	–	3.26	
		Desmethylcitalopram	–	6.60	
		Norsertraline	–	6.68	
		Paroxetine	–	<LOD	
		Sertraline	–	<LOD	
		Testosterone	0.60–0.84	–	
	25	Diclofenac	–	0.5–4.5	Cunha et al. (2017)
	6	Acetaminophen	<LOD	<LOD	Mezzelani et al. (2016a)
		Diclofenac	14.90 ± 7.89	16.11 ± 14.72	
		Ibuprofen	1.63 ± 1.00	9.39 ± 0.59	
		Ketoprofen	<LOD	<LOD	
		Nimesulide	30.22 ± 13.50	4.18 ± 2.54	
	n/a	Amoxicillin	–	709	Zouiten et al. (2016)
		Oxacilin	–	206	

LOD - limit of detection.
n/a - not available.

sewage and the presence of antibiotic in *M. galloprovincialis* exposed to pharmaceutical effluent. Mussels from the cultured population were exposed to various dilutions of sewage from the pharmaceutical industry in Tunisia. To assess DNA damage, a comet assay was performed. As a result of exposure to sewage, DNA damage was observed in gill cells of mussels. The observed effects of pharmaceutical effluent were dose-depended. Higher DNA damage was observed in the group of mussels from the higher sewage concentrations. Furthermore, two antibiotics, oxicillin and amoxicillin, were detected in mussels exposed to 30% diluted pharmaceutical effluent (Table 2). Chiesa et al. (2018) used Mytilidae mussels for the antibiotic monitoring purposes. The occurrence of 29 selected antibiotics in various Italian areas was investigated. Among others, oxytetracycline was found in mussels (Table 2). In addition, mussels were found to be a better bioindicators for detecting oxytetracycline than fish.

Mezzelani et al. (2016a,b) study was the first to show the presence of diclofenac, nimesulide and ibuprofen in mussels *M. galloprovincialis* sampled from several potentially unpolluted stations in the Central Adriatic Sea, Portonovo Bay (Table 2). Mussels were collected in July, August and September. Acetaminophen and ketoprofen were not detected in mussel tissues. On the other hand, Caban et al. (2016) analyzed the occurrence of NSAIDs (ibuprofen, diclofenac, naproxen, ketoprofen and flurbiprofen), paracetamol and synthetic hormone (EE2) in *M. trossulus*. Mussels were collected from the Gulf of Gdańsk (Baltic Sea) near the discharge of WWTP. The presence of pharmaceuticals was also determined in water. Paracetamol, ketoprofen and flurbiprofen were detected in water, while paracetamol, flurbiprofen and EE2 were detected in mussels (Table 2). EE2 was found in the tissues of mussels, although it was not detected in water. This indicates that *M. trossulus* is a good bioindicator in certain EDCs monitoring. This research also showed a poor condition of mussels, which may resulted from the negative impact of sewage from WWTP. Additionally, the presence of estrogenic (E1, E2, E3) and androgenic (T) hormones in blue mussel tissues inhabiting the WWTP outlet area were reported by Zabrzańska et al. (2015), Smolarz et al. (2018) and Hallmann et al. (2016). The presence of estrogenic hormones (estrone, E2, EE2 and diethylstilbestrol) in native mussels was also tested by Saravanabhavan et al. (2009). For the EDCs monitoring purposes *M. edulis* was used. Mussels were collected in two Canadian ports. The occurrence of these estrogenic hormones in water was also analyzed. Only small traces of estrone were detected in the mussel tissues (Table 2) and trace amounts of estrone and E2 were present in the water. EE2 was not detected in mussels from Canadian ports. However it was detected among others in *M. edulis*

trossulus from the Baltic Sea (Caban et al., 2016; Hallmann et al., 2016) and in *M. edulis* used in accumulation research of EE2 (Ricciardi et al., 2016).

Several species of Mytilidae were used to study the presence of pharmaceuticals in the environment. The most frequently researched areas were the discharges of treated wastewater. Some of these studies indicate the presence of pharmaceuticals in mussels, which may suggest the ability of mussels to accumulate these chemicals. However, the presence of drug metabolites, which may also pose a threat to marine biota has not been tested neither in mussels nor in the environment. Fig. 1 shows the summary of different effects caused by certain classes of pharmaceuticals to different target.

4.2. Laboratory experiments - bioaccumulation and ecotoxicology evaluation

4.2.1. Nonsteroidal anti-inflammatory drugs (NSAIDs)

NSAIDs is a group of pharmaceuticals most frequently detected worldwide due to their high consumption and their pseudo-persistence (Cleuvers, 2004; Ericson et al., 2010; Fent et al., 2006). As numerous studies have shown, the presence of NSAIDs in the marine environment may pose a threat to non-target organisms (Ghelfi et al., 2016; He et al., 2017; Nassef et al., 2010). Therefore, researchers pay a special attention to this group of pharmaceuticals. The following section presents examples of laboratory tests using mussels from culture or native mussels.

Ericson et al. (2010) investigated the bioaccumulation and the ecotoxicological impact of diclofenac and ibuprofen using *M. edulis trossulus.* Mussels were collected by scuba divers from the Baltic Sea, then exposed to diclofenac and ibuprofen for seven days. During experiment mussels were fed with ground fish food and microalgae (*Thalassiosira weissflogii* and *Isochrysis galbana*). The sand filtered sea water collected in Askö was used to obtain a salinity corresponding to the collection site. Bioaccumulation was evaluated using the bioconcentration factor (BCF, L/kg). In addition, the ecotoxicological effects of pharmaceuticals were investigated. The parameters used to assess the toxicity were: scope for growth (SFG), byssus strength and abundance of byssus threads. In the byssus strength experiment carried out over eight days 30 individuals were used and in the SFG experiment 10 mussels were used. The results of this study show the potential for bioaccumulation of diclofenac and ibuprofen in *M. edulis trossulus* (Table 2). Interestingly, the higher BCF was observed for mussels exposed to lower concentration of NSAIDs. This study was the first to show the negative effect of diclofenac and ketoprofen on byssus threads and SFG. Toxic effect of pharmaceuticals was manifested by weakening of strength and reduction of the abundance of byssus threads as well as SFG disorder. In addition, a significant correlation was observed between the concentration of pharmaceuticals and their negative impact on byssus. Mezzelani et al. (2016a) also investigated the ecotoxicological effect and bioaccumulation of the following NSAIDs: acetaminophen, nimesulide, diclofenac, ibuprofen and ketoprofen. The model organism was *M. galloprovincialis* sampled from the Adriatic Sea. In order to assess the toxic potential of the analyzed pharmaceuticals, biomarker analysis, bioaccumulation test and cellular analysis were performed. DNA damage was also evaluated. The results of this study as well as study by Ericson et al. (2010) also show the potential for bioaccumulation of diclofenac, ibuprofen and nimesulide by *M. galloprovincialis.* Ketoprofen and acetaminophen were not detected in exposed mussels and in mussels from the environment (Table 2). The metabolism of these pharmaceutical products and their excretion may explain their absence in mussel tissues. These chemical compounds may also not be bioavailable for mussels. Biomarker analysis showed that all tested NSAIDs significantly induced antioxidant defences (Mezzelani et al., 2016a). NSAIDs affect lysosomal membrane stability and inhibit acyl-coenzyme A oxidase and catalase activities (Mezzelani et al., 2016a). Genotoxic effects of ibuprofen and diclofenac were also observed, which was manifested as an increase in the frequency of micronuclei in mussels.

On the other hand, Schmidt et al. (2012) investigated the effect of diclofenac on biomarkers of cellular detoxification (glutathione S-transferase (GST) and metallothionein (MT)), oxidative stress (lipid peroxidation (LPO) and DNA damage) genotoxicity (DNA damage) and reproduction (vitellin-like proteins) measured in *Mytilus* spp. mussels. In contrast to other studies where mussels were exposed to a chemical added to water, in this case the pharmaceutical was injected (10 μl of solution containing 1 μg/L or 1000 μg/L of diclofenac) into the posteriori adductor muscle of each mussel. No effect on MT expression was observed during the experiment. However, a statistically significant increase in GST activity and a decrease in lipid peroxidation were noted. In case of DNA damage no statistically significant changes were observed after 24 h, but after 72 h this indicator was lower than for control.

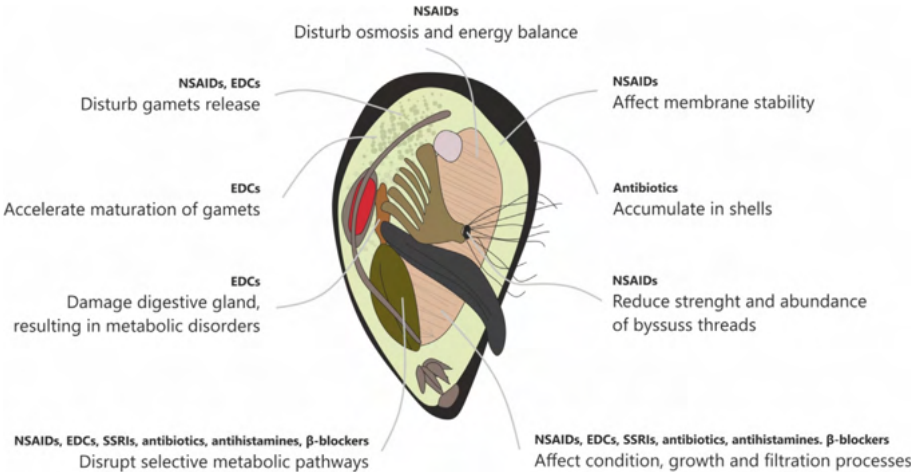


Fig. 1. The most frequent effects of pharmaceuticals on physiological processes in mussels.

On the other hand, no effect on vitellin-like proteins was observed in this study. In a further work by Schmidt et al. (2014), *Mytilus* spp. mussels were exposed for 14 days to diclofenac added to water and then maintained for a further 7 days to monitor their recovery. The organisms were exposed to two concentrations: 1 μg/L and 1000 μg/L. In this study, the influence of the pharmaceutical on biomarkers was also determined, taking into account the activity of GST, LPO and DNA damage. Additionally, changes in protein expression signature (PES) were determined. A significant decrease in both GST expression and LPO was observed in the experiments. In the case of DNA damage, a decrease of this indicator was observed after 14 days for both concentrations, whereas after a 7-day recovery phase, for mussels exposed to 1000 μg/L it was significantly higher. Moreover, proteomic analysis showed that diclofenac induces oxidative stress and seems to have negative effects on the immune response of blue mussels. Several studies showed the impact of diclofenac on early developmental stages of *M. galloprovincialis*, where a significant effect was observed in embryo-toxicity assay (Balbi et al., 2018; Fabbri et al., 2014). It was also observed that diclofenac disturbed osmosis and gametes release in Mytilidae mussels (Bonnefille et al., 2018). Modulation of osmosis in mussels results in disturbance of cell homeostasis, including a reduction in the amount of free amino acids involved in cell volume regulation and an abnormal excretion of nitrogen compounds from the cells (Gosling, 2008). This ultimately results in disruption of the body's energy balance and stress (Bonnefille et al., 2018; Hawkins and Hilbish, 1992).

The fate of pharmaceuticals in the marine environment is not sufficiently investigated, but there are studies confirming uptake of drugs by mussels. However, it is still largely unknown whether these chemical compounds are metabolised, accumulated or perhaps excreted unchanged. Therefore, the research of pharmaceutical metabolites is a priority challenge. Metabolites may be bioavailable and more toxic than the primary compound and may therefore pose a serious threat to the environment. Study by Bonnefille et al. (2017) is one of the first that focused on the analysis of NSAIDs metabolites in mussels. The authors detected 13 metabolites of diclofenac in tissues of *M. galloprovincialis*, five of which were novel, reported for the first time in aquatic organisms (Table 2). Interestingly, two of these metabolites: 4-OH diclofenac and 5-OH diclofenac are the main metabolites excreted by vertebrates including humans. Laboratory tests carried out by Świacka et al. (2019) also proved that a member of Mytilidae - *Mytilus trossulus* metabolizes diclofenac to 4-OH and 5-OH derivatives. What is more, *M. trossulus* accumulated diclofenac and the accumulation of the pharmaceutical was dependent on the exposure time (Table 2).

So far, particular attention has been paid to the negative effects of pharmaceuticals on organisms, but Courant et al. (2018) tested the desired effect of diclofenac, namely the treatment of inflammation, in blue mussels. After 3 days, the anti-inflammatory effect of the pharmaceutical in the body of mussels was observed - the reduction of prostaglandin E2 synthesis (Courant et al., 2018; Lacroix et al., 2017). Higher drug concentrations inhibited the synthesis of prostaglandin, a hormone that contributes to the formation of the inflammation. One of the most valuable studies concerning the effects of pharmaceuticals on organisms are the investigations of metabolic pathways. Metabolic profile studies can provide important knowledge on specific molecular signatures in specific disease conditions, as well as molecular responses resulting from the action of some xenobiotics (Bonnefille et al., 2018; Cappello et al., 2018; Courant et al., 2014; Teixeira et al., 2017). Interesting example of such studies is work by Bonnefille et al. (2018). The target organism was *M. galloprovincialis* exposed to diclofenac at 1 and 100 μg/L. The metabolic pathways of tyrosine and tryptophan were investigated. In the treatment group the signs of tyrosine-dopaminergic pathway were reduced, while the signals of tryptophan metabolic pathway were strengthened. In the metabolism of tryptophan an increase in serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) activity was observed. Interestingly, in comparable studies involving vertebrates there were no changes in the synthesis of serotonin and 5-HIAA due to diclofenac exposure (Guiloski et al., 2017). The disturbance of metabolic pathways of tryptophan and tyrosine may also affect the reproduction and osmosis. Catecholamine and serotonin are indeed important for gametes multiplying, their release and sex maturation of mussels (Bonnefille et al., 2018; Fong et al., 1993b, 1993a; Gibbons and Castagna, 1984).

The negative effects of NSAIDs were manifested mainly through disturbances of osmosis, reproduction, cell homeostasis and energy balance. The modulations of metabolic pathways, weakening of byssus strength and anti-inflammatory potential were also observed. Diclofenac is one of the most tested pharmaceuticals, but more attention should also be paid to others analgesics and their effects. Mytilidae representatives have shown to be an appropriate model organisms to investigate NSAIDs.

4.2.2. Endocrine disrupting compounds (EDCs)

Mytilidae mussels are also successfully used for ecotoxicological studies and monitoring of hormonal compounds (Álvarez-Muñoz et al., 2015; Aris et al., 2014; Ricciardi et al., 2016). Monitoring of EDCs is very difficult because the concentrations of endocrine compounds that occur in the aquatic environment are often below the detection limit. The viable alternative is the use of mussels (Álvarez-Muñoz et al., 2015; Ricciardi et al., 2016). A number of studies have shown that mussels uptake and accumulate these compounds. However, the ratio of the amount of accumulated EDCs in different tissues of mussels is not known and remains the future challenge (Ricciardi et al., 2016). Some studies have shown a negative effect of selected EDCs on Mytilidae mussels. Ricciardi et al. (2016) investigated the uptake and depuration of two EDCs: EE2 and 4-NP (4-nonylphenol) in selected tissues of *M. edulis*. Exposure period was 38 days and depuration period was 35 days. The highest concentration of tested pharmaceutical was found in the digestive gland, then in the gills and remaining viscera (Table 2). This work suggest to use only digestive gland for EDCs monitoring. EDCs can affect the expression level of steroid enzymes, for example 17β-hydroxysteriod dehydrogenase. 17β-hydroxysteriod dehydrogenase is an important enzyme involved in conversion of precursor steroids to estrone, P450 aromatase and testosterone which are involved in reproductive processes. As a result, EDCs disrupt the reproduction of mussels (Janer et al., 2005; Ricciardi et al., 2016; Zhang et al., 2014). However, in various other experiments, none or very minor reproductive effects were observed after exposure of mussels to steroid hormones, indicating insufficient knowledge and scientific disagreement on the origin and role of steroid hormones (and other steroid simulants) in marine mussels (Morthorst et al., 2014; Scott, 2013). Reproduction is one of the most energy consuming processes and can disrupt other functions of organism. During the reproductive cycle mussels are more sensitive to environmental and anthropogenic factors (Pampanin et al., 2005; Travers et al., 2009). Several studies have shown changes in the gene expression, including hormonal response genes, occurring under the influence of E2 in the digestive gland of female mussels (Canesi et al., 2011; Ricciardi et al., 2016). Interestingly, changes in the level of steroid hormones in the glands may lead to adverse effects in gonadal tissues (Canesi et al., 2011; Ricciardi et al., 2016). Study by Ricciardi et al. (2016) showed that

exposure to E2 leads to digestive gland damage, which ultimately results in metabolic disorders (Canesi et al., 2011; Ricciardi et al., 2016). Bioconcentration studies in different organs give an insight into what happens with the pharmaceutical in the body. Study by Ciocan et al. (2010) showed a toxic effect of EE2 on gene expression and gametogenesis. Exposure of *M. edulis* to E2 and EE2 at 5 ng/L and 50 ng/L induced changes in the expression of vitellogenin and vitellin genes, estrogen receptors and specific testis kinase. This suggests that the tested EDCs disturb the reproduction of mussels. Correlation in ER2 expression mRNA with the exposure to EE2 was observed (Ciocan et al., 2011, 2010). Capolupo et al. (2018) also investigated the effect of EE2 on gamete fertilization and embryonic development of *M. galloprovincialis*. To determine the effect of EE2 on embryonic development of *M. galloprovincialis* and gamete fertilization special bio-assays were performed. It was observed that EE2 at environmentally relevant concentration 500 ng/L significantly inhibited gamete fertilization. Fabbri et al. (2014) observed the same effect of EE2 as Capolupo et al. (2018) when the embryos of mussels were treated with EE2 at concentrations 0.01–1000 μg/L. Additionally, Fabbri et al. (2014) revealed these effects were accompanied by a reduction in the number of transcripts of estrogen receptors. Juvenile organisms are the most threatened by the effects of pharmaceuticals, as the organs involved in drug metabolism and detoxification are poorly developed in these forms (Mohammed, 2013). Juvenile individuals create and develop populations, thus they are an important indicators of population condition. The use of the representative species in their most sensitive life-stage should be considered in ecotoxicological studies (Balbi et al., 2016; Capolupo et al., 2018; Estévez Calvar et al., 2017; Franzellitti et al., 2017; Kurihara et al., 2009; Solomon et al., 2008). Cappello et al. (2017a) researched the toxicity of drospirenon, a synthetic hormone with the same mode of action as progesterone. Drospirenon is a popular component of contraceptive pills. *M. galloprovincialis* were exposed for 7 days to drospirenon concentrations ranging from 20 ng/L to 10 μg/L. There were no significant changes in testosterone and progesterone concentrations in the mantle/gonadic tissues and there was no adverse effects on gonadal maturation in treatment group. Nevertheless, disturbances in mussels' metabolic pathways were recorded. In the exposed group, a high percentage of mussels with gonadal resorption was found. Mussels inhabiting the vicinity of the WWPP, similarly to those exposed to EE2, were also characterised by elevated gonadal atresia (Smolarz et al., 2017). Dimastrogiovanni et al. (2015) studied progesterone toxicity at the same concentration as drospirenon in Cappello et al. (2017a) using the same species *M. galloprovincialis*. Exposure of mussels to progesterone caused a significant acceleration of gonadal maturation. In the exposed group most of the mussels were ready to spawn or during spawning. No changes in concentrations of testosterone and E2 in mantle/gonad tissue were found but it was observed that mussels metabolize progesterone to 5α-pregnane-3,20-dione (5α-DHP) and 3β-hydroxy-5α-pregnan-20-one (3β,20-one) in digestive gland. In several studies, the ability of mussels to metabolize E2 was tested and it was revealed that mussels metabolize this natural hormone almost entirely to E2 esters and accumulate the drug in this form (Janer et al., 2005; Labadie et al., 2007).

In order to understand how EDCs affect mussels, the endocrine system of individual Mytilidae species and the bioavailability of hormones should be better studied (Aris et al., 2014; Gilroy et al., 2012). The negative impact of EDCs on gametogenesis and on the reproductive success of Mytilidae may have fatal consequences for the mussels population, whose development and functioning may be disturbed, and consequently may lead to the extinction of these organisms in the affected areas (Aris et al., 2014).

4.2.3. SSRIs (selective serotonin reuptake inhibitor)

Pharmaceuticals belonging to SSRIs that have been most often tested involving Mytilidae representatives are fluoxetine (Brooks et al., 2003b; Gonzalez-Rey et al., 2014; Peters and Granek, 2016) and, to a lesser extent, sertraline. Therefore, this subsection concentrates primarily on these chemicals. Fluoxetine is one of the most widely used antidepressant in the world (Cortez et al., 2019; Metcalfe et al., 2010). Concentrations of fluoxetine detected in surface water from Spain, USA and Canada were ranging from 0.41 ng/L to 141 ng/L (Silva et al., 2012). Moreover, fluoxetine and its metabolite norfluoxetine are the most investigated SSRIs and are considered as the most toxic (Brooks et al., 2003b, 2003a). Norfluoxetine, like other SSRI metabolites, is less polar than the primary compound, making it more susceptible to bioaccumulation (Gelsleichter and Szabo, 2013). Toxic effects of fluoxetine have been observed, even at concentrations an order of magnitude lower than for other SSRIs (Cortez et al., 2019; Estévez Calvar et al., 2017; Silva et al., 2015). Some studies have already investigated the toxic effect of fluoxetine involving Mytilidae (Gonzalez-Rey and Bebianno, 2013; Gros et al., 2006; Metcalfe et al., 2003; Peters and Granek, 2016). The strengthening of lipid peroxidation in the digestive gland and the increase of glutathione-S-transferase and catalase activity in phase II were observed in *M. galloprovincialis* exposed to fluoxetine at a concentration of 75 ng/L. These parameters were 5-times higher in the treatment than in the control (Gonzalez-Rey and Bebianno, 2013). Peters and Granek (2016) tested the effect of fluoxetine on physiology of mussels. *M. californianus* were exposed to fluoxetine at concentrations from 0.3 ng/L to 300 ng/L for 107 days. Mussels kept at higher concentrations, starting from 30 ng/L, were more dispersed in the aquarium and did not attach to each other. Fluoxetine adversely affected the growth of mussels and the filtration processes (Peters and Granek, 2016). The proportion of reproductive tissue to total tissue (GSI) was disturbed by the drug after 47 days of exposure (Peters and Granek, 2016). Fluoxetine may accumulate in the body of mussels over a long period of time and may cause a decrease in reproductive efficiency (Peters and Granek, 2016). Study by Franzellitti et al. (2014) have shown a disorder of cellular signals in *M. galloprovincialis* due to fluoxetine at concentrations of 0.03–300 ng/L. Bioaccumulation of these pharmaceutical was also observed. The obtained BCF values were ranging from 200 to 800 L/kg. Silva et al. (2016), like the above-mentioned studies, investigated the ability of *M. galloprovincialis* to accumulate fluoxetine. Moreover, Silva et al. (2016) was one of the first studies of pharmaceutical metabolism involving mussels. Mussels were exposed at concentration of 75 ng/L for 15 days. *M. galloprovincialis* metabolised fluoxetine. The presence of fluoxetine derivative (norfluoxetine) in tissues was confirmed. As in the above-mentioned studies, the bioaccumulation of fluoxetine has been reported (Table 2). Both, the accumulation of the pharmaceutical and its metabolism increased with the exposure time. Study by Estévez Calvar et al. (2017) revealed negative effect of another SSRI, sertraline, on larval stage of *M. galloprovincialis*. Sertraline inhibited development of larvae, where no fully developed larvae were observed in treatment. In addition, the toxic effect was dependent on the concentration.

Despite the fact that fluoxetine and sertraline are pharmaceuticals with toxic potential, only a few studies investigated their effects on mussels. In addition, the effects of other antidepressants on marine organisms are almost unknown, which is worrying in view of the high consumption of these pharmaceuticals and their presence in the aquatic environment.

4.2.4. Antihistamines, β-blockers and antibiotics

Cetirizine is the most commonly used and most frequently

detected antihistamine in the marine environment (Almeida et al., 2017; Teixeira et al., 2017). However, as in the case of fluoxetine, only a few studies investigated the toxicity of this pharmaceutical to marine mussels (Almeida et al., 2017; Teixeira et al., 2017).

Teixeira et al. (2017) used *M. galloprovincialis* for toxicity studies of cetirizine. Biomarker analysis was performed to assess effects of this compound. The values of energy parameters (protein and glycogen content) indicated disturbances in the energy metabolism due to exposure to pharmaceutical. As the cetirizine concentration increased, mussels consumed more energy and their metabolic rate was also higher. Moreover, inhibition of some antioxidant enzymes and bioaccumulation of cetirizine were observed. Results show that the presence of cetirizine is a stress factor for *M. galloprovincialis*. Cetirizine disturbed the metabolic processes of *M. galloprovincialis* and weakened the condition of mussels. More attention should be paid to studying the effects of cetirizine and other antihistamines, because current knowledge about this group is unsatisfactory.

Campos et al. (2016) tested ecotoxicological effect of propranolol which belongs to β-blockers. Propranolol is frequently detected in the aquatic environment at concentrations ranging from 0.3 ng/L to 6329 ng/L (Campos et al., 2016; Franzellitti et al., 2015). For the purpose of ecotoxicological evaluation *M. edulis* was used. *M. edulis* was exposed to propranolol at concentration of 1000 μg/L for 4 weeks. In addition, it was tested how the decrease of salinity impact the response of mussels to propranolol. Two salinity conditions were incorporated: environmental salinity (6.5) and low salinity (5.5). After exposure of mussels to propranolol a shotgun proteomic analysis was applied. Proteomic analysis revealed a strong relationship between salinity conditions and the impact of propranolol. The stronger effect of propranolol was observed in mussels from lower salinity. This study show that environmental conditions also determine the toxicity and effects of pharmaceuticals. In contrast, Solé et al. (2010) investigated the effect of propranolol on feeding rate and oxidative stress responses of *M. galloprovincialis.* Mussels were exposed to propranolol at concentrations of 11 and 147 μg/L for 10 days. There was no significant effect on the feeding rate at 11 μg/L, while a significant inhibition of feeding was observed at 147 μg/L. Consequently, no-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for propranolol were: 11 and 147 μg/L. Furthermore, the higher concentration of propranolol significantly intensified phase I metabolism, resulting in an increase in the level of lipid peroxidation and severity of the neurotoxic effect (acetylcholinesterase activity was depleted). At lower concentration of propranolol, the activity of phase II glutathione S-transferase in the gills was intensified.

Antibiotics, along with NSAIDs and EDCs, belong to the most frequently consumed pharmaceuticals and are therefore detected worldwide in concentrations ranging from ng/L to μg/L in the aquatic ecosystems (Chiesa et al., 2018; Lacaze et al., 2015; Liu et al., 2018; Matozzo et al., 2016; Siedlewicz et al., 2018; Sui et al., 2015; Zouiten et al., 2016). Several studies have investigated the bioaccumulation and toxic potential of antibiotics involving Mytilidae. Le Bris and Pouliquen (2004) tested the ability of *M. edulis* to uptake and accumulate two antibiotics commonly used in veterinary medicine: oxolinic acid (OA) and oxytetracycline (OTC). The results of this study highlighted that OA and OTC were excreted by mussels rather than accumulated in tissues. BAF for OTC and OA in individual organs was almost always below 0.50 L/kg, except for OTC in viscera, where BAF was 2 L/kg. Taking into account the total body mass, BAF for OTC was 0.7 L/kg and for OA 0.3 L/kg. Due to the ability of antibiotics to bind with mineral substances, accumulation of OA and OTC was observed in shells of *M. edulis*. The detected concentrations of pharmaceuticals in bivalves tissues are presented in Table 2. In addition, it was observed that the rate of excretion of antibiotics from mussel tissues was different. OTC was characterised by a lower rate of elimination than OA. This may be related to the different biotransformation processes of these pharmaceuticals, which have not been investigated so far. Most antibiotics have hydrophilic properties: log K_{ow} (<2), therefore they are poorly accumulated in the tissues of bivalves and their metabolism is also poorly investigated (Le Bris and Pouliquen, 2004). However, shells may also be used for the biomonitoring of antibiotics.

Matozzo et al. (2016) as the first tested the effect of amoxicillin on hemocyte parameters in *M. galloprovincialis*. Amoxicillin is one of the most prescriptive and consumed antibiotics (Alygizakis et al., 2016; Matozzo et al., 2016). Study by Matozzo et al. (2016) showed the significant increase in the haemocyte proliferation in *M. galloprovincialis* exposed to amoxicillin. Moreover, tested antibiotic did not affect the integrity of the mussel cells. The volume and diameter of the mussel cells were not disturbed by amoxicillin. Lacaze et al. (2015) in turn identified the immunotoxic and genotoxic effect of erythromycin, trimethoprim and sulfamethoxazole on *M. edulis*. Selected antibiotics are among the most toxic to microorganisms and are therefore potentially toxic to other groups of organisms (Lacaze et al., 2015). Erythromycin and trimethoprim significantly affected effectiveness of the phagocytosis. These antibiotics therefore have an immuno-modulatory potential, while sulfamethoxazole caused oxidative stress induction. Interestingly, Lacaze et al. (2015) showed that the oxidative potential of antibiotics is related to the number of polar groups that determine the ability to cross cell membranes (Gagné et al., 2006). Moreover, it was revealed that trimethoprim and erythromycin cause DNA damage in *M. edulis* haemocytes.

Antibiotics are the most frequently studied of the abovementioned groups of pharmaceuticals. However, as in other examples, little is known about kinetics of these chemicals in marine organisms. For this purpose mussels from the Mytilidae family seem to be most suitable organisms. More attention should also be paid to research the pharmaceuticals whose impact on aquatic organisms has been poorly investigated.

4.2.5. Mixture toxicity of pharmaceuticals

Because of the fact that many pharmaceuticals are present in the environment at the same time and their effects can be mutually strengthened or weakened, it is also very important to study the toxicity of mixtures of these chemicals. Although there is no doubt that such studies are very important, only a few research have been carried out to analyse this issue. Study by Gonzalez-Rey et al. (2014) tested the toxic effect of pharmaceutical mixtures (diclofenac, ibuprofen and fluoxetine) on *M. galloprovincialis.* Mussels were exposed for seven days, after which, gene expression and multibiomaker analyses were performed. The results of this study highlighted the disruption of metabolic processes, down and upregulation of catalase and CYP4Y1 gene expression and oxidative stress induction in digestive gland. Interestingly, the mixture exhibited xenoestrogenic effect (the formation of vitellogenin proteins was observed), while single compounds did not show such an effect. Moreover, all observed effects differ significantly from the previous single exposure effects. Franzellitti et al. (2015) investigated toxicity of propranolol and fluoxetine mixture using the same species - *M. galloprovincialis*. For this purpose, gene expression analysis and Cyclic AMP (cAMP)-related cascade reactions were performed. cAMP plays an important role in the transmission of signals in many physiological processes. A change in cAMP levels may indicate a disturbance in metabolic processes, therefore this indicator is often used to determine the toxicity of chemicals, including pharmaceuticals. Co-exposure of propranolol and fluoxetine induced significant antagonistic effect even at low

concentration (0.3 ng/L). It was observed that co-exposure to both pharmaceuticals modifies the biological outcome of a single compound by reducing adverse effects. A single pharmaceutical had a significant effect on cAMP content as well as ABCB mRNA level in selected tissues, but the co-exposure did not affect these parameters. The toxic effect of the mixture was however revealed by a significant reduction in PKA activity. Serra-Compte et al. (2018) studied the effect of acidification and water warming on the bioconcentration, metabolism and depuration of a mixture of seven drugs (sotalol, sulfamethoxazole, venlafaxine, carbamazepine, citalopram, methylparaben and triclosan) by *M. galloprovincialis*. Mussels were exposed to a mixture concentration up to 15.7 μg/L for 20 days followed by 20 days of depuration. It was observed that mussels absorb all pharmaceuticals and the concentration in mussel tissues ranged from 1.8 μg/kg dw for methylparaben to 12889.4 μg/kg dw for citalopram. Interestingly, water warming affected the bioconcentration of methylparaben, sulfamethoxazole and sotalol, which was indicated by a higher BCF value for these compounds. In contrast, acidification reduced only the level of trisoclane in mussel tissues. Both acidification and water warming decreased the bioconcentration factor of citalopram and venlafaxine.

5. Summary

The assessment of risks related to pharmaceutical pollution seems to be the necessary step in developing effective management procedures. Worldwide, the legislations and policies regulating emissions of various pharmaceuticals are in the process of developing. However, the frameworks and concepts of general ecological risk analysis for marine pollution already exist. In spite of that, the management of pharmaceuticals is currently recognized as ineffective, mostly because only the most emerging ones are being regulated. The vast majority, however, enters marine ecosystems via various routes without any control. Therefore a proper management strategies are necessary to develop. Risk assessment can include qualitative, semi-quantitative and fully quantitative methods depending on the complexity required for decision making. In principle, the usage of bivalves has a great potential since it provides both, qualitative and quantitative data related to pharmaceuticals toxicity, concentrations and spread.

The discussed *in-situ* studies involving Mytilidae representatives have provided a valuable data on the determination of the pharmaceuticals in different parts of the world. The concentration of pharmaceuticals in the marine environment is often below detection limit. However, the involvement of Mytilidae representatives makes possible detecting even trace amounts of pharmaceuticals. The concentrations of pharmaceuticals may be much higher in mussels than in the environment (Farrington et al., 2016; Moreno-González et al., 2016). On the other hand, the accumulation capacity of individual Mytilidae species needs to be further investigated as well as the bioavailability of pharmaceuticals. The occurrence of tested pharmaceuticals like hormones, analgesic drugs, antibiotics and β-blockers was confirmed in wild mussels from different parts of the world. In addition, the concentrations of pharmaceuticals in the tissues of mussels from different regions are varied. Biomarker analysis also revealed the toxic effect of sewage from WWTPs on mussels placed in cages. Lower condition, disturbance of physiological pathways, morphological changes as well as the oxidative stress induction were observed. However, environmental studies do not provide information on which of the compounds from the sewage have the greatest impact on the test organism. Another problem is overlapping of environmental factors and their diversity. Environmental conditions also contribute to the toxicity and bioavailability of tested substances. In addition, more attention should also be devoted to the study of metabolites of pharmaceuticals.

On the other hand, laboratory studies provide important information on the bioaccumulation of pharmaceuticals and their effect on the morphology, behaviour and physiology of Mytilidae members. The physiology of Mytilidae representatives is well studied and known, but there are large gaps in knowledge when it comes to their hormonal system. Therefore, it is possible to follow the path of the tested pharmaceuticals inside the body of mussels and to evaluate their effects. Toxic effects of analgesic drugs, synthetic hormones, antibiotics, antihistamines and β-blockers have been evaluated mainly by biomarker analysis. The energy budget of organism, morphological changes in tissues, genotoxic effect were also used as indicators of toxic effects. *Ex-situ* studies do indeed give a broad view of the processes to which tested substances are subjected, but never fully reflect environmental conditions. It should always be taken into account that laboratory conditions may cause the processes to have different patterns from those found in the environment.

Mytilidae are one of the most appropriate species for biomonitoring in marine environment. They are involved in many biomonitoring programs (e.g. Mussel Watch) regarding the environmental quality assessment on a global scale (Bajt et al., 2019; Rittschof and McClellan-Green, 2005). The distribution and concentration of pharmaceuticals is constantly changing due to environmental and anthropogenic factors, while monitoring using mussels makes it easy to observe such changes. Involvement of Mytilidae mussels in ecotoxicological studies of pharmaceuticals and biomonitoring programmes could significantly increase knowledge about the impact and the fate of pharmaceuticals in the marine environment. In addition, it can provide valuable information about their determination in the seas and oceans around the world, which will eventually spark off appropriate preventive action based on legal regulations limiting the pharmaceutical emissions to the environment.

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Effects of environmentally relevant concentrations of diclofenac in *Mytilus trossulus*

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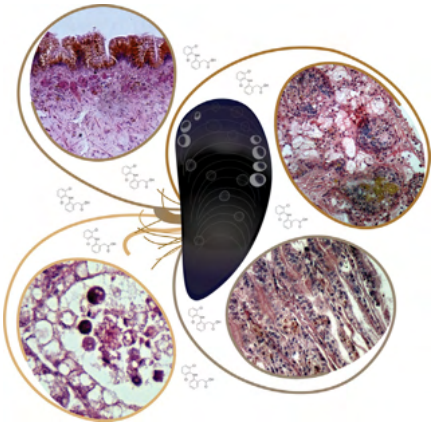
HIGHLIGHTS

- The effect of diclofenac in mussels was related to concentration.
- Diclofenac was completely excreted by mussels during the depuration phase.
- Diclofenac caused an increase in the frequency of lesions in *M. trossulus* tissues.
- No diclofenac metabolites were detected, possibly due to low exposure concentrations.
- Environmentally relevant concentration of drug did not affect the condition of mussels.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of pharmaceuticals in the marine environment is a growing problem of global importance. Although awareness of the significance of this issue is increasing, many questions related to the ecotoxicology of pharmaceuticals remain unclear. Diclofenac is one of the drugs most commonly detected in the marine environment and its potential toxicity has been previously highlighted, thus its impact on organisms deserves a special attention. Therefore, in this study, a thorough analysis of the effects of diclofenac on a condition and tissue level of a model representative of marine invertebrates - *Mytilus trossulus* - was performed. During the 25-day experiment, divided into exposure and depuration phases, bivalves were exposed to two environmentally relevant drug concentrations of 4 and 40 μg/L. The study showed that mussels absorb diclofenac in their tissues and the highest recorded concentration was 1.692 μg/g dw on day 8. Moreover, the content of diclofenac metabolites (4-OH and 5-OH diclofenac) was also examined, but they were not detected either in water or in tissues. Although exposure to low diclofenac concentrations did not significantly affect the condition index of organisms, changes in numerous histopathological parameters were noted. Performed histological examination provided additional valuable information on the influence of drugs on the functioning of invertebrates. Nevertheless, applicability of histopathological techniques in ecotoxicology of drugs requires additional evaluation in future studies.

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1. Introduction

Pharmaceuticals belong to the contaminants of the emerging concern, already present in the surface waters around the world (Borecka et al., 2015; Fisch et al., 2017). Emission of pharmaceuticals and personal care products (PPCPs) to the marine environment, mainly via rivers and surface runoffs, is becoming a serious global threat. However, the fate of pharmaceuticals in the marine ecosystems is still poorly researched. Many of these compounds are characterized by pseudo-persistent in the marine environment, thus they may constantly influence the condition of organisms, posing a serious threat to the marine fauna (Brausch et al., 2012; Fabbri and Franzellitti, 2016; Fent et al., 2006; Kümmerer, 2008; Sanderson et al., 2004). Nevertheless, the legislation controlling the release of PPCPs into the environment is still missing, which may be due to the insufficient number of studies to confirm the seriousness of the threat, as well as its potential long-term consequences (Dodder et al., 2014).

Non-steroidal anti-inflammatory drugs (NSAIDs) are predominant among pharmaceuticals detected in the aquatic environment worldwide. Recent studies have shown the presence of NSAIDs in the wild mussels collected along Spanish and Italian coasts, while many other works have confirmed their presence in different water bodies around the world (Hanif et al., 2020; Sathishkumar et al., 2020; Mezzelani et al., 2016a; Moreno-González et al., 2015). NSAIDs are characterized by anti-inflammatory, antipyretic and analgesic effects (Brune and Patrignani, 2015; He et al., 2017; Kümmerer, 2008; Nikolaou et al., 2007; Paíga et al., 2015). Over-the-counter (OTC) availability favours high consumption of these pharmaceuticals (Cleuvers, 2003; Comeau et al., 2008; Brune and Patrignani, 2015; Paíga et al., 2016; He et al., 2017; McRae et al., 2018). Among the NSAIDs, diclofenac has the highest toxicity and is the most frequently detected, thus it has been added to the European Surface Water Monitoring System according to the Water Framework Directive (Hallgren and Wallberg, 2015). Concentration of diclofenac in the marine environment is relatively low, ranging a few ng/L to several μg/L. However, continuous exposure of marine organisms to low concentrations of diclofenac may pose a risk to them (Dumas et al., 2020; Ryzhkina et al., 2020; Mezzelani et al., 2016b; Araujo et al., 2014). Many short-term laboratory studies have already shown that diclofenac has negative effect on marine organisms (Kloukinioti et al., 2020; Bonnefille et al., 2018; McRae et al., 2018; Freitas et al., 2019a; Freitas et al., 2019b; Du et al., 2016; Balbi et al., 2018; Sanderson et al., 2004). Study by Mezzelani et al. (2018) has shown that diclofenac, at environmentally relevant concentration, causes the modification of immunological parameters, modulation of lipid metabolism, genotoxic effects, as well as changes in cell turnover. On the other hand, Bonnefille et al. (2018) proved that diclofenac causes modulation on tyrosine-dopaminergic and tryptophan metabolic pathways in *M. galloprovincialis,* which consequently results in the disruption of osmotic regulation and reproduction processes. The fate of pharmaceuticals after entering invertebrates is a poorly researched issue. Studies on pharmacokinetics could significantly increase the knowledge concerning toxic properties of tested pharmaceuticals and more importantly, provide valuable information about the structure and activity of metabolites produced by organisms (Sathishkumar et al., 2020). Diclofenac metabolites produced by marine organisms are one of the least recognized concerns (Fu et al., 2020; Sathishkumar et al., 2020; Bonnefille et al., 2017; Świacka et al., 2019). Recently, Bonnefille et al. (2017) demonstrated that *Mytilus* spp. mussels metabolise diclofenac to 13 metabolites, including 4-OH and 5-OH diclofenac. Świacka et al. (2019) also confirmed the metabolism of diclofenac to 4-OH and 5-OH diclofenac by *M. trossulus*. However, data on diclofenac metabolites, their toxicity and potential bioaccumulation is very limited thus it is likely that these compounds may include potentially hazardous substances, and therefore it is worthwhile to scrutinize their structure and biological activity.

The Baltic Sea is characterized by a strong salinity and temperature gradient, also remaining one of the most polluted seas in the world (Voipio, 1981; Albalat et al., 2002; Bonsdorff, 2006; Korpinen et al., 2012). Baltic organisms, such as *M. trossulus* are forced to spend a lot of energy on osmotic regulation, therefore they may be more sensitive to any other external stressors, such as environmental pollution. *M. trossulus*, marine bivalve species that has been adapted to the harsh Baltic conditions, is also one of the most important links in the food chain. Furthermore, mussels are sedentary and filter-feeding organism and can remove various environmental pollutants from the water by accumulating them (James et al., 2020). Moreover, mussels are also often found near sewage outlets. Thus they are one of the most exposed groups of marine fauna to different contaminants (James et al., 2020; Caban et al., 2016; De Solla et al., 2016, Klosterhaus et al., 2013; Marigómez et al., 2013; Gillis, 2012).

The main aim of this work was to provide a more detailed information on the effect of diclofenac on marine fauna - *M. trossulus*, by performing exposure experiment in the laboratory controlled conditions. For the first time histological analysis was used to determine the toxicity of diclofenac in mussels. The effect of diclofenac on the general condition of mussels was also tested. A preliminary pharmacokinetic study was performed through the determination of diclofenac and its two metabolites (4-OH and 5-OH diclofenac) concentration in tissues and water during exposure to environmentally relevant concentrations of diclofenac and further depuration phases. The effect of different diclofenac concentrations has also been included. In addition, screening of selected NSAIDs (ketoprofen, ibuprofen, flurbiprofen and naproxen) in *M. trossulus* and water collected from the Gulf of Gdańsk was performed. Basing on previous experimental work, this study assumed that diclofenac effects may be revealed by increased frequency of tissue changes without necessarily affecting the overall condition. Although absorption of the drug in mussel tissues was expected, taking into account their ability to metabolise diclofenac, it was also anticipated that the drug could be completely excreted from the organisms during depuration. Moreover, it was assumed that the observed effects of diclofenac will be related to its concentration and seen not only at the genetic and biochemical levels but also at the tissue level, thus even more affecting the physiology of an organism.

2. Materials and methods

2.1. Sampling

Material (1000 individuals of *M. trossulus*) was collected from the Gulf of Gdańsk - at Orłowo station (54°29,40 N; 18°38,60 E) in January 2019. During the sampling, temperature and salinity were measured and their values were respectively: 5.1 °C and 7.1. Mussels were sampled using the benthic drag and water samples were collected using bathometer. Additionally, 300 individuals of *M. trossulus* and two litres of seawater were collected for analysis of diclofenac, its metabolites (4-OH diclofenac and 5-OH diclofenac) and other NSAIDs (flurbiprofen, ketoprofen, ibuprofen and naproxen) at the collection site. Next, mussels were transported to the laboratory and prepared for the toxicity and bioconcentration experiment.

2.2. Chemicals preparation

Diclofenac sodium salt (CAS 15307-79-6, analytical standard), 4-hydroxydiclofenac (CAS 64118-84-9, analytical standard) and 5-hydroxydiclofenac (CAS 69002-84-2, analytical standard) were purchased from Sigma-Aldrich, with a purity of >98%. Stock solutions of diclofenac, 4-hydroxydiclofenac and 5-hydroxydiclofenac at 5 mg/mL were prepared by dissolving in methanol (HPLC grade, POCH, Poland). To prepare the artificial salt water with a final salinity equal to 7 PSU

(salinity in the sampling site) distilled water and Aquaforest® salts standards were used.

2.3. Acclimation

Prior to acclimation, epiphytes were removed from mussels' shells. During the acclimation phase, the temperature was increased by 0.5° every 2 days from 5 to 8 °C. After stabilisation of laboratory conditions, the mussels were allowed to acclimatise to new conditions for additional two weeks. Hence, the overall acclimation phase took three weeks. During this stage, water was changed every four days while mussels were kept in dark. After each change of water, mussels were fed with *Chlorella vulgaris* in a volume of 3 mL and a concentration of 13 million cells/mL obtained from CCBA (Culture Collection of Baltic Algae).

2.4. Exposure experiment

Nine glass tanks (two different concentrations of diclofenac and control, each in triplicate) with 15 L of artificial sea water with 7 PSU were prepared (Fig.1). Two nominal concentrations of diclofenac: 4 and 40 μg/L were prepared by adding the following volumes: 12 μL and 120 μL of diclofenac solution at concentration of 5 mg/mL to the six tanks. The other three tanks (control-without solvent) were prepared without addition of diclofenac. Although in the previous experiment (Świacka et al., 2019) a solvent control was performed, for this study the procedure modified and instead of solvent control, stock solutions were prepared in a way that the volumes of methanol added to the aquariums were minimal. After dissolving the added solutions in 15 L of seawater, the maximum concentration of methanol was 8 mg/L. This value is up to 3 orders of magnitude lower than the NOEC value reported by ECHA (European Chemicals Agency) for aquatic organisms, thus still in accordance with the state-of-the-art of experimental studies.

To each tank 73 individuals of *M. trossulus* were placed. Laboratory experiment was divided into two parts: 12-day exposure of mussels to diclofenac followed by a 13-day depuration phase (Fig.1). During the exposure of mussels, water was changed every four days and after change the concentration of the pharmaceutical was renewed (Fig.1). In addition, 0.5 L of water was taken from the tanks with the pharmaceutical, before the water change, every four days. At the beginning of the depuration phase, the glass tanks were refilled with artificial sea water with 7 PSU and no more pharmaceutical was added. This phase lasted 13 days. The mussels were fed in the same way as during the acclimation. Before the exposure, five individuals were taken for histological analysis and two mussels for other testing. During the exposure to diclofenac four individuals were taken from all tanks (repetitions) every four days, before the water change. Four mussels were used in the chemical analysis of diclofenac, 4-OH diclofenac and 5-OH diclofenac presence. In addition, on days: 0 and 12 two individuals were taken from all tanks for histological analysis. On the last day of depuration phase four mussels were collected to chemical analysis.

2.5. Chemical analysis

The complete procedures concerning the determination of diclofenac concentration in water and in mussel tissues are described in our previous work (Świacka et al., 2019). Briefly, pharmaceuticals extraction from mussels' tissues was performed by the accelerated solvent extraction (ASE, Dionex ASE 350 model) with purification of the extracts by solid phase extraction (SPE, Strata-X; Phenomenex; Torrance,

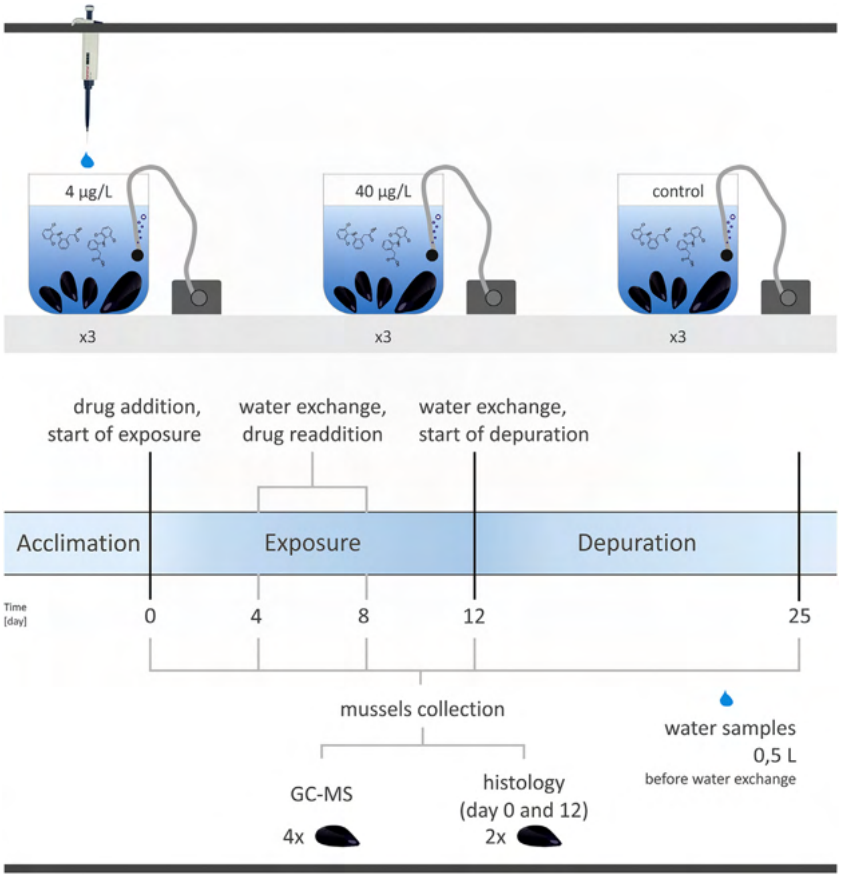

Fig. 1. Experimental procedure.

USA). In order to obtain pharmaceuticals from water samples, SPE was performed using disks H_2O-philic DVB Speedisk® (Bakerbond). Prior to SPE, water samples were filtered with glass fibre filters. Previously, it was proven that the filtration step have not impact the quantification of diclofenac anion in complex samples (Kołecka et al., 2019). Then, extracts form water and mussel tissues were derivatized and analyzed by gas chromatography with mass spectrometry (GC–MS, GCMSQP2010SE, Shimadzu; Kyoto, Japan). The method detection limits (MDL) of GC–MS for diclofenac, 4-hydroxydiclofenac and 5-hydroxydiclofenac are respectively: 1 ng/L/0.01 μg/g; 1 ng/L/0.08 μg/g and 2 ng/L/0.17 μg/g (water/tissue).

Diclofenac-13C6 sodium salt (CAS 1261393-73-0, Sigma-Aldrich) was used as internal standard added before each extraction.

2.6. Histology

The soft tissues of mussels were removed from the shells and placed in Davidson fixative solution (33% distilled water, 33% ethanol [96%], 22% formaldehyde, and 11% acetic acid). After two days mussels tissues were placed in 10% formaldehyde solution buffered with sodium phosphate (6.5 g/L dibasic Na_2HPO_4 and 4 g/L monobasic NaH_2PO_4) and stored for 10 days. Next, tissues were put in histological blocks, dehydrated in ascending ethanol series (from 70% to absolute ethanol) and embedded in paraffin. Two 3 μm sections were obtained from each individual, then left overnight at 60 °C, deparaffinized in three xylene baths, hydrated in descending ethanol series (from absolute ethanol to 70% ethanol followed by water bath) and finally stained according to the Harris' haematoxylin and eosin (H&E) standard protocol. The prepared samples were analyzed under light microscope for lesions in gonads, gills and digestive glands. The binary (0 for absent and 1 for present) scoring of lesions was used to assess the effect of diclofenac on mussel tissues. Similarly to Larsson et al. (2018), the frequency of lesions pooled by tissue type (i.e., gills, gonads and digestive gland) and the number of lesions was analyzed assuming a binomial error distribution.

2.7. Body mass index, BMI

To determine the condition of mussels, body mass index (BMI) showing the relation between the shell length (L) and tissue wet weight (W) was used. BMI was calculated as follows:

$BMI = W/L^3$ [mg/cm^3].

2.8. Statistical analysis

First, normality of data distribution and homogeneity of variances were verified using Shapiro–Wilk and Levene's tests. Statistical comparison of the BMI between mussels from the control and exposure groups and between replicates of exposure groups was performed using Mann–Whitney *U* test (STATISTICA software). Kruskal-Wallis test was used to compare the measured concentration of pharmaceutical in water and mussel tissues between experimental days and repetitions. The significance level was set to $p < .05$.

3. Results and discussion

3.1. Concentration of diclofenac and its metabolites in the mussels and water from the environment

Diclofenac, its metabolites (4-OH and 5-OH diclofenac) and other NSAIDs (ketoprofen, ibuprofen, flurbiprofen, naproxen and nimesulide) were not detected in mussels or in water collected from Puck Bay at Orłowo station. This may indicate that there are no NSAIDs at Orłowo station or their concentrations were too low (below a few ng/L) to be detected by GC–MS.

3.2. Concentration of diclofenac measured in water and tissues of mussels

The final measured concentrations of diclofenac in the tanks with nominal concentration of 40 μg/L were as follows: 19.859, 25.314 and 24.339 μg/L on days 4, 8 and 12, respectively (Fig.2). Four days after adding diclofenac, its measured concentration was almost 50% lower, which may indicate that pharmaceutical was taken by mussels or adsorbed on shells biofilm. The solubility of diclofenac could be also lower in salt water than in fresh water (about 4.8 mg/L [drugbank.ca]). According to numerous studies, Mytilidae mussels can absorb diclofenac from water as evidenced by its presence in their tissues (Ericson et al., 2010; Świacka et al., 2019). Decreased concentration of pharmaceutical may also indicate its instability in the water matrix due to its biodegradation or adsorption on the walls of the tank (Packer et al., 2003). Our previous study (Świacka et al., 2019) showed that the determined concentration of diclofenac in artificial seawater decreased by almost 50% after ten days, which was most probably related to the presence of bacteria and microalgae that metabolized diclofenac, or to its adsorption on glass (Packer et al., 2003; Jewell et al., 2016; Avetta et al., 2016; Xiong et al., 2018). However, the influence of photodegradation was excluded, as the water tank was kept in the dark. There were no statistical differences in diclofenac concentration in water between the replications and days of exposure which indicates good repeatability of experimental conditions.

The final determined concentration of diclofenac in the tanks with nominal concentration 4 μg/L was: 2.697, 3.585 and 4.205 μg/L on days 4, 8 and 12, respectively (Fig.2). Each four days after adding diclofenac, its measured concentration was similar to the nominal concentration. This may be connected to the lower filtration rate and absorption of diclofenac by mussels, as also indicated by the lower concentration of diclofenac in the tissues of mussels exposed to 4 μg/L (Fig.2). Lower concentrations of pharmaceuticals may have been a minor stress factor for mussels and therefore their filtration rate has

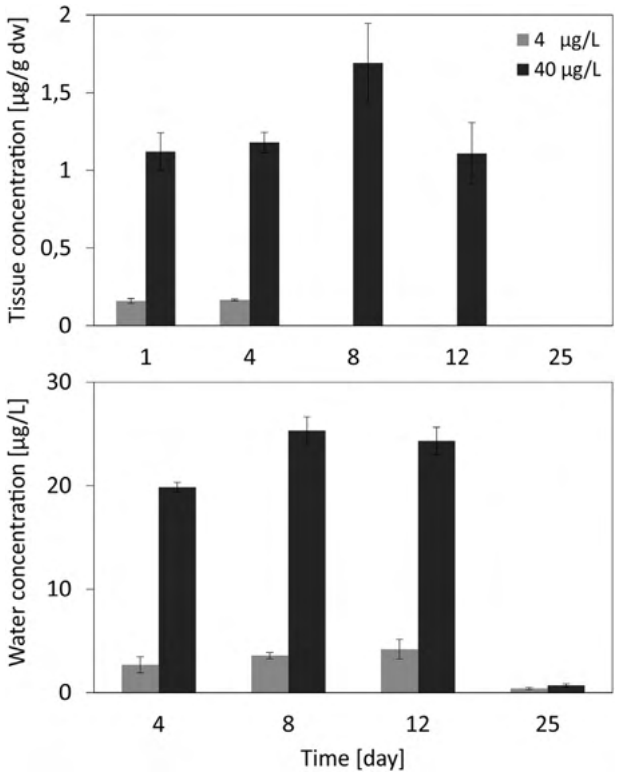

Fig. 2. Diclofenac concentration in water and mussel tissues treated with 4 and 40 μg/L during 25 days of experiment (mean ± standard deviation SD; n = 12). Statistical comparison (Kruskal-Wallis test).

not increased. During the last day of the purification phase, diclofenac was also detected in water. The detected concentration of diclofenac was 0.726 μg/L and 0.398 μg/L in the tanks with nominal concentrations 40 and 4 μg/L respectively (Fig.2). It may indicate that mussels eliminated the pharmaceutical from the tissues or it was desorbed from the shells.

In present study *M. trossulus* mussels were exposed to environmentally relevant concentrations: 4 and 40 μg/L yet it became clear that despite this low levels diclofenac concentration in tissues was dose-dependent. In the group exposed to 4 μg/L diclofenac was detected only on days 1 and 4, with the highest concentration of 0.166 μg/g dw on day 4 (Fig.2). This may indicate that mussel managed to metabolise and excrete smaller amounts of the pharmaceutical faster and more efficiently. Therefore, it confirms that kinetic of pharmaceutical after entering the mussels is strongly related to its concentration. Diclofenac was detected in mussels exposed to 40 μg/L on each day of exposure, with the highest concentration of 1.692 μg/g dw on day 8 (Fig.2). This proves that mussels absorb the pharmaceutical into tissues. Diclofenac is uptaken by bivalves mainly through the gills, but also through the whole body surfaces (Turja et al., 2020). The gills also have another function and are involved in the metabolism and elimination of xenobiotics, including pharmaceuticals (Gomez et al., 2010; Widdows et al., 1983). The degree to which pharmaceuticals are removed by the gills may depend on their concentration.

In the group exposed to 40 μg/L the concentration of diclofenac in tissues was up to 10 times higher than in the group exposed to 4 μg/L, which may be caused by the slower rate of detoxification and excretion of xenobiotic by *M. trossulus*, as well as the higher amount of diclofenac in water available for mussels. On day 8, the concentration of pharmaceutical in mussel tissues was higher than on the remaining days, but it was not statistically significant. This may suggest an increased rate of filtration of mussels due to the stress associated with the permanent presence of diclofenac and thus higher absorption into tissues (Ericson et al., 2010). On day 12, the concentration of pharmaceutical in the tissues decreased. However, it was not statistically significant. This may be related to the increased metabolism of diclofenac and its faster excretion from the body (Bonnefille et al., 2017; Świacka et al., 2019).

The metabolic processes of pharmaceuticals in Mytilidae mussels are still poorly understood and require more attention. The information available so far does not allow a full understanding of the detoxification in marine invertebrates. Therefore, only after filling the knowledge gaps it will be possible to make a realistic assessment of the consequences associated with pharmaceutical contamination.

Freitas et al. (2019a) have shown that salinity significantly affects the concentration of diclofenac in mussel tissues. *M. galloprovincialis* individuals were exposed to the same concentration of 1 μg/L for 28 days. However, the content of diclofenac in their tissues differed by up to 60% between salinity 25 and 30 (environmental salinity). Interestingly, in this study the authors observed that mussels from environmental salinity reduce their metabolic rate after exposure to diclofenac, which may explain lower concentration of diclofenac in their tissues compared to mussels from salinity 25. That highlights the importance of the environmental conditions in which bivalves live and are adapted to, as there may be differences in bioaccumulation, metabolism and toxicity of the compound in species from areas varying in, for example, salinity (like the Baltic Sea and the Atlantic). Both salinity and temperature have a significant influence on the metabolism of bivalves, which in turn contributes to their ability to absorb and transform the compound. Diclofenac concentrations in tissues may also vary between Mytilidae species. Various mussel species may have different absorption, metabolism and excretion capacities. For this reason, it is important to have a better understanding of what happens to the pharmaceutical when it enters the body. The present study has shown that the diclofenac concentration has a significant effect on bioconcentration, as also reported in Ericson et al. (2010) (Table 1), where diclofenac content was higher in the tissues of individuals exposed to the higher concentrations of the compound. Nevertheless, not all toxicity studies of diclofenac have analyzed its concentration in tissues, as shown in Table 1. However, this combination would be very valuable as it could enable verification whether there is a relationship between the drug concentration in tissues and its toxicity, and further risk assessment.

During the experiment, no diclofenac metabolites (4-OH and 5-OH diclofenac) were detected in water and tissues, which may be associated with the low (environmentally relevant) doses of diclofenac and detection limits of GC–MS. However, in our previous study (Świacka et al., 2019) 4-OH and 5-OH diclofenac were detected in the mussels exposed to much higher levels of diclofenac (133.33 μg/L) and its concentration in tissues was around 1 μg/L. In the present study mussels were treated with three times lower concentration that was environmentally relevant, therefore diclofenac metabolites could not be detected. Moreover, 4-OH and 5-OH diclofenac may have been transformed into other derivatives that we have not tested. According to Bonnefille et al. (2017), Mytilidae mussels transform diclofenac to up to 13 different metabolites from both the first and the second phase of detoxification processes. The absence of 4-OH and 5-OH diclofenac in water and mussel tissues may therefore indicate further metabolism in the second phase of detoxification.

3.3. BMI

BMI values representing the condition of mussels during the exposition and depuration are shown in the Fig. 3. The condition of mussels from 4 μg/L tank decreased during 12 days of the exposure. In the mussels exposed to 4 μg/L the highest average BMI was observed on day 1, while in mussels treated with 40 μg/L the highest average BMI was observed on day 4. However, no statistically significant differences in the BMI were observed between the control and the treatment groups during exposition and depuration. Study by Gonzalez-Rey and Bebianno (2014) also investigated the effect of diclofenac on the overall condition of mussels. *Mytilus galloprovincialis* were exposed at environmentally relevant concentration of 250 ng/L for 15 days. During the exposure, no significant differences regarding the condition index (CI) were observed between treatment and control groups. Similarly to our research, diclofenac did not affect the condition of mussels. The exposure time may have been too short to observe the effect of pharmaceutical at environmental concentration on the condition of *M. trossulus*. However, in our previous research, statistically significant effect of diclofenac on mussel condition during depuration phase was observed, but the exposure concentration was three times higher (Świacka et al., 2019). Moreover, no correlation was observed between BMI value and exposure time.

To date, the impact of diclofenac or other NSAIDs on the general condition of mussels has not been tested in any study other than those mentioned. Some studies have evaluated differences in BMI values for mussels collected from different stations including points localizated near sewage treatment plant outlets (Larsson, 2017; Larsson et al., 2018). However, the observed differences in BMI values in *M. trossulus* were more related to the availability of food rather than to the presence of contaminants.

3.4. Histological examination

Fig. 4. shows tissue abnormalities occurring in *M. trossulus* after exposure experiment. Only regressive changes often occurring in bivalves inhabiting poor environmental conditions were found. Accumulation of brown granules, which are most likely to be lipofuscin, was observed in the mantle epithelium, as shown in Fig. 4.A. The accumulation of this pigment was observed only in the epithelium of different tissues (gills, mantle and digestive gland).

Fig. 4.B illustrates atresia in male gonads indicated by hemocytic digestion and degradation of atretic gametes in follicle. Gonadal atresia occurred both in males and females. Atrophy and vacuolisation in

Table. 1
Toxic effects of diclofenac in Mytilidae mussels.

Species	Exposure concentration [μg/L]	Exposure time [day]	Tissue concentration [ng/g dw]	Biological effects	References
Mytilus spp.	1; 1000	1; 4	na	Disorders of glutathione S-transferase (GST) and lipid peroxidase (LPO) activities, increase in DNA damage	Schmidt et al. (2011)
	1; 1000	7; 4; 21	na	Disorders of glyceraldehyde - 3 phosphate dehydrogenase, aconitase-1 and GST activities	Schmidt et al. (2014)
M. galloprovincialis	25	14	14.90 ± 7.89	Induction of antioxidative defence, disorder of lysosomal membrane stability (LMS), inhibition of acyl-coenzyme A oxidase and catalase (CAT) activities, genotoxic effect	Mezzelani et al. (2016a)
	2.5	60	2.25 ± 0.810	Diclofenac accumulation in mussel tissues, genotoxic effect, modulation of immunological parameters and cellular turnover, disturbance of lipid metabolism	Mezzelani et al. (2018)
	0.05; 0.50	14	na	Modulation of CAT activity, genotoxic effect	Munari et al. (2018)
	122.6	7	na	Modulation of metabolic pathways of tyrosine-dopamine and tryptophan, disruption of osmosis and gametes release	Bonnefille et al. (2018)
	1; 100	3	26; 2080	Modulation of prostaglandin sythesis	Courant et al. (2018)
	1	28	47.0 ± 1.4	Induction of antioxidative defence (modulation of GPx and CAT activities, reduction of GSH/GSSG ratio)	Freitas et al. (2019a)
	0.25	15	na	Alteration in estrogens level, induction of antioxidative defence (modulation of the activity of superoxide dismutase, acetylcholinesterase and glutathione reductase)	Gonzalez-Rey and Bebianno (2014)
	1; 10	2	na	Induction of shell deformation, influence on transcription of genes involved in shell formation and biotransformation	Balbi et al. (2018)
M. trossulus	1; 10,000	8; 14; 19; 21	82 ± 6; 0.18 ± 0.02	Decrease of scope for growth and weakening strength and abundance of byssus threads	Ericson et al. (2006)
	133.33	5	0.86	Significant impact on the condition of mussels during the purification phase	Świacka et al. (2019)

na - not available.

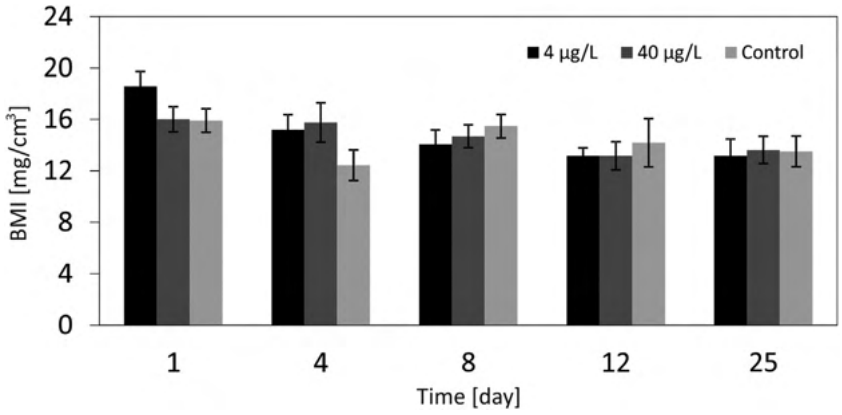

Fig. 3. Body mass index (BMI) calculated for *M. trossulus* during 25 days of experiment (mean ± standard deviation SD; n = 12). Statistical comparison (Mann–Whitney *U* test).

hepatopancreas are shown in Fig. 4.C. Atrophy was defined as the presence of necrotic cells, enlargement of the vacuole in the epithelium and deformation of the tubular cells, which leads to abnormal tubule structure. Fig. 4.D shows gills deformations indicated by numerous swellings and presence of cavities in the epithelium.

M. trossulus individuals treated with diclofenac, as well as those from the control group, were characterized by the frequency of tissue abnormalities such as: gill deformations, gonadal atresia, atrophy and vacuolisation in digestive glands (Fig.4; Fig.5). Brown pigment accumulation and necrosis were observed only in individuals exposed to diclofenac, while inflammations were noted only in the mussels from the control tanks (Fig.5).

Lipofuscin is an end product formed due to the oxidation of lipids and proteins during oxidative stress, therefore the brown pigment that we have observed in treated mussels is likely to be lipofuscin. Lipofuscin accumulation in the exposed groups may indicate metabolism disorders caused by diclofenac. Lipofuscin accumulation can be a marker of metabolic disorders and oxidative stress (Brenner et al., 2014; Mezzelani et al., 2016a; Bignell et al., 2011). According to Martín-Díaz et al. (2009) exposure of mussels to carbamazepine (which belongs to NSAIDs) at 10 μg/L for seven days resulted in lipofuscin accumulation in digestive gland. Study by Mezzelani et al. (2016b) also confirmed significant accumulation of lipofuscin in digestive gland in mussels exposed to diclofenac and ibuprofen. However, the brown pigment accumulation similar to lipofuscin is constantly observed in mussels from the environment (Bignell et al., 2011; Brenner et al., 2014; Raftopoulou and Dimitriadis, 2012). Study by Bignell et al. (2011) showed differences in lipofuscin content in the epitelium of kidney in *Mytilus* spp. from different stations. Level of lipofuscin was higher in stations where higher levels of metals were observed (Shaw et al., 2011). On the other hand, study conducted by Brenner et al. (2014) showed seasonal variations in the lipofuscin levels in the digestive gland of Mytilidae mussels. The changes in lipofuscin content may be related to the reproductive processes of these animals occurring during warm seasons. Changes in lipofuscin levels between mussels submerged and those living in intertidal zones were also found. This in turn may be related to the availability of food and feeding mode. Lipofuscin accumulation can therefore be a good biomarker to

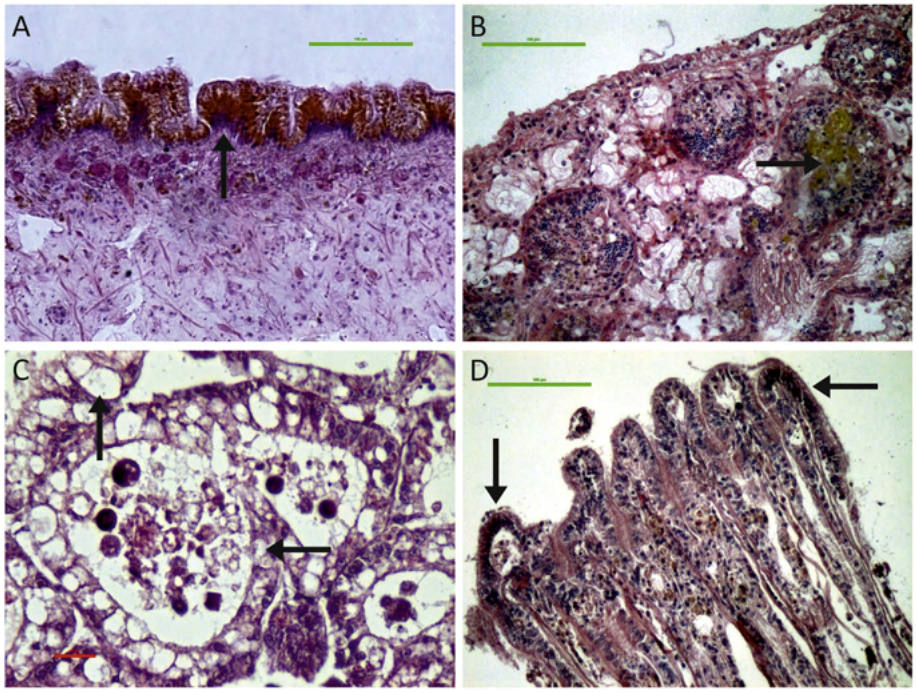

Fig. 4. Cross sections through *M. trossulus* tissues treated with diclofenac after 12 days of exposure. Accumulation of lipofuscin-like granules in mantle' epithelium, scale 100 μm (A); atresia in male gonads manifested by the presence of brown cells (arrow), scale 100 μm (B); Atrophy and vacuolisation of the digestive tubules containing various digestive cells (B-, R- and F-cells) showing an enlargement of the lumen area and reduced epithelial layer, scale 20 μm (C); Gill deformations including oedematous changes and local necrosis (arrows), scale 100 μm (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

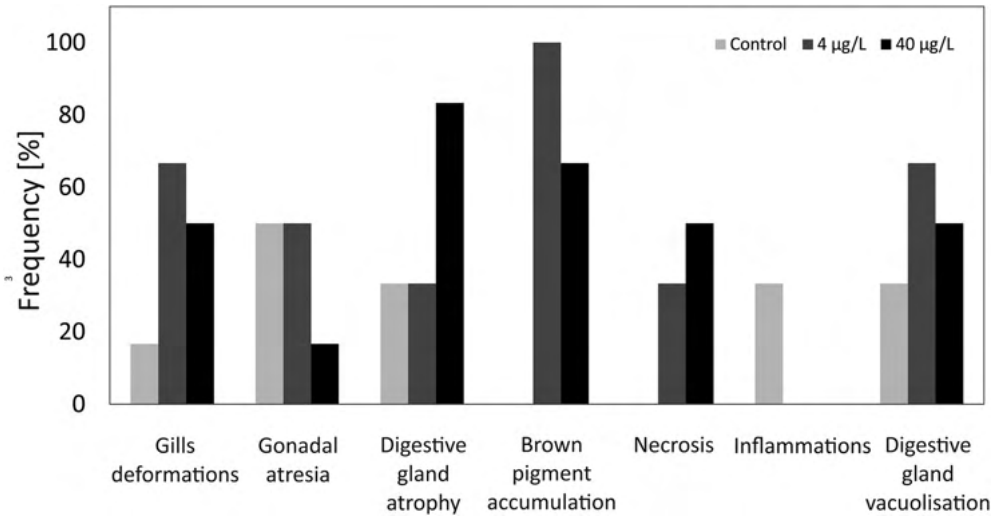

Fig. 5. The frequency of tissue lesions of *M. trossulus* after 12 days of exposure to diclofenac expressed as a percentage.

determine the condition of the environment, and to assess toxic potential of tested compounds including pharmaceuticals. However, the potential influence of seasonal changes, which also affect the lipofuscin content in individual tissues of mussels, should be eliminated.

Most of tissue abnormalities were observed both in experimental groups and controls, which means that diclofenac did not cause these lesions exclusively and the observed lesions are non-specific. However, when looking at the Fig. 5, it can be concluded that diclofenac contributed to an increase in frequency of abnormalities. Several laboratory studies confirmed that diclofenac causes metabolism disorders as evidenced by disruption of glutathione S-transferase (GST), lipid peroxidase (LPO) activity, as well as activity of other enzymes involved in cell metabolism (Table 1). On the other hand, inflammation was observed only in the control tanks which may reflect anti-inflammatory effect of diclofenac in the non-target organisms. Study by Courant et al. (2018) as first revealed anti-inflammatory action of diclofenac in Mytilidae mussels (Table 1). However, in present study very few mussels were tested for the presence of inflammation in tissues, therefore, the absence of inflammation cannot be certainly attributed to diclofenac. Atrophy in digestive gland and necrosis occurred more frequently in mussels exposed to 40 μg/L. This may point to relation between the concentration of diclofenac and its toxicity. However, similar histopathological examination using mussels have not yet been performed. There are some studies that have revealed abnormalities in fish tissues due to diclofenac. Necrotic changes in kidney and gills were observed in *Oncorynhus mykiss* which were exposed to diclofenac

(Schwaiger et al., 2004; Mehinto et al., 2010). Hoeger et al. (2005) also revealed mild kidney necrosis of *Salmo trutta f. fario* exposed to 5 μg/L diclofenac for 21 days. On the other hand, Praskova et al. (2014) did not observe any lesions in *Danio rerio* exposed to high concentrations of diclofenac (up to 60 mg/L) for 28 days. This may be due to the lower sensitivity of *D. rerio* to pharmaceutical contamination.

Unfortunately, the histopathological effect of diclofenac and other NSAIDs on Mytilidae bivalves is almost unknown. There are several studies which tested the condition of tissues in bivalves taken from potentially polluted stations. However, there can be many contaminants and other factors in the environment that can cause lesions. Therefore it is important to investigate the effect of a pharmaceutical on mussel tissues also under laboratory conditions. Considering that lesions occur also in mussels from the environment, histopathological examination cannot be a specific marker of toxicity of diclofenac. In order to have a broader view of the toxicity of diclofenac, in addition to histopathological studies, it is worthwhile to include multibiomarker approach to assess the effects of the pharmaceutical at other levels of biological organisations.

4. Conclusion and future perspectives

Pharmaceutical was absorbed in the tissues of mussels during the exposure time, but completely eliminated after depuration phase. Although no diclofenac metabolites were detected in the study, probably due to too low (but environmentally relevant) concentration, it appears that mussels have successfully detoxified. However, it should be emphasised that the detoxification process in marine invertebrates still requires further research. The exposure to environmental concentrations of diclofenac during this relatively short experiment did not affect the general condition of *M. trossulus*. Yet, various histopathologies were observed in the digestive gland, mantle and gills, thus organs taking part in the pollutants uptake via respiratory and dietary routes. They included gill deformations, necrosis, brown pigment accumulation, gonadal atresia, digestive gland atrophy and digestive gland vacuolistation. Observed changes are non-specific and appear fully reversible at this stage, however, when the exposure is chronic (as it is in the environment), their progression to adverse and non-reversible ones is expected. That is in particular the case for the digestive gland atrophy, reversible at first, but later stages are characterized by irreversible loss of digestive gland absorptive cells. Thus, observed tissue alterations in response to diclofenac may lead to different effects on bivalve physiology at first affecting respiratory system, reproduction and food uptake. Chronic exposure to DCF may, however, result in more complex multiple system abnormalities leading not only to poor nutrition (despite availability of feed), metabolic disorders and compromised reproduction but also to eventual death of an organism. Finally, both disturbed reproduction and increased mortality rate can have a direct consequence on higher levels of biological organization, as the mussels are considered to be an important species from both ecological and economical point of views. Therefore, future research should primarily focus on the analysis of chronic, long-term effects of exposure to diclofenac.

Nevertheless, our findings suggest that even a short exposure to environmental concentration of diclofenac may increase the frequency of changes at tissue level. However, applicability of histopathological techniques in ecotoxicology of drugs requires additional evaluation in future studies.

CRediT authorship contribution statement

Klaudia Świacka: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft. **Katarzyna Smolarz:** Conceptualization, Methodology, Supervision, Writing - review & editing. **Jakub Maculewicz:** Investigation, Visualization, Data curation, Writing - review & editing. **Magda Caban:** Methodology, Validation, Supervision, Writing - review & editing.

Declaration of competing interest

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

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Effects of environmentally relevant concentrations of diclofenac in *Mytilus trossulus*

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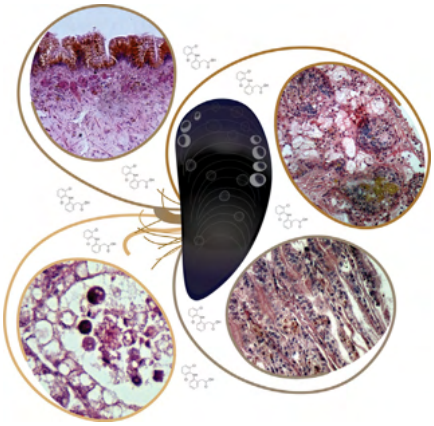
HIGHLIGHTS

- The effect of diclofenac in mussels was related to concentration.
- Diclofenac was completely excreted by mussels during the depuration phase.
- Diclofenac caused an increase in the frequency of lesions in *M. trossulus* tissues.
- No diclofenac metabolites were detected, possibly due to low exposure concentrations.
- Environmentally relevant concentration of drug did not affect the condition of mussels.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of pharmaceuticals in the marine environment is a growing problem of global importance. Although awareness of the significance of this issue is increasing, many questions related to the ecotoxicology of pharmaceuticals remain unclear. Diclofenac is one of the drugs most commonly detected in the marine environment and its potential toxicity has been previously highlighted, thus its impact on organisms deserves a special attention. Therefore, in this study, a thorough analysis of the effects of diclofenac on a condition and tissue level of a model representative of marine invertebrates - *Mytilus trossulus* - was performed. During the 25-day experiment, divided into exposure and depuration phases, bivalves were exposed to two environmentally relevant drug concentrations of 4 and 40 μg/L. The study showed that mussels absorb diclofenac in their tissues and the highest recorded concentration was 1.692 μg/g dw on day 8. Moreover, the content of diclofenac metabolites (4-OH and 5-OH diclofenac) was also examined, but they were not detected either in water or in tissues. Although exposure to low diclofenac concentrations did not significantly affect the condition index of organisms, changes in numerous histopathological parameters were noted. Performed histological examination provided additional valuable information on the influence of drugs on the functioning of invertebrates. Nevertheless, applicability of histopathological techniques in ecotoxicology of drugs requires additional evaluation in future studies.

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1. Introduction

Pharmaceuticals belong to the contaminants of the emerging concern, already present in the surface waters around the world (Borecka et al., 2015; Fisch et al., 2017). Emission of pharmaceuticals and personal care products (PPCPs) to the marine environment, mainly via rivers and surface runoffs, is becoming a serious global threat. However, the fate of pharmaceuticals in the marine ecosystems is still poorly researched. Many of these compounds are characterized by pseudo-persistent in the marine environment, thus they may constantly influence the condition of organisms, posing a serious threat to the marine fauna (Brausch et al., 2012; Fabbri and Franzellitti, 2016; Fent et al., 2006; Kümmerer, 2008; Sanderson et al., 2004). Nevertheless, the legislation controlling the release of PPCPs into the environment is still missing, which may be due to the insufficient number of studies to confirm the seriousness of the threat, as well as its potential long-term consequences (Dodder et al., 2014).

Non-steroidal anti-inflammatory drugs (NSAIDs) are predominant among pharmaceuticals detected in the aquatic environment worldwide. Recent studies have shown the presence of NSAIDs in the wild mussels collected along Spanish and Italian coasts, while many other works have confirmed their presence in different water bodies around the world (Hanif et al., 2020; Sathishkumar et al., 2020; Mezzelani et al., 2016a; Moreno-González et al., 2015). NSAIDs are characterized by anti-inflammatory, antipyretic and analgesic effects (Brune and Patrignani, 2015; He et al., 2017; Kümmerer, 2008; Nikolaou et al., 2007; Paíga et al., 2015). Over-the-counter (OTC) availability favours high consumption of these pharmaceuticals (Cleuvers, 2003; Comeau et al., 2008; Brune and Patrignani, 2015; Paíga et al., 2016; He et al., 2017; McRae et al., 2018). Among the NSAIDs, diclofenac has the highest toxicity and is the most frequently detected, thus it has been added to the European Surface Water Monitoring System according to the Water Framework Directive (Hallgren and Wallberg, 2015). Concentration of diclofenac in the marine environment is relatively low, ranging a few ng/L to several μg/L. However, continuous exposure of marine organisms to low concentrations of diclofenac may pose a risk to them (Dumas et al., 2020; Ryzhkina et al., 2020; Mezzelani et al., 2016b; Araujo et al., 2014). Many short-term laboratory studies have already shown that diclofenac has negative effect on marine organisms (Kloukinioti et al., 2020; Bonnefille et al., 2018; McRae et al., 2018; Freitas et al., 2019a; Freitas et al., 2019b; Du et al., 2016; Balbi et al., 2018; Sanderson et al., 2004). Study by Mezzelani et al. (2018) has shown that diclofenac, at environmentally relevant concentration, causes the modification of immunological parameters, modulation of lipid metabolism, genotoxic effects, as well as changes in cell turnover. On the other hand, Bonnefille et al. (2018) proved that diclofenac causes modulation on tyrosine-dopaminergic and tryptophan metabolic pathways in *M. galloprovincialis,* which consequently results in the disruption of osmotic regulation and reproduction processes. The fate of pharmaceuticals after entering invertebrates is a poorly researched issue. Studies on pharmacokinetics could significantly increase the knowledge concerning toxic properties of tested pharmaceuticals and more importantly, provide valuable information about the structure and activity of metabolites produced by organisms (Sathishkumar et al., 2020). Diclofenac metabolites produced by marine organisms are one of the least recognized concerns (Fu et al., 2020; Sathishkumar et al., 2020; Bonnefille et al., 2017; Świacka et al., 2019). Recently, Bonnefille et al. (2017) demonstrated that *Mytilus* spp. mussels metabolise diclofenac to 13 metabolites, including 4-OH and 5-OH diclofenac. Świacka et al. (2019) also confirmed the metabolism of diclofenac to 4-OH and 5-OH diclofenac by *M. trossulus*. However, data on diclofenac metabolites, their toxicity and potential bioaccumulation is very limited thus it is likely that these compounds may include potentially hazardous substances, and therefore it is worthwhile to scrutinize their structure and biological activity.

The Baltic Sea is characterized by a strong salinity and temperature gradient, also remaining one of the most polluted seas in the world (Voipio, 1981; Albalat et al., 2002; Bonsdorff, 2006; Korpinen et al., 2012). Baltic organisms, such as *M. trossulus* are forced to spend a lot of energy on osmotic regulation, therefore they may be more sensitive to any other external stressors, such as environmental pollution. *M. trossulus*, marine bivalve species that has been adapted to the harsh Baltic conditions, is also one of the most important links in the food chain. Furthermore, mussels are sedentary and filter-feeding organism and can remove various environmental pollutants from the water by accumulating them (James et al., 2020). Moreover, mussels are also often found near sewage outlets. Thus they are one of the most exposed groups of marine fauna to different contaminants (James et al., 2020; Caban et al., 2016; De Solla et al., 2016, Klosterhaus et al., 2013; Marigómez et al., 2013; Gillis, 2012).

The main aim of this work was to provide a more detailed information on the effect of diclofenac on marine fauna - *M. trossulus*, by performing exposure experiment in the laboratory controlled conditions. For the first time histological analysis was used to determine the toxicity of diclofenac in mussels. The effect of diclofenac on the general condition of mussels was also tested. A preliminary pharmacokinetic study was performed through the determination of diclofenac and its two metabolites (4-OH and 5-OH diclofenac) concentration in tissues and water during exposure to environmentally relevant concentrations of diclofenac and further depuration phases. The effect of different diclofenac concentrations has also been included. In addition, screening of selected NSAIDs (ketoprofen, ibuprofen, flurbiprofen and naproxen) in *M. trossulus* and water collected from the Gulf of Gdańsk was performed. Basing on previous experimental work, this study assumed that diclofenac effects may be revealed by increased frequency of tissue changes without necessarily affecting the overall condition. Although absorption of the drug in mussel tissues was expected, taking into account their ability to metabolise diclofenac, it was also anticipated that the drug could be completely excreted from the organisms during depuration. Moreover, it was assumed that the observed effects of diclofenac will be related to its concentration and seen not only at the genetic and biochemical levels but also at the tissue level, thus even more affecting the physiology of an organism.

2. Materials and methods

2.1. Sampling

Material (1000 individuals of *M. trossulus*) was collected from the Gulf of Gdańsk - at Orłowo station (54°29,40 N; 18°38,60 E) in January 2019. During the sampling, temperature and salinity were measured and their values were respectively: 5.1 °C and 7.1. Mussels were sampled using the benthic drag and water samples were collected using bathometer. Additionally, 300 individuals of *M. trossulus* and two litres of seawater were collected for analysis of diclofenac, its metabolites (4-OH diclofenac and 5-OH diclofenac) and other NSAIDs (flurbiprofen, ketoprofen, ibuprofen and naproxen) at the collection site. Next, mussels were transported to the laboratory and prepared for the toxicity and bioconcentration experiment.

2.2. Chemicals preparation

Diclofenac sodium salt (CAS 15307-79-6, analytical standard), 4-hydroxydiclofenac (CAS 64118-84-9, analytical standard) and 5-hydroxydiclofenac (CAS 69002-84-2, analytical standard) were purchased from Sigma-Aldrich, with a purity of >98%. Stock solutions of diclofenac, 4-hydroxydiclofenac and 5-hydroxydiclofenac at 5 mg/mL were prepared by dissolving in methanol (HPLC grade, POCH, Poland). To prepare the artificial salt water with a final salinity equal to 7 PSU

(salinity in the sampling site) distilled water and Aquaforest® salts standards were used.

2.3. Acclimation

Prior to acclimation, epiphytes were removed from mussels' shells. During the acclimation phase, the temperature was increased by 0.5° every 2 days from 5 to 8 °C. After stabilisation of laboratory conditions, the mussels were allowed to acclimatise to new conditions for additional two weeks. Hence, the overall acclimation phase took three weeks. During this stage, water was changed every four days while mussels were kept in dark. After each change of water, mussels were fed with *Chlorella vulgaris* in a volume of 3 mL and a concentration of 13 million cells/mL obtained from CCBA (Culture Collection of Baltic Algae).

2.4. Exposure experiment

Nine glass tanks (two different concentrations of diclofenac and control, each in triplicate) with 15 L of artificial sea water with 7 PSU were prepared (Fig.1). Two nominal concentrations of diclofenac: 4 and 40 μg/L were prepared by adding the following volumes: 12 μL and 120 μL of diclofenac solution at concentration of 5 mg/mL to the six tanks. The other three tanks (control-without solvent) were prepared without addition of diclofenac. Although in the previous experiment (Świacka et al., 2019) a solvent control was performed, for this study the procedure modified and instead of solvent control, stock solutions were prepared in a way that the volumes of methanol added to the aquariums were minimal. After dissolving the added solutions in 15 L of seawater, the maximum concentration of methanol was 8 mg/L. This value is up to 3 orders of magnitude lower than the NOEC value reported by ECHA (European Chemicals Agency) for aquatic organisms, thus still in accordance with the state-of-the-art of experimental studies.

To each tank 73 individuals of *M. trossulus* were placed. Laboratory experiment was divided into two parts: 12-day exposure of mussels to diclofenac followed by a 13-day depuration phase (Fig.1). During the exposure of mussels, water was changed every four days and after change the concentration of the pharmaceutical was renewed (Fig.1). In addition, 0.5 L of water was taken from the tanks with the pharmaceutical, before the water change, every four days. At the beginning of the depuration phase, the glass tanks were refilled with artificial sea water with 7 PSU and no more pharmaceutical was added. This phase lasted 13 days. The mussels were fed in the same way as during the acclimation. Before the exposure, five individuals were taken for histological analysis and two mussels for other testing. During the exposure to diclofenac four individuals were taken from all tanks (repetitions) every four days, before the water change. Four mussels were used in the chemical analysis of diclofenac, 4-OH diclofenac and 5-OH diclofenac presence. In addition, on days: 0 and 12 two individuals were taken from all tanks for histological analysis. On the last day of depuration phase four mussels were collected to chemical analysis.

2.5. Chemical analysis

The complete procedures concerning the determination of diclofenac concentration in water and in mussel tissues are described in our previous work (Świacka et al., 2019). Briefly, pharmaceuticals extraction from mussels' tissues was performed by the accelerated solvent extraction (ASE, Dionex ASE 350 model) with purification of the extracts by solid phase extraction (SPE, Strata-X; Phenomenex; Torrance,

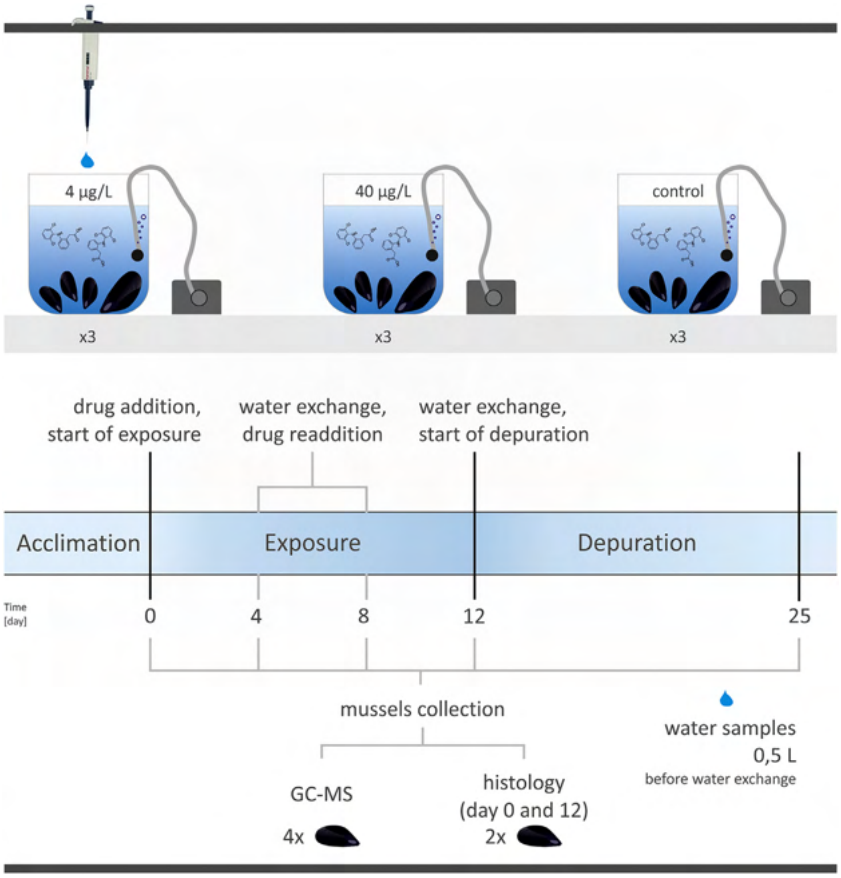

Fig. 1. Experimental procedure.

USA). In order to obtain pharmaceuticals from water samples, SPE was performed using disks H_2O-philic DVB Speedisk® (Bakerbond). Prior to SPE, water samples were filtered with glass fibre filters. Previously, it was proven that the filtration step have not impact the quantification of diclofenac anion in complex samples (Kołecka et al., 2019). Then, extracts form water and mussel tissues were derivatized and analyzed by gas chromatography with mass spectrometry (GC–MS, GCMSQP2010SE, Shimadzu; Kyoto, Japan). The method detection limits (MDL) of GC–MS for diclofenac, 4-hydroxydiclofenac and 5-hydroxydiclofenac are respectively: 1 ng/L/0.01 μg/g; 1 ng/L/0.08 μg/g and 2 ng/L/0.17 μg/g (water/tissue).

Diclofenac-13C6 sodium salt (CAS 1261393-73-0, Sigma-Aldrich) was used as internal standard added before each extraction.

2.6. Histology

The soft tissues of mussels were removed from the shells and placed in Davidson fixative solution (33% distilled water, 33% ethanol [96%], 22% formaldehyde, and 11% acetic acid). After two days mussels tissues were placed in 10% formaldehyde solution buffered with sodium phosphate (6.5 g/L dibasic Na_2HPO_4 and 4 g/L monobasic NaH_2PO_4) and stored for 10 days. Next, tissues were put in histological blocks, dehydrated in ascending ethanol series (from 70% to absolute ethanol) and embedded in paraffin. Two 3 μm sections were obtained from each individual, then left overnight at 60 °C, deparaffinized in three xylene baths, hydrated in descending ethanol series (from absolute ethanol to 70% ethanol followed by water bath) and finally stained according to the Harris' haematoxylin and eosin (H&E) standard protocol. The prepared samples were analyzed under light microscope for lesions in gonads, gills and digestive glands. The binary (0 for absent and 1 for present) scoring of lesions was used to assess the effect of diclofenac on mussel tissues. Similarly to Larsson et al. (2018), the frequency of lesions pooled by tissue type (i.e., gills, gonads and digestive gland) and the number of lesions was analyzed assuming a binomial error distribution.

2.7. Body mass index, BMI

To determine the condition of mussels, body mass index (BMI) showing the relation between the shell length (L) and tissue wet weight (W) was used. BMI was calculated as follows:

$BMI = W/L^3$ [mg/cm^3].

2.8. Statistical analysis

First, normality of data distribution and homogeneity of variances were verified using Shapiro–Wilk and Levene's tests. Statistical comparison of the BMI between mussels from the control and exposure groups and between replicates of exposure groups was performed using Mann–Whitney *U* test (STATISTICA software). Kruskal-Wallis test was used to compare the measured concentration of pharmaceutical in water and mussel tissues between experimental days and repetitions. The significance level was set to $p < .05$.

3. Results and discussion

3.1. Concentration of diclofenac and its metabolites in the mussels and water from the environment

Diclofenac, its metabolites (4-OH and 5-OH diclofenac) and other NSAIDs (ketoprofen, ibuprofen, flurbiprofen, naproxen and nimesulide) were not detected in mussels or in water collected from Puck Bay at Orłowo station. This may indicate that there are no NSAIDs at Orłowo station or their concentrations were too low (below a few ng/L) to be detected by GC–MS.

3.2. Concentration of diclofenac measured in water and tissues of mussels

The final measured concentrations of diclofenac in the tanks with nominal concentration of 40 μg/L were as follows: 19.859, 25.314 and 24.339 μg/L on days 4, 8 and 12, respectively (Fig.2). Four days after adding diclofenac, its measured concentration was almost 50% lower, which may indicate that pharmaceutical was taken by mussels or adsorbed on shells biofilm. The solubility of diclofenac could be also lower in salt water than in fresh water (about 4.8 mg/L [drugbank.ca]). According to numerous studies, Mytilidae mussels can absorb diclofenac from water as evidenced by its presence in their tissues (Ericson et al., 2010; Świacka et al., 2019). Decreased concentration of pharmaceutical may also indicate its instability in the water matrix due to its biodegradation or adsorption on the walls of the tank (Packer et al., 2003). Our previous study (Świacka et al., 2019) showed that the determined concentration of diclofenac in artificial seawater decreased by almost 50% after ten days, which was most probably related to the presence of bacteria and microalgae that metabolized diclofenac, or to its adsorption on glass (Packer et al., 2003; Jewell et al., 2016; Avetta et al., 2016; Xiong et al., 2018). However, the influence of photodegradation was excluded, as the water tank was kept in the dark. There were no statistical differences in diclofenac concentration in water between the replications and days of exposure which indicates good repeatability of experimental conditions.

The final determined concentration of diclofenac in the tanks with nominal concentration 4 μg/L was: 2.697, 3.585 and 4.205 μg/L on days 4, 8 and 12, respectively (Fig.2). Each four days after adding diclofenac, its measured concentration was similar to the nominal concentration. This may be connected to the lower filtration rate and absorption of diclofenac by mussels, as also indicated by the lower concentration of diclofenac in the tissues of mussels exposed to 4 μg/L (Fig.2). Lower concentrations of pharmaceuticals may have been a minor stress factor for mussels and therefore their filtration rate has

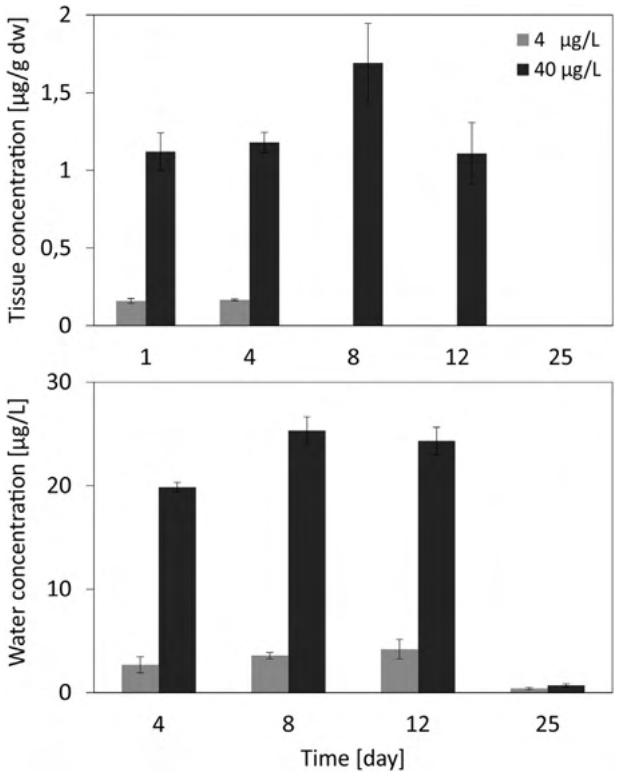

Fig. 2. Diclofenac concentration in water and mussel tissues treated with 4 and 40 μg/L during 25 days of experiment (mean ± standard deviation SD; n = 12). Statistical comparison (Kruskal-Wallis test).

not increased. During the last day of the purification phase, diclofenac was also detected in water. The detected concentration of diclofenac was 0.726 μg/L and 0.398 μg/L in the tanks with nominal concentrations 40 and 4 μg/L respectively (Fig.2). It may indicate that mussels eliminated the pharmaceutical from the tissues or it was desorbed from the shells.

In present study *M. trossulus* mussels were exposed to environmentally relevant concentrations: 4 and 40 μg/L yet it became clear that despite this low levels diclofenac concentration in tissues was dose-dependent. In the group exposed to 4 μg/L diclofenac was detected only on days 1 and 4, with the highest concentration of 0.166 μg/g dw on day 4 (Fig.2). This may indicate that mussel managed to metabolise and excrete smaller amounts of the pharmaceutical faster and more efficiently. Therefore, it confirms that kinetic of pharmaceutical after entering the mussels is strongly related to its concentration. Diclofenac was detected in mussels exposed to 40 μg/L on each day of exposure, with the highest concentration of 1.692 μg/g dw on day 8 (Fig.2). This proves that mussels absorb the pharmaceutical into tissues. Diclofenac is uptaken by bivalves mainly through the gills, but also through the whole body surfaces (Turja et al., 2020). The gills also have another function and are involved in the metabolism and elimination of xenobiotics, including pharmaceuticals (Gomez et al., 2010; Widdows et al., 1983). The degree to which pharmaceuticals are removed by the gills may depend on their concentration.

In the group exposed to 40 μg/L the concentration of diclofenac in tissues was up to 10 times higher than in the group exposed to 4 μg/L, which may be caused by the slower rate of detoxification and excretion of xenobiotic by *M. trossulus*, as well as the higher amount of diclofenac in water available for mussels. On day 8, the concentration of pharmaceutical in mussel tissues was higher than on the remaining days, but it was not statistically significant. This may suggest an increased rate of filtration of mussels due to the stress associated with the permanent presence of diclofenac and thus higher absorption into tissues (Ericson et al., 2010). On day 12, the concentration of pharmaceutical in the tissues decreased. However, it was not statistically significant. This may be related to the increased metabolism of diclofenac and its faster excretion from the body (Bonnefille et al., 2017; Świacka et al., 2019).

The metabolic processes of pharmaceuticals in Mytilidae mussels are still poorly understood and require more attention. The information available so far does not allow a full understanding of the detoxification in marine invertebrates. Therefore, only after filling the knowledge gaps it will be possible to make a realistic assessment of the consequences associated with pharmaceutical contamination.

Freitas et al. (2019a) have shown that salinity significantly affects the concentration of diclofenac in mussel tissues. *M. galloprovincialis* individuals were exposed to the same concentration of 1 μg/L for 28 days. However, the content of diclofenac in their tissues differed by up to 60% between salinity 25 and 30 (environmental salinity). Interestingly, in this study the authors observed that mussels from environmental salinity reduce their metabolic rate after exposure to diclofenac, which may explain lower concentration of diclofenac in their tissues compared to mussels from salinity 25. That highlights the importance of the environmental conditions in which bivalves live and are adapted to, as there may be differences in bioaccumulation, metabolism and toxicity of the compound in species from areas varying in, for example, salinity (like the Baltic Sea and the Atlantic). Both salinity and temperature have a significant influence on the metabolism of bivalves, which in turn contributes to their ability to absorb and transform the compound. Diclofenac concentrations in tissues may also vary between Mytilidae species. Various mussel species may have different absorption, metabolism and excretion capacities. For this reason, it is important to have a better understanding of what happens to the pharmaceutical when it enters the body. The present study has shown that the diclofenac concentration has a significant effect on bioconcentration, as also reported in Ericson et al. (2010) (Table 1), where diclofenac content was higher in the tissues of individuals exposed to the higher concentrations of the compound. Nevertheless, not all toxicity studies of diclofenac have analyzed its concentration in tissues, as shown in Table 1. However, this combination would be very valuable as it could enable verification whether there is a relationship between the drug concentration in tissues and its toxicity, and further risk assessment.

During the experiment, no diclofenac metabolites (4-OH and 5-OH diclofenac) were detected in water and tissues, which may be associated with the low (environmentally relevant) doses of diclofenac and detection limits of GC–MS. However, in our previous study (Świacka et al., 2019) 4-OH and 5-OH diclofenac were detected in the mussels exposed to much higher levels of diclofenac (133.33 μg/L) and its concentration in tissues was around 1 μg/L. In the present study mussels were treated with three times lower concentration that was environmentally relevant, therefore diclofenac metabolites could not be detected. Moreover, 4-OH and 5-OH diclofenac may have been transformed into other derivatives that we have not tested. According to Bonnefille et al. (2017), Mytilidae mussels transform diclofenac to up to 13 different metabolites from both the first and the second phase of detoxification processes. The absence of 4-OH and 5-OH diclofenac in water and mussel tissues may therefore indicate further metabolism in the second phase of detoxification.

3.3. BMI

BMI values representing the condition of mussels during the exposition and depuration are shown in the Fig. 3. The condition of mussels from 4 μg/L tank decreased during 12 days of the exposure. In the mussels exposed to 4 μg/L the highest average BMI was observed on day 1, while in mussels treated with 40 μg/L the highest average BMI was observed on day 4. However, no statistically significant differences in the BMI were observed between the control and the treatment groups during exposition and depuration. Study by Gonzalez-Rey and Bebianno (2014) also investigated the effect of diclofenac on the overall condition of mussels. *Mytilus galloprovincialis* were exposed at environmentally relevant concentration of 250 ng/L for 15 days. During the exposure, no significant differences regarding the condition index (CI) were observed between treatment and control groups. Similarly to our research, diclofenac did not affect the condition of mussels. The exposure time may have been too short to observe the effect of pharmaceutical at environmental concentration on the condition of *M. trossulus*. However, in our previous research, statistically significant effect of diclofenac on mussel condition during depuration phase was observed, but the exposure concentration was three times higher (Świacka et al., 2019). Moreover, no correlation was observed between BMI value and exposure time.

To date, the impact of diclofenac or other NSAIDs on the general condition of mussels has not been tested in any study other than those mentioned. Some studies have evaluated differences in BMI values for mussels collected from different stations including points localizated near sewage treatment plant outlets (Larsson, 2017; Larsson et al., 2018). However, the observed differences in BMI values in *M. trossulus* were more related to the availability of food rather than to the presence of contaminants.

3.4. Histological examination

Fig. 4. shows tissue abnormalities occurring in *M. trossulus* after exposure experiment. Only regressive changes often occurring in bivalves inhabiting poor environmental conditions were found. Accumulation of brown granules, which are most likely to be lipofuscin, was observed in the mantle epithelium, as shown in Fig. 4.A. The accumulation of this pigment was observed only in the epithelium of different tissues (gills, mantle and digestive gland).

Fig. 4.B illustrates atresia in male gonads indicated by hemocytic digestion and degradation of atretic gametes in follicle. Gonadal atresia occurred both in males and females. Atrophy and vacuolisation in

Table. 1
Toxic effects of diclofenac in Mytilidae mussels.

Species	Exposure concentration [μg/L]	Exposure time [day]	Tissue concentration [ng/g dw]	Biological effects	References
Mytilus spp.	1; 1000	1; 4	na	Disorders of glutathione S-transferase (GST) and lipid peroxidase (LPO) activities, increase in DNA damage	Schmidt et al. (2011)
	1; 1000	7; 4; 21	na	Disorders of glyceraldehyde - 3 phosphate dehydrogenase, aconitase-1 and GST activities	Schmidt et al. (2014)
M. galloprovincialis	25	14	14.90 ± 7.89	Induction of antioxidative defence, disorder of lysosomal membrane stability (LMS), inhibition of acyl-coenzyme A oxidase and catalase (CAT) activities, genotoxic effect	Mezzelani et al. (2016a)
	2.5	60	2.25 ± 0.810	Diclofenac accumulation in mussel tissues, genotoxic effect, modulation of immunological parameters and cellular turnover, disturbance of lipid metabolism	Mezzelani et al. (2018)
	0.05; 0.50	14	na	Modulation of CAT activity, genotoxic effect	Munari et al. (2018)
	122.6	7	na	Modulation of metabolic pathways of tyrosine-dopamine and tryptophan, disruption of osmosis and gametes release	Bonnefille et al. (2018)
	1; 100	3	26; 2080	Modulation of prostaglandin sythesis	Courant et al. (2018)
	1	28	47.0 ± 1.4	Induction of antioxidative defence (modulation of GPx and CAT activities, reduction of GSH/GSSG ratio)	Freitas et al. (2019a)
	0.25	15	na	Alteration in estrogens level, induction of antioxidative defence (modulation of the activity of superoxide dismutase, acetylcholinesterase and glutathione reductase)	Gonzalez-Rey and Bebianno (2014)
	1; 10	2	na	Induction of shell deformation, influence on transcription of genes involved in shell formation and biotransformation	Balbi et al. (2018)
M. trossulus	1; 10,000	8; 14; 19; 21	82 ± 6; 0.18 ± 0.02	Decrease of scope for growth and weakening strength and abundance of byssus threads	Ericson et al. (2006)
	133.33	5	0.86	Significant impact on the condition of mussels during the purification phase	Świacka et al. (2019)

na - not available.

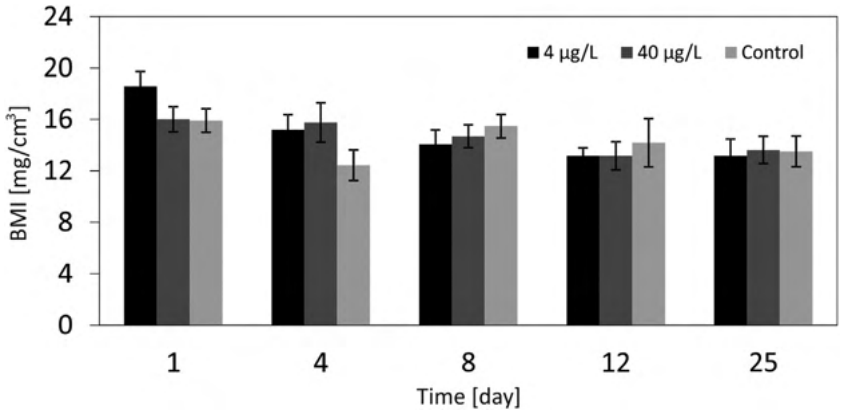

Fig. 3. Body mass index (BMI) calculated for *M. trossulus* during 25 days of experiment (mean ± standard deviation SD; n = 12). Statistical comparison (Mann–Whitney *U* test).

hepatopancreas are shown in Fig. 4.C. Atrophy was defined as the presence of necrotic cells, enlargement of the vacuole in the epithelium and deformation of the tubular cells, which leads to abnormal tubule structure. Fig. 4.D shows gills deformations indicated by numerous swellings and presence of cavities in the epithelium.

M. trossulus individuals treated with diclofenac, as well as those from the control group, were characterized by the frequency of tissue abnormalities such as: gill deformations, gonadal atresia, atrophy and vacuolisation in digestive glands (Fig.4; Fig.5). Brown pigment accumulation and necrosis were observed only in individuals exposed to diclofenac, while inflammations were noted only in the mussels from the control tanks (Fig.5).

Lipofuscin is an end product formed due to the oxidation of lipids and proteins during oxidative stress, therefore the brown pigment that we have observed in treated mussels is likely to be lipofuscin. Lipofuscin accumulation in the exposed groups may indicate metabolism disorders caused by diclofenac. Lipofuscin accumulation can be a marker of metabolic disorders and oxidative stress (Brenner et al., 2014; Mezzelani et al., 2016a; Bignell et al., 2011). According to Martín-Díaz et al. (2009) exposure of mussels to carbamazepine (which belongs to NSAIDs) at 10 μg/L for seven days resulted in lipofuscin accumulation in digestive gland. Study by Mezzelani et al. (2016b) also confirmed significant accumulation of lipofuscin in digestive gland in mussels exposed to diclofenac and ibuprofen. However, the brown pigment accumulation similar to lipofuscin is constantly observed in mussels from the environment (Bignell et al., 2011; Brenner et al., 2014; Raftopoulou and Dimitriadis, 2012). Study by Bignell et al. (2011) showed differences in lipofuscin content in the epitelium of kidney in *Mytilus* spp. from different stations. Level of lipofuscin was higher in stations where higher levels of metals were observed (Shaw et al., 2011). On the other hand, study conducted by Brenner et al. (2014) showed seasonal variations in the lipofuscin levels in the digestive gland of Mytilidae mussels. The changes in lipofuscin content may be related to the reproductive processes of these animals occurring during warm seasons. Changes in lipofuscin levels between mussels submerged and those living in intertidal zones were also found. This in turn may be related to the availability of food and feeding mode. Lipofuscin accumulation can therefore be a good biomarker to

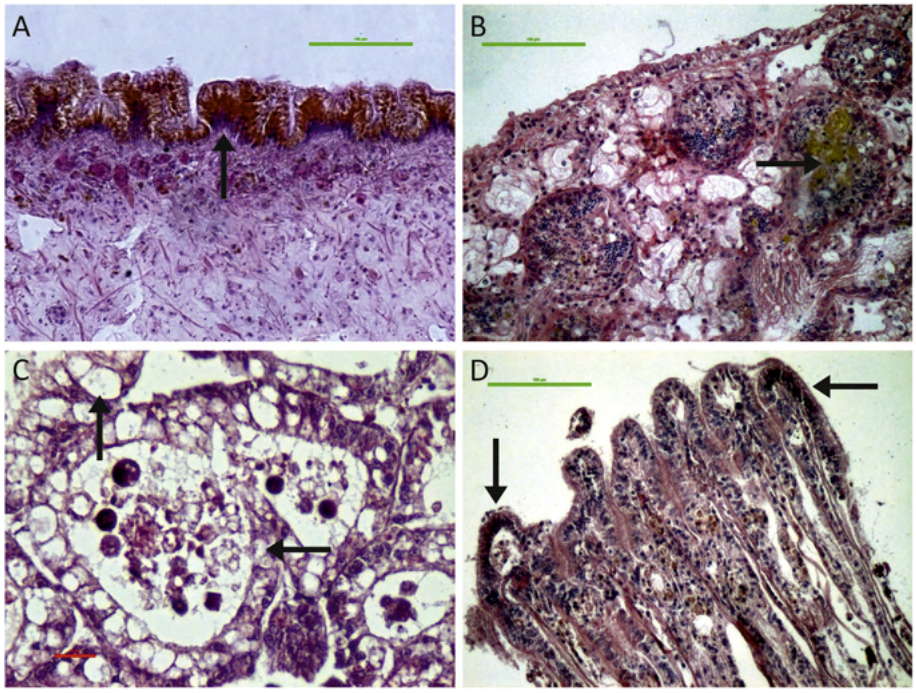

Fig. 4. Cross sections through *M. trossulus* tissues treated with diclofenac after 12 days of exposure. Accumulation of lipofuscin-like granules in mantle' epithelium, scale 100 μm (A); atresia in male gonads manifested by the presence of brown cells (arrow), scale 100 μm (B); Atrophy and vacuolisation of the digestive tubules containing various digestive cells (B-, R- and F-cells) showing an enlargement of the lumen area and reduced epithelial layer, scale 20 μm (C); Gill deformations including oedematous changes and local necrosis (arrows), scale 100 μm (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

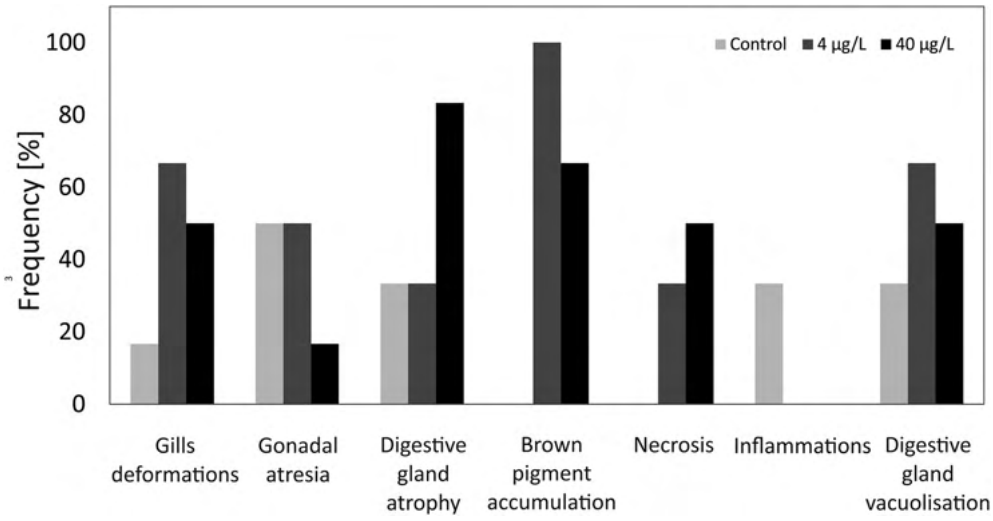

Fig. 5. The frequency of tissue lesions of *M. trossulus* after 12 days of exposure to diclofenac expressed as a percentage.

determine the condition of the environment, and to assess toxic potential of tested compounds including pharmaceuticals. However, the potential influence of seasonal changes, which also affect the lipofuscin content in individual tissues of mussels, should be eliminated.

Most of tissue abnormalities were observed both in experimental groups and controls, which means that diclofenac did not cause these lesions exclusively and the observed lesions are non-specific. However, when looking at the Fig. 5, it can be concluded that diclofenac contributed to an increase in frequency of abnormalities. Several laboratory studies confirmed that diclofenac causes metabolism disorders as evidenced by disruption of glutathione S-transferase (GST), lipid peroxidase (LPO) activity, as well as activity of other enzymes involved in cell metabolism (Table 1). On the other hand, inflammation was observed only in the control tanks which may reflect anti-inflammatory effect of diclofenac in the non-target organisms. Study by Courant et al. (2018) as first revealed anti-inflammatory action of diclofenac in Mytilidae mussels (Table 1). However, in present study very few mussels were tested for the presence of inflammation in tissues, therefore, the absence of inflammation cannot be certainly attributed to diclofenac. Atrophy in digestive gland and necrosis occurred more frequently in mussels exposed to 40 μg/L. This may point to relation between the concentration of diclofenac and its toxicity. However, similar histopathological examination using mussels have not yet been performed. There are some studies that have revealed abnormalities in fish tissues due to diclofenac. Necrotic changes in kidney and gills were observed in *Oncorynhus mykiss* which were exposed to diclofenac

(Schwaiger et al., 2004; Mehinto et al., 2010). Hoeger et al. (2005) also revealed mild kidney necrosis of *Salmo trutta f. fario* exposed to 5 μg/L diclofenac for 21 days. On the other hand, Praskova et al. (2014) did not observe any lesions in *Danio rerio* exposed to high concentrations of diclofenac (up to 60 mg/L) for 28 days. This may be due to the lower sensitivity of *D. rerio* to pharmaceutical contamination.

Unfortunately, the histopathological effect of diclofenac and other NSAIDs on Mytilidae bivalves is almost unknown. There are several studies which tested the condition of tissues in bivalves taken from potentially polluted stations. However, there can be many contaminants and other factors in the environment that can cause lesions. Therefore it is important to investigate the effect of a pharmaceutical on mussel tissues also under laboratory conditions. Considering that lesions occur also in mussels from the environment, histopathological examination cannot be a specific marker of toxicity of diclofenac. In order to have a broader view of the toxicity of diclofenac, in addition to histopathological studies, it is worthwhile to include multibiomarker approach to assess the effects of the pharmaceutical at other levels of biological organisations.

4. Conclusion and future perspectives

Pharmaceutical was absorbed in the tissues of mussels during the exposure time, but completely eliminated after depuration phase. Although no diclofenac metabolites were detected in the study, probably due to too low (but environmentally relevant) concentration, it appears that mussels have successfully detoxified. However, it should be emphasised that the detoxification process in marine invertebrates still requires further research. The exposure to environmental concentrations of diclofenac during this relatively short experiment did not affect the general condition of *M. trossulus*. Yet, various histopathologies were observed in the digestive gland, mantle and gills, thus organs taking part in the pollutants uptake via respiratory and dietary routes. They included gill deformations, necrosis, brown pigment accumulation, gonadal atresia, digestive gland atrophy and digestive gland vacuolistation. Observed changes are non-specific and appear fully reversible at this stage, however, when the exposure is chronic (as it is in the environment), their progression to adverse and non-reversible ones is expected. That is in particular the case for the digestive gland atrophy, reversible at first, but later stages are characterized by irreversible loss of digestive gland absorptive cells. Thus, observed tissue alterations in response to diclofenac may lead to different effects on bivalve physiology at first affecting respiratory system, reproduction and food uptake. Chronic exposure to DCF may, however, result in more complex multiple system abnormalities leading not only to poor nutrition (despite availability of feed), metabolic disorders and compromised reproduction but also to eventual death of an organism. Finally, both disturbed reproduction and increased mortality rate can have a direct consequence on higher levels of biological organization, as the mussels are considered to be an important species from both ecological and economical point of views. Therefore, future research should primarily focus on the analysis of chronic, long-term effects of exposure to diclofenac.

Nevertheless, our findings suggest that even a short exposure to environmental concentration of diclofenac may increase the frequency of changes at tissue level. However, applicability of histopathological techniques in ecotoxicology of drugs requires additional evaluation in future studies.

CRediT authorship contribution statement

Klaudia Świacka: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft. **Katarzyna Smolarz:** Conceptualization, Methodology, Supervision, Writing - review & editing. **Jakub Maculewicz:** Investigation, Visualization, Data curation, Writing - review & editing. **Magda Caban:** Methodology, Validation, Supervision, Writing - review & editing.

Declaration of competing interest

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

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Exposure of *Mytilus trossulus* to diclofenac and 4′-hydroxydiclofenac: Uptake, bioconcentration and mass balance for the evaluation of their environmental fate

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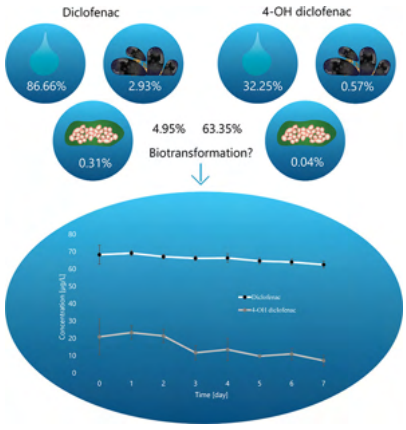
HIGHLIGHTS

- Diclofenac and 4-hydroxydiclofenac are uptaken from water by *M. trossulus*.
- Diclofenac and 4-hydroxydiclofenac are bioconcentrated in mussel tissues and biofilm.
- The bioconcentration of 4-hydroxy-diclofenac is significantly different from that of diclofenac.
- Diclofenac is metabolised by *M. trossulus* to 5-hydroxydiclofenac.
- 5-hydroxydiclofenac was detected only in *M. trossulus* females.

GRAPHICAL ABSTRACT



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ABSTRACT

Diclofenac (DIC) is one of the most widely consumed drugs in the world, and its presence in the environment as well as potential effects on organisms are the subject of numerous recent scientific works. However, it is becoming clear that the risk posed by pharmaceuticals in the environment needs to be viewed more broadly and their numerous derivatives should also be considered. In fact, already published results confirm that the transformation products of NSAIDs including DIC may cause a variety of potentially negative effects on marine organisms, sometimes showing increased biological activity. To date, however, little is known about bioconcentration of DIC and DIC metabolites and the role of sex in this process. Therefore, the present study for the first time evaluates sex-related differences in DIC bioconcentration and estimates bioconcentration potential of DIC metabolite, 4-OH DIC, in the *Mytilus trossulus* tissues. In the experiment lasting 7 days, mussels were exposed to DIC and 4-OH DIC at concentrations 68.22 and 20.85 μg/L, respectively. Our study confirms that DIC can be taken up by organisms not only in its native form, but also as a metabolite, and metabolised further. Furthermore, in the present work, mass balance was performed and the stability of both studied compounds under experimental conditions was analysed. Obtained results suggest that DIC is more stable than its derivative under the tested conditions, but further analyses of the environmental fate of these compounds are necessary.

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1. Introduction

Diclofenac (DIC) is a non-steroidal anti-inflammatory drug (NSAID) commonly used to treat a variety of health problems such as inflammation and pain of various origins, e.g. headaches, rheumatic and spinal diseases, post-operative and post-traumatic pain, gout and inflammatory reactions caused by infections (Nair and Taylor-Gjevre, 2010; Kołodziejska and Kołodziejczyk, 2018; Zhang et al., 2014; Kantor, 1986). The highest national consumption of DIC is estimated in Brazil, India, and Germany (>60 ton per year) followed by Egypt, Russia, USA, France, Ukraine, and Turkey (30–60 ton per year) (Acuña et al., 2015). The widespread use of this compound favours its release into the environment mainly through wastewater treatment plant effluent (WWTPs), household disposal, farm landings and then, through surface runoff and rivers, entering lakes, seas, and oceans (Zhang et al., 2008; Nikolaou et al., 2007; Lonappan et al., 2016). Therefore, DIC is among the most frequently detected pharmaceuticals in the aquatic environments (Nikolaou et al., 2007; Zhang et al., 2011; Coelho et al., 2009; Hallare et al., 2004; Sathishkumar et al., 2020). It has been found in surface waters from many countries such as Pakistan, Canada, USA, Germany, Spain, Poland, and France (Hanif et al., 2020; Letzel et al., 2009; Lonappan et al., 2016; López-Serna et al., 2012; Vulliet et al., 2011; Styszko et al., 2021). In addition, DIC is frequently detected in marine biota. As an example, DIC was found in certain fish species (*Liza aurata* and *Platichthys flesus*) sampled from the Mar Menor lagoon in Spain, the Tagus estuary in Portugal and the Scheldt estuary in Netherlands (Álvarez-Muñoz et al., 2015; Moreno-González et al., 2016). DIC was also found in Mytilidae bivalves. Mytilidae family includes long-living sedentary bivalves filtering large amounts of water to obtain food, but they are also known from their ability of contamination uptake from the ambient environment. Also, they inhabit coastal waters characterized by a wide range of salinities and temperatures. For these reasons they are suitable organisms for biomonitoring of coastal water quality including biomonitoring of pharmaceuticals (Pagano et al., 2020; Abo-Al-Ela and Faggio, 2021; Freitas et al., 2019; Freitas et al., 2020a, 2020b; Stara et al., 2020a, 2020b; Capillo et al., 2018; Pagano et al., 2017).

Recently, DIC was detected in *M. galloprovincialis* collected from known as unpolluted Italian coastal areas at concentrations ranging from 1.21 to 280 ng/g (Mezzelani et al., 2020). In addition, DIC was also detected in *Mytilus* spp. collected from the coast of Italy, Ireland, and Portugal (McEneff et al., 2014; Capolupo et al., 2017; Cunha et al., 2017). Wide occurrence of DIC in the aquatic environment as well as in marine organisms is of a great concern. As a biologically active compound, DIC affects various mechanisms in the body (Trombini et al., 2020; Fent et al., 2006; Lonappan et al., 2016). An exposure to this compound results in a number of adverse effects in various groups of aquatic organisms (Świacka et al., 2020). Among those are oxidative stress, endocrine and reproduction disruption, impaired function of certain organs, lesions in various tissues, as well as disorders of metabolism and immune response. Genotoxicity, cytotoxicity and embryotoxicity have also been reported in organisms exposed to this pharmaceutical (Mezzelani et al., 2016a, 2016b; Mezzelani et al., 2018a, 2018b; Świacka et al., 2020; Ericson et al., 2010; Lee et al., 2011; Bonnefille et al., 2018; Van den Brandhof and Montforts, 2010). As reported by numerous papers DIC is removed from the aquatic environment mainly by photodegradation (Tixier et al., 2003; Zhang et al., 2011; Buser et al., 1998). Some works also points to the large contribution of biofilm from rivers and streams to DIC biodegradation (Lee et al., 2012; Li et al., 2014; Gröning et al., 2007). Recently, it has been shown that in addition to fungi and bacteria, various strains of algae can also effectively remove DIC from water (Ouada et al., 2019). On the other hand, biodegradation of DIC results in the formation of hydroxy metabolites like 4-hydroxydiclofenac (4-OH DIC) and 5-hydroxydiclofenac (5-OH DIC) (Grandclément et al., 2020; Ouada et al.,2019; Li et al.,2014). 4-OH DIC and 5-OH DIC are also the main metabolites excreted by humans and most of vertebrates (Sawchuk et al., 1995; Stülten et al., 2008; Lonappan et al., 2016) and are therefore frequently detected in sewage effluents, open drainages, or rivers (Stülten et al., 2008; Osorio et al., 2014; Schmidt et al., 2018; Scheurell et al., 2009). In some cases, the concentration of 4-OH DIC was found to be higher than that of DIC in raw wastewater, and increase after wastewater treatment (Kołecka et al., 2019, 2020). It has recently been revealed that even some invertebrates and plants exposed to DIC also metabolize it to 4-OH and 5-OH DIC (Bonnefille et al., 2017; Świacka et al., 2019; Fu et al., 2020; Bartha et al., 2014). Hence, not only parent compounds but also their metabolites may pose a threat to the aquatic environment. However, data on the uptake of these compounds by organisms directly from their ambient environment, their accumulation in biota, toxicity, fate, and distribution in the aquatic environment are minor. There are some data on the toxicity of hydroxy metabolites of DIC towards bacteria, but there is no data regarding important groups of higher aquatic organisms (Grandclément et al., 2020). Without this fundamental background information it is not possible to assess the risks associated with the presence of metabolites in the aquatic environment.

Therefore, the main objective of this study was to evaluate the (toxico)kinetics of 4-OH DIC and DIC in artificial brackish water. For this purpose, an important Baltic species, *Mytilus trossulus*, was exposed to DIC and its metabolite under controlled laboratory conditions. The uptake, metabolism, and gender-related bioconcentration of DIC and 4-OH DIC in mussel tissues were evaluated. Using the results obtained, a mass balance was calculated, allowing for a better visualization, and understanding of the kinetics of 4-OH DIC and DIC. In addition, the BCF was determined to show the accumulation potential of DIC and 4-OH DIC in mussel tissues and biofilm.

2. Materials and methods

2.1. Sampling

Mussels (*M. trossulus*) and water were collected on-board of *R/V* Oceanograf via benthic dredge and a Niskin bottle from the Baltic Sea at 17 m depth Orłowo station (Gulf of Gdańsk) on July 7th 2020. The temperature and salinity measured during sampling day were 14.3 °C and 7.1 PSU, respectively. After sampling, mussels (about 1300 individuals) were transported to the laboratory and acclimated to the laboratory conditions before the exposure experiment begun. In addition, 100 individuals and 2 L of brackish water were collected in order to verify the presence of DIC in the environmental samples.

2.2. Chemicals preparation

Diclofenac sodium salt (CAS 15307-79-6, analytical standard, purity>98%) 5-hydroxydiclofenac (CAS 69002-84-2, analytical standard, purity>98%), 4-hydroxydiclofenac (CAS 64118-84-9, analytical standard, purity1>98%) and internal standard diclofenac-13C6 sodium salt (CAS 1261393-73-0) were purchased from Sigma-Aldrich. To evaluate the other than 4-OH DIC metabolites of DIC, the 5-OH DIC metabolite was analysed in the mussels' extract (same metabolites was found previously by Bonnefille et al., 2017 work as well as in our previous study (Świacka et al., 2019). The internal standard was used to correct the variable recovery of targets (DIC and hydroxy-metabolites) from waters and biological extracts. This is a standard procedure in the analysis of complex matrices to obtain accurate results, not burdened by matrix effects (Caban et al., 2012).

Stock solutions of DIC and 4-OH DIC with a final concentration of 1000 μg/mL, were obtained by dissolving them in methanol (HPLC grade, POCH, Poland). Aquaforest® salt standard was used to prepare artificial sea water with a final salinity 7 PSU, corresponding to salinity at place of sampling.

2.3. Acclimation

The acclimation phase lasted two weeks and was performed in 10 °C in order to prevent reproductive processes. Epiphytes were removed from the shells and the mussels were placed in 15 L aquaria filled with artificial brackish water of 7 PSU salinity and pH 8.9. The water in the aquaria was changed every four days. During acclimation phase as well as experimental phase *M. trossulus* were kept in the dark to eliminate the influence of UV–Vis spectrum on DIC and 4-OH DIC degradation and fed with *Chlorella vulgaris* (5 mL of culture containing 4.24×10^7 cell/mL) obtained from CCBA (Culture Collection of Baltic Algae).

2.4. Exposure experiment

Exposure experiment lasted seven days. For this purpose 12 tanks filled with 15 L of artificial brackish water were prepared and 50 mussels were placed in each tank. To three tanks 1.5 mL of DIC at 1000 μg/mL was added giving a nominal concentration of 100 μg/L. The same volume and concentration of 4-OH DIC was added to another three tanks.

Three tanks with 1.5 mL of methanol served as solvent control. The remaining three tanks were used as a proper control. After adding DIC, 4-OH DIC and methanol the water in tanks was mixed and 100 mL of water was collected after half an hour. Each day of experiment, 100 mL of water was sampled from the tanks with DIC and 4-OH DIC. In addition, the ammonia level in each tank was checked daily using special test (Salifert ®). During experiment mussels were fed twice (day 0 and 4) with the same amount of *C. vulgaris* as during acclimation. On the last 7th day of the experiment, mussel were collected. 10 mussels were taken from each tank in order to perform chemical analysis while the remaining mussels were taken for measurement of responses of various biochemical and physiological biomarkers that will be in detail evaluated separately. During sampling water from the tanks was gently poured so the biofilm was not disturbed. Then, using a clean toothbrush, the biofilm was collected from the tank walls as well as from the mussel shells.

2.5. Chemical analysis

2.5.1. Mussel tissues and water

Shortly after collection, the sex of the mussels was microscopically determined based on a sub-sample of gonadal tissue collected from the mantle area (1 mm × 1 mm). Sexing was based on the presence of ovarian (females) and testis (males) tissues. Thus, five males and five females were selected from each tank for chemical analysis. The wet weight of each individual was weighted and the shell length was measured. The tissues, which were divided and pooled based on the sex (males and females' tissues separately), were frozen at −80 °C and lyophilised. Subsequently, the dry weight of mussel tissues was weighted and all dry tissue obtained after lyophilization was used for pharmaceutical extraction. The full procedure (ASE, accelerated solvent extraction; SPE, solid phase extraction and GC–MS, gas chromatography–mass spectrometry) for determination of non-steroidal pharmaceuticals in mussel tissues and water is described in detail in Świacka et al. (2019). In brief, the lyophilised mussel tissues were extracted using ASE. Next, the obtained extract was diluted and subjected to SPE. The analyte was then purified and eluted from the columns, dried under gaseous nitrogen and derivatised. The samples prepared in this way were analysed using GC–MS. The method detection limits (MDL) of GC–MS for DIC, 4, and 5-OH DIC are: 1 ng/L/0.01 μg/g; 1 ng/L/0.08 μg/g and 2 ng/L/0.17 μg/g (water/tissue), respectively.

2.5.2. Biofilm

In order to obtain biofilm from the mussel shells, the shells were immersed in methanol and ultrasonicated twice for 10 min. Obtained extract was transferred into 500 mL amber glass bottles to which the previously obtained biofilm from the tank walls was added. Then methanol was evaporated in rotary evaporator and the dry mass of biofilm was weighted. To each sample with biofilm 100 mL of methanol and 100 μL of DIC 13C6 at 5 μg/mL were added. Next, samples were sonicated for 15 min. Obtained biofilm extract was filtered on glass fiber filters and the final volume of extract was measured. Then 10 mL of extract was collected, dissolved in 190 mL of distilled water. Another steps (SPE and GC–MS) were the same as during tissue extraction.

2.5.3. DIC and 4-OH DIC hydro-stability test

For determination of DIC and 4-OH DIC hydro-stability in test water, the following experiment was performed. The appropriate methanol solution of each analyte was added to glass vials (10 mL), evaporated to dryness, re-constructed with 10 mL of 7 PSU water (filtrated using cellulose sterile filters: CA 13 mm, 0.22 μm, CHROMAFIL, pH 8.9) to obtain 1 μg/mL Samples were kept in thermostatic chamber at the same temperature as during experiment (10 °C) for 7 days without light. Each day 100 μL of solution was taken and analytes concentration was measured by HPLC-DAD (Gemini NX C18 column, mobile phase 10 mM acetate buffer at pH 4.0 and acetonitrile, isocratic (65% and 30% of organic modifier for DIC and 4-OH DIC, respectively), flow 1.5 mL/min, detection at 270 nm, 25 μL injection volume).

2.5.4. Experimental determination of octanol-water distribution coefficient (logDOW)

Both the octanol-water partition coefficient (K_{OW}) and the octanol-water distribution coefficient (D_{OW}) indicate the degree of partitioning of the compound between the aqueous phase and the octanol phase mimicking lipids in the tissues of organisms. The difference is that K_{OW} considers only the neutral form of the compound, while D_{OW} shows the distribution of dissociated and non-dissociated species of analyte as pH function (ECETOC, 2013). The measuring of $\log D_{OW}$ in real water condition is essential for ionisable compound. For the experimental conditions used in this study (pH = 8.9), diclofenac and 4-hydroxydiclofenac are mainly found in ionised forms, so D_{OW} describes their affinity for lipids more accurately. The octanol-water distribution coefficient was determined experimentally according to the shake flask method proposed by OECD, (1995). Briefly, in a first step, artificial brackish water was prepared as described above (salinity = 7 PSU, pH = 8.9) and then octanol was pre-saturated in this water. The experimental samples, which were prepared in triplicate, contained 15 μg of the test compound (DIC or 4-OH DIC), 1 mL of artificial brackish water and 0.5 mL of water-saturated octanol. Control samples contained the same amount of analyte and 1.5 mL of water. In addition, a blind sample containing 1 mL of water and 0.5 octanol was performed to ensure that reagent have not disturb quantification of analytes by giving additional signals during HPLC-DAD measurements. The samples were shaken for 24 h at a constant temperature of 23 °C. After equilibrium was reached, the octanol phase was separated from the aqueous phase and then the aqueous phase was analysed by HPLC. The distribution coefficient was calculated according to the formula proposed by OECD guideline 107 and then presented in logarithmic form.

2.6. Bioconcentration factor, BCF

To determine the bioconcentration of DIC and 4-OH DIC in mussel tissues, BCF was calculated for the last day of the experiment. The BCF was calculated as:

$$BCF = concentration\ in\ tissues\ (mg/kg)/concentration\ in\ water\ (mg/L)$$

2.7. Statistical analysis

First, data distribution and homogeneity of variance were checked using Shapiro-Wilk and Levene's tests. Then, the Mann-Whitney *U* test

($p < 0.05$) (STATISTICA 13.3 program) was used to compare the concentration of DIC and 4-OH DIC between males and females and the concentration of DIC and 4-OH DIC in water and mussels.

3. Results and discussion

3.1. Detection DIC and its metabolites in the mussels and water from the environment

DIC, 4-OH DIC and 5-OH DIC were detected neither in water nor in mussels collected from the Orłowo station at Gulf of Gdańsk. This indicates that these compounds are not present at the selected station or their concentrations are below MDL.

3.2. The concentration of DIC and 4-OH DIC in mussels and biofilm on day 7 of the experiment

The ammonia concentration in all tanks was checked daily and did not exceed 0.25 mg/L in any of the tank. Our study is the first to reveal the uptake and bioconcentration of 4-OH DIC by aquatic organisms. In addition, sex-related differences in the bioconcentration of DIC and 4-OH DIC are evaluated here for the first time. As shown in Fig. 1, the bioconcentration of 4-OH DIC differed between the tanks, but these differences were not statistically significant. Moreover, the concentration of the metabolite was always higher in females. However, the observed differences were statistically insignificant (Fig. 1). The observed changes may be due to the accumulation of the pharmaceutical in female gametes. According to some studies, female mussels may accumulate some xenobiotic in eggs as a defence mechanism, which in turn allows the drug to be removed from the body (van Haren et al., 1994). However, the calculated $\log D_{OW}$ for 4-OH DIC (-0.183 ± 0.043) (Table 2) indicates its high hydrophilicity, which in turn may reduce the chance of its accumulation in gametes primarily made of lipids. We also observed differences in metabolite concentrations among females between replicates, but there were not statistically significant.

No statistically significant differences in the concentration of DIC between females and males were observed ($p > 0.05$). Nevertheless, the bioconcentration of 4-OH DIC was always lower compared to parent compound, and these differences were statistically significant ($p = 0.008$). Furthermore, significantly lower 4-OH DIC concentration compared to DIC may result from its enhanced metabolism by mussels. DIC metabolite may undergo further metabolic processes more easily than the parent compound or be less efficiently taken up from water due to its lower lipophilicity (Table 2). In addition, the concentration of the metabolite in water was much lower than that of DIC. This suggests that a substantial portion of the added 4-OH DIC was unavailable to the mussels, possibly affecting the bioconcentration of metabolite.

In our previous work (Świacka et al., 2020), the highest measured DIC concentrations in mussels exposed to 4 and 40 μg/L for 12 days were 0.166 μg/g dw and 1.692 μg/g dw, respectively. These concentrations are much lower than those of recent study (Fig. 1), which is most likely related to the lower exposure concentration. Ericson et al. (2010) also

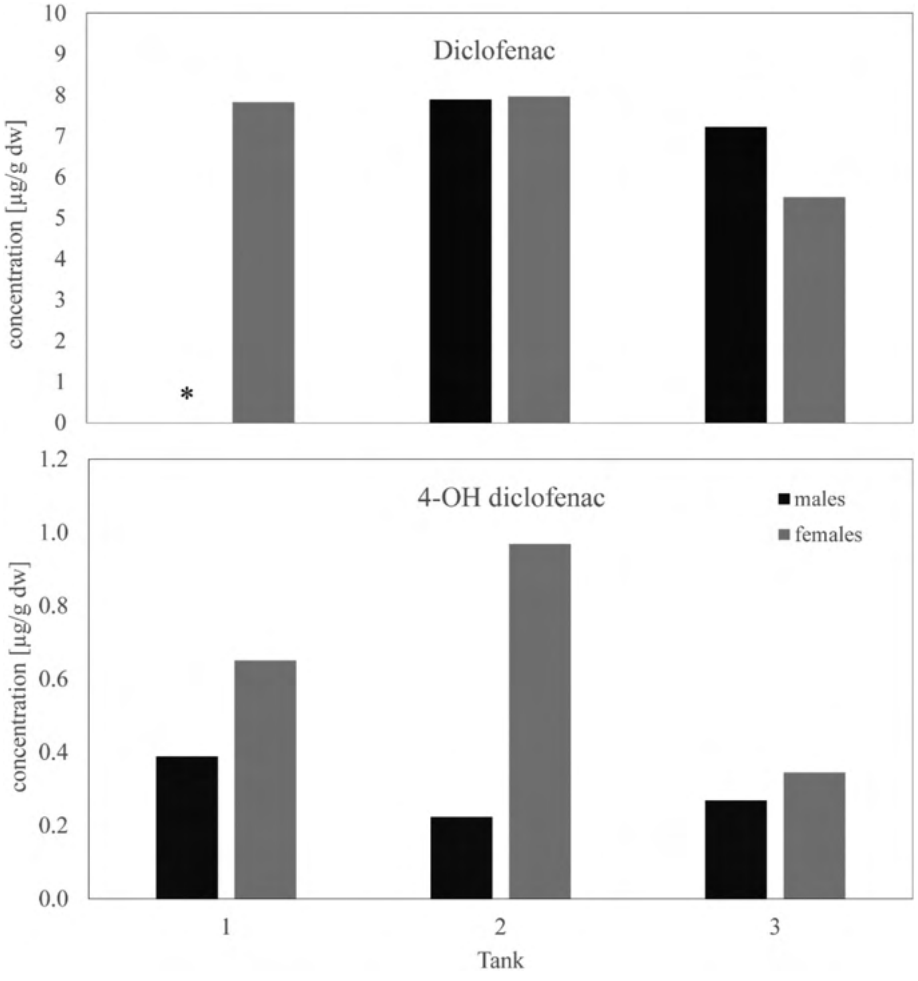

Fig. 1. The concentration of DIC and 4-OH DIC in *M. trossulus* males and females treated with 68.22 and 20.85 μg/L, respectively after 7 days of the exposure ($n = 5$). Statistical comparison (Mann–Whitney U, $p < 0.05$; Statistica 13.3).
* no quantitative analysis due to the matrix effect

observed higher DIC concentration in mussels treated with higher dose of this pharmaceutical. In Świacka et al. (2019) the highest measured concentration of DIC in mussels exposed to 133.33 μg/L for 5 days was 7.79 μg/g dw. This result is similar to those from present work (Fig. 1). However, in Świacka et al. (2019), the highest concentration was observed on day 3, while on day 5 DIC concentration was significantly lower (0.86 μg/g dw). Conversely in a recent study, DIC concentration of 7.375 ± 0.875 μg/g dw was measured on day 7. These differences may be related to detoxification processes, which are poorly investigated in aquatic invertebrates. Interestingly, in mussels exposed to DIC, 5-OH DIC, was detected, but only in females (Table 1). This observation, as well as differences in 4-OH DIC bioconcentration, may indicate differences in pharmaceutical detoxification between *M. trossulus* males and females.

3.3. Determination of selected metabolites of DIC in different matrix (water, biofilm, and mussels)

After 7 days of exposure 5-OH DIC was detected in two tanks with DIC (1 and 3), but only in female tissues (Table 1). However, measured concentrations were close to MDL. In tanks with 4-OH DIC no metabolites were detected in any tested matrices (Table 1). In our previous study (Świacka et al., 2019), in which *M. trossulus* were exposed for 5 days to DIC at a concentration of 133.33 μg/L, two hydroxy metabolites of DIC (4 and 5-OH DIC) were detected in the tissues. Interestingly, 5-OH DIC was detected more often than 4-OH DIC. Moreover, the concentrations of these metabolites in mussel' tissues were higher (4-OH DIC: 0.59–0.73 μg/g dw; 5-OH DIC: 0.38–1.18 μg/g dw) compared to the present results. This is most probably related to higher DIC concentration in water. In addition, in previous work 5-OH DIC was detected in water, while in present work no hydroxy metabolites were detected in water samples, probably resulting from its biodegradation or concentration below detection limits. Bonnefille et al. (2017) as the first detected DIC hydroxy metabolites in mussels *M. galloprovincialis*. Mussels were exposed to DIC concentrations 122.6 μg/L and 647.8 μg/L for 7 days. Interestingly, in addition to the hydroxy metabolites of DIC, the Bonnefille et al. (2017) detected 10 other metabolites from the second phase of detoxification, 5 of which were for the first time detected in aquatic organisms. The results obtained by us confirm that in order to analyse the metabolism of pharmaceuticals in mussels more accurately, exposure concentrations well above environmentally-relevant are needed. If too low experimental concentrations are used, the amount of metabolites excreted by molluscs may be below the limits of quantification. This means that although the drug testing at environmentally relevant concentrations can provide the most precise information in the context of the ecotoxicological implications, it also carries significant limitations. Therefore, a full understanding of the fate of these compounds in aquatic organisms requires a compromise between testing in the most realistic scenarios and the limitations imposed by analytical methods.

3.4. The concentration and mass balance of DIC and 4-OH DIC in different matrices

As the water concentrations measured 30 min after the addition of DIC and 4-OH DIC were much lower than nominal concentrations, it was decided to use these concentrations for all the calculations. Namely: 68.22 μg/L for DIC and 20.85 μg/L for 4-OH DIC.

Both, the concentration of DIC and 4-OH DIC gradually decreased during the experiment. At the day 0 the concentration of DIC and 4-OH DIC were 68.22 ± 5.49 and 20.85 ± 10.38, respectively (Fig. 2). At the end of the experiment the concentrations decreased by 8.46% and 65.94%, respectively, when compared to the concentrations on the day 0. In order to make sure that no hydrolysis occurred in the settled experimental conditions, a stability tests with DIC and 4-OH DIC were performed that resulted in no measured hydrolyses of any of the compounds (Fig. S1).

Similarly, Toński et al. (2019) did not observe hydrolysis of 4-OH DIC during 30 days at 20 °C and pH = 9. Furthermore, as the tanks were kept in the darkness, the photodegradation of the tested chemicals was also excluded. Therefore, the decrease in the concentration of both compounds in the aqueous matrix could be due to their biodegradation, accumulation and metabolism by mussels or algae used to feed the mussels, adsorption and absorption in the biofilm and tank walls (Fig. 3).

According to our knowledge, mass balance of DIC and 4-OH DIC in the experimental setup with artificial brackish water was evaluated here for the first time. A significant decrease in the mass of both compounds was observed in all tanks, which was not related to their absorption by mussels and biofilm. As reported in numerous papers, DIC is readily biodegradable by bacteria, algae and fungi present in environment as well as bacteria and fungi isolated from the WWTPs (Poirier-Larabie et al., 2016; Chen et al., 2020; Facey et al., 2018; Li et al., 2014; Ouada et al., 2019). Reported efficiency of biodegradation is often high, even up to 90% (Murshid and Dhakshinamoorthy, 2019; Tran et al., 2010; Chen et al., 2020; Facey et al., 2018). Although the presence of bacteria and fungi in the test tanks was not analysed here, we assume that they were accommodated along with mussels taken from the environment.

The higher mass loss was observed in the test with 4-OH DIC, which was 67.14% in relation to the measured mass (312.74 μg), while the mass loss of DIC was about 4.95% (Fig. 2). This might indicate a higher biodegradability of the metabolite. However, data concerning biodegradation of DIC metabolites is limited. Lee et al. (2012) reported biodegradation of 4-OH DIC which was more efficient compared to parent compound. There was no significant change in DIC concentration over 28 days and it was ~1.3 μmol/L, whereas slow degradation of 4-OH DIC was observed with a pseudo-first order rate constant of 0.018 d^{-1}. The metabolite concentration decreased from ~1.1 μmol/L to ~0.7 μmol/L over 28 days. To assess the biodegradability of DIC and its metabolite, a modified closed-bottle biodegradability test (OECD 301C) was performed (Lee et al., 2012). Also, Li et al. (2014) investigated environmental fate of DIC and other selected pharmaceuticals in the river. For this purpose, special system with sediment collected from two rivers and artificial river water previously spiked with DIC was build. Experiment was conducted in the dark at temperature 15 °C to reflect the environmental conditions. The pharmaceutical concentrations used in the water/sediment test were 200 and 500 μg/L. Results of this study showed quick microbial degradation of DIC by biofilm present on river sediments resulting in 4-OH DIC formation. Similar results were obtained by Gröning et al. (2007), who also observed degradation of DIC in the concentration range of 3–35 μM by biofilm from the creek sediments. However, the main DIC metabolite detected in this work was p-benzoquinone imine of 5-OH DIC, whereas 4-OH DIC was not detected. Similarly, in the present study 4-OH DIC was not detected in DIC tanks, which may be due to the concentration of this metabolite below the detection limit or the formation of other DIC metabolites that were not detected. Presumably, the species composition of a biofilm can determine the formation of different types of metabolites, so it would be interesting to explore the diversity of biofilm that inhabit shells, bivalve tissues, and marine sediments.

According to octanol-water distribution coefficients provided in the Table 2, in experimental conditions 4 -OH DIC is more hydrophilic than

Table 1
Diclofenac hydroxy metabolites determined in mussel tissues, water and biofilm at day 7 in the tanks with DIC and 4-OH DIC solutions.

Metabolites	Tank	Water [μg/L dw]	Biofilm [μg/g dw]	Mussels [μg/g dw]	
				Males	Females
4-OH DIC	DIC	n.d.	n.d.	n.d.	n.d.
5-OH DIC	DIC	n.d.	n.d.	n.d.	0.202 ±0.02
	4-OH DIC				n.d.

n.d. - not detected.

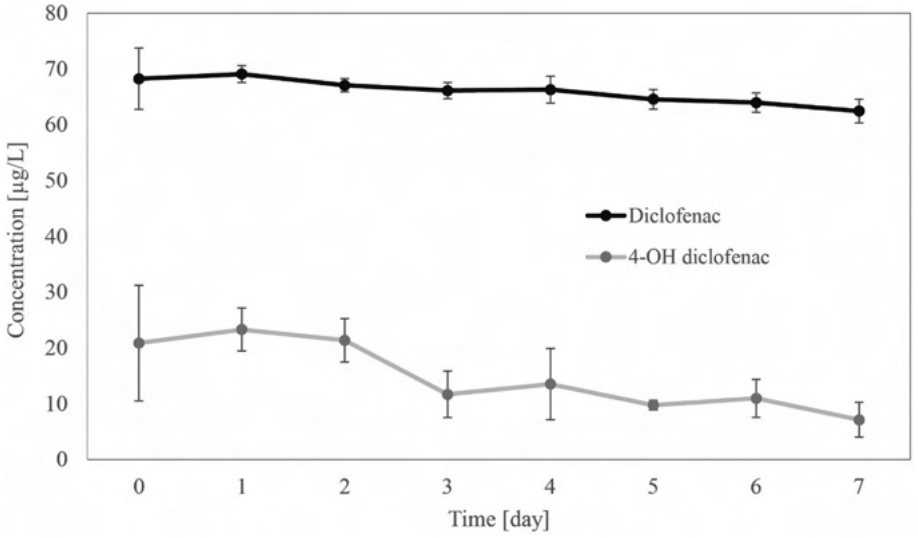

Fig. 2. The concentration of DIC and 4-OH DIC in water during the experiment. Statistical comparison (Mann–Whitney U; $p < 0.05$; Statistica 13.3).

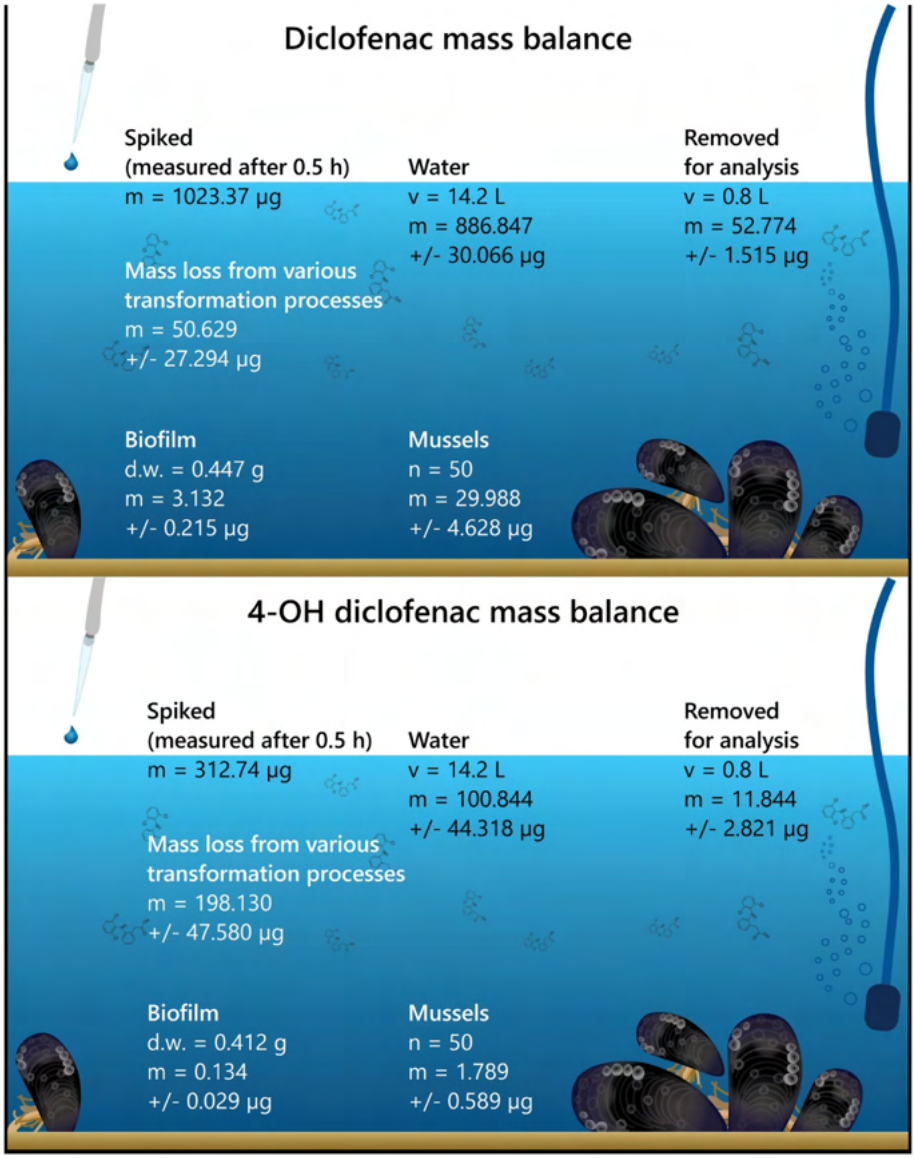

Fig. 3. Mass balance of DIC and 4-OH DIC on the 7th day of the experiment.

Table 2
Chemical characteristic of DIC and 4-OH DIC.

Chemical parameters	DIC	4-OH DIC
$logD_{OW}$[c]	0.647 ±0.001	−0.183 ±0.043
$logK_{OW}$[d]	4.5[b]	n.a.
Solubility [mg/L]	17.8[a]	n.a.
pKa	4[a]	n.a.
Molecular weight [g/mol]	296.1	312.1

n.a. not available.
[a] Sahar et al. (2011).
[b] Tran et al. (2010).
[c] D_{ow}: octanol-water distribution coefficient. Determined at pH 8.9.
[d] K_{ow}: octanol-water partitioning coefficient.

its parent compound as indicated by the lower $logD_{OW}$ value. Thus, hydrophilic nature of DIC metabolite may encourage its biodegradation. Banerjee et al. (1984) created model showing the correlation between $logK_{OW}$ of the substance and its biodegradation rate. This correlation is based on penetration of xenobiotic substrates into the cells which limits the biodegradation processes (Banerjee et al.1984; Sinton et al., 1986). Thus biodegradation rate of highly hydrophilic compound like 4-OH DIC is expected to be higher than its parent compound, what is also confirmed in our study.

3.5. Bioconcentration factor

According to OECD Guideline 305 and the Toxic Substances Control Act (TSCA) obtained BCF values of DIC and its metabolite show low potential of these compounds to bioaccumulate in mussel tissues or in biofilm (Table 3) (OECD, 2016; OECD, 1992a, 1992b). Compounds with BCF > 1000 and $logK_{OW} > 3$ have a high capacity to accumulate in organisms (OECD, 2016). However, a more reliable indication of the compound's ability to bioaccumulate could be provided by the achievement of an equilibrium state between the concentration in the organisms' tissues and the environment after prolonged exposure to the test substance or renewal of tanks by fresh analytes solution. In our study mussels were exposed to DIC and 4-OH DIC too short to observe their accumulation in equilibrium, as presented by kinetic change of concentration in Fig. 2." In comparison to other studies, BCF of DIC obtained by us are relatively high. In Bonnefille et al. (2017), BCF for *M. galloprovincialis* exposed at 122.6 μg/L and 647.8 μg/L for 7 days were 16.5 and 11.3 respectively, while in our previous work (Świacka et al., 2019) BCF was 57.4. Furthermore it was noted that the BCF values of DIC are similar between biofilm and mussels.

4. Conclusion and future perspectives

This study is the first to show the uptake and bioconcentration potential of DIC metabolite, 4-OH DIC in *M. trossulus* mussels and biofilm in comparison to DIC. Moreover, this work was the first to investigate the bioconcentration of DIC and 4-OH DIC in relation to mussel sex. However, no statistically significant differences in DIC and 4-OH DIC bioconcentration were observed according to sex of mussels. The bioconcentration of 4-OH DIC in mussels and biofilm was significantly different from that of DIC, which was probably related to the lower amount of metabolite available in water. In addition, the metabolism of DIC by *M. trossulus* females was observed, as indicated by the detection of 5-OH DIC in their tissues. Yet, the measured concentration was close to MDL value. Interestingly, a significant decrease (67.14%) in the concentration of the metabolite during 7 days was observed which is most probably related to its biodegradation. Conversely, only slight decrease (4.95%) was observed for DIC suggesting higher stability of parent compound.

The current state of knowledge on the pharmacokinetic of pharmaceutical metabolites and in particular NSAIDs metabolites in biota and the environment is very poor. Knowing the (toxico)kinetics of metabolites in the aquatic environment, namely what processes they undergo, whether they are taken up and accumulated by organisms and whether they are toxic to them, would allow an appropriate assessment of the risks associated with their introduction into the environment.

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Table 3
Bioconcentration factor (BCF) of diclofenac (DIC) and 4-hydroxy diclofenac (4-OH DIC) calculated for the mussels and biofilm.

Tank	BCF [L/kg]			
	Biofilm	Mussels[a]	*M. trossulus* Males	*M. trossulus* Females
DIC	119.2 ± 36.9	118.5 ± 17.5	119.5 ± 11.4	114.3 ± 25.3
4-OH DIC	49.8 ± 12.5	69.7 ± 18.2	47.8 ± 24.1	91.5 ± 21.3

[a] Average BCF value for females and males.

CRediT authorship contribution statement

Klaudia Świacka: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Katarzyna Smolarz:** Conceptualization, Supervision, Investigation, Writing – review & editing. **Jakub Maculewicz:** Investigation, Formal analysis, Visualization, Writing – original draft. **Alicja Michnowska:** Investigation. **Magda Caban:** Conceptualization, Supervision, Investigation, Writing – review & editing.

Declaration of competing interest

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

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Review

Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms – Current state of knowledge

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ABSTRACT

In the last decades an increasing number of studies has been published concerning contamination of aquatic ecosystems with pharmaceuticals. Yet, the distribution of these chemical compounds in aquatic environments raises many questions and uncertainties. Data on the presence of selected pharmaceuticals in the same water bodies varies significantly between different studies. Therefore, since early 1990 s, wild organisms have been used in research on environmental contamination with pharmaceuticals. Indeed, pharmaceutical levels measured in biological matrices may better reflect their overall presence in the aquatic environments as such levels include not only direct exposure of a given organisms to a specific pollutant but also processes such as bioaccumulation and biomagnification. In the present paper, data concerning occurrence of pharmaceuticals in aquatic biota was reviewed. So far, pharmaceuticals have been studied mainly in fish and molluscs, with only a few papers available on crustaceans and macroalgae. The most commonly found pharmaceuticals both in freshwater and marine organisms are antibiotics, antidepressants and NSAIDS while there is no information about the presence of anticancer drugs in aquatic organisms. Furthermore, only single studies were conducted in Africa and Australia. Hence, systematization of up-to-date knowledge, the main aim of this review, is needed for further research targeting.

1. Introduction to emerging problem of pharmaceuticals presence in the environment

Human and veterinary pharmaceuticals across a broad spectrum of therapeutic classes are widely used around the world (Monteiro and Boxall, 2010). Human consumption is one of the major causes of their residues entering the aquatic environment (Nunes et al., 2005; Wang et al., 2020). It is estimated that due to the continuous development of medicine, the overall availability of pharmaceuticals, and an aging population, the amount of medication ingested globally continues to increase (Pereira et al., 2020). According to the data published by the OECD, there was an increase of nearly 70% in the consumption of anti-hypertensive drugs between 2000 and 2017. The use of cholesterol-lowering agents and antidepressants recorded a threefold and doubling increase, respectively (OECD, 2019). However, in addition to consumption itself, inappropriate disposal of medicines in households has an inestimable large impact on the release of pharmaceuticals into the aquatic environment (Caban and Stepnowski, 2021). People, not realizing the dangers of doing so, dispose medications by dropping them at various not suitable locations, including toilets. According to surveys conducted, even 20% of people dispose of unnecessary and outdated medicines in such a way (Götz and Keil, 2007; Constantino et al., 2020; Zorpas et al., 2018). Equally alarming is the fact that many medicines are disposed of together with household wastes on waste disposal sites from where they can easily get to e.g. groundwater. Another important problem is related to the overuse of veterinary drugs in animals handling, specifically in mass cultures on both, terrestrial and aquatic organisms. They are used both to treat diseases and to improve the growth rate of organisms. Their entry into the aquatic environment can be direct (e.g. the use of drugs in fish farms) and/or indirect, e.g. through the use of animal manure containing excreted pharmaceutical residues (Boxall, 2010; Kim et al., 2017; Faruk et al., 2021). However, the presence of pharmaceuticals in the aquatic environment is not solely due to global consumption and improper handling. Although good

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manufacturing practices (GMP) exist, bioactive compounds prices are high, and manufacturers are deliberately trying to avoid losses, various pharmaceutical residues still enter the environment during the manufacturing process. Despite the fact that this way of drugs introduction into the aquatic environment may not be the most important, elevated levels of some compounds have already been confirmed in industrial wastewater (at concentrations up to a few mg/L) in, for example, Asian countries (Kümmerer, 2009; Larsson et al., 2007; Li et al., 2008a, 2008b). The presence of pharmaceuticals is also unavoidable in hospital wastewater (Brown et al., 2006; Verlicchi et al., 2010; Macías-García et al., 2019; Zhang et al., 2020), but their contribution to the total municipal wastewater load is less than 10% for most bioactive substances (Kümmerer, 2010). However, as shown in the Table 1 it should be kept in mind that, depending on their polarity, water solubility and persistence, some drugs are not completely eliminated or transformed during sewage treatment. Therefore, a large amount of them enter the environment in this way (Kolpin et al., 2002; Filali-Meknassi et al., 2004).

The presence of pharmaceuticals has been confirmed worldwide in surface waters, wastewaters, groundwaters and even drinking water at concentrations ranging from a few ng/L to a few μg/L (Almeida et al., 2020; Mezzelani et al., 2018). According to data presented by Aus der Beek et al. (2016), environmental water samples were tested for 713 pharmaceuticals and related compounds, of which 631 were determined. Globally, one of the most often detected drug is the commonly used analgesic - diclofenac. Carbamazepine, sulfamethoxazole, ibuprofen, and naproxen were also detected at a similar frequency as diclofenac (Aus der Beek et al., 2016). Although the presence of pharmaceuticals in the aquatic environment is a well-known problem (Seifrtová et al., 2010; Santos et al., 2013; Paíga et al., 2016; Andreu et al., 2016; Białk-Bielińska et al., 2017; Rzymski et al., 2017; Grabicová et al., 2020), according to recent reports also their metabolites and transformation products can reach similar or even higher concentrations in the environment (López-Serna et al., 2013; Kołecka et al., 2019, 2020; Grabarczyk et al., 2020; de de de Oliveira Klein et al., 2021). Yet additional problem is related to the toxicity of mixtures related to the presence of number of compounds in a given environment at the same time and location (Pomati et al., 2008; Białk-Bielińska et al., 2017).

It is clear that the presence of pharmaceuticals in the aquatic environment is not without impact on organisms (Brooks et al., 2003; Cunha et al., 2017; Arnnok et al., 2017; Ruan et al., 2020). They can be absorbed by organisms and adversely affect fundamental life functions. Organisms exposed to pharmaceuticals may experience lower growth rates, higher mortality rates and increased oxygen consumption (Caban et al., 2016; Kuehr et al., 2021). In addition, long-term exposure can lead to the accumulation of compounds in organisms and their further spread through the food chain (Ruhí et al., 2016; Arnnok et al., 2017; Ruan et al., 2020). Of course, the sensitivity of species to pollution depends on many biological factors such as life cycle, reproductive strategy, food type, and habitat (Baird and Van den Brink (2007). In the aquatic environment, commonly used group of organisms in cause-and-effect studies of pollution are fish. This is due to the fact that they are widespread throughout the aquatic environment and are important components of freshwater and marine food webs (Huerta et al., 2012). Nevertheless, the role of invertebrates and algae, which also play an important role in the flow of nutrients in aquatic ecosystems, cannot be overlooked (Huerta et al., 2012). Moreover, algae respond quickly to any environmental changes, which makes them excellent indicators for monitoring pollution in the environment (Franzellitti et al., 2014). Among invertebrates, mussels are particularly noteworthy for such studies as by filtering they are responsible for energy fluxes and suspended matter uptake from the water column therefore influencing the concentration of contaminants (Berny et al., 2002; Hayashi et al., 2008; Caban et al., 2016; Yuan et al., 2020; Świacka et al., 2020; Kuehr et al., 2021).

As for experimental studies, a selection of appropriate species is extremely important. Yet, in cause-and-effect studies with pharmaceuticals using aquatic organisms equally significant is the condition of the experiment. It should be noted that the vast majority of experiments are conducted under controlled laboratory conditions that will never fully represent environmental conditions. In addition, the concentrations used are usually one or more orders of magnitude higher than those found in the environment. Also, the route of exposure is very different from that of the natural environment. Moreover, the very often observed lack of correlation between effects and tissue levels of a given pharmaceutical may be related to the so called delayed toxicity specifically observed for those drugs that are stored in fat or to the occurrence of delayed negative effects manifesting only at later stages of life. The latter problem particularly concerns organisms with longer life cycles (Brooks, 2011. In addition, it should be noted that the immense majority of studies on the effects of pharmaceuticals on aquatic organisms involve freshwater environments (Fig. 1). A probable reason may be that one of the major sources of drug exposure include wastewaters directly dumped into freshwater systems (Huerta et al., 2012). Marine waters were neglected for a long time because it was assumed that "*the solution to pollution is dilution*" thus final dilution of the effluent neglected the risk of pharmaceutical residues leaking into the environment (Fabbri and Franzellitti, 2016). However, recent years have seen a marked increase in research on saltwater and thus on the organisms living in it (Fig. 1) (Li et al., 2012; Granek et al., 2016; Meador et al., 2016; Mezzelani et al., 2020).

The number of publications describing concentration of pharmaceuticals in plants and animals is relatively low compared to the number of publications dealing with analysis of pharmaceuticals in water media. The main reason is that it is easier to sample and analyze these compounds in water compared to biota matrix. The available mass of biota sample is frequently low, and its complexity forces multi-step extraction process with high matrix impact into final determination by mass spectrometry (Puckowski et al., 2016). Moreover, pharmaceuticals are often found in biota as metabolites, for example complexes with glucuronides and sulfates, for which analytical standards are unavailable, or, if available, expensive and not stable. Furthermore, because of the lack of reference materials the quality assurance is more difficult for biota sample. Also, most pharmaceuticals are polar and occur in an ionic form in water what impact their bioaccumulation potential. Generally, polar chemicals are less bioaccumulative in fat tissue and have lower potential for skin barrier transfer. Nevertheless, the bioaccumulation in other organs (liver, kidney, gills, brain) can occur after absorption with food. Therefore, uneven distribution of pharmaceuticals within organism' organs is highly expected. Of course in case of animals of small size measuring the drugs concentration in various organs is very difficult and a whole-body study is performed instead. Also, pharmacokinetic processes in the human body (rate and speed of absorption, binding to proteins, metabolizing organs, metabolites, halftime of elimination etc.) are known, but in the case of non-target organism this knowledge is less than basic. A problem generating issue is the fact that sometimes in the non-target species the metabolites found are different from those known in human (Mulkiewicz et al., 2021). For example, metabolite of diclofenac (OH-DCF - ethanoloamine) not previously identified in mammals was detected in bivalves (Bonnefille et al., 2017). Also Fu et al. detected in selected crustaceans the presence of a methylated derivative of diclofenac, the presence of which had not been previously found in other organisms (Fu et al., 2020). In view of these reports, measurement of a concentration of native pharmaceutical compounds may not be enough to draw a clear conclusion about their presence and specifically their effect on wildlife. Strong differences in behavior and metabolism between species, but also between organism of the same species (connected with sex, stage of development, food availability, temperature, and other factors), hinder generalization about single compound bioaccumulation potential in wild-living organisms. Furthermore, some pharmaceuticals are overrepresented compared to other in in-situ tests (for example NSAIDs, carbamazepine, selected antibiotics, and

Table 1
Physicochemical properties of selected drugs and their rate of biodegradation in sewage sludge.

Compound	pKa	log K_{ow}	Solubility [mg L^{-1}]	Biodegradation rate in sewage sludge	References
NSAIDs					
Ibuprofen	4.52[1]	2.48[2]	21 (20 °C)[3]	100% of biodegradation after 4 days[4]	[1]Ràfols et al. (1997a) [2]Scheytt et al. (2005) [3]Yalkowsky and Dannenfelser (1992) [4]Langenhoff et al. (2013)
Diclofenac	4.16[5]	1.90[2]	2.37 (20 °C)[6]	75% biodegradation after 3 weeks[4]	[5]Ràfols et al. (1997b) [2]Scheytt et al. (2005) [6]Fini et al. (1993) [4]Langenhoff et al. (2013)
Naproxen	4.2[8]	3.18[7]	15.9 (25 °C)[9]	100% biodegradable after 10 days[16]	[16]Mascolo et al. (2010) [7]Tang et al. (2014) [8]Hashim and Khan (2011) [9]https://go.drugbank.com/drugs/DB8 (0078)
Opioids					
Tramadol	9.41[10]	2.51[11]	1.15 (25 °C)[12]	Not readily biodegradable[13]	[10]Kitamura et al. (2014) [11]Thiebault et al. (2017) [12]Rúa-Gómez and Püttmann (2012) [13]Bergheim et al. (2012)
SSRIs					
Fluoxetine	9.8[15]	4.05[14]	38.4 (20 °C)[14]	100% of biodegradation after 15 days[16]	[14]Nentwig (2007) [15]Izadyar et al. (2016) [16] Iranzo et al. (2018)
Sertraline	9.48[18]	1.37[17]	3022 (22 °C)[19]	Partially biodegradable[36]	[17]Black et al. (2005) [18]Deák et al. (2006) [19]Kwon and Armbrust (2008) [36]Gornik et al. (2020)
Citalopram	9.78[15]	1.39[17]	15560[19](22 °C)	10% of biodegradation after 15 days[16]	[17]Black et al. (2005) [15]Izadyar et al. (2016) [19]Kwon and Armbrust (2008)
Anticancer drug					
Tamoxifen	8.76[47]	7.88[20]	0.1916 (25 °C)[22]	91% of biodegradation after 8 days[38]	[20]Galaon et al. (2017) [22] (MIHÇIOKUR, 2021) [38]Ferrando-Climent et al. (2015) [47]Michalides et al. (2004)
5 – fluorouracil	8.0[21]	-0.9[21]	25900 (25 °C)[22]	No biodegrdation observed after 28 days[39]	[21]Lehnberg et al. (2009) [22] (MIHÇIOKUR, 2021) [39] (Kümmerer and Al-Ahmad, 1997)
Methotrexate	2.95[48]	-1.85[22]	2600 (25 °C)[22]	No biodegradation observed after 8 day[38]	[22] (MIHÇIOKUR, 2021) [38]Ferrando-Climent et al. (2015) [48]Mioduszewska et al. (2017)
Anibiotics					
Amoxicilin	7.4; 9.6; 2.4[23]	0.87[23]	3430 (20 °C)[23]	81% of biodegradation after 8 days[40]	[23]Aljeboree and Alshirifi (2019) [40]Yang et al. (2020)
Clarithromycin	8.8[25]	1.17[24]	9220 (25 °C)[26]	n.a.	[24]Kumar et al. (2011) [25]Sein et al. (2009) [26]Salem and Düzgünes (2003)
Sulfamethoxazole	1.7; 6.56[27]	0.89[27]	3942 (25 °C)[27]	100% of biodegradation after 14 days[37]	[27]Chang et al. (2015) [37]Yang et al. (2012)
Antihypertensives					
Atenolol	9.60[28]	3.97[28]	21 (25 °C)[28]	40% of biodegradation after 240 h[41]	[28] Delgado et al. (2015) [41]Xu et al. (2017)
Metoprolol	9.67[29]	1.88[29]	14000 (25 °C)[29]	100% of biodegradation after 96 h[42]	[29] (Velázquez and Nacheva, 2017) [42]Rubirola et al. (2014)
Propranolol	9.15[30]	2.60[30]	61.7 (25 °C)[49]	32% of bidegradation after 24 h[43]	[30]Ribeiro et al. (2013) [43]Bendz et al. (2005) [49]https://go.drugbank.com/drugs/DB1 (0057)
Hormones					
Estrone	10.4[31]	3.4[31]	13 (25 °C)[31]	92.9% of biodegradation after 168 h[44]	[31]Prater (2012) [44]Zhang et al. (2015)
17β – Estradiol	10.2[31]	4.0[31]	21.6 (25 °C)[31]	100% of bidegradation after 2 h[45]	[31]Prater (2012) [45]Zeng et al. (2009)
Antihistamine					
Diphenhydramine	9.0[33]	0.52[32]	n.a.	n.a.	[32]Alonso et al. (2019) [33]Zargarian et al. (2015)
Ranitidine	8.2[35]	0.98[34]	Very freely soluble[50]	76% of biodegradation after 14 days[46]	[50]Hohnjec et al. (1986) [34]Aminot et al. (2015) [35]Balasubramaniam et al. (2008) 46Carballa et al. (2006)
Antiepileptic					
Carbamazepine	17.66[2]	14.0[2]	1.51[2]	No biodegradation observed[46]	[2] Scheytt et al. (2005) [46]Carballa et al. (2006)

n.a. – not available

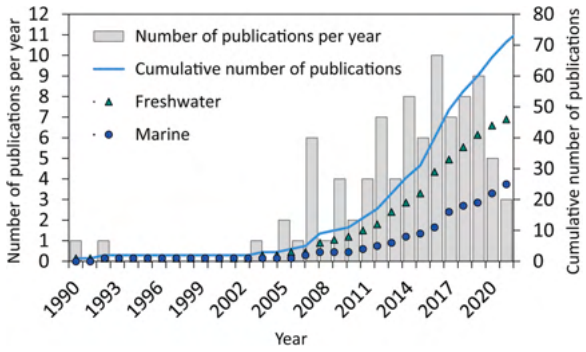

Fig. 1. Number of new papers reporting the occurrence of human and veterinary pharmaceutical residues in wild aquatic organisms published per year. The presented data refers only the original research papers and does not include review papers.

psycho-active drugs), and for majority of known biologically active substances currently there is no information about their presence in aquatic organisms. Additionally to that, in the natural aquatic environments pollutants mixtures, and specifically pharmaceuticals and their metabolites, do occur and various cumulative effects related to that is often observed (Watanabe et al., 2016; Nowakowska et al., 2020; Cerveny et al., 2020). Hence, systematization of up-to-date knowledge is needed for further research targeting.

The main aim of this review is therefore to combine the data related to the levels of pharmaceuticals in aquatic organisms (tissue matrix) not only to reflect the contamination levels of fresh and marine ecosystem globally, but also to highlight their potential for bioaccumulation and therefore the potential threat to aquatic biodiversity. Also, the review covers historical perspective of the problem taking into account years between 1990 and 2021. This paper collects data from studies on the effects of pharmaceuticals in aquatic environments involving different organisms, taking place under various conditions, and considering different environmental variables. More precisely, it is intended to summarize the information available to date and to guide further research to estimate as accurately as possible the effects of pharmaceutical residues on wild-living aquatic organisms. In addition, the differences in the number of pharmaceuticals detected between organisms from different taxonomic group or between different species from the same taxonomic class (e.g. Bivalvia) were discussed.

2. Materials and methods

For the purposes of this review, it was decided to describe pharmaceuticals that were only detected in wild aquatic organisms. Non-native organisms, such as those raised in cages, from aquaculture, as well as those bought on the market, were excluded. Water birds and other water-land animals were also excluded because of the unclear origin of detected pharmaceuticals. In this review we focused only on the pharmaceuticals excluding personal care products. The present paper was divided into two chapters: freshwater environment (lakes, rivers, ponds, wetlands, streams, and springs) and saltwater environment (in which brackish environment like estuaries, lagoons, rias, harbors and bays were included). Each of the chapter was divided into subchapters in which the presence of pharmaceuticals in biota matrix is decided from a geographical perspective. Moreover, data on pharmaceuticals tissues levels in wild biota grouped by therapeutic domains were reported in Table 2 (freshwater environment) and Table 3 (saltwater environment). To better illustrate the differences between geographic regions, the results were also presented in figures - Fig. 1 for freshwater environment and Fig. 2 for saltwater environment.

In order to acquire the literature data to prepare this review, a search for publications was conducted between March and July 2021. Publicly available databases of scientific articles were used, especially Google Scholar, Science Direct, and Scopus. Various combinations of keywords directly relevant to the main topic were used to search these databases, including words 'pharmaceuticals' and 'drugs', but also the names of individual groups of pharmaceuticals or single compounds' names in combination with types of environments, e.g. 'marine', 'aquatic', 'freshwater', 'brackish', 'river', 'lake', 'estuary', 'sea', 'ocean' and groups of organisms, e.g. 'fish', 'mussels', 'crustaceans', 'invertebrates'. This resulted in formulation of three-level keywords based on the scheme "Search = group of pharmaceutics + type of environment + group of organisms". The selection of articles for inclusion in this work was done by evaluating their relevance to the main topic. The analysis of wild living organisms was a key factor in the inclusion of data in this work. Also, the literature search was focused only on original research papers in order to present and review original data sources thus the review papers are not included here. In total, 73 articles concerning the occurrence of pharmaceuticals in the aquatic organisms were selected for use in this study. The data were complemented by 111 articles concerning general information regarding pharmaceuticals in the aquatic environment.

3. Current knowledge of pharmaceuticals presence in aquatic biota

3.1. Freshwater environment

Freshwater bodies are characterized by greater diversity compared to seas and oceans, having different physico-chemical parameters depending on climate, geological background, season and geographical location. They can be a close, stagnant systems (in case of lakes) or highly variable in space and time (in case of rivers). As catchment area they participate in pollution transfer or can be a semi-final sink. Pharmaceutical pollution is recorded in each freshwater type of reservoir, but monitoring is mostly performed in the municipal areas, close to the sewage treatment plant effluent discharge and in estuaries of the greatest catchment areas (Table 2). While screening of pharmaceutical concentration in freshwater biota, vast majority of reports contain also information on the concentration and/or accumulation levels of selected compounds in water (surface, bottom) and sediments (Huerta et al., 2018; Koba et al., 2018).

3.1.1. Asia

Liu et al. (2011) examined the status of endocrine disrupting compounds in Dianchi Lake in China and detected four steroid hormones: estrone, 17β -estradiol, estriol and 17α-ethinylestradiol in wild fish muscles. Similarly, another study area located in the vicinity of WWTPs in Chinese streams reported the occurrence of steroid hormones (17α-ethinylestradiol, EE2) in wild fish species (Liu et al., 2015). This study also showed differences in levels of accumulation of steroids among different fish organs, proving that liver is the main organ responsible for accumulation of these compounds, followed by brain. Yet, another study showed the selective uptake of pharmaceuticals by specific fish tissues and organs in Japanese streams impacted by runoffs from the treated wastewaters. Tanoue et al. (2015) detected 18 pharmaceuticals (antihistamine, calcium channel blockers, non-steroidal anti-inflammatory and psychoactive drugs) differently accumulating in fish plasma, brain, kidney, liver, muscle and gills. Additionally, this study also provides accumulation levels in biofilm.

Based on previous research proving the presence of pharmaceutical compounds in aquatic consumers, Xie et al. (2017) investigated trophic transfer of such compounds among different functional groups in aquatic ecosystems. The authors sampled in multiple stations of the second largest lake in China (Taihu Lake) affected by eutrophication and significant pharmaceutical pollution due to surrounding runoffs. The results of the study revealed presence of various pharmaceuticals (antibiotics, NSAIDs, hormones) in plankton, zoobenthos, shrimps and fish. Importantly, no biomagnification of any of the tested compounds was

Table 2
The occurrence of drugs in wild freshwater biota grouped by therapeutic classes. Studies reporting quantitative analyses giving results within detection limits (different in each of reported cases); [e] – estimated concentrations based on graphical data sources. Concentrations reported in ng/g dry weight (ng/g dw) unless stated otherwise; in plasma (P) and bile (Bi) concentrations are expressed as ng/ml. In case of reports regarding studies conducted in Canada or the United States of America, the regions (states) are indicated in the parenthesis by their universal abbreviations. The impact by specific sources of pharmaceutical pollution is indicated in the table with abbreviations: wastewater treatment plants discharge (WWTP), pharmaceutical industry discharge (PHAR), highly urbanized area (URB) or harbor (HAR). Symbols used: [w] – wet weight; LOQ – limit of quantification; N.D. – not detected; MDL – method detection limit; B – brain, Bi – bile, G – gills, Go – gonads; K – kidneys; L – liver; M – muscle, P – plasma; & - comprised of different phyla, not specified.

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
Non-Steroidal Anti-Inflammatory drugs						
Diclofenac	biofilm	–	N.D.–23.5	Spain	River (WWTP)	Ruhí et al. (2016)
		–	< 1.43–4.4	UK	Rivers (WWTP)	Wilkinson et al. (2018)
	birds	1	N.D.–3730	USA (DE)	River	Bean et al. (2018)
	bivalves		N.D.–0.2[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
	crustacean		0.7	UK	Rivers (WWTP)	Wilkinson et al. (2018)
	fish	2	N.D.–20[e w]	USA (VA)	River (WWTP)	Arya et al. (2017)
		7	N.D.–11930	USA (DE)	River	Bean et al. (2018)
		2	N.D.–148 (Bi)	Finland	River (WWTP)	Brozinski et al. (2013)
		11	N.D.–15.4	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
		2	<MDL–1.8 (P)[w] <MDL–0.86 (B)[w] <MDL–2.86 (L)[w] <MDL–1.57 (K)[w] <MDL–0.55 (M)[w] 0.53–5.31 (G)[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
		2	N.D.–< 1.66	Argentina	River (WWTP)	Valdés et al. (2016)
		2	4.1-8.8	Spain	Rivers	Huerta et al. (2013)
		1	0.7	USA	Rivers (WWTP)	Huerta et al. (2018)
	gastropod	1	1.4	UK	Rivers (WWTP)	Wilkinson et al. (2018)
	Insects	1	10.2–93.7[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
		1	N.D.–12.4	Spain	River (WWTP)	Ruhí et al. (2016)
		1	< 100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
	periphyton	–	N.D.–35.1[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
	plants	2	<LOQ–0.42	UK	Rivers (WWTP)	Wilkinson et al. (2018)
	zoobenthos[&]	3	N.D–8.5	China	Lake (WWTP)	Xie et al. (2017)
Ibuprofen	fish	2	N.D.–38[e w]	USA (VA)	River (WWTP)	Arya et al. (2017)
		2	N.D.–16.5 (Bi)	Finland	River (WWTP)	Brozinski et al. (2013)
		1	95.3 ± 4.5	Nigeria	River, lagoon (PHAR)	Lan et al. (2019)
	insects	1	N.D.–182.7	Spain	River (WWTP)	Ruhí et al. (2016)
	platyhelminths	1	N.D.–30.9	Spain	River (WWTP)	Ruhí et al. (2016)
	zoobenthos[&]	3	16–28	China	Lake (WWTP)	Xie et al. (2017)
Indomethacin	fish	2	0.50–5.09 (P)[w] 0.17–2.10 (B)[w] 0.74–5.44 (L)[w] 1.84–11.3 (K)[w] <MDL–1.41 (M)[w] 3.56–12.2 (G)[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
	periphyton	–	0.93[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
Mefenamic acid	fish	2	0.13–1.47 (P)[w] 0.063–0.90 (B)[w] 0.37–19.2 (L)[w] <MDL–7.16 (K)[w] <MDL–0.092 (M)[w] 0.20–1.23 (G)[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
	periphyton	–	1.7[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
Naproxen	fish	2	6–103 (Bi)	Finland	River (WWTP)	Brozinski et al. (2013)
Analgesics						
Acetaminophen	birds	1	<MDL–3950 (P)	USA (DE)	River	Bean et al. (2018)
	crustaceans	1	0.5	UK	Rivers (WWTP)	Wilkinson et al. (2018b)
	fish	10	N.D. (B) N.D–4.7 (Go) N.D. (L) N.D. (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		2	N.D.–23[e w]	USA (VA)	River (WWTP)	Arya et al. (2017)
		7	N.D.–4660 (P)	USA (DE)	River	Bean et al. (2018)
	gastropod	1	0.7	UK	Rivers (WWTP)	Wilkinson et al. (2018b)
	plants	2	<LOQ–0.38	UK	Rivers (WWTP)	Wilkinson et al. (2018b)
Codeine	bivalve	1	<MDL–8.2[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	3	0.13[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	N.D.–163	Argentina	River (WWTP)	Valdés et al. (2016)
	insects	1	200[e]	Australia	Streams (WWTP)	Richmond et al. (2018)

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Table 2 (*continued*)

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
Oxycodone	bivalves	1	0.1–5.6[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	insects	1	850[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Antiarrhytmics						
Flecainide	fish	3	< 0.1–2.8 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
	insects	1	100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Antiasthmatics						
Salbutamol	fish	2	3.4	USA	Rivers (WWTP)	Huerta et al. (2018)
		11	2.6	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
		3	0.082[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	6–47	Argentina	River (WWTP)	Valdés et al. (2016)
Antibiotics						
Amoxicilin	periphyton	–	N.D.–58.5[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
	fish	9	N.D. (Bi) N.D. (P) N.D.–326 (L) N.D. (M)	China	Rivers (WWTP)	Zhao et al. (2015)
	fish	9	N.D.–52.3 (Bi) N.D.–< 4.55 (P) N.D.–2.44 (L) N.D.–1.26 (M)	China	Rivers (WWTP)	Zhao et al. (2015)
	periphyton	–	N.D.–211[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Anhydrotetracycline	bivalve	1	5.4–15.9[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
Azithromycin	bivalve	1	1.8–10[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	3	N.D.–19 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
Ciprofloxacin	decapods, fish	3, 4	3.08	China	River (URB)	Bai et al. (2014)
	fish	3	4.2	China	River (URB)	Gao et al. (2012)
Clarithromycin	bivalve	1	0.9–7.8[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	3	N.D.–11 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
Clindamycin	insects	1	250[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
	fish	4	0.69–1.90 (M)[w] 0.92–5.6 (L)[w] 0.68–2.0 (G)[w]	Argentina	Rivers	Ondarza et al. (2019)
Difloxacin	fish	3	0.9	China	River (URB)	Gao et al. (2012)
Erythromycin	bivalves	1	0.3–0.8[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	10	N.D.–54.1 (B) N.D.–28.7 (Go) 5.2–46.9 (L) 1.5–2.9 (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		3	7.8	China	River (URB)	Gao et al. (2012)
Erythromycin dehydrated[+]	fish	9	N.D.–285 (Bi) N.D.–545 (P) N.D.–2390 (L) N.D.–2.36 (M)	China	Rivers (WWTP)	Zhao et al. (2015)
	decapods, fish	3, 4	1.66	China	River (URB)	Bai et al. (2014)
Lincomycin	fish	9	N.D.–58.6 (Bi) N.D.–567 (P) N.D.–1384 (L) N.D.–26.2 (M)	China	Rivers (WWTP)	Zhao et al. (2015)
	fish	9	N.D. (Bi) N.D.–23.0 (P) N.D. (L) N.D. (M)	China	Rivers (WWTP)	Zhao et al. (2015)
	fish	1	10.9–11.7	Nigeria	River, lagoon (PHAR)	Lan et al. (2019)
Lomefloxacin	fish	3	0.3	China	River (URB)	Gao et al. (2012)
Minocycline	bivalves	1	<MDL–11.4[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
Norfloxacin	crustacean	2	31–37	China	Lake (WWTP)	Xie et al. (2017)
	fish	3	16.8	China	River (URB)	Gao et al. (2012)
		9	N.D.–83.9 (Bi) N.D. (P) N.D.–7.67 (L) N.D. (M)	China	Rivers (WWTP)	Zhao et al. (2015)
	zoobenthos[&]	3	17–28	China	Lake (WWTP)	Xie et al. (2017)
	fish	9	N.D.–37.8 (Bi) N.D.–11.9 (P) N.D.–9.85 (L) N.D.–16.8 (M)	China	Rivers (WWTP)	Zhao et al. (2015)

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Table 2 (*continued*)

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
	periphyton	–	N.D.–105.9[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
	plankton	–	20	China	Lake (WWTP)	Xie et al. (2017)
	zoobenthos[&]	3	12–23	China	Lake (WWTP)	Xie et al. (2017)
Ofloxacin	decapods, fish	3, 4	2.72	China	River (URB)	Bai et al. (2014)
	fish	3	1	China	River (URB)	Gao et al. (2012)
Roxithromycin	decapods, fish	3, 4	2.77	China	River (URB)	Bai et al. (2014)
	plankton	–	6.8	China	Lake (WWTP)	Xie et al. (2017)
	zoobenthos[&]	3	9.4–15	China	Lake (WWTP)	Xie et al. (2017)
Sarafloxacin	fish	3	0.6	China	River (URB)	Gao et al. (2012)
Spiramycin	fish	3	82.8	China	River (URB)	Gao et al. (2012)
Sulfamerazine	decapods, fish	3, 4	0.53	China	River (URB)	Bai et al. (2014)
	zoobenthos[&]	3	< 0.62	China	Lake (WWTP)	Xie et al. (2017)
Sulfamethazine	fish	3	13.9	China	River (WWTP)	Gao et al. (2012)
		9	N.D.–49.9 (Bi) N.D.–144 (P) N.D.–3.12 (L) N.D.–8.0 (M)	China	Rivers (WWTP)	Zhao et al. (2015)
Sulfamethoxazole	decapods, fish	3, 4	0.91	China	River (URB)	Bai et al. (2014)
	fish	4	<MDL–1.87 (M)[w] <MDL–2.4 (L)[w] <MDL–1.87 (G)[w]	Argentina	Rivers	Ondarza et al. (2019)
		9	N.D. (Bi) N.D.–5.5 (P) N.D. (L) N.D. < 0.91 (M)	China	Rivers (WWTP)	Zhao et al. (2015)
	insects	1	200[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Sulfamonomethoxine	fish	9	N.D.–20.4 (Bi) N.D. (P) N.D. (L) N.D. (M)	China	Rivers (WWTP)	Zhao et al. (2015)
Sulfathiazole	fish	3	0.3	China	River (URB)	Gao et al. (2012)
		4	<MDL–2.07 (M)[w] <MDL–2.43 (L)[w] <MDL–2.07 (G)[w]	Argentina	Rivers	Ondarza et al. (2019)
Sulfisoxazole	fish	3	101	China	River (URB)	Gao et al. (2012)
Tetracycline	fish	1	35.1–44.6	Nigeria	River, lagoon (PHAR)	Lan et al. (2019)
	plankton	–	29–35	China	Lake (WWTP)	Xie et al. (2017)
	zoobenthos[&]	3	26–49	China	Lake (WWTP)	Xie et al. (2017)
Triclosan	bivalves	1	12.5–96.3[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	2	37–39[e w]	USA (VA)	River (WWTP)	Arya et al. (2017)
Trimethoprim	fish	10	N.D. (B) N.D. (Go) N.D. (L) N.D.–0.7 (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		4	<MDL–8.8 (M)[w] <MDL–2.8 (L)[w] <MDL–5.5 (G)[w]	Argentina	Rivers	Ondarza et al. (2019)
		9	N.D.–4.01 (Bi) N.D.–6.13 (P) N.D.–2.13 (L) N.D.–0.39 (M)	China	Rivers (WWTP)	Zhao et al. (2015)
Anticholinergics						
Biperiden	insects	1	< 100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
	fish	3	N.D.–0.11 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
	insects	1	2850[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Anticoagulants						
Warfarin	bivalves	1	<MDL–0.2[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
Antidiabetics						
Metformin	fish	10	N.D. (B) N.D. (Go) N.D.–1.5 (L) N.D. (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
Repaglinide	fish	3	< 0.05–1.8 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
Antifungals						
Clotrimazole	bivalves	1	<MDL–0.6[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	3	< 1–3.4 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)

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Table 2 (*continued*)

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
	insects	1	6900[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Fluconazole	insects	1	1800[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Ketoconazol	insects	1	250[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Miconazole	bivalves	1	0.1–0.2[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	3	N.D.–12 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
	insects	1	500[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Antihistamines						
Azelastine	insects	[1]	700[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Desloratadine	fish	3	< 0.5–2.6 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
	insects	1	< 100	Australia	Streams (WWTP)	Richmond et al. (2018)
	periphyton	–	N.D.–8.6	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Diphenhydramine	bivalves	1	10.4–52.2[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	10	N.D.–7.3 (B) <LOQ–2.4 (Go) N.D.–5.2 (L) N.D.–0.5 (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		2	62–90[e w]	USA (VA)	River (WWTP)	Arya et al. (2017)
		7	N.D.–390 (P)	USA (DE)	River	Bean et al. (2018)
		3	N.D.–0.19 (P)	Germany, UK, Czech Republic USA (UT)	River (WWTP)	Cerveny et al. (2021)
		1	0.14–0.31 (M)[w] <MDL–8.6 (L)[w]	USA (TX)	River (WWTP)	Du et al. (2012)
		10	0.66–1.32[w]	USA (AZ, FL, IL, PA, TX)	Streams (WWTP)	Ramirez et al. (2007)
		1	1.2–1.7 (M) < MDL–11.1 (L)		Rivers (WWTP)	Ramirez et al. (2009)
			0.17	Germany		
		1	0.04 – 0.07[w]	Japan	Rivers (WWTP)	Subedi et al. (2012)
		2	0.18–1.82 (P) 0.84–8.34 (B)[w] 1.29–19.8 (L)[w] 1.60–58.9 (K)[w] 0.12–0.54 (M)[w] 0.24–3.07 (G)[w]		Streams (WWTP)	Tanoue et al. (2015)
	insects	1	700[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
	periphyton	–	11[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
Fexophenadine	insects	1	600[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Hydroxyzine	fish	3	N.D.–0.53 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
Antilipemics						
Bezafibrate	fish	2	<MDL–0.13 (P) <MDL (B)[w] <MDL (L)[w] <MDL (K)[w] <MDL (M)[w] <MDL–0.21 (G)[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
	periphyton	–	0.25[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
Fenofibric acid	insects	1	1750[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
	periphyton	–	0.55[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
Gemfibrozil	biofilm	–	N.D.–4	Spain	River (WWTP)	Ruhí et al. (2016)
	fish	2	N.D.–12[e w]	USA (VA)	River (WWTP)	Arya et al. (2017)
		1	ND (M) 11–34 (L)[w]	USA (UT)	River (WWTP)	Du et al. (2012)
		1	N.D.–90.0 (L)	USA (AZ, FL, IL, PA, TX)	Rivers (WWTP)	Ramirez et al. (2009)
	periphyton	–	N.D.–21.5	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Antiplatelet agents						
Clopidogrel	fish	2	7.7	USA	Rivers (WWTP)	Huerta et al. (2018)
		3	0.066[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	N.D.–14	Argentina	River (WWTP)	Valdés et al. (2016)
Antipruritic agents						
Crotamiton	fish	2	<MDL–1.61 (P) <MDL (B)[w] <MDL–4.28 (L)[w] <MDL–2.92 (K)[w] <MDL (M)[w] <MDL–2.46 (G)[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
Antitussives						
Dextromethorphan	fish	2	300–460[e w]	USA (VA)	River (WWTP)	Arya et al. (2017)
α–β blockers, antagonists, inhibitors						
Alfuzosin	fish	3	N.D.–0.33 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)

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Table 2 (*continued*)

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
Atenolol	fish	3	0.088[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	11–57	Argentina	River (WWTP)	Valdés et al. (2016)
Atorvastatin	insects	1	< 100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Bisoprolol	fish	3	N.D.–0.11	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
	Insects	1	200[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Cilazapril	insects	1	< 100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Carazolol	fish	3	0.054[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	N.D.–13	Argentina	River (WWTP)	Valdés et al. (2016)
Diltiazem	biofilm	–	N.D.–11.8	Spain	River (WWTP)	Ruhí et al. (2016)
	bivalves	1	0.4–1.8[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	10	N.D–17.2 (B) N.D.–0.4 (Go) N.D. (L) N.D.–1.2 (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		7	N.D.–260 (P)	USA (DE)	River	Bean et al. (2018)
		1	<MDL (M) <MDL–0.86 (L)[w]	USA (UT)	River (WWTP)	Du et al. (2012)
		1	0.11–0.27[w]	USA (TX)	Streams (WWTP)	Ramirez et al. (2007)
		1	0.13–0.15 (M) 0.30–0.70 (L)	USA (AZ, FL, IL, PA, TX)	Rivers (WWTP)	Ramirez et al. (2009)
		2	<MDL–0.19 (B)[w] 0.048–0.44 (L)[w] 0.048–1.94 (K)[w] <MDL–0.032 (M)[w] <MDL–0.072 (G)[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
	insects	1	400[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
	periphyton	–	0.17[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
Donepezil	insects	1	700[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Irbesartan	insects	1	100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Levamisole	bivalve	1	N.D.–6.6[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Memantine	fish	2	N.D.–22	Argentina	River (WWTP)	Valdés et al. (2016)
	insects	1	300[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Metoprolol	fish	3	0.37[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	N.D.–< 0.67	Argentina	River (WWTP)	Valdés et al. (2016)
	insects	1	2600[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
	periphyton	–	N.D.–181[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Metoprolol acid[+]	periphyton	–	N.D–169.1[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Nadolol	fish	2	2.1	USA	Rivers (WWTP)	Huerta et al. (2018)
		3	0.059[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	N.D.–23	Argentina	River (WWTP)	Valdés et al. (2016)
Pizotifen	fish	3	N.D.–0.55 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
Propanolol	bivalves	1	3.4–8.3[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	1	0.4	USA	Rivers (WWTP)	Huerta et al. (2018)
		11	N.D.–4.5[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
		3	0.19[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	N.D.–85	Argentina	River (WWTP)	Valdés et al. (2016)
	zoobenthos[&]	3	28–33	China	Lake (WWTP)	Xie et al. (2017)
Sotalol	fish	3	N.D.–1.8 (P)	Germany, UK, Czech Republic USA	Rivers (WWTP)	Cerveny et al. (2021)
		2	21.4	Argentina/Uruguay	Rivers (WWTP)	Huerta et al. (2018)
		3	0.02	Argentina	River	Rojo et al. (2019)
		2	N.D.–< 0.88		River (WWTP)	Valdés et al. (2016)
Venlafaxine	biofilm	–	N.D.–43.7	Spain	River (WWTP)	Ruhí et al. (2016)
	bivalves	1	5.1–20.1[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	10	N.D. (B) N.D.–5.5 (Go) N.D.–56.8 (L) N.D.–0.6 (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		3	N.D.–11 (P)	Germany, UK, Czech Republic USA (FL)	Rivers (WWTP)	Cerveny et al. (2021)
		1	0.24	Argentina/Uruguay	Rivers (HAR)	Gelsleichter and Szabo (2013)
		3	0.21[w]		River	Rojo et al. (2019)
	insects	1	5950[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Verapamil	biofilm	–	N.D–20.2	Spain	River (WWTP)	Ruhí et al. (2016)
	bivalves	1	<MDL–0.3[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
Norverapamil[+]	biofilm	–	N.D.–18.7	Spain	River (WWTP)	Ruhí et al. (2016)

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Table 2 (*continued*)

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
	bivalves	1	<MDL–0.09[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
Diuretics						
Hydrochlorothiazide	fish	3	0.8	USA	Rivers (WWTP)	Huerta et al. (2018)
		3	0.31[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	5–67	Argentina	River (WWTP)	Valdés et al. (2016)
	periphyton	–	N.D.–36.9[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Hormones						
17α-Ethinylestradiol	fish	2	N.D.–38[e w]	USA (VA)	River (WWTP)	Arya et al. (2017)
		1	17–29 (Bi)	China	Lake (WWTP)	Liu et al. (2011)
Psychoactive drugs						
Acridone	periphyton	–	N.D.–23	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Alprazolam	insects	1	450[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Amitriptyline	bivalves	1	9.4–30.1[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	insects	1	< 100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
10-HO-amitriptyline	bivalve	1	0.8–1.2[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
Bupropion	fish	10	N.D.–1.6 (B) N.D.–13.5 (Go) N.D.–1.0 (L) N.D. (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		3	< 0.1–1.4 (P)	Germany, UK, Czech Republic USA (CO, IA)	Rivers (WWTP)	Cerveny et al. (2021)
		1	N.D.–0.07 (B)[w]		Streams (WWTP)	Schultz et al. (2010)
Carbamazepine	fish	10	N.D.–1.6 (B) N.D.–6.6 (Go) N.D. (L) N.D.–3.8 (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		2	N.D.–62[e w]	USA (VA)	River (WWTP)	Arya et al. (2017)
		3	N.D.–4.4 (P)	Germany, UK, Czech Republic USA (UT)	Rivers (WWTP)	Cerveny et al. (2021)
		1	<MDL–0.6 (M)[w] <MDL–1.1 (L)[w]	USA (TX)	River (WWTP)	Du et al. (2012)
		1	0.83–1.44[w]	USA	Streams (WWTP)	Ramirez et al. (2007)
		10	0.30–0.70 (M) 90.0 (L)	Spain	Rivers (WWTP)	Ramirez et al. (2009)
		11	N.D.–17.9 (L)[w]	Argentina/Uruguay	Rivers	Rodríguez-Mozaz et al. (2015)
		3	0.19[w]	Japan	River	Rojo et al. (2019)
		2	<MDL–0.054 (P) 0.021–0.16 (B)[w] 0.052–0.20 (L)[w] <MDL–0.18 (K)[w] <MDL–0.050 (M)[w] <MDL–0.12 (G)[w]	Argentina	Streams (WWTP)	Tanoue et al. (2015)
		2	N.D.–33		River (WWTP)	Valdés et al. (2016)
	insects	1	< 50[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
OH-carbamazepine[+]	periphyton	–	N.D.–1.4[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
2-hydroxy carbamazepine[+]	Fish	4	0.7	USA	Rivers (WWTP)	Huerta et al. (2018)
		3	0.44[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	N.D.–17	Argentina	River (WWTP)	Valdés et al. (2016)
10,11-Epoxycarbamazepine[+]	fish	3	0.53[w]	Argentina/Uruguay	River	Rojo et al. (2019)
Epoxycarbamazepine[+]	fish	2	N.D.–9	Argentina	River (WWTP)	Valdés et al. (2016)
	periphyton	–	N.D.–1.5[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Chlorprothixene	insects	1	< 100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Citalopram	bivalves	1	10.2–33.3[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
		1	N.D.–0.9[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
	fish	10	N.D.–3.3 (B) N.D.–1.7 (Go) N.D.–3.6 (L) N.D.–0.4 (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		1	N.D.–0.4 (P)	USA (FL)	Rivers (HAR)	Gelsleichter and Szabo (2013)
		3	1.2	USA	Rivers (WWTP)	Huerta et al. (2018)
		11	N.D.–1.4[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
		1	N.D.–0.07 (B)[w]	USA (CO, IA)	Streams (WWTP)	Schultz et al. (2010)
		2	N.D.–51	Argentina	River (WWTP)	Valdés et al. (2016)
	insects	1	6500	Australia	Streams (WWTP)	Richmond et al. (2018)

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Table 2 (*continued*)

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
	periphyton	–	N.D.–17.2[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Clomipramine	fish	3	< 0.5–13 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
	insects	1	< 100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
	insects	1	1750[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Desmethylsertraline[+]	fish	4	15.6 ± 14.3 (B) 12.94 ± 10.45 (L) 0.69 ± 0.59 (M)	USA (TX)	Stream (WWTP)	Brooks et al. (2005)
		1	8.5–12 (M)[w] 140–600 (L)[w]	USA (UT)	River (WWTP)	Du et al. (2012)
Diazepam	fish	1	<MDL (M) <MDL–10 (L)[w]	USA (UT)	River (WWTP)	Du et al. (2012)
		1	1.9	USA	Rivers (WWTP)	Huerta et al. (2018)
		1	1.3 ± 0.27	USA (WA)	River, lagoon (PHAR)	Lan et al. (2019)
		3	0.048[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	6–41	Argentina	River (WWTP)	Valdés et al. (2016)
Duloxetine	insects	1	700[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Escitalopram	fish	2	44–175[e w]	USA (VA)	River (WWTP)	Arya et al. (2017)
Fluoxetine	bivalves	1	1.3–7.2[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	4	1.58 ± 0.74 (B) 1.34 ± 0.65 (L) 0.11 ± 0.03 (M)	USA (TX)	Stream (WWTP)	Brooks et al. (2005)
		3	N.D.–1.02[w]	Canada (ON)	Lake (HAR)	Chu and Metcalfe (2007)
		1	N.D. (M) 18–86 (L)[w]	USA (UT)	River (WWTP)	Du et al. (2012)
		1	N.D.–19.0 (L)	USA (AZ, FL, IL, PA, TX)	Rivers (WWTP)	Ramirez et al. (2009)
		1	N.D.–0.6 (B)[w]	USA (CO, IA)	Streams (WWTP)	Schultz et al. (2010)
	insects	1	1900[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Flupentixol	fish	3	N.D.–12	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
	insects	1	100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Fluphenazine	insects	1	100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Fluvoxamine	fish	1	0.83	USA (FL)	Rivers (HAR)	Gelsleichter and Szabo (2013)
Haloperidol	fish	3	N.D.–0.6 (P)	Germany, UK, Czech Republic Japan	Rivers (WWTP)	Cerveny et al. (2021)
		2	<MDL–0.042 (P) 0.050–0.33 (B)[w] 0.12–3.25 (L)[w] 0.067–1.28 (K)[w] <MDL–0.067 (M)[w] 0.041–0.30 (G)[w]		Streams (WWTP)	Tanoue et al. (2015)
	insects	1	800[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
	periphyton	–	1.9[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
Lorazepam	fish	3	0.21[w]	Argentina/Uruguay	River	Rojo et al. (2019)
		2	< 1.62–56	Argentina	River (WWTP)	Valdés et al. (2016)
Methylphenidate	fish	7	N.D.–150 (P)	USA DE)	River	Bean et al. (2018)
Mianserin	fish	3	N.D.–12 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
	insects	1	500[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Mirtazapine	insects	1	1500[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Norfluoxetine[+]	bivalves	1	0.1–1.5[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	10	N.D.–33.9 (B) N.D.–12.7 (Go) N.D. (L) N.D.–1.8 (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		4	8.86 ± 5.9 (B) 10.27 ± 5.73 (L) 1.07 ± 0.41	USA (TX)	Stream (WWTP)	Brooks et al. (2005)
		3	N.D.–1.08	Canada (ON)	Lake (HAR)	Chu and Metcalfe (2007)
		1	N.D. (M) 15–110 (L)[w]	USA (UT)	River (WWTP)	Du et al. (2012)
		1	N.D.–4.08 (P)	USA (FL)	Rivers (HAR)	Gelsleichter and Szabo (2013)
		4	1.1–3.9 (M)[w] <MDL–3.3 (G)[w] <MDL–9.1 (L)[w]	Argenina	Rivers	Ondarza et al. (2019)
		1	3.49–5.14[w]	USA (TX)	Streams (WWTP)	Ramirez et al. (2007)
		1	3.2–4.8 (M) 33.0–73.0 (L)	USA (AZ, FL, IL, PA, TX)	Rivers (WWTP)	Ramirez et al. (2009)
		1	N.D.–0.5 (B)[w]	USA (CO, IO)	Streams (WWTP)	Schultz et al. (2010)
Norsertraline[+]	fish	1	N.D.–3.0 (B)[w]	USA (CO IA)	Streams (WWTP)	Schultz et al. (2010)

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Table 2 (*continued*)

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
Oxazepam	fish	3	N.D.–25 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
	insects	1	100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Paroxetine	bivalves	1	1.3–2.4	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	3	N.D.–0.58[w]	Canada (ON)	Lake (HAR)	Chu and Metcalfe (2007)
		1	<MDL (M) 6.8–13 (L)[w]	USA (UT)	River (WWTP)	Du et al. (2012)
		1	N.D.–0.55	USA (FL)	Rivers (HAR)	Gelsleichter and Szabo (2013)
		1	N.D.–0.02 (B)[w]	USA (CO, IA)	Streams (WWTP)	Schultz et al. (2010)
	insects	1	< 100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Perphenazine	insects	1	200[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Risperidone	fish	3	< 0.1–10 (P)	Germany, UK, Czech Republic	Rivers (WWTP)	Cerveny et al. (2021)
	insects	1	< 100[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
Sertraline	bivalves	1	34.2–58.7[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	10	N.D.–16.9 (B) N.D.–6.7 (Go) N.D. (L) N.D. (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
			4.27 ± 1.40 (B) 3.59 ± 1.67 (L) 0.34 ± 0.09 (M)	USA (TX)	Stream (WWTP)	Brooks et al. (2005)
		1	N.D. (M) 75–110 (L)[w]	USA (UT)	River (WWTP)	Du et al. (2012)
		1	N.D.–0.48	USA (FL)	River (HAR)	Gelsleichter and Szabo (2013)
		1	17.1	USA	Rivers (WWTP)	Huerta et al. (2018)
		1	< MDL–11.0 (M) 27.0–84.0 (L)	USA (AZ, FL, IL, PA, TX)	Rivers (WWTP)	Ramirez et al. (2009)
		1	N.D.–1.8 (B)[w]	USA (CO, IA)	Streams (WWTP)	Schultz et al. (2010)
		2	0.049–0.79 (P) 0.52–9.51 (B)[w] 0.18–7.86 (L)[w] 0.77–23.4 (K)[w] 0.035–1.15 (M)[w] 0.23–3.40 (G)[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
		2	N.D.–58	Argentina	River (WWTP)	Valdés et al. (2016)
	insects	1	500[e]	Australia	Streams (WWTP)	Richmond et al. (2018)
	periphyton	–	0.97[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
Methylsertraline[+]	fish	1	1.65 – 3.28[w]	Germany	Rivers (WWTP)	Subedi et al. (2012)
	periphyton	–	N.D.–229.8[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Norsertraline[+]	fish	10	N.D.–400.1 (B) N.D.–44.0 (Go) N.D.–647.3 (L) N.D.–72.6 (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
		2	<MDL–2.78 (P) 5.55–44.7 (B)[w] 6.39–54.0 (L)[w] <MDL–52.4 (K)[w] <MDL–11.8 (M)[w] <MDL (G)[w]	Japan	Streams (WWTP)	Tanoue et al. (2015)
Venflaxine	fish	6	4.6	USA	Rivers(WWTP)	Huerta et al. (2018)
		11	N.D.–0.6[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
		1	N.D.–0.1 (B)[w]	USA (CO, IA)	Streams (WWTP)	Schultz et al. (2010)
		2	N.D.–35	Argentina	River (WWTP)	Valdés et al. (2016)
	periphyton	–	N.D.–4.1[w]	Spain	Rivers	Rodríguez-Mozaz et al. (2015)
Radiocontrasts						
Iopamidol	bivalves	1	74.3–110.9[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	fish	10	N.D.–1058.5 (B) N.D.–749.8 (Go) N.D. (L) N.D. (M)	Canada (ON)	River (WWTP)	Arnnok et al. (2017)
Veterinary drugs						
Enrofloxacin	bivalves	1	<MDL–0.4[w]	Canada (ON)	River (WWTP)	de Solla et al. (2016)
	decapods, fish	3, 4	762.34	China	River (URB)	Bai et al. (2014)
	fish	3	5.5	China	River (URB)	Gao et al. (2012)

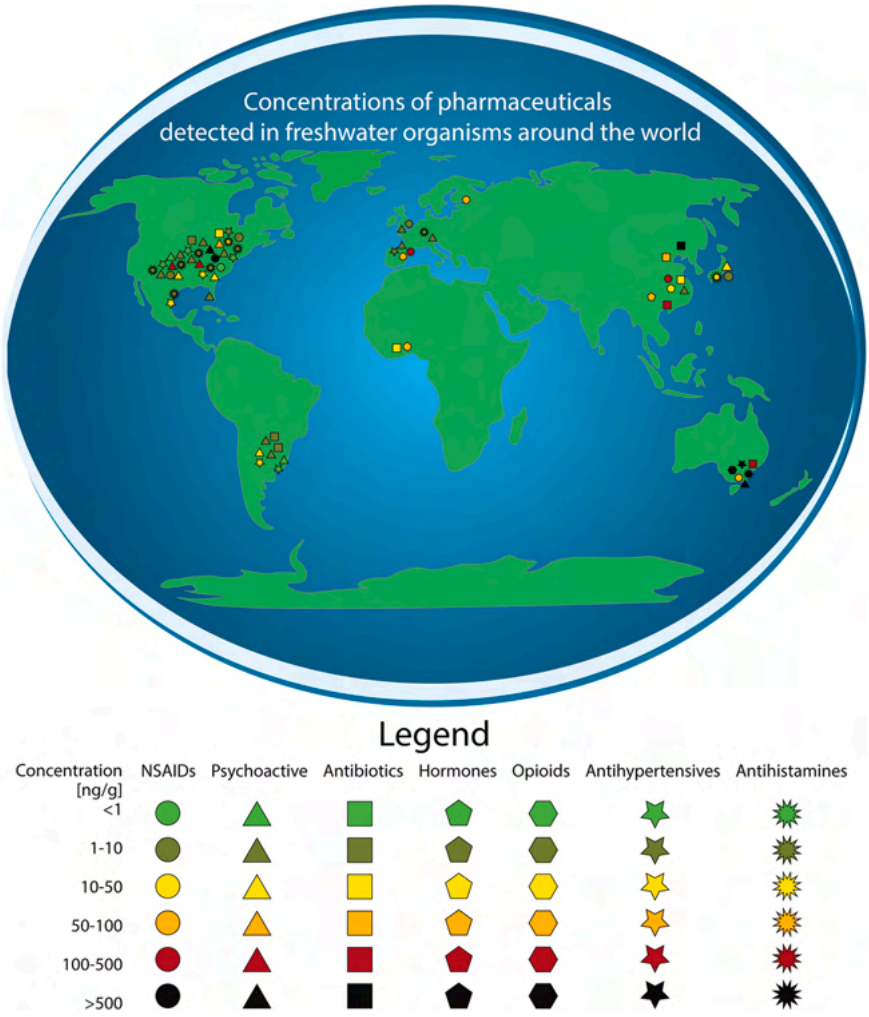

Fig. 2. Range of concentrations of selected groups of pharmaceuticals recorded in freshwater organisms worldwide.

observed in the present study. The highest concentrations were recorded in zoobenthic organisms, confirming that they deserve special attention in the context of bioaccumulation exposure.

Also in China, a large delta of Pearl River has been subjected to studies on pharmaceutical concentrations in wild fish. Four creeks of the river located in highly urbanized area were chosen as representative targets for pharmaceutical pollution screening. In total, 12 human and veterinary antibiotic agents were found in native fish bile, plasma, liver and muscle (Zhao et al., 2018).

A broad target analysis of 31 endocrine-disrupting compounds (EDCs) was conducted by Bao et al. (2020), analyzing five fish species from Taihu Lake, one of the largest lakes in China. Fish plasma was collected for analysis in this study, and BCFs were calculated relative to the concentrations of drugs detected in the water. Interestingly, the paper reported very large differences in bioaccumulation among fish species. In the case of *Cyprinus carpio*, medroxy-progresterone was characterized by the highest bioaccumulation factor (BAF = 1474), in the case of *Carassius auratus* it was hexesestrol (BAF = 1400), for *Hypophthalmichthys molitrix* and *Aristichthys nobilis*. it was dienoestrol (BAF = 893), and for *Anabarilius* sp. D-norgestrel (BAF = 2460). These results clearly demonstrate that species-related concentration of EDCs in plasma of wild fish can vary dramatically, and compounds with low concentrations in one species, can reach very high concentrations in another.

Moreover, Bai et al. (2014) analyzed the presence of 19 antibiotics from four different groups: sulfonamides, quinolones, tetracyclines, macrolides in environmental samples from the Liao River Basin, which receives wastewater from an area with a population of more than 40 million people. In this study, four fish species (*Carassius auratus, Acheilognathus chankaensis, Culter alburnus, Slurus asotus*) and three crustacean species (*Eriocheir sinensis, Pinnotheres sinensis, Hemigraspus sanguineus*) were analyzed together with water and sediment. In the case of fish, muscles were used for analysis. The results indicated that enrofloxacin is a compound requiring special attention. Although the water and sediment concentrations for this compound were lower than for many other antibiotics, it reached concentrations several orders of magnitude higher than other antibiotics in the tissues of all organisms tested, reaching bio-concentration factors (BCF) ranging from 10047 for *P. sinensis* to 45407 for *A. chankaensis*. Roxithromycin was also found to be a bioaccumulative compound with its BCF reaching 5290 in *A. chankaensis*, but this result should be treated with caution as no bioaccumulation of this compound was observed in any of the other organisms. Bioaccumulation was not observed for any of the other 17 antibiotics for which the BCFs were less than 1000, and most of them were not detected in the tissues of the test organisms at all. Also, the work of Gao et al. (2012) analyzed the distribution of antibiotics in the environment of one Chinese river (Haihe) by analyzing the tissues of three carp species (*C. carpio, H. molitrix, C. auratus*). Unlike the work of Bai et al. (2014), however, no significant bioaccumulation and/or biomagnification of enrofloxacin was observed (although it was present in the tissues and in water samples), and the antibiotics that accumulated most strongly in the tissues of the studied fish were ciprofloxacin and

erythromycin (BAF 3262 and 4492, respectively). However, it is worth noting that a total of 13 different antibiotics were detected in fish tissues.

3.1.2. Europe

Koba et al. (2018) studied the occurrence of drugs in fish inhabiting a pond used as tertiary treatment of wastewaters in Czech Republic, detecting eleven pharmaceuticals and their metabolites. In this study authors reported occurrence of psychiatric drugs and their metabolites, i.e. sertraline, metoprolol/atenolol acid, *N*-desmethylcitalopram, and citalopram in fish livers. High bioaccumulation was observed for sertraline, for which BAF was above 10000, but also for *N*-desmethylcitalopram, for which BAF exceeded 1000. In Germany there was a national study of potential pharmaceutical contamination in rivers with WWTP effluent discharge in relation to bioaccumulation in fish tissues. The results of this study were synthesized by Subedi et al. (2012) and only 2 out of 15 selected pharmaceuticals, diphenhydramine and desmethylsertraline, were found in the tissues of fish at measurable levels.

A very extensive analysis related to the presence of pharmaceuticals in European waters was recently carried out by Cerveny et al. (2021) in order to examine the concentrations of drugs present in the plasma of the aquatic organisms. Wild fish collected from 18 stations (including both effluent dominated sites and less impacted ones) located in the Czech Republic, Germany and the UK were analyzed for concentrations of 94 pharmaceuticals in blood. Interestingly, determination of any of the targeted compounds in blood of sampled fish from the German waters was not possible (concentrations below the LOQ in all samples), while in the individuals collected in the Czech Republic and the UK a total of 23 different substances were determined. Next, the determined levels of drugs were compared to their concentrations in human blood that produce a therapeutic effect. The analysis showed that flupentixol was the only drug whose blood concentration in fish was higher than the therapeutic level (>0.5 ng/ml), while azithromycin, haloperidol and risperidone were found to be above 10% of the therapeutic level.

The work of Grabicova et al. (2015) analyzed the presence of pharmaceuticals in two benthic freshwater organisms, *Hydropsyche* sp. and *Erpobdella octoculata,* collected from a small stream in Czech Republic. Of the 70 pharmaceuticals analyzed, six were determined in *Hydropsyche* sp. (azithromycin, citalopram, clarithromycin, clotrimazole, sertraline, verapamil), while only four were determined in *E. octoculata* (clotrimazole, diclofenac, sertraline, valsartan). The calculated BAFs indicate that among these compounds, azithromycin (BAF up to 34000) and sertraline (BAF up to 2100) may bioaccumulate particularly strongly. Recently, the same research group analyzed selective serotonin reuptake inhibitors (SSRIs) in 10 different streams located in the Czech Republic (Grabicová et al., 2020). As much as 5 compounds were detected in the fish tissues (*Squalius cephalus*), namely n-desmethyl citalopram, sertraline, mirtazapine, venlafaxine and o-desmethyl venlaxaine, but the BAF was low and did not exceed 600.

Several studies refer to Mediterranean region. For example, Rodríguez-Mozaz et al. (2015) did the research of pharmaceutical occurrence in four rivers (Ebro, Llobregat, Jucar, and Guadalquivir) that may be seasonally affected by water scarcity but receive treated wastewater all year round. In this study, biota samples from different trophic levels – periphyton, macroinvertebrates (*Hydropsyche* sp. larvae and mussels *Dreissena polymorpha*) and different species of fish – were collected from four Iberian rivers basins. Diclofenac (NSAIDs) was found to be most prevalent among study sites and trophic groups. Pharmaceuticals occasionally detected were sertraline (in periphyton), diclofenac (in macroinvertebrates) or carbamazepine (in fish liver). Ruhí et al. (2016) also selected Ebro River (Spain) as target in studying the influence of WWTPs runoff on the uptake of selective pharmaceuticals by biofilm and three invertebrate species: gastropod *Ancylus fluviatilis*, insects *Hydropsyche* sp. larvae, platyhelminth *Phagocata vitta*. Eight different pharmaceuticals were detected in these organisms: diclofenac (biofilm, insects), ibuprofen (insects, platyhelminths), estrone (platyhelminths), verapamil and norverapamil, gemfibrozil, diltiazem and venlafaxine (biofilm).

In an extensive study conducted in United Kingdom rivers (Hogsmill River, Chertsey Bourne River Blackwater River) affected by five WWTPs, occurrence of pharmaceuticals was determined in different biota groups including biofilm, benthic fauna and flora (Wilkinson et al., 2018). Of the three pharmaceuticals analyzed (acetaminophen, diclofenac, ethinylestradiol), no significant accumulation was observed for any of them, as the BCFs reached a maximum of a few tens for diclofenac and acetaminophen, while ethinylestradiol was not detected in any of biota tested.

Although works on environmental bioaccumulation rarely use small crustaceans, which, due to their size, lifestyle and diet, are difficult to analyze and require methodology of elevated sensitivity, Miller et al. (2015) analyzed the content of selected drugs in the freshwater amphipod *Gammarus pulex* from the River Thames (UK). Of the seven pharmaceuticals tested, most were not detected at any station or were detected at levels that prevented quantification. The maximum concentration was recorded for nimesulide and was only 36 ng/L.

Studies on the occurrence of pharmaceuticals in aquatic biota are still at a developing phase and focus on many taxonomic and functional groups. Clearly vast majority of the reported cases were found in benthic aquatic invertebrates and fish. However, Richards et al. (2011) found traces of pharmaceutical compounds (diclofenac and ibuprofen) in the hair of Eurasian otters (*Lutra lutra*) from UK, raising questions of the ways of exposure, transfer and potential effects of pharmaceuticals on large aquatic mammals.

On the other hand, in the context of antibiotic contamination of water, special attention should be paid to fish farms, mostly localized in the northern Europe. These farms are using large amount of antibiotics in order to increase breeding efficiency. In the work of Björklund et al. (1990), it was shown that when farmed fish are treated with oxytetracycline, the compound also easily enters wild fish feeding nearby and into sediments. In the conducted analyses, it was found that oxytetracycline could still be detected in wild fish 13 days after treatment and, importantly, antibiotic-resistant bacteria were also detected in their guts.

An interesting approach was presented by Brozinski et al. (2013), where the bile from fish collected at a polluted stream (Rakkolanjoki river) was analyzed. Diclofenac, naproxen and ibuprofen were successfully determined and their concentrations in bile were found to be about 1000 times higher than in water. This high degree of bioaccumulation of selected compounds in bile may mean that it is a good choice in the context of monitoring pharmaceutical contamination, at least for acidic NSAIDs.

3.1.3. North America

Rivers and streams are also important water bodies as they often receive wastewater treatment plant residues at first, so organisms living in them may be exposed to the highest concentrations of drugs. For example, the Potomac River receives wastewater from most of the 37 treatment plants located in the Washington, DC (USA) metropolitan area, carrying a huge load of contaminants such as pharmaceuticals. The work of Arya et al. (2017) analyzed the presence of pharmaceuticals in two fish species inhabiting this river, banded killifish and white perch, detecting 19 and 16 different compounds in them, respectively. Importantly, water and sediment were also analyzed in this study, but many compounds were detected only in fish tissues. Among these were naproxen, diclofenac, carbamazepine, estriol, testosterone, norethindrone or gemfibrozil, and their presence in fish tissues in the absence of other matrices may indicate significant deposition in organisms relative to the environment.

Ramirez et al. (2007) reported the occurrence of four (diphenhydramine, diltiazem, carbamazepine, and norfluoxetine) out of 24 pharmaceuticals tested in the fish inhabiting WWTP effluent-dominated part of a stream in the USA, Texas. Similarly, Tanoue et al. (2014) detected

certain pharmaceuticals in wild biota organs, i.e. fish and fish-eating birds while developing an isotope dilution method for the simultaneous determination of 17 polar pharmaceutical and personal care product (PPCP) residues in biological samples. Non-steroidal anti-inflammatory drugs, antibacterial agents, and psychotropic agents were frequently detected in the fish tissues, including their brains.

One of the earliest complex report of pharmaceutical residues occurring in aquatic biota were delivered by Ramirez et al. (2009). In this study the authors conducted a national pilot study of WWTP effluent and suburban river areas across different US regions surveying the levels of selected bioactive drugs. The results showed that out of 24 target pharmaceuticals seven were present in native fish tissues (in both fillets and separated livers). Those were psychoactive (carbamazepine, fluoxetine, norfluoxetine, sertraline), antihistamine (diphenhydramine), antihypertension (diltiazem) and antilipemic (gemfibrozil) agents. The highest concentrations were observed for SSRIs, reaching up to 545 ng/g for sertraline.

As shown in the work of Garcia et al. (2012), carbamazepine, which is the most widely used anticonvulsant worldwide, shows rather low potential for bioaccumulation. A study using fish collected from a sewage-polluted stream (Pecan Creek, Texas, USA) showed that none of the tissues accumulated the compound, and the BCF value was determined to be only 2.5–3.8.

Meanwhile, the work of de Solla et al. (2016) analyzed the potential presence of 145 drugs in Grand River, Ontario, Canada receiving wastewater effluent, finding 27 of them in the tissues of wild mussels of the species *Lasmigona costata*. Among these compounds, sertraline, amitriptyline, amlodipine, iopamidol, triclosan, and triclocarban were observed to be particularly accumulated in mussel tissues.

Further on, Schultz et al. (2010) reported the uptake of antidepressant pharmaceuticals by native fish neural tissue in two streams (Boulder Creek, Colorado, USA; Fourmile Creek, Iowa, USA). The model organism, white sucker (*Catostomus commersoni*), was found to accumulate bupropion, citalopram, fluoxetine, paroxetine, sertraline, venlafaxine and two drug metabolites: norfluoxetine and norsertraline.

Likewise, Huerta and colleagues made a major reconnaissance of pharmaceutical residues in biota of several US national rivers and streams over the years 2008–2009 (Huerta et al., 2018). The authors investigated the occurrence of pharmaceutical compounds in wild fish from 25 ranked as most polluted river sites located downstream from wastewater treatment plants (WWTPs). In this study 13 out of 20 (65% frequency) selected pharmaceuticals were detected in eight fish species representing various feeding strategies. Psychoactive drugs (venlafaxine, sertraline, carbamazepine) and compounds belonging to β-blocker therapeutic family (sotalol, nadolol) occurred in their tissues most frequently. Out of the latter drug group, sotalol reached greatest concentration of all detected compounds but was fund in two out of eight fish species only, whereas venlafaxine, carbamazepine and its metabolite (2-hydroxycarbamazepine) were found in more fish species at relatively low concentrations. Interestingly, carbamazepine metabolites were not detected in the same fish species as the original compound.

Although it is consistent among studies that terrestrial water bodies are generally closer to focal pollution from sewage reclamation points and thus become more affected by pharmaceutical load, coastal systems are anticipated to be gradually influenced by continued urbanization and contaminated river runoff. It has already been observed in the estuaries of the Gulf of Mexico, based on the study that determined pharmaceutical concentrations of diphenhydramine and diltiazem in plasma of the fish living in four tidally influenced river estuaries (Scott et al., 2016).

Drug concentration analysis in fish plasma was also conducted by Bean et al. (2018) on samples collected from the Delaware River (USA). Fourteen compounds were determined in the blood of fish out of the 17 sought, and acetaminophen and diclofenac reached the highest concentrations (up to a maximum of 4660 ng/l and 3620 ng/l, respectively).

The work of Arnnok et al. (2017) analyzed the presence of SSRI drugs in fish tissues from the Niagara River, which receives wastewater from multiple treatment plants. The study examined as many as 10 fish species with diverse biology and ecology, separating the tissues into brain, gills, gonads, and muscle during the analysis. The study showed the highest bioaccumulation of norsertraline (a metabolite of sertraline), for which the BCF was up to 3000, and accumulated mainly in the liver and brain. Interestingly, bioaccumulation of the more lipophilic parent compound - (BCF < 100 in all trials) was not observed at the same time. This may be due to the fact that sertraline is metabolized by fish and only as norsertraline accumulated in tissues, but this finding requires further studies. It is worth noting that the same mechanism was not noted for norfluoxetine, whose BCF reached a maximum of 130, and in many cases this compound was not detected in tissues at all.

An extensive analysis determining the concentration of 23 different pharmaceutical compounds in a freshwater snail and periphyton was conducted by Du et al. (2015b). The study was conducted in one of the streams located from Texas (USA) during the drought period, hoping to determine the concentrations in a worst case scenario. As many as 11 compounds were detected in snail tissues, while only three were detected in periphyton. The highest bioaccumulation was recorded for SSRI drugs, with BAFs of 3000 and 16000 for fluoxetine and norsertraline, respectively. It is worth noting that also in an earlier works by these authors (Du et al., 2012; Brooks et al., 2005), high concentrations of SSRIs were noted in organisms from different sites located in USA. Du et al. (2012) analyzed 17 different pharmaceuticals in fish (*Salmo trutta*) from one of the streams located in Utah, showing the highest concentrations for SSRIs and their metabolites, norsertraline (440 ng/g), sertraline (92 ng/g), norfluoxetine (57 ng/g) and fluoxetine (44 ng/g). Brooks et al. (2005) analyzed three species of fish collected from the stream located in Texas, finding the same four compounds in each of them, with the highest concentration exceeding 15 ng/g for norsertraline. Interestingly, as many as seven different antidepressants (citalopram, fluoxetine, norfluoxetine, fluvoxamine, paroxetine, sertraline, venlafaxine) were detected in the blood of bull shark (*Carcharhinus leucas*) from Caloosahatchee River (USA) (Gelsleichter and Szabo, 2013). The highest concentration was determined for norfluoxetine and was over 4 ng/ml of blood, but it is worth noting that this compound was detected in only one of 10 samples. In contrast, sertraline was the only compound detected in all samples. Unfortunately, norsertraline was not analyzed in the above mentioned study, but it once again confirmed that SSRI metabolites, in this case norlfuoxetine, can reach higher concentrations than parent compounds in tissues of aquatic organisms. As shown, compounds belonging to the SSRIs represent one of the best studied groups of pharmaceuticals for North America. A target analysis focused on this drug group was also conducted by Chu and Metcalfe (2007) using fish collected from Hamilton Harbor in Ontario (Canada), which is an urbanized and industrialized embayment of Lake Ontario. Concentrations recorded in this work were relatively low compared to those recorded in organisms collected from rivers in the US, with only two samples exceeding the 1 ng/g wet weight level for fluoxetine and norfluoxetine.

3.1.4. South America

Valdés et al. (2016) studied the spatial and temporal occurrence of commonly prescribed pharmaceuticals in Córdoba River basin taking into account the presence of pharmaceutical residues originating from WWTP effluents in wild fish. Two studied fish species (*Gambusia affinis* and *Jenynsia multidentata*) showed divergent pharmaceutical uptake patterns for polar compounds. *G. affinis* accumulated 20 pharmaceutical compounds belonging to several treatment groups and those were psychiatric drugs (and metabolites), β-blockers, analgesics, non-steroidal anti-inflammatory agents and others. Growing literature on increased pharmaceutical uptake by wild fauna raised concerns over protected areas and their fate as the urbanization progress is more evident. Thus Ondarza et al. (2019) examined bioaccumulation of pharmaceuticals

and their metabolites in muscle, liver and gills of multiple fish species (*Rhamdia quelen*, *Hypostomus commersoni*, *Hoplias lacerdae*, *Prochilodus lineatus*) from an urban river receiving wastewater discharges (Parana) and a lotic system (Acaragua). Antibiotics such as erythromycin, sulfamethoxazole, sulfathiazole, trimethoprim and the psychoactive metabolite norfluoxetine occurred in various concentrations in different examined tissues. Alarmingly, some of these occurred in biota from theoretically unaffected and protected areas. Rojo et al. (2019) studied three main fish species of Uruguay River (*Megaleporinus obtusidens*, *Salminus brasiliensis*, and *Prochilodus lineatus*) that are important for human consumption. This study reported presence of pharmaceuticals in common fish and elucidated the differences in concentrations of pharmaceutical residues in fish tissues related to their feeding habits. The most recurrent ones were psychiatric drugs: carbamazepine and atenolol (present in all species). Other reported pharmaceuticals occurred exclusively in one species.

3.1.5. Africa

As compared to other geographical locations, overall very few studies analyzing the levels of drugs in the biota matrix were performed so far. Only recently, African water bodies (small rivers) have been subjected to major examination of pharmaceutical residues in the wild, including biota, by Lan et al. (2019). This study revised the possible environmental impact of wastewater effluents from pharmaceutical industry in Nigeria, near Lagos Lagoon. Among the studied pharmaceuticals, antibiotic agents such as metronidazole and tetracycline, the psychotropic diazepam and NSAID ibuprofen were detected in Tilapia fish (*Oreochromis niloticus*). Authors concluded that ibuprofen found at concentration of 95 ng/g in fish may impose environmental risk.

3.1.6. Australia

Similarly, to Africa, also in Australia studies analyzing the levels of drugs in the biota matrix are lacking. To our knowledge, there is only one study regarding the presence of pharmaceuticals in freshwater fauna of Australia. Richmond et al. (2018) detected 69 pharmaceutical compounds belonging to diverse pharmaceutical groups in riparian invertebrates collected from streams (six study sites) located near urbanized areas of Melbourne. Alarmingly, the concentrations detected in this work reached levels rarely seen in studies from other continents, and many of the compounds tested exceeded levels of 1000 ng/g d.w. in the tissues of *Hydropsyche* sp. larvae. Among these were as many as three compounds from the SSRI group - citalopram, fluoxetine and venlafaxine.

3.1.7. Summary

Given increasing numbers of investigations led across the world, the main factors influencing the occurrence and fate of pharmaceutical compounds in freshwater habitats seems to greatly vary between geographic locations. The type of pharmaceutical residues found in freshwater biota depends primarily on the levels of human and veterinary usage, including distinctive prescription regulations among countries. The most commonly used pharmaceutical classes of prescription (e.g. psychoactive drugs, antibiotics) and over-the-counter medication (e.g. mild analgesics and NSAIDs) are accurately reflected in the trends of detection levels of these classes in the natural environment (Rodríguez-Mozaz et al., 2015; Ruhí et al., 2016). Within a regional scale, the highest levels of pharmaceutical concentrations in biotic matrices are expected and usually found in water bodies (rivers, streams, lakes) adjacent to or receiving significant water runoff from highly urbanized and densely populated areas (agglomerations) (Bai et al., 2014; Ruhí et al., 2016; Huerta et al., 2018; Richmond et al., 2018; Zhao et al., 2018; Bao et al., 2020). In such conditions uncontrolled and improper disposal of pharmaceuticals may take place but yet the main source of drugs and their active metabolites is attributed to direct discharge of treated municipal water into aquatic systems. On the organisms level pharmaceuticals have been shown to accumulate in freshwater biota, particularly in fish, yet their effects can be observed on various levels of biological organization, from genes to ecosystems. In these vertebrates also distinctive uptake/accumulative patterns between certain organs has been observed. Although primarily it was believed that in dynamic freshwater environments (rivers and streams) the pharmaceutical pollution fades along with increasing distance from discharge points, recent research showed that drug residues may still be found in the areas located far from the source of contamination (Ondarza et al., 2019), including river mouth and final sinks such as marine ecosystems. This raises questions on the complexity of transportation pathways and persistence of these compounds in the light of global environmental health risk.

3.2. Saltwater environment

Saltwater environment include various types of basins spanning in the salinity level from brackish (e.g. Baltic Sea, estuaries) to fully marine ecosystems (oceans and seas). From a global perspective they cover over 70% of the Earth surface and contain over 95% of Earth's water supply (https://www.un.org/sustainabledevelopment/oceans/). Also, approx. 40% of global population inhabits coastline or areas or not further then 100 km of the coast (https://www.un.org/esa/sustdev/natlinfo/indicators/methodology_sheets/oceans_seas_coasts/pop_coastal_areas.pdf). Seawaters are vital for human well-being as they provide services such as provisioning (as food, habitats, energy sources), regulating/maintaining (as climate protection) and supportive (as pollution control) (Schuhmann and Mahon, 2015). Large marine ecosystems, in contrast to freshwater ones, serve as final sinks of natural and anthropogenic contaminants, including pharmaceuticals. Despite receiving pollution from diverse direct sources (WWTP outlets, agriculture etc.), the main transport routes of pollution in marine and brackish environments include atmospheric deposition and a variety of semi-final sinks such as rivers, some of which with large drainage basins covering enormous land areas. Water salinity and a large water volume of these reservoirs generally lower the anthropogenic pollutants concentration, but there is high disproportion between data from monitoring of fresh and saltwater ecosystems. The minority of open ocean waters were tested in order of pharmaceuticals concentration, and most of studies were performed in coastal zones (Table 3). Also, similarly to freshwater ecosystems, monitoring of pharmaceuticals and other pollution is mostly performed in the municipal areas, close to the sewage treatment plant effluent discharge zones and in estuaries of the greatest catchment areas.

3.2.1. Asia

Ali et al. (2018) searched for selected pharmaceuticals and personal care products (PPCPs) in coastal waters of the Saudi Red Sea. For this purpose, native to that area fish (*Oreochromis niloticus, Chanos chanos, Gerres oyena, Lutjanus Johni, Lethrinus nebulosus*), barnacles (*Balanus perforatus*), and macroalgae (*Ulva lactuca*, *Enteromorpha* sp., *Turbinaria conoides*, *Hydroclathrus clathratus*) were collected. Two of three selected stations are known to be affected by sewage effluent from Jeddah city. The latter is potentially unpolluted and located relatively far from the closest discharge point (9 km). 15 pharmaceuticals from different group: nonsteroidal anti-inflammatory drugs (NSAIDs) and analgesic (diclofenac, ibuprofen, acetaminophen), antihistamine (chlorpheniramine malate), antidepressant (amitriptyline, fluoxetine), bacteriostatic and antibiotic (trimethoprim, ciprofloxacin, metronidazole, sulfamethoxazole, cephalexin), antiepileptic (carbamazepine) β-blockers (atenolol) H2-antihistamine (ranitidine) and anticoagulant (warfarin) were analyzed in biota and water. Interestingly, most pharmaceuticals (13) were detected in fish followed by barnacles (11) and macro algae (6). However, most of compounds detected in barnacles were below the limit of quantification, except for amitriptyline, which concentration was 17.9 ng/g dw. Worryingly, high level of carbamazepine (1.7 ng/g) was detected in macroalgae. In fish, the highest level was observed for metronidazole, which was 82.1 ng/g dw. Specifically in the case of fish,

Table 3
The occurrence of drugs in marine wildlife (including brackish ecosystems and estuaries) grouped by therapeutic classes. Studies reporting quantitative analyses giving results within detection limits. [e]–estimated concentrations. Concentrations reported in ng/g dry weight (ng/g dw) unless stated otherwise; in plasma concentrations expressed as ng/ml. In case of reports regarding studies conducted in Canada or the United States of America, the regions (states) are indicated in the parenthesis by their universal abbreviations. The impact by specific sources of pharmaceutical pollution is indicated in the table with abbreviations: wastewater treatment plants discharge (WWTP), highly urbanized area (URB), aquaculture (FARM) or harbor (HAR). Symbols used: RL–reporting limit; [w]–wet weight; LOQ–limit of quantification; N.D.–not detected; MDL–method detection limit; H–hemolymph, L–liver; M–muscle, P–plasma, V–viscera.

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
Non-Steroidal Anti-Inflammatory drugs						
Diclofenac	bivalves	1	<MDL–16.11	Italy	Coastal	Mezzelani et al. (2016)
		1	0.5–4.5	Portugal	Coastal, estuary	Cunha et al. (2017)
		1	< 1.4–171.1	Italy	Coastal (WWTP)	Mezzelani et al. (2020)
	fish	1	0.5–5.2[w]	Sweden	Coastal Estuary (URB)	UNESCO and HELCOM (2017)
		2	1.10–10.76	Malaysia		Omar et al. (2019)
	gastropod	1	0.93–7.41	Malaysia	Estuary (URB)	Omar et al. (2019)
Ibuprofen	bivalves	1	N.D–10.5[w]	USA (OR)	Estuary (WWTP)	Granek et al. (2016)
		1	<MDL–9.39	Italy	Coastal	Mezzelani et al. (2016)
		2	< 8.0–143.7	Italy	Coastal (WWTP)	Mezzelani et al. (2020)
	fish	1	2.4[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Naproxen	bivalve	1	N.D–3.39[w]	USA (OR)	Estuary (WWTP)	Granek et al. (2016)
Nimesulide	bivalve	1	2.99–6.04	Italy	Coastal	Mezzelani et al. (2016)
		1	< 2.0–80.6	Italy	Coastal (WWTP)	Mezzelani et al. (2020)
Analgesics						
Codeine	fish	1	83.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Flurbiprofen	bivalves	1	210	Poland	Coastal (WWTP)	Caban et al. (2016)
Paracetamol	bivalves	1	80	Poland	Coastal (WWTP)	Caban et al. (2016)
Tramadol	bivalves	1	128–179[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Antibiotics						
Amoxicilin	fish	2	0.29 3.66	Malaysia	Estuary (URB)	Omar et al. (2019)
	gastropod	1	0.29–0.58	Malaysia	Estuary (URB)	Omar et al. (2019)
Azithromycin	bivalves	5	1.3–13.3	Portugal	Estuary (URB)	Álvarez-Muñoz et al. (2015b)
		3	1.2–3.0	Spain	Estuary (WWTP)	Álvarez-Muñoz et al. (2015a)
		1	0.9–3.2[w]	USA (OR)	Estuary (WWTP)	Granek et al. (2016)
	fish	2	<RL–1.7[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Chloramphenicol	bivalves	1	2.64 ± 0.47	China	Coastal (URB)	Ruan et al. (2020)
	crustaceans	5	0.25–1.88	China	Coastal (URB)	Ruan et al. (2020)
	fish	15	0.12–1.23	China	Coastal (URB)	Ruan et al. (2020)
	gastropods	3	0.44–0.71	China	Coastal (URB)	Ruan et al. (2020)
	fish	2	160.0–590.0 (V)	Spain	Coastal (URB)	Fernandez-Torres et al. (2011)
Ciprofloxacin	bivalves	11	N.D.–208.0	China	Coastal (URB)	Li et al. (2012)
	fish	2	<RL–17.0[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
		1	7.0–8.5[w]	Sweden	Marine	UNESCO and HELCOM (2017)
Clarithromycin	fish	7	N.D.–0.44	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Cloxacillin	bivalves	1	N.D.–5.1[e]	USA (OR)	Estuary (WWTP)	Granek et al. (2016)
Difloxacin	bivalves	11	N.D–57.0	China	Coastal (URB)	Li et al. (2012)
Dimetridazole	bivalves	1	7.7 ± 2.5	Portugal	estuary (URB)	Álvarez-Muñoz et al. (2015b)
Erythromycin	bivalves	1	12.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
		11	N.D.–31.3	China	Coastal (URB)	Li et al. (2012)
	fish	2	<RL–0.9[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
		7	N.D–0.17	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Fleroxacin	bivalves	11	N.D.–250.0	China	Coastal (URB)	Li et al. (2012)
Josamycin	bivalves	11	N.D.–1.1	China	Coastal (URB)	Li et al. (2012)
Lomefloxacin	bivalves	1	N.D.–177.0			
		11	N.D.–141.0	China	Coastal (URB)	Li et al. (2012)
Metronidazole	barnacles	1	<LOQ–5.9	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
	fish	5	N.D.–82.1	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
		7	N.D.–1.92	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Norfloxacin	bivalves	11	N.D.–370.0	China	Coastal (URB)	Li et al. (2012)
		1	9.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Ofloxacin	bivalves	1	0.0 –18.0	USA (CA)	Coastal (URB)	Dodder et al. (2014)
		11	N.D.–242.0	China	Coastal (URB)	Li et al. (2012)
	fish	7	N.D.–3.43	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Oxytetracycline	fish	1	200–1300[w]	Finland	Brackish (FARM)	Björklund et al. (1990)
		4	N.D.–50.0 (V)	Spain	Coastal (URB)	Fernandez-Torres et al. (2011)
		3	N.D.–87 ppb	Chile	Coastal (FARM)	Fortt et al. (2007)
Ronidazole	bivalves	3	N.D–1.8	Spain	Estuary (WWTP)	Álvarez-Muñoz et al. (2015a)
		1	2.26 ± 0.51	Spain	Coastal (WWTP)	Martínez-Morcillo et al. (2020)
Roxithromycin	bivalves	11	N.D.–1.9	China	Coastal (URB)	Li et al. (2012)
Trimethoprim	barnacles	1	0.84–1.50	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
	fish	5	N.D.–44.90	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
Spiramycin	bivalves	11	N.D.–23.2	China	Coastal (URB)	Li et al. (2012)
Sulfadiazine	bivalves	1	0.70–0.78	USA (OR)	Estuary (WWTP)	Granek et al. (2016)
		11	N.D–2.72	China	Coastal (URB)	Li et al. (2012)
	fish	3	N.D.–190.0 (V) N.D.–50.0 (M)	Spain	Coastal (URB)	Fernandez-Torres et al. (2011)
		2	< RL–0.88[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Sulfamerazine	bivalves	11	N.D.–6.0	China	Coastal (URB)	Li et al. (2012)

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Table 3 (*continued*)

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
	fish	4	N.D.–70.0	Spain	Coastal (URB)	Fernandez-Torres et al. (2011)
		2	< RL–0.51[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
N^4-acetylsulfamerazine$^+$	fish	4	N.D.–20.0	Spain	Coastal (URB)	Fernandez-Torres et al. (2011)
Sulfamethazine	bivalves	1	N.D.–430.0	USA (CA)	coastal (URB)	Dodder et al. (2014)
		11	N.D.–29.8	China	Coastal (URB)	Li et al. (2012)
	fish	1	N.D.–70.0	Spain	Coastal (URB)	Fernandez-Torres et al. (2011)
Sulfamethizole	bivalves	1	<RL–0.20	USA (CA)	Estuary (URB)	Klosterhaus et al. (2013)
	fish	1	N.D.–70.0	Spain	Coastal (URB)	Fernandez-Torres et al. (2011)
Sulfamethoxazole	barnacles	1	N.D.–13.1	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
	bivalves	1	N.D.–2.76[w]	USA (OR)	Estuary (WWTP)	Granek et al. (2016)
		11	N.D.–21.0	China	Coastal (URB)	Li et al. (2012)
	fish	5	N.D.–11.2	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
		1	51.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
		2	0.09–0.27	Malaysia	Estuary (URB)	Omar et al. (2019)
	gastropods	1	0.10–1.13	Malaysia	Estuary (URB)	Omar et al. (2019)
Sulfamonomethoxine	bivalves	11	N.D.–15.4	China	Coastal (URB)	Li et al. (2012)
Sulfathiazole	bivalves	11	N.D.–35.2	China	Coastal (URB)	Li et al. (2012)
Sulfisoxazole	bivalves	11	N.D.–71.6	China	Coastal (URB)	Li et al. (2012)
Anticholinergics						
Bromocriptine	bivalve	1	110.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Dicycloverine	fish	1	7.0–26.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Antidiabetics						
Metformin	fish	2	<RL–28.0[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Antidiarrheals						
Loperamide	bivalve	1	167.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Antifungals						
Clotrimazole	fish	1	77.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Ketoconazol	bivalves	1	9.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
	fish	1	70.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Miconazole	fish	2	<RL–1.8[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
	bivalves	1	115.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Antihelmintics						
Thiabendazole	fish	7	N.D.–2.09	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Antihistamines						
Chlorpheniramine malate	barnacles	1	N.D.–< 4.0	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
Clemastine	fish	1	3.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Cyproheptadine	bivalve	1	7.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Diphenhydramine	bivalve	1	0.26[w]	USA (OR)	Estuary (WWTP)	Granek et al. (2016)
		1	153.0[w]	Sweden	marine	UNESCO and HELCOM (2017)
	fish	5	N.D.–62.10	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
		2	0.24–2.7[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
		10	N.D.–2.83 (P)	USA (TX)	Estuary (WWTP)	Scott et al. (2016)
Orphenadrine	fish	1	< 1.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Ranitidine	bivalves	1	10.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
		1	<RL–0.3[w]	USA (CA)	Estuary (URB)	Klosterhaus et al. (2013)
	fish	5	N.D.–27.0	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
		2	0.82–1.1[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Anti-inflammatory glucocorticoids						
Fluocinonide	fish	2	<RL–6.5[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Antilipemics						
Gemfibrozil	fish	2	<RL–1.3[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Antimuscarinics						
Trihexyphenidyl	bivalves	1	135–187[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
	fish	1	5.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
α–β Blockers, antagonists, inhibitors						
Alfuzosin	fish	2	5–13[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Amlodipine	fish	2	<RL–1.0[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Atenolol	barnacles	1	1.3–< 8.1	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
	bivalves	1	N.D.–13.0	USA (CA)	Coastal (URB)	Dodder et al. (2014)
		1	1.98 ± 0.33	China	Coastal (URB)	Ruan et al. (2020)
	crustaceans	5	1.22–21.1	China	Coastal (URB)	Ruan et al. (2020)
	fish	5	N.D.–63.0	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
		15	< 0.2–2.61	China	Coastal (URB)	Ruan et al. (2020)
	gastropods	3	< 0.2–1.91	China	Coastal (URB)	Ruan et al. (2020)
Atorvastatin	fish	1	2[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Bisoprolol	bivalves	1	102.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
	fish	7	N.D.–0.23	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Cilazapril	bivalves	1	7.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Diltiazem	fish	2	< RL–1.6[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
		10	1.71–42.73 ng/ml (P)	USA (TX)	Estuary (WWTP)	Scott et al. (2016)
Diltiazem desmethyl$^+$	fish	2	0.06–1.5[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Dipyridamole	bivalve	1	159.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Donepezil	fish	1	3.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Eprosartan	bivalve	1	95.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Felodipine	bivalve	1	147.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
	fish	1	65.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)

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Table 3 (*continued*)

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
Irbesartan	bivalve	1	9.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
	fish	1	38.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Levamisole	bivalve	1	1.6–6.0	Spain	Lagoon (WWTP)	Moreno-González et al. (2016)
		1	0.93 ± 0.02	China	Coastal (URB)	Ruan et al. (2020)
Memantine	fish	1	1.5[w]	Sweden	Coastal	UNESCO and HELCOM (2017),
Metoprolol	crustaceans	5	0.8–3.82	China	Coastal (URB)	Ruan et al. (2020)
	fish	15	< 0.15–5.59	China	Coastal (URB)	Ruan et al. (2020)
	gastropod	3	0.24–0.74	China	Coastal (URB)	Ruan et al. (2020)
Pizotifen	fish	1	< 1.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Tamoxifen	fish	1	58.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Tamsulosin	bivalves	2	2.9–3.0	Portugal	Estuary (URB)	Álvarez-Muñoz et al. (2015b)
Valsartan	bivalve	1	< 0.5–6.7	Italy	Coastal (WWTP)	Mezzelani et al. (2020)
Venlafaxine	bivalves	1	N.D.–2.7	France	Coastal (URB)	Bueno et al. (2014)
		5	2.1–36.1	Portugal	Estuary (URB)	Álvarez-Muñoz et al. (2015)
		1	N.D.–1.6	Spain		Moreno-González et al. (2016)
		1	0.78 ± 0.24	China	Lagoon (WWTP) Coastal (URB)	Ruan et al. (2020)
	crustaceans	5	0.23–0.40	China	Coastal (URB)	Ruan et al. (2020)
	fish	1	2.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
		15	0.27–0.88	China	Coastal (URB)	Ruan et al. (2020)
	gastropods	3	0.28–0.56	China	Coastal (URB)	Ruan et al. (2020)
N-desmethyl-Venlafaxine[+]	bivalves	1	N.D–3.0	France	Coastal (URB)	Bueno et al. (2014)
O-desmethyl-venlafaxine[+]	bivalves	1	N.D–3.7	France	Coastal (URB)	Bueno et al. (2014)
N,N-didesmethyl-venlafaxine[+]	bivalves	1	N.D–3.8	France	Coastal (URB)	Bueno et al. (2014)
N,O-didesmethyl-venlafaxine[+]	bivalves	1	N.D–3.5	France	Coastal (URB)	Bueno et al. (2014)
Warfarin	fish	5	<LOQ–8.7	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
Diuretics						
Hydrochlorothiazide	bivalve	1	N.D.–6.8[w]	USA (OR)	Estuary (WWTP)	Granek et al. (2016)
		1	N.D.–1.6	Spain	Lagoon (WWTP)	Moreno-González et al. (2016)
Furosemide	bivalve	1	N.D.–18.7[w]	USA (OR)	Estuary (WWTP)	Granek et al. (2016)
Triamterene	bivalve	1	<RL–06[w]	USA (CA)	Estuary (URB)	Klosterhaus et al. (2013)
Ergot alkaloids						
Dihydroergotamine	fish	1	32.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Hormones						
17α-Ethinylestradiol	bivalves	1	3.42	China	Lagoon (WWTP)	Zhang et al. (2011)
		1	310	Poland	Coastal (WWTP)	Caban et al. (2016)
		1	< 3.0–38.0	Italy	Lagoon (URB)	Pojana et al. (2007)
		1	N.D.–9.7	China	Coastal (WWTP)	Xu et al. (2016)
	fish	3	<MDL–3.04	China	Lagoon (WWTP)	Zhang et al. (2011)
17β-Estradiol	bivalves	1	3.62	China	Lagoon (WWTP)	Zhang et al. (2011)
	fish	3	<MDL–3.03	China	Lagoon (WWTP)	Zhang et al. (2011)
Diethylstilbestrol	bivalves	1	11.41	China	Lagoon (WWTP)	Zhang et al. (2011)
	fish	3	2.85–3.65	China	Lagoon (WWTP)	Zhang et al. (2011)
Etonogestrel	bivalves	1	7.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Flutamide	bivalves	1	15.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Levonorgestrel	bivalves	2	6.5–15.1	Portugal	Estuary (URB)	Álvarez-Muñoz et al. (2015b)
Progesterone	bivalves	2	1.5–2.6	Portugal	Estuary (URB)	Álvarez-Muñoz et al. (2015b)
	fish	2	0.81–1 1.9	Malaysia	Estuary (URB)	Omar et al. (2019)
	gastropod	1	0.73–9.57	Malaysia	Estuary (URB)	Omar et al. (2019)
Testosterone	fish	2	0.33–0.70	Malaysia	Estuary (URB)	Omar et al. (2019)
	gastropods	1	0.29	Malaysia	Estuary (URB)	Omar et al. (2019)
Psychoactive drugs						
Alprazolam	bivalves	1	0.80 ± 0.04	China	Coastal (URB)	Ruan et al. (2020)
	cephalopods	1	0.31 ± 0.33	Spain	Coastal (WWTP)	Martínez-Morcillo et al. (2020)
	fish	2	<RL–0.38[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
		1	19[w]	Sweden	Marine	UNESCO and HELCOM (2017)
Amitriptyline	barnacle	1	7.3–17.9	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
	fish	5	N.D.–44.2	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
		2	<RL–0.68[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
10-OH-amitriptyline[+]	fish	2	0.09–0.13[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Benztropine	fish	2	<RL–0.20[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Carbamazepine	barnacles	1	N.D.–5.5	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
	bivalves	1	140.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
		1	1.30–5.30	USA (CA)	Estuary (URB)	Klosterhaus et al. (2013)
		1	< 1.0–299.7	Italy	Coastal (WWTP)	Mezzelani et al. (2020)
		1	N.D.–0.2[w]			
	fish	5	N.D.–33.8	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
		7	N.D.–1.18	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Citalopram	bivalves	1	20.6 ± 0.2	Portugal	estuary (URB)	Álvarez-Muñoz et al. (2015b)
		1	N.D–4.97	Portugal	Coastal, estuary	Silva et al. (2017)
		1	9.38 ± 0.15	Spain	Coastal (WWTP)	Martínez-Morcillo et al. (2020)
	cephalopod	1	14.10 ± 4.15	Spain	Coastal (WWTP)	Martínez-Morcillo et al. (2020)
N-Desmethyl-citalopram[+]	bivalves	1	N.D.–10.17	Portugal	Coastal, estuary	Silva et al. (2017)
Clonazepam	bivalves	1	12.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Diazepam	fish	2	0.25–0.39[w]	Nigeria	Estuary (WWTP)	Meador et al. (2016)

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Table 3 (*continued*)

Compound	Biota	No. of species	Concentration (ng/g dw)	Country	Environment	Reference
Duloxetine	bivalves	1	89.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Fluoxetine	bivalves	1	N.D–9.93	Portugal	Coastal, estuary	Silva et al. (2017)
	fish	5	N.D.–9.4	Saudi Arabia	Coastal (WWTP)	Ali et al. (2018)
		1	4.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
		2	<RL–4.9[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
		7	N.D.–0.57	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Haloperidol	fish	1	7.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Lormetazepam	bivalves	1	< 0.4–439.5	Italy	Coastal (WWTP)	Mezzelani et al. (2020)
Maprotiline	bivalves	1	173.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
	fish	1	4.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Mianserin	bivalves	1	12.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Mirtazapine	bivalves	1	121.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Norfluoxetine^{+}	bivalves	1	N.D.–21.66	Portugal	Coastal, estuary	Silva et al. (2017)
	fish	2	<RL–3.2[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Norsertraline^{+}	bivalves	1	N.D.–6.68	Portugal	Coastal, estuary	Silva et al. (2017)
Ormetoprim	fish	5	<RL–1600[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Oxazepam	fish	2	< 0.1–7.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Paroxetine	bivalve	1	< 1.0–49.9	Italy	Coastal (WWTP)	Mezzelani et al. (2020)
	fish	1	5.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Primidone	gastropod	1	0.11–3.59	Malaysia	Estuary (URB)	Omar et al. (2019)
	fish	2	0.45–1.05	Malaysia	Estuary (URB)	Omar et al. (2019)
Promazine	fish	7	N.D.–1.56	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Sertraline	bivalves	1	10.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
		1	0.10–1.40	USA (CA)	Estuary (URB)	Klosterhaus et al. (2013)
	fish	1	0.8[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
		2	0.2–17.0[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Tianeptine	fish	7	N.D–0.53	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Venflaxine	bivalves	3	2.1–2.7	Spain	Estuary (WWTP)	Álvarez-Muñoz et al. (2015a)
		1	2.91 ± 3.47	Spain	Coastal (WWTP)	Martínez-Morcillo et al. (2020)
Verapamil	fish	2	0.07–0.6[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Norverapamil^{+}	fish	2	0.12–0.47[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
Zolpidem	bivalves	1	10–15[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
	fish	1	45.0[w]	Sweden	Coastal	UNESCO and HELCOM (2017)
Veterinary drugs						
Enrofloxacin	bivalves	11	N.D.–147.0	China	Coastal (URB)	Li et al. (2012)
Oxolinic acid	bivalves	1	N.D.–650.0	Norway	Coastal (FAR)	Samuelsen et al. (1992)
	decapods	1	30.0–190 (H) 30.0–810 (M)	Norway	Coastal (FAR)	Samuelsen et al. (1992)
	fish	8	10.0–1560 (P) 10.0–5190 (M) 10.0–6450 (L)	Norway	Coastal (FAR)	Samuelsen et al. (1992)
Sulfadimethoxine	bivalves	11	N.D.–1.7	China	Coastal (URB)	Li et al. (2012)
	fish	2	< RL–17.0[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)
		7	N.D.–0.37	Poland	Coastal	Bobrowska-Korczak et al. (2021)
Sulfapyridine	bivalves	11	N.D.–3.6	China	Coastal (URB)	Li et al. (2012)
Tylosin	bivalves	11	N.D.–1.8	China	Coastal (URB)	Li et al. (2012)
Virginiamycin M1	bivalves	1	N.D.–3.9[w]	USA (OR)	Estuary (WWTP)	Granek et al. (2016)
	fish	2	8.0–34.0[w]	USA (WA)	Estuary (WWTP)	Meador et al. (2016)

an important factor determining the uptake, bioconcentration and biomagnification of pharmaceuticals was their high lipophilicity. Also, drugs with high lipophilicity were characterized by higher BAF values. For all five fish species, the highest BAF value was observed for chlorophenamine, which has one of the highest lipophilicity coefficients (logDow) among the other studied compounds. Similarly, Omar et al. (2019) studied the distribution of contaminants of emerging concern (CECs) including pharmaceuticals (primidone, dexamethasone, sulfamethoxazole, amoxicillin, diclofenac and 17α-ethinylestradiol) in Klang River estuary, Malaysia. For this purpose wild and commercially important fish (*Arius thalassinus* and *Pennahia anea*) were collected from five stations and mollusc (*Nerita lineata*) from 12 stations. In both fish and snails, 4 out of 6 tested pharmaceuticals were detected. The results indicated that both fish species accumulated the same pharmaceuticals, namely primidone (anticonvulsant), sulfamethoxazole (antibiotic), amoxicillin (antibiotic) and diclofenac (NSAIDs). The same pharmaceuticals have also been detected in snails. In addition, diclofenac was characterized by the highest concentrations in fish and snails, which ranged from 1.42 ng/g to 10.76 ng/g in fish and from < 0.35 [Method Quantitation Limit (MQL)] to 7.41 ng/g in snail. Interestingly, dexamethasone and 17α-ethinylestradiol were not detected nor in fish or snails.

On the other hand, Li et al. (2012) studied the distribution of 22 antibiotics (eight quinolones, nine sulfonamides and five macrolides) in eleven species of bivalves (*Crassostera talienwhanensis, Chlamys farreri, Amussium, Scapharca subcrenata, Meretrix merehjgntrix Linnaeus, Mactra veneriformis, Mactra chinesis, Mya arenaria, Neverita didyma, Rapana venosa,* and *Mytilus edulis*) from the Bohai Sea of China. Nine stations, which were localized by the cities along the Bohai Sea, were selected. Bohai Sea was chosen for this study because of rapid economic growth in this area, which goes hand in hand with serious pollution issues. Samples were collected in 2006, 2007 and 2009 to see if there were any annual trends in antibiotic distribution. Of the 22 antibiotics tested, 21 were found in at least one species of mollusc, which is very frightening data. Quinolones were the predominant antibiotics detected in shellfish, while macrolides were rarely detected in bivalves. The distribution of quinolones and macrolides in bivalves' tissues did not show any annual trends, while sulfonamides decreased significantly from 2006 to 2009. Interestingly, different antibiotics were found depending on the station. For example, Dalian city was more impacted by quinolones, while Beidaihe was more polluted with erythromycin and sulfapyridine. These antibiotics are only sold as prescribed, thus the observed spatial differences in their concentration in wild bivalve species may be related to different medical practices in different cities. *Mactra veneriformis* and

Meretrix merehjgntrix Linnaeus species had higher concentrations of quinolones and sulfonylmethoxine, while *Mytilus edulis* had higher concentrations of erythromycin and sulfapyridine.

Xu et al. (2016) checked the presence of the most frequently detected EDCs including one pharmaceutical 17α-ethinylestradiol. The presence of 17α-ethinylestradiol was verified in four Marine Protected Areas in Hong Kong which are close to urbanized areas and are exposed to contaminations from WWTPs. The pharmaceutical was quantified in mussel (*Perna viridis*) and fish (*Cephalopholis boenak, Neopomacentrus bankieri* and *Bathygobius fuscus*). The 17α-ethinylestradiol was detected only in mussels at two stations at levels of 5.4 and 9.7 ng/g dw. Interestingly, the compound was not detected in waters but only in the sediments at all four stations, which may have contributed to the mussels' greater exposure to the compound compared to fish. Ruan et al. (2020) checked the presence of four most commonly used pharmaceuticals namely atenolol (β-blocker), metoprolol (β-blocker), venlafaxine (antidepressant), and chloramphenicol (antibiotic) in 24 species (molluscs, fish and crustaceans). Similar to Xu et al. (2016), the research area were water surrounding Hong Kong. Three sampling areas located in eastern, southern, and western Hong Kong were designated. The most frequently detected pharmaceuticals in biota sample were venlafaxine and chloramphenicol (0.23–0.88 ng/g dw and 0.12–2.64 ng/g dw respectively), followed by metoprolol (22 species, < 0.20 [MQL] to 5.59 ng/g dw) and atenolol (17 species, < [MQL] 0.15–21.1 ng/g dw). The frequency of detection of all pharmaceuticals tested is at a high level, suggesting their widespread occurrence. In addition, the observed pharmaceutical concentrations were generally higher in crustaceans than in fish. Unlike other works, the present study reported significantly higher concentrations of the tested β-blockers (atenolol and metoprolol) in crustaceans than in fish, while higher values of venlafaxine (antidepressant), and chloramphenicol were reported in fish and molluscs.

On the other hand, Xie et al. (2019) checked the contamination with PPCPs in fish (*Culter alburnus*, *Ephippus orbis* and *Epinephelus awoara*) and shellfish (*M. edulis, Mimachlamys nobilis, Bufonaria perelegans* and *O. gigas*) from Pearl River Delta, China. The results showed the main pollutants in aquatic organisms were ketoprofen and ibuprofen (NSAIDs) with detection frequencies of 100% and 97%, respectively. Norfloxacin, trimethoprim, tetracycline (antibiotics) and diltiazem (antihypertensive) were also frequently detected in biota, however their concentrations were much lover compared to NSAIDs. In addition, BCF was calculated for five detected pharmaceuticals in biota. The highest BCF values (3000–5000) were observed for ketoprofen in five species (*M. edulis, O. gigas, M. nobilis*, *C. alburnus* and *E. awoara*), which indicates that this pharmaceutical was the most easily bioaccumulated among the remaining ones. In addition, paracetamol and norfluoxetine were easily bioaccumulated in *E. awoara* (BCF: 2000–5000) and spectinomycin (antibiotic) in *M. edulis*. Moreover, there was no statistically significant difference in BCF values between the studied species.

3.2.2. Europe

Álvarez-Muñoz et al. (2015a) studied the occurrence of selected pharmaceuticals in macroalgae (*Saccharina latissima* and *Laminaria digitata*), bivalves (*Mytilus galloprovincialis, Mytilus* spp., *Chamalea gallina* and *Crassostrea gigas*) and fish (*Liza aurata* and *Platichthys flesus*) which were sampled from Portugal (Tagus estuary), Spain (Ebro delta), Italy (Po delta), Netherlands (Scheldt estuary), and Norway (Fureholmen, Solund). Interestingly, the highest number of pharmaceuticals was detected in mussels, which is most probably related to their filter-feeding behavior and sedentary lifestyle. Most of these chemicals belonged to psychiatric drugs and antibiotics. The highest level was corresponding to venlafaxine (up to 36.1 ng/g dw) and the azithromycin (up to 13.3 ng/g dw) in bivalves from Po delta. The lowest number of pharmaceuticals was found in macroalgae sampled from Fureholmen, Solund and their concentrations were often below the MQL. Interestingly, the number of detected pharmaceuticals differed between bivalve's species sampled from the same region (Ebro delta). The highest pharmaceutical number was detected in *C. gigas* and the lowest in blue mussel *Mytilus* spp. Considering the places from which tested organisms were collected, some differences were observed. For example, the number of pharmaceuticals detected in bivalve molluscs collected from the Ebro and the Po delta differed, as did those from the Tagus estuary and the Ebro delta.

In the other work by the same author (Alvarez-Muñoz et al., 2015b) the presence of 23 pharmaceuticals in bivalves, including metabolites from eight therapeutic class, was analyzed. For this purpose, three species: *C. gigas*, *M. galloprovincialis* and *C. gallina* were collected from two sites localized in Ebro delta. As in the previous study (Álvarez-Muñoz et al., 2015a), the highest number of pharmaceuticals was detected in *C. gigas* with the prevalence of psychiatric drugs followed by antibiotics, while the lowest number of pharmaceuticals was detected in *M. galloprovincialis.* Venlafaxine was detected at the highest concentration of 2.7 ng/g dw in *M. galloprovincialis*, while azithromycin at the highest concentration of 3.0 ng/g dw in oysters. Pojana et al. (2007) focused on the detection of EDCs including pharmaceuticals (mestranol, 17α-ethinylestradiol and diethylstilbestrol) in *M. galloprovincialis* sampled from the Venice lagoon, Italy. Similar to previous studies, a highly urbanized area receiving effluents from wastewater treatment plants (WWTPs) was selected as a model area where four sampling stations were chosen. Among three studied pharmaceuticals 17α-ethinylestradiol and diethylstilbestrol were detected in mussels. Diethylstilbestrol was detected at two sites at concentrations of 9.6 and 13 ng/g dw, while 17α-ethinylestradiol was detected at all four sites in the concentration range < 3[MQL]− 38 ng/g dw. In addition, all pharmaceuticals except mestranol were detected in water samples from the Venice.

On the other hand, Cunha et al. (2017) investigated diclofenac contamination of the Portuguese seaside using blue mussels (*M. galloprovincialis* and *Mytilus edulis*). For this purpose, eight sites located along the entire length of the Portuguese coast (1115 miles) were selected. The annual variation in diclofenac bioconcentration was also verified. Alarmingly, diclofenac was detected in mussels in seven out of the eight sites and potential diclofenac source turned out to be harbors, WWTPs as well as seaport adjacent to research sites. Not surprisingly, diclofenac levels were strongly related to the population density in collected sites. However, no seasonal variation in diclofenac level was observed. The highest mean diclofenac concentration (4.5 ng/g dw) was found in July at the Costa da Caparica station, while the lowest (0.5 ng/g dw) in the same area but at a different station (Peniche). Similarly, Silva et al. (2017) check the presence of pharmaceuticals along the entire coast of Portugal. The same model species *M. galloprovincialis* was involved in this research. Tested pharmaceuticals included selective serotonin reuptake inhibitors (SSRI) with metabolites (fluoxetine, norfluoxetine, citalopram, n-desmethylcitalopram, norsertraline, paroxetine and sertraline) and not NSAID as in Cunha et al. (2017) study. Among seven verified SSRI and their metabolites five were detected in mussel tissues. Only paroxetine and sertraline were not detected. Based on the detection frequency the predominant drug was norfluoxetine with the highest detected mean concentration of 13.52 ng/g dw. followed by fluoxetine (4.83 ng/g dw), *N*-desmethylcitalopram (6.60 ng/g dw), citalopram (3.26 ng/g dw), and norsertraline (6.68 ng/g dw). Additionally, three SSRI metabolites were detected in fish tissues possibly indicating that the metabolites are more persistent than the primary compounds in the organism tissue. (Bueno et al., 2014) tested the presence of venlafaxine (antidepressant) along with its common metabolites in *M. galloprovincialis*. For this purpose, four sites along the coast of south-eastern France potentially contaminated with pharmaceuticals from harbors, highly urbanized regions and urban discharges were selected. Venlafaxine metabolites were most frequently detected compared to the parent compound with N-desmethylvenlafaxine (<2.5[MQL]−3.0 ng/g dw) predominating, followed by N, O-di-desmethylvenlafaxine (<2.5[MQL]−3.5 ng/g dw). These results are consistent with the study of Silva et al. (2017) described above, in

which metabolites of antidepressant drugs were most frequently detected.

On the other hand, Fernandez-Torres et al. (2011) was looking for selected veterinary antibiotics in wild fish (*Trachynotus ovatus*, *Sarpa salpa*, *Oblada melanura and Liza ramada*) which were collected near the fishing town of Aguilas (Spain). From 16 analyzed veterinary antibiotics seven were found in fish viscera or tissue. Sulfadiazine was detected at concentration range 10–190 ng/g in three species, oxytetracycline in one species (50 ng/g), sulfamerazine in two species (40 and 70 ng/g), sulfamethazine in one species (70 ng/g), chlortetracycline in three species (160–590 ng/g) and N4-acetylsulfamerazine in one species (20 ng/g). The highest number of antibiotics were detected in *T. ovatus*, while the lowest in *O. melanura*. Martínez-Morcillo et al. (2020) studied pharmaceutical pollution in north-western Spain. The selected study sites were located along the coastal waters of the Rías Baixas, which is highly urbanized area with more than a dozen sewage treatment plants. Five therapeutic classes (cardiovascular, antipruritic, psychoactive, diuretic, analgesic/anti-inflammatory, antibiotic) were checked in economically important species (*M. galloprovincialis, Ensis siliqua* and *Cerastoderma edule*), cephalopods (*Octopus vulgaris*), arthropods (*Pollicipes pollicipes*, and *Necora púber*), and fish (*Sardina pilchardus*). Of the 16 pharmaceuticals tested, only four were detected in marine organisms, three of which were psychoactive. Alprazolam (psychoactive) and benzodiazepine citalopram (psychoactive, SSRI) were detected in *O. vulgaris* at concentrations of 0.3 and 14.1 ng/g dw, respectively. The benzodiazepine citalopram was found at 9.4 ng/g dw in *E. siliqua*. In turn, anxiolytic venlafaxine (psychoactive) was detected at 2.9 ng/g dw in *C. edule*. Ronidazole which belongs to veterinary antiparasitic drugs was found in *E. siliqua* (2.3 ng/g dw). Martínez-Morcillo et al. (2020) is one of the few studies that examined pharmaceutical content in cephalopods. Interestingly, unlike the previously discussed works conducted in Spain (Álvarez-Muñoz et al., 2015a, 2015b; Fernandez-Torres et al., 2011), none of the antibiotics tested were detected in marine organisms. Furthermore, contrary to the other studies, no pharmaceuticals were detected in fish. In addition, the number of drugs detected here was visibly lower when compared to similar study of Moreno-González et al. (2016) in which 18 out of the 20 tested pharmaceuticals were detected. The above mentioned differences in pharmaceuticals detection may most likely be linked to the fact that species from both works were sampled from different ecosystem. In referred study organisms were sampled from rias connected to open oceanic waters, and subject to tides, while in Moreno-González et al. (2016), organisms were collected from the enclosed coastal lagoon Mar Menor, Spain, in which contaminants may persist longer. Moreno-González et al. (2016) observed differences in pharmaceutical distribution between tested groups of marine organisms. The highest number of pharmaceuticals was detected in fish (18) followed by mollusc, bivalves, and gastropods (8). Furthermore, β-blockers and psychiatric drugs were more readily accumulated in fish than in mussels, while hydrochlorothiazide which belong to diuretic has a higher frequency of detection for wild mollusc (*Cerastoderma glaucum*). In contrast to frequency of detection, the highest noted concentration of hydrochlorothiazide (10.5 ng/g dw) was found in fish. Among 18 pharmaceuticals detected in marine organisms were such therapeutic classes like β-blockers, psychiatric drugs, antiplatelet agents, analgesics/anti-inflammatories, diuretic, antihelmintics and anti-asthmatics. Also, seasonal variation in pharmaceutical frequency in fish and mollusc were observed.

In contrast to above-described works Mezzelani et al. (2020) studied pharmaceutical distribution from relatively "unpolluted" 14 sites localized along the Italian coast (Senigallia, Torrette and Portonovo). Nine commonly used pharmaceuticals belonging to the six therapeutic classes: NSAID (diclofenac, ibuprofen, ketoprofen and nimesulide), the analgesic (acetaminophen), the antiepileptic (carbamazepine), the antihypertensive (valsartan), the anxiolytic (lormetazepam) and the antidepressant (paroxetine) were analyzed in mussel *M. galloprovincialis* tissues. Among all tested pharmaceuticals only ketoprofen and acetaminophen were always below the limit of quantification. In addition, Mezzelani et al. (2020) observed the seasonal variation of pharmaceutical distribution in mussel tissue. For example, the higher diclofenac values in mussels were always measured between late spring and early autumn while in late autumn and winter diclofenac values were almost always below the detection limits. Furthermore, from NSAIDs diclofenac was the most frequently detected in mussel tissue. Another interesting observation was predominance of carbamazepine which was detected in more than 95% of all analyzed mussel samples. In contrast to diclofenac, no seasonal variations were observed for carbamazepine. Previous work by the same author (Mezzelani et al., 2016) also reveal the occurrence of NSAIDs (diclofenac, nimesulide and ibuprofen) in *M. galloprovincialis* tissues, which were sampled during summer from an "unpolluted" touristic area of Central Adriatic Sea (Portonovo Bay). Ketoprofen and acetaminophen, on the other hand, were not detected in mussels.

According to the UNESCO and HELCOM (2017), Baltic Sea is among the one of the most contaminated seas in the world by emerging pollutant including pharmaceuticals. These organic contaminants are frequently detected not only in water, sediment, WWTPs effluents but also in marine biota. 116 different pharmaceuticals were tested in marine organisms and 35 were detected (Zandaryaa and Frank-Kamenetsky, 2021). For example, 13 pharmaceuticals were detected in *Platichthys flesus* and *Anguilla anguilla*. However, the largest number of pharmaceuticals (27) and the highest concentrations were detected in *Mytilus trossulus* making the species the promising model species in pharmaceutical detection in the Baltic Sea region. In similar study conducted by Bobrowska-Korczak et al. (2021) the presence of selected pharmaceuticals in several fish species (*Perca fluviatilis, Platichthys flesus, Scophthalmus maximus, Pleuronectes platessa, Gadus morhua callarias, Abramis brama* and *Carassius carassius*) was tested. Among 98 tested pharmaceuticals were cardiovascular drugs, antidepressants, hypnotics, antibiotics, and sulfonamides. 10 out of 98 pharmaceuticals were detected in fish muscle. Among those four were antibiotics (clarithromycin, erythromycin, metronidazole and ofloxacin), one cardiovascular (bisoprolol), three antidepressants (carbamazepine, fluoxetine and tianeptine), one hypnotic (promazine) and one sulfonamide (sulfadimethoxine). There were also differences in the type and number of pharmaceuticals detected in different fish species. The highest number of pharmaceuticals (8) was detected in *G. morhua callarias* and the lowest *in P. fluviatilis* and *P. platessa* while in *A. brama* and *C. Carassius* pharmaceuticals were not detected. Samuelsen et al. (1992) is one of the first studies to address the presence of pharmaceutical compounds in wild aquatic organisms. Samuelsen et al. (1992) focused on the presence of oxolinic acid (antibiotic) in several wild fish (*Pollachius virens*, *G. morhua*, *Scomber scombrus*, *Labrus bergylta* and *Melanogramrnus aeglefinus*) crab (*Cancer pagurus*) and mussels (*M. edulis*). Oxolinic acid is known to be commonly used in aquaculture, therefore samples were collected in the vicinity of two marine aquaculture facilities on the west coast of Norway. The antibiotic was detected in all tested organisms. The highest values of oxolinic acid were detected in fish followed by mussels and crabs.

3.2.3. North America

Dodder et al. (2014) searched for different CECs including pharmaceutical compounds along the California coast. For this purpose 68 sampling stations were chosen from which *Mytilus* spp. was collected. The selected stations were categorized as: agricultural, low development, mixed development and urban. Interestingly, depending on the category of station different pharmaceuticals were detected and their detection frequency varied. For example, high concentrations of diphenhydramine (an antihistamine) were more frequently detected in mussels sampled from urban locations and mix development stations (9 out of 30 stations) compared to low development and agricultural stations (2 stations). Antibiotics was the most commonly detected class of pharmaceuticals in mussel tissues. In addition, the antihistamine, SSRI

and β-blockers were also detected. Similarly, Klosterhaus et al. (2013) checked the pharmaceutical contamination of San Francisco Bay, USA. However, the model species was *Geukensia demissa* mussels. Sample sites were spatially distributed throughout the major urbanized places of the Bay impacted with a variety of potential contaminant sources like municipal and industrial wastewater, oil refineries and stormwater runoff. Several pharmaceuticals were detected in mussel tissues, but only carbamazepine (anticonvulsant or anti-epileptic), sertraline (SSRI), and dehydronifedipine (Calcium channel blocker) were detected in all five analyzed stations. Among others pharmaceuticals found in mussel tissues were psychotropic (SSRI and others), calcium channel blockers, antihypertensives, antibiotics, and H2-blockers. Interestingly, frequently detected pharmaceuticals in water samples from the San Francisco Bay like atenolol (β-blocker), gemfibrozil, sulfamethoxazole (antibiotic), meprobamate (psychotropic) and valsartan (antihypertensive drug) were not detected in mussel tissues, suggesting their low bioaccumulation potential or efficient metabolism by mussels.

Granek et al. (2016) also studied the contamination with CECs including pharmaceuticals in bivalves. For this purpose, *Ostrea lurida* was collected from two high urbanized Oregon coastal estuaries (the Coos Bay and Netarts Bay). The oysters were sampled in various months to see if there is a seasonal variation in pharmaceutical bioconcentration. Interestingly, the content of pharmaceuticals in oyster tissue differed between summer and spring months at the same station. Diphenhydramine and naproxen were detected only during summer, while azithromycin and sulfamethoxazole only during spring, and no pharmaceuticals were detected in oyster tissue collected during fall. There were also visible differences in the tissue content of pharmaceuticals between two research sites (the Coos Bay and Netarts Bay) in the same seasons. Similar to the above-described studies, antibiotics were predominant in oyster tissues in Granek et al. (2016). Antihistamine and anti-inflammatory drugs were also detected. In work of Meador et al. (2016), the occurrence of wide range of CECs including pharmaceuticals in three estuaries: Sinclair Inlet, Puyallup River, and the Nisqually estuary (Washington, USA) was assessed. Sinclair Inlet and Puyallup River are receiving effluent from WWTPs. Nisqually estuary was selected as reference site "minimally-contaminated" with CECs according to various studies. Pharmaceutical content was checked in both wastewater effluent and estuarine water and in two juvenile fish species *Oncorhynchus tshawytscha* and *Leptocottus armatus*. Interestingly, several pharmaceuticals which were detected in fish tissues and effluents were not detected in estuarine waters what may indicate their significant bioaccumulation potential.

3.2.4. South America

Pemberthy et al. (2020) was the first study testing the presence of two commonly used pharmaceuticals namely ibuprofen and diclofenac (NSAIDs) in water and commercially important fish (*Scomberomorus cavalla*, *Oligoplites saurus*, *Mugil incilis*, *Bagre marinus*, *Genidens barbus* and *Caranx hippos*) in the Gulf of Urabá, Colombia. Despite the fact that both tested pharmaceuticals were detected in water matrix, none of them was detected in fish samples which is surprising and not aligned with the results of other studies. However, fish are mobile organisms and therefore they could be exposed to those drugs for a short time period not allowing to bioaccumulate or the drugs, once entered the organisms, could have been effectively metabolized. Yet another explanation of the lack of tested drugs in the animals tissue is related to detection limit of the method. On the other hand, (Fortt et al., 2007) detected oxytetracycline and quinolones, which belong to antibiotics group, in wild economically important fish species (*Sebastes capensis* and *Elginops maclovinus*). Fish were captured near salmon aquaculture pens in Cochamó, Region X, Chile. Among five captured *S. capensis*, in one detected oxytetracycline and in second one quinolone, while in *E. maclovinus* only quinolones was detected in one individual.

3.2.5. Summary

As shown in the Fig. 3 there are significant differences in the types of pharmaceuticals found in marine organisms from various geographical areas. In Europe, the most frequently detected class of pharmaceuticals are psychoactive drugs and antibiotic, while in the United States antihistamines followed by antibiotics, psychoactive drugs and antihypertensives. In Asia the predominant classes include antibiotics followed by NSAIDs. These differences may result from various medical practises and health-related precautionary measures between geographical areas as well as from different health problems, at least those that are most often diagnosed. For example, in the United States commonly diagnosed and treated health issue is hypertension (Chobufo et al., 2020). Interestingly, in Asia most of cases of hypertension are undiagnosed and as a consequence lower number of antihypertensives drugs is prescribed (Kim et al., 2018). Furthermore, in Asia the significant amount of antibiotics along with NSAIDs are used to treat not only human health issues but also in livestock industries including mass farming and aquaculture. In addition, in many Asian countries the use of antibiotics in animal husbandry is also not regulated (Quaik et al., 2020). However, the number of studies differ significantly between geographical areas influencing the detection frequency of different drugs. Coastal areas of Europe and the west coast of the United States of America seem to be well-studied already, however, there are no single data or data are scarce regarding the presence of pharmaceuticals in marine biota available from areas such as Australia and south America. On the other hand, looking for the concentration levels in marine biota, the most relevant class are antibiotics and NSAIDs. Tissue concentrations of these compounds exceed 500 ng/g which is very concerning. NSAIDs is a class of pharmaceuticals often sold without prescription. They are used to treat the most common health problems, such as pain of various origins and inflammation. For this reason, they are also often overused. Psychoactive drugs and hormones were also found in marine organisms at significant levels (100–500 ng/g). On the other hand, analyzing the data obtained worldwide, it is surprising that there are no reports of anti-cancer compounds detected in wild organisms, despite the fact that cancer is one of the most common diseases and large amounts of chemotherapeutics are used to treat it (Bray et al., 2018). However, this group of compounds was neglected in most of the reviewed works, resulting in a lack of data on their presence in organisms.

As already mentioned, the main transport routes of pollution in marine and brackish environments include, depending on the substance, agricultural, urban and industrial run-offs (mainly via WWTPs outlets), but also rivers and streams flowing into the sea. Moreover, marine environments were historically used as dumping areas with various pollution types released to the seas. Nowadays, there are various data about long-range pharmaceuticals transport via atmospheric deposition and ocean currents, yet the majority of studies are performed in the coastal areas while open waters are almost neglected. Similarly to freshwater ecosystems, pharmaceuticals, as biologically active compounds, already at very low doses exert negative effects on marine life at various levels of biological organization. Some differences were also observed in pharmaceuticals uptake, storage and metabolism depending on the studied group of marine organisms. Not surprisingly, some fish, crustaceans and mollusc species are mostly used in pharmaceuticals monitoring while there are various marine organism' groups being deeply understudied.

4. Conclusions and future perspectives

The papers discussed herein examined the presence of selected pharmaceuticals in wild aquatic organism tissues. Studies on pharmaceuticals range from single compounds and single species, through selected groups of pharmaceuticals in single species, to multi-species studies conducted to determine differences in pharmaceutical uptake by organisms representing different ecological functions, feeding habits, and habitats. The first studies on the presence of pharmaceuticals in wild

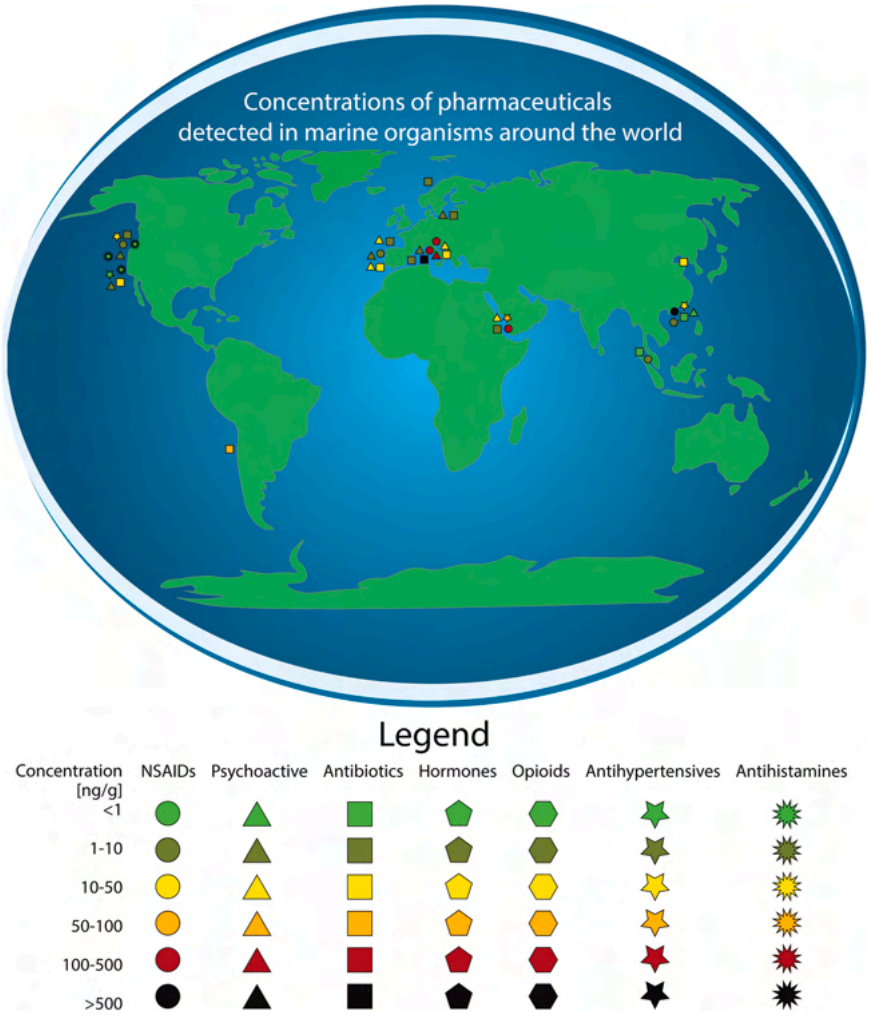

Fig. 3. Range of concentrations of selected groups of pharmaceuticals recorded in marine organisms worldwide.

aquatic organisms were conducted as early as 1990. Since then, the number of works concerning pharmaceutical detection in biota has slowly increased, with a surge in studies in both marine and freshwater environments occurring in 2004–2005. This was most likely due to improved methods for the detection of pharmaceutical compounds, which are present at very low levels in the environment.

Most of described works focused on organisms that live close to local sources of pharmaceutical contamination such as aquaculture, urban estuaries, sewage treatment plant effluents, or agricultural sites. It is of great concern that only a handful of papers have examined the presence of pharmaceuticals in organisms living in potentially "uncontaminated" locations like open marine and oceanic waters, to see how far from the source these contaminants are transported. Interestingly, ecosystem type also influences the presence and number of pharmaceuticals in organisms. In enclosed ecosystems as coastal lagoons the number of pharmaceuticals detected in organisms tended to be higher when compared to open reservoirs. Waves and currents help transporting and mixing pharmaceuticals in the open seas and oceanic waters, while closed lagoons are more susceptible to contamination from pharmaceutical compounds.

The most researched, as well as detected, pharmaceuticals in aquatic organisms (both marine and freshwater) are antibiotics, antidepressants, NSAIDs and antihypertensives. On the other hand, infrequently studied groups of pharmaceuticals in aquatic biota are, for example, anticancer drugs, which is of great concern because the consumption of these chemicals is significant, and their high toxicity to aquatic biota has been revealed.

Fish were the most studied organisms, followed by bivalves, while macroalgae and crustaceans were used in studies occasionally. Therefore, it is needed to determine the presence of pharmaceuticals in other commercially important groups of aquatic organisms like macroalgae, cephalopods and echinodermata. Furthermore, differences were observed in the number of detected drugs as well as in their concentrations in organisms of the same taxonomic group collected from the same station, e.g., mussels and fish, which may be related to differences in lipid concentration of the examined biota (fish or bivalves with a higher lipid level have a higher ability to accumulate hydrophobic compounds) and life stage (the elimination rate of contaminants by young organisms is faster than older organisms) which would also be worth investigating. In addition, studies that incorporate seasonal changes are very valuable since they show annual fluctuations in environmental concentrations of pharmaceutical compounds depending on water regime (especially inland water creeks) and these are quite well reflected in the concentrations observed in biota. However, worrying fact is that only a few works calculated BCF or BAF, factors which describe the readiness of chemicals to bioconcentrate and biomagnify. In some cases, pharmaceuticals were detected only in wildlife and not in water, which supports the use of organisms in monitoring studies.

Pharmaceuticals and their transformation products (metabolites and degradation products) are found in all available fractions of aquatic environments, from abiotic to biotic components. Like many other known xenobiotic pollutants, pharmaceutical compounds are present in

these components (matrices) in differing concentrations and enter food webs through various feeding strategies of resident biota leading to potential bioaccumulation. The knowledge on trophic transfer of pharmaceutical residues is still insufficient, however available studies emphasize that filter-feeding benthic biota seems to concentrate higher amounts of pharmaceuticals due to biomagnification processes and relatively higher ambient exposure risk, including ingestion of organic matter from polluted sediments that tend to accumulate pharmaceutical compounds (Oetken et al., 2005; Lagesson et al., 2016; Xie et al., 2017; Wilkinson et al., 2018). Congruently, according to Lagesson et al. (2016) benthic primary consumers, i.e. detritivores and herbivores at lower trophic levels seem to be also the primary bioaccumulators of pharmaceutical contamination in aquatic ecosystems. A study focusing just on fish representing different trophic niches proved that the uptake of pharmaceuticals may also be selective with regards to the drug therapeutic class. For example, freshwater omnivorous fish of the U.S. rivers and streams accumulate greater variety of pharmaceutical compounds of different therapeutic categories than the co-habitant carnivores and invertivores (Huerta et al., 2018). Having studied human pharmaceuticals prevalence in three fish species from the Uruguay River (South America) with different feeding habits Rojo et al. (2019) came to similar conclusions. On the other hand, some studies may argue that waterborne exposure could be more important for uptake and bioaccumulation rates than the dietary one (e.g. snails, Du et al., 2015a).

Comparing results obtained from different locations, it must be remembered that environmental background, namely pH, oxygen concentration, or salinity also determines the bioaccumulation of pharmaceuticals. Depending on pH, pharmaceuticals exist in a more or less ionized form which affects their affinity to lipids including lipid membranes and thus their ability to bioaccumulate. Furthermore, differences in used detection methods may lead to problems with comparability of the results (different detection limits and units (some expressed in dry and other in wet weight).

In some works, both in fresh and marine, the frequency of detection as well as concentration of metabolites was higher than that of primary compounds, which may indicate their higher persistence in the aquatic biota. A prime example are antidepressants, for which environmental BAFs were up to tens of times higher than for the parent compound. The study by Arnnok et al. (2017) showed high bioaccumulation of norsertraline (a metabolite of sertraline), for which the BCF was up to 3000, and accumulated mainly in the liver and brain. Interestingly, bioaccumulation of more lipophilic parent compound - (BCF $<$ 100 in all trials) was not observed at the same time. This may be due to the fact that sertraline is metabolized by fish and only as norsertraline accumulated in tissues, but this finding requires further studies. It is worth noting that the same mechanism was not observed for norfluoxetine, whose BCF reached a maximum of 130, and in many cases this compound was not detected in tissues at all. Similar results were obtained in the work of Du et al. (2012), where almost five times higher concentrations of norsertraline than sertraline were detected in fish livers, while concentrations of fluoxetine and norfluoxetine were very close to each other. The increased bioaccumulation potential for norsertraline is also confirmed by the work of Du et al. (2015b) in which it was shown that the BAF of this compound in the tissues of the freshwater *Planorbid* sp. was as high as 16000, while for sertraline it did not exceed 1000. On the other hand, a different trend was observed for fluoxetine, where the parent compound reached a BAF of 3000 and the metabolite, norfluoxetine, only 510. However, different results were obtained in the work of Brooks et al. (2005), where also for norfluoxetine, higher concentrations of this compound than parent compound were observed in fish tissues. Although bioaccumulation factors were not calculated in this study, concentrations of norfluoxetine and norsertraline in fish tissues exceeded 10 ng/g, and these compounds were predominantly deposited in the liver and brain. Although the reasons for this phenomenon have not yet been fully elucidated, it is possible that some drugs may be first metabolized by aquatic organisms and then accumulated in tissues only in the form of metabolites. However, the knowledge on bioaccumulation of drug metabolites is very scarce. This is partly due to the fact that all works discussed in this review are target analyses, in which tissues of organisms are evaluated for specific compounds, almost exclusively native pharmaceuticals. An enormous gap in knowledge, therefore, is related to the non-target analysis, which would allow a broader view of the bioaccumulation problem, looking not only for native pharmaceuticals, but also for their derivatives that may be formed in the wild organism and only accumulated in that form. Realistically speaking, however, one should be aware that current analytical methods may not be sufficient to conduct effective non-target analyses in such rich and complex matrices as aquatic organism tissues. Nevertheless, a good start would be to make more frequent use of drug derivatives that are already known and available as analytical standards.

On the other hand, guided by geography, it is important to note that to date only single works have been conducted in Africa and Australia. Despite this sparse number of reports, particularly alarming data have come from Australia, where concentrations of pharmaceuticals measured in organisms have reached levels rarely seen elsewhere. A more accurate scale of the problem within these continents, however, certainly requires more extensive data from more places.

When comparing freshwater and saltwater organisms from a global perspective, there are significant differences in the detection frequency of different pharmaceutical classes. For example, in freshwater organisms from the United States, psychoactive drugs were most frequently detected followed by antihistamines, while antihistamines predominated in saltwater organisms. However, it should be noted that a much higher number of studies were conducted on freshwater organisms compared to saltwater organisms. For Asia and Europe, the same groups of pharmaceuticals predominated in saltwater and freshwater organisms, namely antibiotics and psychotropic drugs. Interestingly, pharmaceutical concentrations in freshwater organisms were more often $>$ 500 ng/g compared to saltwater organisms. Spatial (both, local and global) and temporal differences in pharmaceutical presence were observed in both environment types. Antibiotics were the most frequently detected in Asia, NSAIDs and psychoactive drugs in North America and in Australia opioids, psychoactive, antihypertensives and antihistamines. Yet, the latter continent is by far the least studied when it comes to pharmaceutical presence in the wild aquatic species. Antibiotics have been detected in freshwater organisms from all continents (except Antarctic - no studies in this direction) as well as in saltwater organisms. In contrast to saltwater organisms in which level of hormones exceed 500 ng/g, the levels of hormones detected in freshwater organisms was low (did not exceed 100 ng/g).

Declaration of Competing Interest

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

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A multi-biomarker approach to assess toxicity of diclofenac and 4-OH diclofenac in *Mytilus trossulus* mussels - First evidence of diclofenac metabolite impact on molluscs☆

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ABSTRACT

Although the presence of pharmaceuticals in the environment is an issue widely addressed in research over the past two decades, still little is known about their transformation products. However, there are indications that some of these chemicals may be equally or even more harmful than parent compounds. Diclofenac (DCF) is among the most commonly detected pharmaceuticals in the aquatic environment, but the potential effects of its metabolites on organisms are poorly understood. Therefore, the present study aimed to evaluate and compare the toxicity of DCF and its metabolite, 4-hydroxy diclofenac (4-OH DCF), in mussels using a multi-biomarker approach. *Mytilus trossulus* mussels were exposed to DCF and 4-OH DCF at 68.22 and 20.85 μg/L (measured concentrations at day 0), respectively, for 7 days. In our work, we showed that both tested compounds have no effect on most of the enzymatic biomarkers tested. However, it has been shown that their action can affect the protein content in gills and also be reflected through histological markers.

Environmental implication: Studies in recent years clearly prove that pharmaceuticals can negatively affect aquatic organisms. In addition to parent compounds, metabolites of pharmaceuticals can also be a significant environmental problem. In the present work, the effects of diclofenac and its main metabolite, 4-hydroxy diclofenac, on marine mussels were evaluated. Both compounds showed negative effects on mussels, which was primarily observed through histological changes. The present study therefore confirms that not only diclofenac, but also its main metabolite can have negative effects on aquatic organisms.

1. Introduction

Recently, the problem of pharmaceutical pollution has begun to be considered in a broader context, with a particular focus on non-steroidal anti-inflammatory drugs (NSAIDs). Diclofenac (DCF), one of the most commonly consumed drugs from this group, is released into aquatic environments around the world day after day, making it one of the most frequently detected pharmaceuticals (Acuña et al., 2015; Nikolaou et al., 2007; Coelho et al., 2009). DCF concentration in places like sewage outlets may reach up to several μg/L (Ali et al., 2018; Madikizela et al., 2017; González-Alonso et al., 2017). Alarmingly, this compound is being found even in marine organisms like fish and mussels in concentrations ranging from several ng/g dw to even a few μg/g dw (Álvarez-Muñoz et al., 2015; Moreno-González et al., 2016; Mezzelani et al., 2018a, 2020; McEneff et al., 2014; Capolupo et al., 2017; Cunha et al., 2017).

Currently, it is becoming clear that when assessing the potential environmental impact of pharmaceuticals, not only the effect of parent compound should be considered, but also its derivatives (Maculewicz et al., 2022). Pharmaceuticals including DCF enter the aquatic environment only partially in an unchanged form. They undergo transformation processes, both those being the effect of metabolic activity of organisms and abiotic ones, resulting from various environmental factors (e.g. photolysis, hydrolysis) (Maculewicz et al., 2022). As a result, new chemical compounds are formed that may have radically different properties from those of the parent drug. Also, some pharmaceutical derivatives can reach even higher concentration in water compared to

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parent compounds (Kołecka et al., 2019, 2020).

To date, however, knowledge of the ecotoxicology of pharmaceutical transformation products is very limited, and such studies are only beginning to emerge. Nevertheless, there are indications that these compounds may in some cases be more harmful than the parent molecules. There are only a few papers confirming the toxicity of DCF metabolites in algae, crustaceans and bacteria but most of them focused mainly on acute toxicity (Maculewicz et al., 2022; Schulze et al., 2010; Schmitt-Jansen et al., 2007). As shown by Schulze et al. (2010), the phototransformation products of DCF can exhibit up to 10 times greater toxicity to algae. Comparable results were obtained by Schmitt-Jansen et al. (2007) who reported toxic effect of DCF phototransformation products mixture towards algae *Scenedesmus vacuolatus*, which was manifested by growth inhibition. Several works have also demonstrated a similar phenomenon for other NSAIDs (ibuprofen, naproxen and salicylic acid) phototransformation products that showed greater toxicity to algae and bacteria compared to parent compound (Cazzaniga et al., 2020; Isidori et al., 2005; DellaGreca et al., 2003). In addition, Grabarczyk et al. (2020) observed 38% inhibition of *Vibrio fischeri* luminescence and 45% inhibition of algae (*Raphidocelis subcapitata*) growth upon exposure to 4-hydroxydiclofenac (4-OH DCF). Furthermore, studies showing the effects of DCF metabolites on invertebrates are limited. Only a few papers have examined the effects of metabolites on invertebrates such as *Daphnia magna* (Grabarczyk et al., 2020). Increased toxicity and accumulation of DCF metabolite, diclofenac methyl ester, was observed in two keystone aquatic crustacean species: *Gammarus pulex* and *Hyalella Azteca* (Fu et al., 2021).

Among DCF metabolites 4-hydroxydiclofenac (4-OH DCF) is most often detected in aquatic environment (Stülten et al., 2008; Scheurell et al., 2009; Osorio et al., 2014; Schmidt et al., 2018). 4-OH DCF is one of the main DCF metabolites excreted by humans but is formed through environmental processes including biodegradation (Li et al., 2014). Alarmingly, in our previous study (Świacka et al., 2022) the high stability of 4-OH DCF was found under laboratory conditions mimicking the seafloor zone. Moreover, it has been revealed that mussels from Mytilidae family absorb 4-OH DCF in the tissues (Świacka et al., 2021). As sedentary and water-filtering organisms, mussels are the most susceptible to the action of pharmaceuticals and its metabolites. To date, however, it is still unknown what effect 4-OH DCF may have on the general health of molluscs.

Therefore, the aim of the present study was to examine and compare the effect of DCF and its metabolite, 4-OH DCF on *Mytilus trossulus* mussels. Although the effects of DCF have been studied and determined in many aquatic species, in the case of its main metabolite, this knowledge is very limited. Thus, the present study was designed to fill this important gap by thoroughly characterizing the effects of 4-OH DCF on marine bivalves. For this purpose, mussels sampled from the Baltic Sea were exposed in laboratory conditions to DCF and 4-OH DCF at 68.22 and 20.85 μg/L (measured concentrations at day 0), respectively, for 7 days. Detailed results of chemical analyses, including daily measured concentrations of analytes in water and mussel tissues, mass balance and bioconcentration factors are presented in our previous work (Świacka et al., 2021). In this work we present the results of a multi-biomarker approach including enzymatic and histological evaluation, as well as BMI and cytosolic protein content analysis.

2. Materials and methods

2.1. Sampling

Mussels were sampled aboard the *R/V Oceanograf* using a benthic dredge at Orłowo station (Gulf of Gdańsk, Baltic Sea) on July 7th' 2020. Sampling depth was 17 m. The water temperature and salinity measured were 14.3 °C and 7.1 PSU, respectively. After sampling, about 1300 *M. trossulus* individuals were transported to the laboratory, where they were acclimated to laboratory conditions.

2.2. Chemical solution preparation

Chemical preparation was described in detail in our previous work (Świacka et al., 2021). In short, diclofenac sodium salt (CAS 15307-79-6, analytical standard, purity>98%) and 4-hydroxydiclofenac (CAS 64118-84-9, analytical standard, purity>98%) used in exposure experiment were purchased from Sigma-Aldrich. Ice-cold Tris sulfate buffer (pH 7.8, 500 mL) containing 50 mM TRIS-H_2SO_4; 0.1 mM EDTA; 1 mM PMFS; 2 mM DTT and 0.1% Triton-X was prepared according to method described in Sokołowski et al. (2021).

2.3. Acclimation and exposure experiment

Acclimation and experimental set-up were described in detail in our previous work (Świacka et al., 2021). Briefly, mussels were acclimated for two weeks to laboratory conditions (artificial brackish water with salinity 7 PSU and temperature 10 °C). During the acclimation and exposure experiment mussels were kept in the dark and fed with *Chlorella vulgaris* (5 mL of culture containing 4.24×10^7 cell/mL). *M. trossulus* were exposed to DCF and 4-OH DCF at concentrations 68.22 and 20.85 μg/L (measured at day 0), respectively for 7 days. The concentrations of both compounds were analysed daily, and the results of the chemical analyses, as well as validation of methods, are presented in detail in our previous work (Świacka et al., 2021). The compound concentrations of both DCF and 4-OH DCF that we used in the experiment are higher than their concentrations recorded in the environment because this research is a continuation of already published work (Świacka et al., 2021) in which we compared the bioconcentration of DCF and its metabolite in mussel tissues and biofilm, determined their fate in the marine environment based on mass balance and also determined their subsequent metabolism. We used higher concentrations than in the environment to be able to detect both compounds in mussel tissues and biofilm, as well as potential metabolites.

On the last 7th day of the experiment, 10 mussels from each tank were taken for measurement of biomarkers responses, 10 mussels (divided into 5 females and 5 males) for BMI and chemical evaluation (see Świacka et al., 2021) and another 10 for histological evaluation.

2.4. Biochemical evaluation

For the purpose of prophenoloxidase (ProPO) activity evaluation, the haemolymph from 10 mussels was collected. Haemolymph was extracted from the adductor muscle using 50 μL chromatographic syringe. The collected haemolymph from 10 mussels was pooled in 2.5 mL eppendorf's held on ice, frozen in liquid nitrogen, and then stored at −80 °C until enzymatic analyses. In addition to haemolymph, gills were immediately collected from five individuals to assess glutathione reductase (GR) and glutathione transferase (GST) activities and from another five individuals to assess catalase (CAT) and acetylcholinesterase (AChE) activities. Next, isolated tissues were frozen in liquid nitrogen and then stored at −80 °C prior to bioassays. For GR and GST analysis dedicated assay kits (GST Colorimetric Activity Assay Kit 26 K263 100 BioVision; GR Activity Colorimetric Assay Kit, 26-K761-20 BioVision) were used.

2.4.1. Prophenoloxidase (proPO) activity

Haemolymph was centrifuged at 400×*g* for 5 min at 4 °C, and the obtained supernatant was transferred and used as plasma. 100 μL of plasma from each sample with 50 μL of 5 mg/L Sodium Dodecyl Sulfate (SDS dissolved in tris buffer pH 6.5) was incubated for 15 min at 25 °C. Next, 100 μl of substrate was taken and then 1 mL of 10 mM L-DOPA (Sigma-Aldrich) solution (3 mg/mL in 0.5 M HCl containing 10 mM $CaCl_2$) was added to each well. Samples were immediately analysed spectrophotometrically at $\lambda = 480$ for 15 min with 1-min intervals on Multiskan SkyHigh Microplate Spectrophotometer (Thermofisher). Readings were blanked against L-dopa solution (Muñoz et al., 2006).

The activity of ProPO was calculated per amount of protein determined in the haemolymph (unit/mg protein).

2.4.2. Glutathione reductase (GR) and glutathione S-transferase (GST) activities

Enzymatic activity assays were performed according to manufacturers' instructions with slight modifications. Briefly, about 0.1 g of gill tissue was homogenized in four volumes of assay buffer using glass hand-held homogenizer (Bionovo). Next, obtained homogenate was centrifuged at 10,000×*g* for 15 min at 4 °C, and obtained supernatant was transferred to 1.5 mL tubes. The supernatant volume within range of 10–100 μL was used in GR and GST assays and transferred to well in tree replicates. After addition of the appropriate kits' reagents to each sample's well absorbance was read on Multiskan SkyHigh Microplate Spectrophotometer (Thermofisher). To evaluate GST activity, absorbance was monitored for 10 min with 1-min intervals at 340 nm. For GR evaluation OD was read every minute at 405 nm for 30 min. The activity of both enzymes was calculated per amount of protein that was determined in the gills (GST: mmol/min/mg protein; GR: mU/mg protein).

2.4.3. Acetylcholinesterase (AChE) and catalase (CAT) activities

AChE was measured according to Ellman et al. (1961) method. Briefly, about 0.1 g of gill tissue was homogenized in eight volumes of ice-cold Tris sulfate buffer (pH 7.8) using glass hand-held homogenizer (Bionovo). Next, homogenate was centrifuged at 4 °C, 13,000×*g* for 10 min and obtained supernatant was transferred to clean tubes. Such obtained supernatant was used for AChE and CAT activity measurement. For AChE activity, 50 μL of supernatant was dissolved in 100 μL of ice-cold Tris sulfate buffer and 30 μL of it was transferred to microplate well. Subsequently, 70 μL of 0.6 mM Ellman's reagent (DTNB) and 1.2 mM acetylthiocholine iodine mixture was added to each well and the absorbance was read in Multiskan SkyHigh Microplate Spectrophotometer (Thermofisher) every minute for 20 min at 415 nm.

CAT was determined according to Cohen et al. (1996). In brief, the reaction between H_2O_2 and standard excess of 2 mM $KMnO_4$ was carried out and then decomposition of H_2O_2 was measured spectrophotometrically after 3 min at 480 nm. The reaction was maintained in an ice-water bath. The activity of both enzymes was calculated per amount of cytosolic protein that was determined in the gills (AChE and CAT: U/mg protein).

2.4.4. Protein concentration

Protein concentration in gills and haemolymph was measured using phenol method (Lowry et al., 1951) modified for microplate reader. The protein concentration measured in the haemolymph was only used to calculate proPO activity, while the amount of protein in the gills was used for the other biomarkers. A standard curve was prepared from bovine serum albumin (BSA, Sigma-Aldrich) as follows. BSA powder was diluted in distilled water to obtain a concentration 2 mg/mL. Next, the following dilutions: 0, 1, 2, 5, 10, 20 and 40 μg/tube were prepared with a final volume of 400 μL in eppendorf tubes (2.5 mL). 5–20 μL of supernatant from each test samples was transferred to eppendorf tubes in tree replicates and diluted with distilled water to a final volume of 400 μL. Biuret reagent at volume 500 μL was added to each eppendorf tube and was incubated at room temperature for 15 min. Biuret reagent was prepared by mixing 250 mL 5% SDS, 125 mL of mixture (10% Na_2CO_3 + 0.8N NaOH) and 125 mL (0.1% sodium potassium tartrate and 0.2% $CuSO_4$). Next, Folin & Ciocalteu's reagent (v:v; 1:2; folin:distilled water; Chempur) at volume 250 μL was added to each eppendorf tube and incubated for 30 min until a colour reaction occurred. Finally, 100 μL of each sample was transferred to well in tree replicates. Absorbance was measured at 650 nm (Multiskan SkyHigh Microplate Spectrophotometer; Thermofisher).

2.5. Histology

Whole mussel tissues were removed from the shells, immersed in Davidson fixative solution (volume: 33% distilled water, 33% ethanol [96%], 22% formaldehyde, and 11% acetic acid) and kept for two days. Subsequently, tissues were placed in formaldehyde solution (10%) buffered with sodium phosphate (6.5 g/L dibasic Na_2HPO_4 and 4 g/L monobasic NaH_2PO_4; pH 7,2-7,4) and stored for one month. During this time, the formaldehyde solution was changed twice. In the next step tissues were placed in tissue cassettes, dehydrated with increasing concentrations of ethanol and xylene and subsequently immersed in paraffin. Paraffin-embedded tissues were cut into 3 μm sections, which were then placed on a glass slide. Glass slides with tissues were kept for 2 days at 60 °C. Finally, samples were stained according to the Harris' hematoxylin and eosin (H&E) standard protocol. Histological slides were also used for sexing of tested blue mussel populations. After that, sex ratio (SR) was calculated for each experimental treatment based on formula 1:

$$SR = \frac{females}{males}$$

Also, gonadal index (GI) was calculated as follows (after Wenne, 1985):

$$GI = \frac{1n + 2n + 3n + 4n + 5n}{N}$$

where n resembles the number of individuals in each stage of gonad development (1 gametogenesis start, 2 and 3 developing gonads, 4 developed gonads and spawning and 5 resting phase), and N resembles the number of all individuals per treatment.

2.6. Body mass index, BMI

BMI (body mass index) was used to determine the condition of mussels during the experiment. For BMI evaluation 5 males and 5 females were selected from each tank (replicate) shortly after collection. Mussels which were used to assess BMI, were then used in pharmaceutical content evaluation published in Świacka et al. (2021). Additionally to sexing performed based on histology, also sex of mussels taken for evaluation of DCF and 4-OH DCF content was performed. For this purpose, a sub-sample of gonadal tissue was collected from the mantle area (1 mm × 1 mm), smeared on the microscopic slide and covered with a cover slide. The presence of ovarian (females) and testis (males) tissues determined mussel' sex. Then, the wet weight of each individual was taken and the shell length was measured. Next, BMI was calculated according to Honkoop et al. (1999): W-weight, L-shell length

$$BMI = \frac{W}{L3}[mg / cm3]$$

2.7. Statistical analysis

Statistical tests were performed in Statistica 12. The distribution and homogeneity of variance were checked before statistical comparison. Differences between replicates were checked first and then between experimental tanks. Kruskal-Wallis, Mann Whitney U, and one-way ANOVA were used for statistical comparisons. The significance level was set at $p < 0.05$. Pairwise Fisher's comparison was used to evaluate the effect of compounds on the observed histological changes.

3. Results and discussion

3.1. Biomarker responses

During the experiment, no significant mortality was observed in the tested blue mussels. In fact, only one dead individual was found.

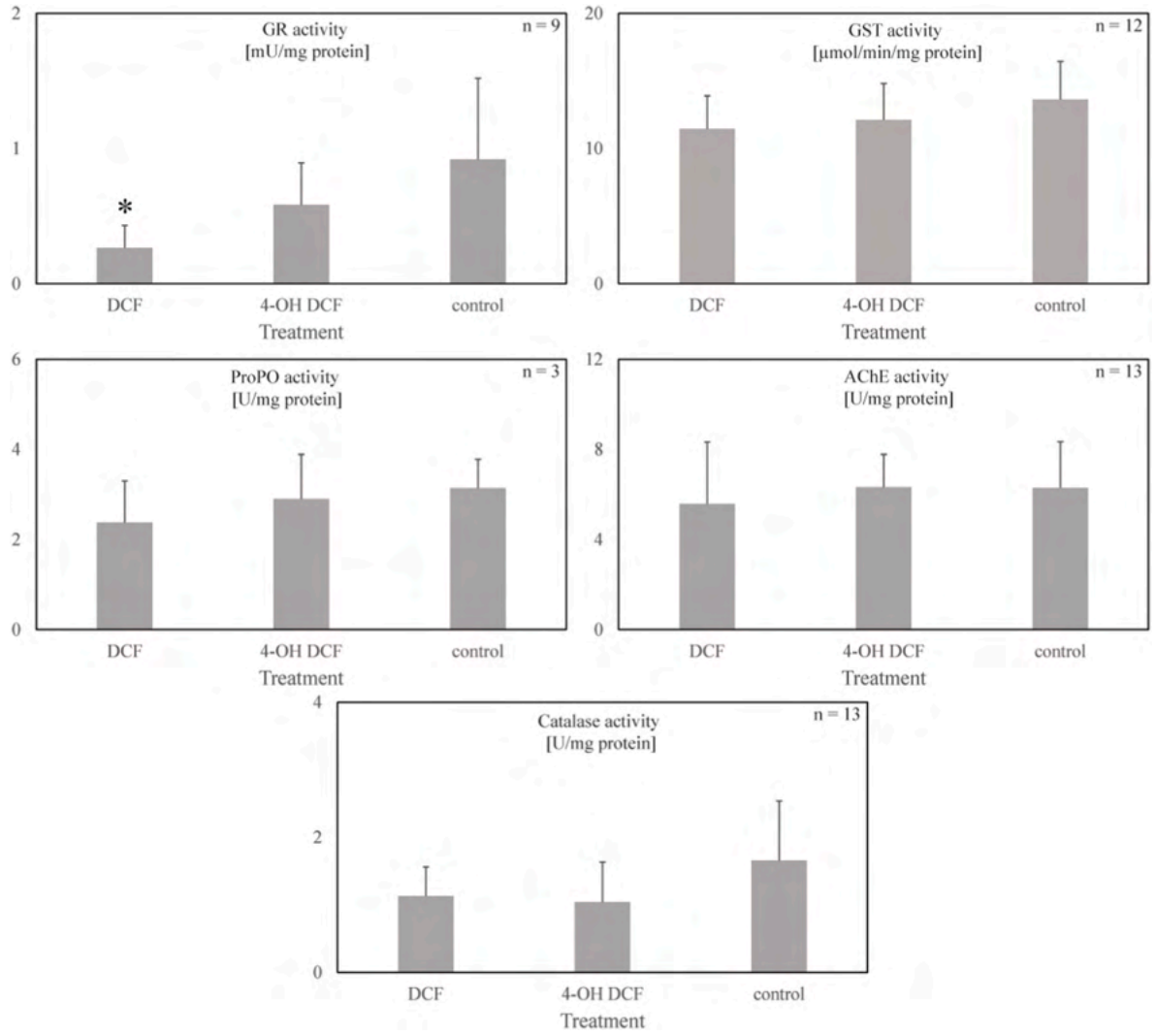

Fig. 1. Measured enzymatic activities in *M. trossulus* mussels exposed to DCF and 4-OH DCF for seven days. Statistical comparison (Mann-Whitney U, $p < 0.05$; Statistica 13.3). *Statistically significant differences $p < 0.05$.

There were no statistically significant differences in the activity of all biomarkers tested between the replicates (Mann Whitney U; One-way ANOVA, $p > 0.05$). Therefore, the mean values with standard deviation of these biomarkers for each tank were presented (Fig. 1).

Among enzymatic biomarkers, statistically significant differences between bivalves from the control and treatment tanks were observed only for GR activity. GR activity in mussels exposed to DCF and 4-OH DCF was lower compared to mussels from the control tanks with statistical significance for DCF (Mann Whitney U, $p = 0.013$). The function of GR in organism is to catalyse the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), using NADPH as a coenzyme (Ramos-Martinez et al., 1983; Manduzio et al., 2004). Gonzalez-Rey and Bebianno (2014) observed significant induction of GR activity in *Mytilus galloprovincialis* treated with DCF at 250 ng/L. In a study by Jaafar (2015), GR activity was also significantly increased in the gills of *Mytilus edulis* treated with DCF at a concentration of 1 mg/L, while in digestive glands of mussels GR activity was significantly decreased. On the other hand, Mezzelani et al. (Mezzelani et al., 2016a,b, 2018b) did not observe any significant GR modulation in mussels exposed to DCF at 25 and 2.5 µg/L, respectively. Furthermore, Cossu et al. (1997) observed significant reduction in GR activity in freshwater Bivalves, *Unio Tumidus* transplanted for 15 days to site exposed to domestic and industrial pollution.

There were no statistically significant alterations in activity of remaining oxidative biomarkers (GST, CAT and Pro-PO) in treatment groups compared to control (One-way ANOVA, $p < 0.05$) (Fig. 1). Given that both DCF and 4-OH DCF have been found in mussel tissues (Świacka et al., 2021), the lack of visible effect on the activity of aforementioned biomarkers in treatment groups may indicate a negligible role of prooxidant mechanisms in the mode of action of tested chemicals. This observation is consistent with the results obtained by Mezzelani et al. (2018b). In this study DCF at environmentally relevant concentrations (2.5 µg/L) during 60 days of exposure did not cause alterations in the activity of oxidative stress biomarkers including CAT, GST and other glutathione-dependent enzymes tested in *M. galloprovincialis*. Even though we used 30 times higher DCF concentration compared to Mezzelani et al. (2018b) we also did not observe effects of DCF on detoxification and oxidative stress markers in Mytilidae mussels. Furthermore, the lack of visible effect on oxidative biomarkers was reported in *M. galloprovincialis* exposed to 25 µg/L and 0.5 µg/L of DCF for 14 days (Mezzelani et al., 2016a, b). In addition, Mezzelani et al. (2016a, 2018b) studied the total oxygen radical scavenging capacity and obtained almost constant values against peroxyl and hydroxyl radicals in the treatment groups, suggesting the absence of pro-oxidant activity of the tested anti-inflammatory drugs including DCF in *M. galloprovincialis*. Similarly, Jaafar (2015) did not observe GST and CAT activity modulation in *M. edulis* gills exposed to DCF at 0.2 and 1 mg/L for 7 days.

On the other hand, Freitas et al. (2019) showed pro-oxidant activity of DCF already at 1 µg/mL after 28 days of exposure in

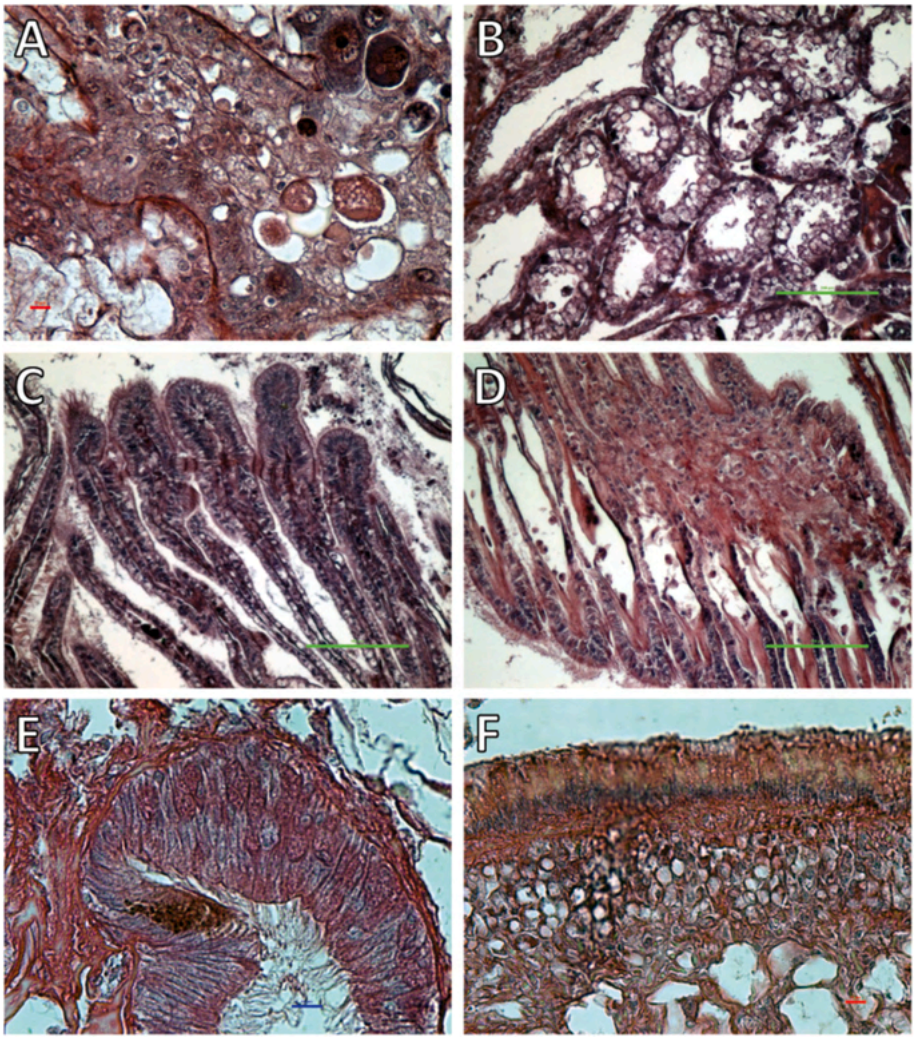

Fig. 2. Cross section of tested blue mussels indicating female gonad atresia (a), atrophy and necrosis of digestive tubules (damages of the digestive tubule walls marker with an arrowhead) (b), hypertrophy and edema of lamellar epithelium (c), gill deformities manifested by lamellar fusion and edema of gill' filaments (d), single brown spot in the stomach mantle (e), brownish appearance of mantle epithelium (f). Hematoxylin and eosin staining. The dimensions of the scale bars are as follows: 100 μm (B, C, D); 10 μm (A, E, F). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

M. galloprovincialis, which was reflected as superoxide dismutase (SOD), GST, CAT and glutathione peroxidase (GPx) activities modulation in treated groups. Schmidt et al. (2011) also observed GST activity induction in *Mytilus* spp. Exposed by injection to DCF at 1 and 1000 μg/L after 96 h. In another acute toxicity study by Parolini et al. (2010), the effect of DCF on GST activity was observed in *Dreissena polymorpha* treated with 1 and 2 nM for 24 and 72 h respectively. However, DCF had no effect on CAT activity in any time or dose scenario.

Some studies (Gonzalez-Rey and Bebianno, 2014; Fontes et al., 2018) showed that the duration of exposure plays a crucial role in assessing toxicity via biomarker responses. In Fontes et al. (2018) DCF affected GST activity in *Perna perna* mussels at each concentration (20; 200 and 2000 ng/L) after 28 h, whereas after 96 h, DCF induced GST activity only at the highest concentration of 2000 ng/L. Similarly, Gonzalez-Rey and Bebianno (2014) observed changes in the activity of some biomarkers (GR, AChE, SOD and CAT) in DCF treatment group over the duration of exposure, and these results were significantly different compared to control, depending on the day of exposure. In addition, DCF toxicity for selected biomarkers was dependent on the analysed tissues. For example, changes in CAT activity were observed only in the digestive gland and not in gills. Interestingly, some studies reported that physicochemical conditions in the aquatic environment have a greater effect on biomarker activity than pharmaceuticals. Munari et al. (2018) showed that reduced pH affected oxidative enzymes such as CAT, cyclooxygenase and SOD significantly more than DCF or the pH*DCF interaction, although the activity of the biomarkers differed between the species (*M. galloprovincialis* and the clam *Ruditapes philippinarum*) and tissues (gills and digestive gland) analysed.

In summary, enzymatic biomarker activity can be related to many factors, for example, DCF concentration, exposure time, model species or tissue analysed. Moreover, enzymatic biomarker response is more frequently observed in DCF acute toxicity studies compared to chronic toxicity studies. Therefore, the chronic effects of DCF and 4-OH DCF on mussels may be difficult to observe using only this tool.

We also investigated the effect of DCF and its metabolite on AChE which is an enzyme involved in neurotransmission (Gonzalez-Rey and Bebianno, 2014). AChE, similarly, to oxidative enzymes' activity, is a common biomarker used for toxic effect assessment (Gonzalez-Rey and Bebianno, 2014; Mezzelani et al., 2018b; Solé et al., 2010). However, we did not observe any effect of DCF and 4-OH DCF on AChE activity. This result is congruent with the findings of Mezzelani et al. (2016a,b, 2018a, b) who did not observe the influence of DCF on neurotransmission (AChE) in mussels either. In contrast, Gonzalez-Rey and Bebianno (2014) showed a significant increase in AChE activity in *M. galloprovincialis* exposed to DCF at 250 ng/L on days 3 and 7 of exposure. However, the increase in AChE activity in DCF treatment group was related to ALP levels in the female gonads rather than to neurotransmission dysfunction (Gonzalez-Rey and Bebianno, 2014). Similarly, Fontes et al. (2018) observed induction of AChE activity in *Perna perna* gills exposed to DCF at 2000 ng/L after 48 h of exposure. Apart from the aforementioned studies there are no other reports describing AChE activity modulation due to DCF. Thus, AChE function

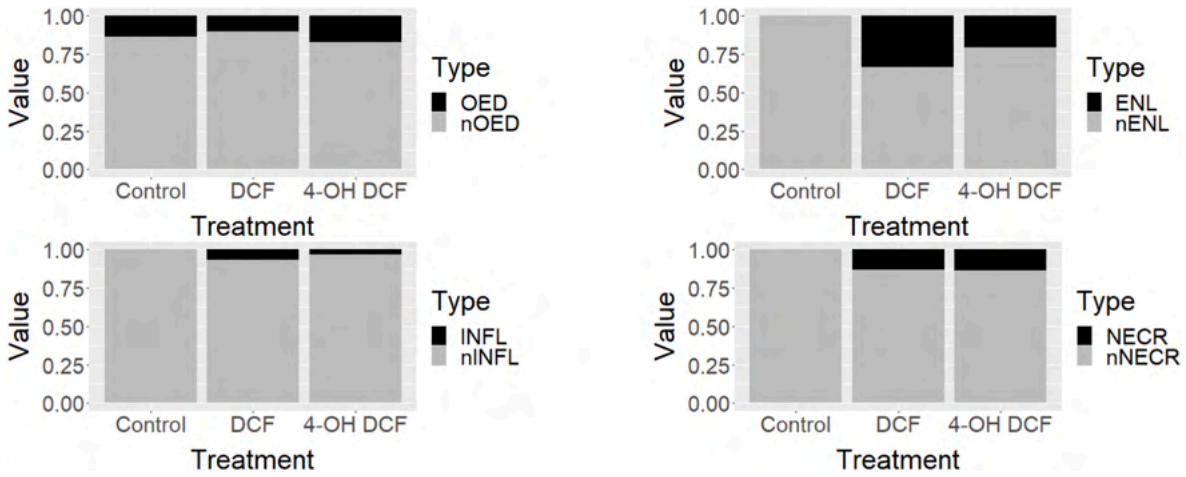

Fig. 3. Proportions of pathological changes in the mussels' gills; OED - individuals with clear edema, nOED - individuals with no edema; ENL - individuals with enlarged gills, nENL - individuals with no gill enlargement; INFL - individuals with inflammations, nINFL - individuals with no signs of inflammation; NECR - individuals with necrosis, nNECR - individuals with no signs of necrosis.

in invertebrates still needs to be further scrutinized.

Prophenolooxidase (proPO) has three main functions in invertebrates, including mussels: it works as antioxidant and detoxicant, being also involved in immune defence mechanisms such as melanisation (Coles and Pipe, 1994; Luna-Acosta et al., 2017). Its role is quite well understood in crustaceans, whereas still little is known about its function in molluscs (Coles and Pipe, 1994; Luna-Acosta et al., 2017). Some works reported significant influence of pollution on proPO activity in invertebrates (Laitano and Fernández-Gimenez, 2016). In our previous ((Świacka et al., 2020) and recent study the lipofuscin/melanin accumulation in different tissues of mussels treated with DCF was revealed. Therefore, we decided to use proPO for the first time to evaluate DCF and 4-OH DCF effect on mussel immunity (induction of melanisation processes). However, we did not observe any effect of both tested chemicals on this marker. To our knowledge there are no other studies that investigated the effect of DCF on this marker in invertebrates. Furthermore, few studies reported significant effect of ibuprofen, also belonging to NSAIDs, on proPO activity (Luna-Acosta et al., 2017; Matozzo et al., 2012).

3.2. Histology

Sex structure of the tested blue mussels was varying among the treatment. In the control and 4-OH DCF sex ratio (SR) was around one indicating similar number females and males in both experimental conditions. As for DCF treatment, SR was 3.66 highlighting the fact that most blue mussels in this condition were females (22 females and six males). Calculated gonadal index (GI) was the highest in mussels from the control (2.83), while the lowest in mussels from DCF treatment (GI 1.89). Sex-related differences in GI among tested mussels were also observed. In the control, males developed gonads faster than females (IG_{males} 3.13 and $IG_{females}$ 2.53). Analogous situation was observed in the DCF treatment, yet the difference was that both males and females were developing gonads much slower when compared to the control (IG_{males} 2.16 and $IG_{females}$ 1.91). No sex-related differences in the 4-OH DCF condition were observed.

Based on histological analyses, several types of alterations in tissue architecture in tested mussels were found. These alterations included regressive changes occurring in the digestive, respiratory, and reproductive systems. Among the most identified changes gonadal atresia (Fig. 2a.), digestive tubules atrophy and necrosis (Fig. 2b.) and locally occurring diffuse infiltrations of hemocytes (inflammation) occasionally forming granulocytomas were found. Histopathologies found in gills included hypertrophy of lamellar epithelium (Fig. 2c.) the presence of degenerative lesions, edema (Fig. 2d.), fusion of gill filaments, partial loss of the lamella and epithelial hyperplasia. Gill edema occurred at similar frequency in all experimental conditions (Fig. 3.). Gill necrosis was only observed in mussels from both experimental conditions and occurred at the frequency of approx. 17% (Fig. 3.). In mussels exposed to 4-OH DCF and DCF fusion of gill filaments, partial loss of the lamella and epithelial hyperplasia occurred the most often. These changes were not observed in mussels from the control (Fig. 3.).

Following the result of Fisher's exact test, the gill enlargement was caused by presence of parent compound and the metabolite (p-value = 0.0013, pairwise Fisher's comparison: Control vs. DCF adjusted p-value = 0.002, Control vs. 4-OH DCF adjusted p-value = 0.035). Although there were other changes observed in gills of mussels exposed to DCF and 4-OH DCF, they appeared sporadically and cannot be attributed solely to the effects of the pharmaceutical treatment (Fig. 3.).

Additionally, the presence of brown cells (BC), containing melanin and/or lipofuscin pigmentation, was detected (Fig. 2e and f.). Pigmented cells were most commonly occurring in the mantle epithelium and this observation was statistically significant for 4-OH DCF-treated mussels (pairwise Fisher's comparison: Control vs. 4-OH DCF adjusted p-value = 0.0001), but also in the digestive gland' and gill' epithelium. In such cases, brown pigmentation occurred in all the cells forming epithelial layer of a given organ/structure.

In digestive gland various pathological alterations were more apparent, although only the increase of frequency of necrotic changes in these regions was statistically significant (Fisher's exact test, p-value = 0.026; pairwise Fisher's test: Control vs. 4-OH DCF p-value = 0.020, adjusted p-value = 0.061) (Fig. 5.). Lesions observed in the digestive system included digestive tubules atrophy (DTA), epithelial hypertrophy or necrosis, local inflammations, and the presence of BC. These changes were often found co-occurring with tubular cell necrosis (dead or decayed cells and/or tissue areas) and degenerative changes of digestive cells. They were observed in 20% of bivalves from the control condition, 41% of bivalves exposed to 4-OH DCF and 38% of bivalves exposed to DCF (Fig. 5.). Local inflammatory reactions occurring in the digestive system area occurred in 6.5% of bivalves from the control, 13.7% of bivalves exposed to 4-OH DCF and in 21% of those exposed to DCF (Fig. 5.). Digestive system necrosis occurred at similar frequency in mussels exposed to 4-OH DCF and DCF and was higher than in those from the control (Fig. 5.).

In single cases a tumor, gonadal germinoma (characterized by cellular atypia and hypertrophy and poorly differentiated germ cells) was found (1 case in the control and one case in the 4-OH DCF). Other occurring lesions included granulocytoma (1 case from 4-OH DCF) and

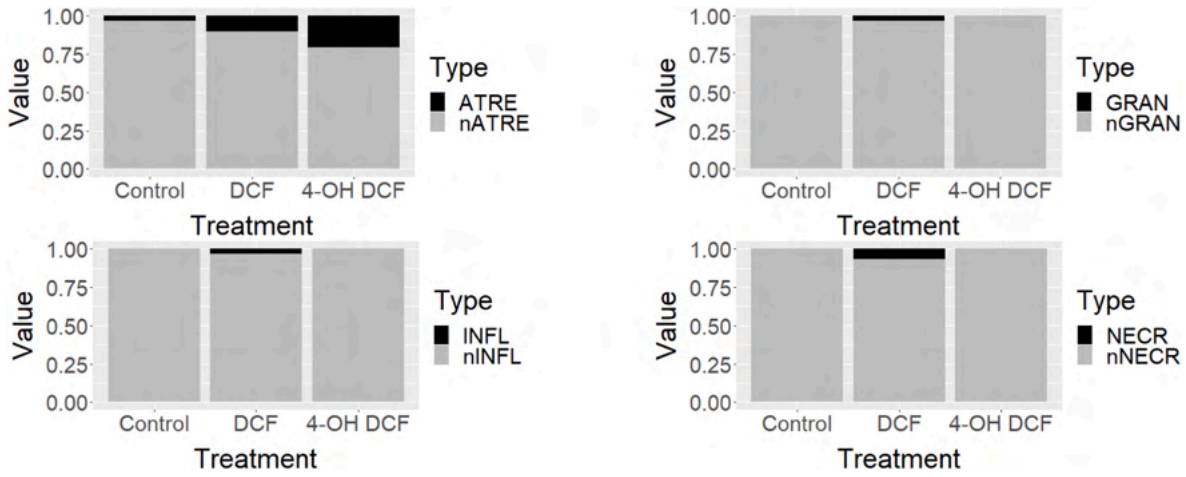

Fig. 4. Proportions of pathological changes in the mussels' gonads; ATRE - individuals with atresia, nATRE - individuals with no atresia; GRAN - individuals with granulocytoma, nGRAN - individuals without granulocytoma; INFL - individuals with inflammations, nINFL - individuals with no signs of inflammation; NECR - individuals with necrosis, nNECR - individuals with no signs of necrosis.

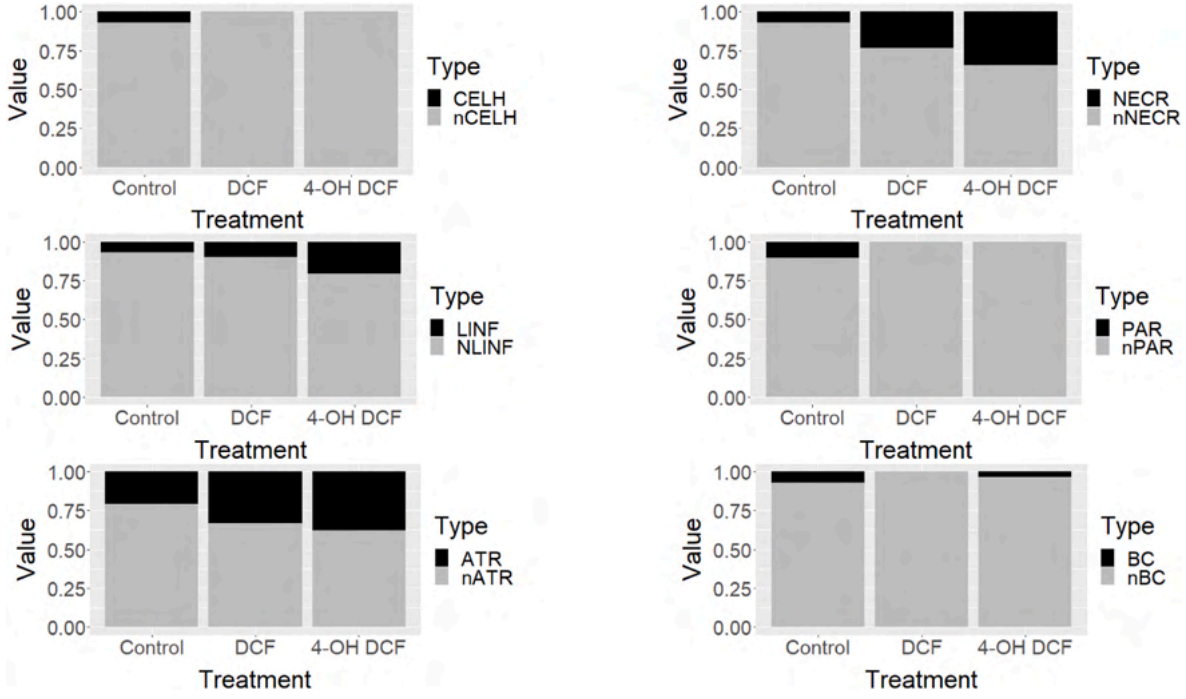

Fig. 5. Proportions of pathological changes in the mussels' digestive gland; CELH - individuals with cellular hypertrophy, nCELH - individuals with no cellular hypertrophy; NECR - individuals with necrosis, nNECR - individuals with no signs of necrosis. LINF - individuals with local inflammations, nLINF - individuals with no signs of local inflammation; PAR - individuals with potential parasites, nPAR - individuals without parasites; ATR - individuals with atrophied digestive tubules; nATR - individuals with normal digestive tubules; BC - individuals with brown cells in the digestive gland; nBC - individuals without brown cells in the digestive gland.

gonadal necrosis (Fig. 4.). In gonads none of observed lesions can be attributed solely to the treatment (Fisher's exact test $p > 0.05$) but seeing gradual increase of atretic changes towards the presence of DCF and its metabolite, the pharmaceuticals may exacerbate pathological processes leading to gonadal atresia (Fig. 4.). In conclusion, based on Fisher's exact test, both DCF and 4-OH DCF caused gill deformations. In addition, 4-OH diclofenac caused gill necrosis and accumulation of brown cells in the mantle. As for the other observed changes, both compounds only increased the frequency of their observation.

Similar histopathological changes were reported in our previous work (Świacka et al., 2020). DCF already at environmentally relevant concentrations 4 μg/L caused gills deformations, vacuolization of digestive gland and brown pigment accumulation. In addition, exposure to DCF at 40 μg/L caused digestive gland atrophy and necrosis. To our knowledge, there are no other studies that have performed histological evaluation of mussels exposed to DCF. In addition, this study is the first to investigate the histopathological effect of 4-OH DCF. There are few works showing tissue abnormalities in fish exposed to DCF. Pharmaceutical triggered necrotic changes in kidneys and gills of *Oncorhynchus mykiss* and *Salmo trutta f. fario* (Schwaiger et al., 2004; Hoeger et al., 2005; Mehinto et al., 2010; Triebskorn et al., 2004). On the other hand, DCF at 4.6 μg/L caused renal hematopoietic hyperplasia in *Gasterosteus aculeatus* (Näslund et al., 2017). Zhang et al. (2021) reported damage of intestines (the lamina propria's and intestinal mucosa epithelial cells) in

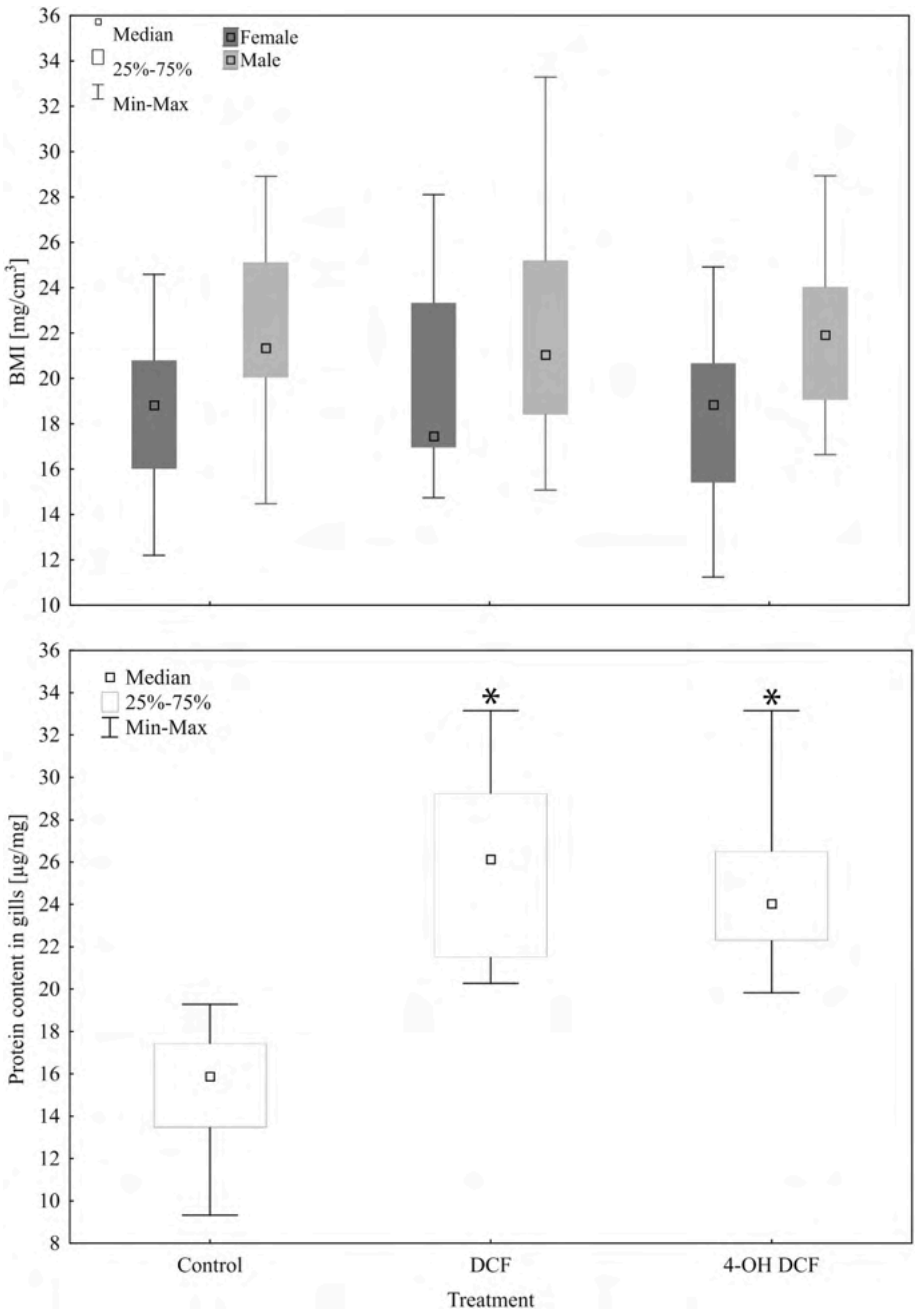

Fig. 6. BMI index and cytosolic protein content in *M. trossulus* gills per wet weight of gills. Statistical comparison (Mann Whitney U, $p < 0.05$; $n = 30$ for BMI; $n = 15$ for protein) *statistically significant differences.

freshwater crayfish (*Procambarus clarkii*) exposed to DCF at 1 and 10 mg/L. Nevertheless, the histopathological effects of NSAIDs on aquatic organisms are very poorly understood. However, this tool may prove to be especially useful in better understanding the toxicity of pharmaceuticals.

3.3. Cytosolic protein content and BMI

Changes in the cytosolic protein content in the gills between tanks were observed. There was significantly lower content of protein in the mussels' gills from the control tank as compared to DCF and metabolite exposure (Fig. 6.). Statistical tests (Kruskall-Wallis; Mann-U Whitney $p < 0.05$) also revealed significant differences in the cytosolic protein content between control and DCF ($p = 0.000002$) and between control and metabolite ($p = 0.000005$). This observation may suggest that the mode of action of both compounds may contribute to increased protein synthesis in gills. Similarly, in Freitas et al. (2019) significant influence of DCF on cytosolic protein content in *M. galloprovincialis* soft tissues was observed. In mentioned studies mussels were exposed at 1 µg/L in three different salinity scenarios (25, 30 and 35) or temperature scenarios (natural: 17 °C and raised: 21 °C). In each salinity scenario including environmental salinity (30) cytosolic protein content was significantly lower in control compared to DCF (Freitas et al., 2019), while only in environmental temperature protein content was significantly higher in DCF compared to control (Freitas et al., 2019). Interestingly, Jaafar (2015) observed reduction of protein thiols in mussel gills exposed to DCF at 0.2 and 1 mg/L compared to control.

So far, there are no other studies investigating the effect of 4-OH DCF on cytosolic protein content. Given the lack of effect of the tested chemicals on main oxidative biomarkers excluding GR, it can be

speculated that the proteins affected by DCF and its metabolite are not involved in phase I and phase II detoxification mechanisms. These may be proteins involved in energy reserves or inflammation, but this would imply that 4-OH DCF, like DCF, has anti-inflammatory effects. Therefore, it would be worthwhile to find out what type of proteins are affected by DCF and 4-OH DCF.

No statistically significant changes in mussel condition between DCF, control and metabolite both with and without sex division of mussels were observed (Kruskall-Wallis $p > 0.05$) (Fig. 6). Short exposure did not show an effect of DCF and its metabolite on the mussel condition. Interestingly, statistically significant differences were observed based on sex distribution. As can be seen in Fig. 6 BMI values were higher for males, with statistically significant changes observed in control and metabolite (Mann at Whitney $p < 0.05$). The lower BMI value for females may be related to the release of gametes during the experiment. The production and release of gametes is associated with a large energy cost that females had to spend for these processes.

4. Conclusions

The present study demonstrated for the first time the impact of 4-OH DCF on Mytilidae mussels. Toxic effect was manifested by the presence of lesions including numerous gill deformations (enlargement), necrosis and local inflammation in digestive gland, as well as atresia in gonads and atrophy. Although in some cases statistical evaluation showed no significant differences in lesion occurrence, it should be noted that the frequency of lesions observed was higher in the drug exposure tanks than in the control group. Worryingly, some of these changes were more frequently observed in 4-OH DCF exposure, even though the measured concentration of this chemical in water was three times lower compared to DCF and was more rapidly decreasing during the experiment (Świacka et al., 2021). Moreover, both DCF and 4-OH DCF significantly affected gill protein content. Therefore, cytosolic protein content in gills has proven to be a good marker in toxicity evaluation for both tested chemicals.

Considering the response of the enzymatic biomarkers used in this study, the effects of DCF and 4-OH DCF on bivalves may be difficult to observe using only this tool. Among all biomarkers tested, only GR activity inhibition due to DCF was noted. Moreover, given the results of other studies on DCF toxicity, such biomarker analysis appears to be more suitable for acute toxicity assessment, as the biomarker response is generally visible in the first hours of drug exposure and usually weakens thereafter.

So far, only a few studies have investigated the toxicity of DCF transformation products. Some of them showed higher toxicity of DCF photodegradation products compared to the parent compound. However, these studies focused on acute toxicity to algae and bacteria. To our knowledge, there are no other works addressing the toxic effect of DCF metabolite in bivalves. Consequently, our study fills a significant gap in the knowledge regarding the NSAIDs metabolites toxicity in aquatic invertebrates.

Author statement

Klaudia Świacka: Conceptualization, Investigation, Data Analysis, Writing - Original Draft; **Jakub Maculewicz:** Investigation, Data Analysis, Visualisation, Writing - Original Draft; **Justyna Świeżak:** Investigation; Visualisation; Writing - Review & Editing; **Magda Caban:** Supervision, Writing - Review & Editing; **Katarzyna Smolarz:** Conceptualization, Supervision, Investigation, Writing - Review & Editing;

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Long-term stability of diclofenac and 4-hydroxydiclofenac in the seawater and sediment microenvironments: Evaluation of biotic and abiotic factors☆

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ABSTRACT

Studies in recent years have shown that significant amounts of diclofenac (DCF) and its metabolites are present in marine coastal waters. Their continuous flow into the environment may be associated with numerous negative effects on both fauna and flora. Although more and more is known about the effects of pharmaceuticals on marine ecosystems, there are still many issues that have not received enough attention, but are essential for risk assessment, such as long term stability. Furthermore, interaction of pharmaceuticals with sediments, which are inhabited by rich microbial, meiofaunal and macrobenthic communities need investigation. Therefore, we undertook an analysis of the stability of DCF and its metabolite, 4-hydroxy diclofenac, in seawater and sediment collected from the brackish environment of Puck Bay. Our 29-day experiment was designed to gain a better understanding of the fate of these compounds under experimental conditions same as near the seafloor. Diclofenac concentration decreased by 31.5% and 20.4% in the tanks with sediment and autoclaved sediment, respectively during 29-day long experiment. In contrast, the concentration of 4-OH diclofenac decreased by 76.5% and 90.2% in sediment and autoclaved sediment, respectively. The concentration decrease of both compounds in the sediment tanks resulted from their sorption in the sediment and biodegradation. Obtained results show that marine sediments favour DCF and 4-OH DCF removal from the water column.

1. Introduction

As the world's population continues to grow, so does the demand for pharmaceutical compounds, including non-steroidal anti-inflammatory drugs (NSAIDs), which are the most commonly used group of pharmaceuticals (Mlunguza et al., 2019; Pap et al., 2021; Pawłowska et al., 2021). In highly developed or highly populated developing countries, the consumption of pharmaceutical compounds is already high and the numbers are growing (Rehman et al., 2015; Branchet et al., 2021). NSAIDs, due to specific biological action, are widely used in many economic sectors, hence they are released into the environment from many sources like sewage from industrial and domestic wastewater treatment plants (WWTPs), landfill sites, pharmacies disposals, households' wastes, farms, and aquaculture (Zhang et al., 2008; Nikolaou et al., 2007; Lonappan et al., 2016). The conventional technologies of urban WWTPs are inefficient in both DCF and its metabolites removal, while advanced technologies are still not the common practise (Kołecka et al., 2022). Worryingly, these compounds are being found even in pristine ecosystems such as Antarctic and Arctic, where the absence of modern wastewater treatment plants plays a key role in the release of these chemicals into the environment (Kallenborn et al., 2018; González-Alonso et al., 2017).

As a consequence, the drugs are commonly detected in different environmental matrices around the world (Lolić et al., 2015; Branchet et al., 2021; Świacka et al., 2022; Caban et al., 2021a, b). Diclofenac (DCF) belongs to the most commonly found NSAIDs worldwide (Bonnefille et al., 2018; aus der Beek et al., 2015; Ajibola et al., 2021; Pap et al., 2021). DCF is known to cause dysfunctions in aquatic organisms such as metabolic and osmotic regulation disorders, reproduction disruption, modification of immune parameters, histopathological and genotoxic effects. These effects are observed already at environmentally relevant concentrations ranging from several ng/L to a few μg/L (Świacka et al., 2020; Mezzelani et al., 2018; Bonnefille et al., 2018; Rocco et al., 2010; Fontes et al., 2018; Boisseaux et al., 2017; Trombini et al., 2019; Eades and Waring, 2010; Świacka et al., 2022). Thereby it is crucial to take an action of minimalization of its presence in the

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environment, which are obtained in each step of pharmaceutical life cycle, starting with design, then marketing, authorisation, production, prescription, sales, consumption, and waste handling (Caban et al., 2021a, b).

NSAIDs, including DCF, are found in the environment not only in primary form but also as metabolites (Mulkiewicz et al., 2020; Maculewicz et al., 2021; Stülten et al., 2008; Osorio et al., 2014; Schmidt et al., 2018; Scheurell et al., 2009; Weigel et al., 2004; Celiz et al., 2010). Even though metabolites are found at much lower concentrations than parent compounds, their toxicity to some algae, zooplankton, and bacteria has been revealed (Maculewicz et al., 2021; Schmitt-Jansen et al., 2007; Grabarczyk et al., 2019; Rubasinghege et al., 2018; Schulze et al., 2010). Yet, constantly detected in the aquatic environment DCF metabolites are one of the least recognized concerns (Branchet et al., 2021). Some studies focused on fluoxetine, ibuprofen, and carbamazepine metabolites while DCF metabolites have been neglected so far (Branchet et al., 2021; Weigel et al., 2004).

DCF and 4-hydroxy diclofenac (4-OH DCF) are detected not only in surface waters and tissues of organisms such as fish and bivalves, but also in marine and river sediments (Bonnefille et al., 2018; Branchet et al., 2021). For example, DCF concentrations in sediments ranged from <0.10 to 1.06 ng/g in the sediments collected in the Todos os Santos Bay and north coast of Salvador in Brazil (Beretta et al., 2013). Moreover, 1–390 ng/g of DCF was detected in South African rivers (Mbokodweni river) sediments (Sibeko et al., 2019; Agunbiade and Moodley, 2016). In a study by Zind et al. (2021), DCF and its hydroxymetabolites (4 and 5-OH DCF) were detected in the sediments of three French rivers. Alarmingly, the concentration of DCF in sediments was half that of its hydroxymetabolites, while in river water the concentration of DCF was similar to that of hydroxymetabolites. The reported mean concentration of DCF in river sediments was 51 ± 55 and its hydroxy metabolites was 103 ± 107 ng/g (Zind et al., 2021). Maranho et al. (2015a, b) detected DCF at concentrations up to 1.50 ng/g in sediments collected from the Gulf of Cadiz, Spain, while DCF concentrations reported by Pintado--Herrera et al. (2013) in the same area were up to 10 ng/g dw. On the other hand, Stewart et al. (2014) found DCF in sediments collected along the New Zealand Pacific Ocean coast. The concentrations detected were up to 2.5 ng/g dw.

However, it remains unknown whether the sorption to the marine sediment is an important way of DCF and its metabolites removal from the water column. In addition, the stability of DCF and 4-OH DCF in bottom water is also poorly studied. The bottom environment is characterised by specific conditions - it is more stable in terms of temperature and reduced solar radiation, which may favour the stability of pharmaceuticals in this environment (Stigebrandt, 2001). Therefore, the aim of this study was to investigate the long-term stability of DCF and its metabolite 4-OH DCF in marine bottom waters and their sorption potential in marine sediments using an experimental approach. Used DCF and 4-OH DCF concentrations were above typical environmental levels (up to 1 μg/L for DCF and few hundred ng/L for 4-OH DCF). The usage of higher DCF and 4-OH DCF levels was planned in order to be able to assess biodegradation activity reflected as a decrease in these chemical' concentrations and to detect low-level biodegradation products (metabolites) like 5-OH DCF. As already proved, many pharmaceuticals, including DCF, are biodegradable by various bacteria or fungi strains (Lee et al., 2012; Gröning et al., 2007; Domaradzka et al., 2015; Hasan et al., 2021; Shu et al., 2021; Aulestia et al., 2021; Rastogi et al., 2021; Branchet et al., 2020). As various microorganisms live on or inside the sediments, microbial degradation and final reduction of their levels may thus be expected.

2. Materials and methods

2.1. Sampling of water and sediment

The water and sediment samples were collected at the station with geographical coordinates 54°37′15″ N and 18°32′ 90″E. Bottom water and sediments were collected near the outlet of the "Dębogórze" Wastewater Treatment Plant (WWTP) on board of *rv Oceanograf*. The volume of 40 L of bottom seawater was sampled from a depth of 10 m using bathometer and a 5 cm thick surface layer of sediment (few kg) was taken using Van Veen grab sampler. The water temperature and salinity were measured using an intelligent digital sensor MPP and were 5.6 °C and 7.8 PSU, respectively. The samples (40 L of bottom water in special containers and sediments along with bottom water in four buckets) were then transported to the laboratory. The pH of the seawater was measured and then the samples were placed in a refrigerated incubator with the temperature set at 5 °C to reflect the environmental temperature. In addition, the samples were aerated with pressurized air, because of the aerobic conditions at the depth from which they were collected. Samples were stored for 4 days prior to the experiment.

2.2. Chemicals

Diclofenac sodium salt (DCF, CAS 15307-79-6, analytical standard, purity >98%), stable isotope labelled internal standard diclofenac-$13C_6$ sodium salt (IS, CAS 1261393-73-0), 4-hydroxydiclofenac (4-OH DCF, CAS 64118-84-9, analytical standard, purity >98%) and 5-hydroxydiclofenac (5-OH DCF, CAS 69002-84-2, analytical standard, purity >98%) were purchased from Sigma-Aldrich. Stock solutions of DCF and 5-OH DCF at concentration 1 mg/mL and 4-OH DCF at 0.73 mg/mL were obtained by dissolving them in 100% methanol (HPLC grade, POCH, Poland). The stock solution of internal standard was 0.1 mg/L. Artificial sea water with a final salinity 7.8 was prepared using Aquaforest® salt standard.

2.3. Experimental set-up

Simulated reactors for both aqueous and sediment-water systems were used to investigate the stability of DCF and its metabolite. For the experimental purposes, fifteen 1 L glass bottles with screw caps, previously washed with methanol, for DCF and 4-OH DCF (five experimental variations in three replicates each) were used (Fig. 1).

To be sure that autoclaving would not change the water salinity, a pre-testing was performed. After measuring the salinity of collected seawater, 1 L of seawater subsample was collected and autoclaved (Classic Prestige Medical Autoclave) at 130 °C for 2 h. After autoclaving, the seawater salinity was measured again and the result indicated no change in that parameter. Next, 8 L of seawater was filtered twice using glass fibre filters (Whatman, GF/F grade) and then autoclaved (130 °C for 2 h) to clean of suspended solids and remove microorganisms. For sterilisation purpose, half of the sampled volume of marine sediment was autoclaved twice, at 130 °C for 2 h. Next, autoclaved marine sediment was placed in six glass bottles (three for tests with DCF and three with metabolite) so that each bottle contained about 200 cm^3 of sediment. Bottles with autoclaved sediment were filled with 800 mL of autoclaved and filtered seawater (Fig. 1). Sediment and water sterilisation using sodium azide (NaN_3) was not performed, even though it's an effective way to eliminate microorganisms. NaN_3 can modify the chemistry of sediment what in turn may influence the pharmaceutical concentration. The non-autoclaved sediment, previously cleaned from macrozoobenthos, was placed to another six glass bottles (volume 200 mL) and then each bottle was filled with 800 mL of autoclaved and filtered sea water. Another six glass bottles were filled with 800 mL of seawater (non-filtered) and the last six with 800 mL of filtered artificial seawater. Briefly, five experimental sets were prepared. Two sets, i.e., sediment, and autoclaved sediment, were prepared to test whether the microorganisms present in the sediment could biodegrade DCF and its metabolite and to see these chemicals sorption in sediments. The other three sets were tanks without sediment: "environmental water", "artificial seawater" and "sterilized environmental water". (Fig. 1). The environmental water tanks were prepared to test whether the presence

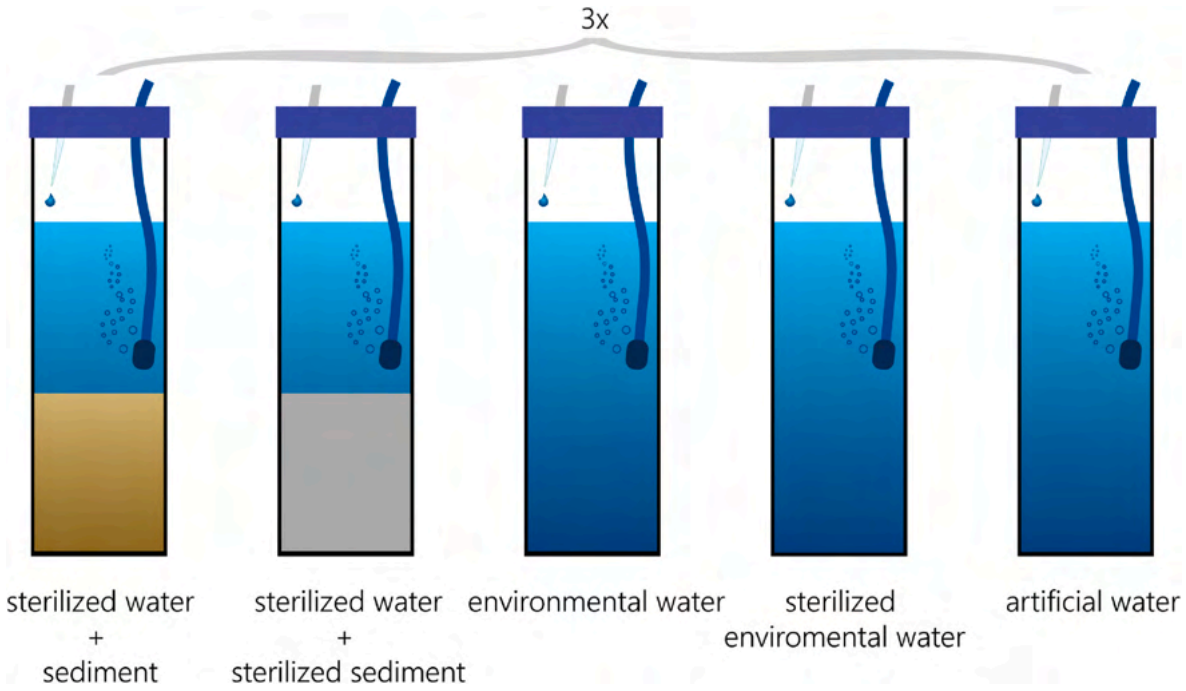

Fig. 1. Experimental set-up.

of microorganisms affects the concentration of the test chemicals. The remaining tanks were controls. Two different controls, namely "artificial seawater" and "sterilized environmental water" were prepared to see if the concentrations of DCF and 4-OH DCF would differ between the natural sterilized water from the environment and the artificial water prepared in the laboratory.

Since the concentrations of DCF metabolites in the marine environment are much lower than those of the parent compounds, it was decided to use a 10 fold lower concentration of 4-OH DCF than DCF in this experiment. DCF was spiked at 1000 μg/L (by addition of 800 μL of 1 mg/mL solution) to each 15 bottles, while 4-OH DCF was spiked at 100 μg/L (110 μL of 0.73 mg/mL) to remaining 15 bottles.

All tanks were aerated during experiment to avoid anaerobic conditions. Experiment was conducted at 5 °C during 29 days in the dark to avoid photodegradation of the tested compounds.

To ensure that bottle conditions were stable, on each day of sample collection for HPLC, pH and OD_{600} (optical density) were measured. OD_{600} was measured to see if bacteria were present and if were growing in the samples (especially autoclaved samples). For this purpose, 1 mL of water from each bottle with DCF and metabolite was taken at days 1, 5, 9, 13, 17, 21, 25 and 29 and measure at a wavelength of 600 nm by spectrophotometer (Prove 300 Spectroquant®, Merck Milipore). pH was measured in each bottle using pH metre (SI Analytics, Lab 850). In addition, on days 8, 15 and 29 dissolved oxygen levels was measured using an oxygen electrode (Elmetron CO-401).

2.4. Quantification of DCF and 4-OH DCF in water by HPLC-DAD

During the first nine days of the experiment, samples for high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) analysis were taken every two days, and then every four days (sampling days: 0, 1, 3, 5, 7, 9, 13, 17, 21, 25 and 29). At day 0 (30 min after adding chemicals) a 1 mL of water from each bottle with DCF was taken and analysed by HPLC (Nexera XR, Shimadzu). As the concentration of the metabolite was 10 times lower, to enable its detection on HPLC directly, 10 mL of water from each bottle was concentrated using SPE (solid-phase extraction), as described in our previous works (Swiacka et al., 2020, 2022). The analyte was eluted from the Strata-X (200 mg, 3 mL, Phenomenex) cartridges with 5 mL of methanol and dried under gaseous nitrogen. Samples with metabolite were then dissolved in 0.5 mL of methanol and analysed by HPLC.

2.5. Quantification of DCF, 4-OH DCF and 5-OH DCF in water and sediment by GC-MS

On the last day of the experiment, all remaining water from DCF and metabolite bottles was collected and prepared for gas chromatography couple with mass spectrometry (GC-MS) analysis. The water was passed through SPE cartridges to concentrate and purify the analytes and thus increase the possibility of detecting other metabolites. Samples were then eluted from the cartridges with 5 mL of methanol and dried under gaseous nitrogen. Next steps were derivatisation, followed by GC-MS analysis. All the steps of procedure are described in detail in our previous work (Świacka et al., 2020). Validation results of the analitycal method is presented in our previous study (Świacka et al., 2020). MDL water/tissue calculated for DCF, 4-OH DCF and 5-OH DCF was 1 ng/L/0.01 μg/g; 1 ng/L/0.08 μg/g and 2 ng/L/0.17 μg/g respectively. MQL water/tissue calculated for DCF, 4-OH DCF and 5-OH DCF was 3 ng/L/0.02 μg/g; 3 ng/L/0.23 μg/g and 7 ng/L/0.36 μg/g respectively (Świacka et al., 2020). MDL and MQL calculated for tissue can be applied to other solid matrices like sediments.

The tested compounds were also extracted from the sediment. For this purpose, 150 mL of methanol was added to each sample with sediment (after removing water for SPE), and sonicated for 15 min, two times. Next, obtained sediment extract was filtered by glass fiber filters and the final volume of extract was measured. The volume of 15 mL of extract was dissolved in 285 mL of distilled water. In turn, 20 mL of extract with 4-OH DCF was dissolved in 380 mL of distilled water. Next, 50 μL of DCF $13C_6$ at 10 μg/mL was then added to each sample with DCF and 4-OH DCF. Such prepared samples were subjected to SPE and GC-MS analysis. Finally, the sediment was dried in a laboratory oven at 100 °C for several days in order to calculate the concentration of the detected compounds in the dry mass of the sediment.

2.6. Statistic

Statistic tests were performed in R studio 8.17 software program. Before statistical comparison, distribution and homogeneity of variances were verified. Statistical comparison of DCF and 4-OH DCF concentration between various tanks on selected days was performed using the Dunnett's test (R studio 8.17 software).

3. Results and discussion

3.1. Parameters measured in water

pH of the environmental seawater after collection was 7.7 (before chemical addition) and didn't change after DCF and 4-OH DCF addition. A slight increase in pH was observed in all tanks during the experiment, which could be related to a decrease in dissolved carbon dioxide in water (Table 1).

Measured OD_{600} values remained low during experiment. Only slight fluctuations, which were not related to the exposure time, in each tank were observed (Table 1). The highest OD_{600} values were observed in tanks with autoclaved sediment, while the lowest values in environmental water (Table 1). Furthermore, slight increase in dissolved oxygen concentration was observed on day 29 (Table 1).

3.2. Concentration of DCF and 4-OH DCF in water

In the tanks with sediment a decrease in the concentration of both compounds tested was observed (Fig. 2). The highest decrease in DCF concentration was observed in the tanks with sediment and the highest decrease in 4-OH DCF concentration in the tanks with autoclaved sediment (Fig. 2). DCF concentration decreased by 31.5% in the tanks with sediment and by 20.4% in the tanks with autoclaved sediment during 29-day long experiment (Fig. 2). The concentration of 4-OH DCF decreased by 76.5% and 90.2% in sediment and autoclaved sediment, respectively (Fig. 2). Interestingly, there were statistically significant differences in DCF concentration between sediment and autoclaved sediment tanks on days 21, 25 and 29 ($p < 0.05$ on days 21 and 25, $p < 0.001$ on day 29). In contrast, no statistically significant differences in 4-OH DCF concentration between sediment and autoclaved sediment tanks were observed (Fig. 2). This finding may suggest a greater role of biodegradation processes for DCF than its metabolite.

Table 1
Range of OD_{600} and pH values measured several times during the experiment.

Compound added to tank	Tank set-up	OD_{600}	pH	Dissolved oxygen concentration [mg/L]
DCF	Water with sediment	0–0.025	7.8–8.2	7–8.8
	Water with sterilized sediment	0.01–0.034	7.7–8.2	6.4–8.7
	Environmental water	0–0.015	7.8–8.1	7.6–9.6
	Sterilized environmental water	0–0.019	7.8–8.2	7.7–9.6
	Artificial seawater	0–0.009	7.2–7.6	7.8–9.7
4-OH DCF	Water with sediment	0–0.027	7.7–8.2	7.4–9.0
	Water with sterilized sediment	0.002–0.033	7.7–8.2	7.2–9.5
	Environmental water	0–0.013	7.8–8.1	6.7–9.9
	Sterilized environmental water	0–0.017	7.8–8.1	7.7–9.7
	Artificial water	0–0.015	7.2–7.5	7.5–9.9

Statistical comparison revealed statistically significant differences in 4-OH DCF concentration between tanks with sediments (autoclaved and non-sterilized) and other tanks, which were present on Fig. 2. For example, there were statistically significant differences in metabolite concentration between sediment, autoclaved sediment, and environmental water tanks on days 7–29 ($p < 0.05$), and between sediment and filtered environmental water tanks on days 1–29 (Fig. 2). These differences were also observed between autoclaved sediment and filtered environmental water tanks on days 1–3 and 7–29 ($p \leqslant 0.005$, Fig. 2). Contrary to metabolite, for DCF, statistically significant differences between the tanks with sediment and the remaining tanks only appear on the later days of the experiment (from day 7 onwards). For example, statistically significant differences in DCF concentration between sediment and artificial sea water were observed on days 7–29, while between autoclaved sediment and filtered environmental water, environmental water only on days 25 and 29 (Fig. 2). Statistically significant differences were also observed between autoclaved sediment and artificial sea water on days 9, 13 and 29 (Fig. 2). Notably, there were no statistically significant differences in DCF and metabolite concentrations between all tanks on day 0 (Fig. 2). Obtained results indicate that in the presence of sediment, DCF and its metabolite are more readily eliminated from the water. Moreover, our observations suggest, that the metabolite may be more readily susceptible to removal than the primary compound. The significantly higher decrease in the concentration of both chemicals in the sediment tanks compared to the other tanks may be due to the adsorption of DCF and 4-OH DCF on the sediment particles and biofilm, or their biotic and abiotic degradation.

Gröning et al. (2007) also observed significant DCF concentration decrease in fixed-bed column bioreactor filled with sediment from the creek Münzbach (Germany). Sediment and water were taken from a location near communal WWTP outlet. Just five days after the addition of DCF, a 93% decrease in its concentration was observed. Subsequently, another load of DCF was added until a final concentration of 1095.2 μg/L was reached, and again a 94% decrease in DCF concentration was observed just 3 days after the second load. The DCF concentration used by Gröning et al. (2007) was similar (1036 μg/L) to our research (1000 μg/L). Despite this, in our study, after 29 days of DCF injection, the DCF depletion was much smaller compared to the work of Gröning et al. (2007), in which a 93% DCF depletion was observed after only 4 days. This may result from higher temperature used in this study. In Gröning et al. (2007) the experiment was conducted at a temperature of 20 °C, while in our study temperature was four times lower (5 °C). Higher temperature may increase the biodegradation activity of microorganisms. Gröning et al. (2007) showed a constant rate of DCF depletion under stable laboratory conditions up to a given DCF concentration of approximately 9000 μg/L. Concentrations of 9000 μg/L and higher caused a decrease in the rate of DCF depletion which was likely due to the toxic effect of this pharmaceutical to microorganisms from sediment. In sterilized sediment, DCF depletion rate was significantly lower than for the non-sterilized sediment and it constituted only 0.8–1.5% of all DCF depletion (Gröning et al., 2007). In contrast, in our work, the decrease in DCF concentration after 29 days was only 10% higher in the non-sterilized sediment than in sterilized sediment. The depletion of DCF in tank with sterile sediment could be due to its adsorption on sediment particles. Thus, these processes played a larger role in our work than in work of Gröning et al. (2007), where biodegradation was the main contributor to DCF depletion. Paje et al. (2002) also observed DCF biodegradation of about 70% by river biofilm within 4 days. For this purpose, river biofilms from the Saale River (Germany) cultured in rotary ring reactors were used instead of river sediments. Similar to Gröning et al. (2007), the biofilm from river also slowly degraded DCF in Paje et al. (2002) (constant DCF depletion rate was observed). Unfortunately, we did not extract and measure the mass of biofilm in the sediment, but it was mainly medium-grained sediment (Tęgowski et al., 2004). Biofilm is an important factor in the elimination of

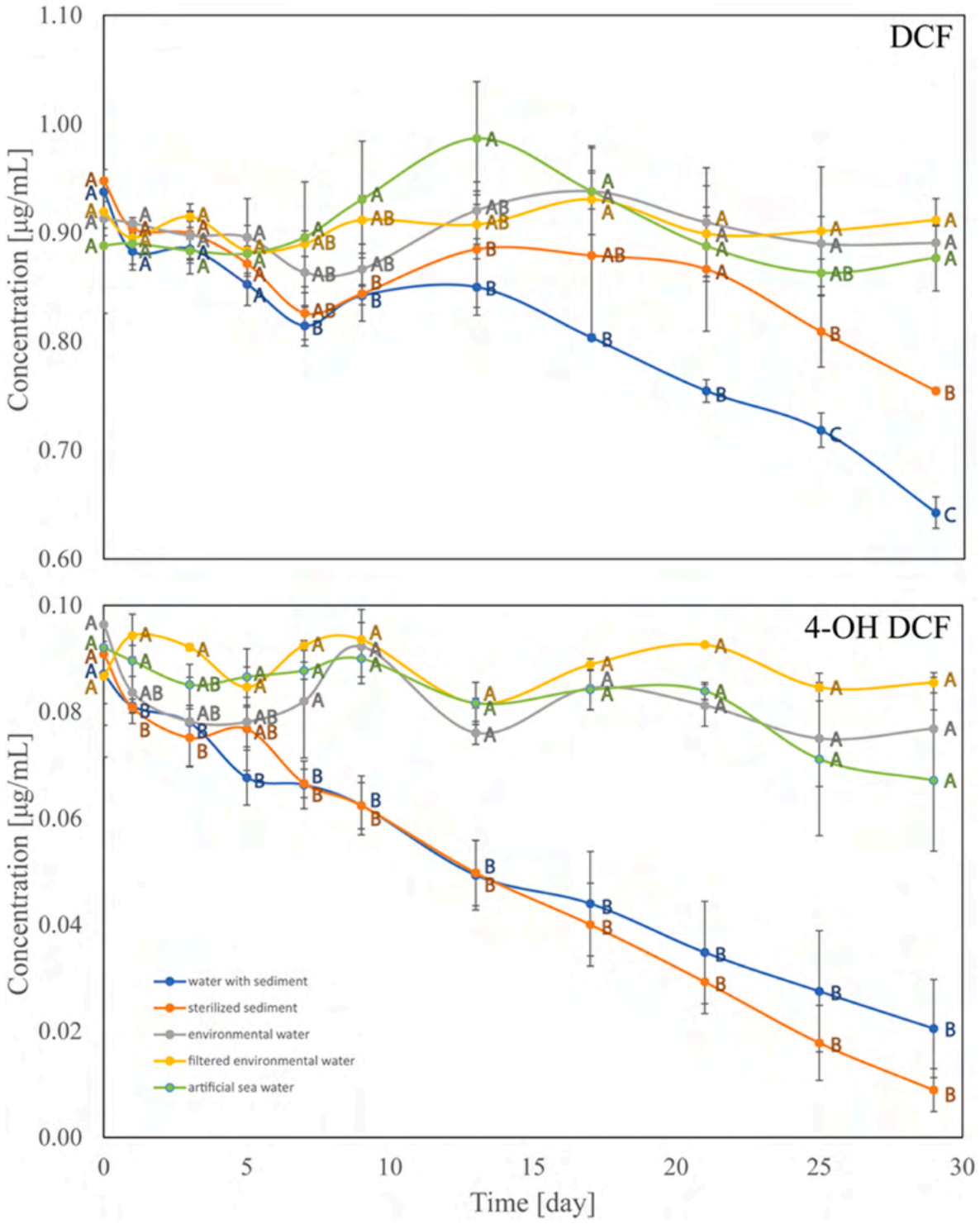

Fig. 2. Concentration of DCF and 4-OH DCF in a 29-day experiment. The letters A, B and C indicate statistically significant difference within each day (Dunnett's test). Treatments with no common letters are significantly different ($p < 0.05$).

pharmaceuticals.

Lee et al. (2012) investigated microbial degradation potential of DCF as well as 4-OH DCF. For this purpose mixed river sediment and sludge from domestic and industrial wastewater treatment plants (WWTPs) was used and modified Closed Bottle Test (OECD 301 D) was conducted. Results of this study showed high stability of DCF. Pharmaceutical concentration did not change significantly over 28 days of the experiment. Contrary, a slow decrease in the concentration of 4-OH DCF of about 40% from the initial concentration was observed over 28 days. Although we didn't use sludge from WWTPs like in Lee et al. (2012), the observed concentration decrease of 4-OH DCF was higher in our work. This is a very interesting result, since it would be expected to see a higher concentration decrease in sludge taken directly from a wastewater treatment plant. Also, Zwiener et al. (2003) did not observe biodegradation of DCF by biofilm grown on sludge from the wastewater treatment plant in the aerobic conditions within 48 h, while a slight decrease in drug concentration was observed under anoxic conditions. Furthermore, analysis of DCF and 4-OH DCF in WWTP outflows often

present low reduction potential for these two chemicals (Kołecka et al., 2020).

In the remaining tanks with DCF and 4-OH DCF (environmental seawater, sterilized environmental seawater and artificial seawater) no significant decrease in drug concentration was observed after 29 days. In addition, no statistical differences in DCF and 4-OH DCF concentrations between these matrices (environmental seawater, sterilized environmental seawater and artificial seawater) were observed ($p > 0.05$). This may indicate negligible role of microorganisms living in environmental seawater in DCF and 4-OH DCF biodegradation. Only slight fluctuations in DCF concentration were noted, which could be caused by matrix effect in HPLC determination. On the other hand, there was a decrease of 27.1% and 20.3% in the concentrations of 4-OH DCF in the artificial and environmental seawater, respectively. Results of the present study show that in the presence of sediment, DCF and its metabolite are more readily degraded by biotic or abiotic factors, while in the seawater these compounds are rather persistent in conditions obtained in bottom zone - low temperature and no UV–Vis radiance.

3.3. Mass balance of DCF and 4-OH DCF

To demonstrate the fate of DCF and 4-OH DCF in microenvironment based on sediment and seawater, a mass balance was calculated (Fig. 3; Fig. 4). Mass balance is defined as the amount or the percentage of the substance that can be analytically recovered after an adsorption test versus the amount of the substance which was added at the beginning of the test (nominal concentration) (OECD, 106). Furthermore, in our calculation the amounts of analytes removed from system for analytical determination of concentration (by HPLC-DAD) was considered.

3.3.1. Tanks with seawater and sediments

The highest mass loss of 4-OH DCF was observed in the thank with sterilized sediment (~82.75%). Only small part of metabolite mass (~1.5%) was accumulated in the sterilized sediment, thus a significant mass loss may be connected to its biotic and abiotic degradation (Fig. 3). Furthermore, the mass of metabolite in the non-sterilized sediment was 18% lower compared to the sterilized sediments, which may be related to the more efficient biodegradation of 4-OH DCF in the non-sterilized sediments. However, observed differences were not statistically significant ($p > 0.05$).

The highest DCF mass loss was observed in tanks with sediment (~26.16%) (Fig. 4). The mass loss of DCF in sediment tanks was almost three times lower than its metabolite. This may indicate lower degradability of DCF than its metabolite in the presence of sediment. About 9% of the initial mass of DCF was adsorbed in the non-sterilized sediment and about 7% in the sterilized sediment (Fig. 4). According to the mass balances, the sediment-bound fraction was four times higher for DCF than for its metabolite. Therefore, our results demonstrate that DCF have higher tendency for adsorption in sediment compared to 4-OH DCF. Sorption probably occurred mainly on the surface of the sediment as it was not mixed. However, such condition imitate real natural environment.

In addition, 5-OH diclofenac (5-OH DCF) was detected in the non-sterilized sediment from each of the three DCF tanks at a concentration of 0.392±0.125 μg/kg dw, while in sterilized sediment none of DCF metabolite (5-OH DCF or 4-OH DCF) was detected. These findings indicate possible biotransformation of DCF by microorganisms from sediment. As reported by numerous studies 5-OH DCF is a common metabolite produced by various bacteria strains, including bacteria from river sediments (Gröning et al., 2007; Palyzová et al., 2019; Lu et al., 2019; Ivshina et al., 2019; Osorio-Lozada et al., 2008; Moreira et al., 2018). Still, little is known about the biotransformation capacity of bacteria from marine sediments for pharmaceuticals.

Mass loss of both DCF and metabolite in tanks with sediment, unrelated to their sorption, was visibly higher compared to the tanks with seawater alone (of 43–69% for metabolite and 4–17% for DCF). This important observation may suggest that the presence of sediment favour the removal of both chemicals from water matrix.

Unfortunately, there are no other similar works that examined the mass balance of DCF and 4-OH DCF in water and sediment collected

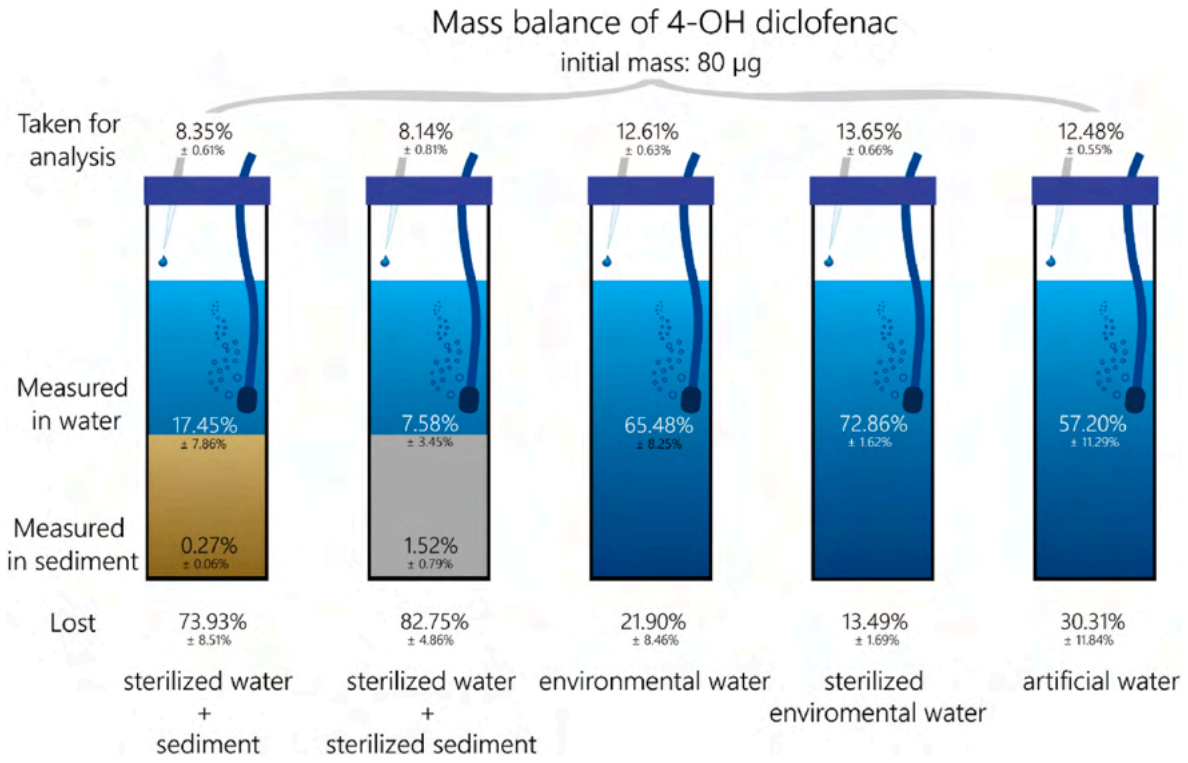

Fig. 3. The % mass balance shows the mass of 4-OH DCF measured by HPLC and calculated in water samples taken for analysis, sediment and water after 29 days. Losses of 4-OH DCF were calculated from the initial mass.

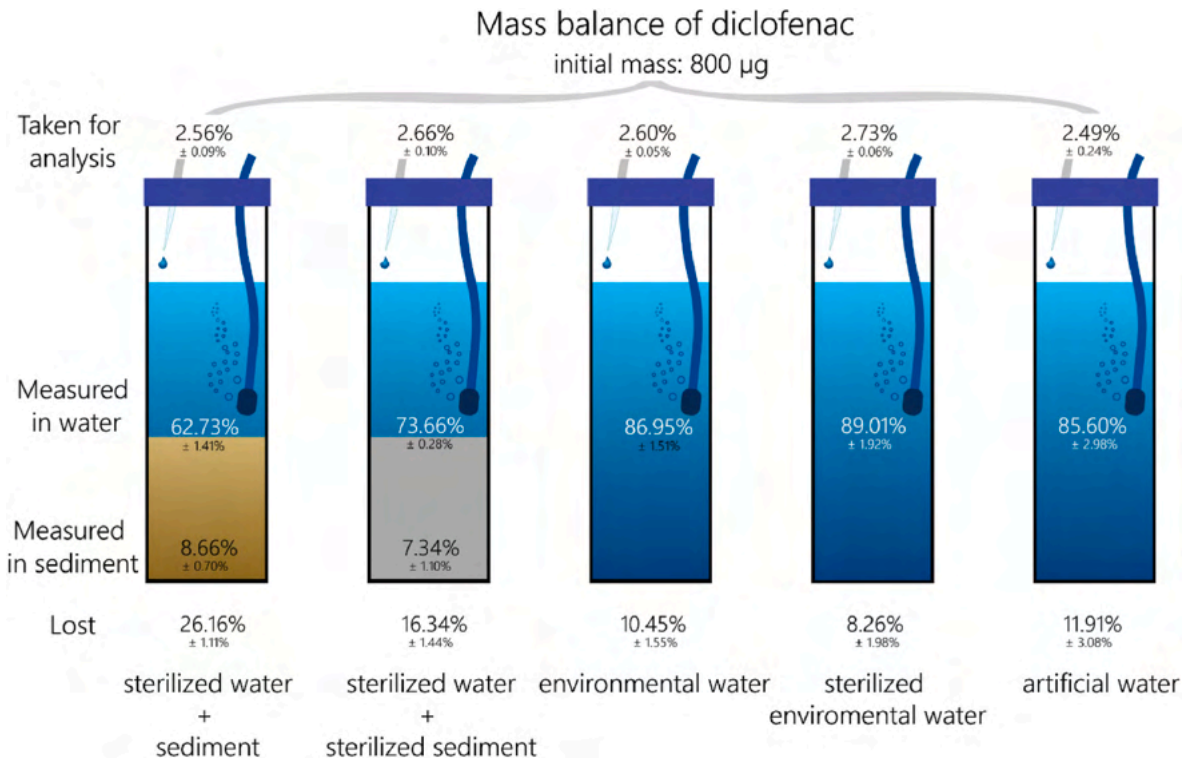

Fig. 4. The % mass balance shows the mass of DCF measured by HPLC and calculated in water samples taken for analysis, sediment and water after 29 days. Losses of DCF were calculated from the initial mass.

from the environment. So far, most works has focused only on changes in concentration and biodegradation rate, leaving out mass balance.

3.3.2. *Tanks with seawater*

For both DCF and 4-OH DCF, the lowest mass loss was observed in the tanks with sterilized environmental seawater (Fig. 3; Fig. 4). For DCF the mass loss in these tanks was only ~8.3% and for its metabolite ~13.5% (Fig. 3; Fig. 4). Interestingly, the highest mass loss of DCF and 4-OH DCF was found in artificial seawater (Fig. 3; Fig. 4). However, only slight differences were found in the mass loss of DCF and its metabolite in the tanks without sediment. The mass loss of DCF in each of the saltwater tanks was 8.3–11.9%, while the mass loss of 4-OH DCF was 13.5–30.3% (Fig. 3; Fig. 4). So far, there are no other similar studies, which investigated pharmaceutical stability in marine bottom water. There are works that have studied the stability of DCF under laboratory conditions that reflect the environmental conditions of marine surface waters. Baena-Nogueras et al. (2017) reported rapid photodegradation of DCF under simulated surface water environmental conditions. In addition, biodegradation by marine microorganisms was not observed.

3.4. *Contribution of sorption of DCF and 4-OH DCF to sediment in their removal process*

The solid-water distribution coefficient (K_d) was calculated to assess the sorption potential of DCF and its metabolite to marine sediments. K_d is defined as the ratio of the concentration of tested compound in sediment to its concentration in water according to OECD Guideline test No. 106: Adsorption - Desorption Using a Batch Equilibrium Method (2000). According to Sedeño-Díaz et al. (2019) $\log K_d > 5$ indicates compounds with high sorption capacity or that remain in sediments; $3 < \log K_d < 4$ correspond to chemicals easily released from the solid to aqueous phase and $\log K_d < 3$ indicate the compounds that predominate in the liquid phase.

For sediment $\log K_d$ obtained for DCF was -0.57 ± 0.04 and for autoclaved sediment -0.75 ± 0.07. For 4-OH DCF, $\log K_d$ was -1.52 ± 0.17 and -0.51 ± 0.29 for sediment and autoclaved sediment, respectively. Our results suggest that both, DCF and 4-OH DCF, are predominant in the aqueous phase with minor affinity for sediment. There are no other studies that have calculated $\log K_d$ for 4-OH DCF.

In Zhou and Broodbank (2014) DCF concentrations were determined in sediment and water samples upstream of a wastewater discharge site, in the River Medway, Kent, UK between December 2009–2010. The $\log K_d$ values ranged from 1.58 to 2.72 (K_d: 38–519 L/kg). These results are higher, than obtained by us, but still indicate a weak DCF affinity to sediment. The variable $\log K_d$ values obtained in this study in 2009–2010 may indicate dynamic processes occurring between the sediment and water, as well as different chemico-biological properties of the collected sediments. Interestingly, the partition coefficient values were decreasing along with an increase in suspended sediment concentration. Koba et al. (2018) studied the distribution of selected pharmaceuticals including DCF between water and sediment in pond designed for tertiary wastewater treatment. For this purpose $\log K_d$ was calculated. Calculated average value of $\log K_d$ for DCF was 1.27. Sibeko et al. (2019) also studied distribution of DCF between sediment and water in Mbokodweni river in South Africa. Log K_d calculated for DCF was ranging from 0.13 to 0.91. These results are also slightly higher than results obtained by us. Similar to our study, Dobor et al. (2012) checked DCF affinity to sediment in laboratory conditions. For this purpose, sediment was collected in the urban area of Danube River. After sampling sediment was dried, sieved through a 1 mm sieve and put into glass bottles. Next, distilled water was added and then, sediment was spiked with DCF to obtain 4×10^{-7} M. Samples were taken few times over 24 h. Obtained $\log K_d$ results for DCF were ranging from -0.70–0.15. These values are higher than in our study.

All aforementioned results indicate poor distribution of DCF in sediment, both in environment like in laboratory conditions. Unfortunately, due to variations in $\log K_d$ values between studies, it is difficult to determine the role of DCF and its metabolite sorption to sediments without knowing the biological and chemical parameters of the

sediments (OECD, 106). Chemico-biological properties of sediments are likely to play a crucial role in the sorption of pharmaceuticals. For example, Scheytt et al. (2005) observed that the organic carbon content of sediments plays a key role in the sorption of pharmaceuticals in sediments. Therefore, the sorption coefficient between organic carbon and water (log K_{oc}) is another important factor illustrating the susceptibility of the studied compound to sorption in sediments. pH and salinity have also key role in pharmaceuticals sorption to sediments. In addition, DCF is negatively charged like the mineral fraction of sediment, hence the repulsion and low sorption when sediment has little organics fraction (Margon et al., 2009; De Ridder et al., 2011). The extrapolation of values obtained in the laboratory to the environment can be difficult due to the high complexity and dynamics of the processes that occur in the field.

4. Conclusions

In order to study the biodegradation potential of DCF and its metabolite in the site that is chronically exposed to the pharmaceutical residues, the sediment and water samples were collected near the "Dębogórze" WWTP outlet. To avoid the photodegradation processes, the exposure experiment was carried out in the darkness under fully controlled laboratory conditions. Interestingly, for both DCF and 4-OH DCF, the mass loss was observed in the tanks with sediment and sterilized sediment, which, however, was not related to the adsorption of chemicals on the sediment particles (only a small amount was detected in the sediment). In addition, 5-OH DCF was detected in sediment with DCF in each of the three replicates, while in autoclaved sediment any hydroxy metabolite was detected. Moreover, DCF concentration was significantly lower in sediment compared to autoclaved sediment on days 17, 21 and 29 and these differences were statistically significant. These findings indicate that microorganisms present in sediment near the "Dębogórze" WWTP outlet may effectively eliminate DCF from the water column. However, it should be remembered that biodegradation does not contribute to the complete elimination of a compound, but to its transformation to completely different compounds, which also may pose a risk to marine organisms.

In contrast, no hydroxy metabolites were detected in water from each DCF tanks after 29 days. This result indicates that biotic degradation may not be as efficient in water as in sediment, or different metabolites are formed in water than in sediment. In our study, we only focused on two metabolites (4-OH DCF and 5-OH DCF). Therefore, in future studies, it would be worthwhile to conduct a non-targeted analysis of metabolites formed in both sediment and water from environment.

For 4-OH DCF, a higher concentration decrease and mass reduction was observed in each tank with sediment compared to DCF. This result, in turn, may indicate that metabolite is more easily degradable (biotically or abiotically) or less stable than parent compound.

High stability of DCF and its common metabolite 4-OH DCF in the saltwater tanks without sediment is of great concern. Only a slight decrease in the concentration of both compounds was found after 29 days under stable close to environmental conditions. Thus, DCF and its metabolite may pose a serious threat to pelagic marine organisms, that may be exposed to both compounds for extended periods of time, absorbing and accumulating them.

So far, most studies have focused on the biodegradation of pharmaceuticals by microorganisms present in sewage treatment plant sludge, and only a few have investigated their biodegradation by microorganisms present in the environment - mainly in river sediments. Therefore, the next step is to investigate the biodegradation potential of microorganisms inhabiting marine sediments. There is also a lack of studies calculating the mass balance of DCF and 4-OH DCF in different environmental matrices. Few works have calculated the solid-water partition coefficient, but the results varied significantly. Mass balance would be, therefore, a more reliable indicator of the fate of pharmaceuticals.

Author statement

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Declaration of competing interest

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

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Appendix A. Supplementary data

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Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Świacka, K., Maculewicz, J., Smolarz, K., Szaniawska, A., & Caban, M. (2019). Mytilidae as model organisms in the marine ecotoxicology of pharmaceuticals-a review. Environmental Pollution, 254, 113082.

wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: koncepcję, edycję manuskryptu oraz opiekę merytoryczną

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Gdynia, 02.02.2023 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Świacka, K., Maculewicz, J., Smolarz, K., Szaniawska, A., & Caban, M. (2019). Mytilidae as model organisms in the marine ecotoxicology of pharmaceuticals-a review. Environmental Pollution, 254, 113082.

wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: edycję manuskryptu

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka, Magda Caban

Analiza danych literaturowych- Klaudia Świacka

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu-Katarzyna Smolarz, Magda Caban, Klaudia Świacka, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban, Anna Szaniawska

Jakub Maculewicz

dr hab. Magda Caban Prof. UG
Katedra Analizy Środowiska
Wydział Chemii, UG

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OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Świacka, K., Smolarz, K., Maculewicz, J., & Caban, M. (2020). Effects of environmentally relevant concentrations of diclofenac in Mytilus trossulus. Science of the Total Environment, 737, 139797.

wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: edycję manuskryptu oraz opiekę merytoryczną

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka, Katarzyna Smolarz

Przeprowadzenie eksperymentu- Klaudia Świacka, Jakub Maculewicz

Przeprowadzenie badań laboratoryjnych- Klaudia Świacka

Analiza i interpretacja danych- Klaudia Świacka, Katarzyna Smolarz

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu- Klaudia Świacka, Jakub Maculewicz, Katarzyna Smolarz, Magda Caban

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

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Świacka, K., Smolarz, K., Maculewicz, J., & Caban, M. (2020). Effects of environmentally relevant concentrations of diclofenac in Mytilus trossulus. Science of the Total Environment, 737, 139797.

wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: koncepcję, pomoc w analizie i interpretacji danych (analiza histologiczna), edycję manuskryptu oraz opiekę merytoryczną

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka, Katarzyna Smolarz

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Analiza i interpretacja danych- Klaudia Świacka, Katarzyna Smolarz

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu- Klaudia Świacka, Jakub Maculewicz, Katarzyna Smolarz, Magda Caban

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

Katarzyna Smolarz

mgr Jakub Maculewicz
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wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: pomoc w przeprowadzeniu eksperymentu oraz edycję manuskryptu

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

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Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

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Analiza danych literaturowych- Klaudia Świacka, Alicja Michnowska, Jakub Maculewicz

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka, Alicja Michnowska, Jakub Maculewicz

Edycja manuskryptu-Katarzyna Smolarz, Magda Caban, Klaudia Świacka, Jakub Maculewicz

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Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

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Analiza danych literaturowych- Klaudia Świacka, Alicja Michnowska, Jakub Maculewicz

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka, Alicja Michnowska, Jakub Maculewicz

Edycja manuskryptu-Katarzyna Smolarz, Magda Caban, Klaudia Świacka, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz

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Koncepcja- Klaudia Świacka, Katarzyna Smolarz, Magda Caban

Przeprowadzenie eksperymentu- Klaudia Świacka

Przeprowadzenie badań laboratoryjnych- Klaudia Świacka, Jakub Maculewicz, Magda Caban, Alicja Michnowska

Analiza i interpretacja danych: Klaudia Świacka

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu- Klaudia Świacka, Katarzyna Smolarz, Magda Caban, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

Caban Magda

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Pisanie wstępnej wersji manuskryptu- Klaudia Świacka, Alicja Michnowska, Jakub Maculewicz

Edycja manuskryptu-Katarzyna Smolarz, Magda Caban, Klaudia Świacka, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz

Jakub Maculewicz

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Zakład Funkcjonowania Ekosystemów Morskich
Instytut Oceanografii Uniwersytetu Gdańskiego

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wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: koncepcję, edycję manuskryptu oraz opiekę merytoryczną

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka, Katarzyna Smolarz, Magda Caban

Przeprowadzenie eksperymentu- Klaudia Świacka

Przeprowadzenie badań laboratoryjnych- Klaudia Świacka, Jakub Maculewicz, Magda Caban, Alicja Michnowska

Analiza i interpretacja danych: Klaudia Świacka

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu- Klaudia Świacka, Katarzyna Smolarz, Magda Caban, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

Katarzyna Smolarz

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Zakład Funkcjonowania Ekosystemów Morskich
Instytut Oceanografii Uniwersytetu Gdańskiego

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Świacka, K., Smolarz, K., Maculewicz, J., Michnowska, A., & Caban, M. (2021). Exposure of Mytilus trossulus to diclofenac and 4′-hydroxydiclofenac: Uptake, bioconcentration and mass balance for the evaluation of their environmental fate. Science of the Total Environment, 791, 148172.

wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: pomoc w przeprowadzeniu badań laboratoryjnych (izolacja materiału do analiz biochemicznych)

Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Koncepcja- Klaudia Świacka, Katarzyna Smolarz, Magda Caban

Przeprowadzenie eksperymentu- Klaudia Świacka

Przeprowadzenie badań laboratoryjnych- Klaudia Świacka, Jakub Maculewicz, Magda Caban, Alicja Michnowska

Analiza i interpretacja danych: Klaudia Świacka

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu- Klaudia Świacka, Katarzyna Smolarz, Magda Caban, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

Alicja Michnowska

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Wydział Chemii, UG

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Świacka, K., Smolarz, K., Maculewicz, J., Michnowska, A., & Caban, M. (2021). Exposure of Mytilus trossulus to diclofenac and 4′-hydroxydiclofenac: Uptake, bioconcentration and mass balance for the evaluation of their environmental fate. Science of the Total Environment, 791, 148172.

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Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka, Katarzyna Smolarz, Magda Caban

Przeprowadzenie eksperymentu- Klaudia Świacka

Przeprowadzenie badań laboratoryjnych- Klaudia Świacka, Jakub Maculewicz, Magda Caban, Alicja Michnowska

Analiza i interpretacja danych: Klaudia Świacka

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

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Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

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Świacka, K., Maculewicz, J., Kowalska, D., Caban, M., Smolarz, K., & Świeżak, J. (2022). Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms–current state of knowledge. Journal of Hazardous Materials, 424, 127350.

wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: edycję manuskryptu

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka

Analiza danych literaturowych- Klaudia Świacka, Jakub Maculewicz, Dorota Kowalska, Justyna Świeżak

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka, Jakub Maculewicz, Dorota Kowalska, Justyna Świeżak

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Opieka merytoryczna- Katarzyna Smolarz

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Opieka merytoryczna- Katarzyna Smolarz

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Dr Justyna Świeżak
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Opieka merytoryczna- Katarzyna Smolarz

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Katedra Analizy Środowiska
Wydział Chemii, UG

Gdynia, 02.02.2023 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Świacka, K., Maculewicz, J., Kowalska, D., Caban, M., Smolarz, K., & Świeżak, J. (2022). Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms–current state of knowledge. Journal of Hazardous Materials, 424, 127350.

wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: analizę danych literaturowych, pisanie wstępnej wersji manuskryptu oraz edycję manuskryptu

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka

Analiza danych literaturowych- Klaudia Świacka, Jakub Maculewicz, Dorota Kowalska, Justyna Świeżak

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka, Jakub Maculewicz, Dorota Kowalska, Justyna Świeżak

Edycja manuskryptu-Katarzyna Smolarz, Magda Caban, Klaudia Świacka, Jakub Maculewicz, Dorota Kowalska, Justyna Świeżak

Opieka merytoryczna- Katarzyna Smolarz

Jakub Maculewicz

dr hab. Magda Caban Prof. UG
Katedra Analizy Środowiska
Wydział Chemii, UG

Gdynia, 02.02.2023 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Świacka, K., Maculewicz, J., Świeżak, J., Caban, M., & Smolarz, K. (2022). A multi-biomarker approach to assess toxicity of diclofenac and 4-OH diclofenac in Mytilus trossulus mussels-First evidence of diclofenac metabolite impact on molluscs. Environmental Pollution, 315, 120384.

wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: edycję manuskryptu i opiekę merytoryczną

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka, Katarzyna Smolarz

Przeprowadzenie eksperymentu- Klaudia Świacka, Jakub Maculewicz

Przeprowadzenie badań laboratoryjnych- Klaudia Świacka, Justyna Świeżak

Analiza i interpretacja danych- Klaudia Świacka, Katarzyna Smolarz, Justyna Świeżak

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu- Klaudia Świacka, Justyna Świeżak, Katarzyna Smolarz, Magda Caban, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

Caban Magda

dr hab. Katarzyna Smolarz Prof. UG
Zakład Funkcjonowania Ekosystemów Morskich
Instytut Oceanografii Uniwersytetu Gdańskiego

Gdynia, 02.02.2023 r.

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Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Świacka, K., Maculewicz, J., Świeżak, J., Caban, M., & Smolarz, K. (2022). A multi-biomarker approach to assess toxicity of diclofenac and 4-OH diclofenac in Mytilus trossulus mussels-First evidence of diclofenac metabolite impact on molluscs. Environmental Pollution, 315, 120384.

wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: koncepcję, pomoc w analizie i interpretacji danych (analiza histologiczna), edycję manuskryptu oraz opiekę merytoryczną

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka, Katarzyna Smolarz

Przeprowadzenie eksperymentu- Klaudia Świacka, Jakub Maculewicz

Przeprowadzenie badań laboratoryjnych- Klaudia Świacka, Justyna Świeżak

Analiza i interpretacja danych- Klaudia Świacka, Katarzyna Smolarz, Justyna Świeżak

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu- Klaudia Świacka, Justyna Świeżak, Katarzyna Smolarz, Magda Caban, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

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wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: pomoc w przeprowadzeniu badań laboratoryjnych, analizie i interpretacji danych oraz edycję manuskryptu

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka, Katarzyna Smolarz

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Przeprowadzenie badań laboratoryjnych- Klaudia Świacka, Justyna Świeżak

Analiza i interpretacja danych- Klaudia Świacka, Katarzyna Smolarz, Justyna Świeżak

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Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

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Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka, Katarzyna Smolarz

Przeprowadzenie eksperymentu- Klaudia Świacka, Jakub Maculewicz

Przeprowadzenie badań laboratoryjnych- Klaudia Świacka, Justyna Świeżak

Analiza i interpretacja danych- Klaudia Świacka, Katarzyna Smolarz, Justyna Świeżak

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu- Klaudia Świacka, Justyna Świeżak, Katarzyna Smolarz, Magda Caban, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

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Świacka, K., Maculewicz, J., Smolarz, K., & Caban, M. (2022). Long-term stability of diclofenac and 4-hydroxydiclofenac in the seawater and sediment microenvironments: Evaluation of biotic and abiotic factors. Environmental Pollution, 304, 119243.

wchodzącej w skład rozprawy doktorskiej Pani mgr Klaudii Świackiej pt. „Los środowiskowy i ocena toksyczności wybranych farmaceutyków i ich metabolitów z wykorzystaniem *Mytilus trossulus* jako gatunku modelowego" obejmował: koncepcję, edycję manuskryptu oraz opiekę merytoryczną

Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

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Analiza i interpretacja danych- Klaudia Świacka

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu- Klaudia Świacka, Katarzyna Smolarz, Magda Caban, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

Katarzyna Smolarz

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Oświadczam również, że wkład pozostałych współautorów w powstanie niniejszej pracy był następujący:

Koncepcja- Klaudia Świacka, Jakub Maculewicz, Katarzyna Smolarz, Magda Caban

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Przeprowadzenie badań laboratoryjnych- Klaudia Świacka, Jakub Maculewicz

Analiza i interpretacja danych- Klaudia Świacka

Pisanie wstępnej wersji manuskryptu- Klaudia Świacka

Edycja manuskryptu- Klaudia Świacka, Katarzyna Smolarz, Magda Caban, Jakub Maculewicz

Opieka merytoryczna- Katarzyna Smolarz, Magda Caban

Jakub Maculewicz