

Wydział Chemii Uniwersytetu Gdańskiego

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**Znaczenie czynników środowiskowych w ocenie
oddziaływania farmaceutyków na organizmy
wodne w kontekście zmiany klimatu**

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*za to, że nauczyli mnie wierzyć w marzenia,
nie poddawać się w chwilach zwątpienia
i odważnie podążać własną drogą.
Za każde słowo otuchy,
gdy najbardziej go potrzebowałam.*

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P1 Bethke Katarzyna, Kropidłowska Klaudia, Stepnowski Piotr, Caban Magda*, Review of warming and acidification effects to the ecotoxicity of pharmaceuticals on aquatic organisms in the era of climate change, *Science of the Total Environment*, 2023, vol. 877, s. 1-24, DOI: 10.1016/j.scitotenv.2023.162829, 200 punktów, IF₂₀₂₃ 8,2 (Q1)

P2 Bethke Katarzyna, Kwidzińska Klaudia, Caban Magda*, Investigation of pharmaceutical bioaccumulation in *Daphnia* sp. living in a wastewater treatment plant, *Science of the Total Environment*, 2024, vol. 950, s. 1-8, DOI: 10.1016/j.scitotenv.2024.174915, 200 punktów, IF₂₀₂₄ 8,0 (Q1)

P3 Bethke Katarzyna*, Caban Magda, Effect of acidification on the chronic toxicity of diclofenac to *Daphnia magna*, *Aquatic Toxicology*, 2025, vol. 287, s. 1-7, DOI: 10.1016/j.aquatox.2025.107497, 140 punktów, IF₂₀₂₅ 4,6 (Q1)

P4 Bethke Katarzyna*, Caban Magda, Exploring the impact of pH on the toxicological response of *Lemna minor* to pharmaceuticals - insights from sulfamethoxazole exposure, *Environmental Toxicology and Chemistry*, 2026, vol. 45, s. 2610-2621, DOI: 10.1093/etoxjnl/vgag136, 100 punktów, IF₂₀₂₅ 3,6 (Q2)

P5 Bethke Katarzyna, Caban Magda*, Context-Dependent Toxicity of Diclofenac in *Daphnia magna*: Roles of Water Chemistry, Neonate Source and Preparation Procedure, *Aquatic Toxicology*, 2026, DOI: 10.1016/j.aquatox.2026.108029, 140 punktów, IF₂₀₂₅ 4,6 (Q1)

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²Sumaryczna liczba cytowań (bez autocytowań) według *Web of Science Core Collection*.

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Wykaz skrótów

2-OH IBF - 2-hydroksydiklofenak

4-OH DCF - 4-hydroksydiklofeefnak

ACN - acetonitryl

ADME - absorpcja, dystrybucja, metabolizm, eliminacja (ang. *Absorption, Distribution, Metabolism and Excretion*)

ANOVA - analiza wariancji (ang. *analysis of variance*)

BAF - współczynnik bioakumulacji (ang. *Bioaccumulation Factor*)

BCF - współczynnik biokoncentracji (ang. *Bioconcentration Factor*)

CFA - kwas klofibrowy

DCF - diklofenak

DHPS - enzym syntaza dihydropteroianowa

EC₅₀ - stężenie badanej substancji chemicznej, które u 50% badanych organizmów wywołuje określony skutek negatywny (ang. *half maximal effective concentration*)

EE-Neo - osobniki *Daphnia magna* wyklute bezpośrednio z efiów

EPA - Amerykańska Agencja Ochrony Środowiska (ang. *Environmental Protection Agency*)

GLM - uogólniony model liniowy (ang. *generalized linear model*)

HELCOM - Komisja Ochrony Środowiska Morskiego Bałtyku (ang. *Baltic Marine Environment Protection Commission*)

HPLC/DAD - wysokosprawna chromatografia cieczowa z detektorem matrycy fotodiodowej (ang. *High Performance Liquid Chromatography with Diode Array Detector*)

IBF - ibuprofen

IC - chromatografia jonowa (ang. *Ion Chromatography*)

LL.4 - czteroparametrowy nieliniowy model log-logistyczny (ang. *four-parameter log-logistic model*)

m.m. - mokra masa

M-DCF - metylowy ester diklofenaku

MRM - tryb wielokrotnego monitorowania reakcji (ang. *Multiple Reaction Monitoring*)

NLPZ - niesteroidowe leki przeciwzapalne

NPX – naproksen

OECD - Organizacja Współpracy Gospodarczej i Rozwoju (ang. *Organisation for Economic Co-operation and Development*)

P-Neo - osobniki *Daphnia magna* pochodzące z długotrwałej hodowli laboratoryjnej, uzyskane w wyniku rozmnażania partenogenetycznego

QSAR - analiza ilościowych zależności między strukturą chemiczną a aktywnością biologiczną (ang. *Quantitative Structure-Activity Relationship*)

ROS - reaktywne formy tlenu (ang. *Reactive Oxygen Species*)

s.m. - sucha masa

SLE — ekstrakcja ciało stałe-ciecz (ang. *Solid-Liquid-Extraction*)

SMX — sulfametoksazol

SPE - ekstrakcja do fazy stałej (ang. *Solid-Phase Extraction*)

UHPLC-MS/MS - ultrawysokosprawna chromatografia cieczowa sprzężona z tandemową spektrometrią mas (ang. *Ultra-high-performance Liquid Chromatography coupled with Tandem Mass Spectrometry*)

WoE - podejście oparte na wadze dowodów (ang. *Weight of Evidence*)

1. Streszczenie

Współcześnie ekosystemy wodne narażone są na czynniki stresowe związane z postępującą zmianą klimatu, takie jak wzrost temperatury oraz zakwaszenie środowiska wodnego. Istotnym zagrożeniem dla środowiska naturalnego są także substancje pochodzenia antropogenicznego, w tym nowo pojawiające się zanieczyszczenia, do których należą farmaceutyki. Związki te trafiają do wód środowiskowych głównie na skutek niepełnego usuwania w konwencjonalnych oczyszczalniach ścieków. Ze względu na aktywność biologiczną mogą one, nawet przy niskich stężeniach, oddziaływać na organizmy niecelowe. Ich toksyczność może ulegać zmianie pod wpływem temperatury i pH środowiska wodnego, co podsumowano w przeglądzie literaturowym stanowiącym część niniejszej rozprawy doktorskiej. W przypadku farmaceutyków jonizowalnych pH wpływa na ich formę jonową i biodostępność, zwłaszcza gdy wartość pKa związku jest zbliżona do pH wód naturalnych. Przykładem są niektóre sulfonamidy - antybiotyki, które są jednymi z najczęściej wykrywanych farmaceutyków w próbkach wód środowiskowych. Inną grupą farmaceutyków, których obecność odnotowuje się w środowisku na poziomie ng/l-μg/l, są niesteroidowe leki przeciwzapalne (NLPZ).

Dlatego też celem niniejszej rozprawy doktorskiej była ocena znaczenia pH, składu medium testowego oraz źródła i sposobu przygotowania organizmów testowych w kształtowaniu wyników ostrej i przewlekłej toksyczności sulfametoksazolu (SMX) i diklofenaku (DCF) wobec organizmów referencyjnych, a także w interpretacji testów opartych na wytycznych Organizacji Współpracy Gospodarczej i Rozwoju (OECD). Realizację postawionych celów rozpoczęto od badań przeprowadzonych w warunkach rzeczywistych, które wykazały możliwość bioakumulacji DCF w organizmach *Daphnia* sp. bytujących w oczyszczalni ścieków. Analiza celowana farmaceutyków z grupy NLPZ, z wykorzystaniem ultrawysokosprawnej chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (UHPLC-MS/MS), wykazała obecność DCF w organizmach rozwielitek oraz wskazała na możliwość funkcjonowania ich populacji w środowisku przewlekłe narażonym na mieszaninę zanieczyszczeń. Badania te stanowiły punkt wyjścia do dalszych badań laboratoryjnych.

W celu oceny wpływu obniżenia pH na toksyczność farmaceutyków oraz weryfikacji przydatności testów opartych na wytycznych OECD przeprowadzono szereg testów ekotoksykologicznych o krótkim i długim czasie ekspozycji, zgodnie z OECD 202, OECD 211 oraz OECD 221. Wykorzystano organizmy referencyjne, takie jak *Daphnia magna* oraz *Lemna minor*. Realizacja celu rozprawy doktorskiej obejmowała również analizę wpływu warunków prowadzenia testów, takich jak skład medium hodowlanego, stabilność parametrów fizykochemicznych, a także źródło i sposób przygotowania organizmów testowych na uzyskane wyniki ekotoksykologiczne.

Badania ostrej toksyczności SMX wobec *L. minor*, ocenianej na podstawie zahamowania wzrostu, wykazały zwiększenie toksyczności leku wraz ze spadkiem pH. Zakwaszenie spowodowało wzrost udziału neutralnej formy leku, bardziej dostępnej biologicznie dla rośliny. W przypadku DCF zakwaszenie zwiększało jego toksyczność przewlekłą, wpływając na parametry reprodukcyjne, m.in. na czas do pojawienia się pierwszego miotu. Co ważne, wyniki wskazały, że obniżenie pH może również modyfikować odpowiedź biologiczną organizmu, a w konsekwencji jego wrażliwość na zanieczyszczenie. Nie bez znaczenia w ocenie toksyczności DCF wobec *D. magna* (OECD 202) okazały się rodzaj medium oraz źródło i sposób przygotowania organizmów testowych. Różnice między zastosowanymi mediami, w tym ich skład jonowy, a także różnice w źródle i sposobie przygotowania organizmów testowych wiązały się z istotnym statystycznie zróżnicowaniem wartości EC₅₀.

Uzyskane wyniki wskazują, że testy OECD stanowią podstawowe narzędzie oceny ekotoksyczności, jednak interpretacja ich wyników w przypadku farmaceutyków jonizowalnych powinna uwzględniać wpływ zmiennych warunków środowiskowych zarówno na właściwości badanych związków, jak i na odpowiedź organizmów wodnych. Szczególne znaczenie ma monitorowanie i raportowanie pH, które wpływa nie tylko na formę jonową farmaceutyków, ale również na odpowiedź biologiczną i wrażliwość badanych organizmów. Ponadto wiarygodna ocena wyników wymaga szczegółowego raportowania historii hodowli organizmów testowych, a także pełnej charakterystyki medium, obejmującej również jego skład jonowy. Podsumowując, przedstawione w niniejszej pracy doktorskiej wyniki wskazują na potrzebę uwzględnienia zmienności warunków środowiskowych i metodycznych oraz szerszej charakterystyki organizmów testowych podczas interpretacji i raportowania wyników testów opartych na wytycznych OECD, co może pozwolić na bardziej rzetelną ocenę ryzyka środowiskowego związanego z obecnością farmaceutyków w warunkach zmieniającego się klimatu.

2. Abstract (w j. angielskim)

Aquatic ecosystems are currently exposed to stressors associated with ongoing climate change, including rising temperatures and acidification of the aquatic environment. Anthropogenic substances, including emerging contaminants such as pharmaceuticals, also pose a significant threat to the natural environment. These compounds enter natural waters mainly because of their incomplete removal in conventional wastewater treatment plants. Due to their biological activity, pharmaceuticals may affect non-target organisms even at low concentrations.

Their toxicity may be modified by changes in water temperature and pH, as summarized in the literature review included in this doctoral dissertation. In the case of ionizable pharmaceuticals, pH affects their ionic form and bioavailability, particularly when the pK_a of a compound is close to the pH of natural waters. Examples include some sulfonamides, a class of antibiotics among the pharmaceuticals most frequently detected in environmental water samples. Another group commonly detected in the environment at concentrations ranging from ng L⁻¹ to µg L⁻¹ is non-steroidal anti-inflammatory drugs (NSAIDs).

Therefore, this doctoral dissertation aimed to assess the importance of pH, test medium composition, and the source and preparation procedure of test organisms in shaping the outcomes of acute and chronic toxicity tests of sulfamethoxazole (SMX) and diclofenac (DCF) in reference organisms, as well as in the interpretation of tests based on the guidelines of the Organisation for Economic Co-operation and Development (OECD).

The research began with studies conducted under environmentally relevant conditions, which indicated the potential for DCF bioaccumulation in *Daphnia* sp. inhabiting a wastewater treatment plant. Targeted analysis of NSAIDs using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS) confirmed the presence of DCF in daphnids, while field observations indicated that their populations could persist in an environment chronically exposed to a mixture of contaminants. These studies provided a basis for subsequent laboratory experiments.

To assess the effect of reduced pH on pharmaceutical toxicity and evaluate the applicability of tests based on OECD guidelines, a series of short- and long-term ecotoxicity tests was conducted according to OECD 202, OECD 211, and OECD 221. The reference organisms *Daphnia magna* and *Lemna minor* were used. The scope of the dissertation also included an analysis of how test conditions, including culture medium composition, the stability of physicochemical parameters, and the source and preparation procedure of test organisms, affected the ecotoxicological results.

Acute SMX toxicity to *L. minor*, assessed based on growth inhibition, increased with decreasing pH. Acidification increased the proportion of the neutral form of SMX, which is more bioavailable to the plant. In the case of DCF, acidification increased its chronic toxicity and affected reproductive endpoints, including the time to first brood production. Importantly, the results indicated that reduced pH may also modify the biological response of the test organism and, consequently, its sensitivity to the contaminant. Test medium type and the source and preparation procedure of test organisms were also associated with differences in DCF toxicity to *D. magna* in the OECD 202 test. Differences in these factors were associated with statistically significant variation in EC₅₀ values.

The results indicate that OECD tests are fundamental tools for assessing ecotoxicity. However, when applied to ionizable pharmaceuticals, their interpretation should account for the effects of variable environmental conditions on both the properties of the tested compounds and the responses of aquatic organisms. Particular attention should be paid to monitoring and reporting pH, as this parameter affects not only the ionic form of pharmaceuticals but also the biological responses and sensitivity of the test organisms. A reliable assessment also requires detailed reporting of the culture history of test organisms and comprehensive characterization of the test medium, including its ionic composition. In conclusion, the findings of this doctoral dissertation highlight the need to consider variability in environmental and methodological conditions, as well as the source and preparation procedure of test organisms, when interpreting and reporting the results of tests based on OECD guidelines. This approach may contribute to a more robust environmental risk assessment of pharmaceuticals under a changing climate.

3. Wstęp

Farmaceutyki i ich metabolity należą do wyzwań współczesnej ekotoksykologii i chemii środowiska. Określa się je mianem nowopojawiających się zanieczyszczeń (ang. *emerging contaminants*). Zaprojektowane w taki sposób, by w jak najmniejszych dawkach wywoływały efekt biologiczny, stanowią poważne zagrożenie dla środowiska naturalnego i żyjących w nim, niebędących ich celem terapeutycznym, organizmów. Powszechne stosowanie farmaceutyków wśród ludzi i zwierząt oraz brak ich efektywnej eliminacji w oczyszczalniach ścieków sprawiają, że są one wykrywane w ściekach oczyszczonych oraz różnych komponentach środowiska, w tym osadach dennych czy wodach powierzchniowych i podziemnych (stężenie rzędu ng/l-μg/l dla wód oraz ng/g w matrycach stałych) (Kołecka et al., 2022; Maszkowska et al., 2013). Szacuje się, że ich wykrywalność w środowisku będzie rosła (Caban & Stepnowski, 2021). Wiąże się to z większą dostępnością metodyk i technik ich analizy oraz wzrastającą liczbą regulacji prawnych wymuszających monitoring.

Pomimo stałego dostarczania dużej ilości informacji potrzebnych do oceny ryzyka i zagrożenia środowiskowego, farmaceutyki, w porównaniu z szerzej opisanymi w literaturze grupami takimi jak pestycydy, trwałe zanieczyszczenia organiczne czy metale ciężkie, stanowią nadal istotny problem badawczy. Farmaceutyki są bowiem bardzo zróżnicowaną grupą analitów, dodatkowo różniących się pod względem budowy i właściwości fizykochemicznych od trwałych zanieczyszczeń typu metali ciężkich czy wielopierścieniowych węglowodorów aromatycznych. Przykładowo, wykazują różne właściwości kwasowo-zasadowe, które determinują ich stopień jonizacji w zależności od pH środowiska. To z kolei wpływa na ich lipofilowość, a tym samym na zdolność do przenikania przez błony komórkowe. Co ważne, różnice we właściwościach fizykochemicznych tej grupy zanieczyszczeń można zaobserwować już w obrębie jednej klasy leków. Przykładem są β-blokery, które pomimo takich samych grup funkcyjnych charakteryzują się szerokim zakresem lipofilowości. Natomiast przedstawiciele sulfonamidów posiadają różne dodatkowe grupy funkcyjne, czego konsekwencją jest duża zmiana stopnia zjonizowania wraz ze zmianą kwasowości środowiska.

Problem obecności farmaceutyków w naturalnych zbiornikach wodnych zaczął się dodatkowo komplikować, odkąd pojawiły się doniesienia naukowe dotyczące wpływu efektów globalnego ocieplenia na ich ekotoksykologię. Wzrost temperatury wód jest jednym z jego skutków i stanowi potencjalny czynnik modyfikujący odpowiedź

organizmów wodnych na obecność zanieczyszczeń. Według Amerykańskiej Agencji Ochrony Środowiska (ang. *Environmental Protection Agency*; EPA) ostatnie dziesięciolecie stanowią najcieplejszy okres w historii pomiarów temperatury, których wartości przekraczają te odnotowane od 1880 roku. Globalnie, średni wzrost temperatury jezior wg Tong et al. (2023) w latach 1981-2020 wynosił ok. $+0,24$ °C/dekadę. Warte odnotowania jest rola procesów hydrologicznych i energetycznych (takich jak parowanie) w procesie ocieplania jezior, ponieważ okazuje się, że ich tempo ocieplania jest wolniejsze niż w przypadku atmosfery ($+0,29$ °C/dekadę). Podwyższenie średnich temperatur odnotowuje się również w wodach słonych, szczególnie w morzach półzamkniętych i śródkontynentalnych, takich jak Bałtyk ($0,2$ - $0,6$ °C/dekadę). Warto zaznaczyć, że zarówno w zbiornikach wody słodkiej, jak i w słonej istotne znaczenie mają zmiany dobowe oraz sezonowe temperatury, które wpływają na rzeczywistą ekspozycję organizmów wodnych. Aklimatyzacja do nowych warunków temperaturowych wymaga dużego nakładu energii. To z kolei wpływa na procesy metaboliczne i aktywność enzymatyczną, a tym samym na biotransformację i eliminację zanieczyszczeń. Ponadto wzrost temperatury może pośrednio oddziaływać na organizmy wodne poprzez zmianę toksyczności i zachowania zanieczyszczeń w środowisku, w tym poprzez zmianę tempa ich wchłaniania oraz eliminacji. Kierunek i wielkość tych zmian pozostają jednak wciąż trudne do określenia [P1], w przeciwieństwie do wspomnianych już, szerzej poznanych trwałych zanieczyszczeń środowiska, dla których powstały modele prognozujące wpływ zmian klimatu na toksyczność.

Jednym ze skutków postępujących zmian klimatu jest także zakwaszenie środowiska wodnego, będące pochodną wzrostu stężenia dwutlenku węgla w atmosferze. Szacuje się, że oceany pochłonęły około 30% antropogenicznej emisji CO₂, czego następstwem okazał się spadek średniego pH powierzchni oceanów z około 8,2 w okresie sprzed epoki industrialnej do 8,1 obecnie (EPA, 2022; Feely et al., 2004). Oceany charakteryzują się średnim tempem zakwaszania wynoszącym około 0,002 jednostki pH rocznie, natomiast w ciągu kolejnych 100 lat przewiduje się spadek pH o 0,3-0,5 jednostki (Caldeira & Wickett, 2005; Häder & Gao, 2019). Zakwaszenie związane ze wzrostem stężenia CO₂ obserwuje się również w wodach słodkich. Jednak złożoność zachodzących w nich procesów biogeochemicznych, zróżnicowana zdolność buforowa oraz właściwości zlewni utrudniają określenie globalnego tempa zakwaszania tych ekosystemów. W niemieckich zbiornikach zaporowych (lata 1981-2015) udokumentowano spadek pH o około 0,3 jednostki, związany ze wzrostem ciśnienia

parcjalnego CO₂ (Weiss et al., 2018). Badania modelowe wskazują również, że wzrost stężenia CO₂ w atmosferze może powodować obniżenie pH Wielkich Jezior oraz jezior znajdujących się w północno-wschodniej części Stanów Zjednoczonych w skali porównywalnej z zakwaszeniem oceanów (Johannesson et al., 2023; Phillips et al., 2015). Wynikające z tych procesów zmiany pH mogą zaburzać równowagę kwasowo-zasadową organizmów oraz wpływać na przebieg procesów metabolicznych, funkcje fizjologiczne i aktywność enzymów (Feely et al., 2004; Lim et al., 2021). Parametr pH jest szczególnie istotny z punktu widzenia farmaceutyków jako związków jonizowalnych, ponieważ wpływa na ich stopień jonizacji, a tym samym na formę, w jakiej występują w środowisku wodnym. Nie można jednoznacznie przewidzieć kierunku zmiany toksyczności wraz ze zmianą pH. Z założenia jednak związki o właściwościach słabych kwasów będą zwiększały toksyczność wraz ze wzrostem zakwaszenia (Alsop & Wilson, 2019; Kim et al., 2010). Natomiast słabe zasady wykazują odwrotną tendencję - charakteryzują się zmniejszoną toksycznością przy niższym pH, co udowodniono dla sertraliny, fluoksetyny, chlorochiny (Alsop & Wilson, 2019; Rendal, 2011; Valenti et al., 2009). Te zależności są ściśle związane z wartościami pK_a poszczególnych farmaceutyków oraz wynikającym z nich udziałem form neutralnych, co bezpośrednio wpływa na przepuszczalność przez błony i biodostępność leku.

Wśród farmaceutyków szczególną uwagę należy zwrócić na grupy, które są najczęściej wykrywane w wodach naturalnych: niesteroidowe leki przeciwzapalne (NLPZ) oraz antybiotyki. Na potrzeby niniejszej pracy jako przedstawicieli tych grup wytypowano odpowiednio diklofenak (DCF) oraz sulfametoksazol (SMX). Leki te są wykrywane w wodach powierzchniowych w stężeniach rzędu ng/l-μg/l (Białk-Bielińska et al., 2012; Felis et al., 2024; Sathishkumar et al., 2020). Oba farmaceutyki charakteryzują się stosunkowo wysokim zużyciem, niepełnym usuwaniem w konwencjonalnych oczyszczalniach ścieków, a co za tym idzie, wykrywalnością w ściekach oczyszczonych i środowisku (Białk-Bielińska et al., 2012; Felis et al., 2024; KołECKA et al., 2019, 2022; Muter et al., 2017).

Diklofenak jest jednym z farmaceutyków monitorowanych w ramach działań HELCOM (Komisji Ochrony Środowiska Morza Bałtyckiego; ang. *Baltic Marine Environment Protection Commission*) i został uwzględniony na liście substancji priorytetowych dla Morza Bałtyckiego (HELCOM, 2025). Chemicznie DCF jest słabym kwasem (pK_a = 4,15; DrugBank). Jego toksyczność ostra i chroniczna wobec organizmów wodnych jest w literaturze dobrze udokumentowana, a większość badań koncentruje się

na małżach (Świacka et al., 2019, 2020, 2022; Świacka, Michnowska, et al., 2021; Świacka, Smolarz, et al., 2021), rybach (Islas-Flores et al., 2017; Pandey et al., 2017) oraz rozwielitkach (De Oliveira et al., 2016; Haap et al., 2008).

Antybiotyk SMX ($pK_{a2} = 5,7$; DrugBank) jest szczególnie istotny z punktu widzenia monitoringu środowiskowego, ponieważ przyczynia się do rozwoju i rozprzestrzeniania się oporności na antybiotyki u mikroorganizmów (Kergoat et al., 2021). Ponadto wykazano, że SMX wywiera działanie fitotoksyczne (Aubakirova et al., 2017; Białk-Bielińska et al., 2018). U bakterii zakłóca on syntezę kwasu foliowego poprzez inhibicję enzymu syntazy dihydropteroianowej (DHPS), a mechanizm ten może występować również u roślin (Brain et al., 2008; Cheong et al., 2020). Dodatkowo, w odpowiedzi na stres komórkowy, SMX może przyczyniać się do produkcji reaktywnych form tlenu (ang. *Reactive Oxygen Species*; ROS) w roślinach oraz indukować sygnalizację stresową za pośrednictwem kwasu salicyłowego, co ostatecznie powoduje zahamowanie wzrostu roślin (Ma et al., 2025).

W klasycznym podejściu do oceny ryzyka, podejmowane są badania mające na celu wyznaczenie wartości toksyczności ostrej i chronicznej względem organizmów referencyjnych. W pracy podjęto badania z wykorzystaniem dwóch tego typu organizmów zgodnie z wytycznymi Organizacji Współpracy Gospodarczej i Rozwoju (ang. *Organisation for Economic Co-operation and Development*; OECD): *Daphnia magna* oraz *Lemna minor*. Skorupiak *D. magna* charakteryzuje się prostotą hodowli i łatwością karmienia (Tkaczyk et al., 2021). Jest to zwierzę, które umożliwia ocenę wpływu zanieczyszczeń na reprodukcję w stosunkowo krótkim czasie, ponieważ cechuje się krótkim cyklem namnażania. Ogromną zaletą *D. magna* jako organizmu referencyjnego jest przezroczysty pancerz, który umożliwia obserwację jej struktur anatomicznych podczas eksperymentu. Okazuje się, że testy toksyczności *D. magna* mają wysoką wartość predykcyjną, porównywalną z eksperymentami cytotoksyczności u ssaków (Guilhermino et al., 2000). Istotną zaletą rośliny *L. minor* jest możliwość obserwacji jej struktur bez użycia mikroskopu, a przeprowadzenie testu nie wymaga dużego nakładu finansowego (Aubakirova et al., 2017). Zdolność *L. minor* do absorpcji i akumulacji zanieczyszczeń czyni ją organizmem odpowiednim do badań nad ich ekotoksycznością, a podobieństwo genetyczne do roślin macierzystych ogranicza zmienność wyników niezwiązaną z działaniem badanej substancji (Yahya Jawad Sharba et al., 2024). Również szybkie podwojenie biomasy przez wzrost wegetatywny bez wątplenia jest zaletą *L. minor* jako organizmu testowego (Ullah et al., 2023).

Podstawowym narzędziem do oceny zagrożeń stwarzanych przez występujące w środowisku naturalnym zanieczyszczenia, w tym farmaceutyki, są standaryzowane protokoły ekotoksykologiczne OECD. W niniejszej pracy do oceny zmian toksyczności DCF korzystano z wytycznych OECD 202 (Ostry test unieruchomienia *Daphnia* sp.) [P5], OECD 211 (Test reprodukcji *D. magna*) [P3] oraz OECD 221 (Test hamowania wzrostu *Lemna* sp.) [P4]. Testy te stanowią podstawę do rejestracji chemikaliów poprzez wyznaczenie progowych wartości toksyczności dla organizmów środowiskowych. Zaletą testów jest standaryzacja warunków eksperymentalnych, która ma na celu umożliwienie odtworzenia i porównania wyników pomiędzy laboratoriami. Eksperymenty prowadzone w warunkach laboratoryjnych niestety nie odwzorowują warunków panujących w środowisku naturalnym. Jedną z przyczyn jest stosowanie sztucznych mediów hodowlanych, które w myśl ustandaryzowania warunków eksperymentalnych zawierają stały, ściśle scharakteryzowany skład chemiczny [P5]. Natomiast wody środowiskowe stanowią otwarty, dynamiczny układ chemiczny, w którym zachodzą procesy takie jak wymiana gazowa z atmosferą, procesy fotosyntezy, interakcja z osadami, rozkład materii organicznej i wiele innych, które wpływają na zmienny chemizm zbiornika. Dobowe i sezonowe zmiany temperatury w zbiornikach wodnych, wahania pH, twardość czy przewodność wody mogą wpływać na fizjologię żyjących w nich organizmów, a także na formę jonową i biodostępność obecnych w nich zanieczyszczeń. W konsekwencji wyniki uzyskane w laboratorium nie zawsze znajdą odzwierciedlenie w środowisku naturalnym. Choć niemożliwe jest uwzględnienie wszystkich potencjalnych korelacji parametrów środowiskowych w badaniach laboratoryjnych, obecnie istotne jest przybliżanie warunków testowych do tych panujących w środowisku. W związku z opisanymi wcześniej skutkami zmian klimatu, konieczne jest uwzględnienie w badaniach ekotoksykologicznych zmian parametru pH czy temperatury [P1, P3, P4]. Źródłem zmienności okazują się również same organizmy referencyjne, nawet w obrębie jednego gatunku [P5]. Warunki hodowli organizmów, mimo ich pochodzenia z tej samej linii rodzicielskiej, mogą skutkować odmienną wrażliwością na badane zanieczyszczenia lub warunki eksperymentalne, co może prowadzić do rozbieżności w uzyskiwanych wynikach badań ekotoksykologicznych, a w konsekwencji wpływać na ocenę ryzyka środowiskowego. W związku z potrzebą urealnienia oceny zagrożenia, jakie niesie za sobą obecność farmaceutyków w środowisku, istnieje wzmożone zapotrzebowanie na przeprowadzanie eksperymentów *in situ*. Praca P2 jest jedyną, według naszej wiedzy, opisującą badania bioakumulacji NLPZ u *Daphnia pulex* w warunkach rzeczywistych,

na podstawie wyznaczonych współczynników bioakumulacji (ang. *Bioaccumulation Factor*; BAF). Biokoncentrację DCF w warunkach laboratoryjnych przedstawiono natomiast w pracy (Nkoom et al., 2019), w której udokumentowano zależność wartości współczynnika biokoncentracji (ang. *Bioconcentration Factor*; BCF) od stężenia ekspozycyjnego DCF. Tendencja ta została zauważona już przez Schwaiger et al. (2004). Należy jednak uwzględnić różnicę między oboma współczynnikami. W przypadku współczynnika BCF (**Równanie 1**), w przeciwieństwie do BAF (**Równanie 2**), nie uwzględnia się innych dróg narażenia niż pobieranie zanieczyszczeń bezpośrednio z wody.

$$\text{BCF [l/kg]} = \frac{C_{\{organizm\}}}{C_{\{woda\}}} \quad (1)$$

$$\text{BAF [l/kg]} = \frac{C_{\{organizm\}}}{C_{\{\text{środowisko}\}}} \quad (2)$$

gdzie:

C_{organizm} - stężenie badanej substancji w organizmie [mg/kg mokrej masy (m.m.) lub suchej masy (s.m.)];

C_{woda} - stężenie badanej substancji w wodzie [mg/l];

$C_{\text{środowisko}}$ - stężenie badanej substancji w otaczającym środowisku (woda, pokarm, cząstki zawieszone) [mg/l lub mg/kg - zależnie od matrycy].

Współczynnik BCF jest parametrem, który jest stosowany głównie do oceny bioakumulacji organicznych substancji chemicznych, najczęściej występujących w formach neutralnych i hydrofobowych. Klasycznie definiuje się go dla warunków stanu równowagi. W przypadku farmaceutyków i innych jonizowalnych związków organicznych interpretacja wyników wymaga uwzględnienia stopnia dysocjacji wynikającego z warunków środowiskowych, takich jak pH (Arnot et al., 2023). Obecnie ocena bioakumulacji, zgodnie z podejściem opartym na wadze dowodów (ang. *Weight of Evidence*; WoE), integruje wiele linii dowodowych, w tym dane uzyskane w laboratorium (np. BCF, współczynnik biomagnifikacji), dane uzyskane w terenie (np. BAF), a także modele kinetyczne oraz modele analiz ilościowych zależności między strukturą chemiczną a aktywnością biologiczną (ang. *Quantitative Structure-Activity Relationship*; QSAR). W przypadku farmaceutyków podejście WoE jest często wspierane

metodologią typu „*read-across*”, która uzupełnia dane eksperymentalne o informacje mechanistyczne i literaturowe pochodzące m.in. z badań dotyczących absorpcji, dystrybucji, metabolizmu i eliminacji (ang. *Absorption, Distribution, Metabolism and Excretion*; ADME) (Armitage et al., 2017). Jest to szczególnie ważne, ponieważ farmaceutyki zaprojektowano z myślą o oddziaływaniu na organizmy ssaków, w związku z czym ich procesy metaboliczne i transportowe są dobrze opisane w literaturze. Jednakże parametry bioakumulacji wyznaczone na podstawie danych pochodzących z badań na kręgowcach nie mogą być bezpośrednio przenoszone na bezkręgowce, takie jak *Daphnia* spp., natomiast analiza porównawcza (*read-across*) może stanowić wsparcie interpretacyjne dla kluczowych procesów zachodzących w organizmach wodnych, w szczególności dotyczących pobierania, biotransformacji i eliminacji farmaceutyków. W literaturze często podaje się niższe wartości BCF w porównaniu z wartościami BAF dla tych samych substancji, co wskazuje, że laboratoryjne pomiary BCF mogą zaniżać rzeczywistą bioakumulację w środowisku, gdy nie uwzględnia się dodatkowych dróg narażenia (Arnot & Gobas, 2006).

W świetle przedstawionych zagadnień istnieje potrzeba uzupełnienia luki w ekotoksykologii dotyczącej wpływu parametrów środowiskowych na zmianę toksyczności farmaceutyków. Jest to szczególnie istotne ze względu na postępujące zmiany klimatu i rosnącą wykrywalność farmaceutyków w środowisku wodnym. W niniejszej pracy doktorskiej zbadano wpływ obniżonego pH wód na zmianę toksyczności DCF oraz SMX jako przedstawicieli farmaceutyków o różnych właściwościach fizykochemicznych. Oceniono również przydatność standardowych procedur OECD do określenia potencjalnych zmian toksyczności wytypowanych leków w warunkach symulowanego obniżonego pH. W tym celu wykonano szereg testów ekotoksykologicznych ostrych oraz chronicznych, zgodnych z OECD 202, 211 oraz 221, z wykorzystaniem organizmów referencyjnych takich jak *D. magna* oraz *L. minor*. Testy te zostały wzbogacone o ocenę wpływu rodzaju medium hodowlanego oraz sposobu uzyskania organizmów testowych na wyniki badań, w tym na parametr EC₅₀ [mg/l] (ang. *half maximal effective concentration*) - stężenie badanej substancji chemicznej, które u 50% badanych organizmów wywołuje określony skutek negatywny. Przeprowadzono również badania *in situ* nad bioakumulacją DCF w *D. pulex* w celu wyznaczenia współczynnika BAF. Umożliwiły one ocenę procesu bioakumulacji DCF w warunkach rzeczywistej ekspozycji.

Przedstawione w niniejszej pracy doktorskiej wyniki badań zostały w całości opublikowane w międzynarodowych recenzowanych czasopismach naukowych.

4. Cel pracy i zakres badań

Celem rozprawy doktorskiej była ocena znaczenia wybranych czynników środowiskowych, metodycznych i biologicznych, w tym pH, składu medium testowego oraz charakterystyki organizmów testowych, w kształtowaniu wyników badań oceny toksyczności ostrej i przewlekłej farmaceutyków wobec wytypowanych organizmów referencyjnych. Głównym analizowanym czynnikiem fizykochemicznym było pH ze względu na jego znaczenie dla występowania różnych form jonowych farmaceutyków oraz odpowiedzi organizmów wodnych, szczególnie w kontekście zmian środowiskowych związanych ze zmianą klimatu. Wpływ obniżenia pH badano dla farmaceutyków o zróżnicowanej budowie i właściwościach fizykochemicznych. Jako modele badawcze wybrano DCF (słaby kwas; $pK_a \approx 4,0$) oraz SMX - antybiotyk sulfonamidowy o właściwościach amfoterycznych ($pK_{a1} \approx 1,6$, $pK_{a2} \approx 5,7$). Farmaceutyki te stanowią istotne zanieczyszczenia środowiska wodnego ze względu na ich częste wykrywanie oraz możliwy długoterminowy wpływ na organizmy wodne. Jako organizmy testowe wybrano rozwielitkę wielką *D. magna* oraz rzęsę drobną *L. minor*, reprezentujące różne poziomy troficzne i wykorzystywane jako organizmy referencyjne w testach ekotoksykologicznych.

Cel główny zrealizowano poprzez przeprowadzenie następujących badań:

1. przegląd i syntezę dotychczasowej wiedzy dotyczącej wpływu wzrostu temperatury oraz zakwaszenia środowiska wodnego na ekotoksyczność farmaceutyków;
2. ocenę bioakumulacji DCF przez organizmy *D. pulex* bytujące w oczyszczalni ścieków w warunkach *in situ*;
3. zbadanie wpływu obniżenia pH na przewlekłą toksyczność DCF wobec *D. magna*;
4. ocenę wpływu obniżenia pH na ostrą toksyczność SMX wobec *L. minor*, z uwzględnieniem degradacji i biodostępności farmaceutyku;
5. analizę wpływu rodzaju medium testowego (środowiskowej wody stawowej i medium M4) oraz źródła i sposobu przygotowania organizmów testowych *D. magna* (EE-Neo - osobników wyklutych bezpośrednio z efipiów oraz P-Neo - osobników uzyskanych z wielopokoleniowej hodowli laboratoryjnej) na wyniki badań ekotoksykologicznych;
6. identyfikację czynników ograniczających porównywalność wyników testów opartych na wytycznych OECD oraz ocenę znaczenia pH, składu medium oraz źródła i sposobu

przygotowania organizmów testowych dla interpretacji toksyczności farmaceutyków jonizowalnych.

5. Opis koncepcji eksperymentalnej i zastosowanych metod badawczych

W badaniach wykorzystano powszechnie uznane protokoły referencyjne oceny toksyczności ostrej i chronicznej względem wybranych organizmów wodnych. Wynikało to z potrzeby porównania uzyskanych wyników z bazą literaturową. W protokołach wprowadzano niezbędne modyfikacje obejmujące zmianę pH roztworów testowych, przy czym każda zastosowana wartość pH mieściła się w zakresie optymalnym dla życia organizmów testowych. Każda taka zmiana została odnotowana w niniejszej pracy. Testy toksyczności ostrej oraz chronicznej weryfikowano pod kątem spełnienia kryteriów ważności określonych w protokołach referencyjnych. W zależności od zastosowanych organizmów testowych wykorzystywano odpowiednie protokoły referencyjne: dla *D. magna* testy toksyczności ostrej (OECD 202) oraz chronicznej (OECD 211), natomiast dla *L. minor* wyłącznie test toksyczności ostrej (OECD 221). Wyjątek stanowiły badania BAF z wykorzystaniem organizmów *D. pulex* pochodzących z oczyszczalni ścieków, narażonych długoterminowo na mieszaninę zanieczyszczeń, w tym DCF. Przeprowadzono także walidację opracowanych metod analitycznych oznaczania DCF oraz SMX przed realizacją eksperymentów. W badaniach, w których wykonywano analizę statystyczną, za poziom istotności przyjęto $p < 0,05$ [P3, P4, P5].

5.1. Toksyczność ostra diklofenaku wobec *Daphnia magna*

Test toksyczności ostrej DCF wobec *D. magna* przeprowadzono zgodnie z wytycznymi OECD 202. Doświadczenie wykonano w dwóch typach medium: w medium M4 oraz w naturalnej wodzie stawowej, pobranej ze zbiornika wodnego kampusu Uniwersytetu Gdańskiego. Matrycę tę scharakteryzowano pod względem podstawowych parametrów fizykochemicznych. Do eksperymentu wykorzystano osobniki *D. magna* pochodzące z komercyjnych efipiów (DAPHTOXKIT F, MicroBio Tests) oraz ze stabilnej kultury laboratoryjnej, utrzymywanej przez ponad trzy lata, co pozwoliło wyróżnić cztery grupy eksperymentalne różniące się źródłem i sposobem przygotowania rozwielitek do testu oraz rodzajem medium. We wszystkich grupach eksperymentalnych do testów wykorzystywano młode osobniki, które nie miały więcej niż 24 godziny życia (zgodnie z protokołem OECD). Przed przystąpieniem do eksperymentu osobniki *D. magna* z hodowli aklimatyzowano do warunków odpowiednich dla danego medium

testowego (M4 vs. woda stawowa). W przypadku osobników pochodzących z efipiów wylęg następował w warunkach kontrolowanych (20-21 °C, 6000 lx), zgodnie z wytycznymi producenta. Test przeprowadzano w 30-dółkowych płytkach plastikowych. W każdym dołku znajdowało się 10 ml roztworu testowego. W celu przygotowania roztworów testowych sól sodową DCF ($C_{14}H_{10}Cl_2NNaO_2$) rozpuszczano w medium testowym bez wykorzystania rozpuszczalników organicznych. Zakres stężeń testowych DCF obejmował 1-150 mg/l. Test wykonano w układzie wielokrotnych powtórzeń, przy czym kontrola biotyczna zawierała wyłącznie odpowiednie medium bez dodatku DCF. W każdym dołku umieszczono po 5 młodych osobników. Płytki testowe zabezpieczano parafilmem oraz pokrywką. Organizmy ekspozowano w ciemności w temperaturze 21 °C przez okres 48 godzin. Każdy z testów wykonano trzykrotnie w ramach niezależnych serii eksperymentalnych, przeprowadzonych w różnych terminach. Przed oraz po zakończeniu testów mierzono parametry fizykochemiczne, takie jak pH, przewodność i zawartość tlenu rozpuszczonego. Pozytywną kontrolę stanowiła substancja referencyjna - dwuchromian potasu. Dodatkowo o spełnieniu kryterium ważności testu świadczyła śmiertelność *D. magna* w kontroli nie większa niż 10% osobników testowych. Stężenia DCF w roztworach testowych oznaczano na początku oraz po 48 godzinach ekspozycji, wykorzystując technikę wysokosprawnej chromatografii cieczowej z detektorem matrycy fotodiodowej (ang. *High Performance Liquid Chromatography with Diode Array Detector*; HPLC/DAD). Na podstawie uzyskanych wyników oceniano zmiany stężenia DCF w trakcie ekspozycji. Dodatkowo oznaczano stężenia DCF w kontrolach abiotycznych bez organizmów testowych (nominalnie 100 mg/l DCF). W celu analizy dawka-odpowiedź wykorzystano program statystyczny R (wersja 4.3.2; R Core Team, 2023). Parametr EC_{50} wyznaczano za pomocą modelu probitowego. Do obliczeń EC_{50} zastosowano stężenia DCF zmierzone na początku eksperymentów. Przed analizą statystyczną wartości EC_{50} zlogarytmowano w celu spełnienia założeń normalności i homogeniczności wariancji. Analizę wykonano w programie Statistica 13.1. W celu oceny normalności danych wykorzystano test Shapiro-Wilka, natomiast do oceny jednorodności wariancji test Levene'a. Wpływ typu medium oraz źródła i sposobu przygotowania organizmów testowych, a także ich interakcje, sprawdzono za pomocą dwuczynnikowej analizy wariancji (ANOVA). Porównania post hoc przeprowadzono testem Tukeya (HSD). Szczegółowy opis metodyki przedstawiono w pracy **P5**.

5.2. Toksyczność chroniczna diklofenaku wobec *Daphnia magna*

Test toksyczności chronicznej DCF wobec *D. magna* przeprowadzono zgodnie z wytycznymi OECD 211. Doświadczenie wykonano w medium M4 przy dwóch poziomach pH: 8,7 (wartość naturalna medium M4) oraz 7,0 (warunki zakwaszenia, uzyskane poprzez dodatek HCl), mieszczących się w zakresie dopuszczalnym według protokołu referencyjnego.

Przed rozpoczęciem testu organizmy macierzyste aklimatyzowano do warunków pH, w których przeprowadzono test właściwy. Do testów wykorzystano młode osobniki *D. magna* (< 24 godziny), nie pochodzące z pierwszego miotu. *Daphnia magna* poddane były ekspozycji (1 organizm/50 ml roztworu) na pięć stężeń DCF (0,6; 1,3; 2,5; 5,0 oraz 10,0 mg/l) w 6 powtórzeniach. Wykonano również kontrolę biologiczną (*D. magna* w medium M4 bez ekspozycji na DCF; 10 powtórzeń). Test trwał 21 dni, przy czym medium M4 wymieniano trzy razy w tygodniu. Warunki ekspozycji obejmowały temperaturę 21 ± 1 °C, cykl świetlny 16:8 (dzień/noc) oraz natężenie światła 1500 lx. Rozwielitki karmiono zielonymi glonami *Chlorella vulgaris* z własnej hodowli.

W trakcie eksperymentu monitorowano parametry fizykochemiczne (pH, przewodnictwo oraz zawartość tlenu rozpuszczonego w roztworach testowych) oraz wskaźniki reprodukcyjne (liczba żywych nowo narodzonych osobników w trakcie trwania testu, liczba miotów, liczba nowo narodzonych osobników w miocie oraz dzień pierwszego miotu w każdym z analizowanych stężeń). Do oceny zmian stężenia DCF w roztworach testowych wykorzystano technikę HPLC/DAD.

Analizy statystyczne przeprowadzono w programie Statistica 13.1 z wykorzystaniem testów nieparametrycznych - testu U Manna-Whitneya oraz testu Kruskala-Wallisa, z uwzględnieniem analiz post hoc. Szczegółowy opis metodyki przedstawiono w pracy P3.

5.3. Toksyczność ostra sulfametoksazolu wobec *Lemna minor*

Ostrą toksyczność SMX wobec *L. minor* oceniano na podstawie zahamowania wzrostu w siedmiodniowym teście opartym na wytycznych OECD 221, którego punktem końcowym była wartość EC₅₀. Test wykonano w medium Steinberga przy dwóch poziomach pH: 5,5, stanowiącym standardowe pH tego medium, oraz 5,0, odpowiadającym warunkom zakwaszenia uzyskanym poprzez dodatek HCl.

Przed przystąpieniem do właściwych testów *L. minor* aklimatyzowano przez trzy tygodnie do wartości pH stosowanych podczas ekspozycji, tj. 5,5 oraz 5,0. Pożywkę wymieniano co tydzień. Hodowlę prowadzono w kolbach Erlenmeyera w fitotronie, w temperaturze 25 °C, przy natężeniu oświetlenia 6000 lx oraz wilgotności względnej 60%.

Lemna minor została poddana ekspozycji na sześć stężeń SMX: 0,31; 0,62; 1,25; 2,50; 5,00 oraz 10,00 mg L⁻¹. W każdym naczyniu zawierającym 10 ml roztworu umieszczano jedną kolonię obejmującą trzy człony pędowe. Dla każdego stężenia i wariantu pH zastosowano dwa powtórzenia. Wykonano również kontrole biotyczne oraz abiotyczne w świetle i w ciemności. Cały test przeprowadzono trzykrotnie jako niezależne eksperymenty. Zmiany stężenia SMX monitorowano z wykorzystaniem techniki HPLC-DAD. Przykładową płytkę testową z *L. minor* przedstawiono na **Rysunku 1**.



Rysunek 1. Zdjęcie płytki testowej *Lemna minor*, przedstawiające zahamowanie wzrostu rośliny w obecności kolejno malejących stężeń sulfametoksazolu, począwszy od górnego lewego dolka

Wartość EC₅₀ wyznaczono na podstawie obliczonego współczynnika zahamowania wzrostu *L. minor* w trakcie eksperymentu. W celu jego określenia wykonano analizę powierzchni członów pędowych (ang. *fronds*) za pomocą programu WinDias 3. Parametr EC₅₀ wyznaczono w środowisku R za pomocą dwóch modeli: uogólnionego modelu liniowego (ang. *generalized linear model*; GLM) z funkcją łączącą probit oraz czteroparametrowego nieliniowego modelu log-logistycznego (ang. *four-parameter log-logistic model*; LL.4). Różnice między wariantami pH oceniano za pomocą nieparametrycznego testu U Manna-Whitneya w programie Statistica 13.1. Dodatkowo określono udział formy niejonizowanej SMX w roztworach w zależności od pH

na podstawie równania Hendersona-Hasselbalcha. Szczegółowy opis metodyki przedstawiono w pracy **P4**.

5.4. Analiza bioakumulacji diklofenaku w warunkach *in situ*

Badania bioakumulacji DCF w warunkach *in situ* przeprowadzono z wykorzystaniem organizmów *D. pulex* bytujących w osadnikach wtórnych oczyszczalni ścieków w Swarzewie. W próbkach wody ze stawów doczyszczających (2019) oraz ścieków oczyszczonych z osadników wtórnych (2019, 2021) wykonano analizę celowaną NLPZ, w tym DCF i jego metabolitów. Dodatkowo w 2021 roku analizą objęto organizmy *D. pulex* bytujące w osadnikach oraz próbki zawiesiny cząstek stałych. Podczas pobierania próbek mierzono podstawowe parametry fizykochemiczne wody panujące w zbiornikach, takie jak temperatura, pH, przewodność elektrolityczna oraz stężenie tlenu rozpuszczonego.

Organizmy odseparowano z wody za pomocą gazy medycznej, przemyto wodą dejonizowaną w celu pozbycia się cząstek stałych i zamrożono. Próbki wody filtrowano i zakwaszono przed analizą (HCl, pH 3). Filtry z osadem zebrany podczas filtracji wody zamrożono. Zarówno osad, jak i organizmy poddano liofilizacji.

Ekstrakcję analitów z próbek wodnych wykonano za pomocą ekstrakcji do fazy stałej (ang. *Solid Phase Extraction*; SPE) na kolumnach Strata-X o wypełnieniu polimerowym. Ekstrakcję analitów z rozwiłitek oraz osadu przeprowadzono metodą ekstrakcji ciało stałe-ciecz (SLE) z zastosowaniem mieszaniny acetonitrylu (ACN) i buforu octanowego (65:35, pH 4,1). Uzyskane ekstrakty po homogenizacji i wirowaniu oczyszczano metodą SPE. Szczegółowy opis procedur przedstawiono w pracy **P2**.

Oznaczenia ilościowe analitów wykonano techniką ultrawysokosprawną chromatografią cieczową sprzężoną z tandemową spektrometrią mas (ang. *Ultra-high-performance Liquid Chromatography coupled with Tandem Mass Spectrometry*; UHPLC-MS/MS) w trybie wielokrotnego monitorowania reakcji (ang. *Multiple Reaction Monitoring*; MRM) z zastosowaniem wzorca wewnętrznego oraz krzywych kalibracyjnych. W próbkach wody oznaczono wybrane NLPZ i ich metabolity, natomiast BAF wyznaczono wyłącznie dla DCF ze względu na jego wykrywalność w *D. pulex*. Na podstawie otrzymanych stężeń DCF obliczono BAF (l/kg) dla suchej i mokrej masy rozwiłitek jako stosunek stężenia farmaceutyku w organizmach do jego stężenia w wodzie. Wyznaczono również współczynnik podziału DCF pomiędzy osadem a wodą

oraz pomiędzy rozwiłkami a osadem. Ogólny opis metody analitycznej UHPLC-MS/MS przedstawiono w podrozdziale 5.5.3. (Analizy UHPLC-MS/MS), natomiast informacje szczegółowe dotyczące walidacji i aparatury w publikacji **P2**.

5.5. Chemiczne analizy ilościowe

5.5.1. Analizy HPLC

Analizy ilościowe DCF w testach toksyczności ostrej [**P5**], chronicznej [**P3**], a także SMX w testach toksyczności ostrej [**P4**] wykonano za pomocą techniki HPLC-DAD (chromatograf Nexera XR, Shimadzu). Rozdziały chromatograficzne prowadzono na kolumnie typu C18 (Gemini NX, 5 μm , 110 Å, 150 $\times$ 4,6 mm, Phenomenex) w warunkach izokratycznych z zastosowaniem mieszaniny buforu octanowego (komponent A pH 4,0-4,1) i ACN (komponent B) jako fazy ruchomej. Każda z metod została zwalidowana, a zakres oznaczanych stężeń dobierano do celu badania i przewidywanego stężenia DCF. Szczegóły dotyczące parametrów analitycznych i walidacyjnych metod przedstawiono odpowiednio w publikacjach oraz materiałach uzupełniających [**P2-P5**]. Wiarygodność uzyskanych wyników monitorowano za pomocą prób kontrolnych obejmujących analizę kontroli abiotycznych, kontroli biotycznych oraz analizę samego medium hodowlanego.

5.5.2. Analizy IC

Do oznaczenia składu jonowego próbek wody wykorzystanej jako medium testowe w publikacji **P5** zastosowano technikę chromatografii jonowej (ang. *Ion Chromatography*; IC) przy użyciu chromatografu Metrohm Vario. Umożliwiła ona oznaczenie takich kationów jak Na^+ , K^+ , Ca^{2+} , Mg^{2+} oraz anionów: F^- , Cl^- , PO_4^{3-} , Br^- , NO_3^- , SO_4^{2-} . Rozdział kationów prowadzono z wykorzystaniem kolumny Metrosep C4 100/0.4 w temperaturze 25 °C, stosując jako fazę ruchomą wodny roztwór kwasu azotowego (V) (1,7 mM) i kwasu dipikolinowego (0,7 mM), przy przepływie 0,8 ml/min. Rozdział anionów prowadzono z wykorzystaniem kolumny Metrosep A Supp 10 100/0.4 w temperaturze 40 °C. Jako fazę ruchomą zastosowano wodny bufor węglanowy (5 mM) przy przepływie 1,0 ml/min. Oznaczenia ilościowe oparto na kalibracji zewnętrznej. W tym celu zastosowano certyfikowane, wieloskładnikowe roztwory wzorcowe. Przed analizą chromatograficzną próbki filtrowano przez filtry strzykawkowe o średnicy porów 0,2 μm . Szczegółowe informacje dotyczące analizy oraz parametrów walidacyjnych przedstawiono w publikacji **P5** oraz jej materiałach uzupełniających.

5.5.3. Analizy UHPLC-MS/MS

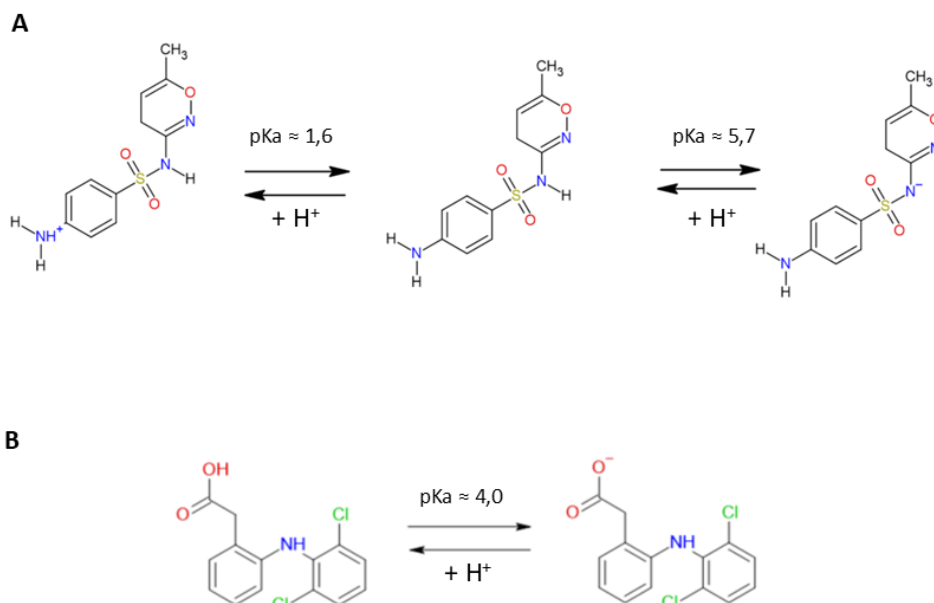
Analizę ilościową DCF, 4-hydroksydiklofenaku (4-OH DCF), estru metylowego diklofenaku (M-DCF), ibuprofenu (IBF), 2-hydroksyibuprofenu (2-OH IBF), naproksenu (NPX) oraz kwasu klofibrowego (CFA) przeprowadzono z wykorzystaniem techniki UHPLC-MS/MS (spektrometr mas z potrójnym analizatorem kwadrurowym, model LCMS-8050, Shimadzu). Rozdział chromatograficzny przeprowadzono na kolumnie typu C18 (Eurospher II 100-3 C18H, 50 x 3 mm) z zastosowaniem wymywania gradientowego, przy czym faza ruchoma składała się z mieszaniny 5 mM buforu octanowego o pH 4,1 i ACN. Detekcja analitów została wykonana w trybie MRM, wykorzystując dodatni oraz ujemny tryb detekcji jonów w zależności od wybranego związku. Reakcja fragmentacji MRM oraz czasy retencji pozwoliły na identyfikację jakościową badanych związków. Do analizy ilościowej wykorzystano metodę wzorca wewnętrznego. Zakres liniowości metody wynosił 31-500 ng/ml dla wszystkich analitów. Krzywe kalibracyjne charakteryzowały się wartościami współczynników determinacji min. 0,9990.

Odzysk analitów dla procedury SPE wynosił 98,4-105,1%, natomiast dla SLE-SPE 61,8-85,6%. Szczegółowe parametry walidacyjne oraz instrumentalne metody UHPLC-MS/MS przedstawiono w materiałach uzupełniających (**Tabela S2**) do publikacji **P2**.

6. Wyniki badań własnych i dyskusja

6.1. Wpływ zmiany pH na wyniki testów ekotoksykologicznych

Wartość pH środowiska naturalnego jest jednym z kluczowych parametrów wpływających na los i oddziaływanie zanieczyszczeń obecnych w środowisku wodnym, w tym szczególnie nowopojawiających się zanieczyszczeń środowiska, jakimi są farmaceutyki. Szacuje się, że obecnie obserwowane zakwaszanie wód może mieć wpływ na toksyczność tego typu zanieczyszczeń [P1]. W związku z tym poświęcono mu szczególną uwagę. W pracach **P3-P4** oceniano wpływ obniżenia pH medium testowego na zmianę toksyczności wybranych farmaceutyków wobec organizmów wodnych. Wpływ pH na toksyczność zanieczyszczeń obecnych w wodach środowiskowych udokumentowano dla takich zanieczyszczeń środowiska jak metale ciężkie, pestycydy czy trwałe zanieczyszczenia organiczne (Alava et al., 2017; Belivermiş et al., 2020; Roberts et al., 2013; Zou et al., 2018). Po dogłębnym przeglądzie literatury [P1] znaleziono lukę w wiedzy dotyczącej wpływu tego parametru na związki jonizowalne, jakimi są farmaceutyki. W przypadku tych związków chemicznych jest to niezwykle ważny parametr, ponieważ może on wpływać na zmianę formy jonowej wybranego farmaceutyku. Szczególnie istotne jest to dla leków, których wartości stałej równowagi kwasowo-zasadowej pK_a są zbliżone do pH naturalnie występujących wód środowiskowych. Przykładem takich związków są amfoteryczne sulfonamidy. Udział neutralnej formy SMX ($pK_{a2} \approx 5,7$) zmieniał się w zależności od pH medium testowego (OECD 221). Forma ta jest bardziej toksyczna dla organizmów wodnych ze względu na większą zdolność przenikania przez błony komórkowe (Almeida et al., 2018; Kim et al., 2010; Nakamura et al., 2008; Sun et al., 2020). Zgodnie z równowagą kwasowo-zasadową SMX (**Rysunek 2A**) niższe pH sprzyja obecności frakcji neutralnej farmaceutyku.



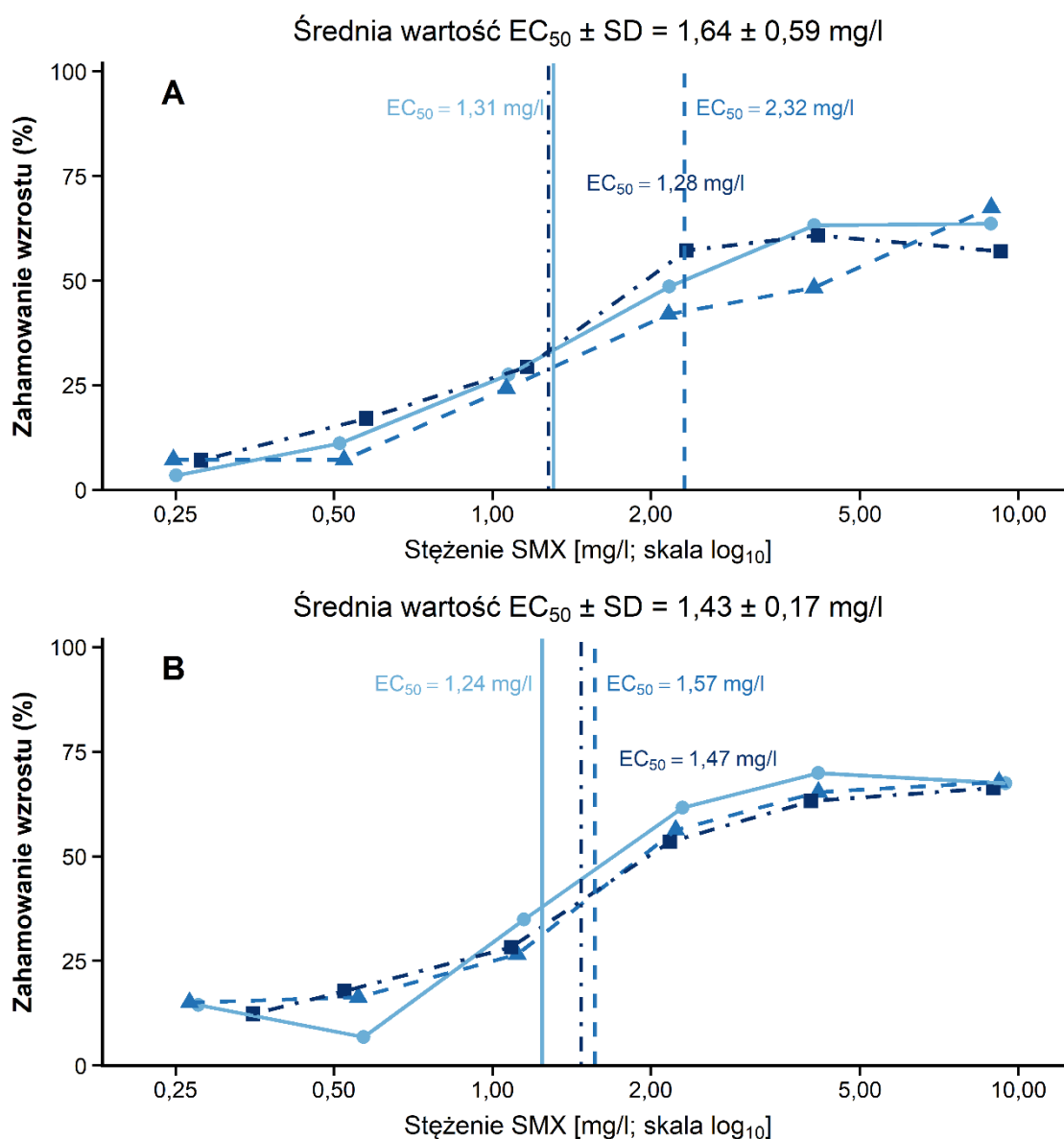
Rysunek 2. Formy kwasowo-zasadowe sulfametoksazolu (A) oraz diklofenaku (B)

Wartość pH wpływa więc na jonizację farmaceutyków, tym samym ingerując w biodostępność leku. Potwierdzają to wyniki badań własnych [P4], które wskazują na tendencję do większego pobierania leku przez *L. minor* przy niższym pH eksperymentu ($\mu\text{g SMX}/\text{mm}^2$ członu pędogowego, **Tabela 1**).

Tabela 1. Pobieranie sulfametoksazolu (SMX) przez *Lemna minor*, w przeliczeniu na powierzchnię członów pędogowych, przy różnych stężeniach SMX i dwóch wartościach pH medium [P4]

Stężenie SMX [mg/l]	pH 5,5 [$\mu\text{g SMX}/\text{mm}^2$]	pH 5,0 [$\mu\text{g SMX}/\text{mm}^2$]
0,31	0,008	0,009
0,62	0,018	0,013
1,25	0,027	0,031
2,50	0,088	0,133
5,00	0,207	0,230
10,00	0,161	0,211

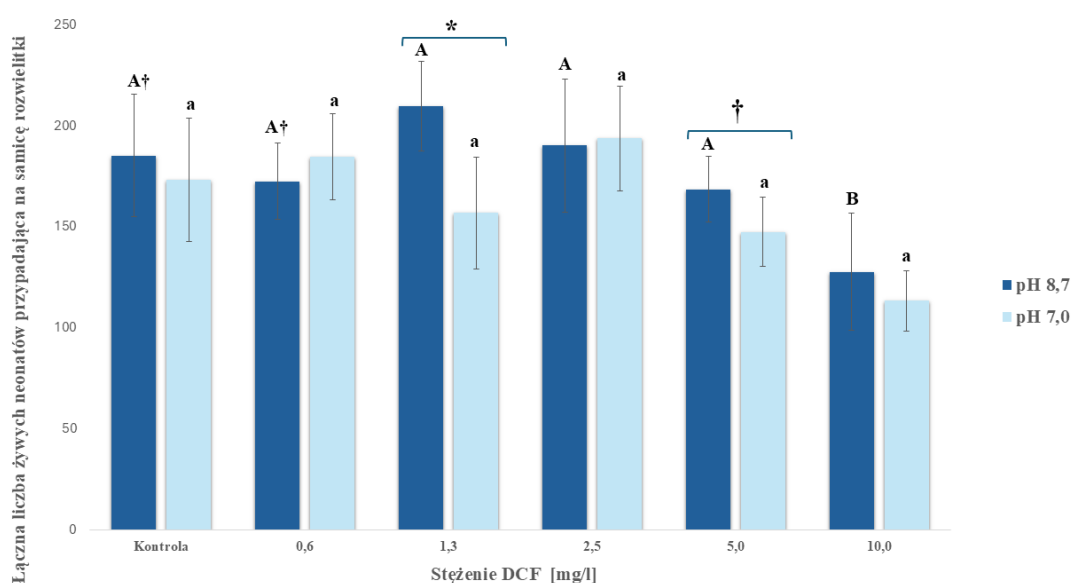
Ważnym aspektem jest również proces fotodegradacji, od którego zależała rzeczywista ekspozycja *L. minor* na SMX. Okazała się ona procesem wieloczynnikowym, zależnym głównie od stężenia analizowanego związku. Wpływ pH na jej przebieg został odnotowany, jednak niemożliwe jest określenie konkretnego trendu tej zmiany. Spośród sześciu analizowanych stężeń SMX statystycznie istotne różnice w stopniu degradacji pomiędzy badanymi wariantami pH odnotowano jedynie dla stężeń 2,5 mg/l oraz 1,25 mg/l. Kierunek zaobserwowanych zmian był przeciwny dla obu stężeń SMX, dlatego uzyskane wyniki nie wskazują jednoznacznie na wpływ badanego zakresu pH na przebieg fotodegradacji. Najbardziej prawdopodobnym czynnikiem wpływającym na zmianę toksyczności SMX wobec *L. minor* była więc zmiana udziału neutralnej formy związku, a fotodegradacja jest procesem towarzyszącym, wpływającym na rzeczywiste stężenie SMX w medium testowym. Podsumowując, średnie wartości EC_{50} wyznaczone za pomocą modelu LL.4 wynosiły $1,64 \pm 0,59$ mg/l przy pH 5,5 oraz $1,43 \pm 0,17$ mg/l przy pH 5,0 (**Rysunek 3**). Obniżeniu pH towarzyszył zatem spadek średniej wartości EC_{50} , wskazujący na zwiększenie toksyczności SMX wobec *L. minor* w warunkach zakwaszenia.



Rysunek 3. Zależność zahamowania wzrostu *Lemna minor* od stężenia sulfametoksazolu (SMX) oraz wartości EC₅₀ [mg/l] wyznaczone przy pH 5,5 (A) i pH 5,0 (B) za pomocą czteroparametrowego modelu nieliniowego log-logistycznego (LL.4). Poszczególne kolory przedstawiają wyniki trzech niezależnych powtórzeń testu, a pionowe linie - odpowiadające im wartości EC₅₀ (n = 3; P4); wykresy wygenerowano za pomocą programu statystycznego R (wersja 4.3.2; R Core Team, 2023)

Okazuje się, że nie dla wszystkich związków chemicznych z grupy farmaceutyków zmiana formy jonowej związku będzie kluczowym mechanizmem wpływającym na toksyczność. Dla DCF (pK_a ≈ 4,0) zmiana pH medium z 8,7 do 7,0 (OECD 211) spowodowała wzrost toksyczności tego leku wobec *D. magna*, przy czym DCF występował przeważnie w formie anionowej w obu wariantach pH [P3]. Równowagę kwasowo-zasadową DCF przedstawiono na **Rysunku 2B**. Istnieje więc inny powód zwiększonej toksyczności DCF - zmiana pH może oddziaływać bezpośrednio na organizm, który staje się tym samym bardziej podatny na działanie tego farmaceutyku. Literatura donosi, że obniżenie pH wpływa negatywnie na organizm rozwielitek poprzez

zaburzenie gospodarki jonowej, oddziałując również na procesy metaboliczne i energetyczne (Alibone & Fair, 1981; Im et al., 2019; Shadi Khudr et al., 2020), a także może prowadzić do uszkodzeń pancerzyka rozwielitek (Weber & Pirow, 2009), zwiększając podatność organizmu na obecność zanieczyszczeń. W pracy **P3** wykazano, że pH miało bezpośredni wpływ na liczbę miotów przypadających na samicę rozwielitki oraz dzień pojawienia się pierwszego miotu (U Mann-Whitney, $p < 0,05$). Odnotowano również wpływ pH na działanie DCF, przy czym zależność ta nie miała charakteru liniowego i była uzależniona od stężenia związku. Najsilniejszą, istotną statystycznie różnicę pomiędzy wariantami pH zaobserwowano w przypadku liczby nowonarodzonych rozwielitek przy stężeniu DCF wynoszącym 1,3 mg/l (U Mann-Whitney, $p = 0,028$). Wpływ stężenia DCF i pH medium na łączną liczbę żywych młodych osobników przypadających na samicę *D. magna* przedstawiono na **Rysunku 3**.



Rysunek 4. Wpływ stężenia diklofenaku (DCF) i pH medium na średnią liczbę żywych młodocianych rozwielitek przypadających na jedną samicę *Daphnia magna* podczas 21-dniowego testu przewlekłego prowadzonego w warunkach standardowych (pH 8,7) oraz obniżonego pH (pH 7,0). Statystycznie istotne różnice ($p < 0,05$) pomiędzy poszczególnymi stężeniami DCF a kontrolą oznaczono różnymi wielkimi literami dla warunków standardowych oraz różnymi małymi literami dla warunków obniżonego pH. Różnice pomiędzy wariantami pH przy tym samym stężeniu DCF oznaczono gwiazdką (*). Symbol † oznacza wynik zbliżony do progu istotności statystycznej ($0,05 < p < 0,10$) (P3)

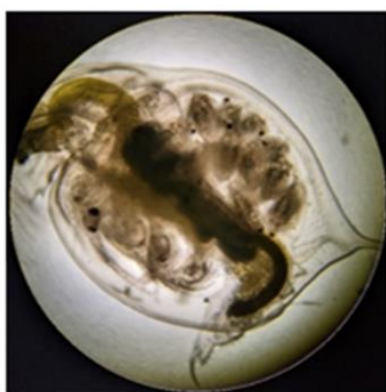
W pracy **P4** nie stwierdzono natomiast negatywnego wpływu obniżenia pH na wzrost *L. minor* w próbach kontrolnych, co pozwala wnioskować, że znaczenie pH w ekotoksykologii nie ma charakteru uniwersalnego i zależy od właściwości związku chemicznego, a także od wrażliwości badanego organizmu na jego zmiany.

Istotnym ograniczeniem obu prac **P3-P4** w ocenie wpływu zakwaszenia na zmianę toksyczności farmaceutyków jest wysoka zmienność pH w trakcie trwania testu. Wynika ona z ograniczonych zdolności buforowych medium Steinberga (OECD 221) oraz medium M4 (OECD 211), a także z ograniczonego zakresu dopuszczalnych metod modyfikacji tego parametru (głównie poprzez dodanie HCl lub NaOH). W obu przypadkach pH nie pozostawało stałe w trakcie ekspozycji. W OECD 211 medium M4 jest dynamicznym układem opartym na równowadze węglanowej; wymiana CO₂ z atmosferą powoduje więc zmienność pH w trakcie trwania testu, mimo cyklicznej odnowy medium. W przypadku testów prowadzonych zgodnie z OECD 221 dodatkowy wpływ na podwyższenie pH medium Steinberga mogą wywierać procesy fotosyntezy oraz asymilacji azotanów przez rośliny. Przewagę procesów fizykochemicznych w kształtowaniu zmian pH w medium M4 oraz odpowiednio procesów związanych z obecnością *L. minor* w medium Steinberga podkreślają również pomiary pH przeprowadzone w kontrolach abiotycznych obu testów. Istotne zmiany pH odnotowano jedynie w kontroli abiotycznej testu przeprowadzonego zgodnie z OECD 211.

Parametr pH jest więc dynamiczny, a jego wartość w trakcie trwania testów ekotoksykologicznych jest wypadkową różnych procesów zachodzących w obrębie buforu stanowiącego medium hodowlane, środowiska, a także organizmów testowych. W przypadku OECD 221 stężenie SMX modyfikowało tempo wzrostu *L. minor*, tym samym wpływając na pH medium testowego, ponieważ szybciej rosnące rośliny bardziej podwyższają pH roztworu. Biorąc pod uwagę charakter jonowy farmaceutyków, związaną z tym możliwą zmianę udziału poszczególnych form leku w trakcie eksperymentu, zmiany metaboliczne i morfologiczne zachodzące w organizmach testowych, wpływ na fotosyntezę czy procesy degradacji zanieczyszczeń, pH powinno być raportowane na początku, na końcu oraz w trakcie trwania testu ekotoksykologicznego. Wszelkie wspomniane procesy zależą od pH, dlatego pH należy traktować jako istotny czynnik modyfikujący ocenę toksyczności farmaceutyków oraz ryzyka środowiskowego. Jak podsumowano w pracach **P3-P4** niezbędne jest wprowadzenie i walidacja strategii buforowych, które nie będą obciążone ryzykiem toksyczności wobec badanych organizmów, a które pozwolą na określenie kierunku zmiany toksyczności zanieczyszczenia.

6.2. Wpływ rodzaju medium na wyniki testów ekotoksykologicznych

Standardowe testy ekotoksykologiczne OECD określają wymagania dotyczące właściwości mediów testowych, w tym ich podstawowych parametrów fizykochemicznych oraz zawartości witamin. Takie wytyczne mają na celu zapewnienie porównywalności i powtarzalności wyników testów ekotoksykologicznych między laboratoriami. Testy OECD 202, OECD 211 oraz OECD 221 są testami, których wyniki dotyczące toksyczności analizowanych zanieczyszczeń są wykorzystywane w ocenie ryzyka środowiskowego. W pracy **P5** analizowano skład chemiczny medium M4 oraz środowiskowej wody stawowej, którą wykorzystano jako medium testowe w teście toksyczności ostrej DCF wg OECD 202. Zgodnie z protokołem referencyjnym istnieje możliwość wykorzystania wody naturalnej, pod warunkiem jej uprzedniego scharakteryzowania oraz zapewnienia odpowiednich wartości parametrów, takich jak pH, przewodność elektrolityczna, stężenie tlenu rozpuszczonego oraz twardość. Wartości tych parametrów dla obu zastosowanych mediów testowych przedstawiono w suplemencie do publikacji **P5** (**Tabela S5**). Twardość wody stawowej wynosiła 197,5 mg/l CaCO₃, natomiast twardość medium M4 - 211 mg/l CaCO₃. Obie wartości mieściły się w zakresie zalecanym przez OECD (140-250 mg/l CaCO₃). Stężenia oznaczonych kationów i anionów w obu rodzajach medium przedstawiono w publikacji **P5** (**Tabela 2**). Obserwacje rozwielitek podczas hodowli w obu typach medium wykazały brak widocznych oznak pogorszenia kondycji *D. magna*, które regularnie wydawały liczne potomstwo. Potwierdzają to obrazy mikroskopowe rozwielitek przedstawione na **Rysunku 3**, na których u osobników z obu hodowli pod karapaksem widoczne są liczne jaja oraz wyklute młode osobniki.



Medium M4



Woda stawowa

Rysunek 5. Obrazy mikroskopowe *Daphnia magna* z różnych mediów hodowlanych [P5]

Pomimo braku widocznych różnic w kondycji organizmów podczas hodowli, udokumentowano istotny statystyczny wpływ rodzaju medium na wartość EC_{50} dla DCF wobec rozwiłitek, jednak nie wskazano konkretnego czynnika mogącego wpływać na tę rozbieżność. Analiza składu jonów obu mediów wykazała różnice w stężeniach jonów sodu, potasu, wapnia oraz jonów fluorkowych, chlorkowych, siarczanowych. Chociaż wszystkie zmierzone stężenia jonów dla wody środowiskowej mieściły się w dopuszczalnych zakresach dla systemów słodkowodnych i były zgodne z odpowiednimi wytycznymi dotyczącymi jakości wody (m.in. w odniesieniu do stężenia chlorków oraz siarczanów; Hong et al., 2023; Ministerstwo Infrastruktury, 2021), różnice w ogólnym składzie jonowym znalazły odzwierciedlenie w wartościach przewodności (**P5, Tabela S5**). Może to wskazywać na różnice w sile jonowej między tymi dwoma układami. Zaobserwowana zmienność toksyczności może być częściowo związana z odmiennymi warunkami osmotycznymi, które mogą wpływać na regulację jonową, alokację energii i procesy transportu błonowego u rozwiłitek, a w konsekwencji oddziaływać na wchłanianie zanieczyszczeń i narażenie wewnętrzne. Reakcje fizjologiczne na zmiany warunków osmotycznych zaobserwowano również u innego skorupiaka - *Panulirus ornatus*, co nie stanowi bezpośredniego potwierdzenia, ale wspiera biologiczne prawdopodobieństwo udziału tych mechanizmów u *D. magna* (Eleanor L. Spencer et al., 2025).

W trakcie trwania testu toksyczności ostrej (48 godzin) nie odnotowano znaczących wahań wartości pH. Jednak biorąc pod uwagę wrażliwość jonizowalnych substancji farmaceutycznych na zmiany pH, parametr ten pozostaje istotny dla interpretacji różnic w toksyczności. Oczekuje się, że DCF, jako słaby kwas, w badanym zakresie pH występuje w formie anionowej. Niemniej jednak, jak opisano w rozdziale 6.1, dominująca forma jonowa DCF nie musi się zmieniać, by zmianie pH towarzyszyła zmiana jego toksyczności wobec organizmu. Wahania pH mogą wpłynąć na interakcje błonowe, fizjologię organizmów oraz biodostępność składników rozpuszczonych w medium. Długoterminowe dane środowiskowe z badania **P5** (pH zbiornika naturalnego w zakresie 6,74-8,40 w okresie 9 miesięcy; **Tabela S2**) dodatkowo pokazują, że zmienność pH w naturalnych systemach wodnych może być znacznie większa niż w warunkach laboratoryjnych. Ze względu na logarytmiczny charakter skali pH, zmiana pH o jedną jednostkę odpowiada dziesięciokrotnej zmianie stężenia jonów wodorowych, co może potencjalnie wpływać na stopień dysocjacji związku i odpowiedź

biologiczną (**P1**; (Häder & Gao, 2019). Różnica pomiędzy skrajnymi wartościami pH danych środowiskowych z badania **P5** wynosi 1,66, co odpowiada około 46-krotnej różnicy stężeń jonów wodorowych.

Wyniki uzyskane w badaniu **P5** wskazują, że nawet wtedy, gdy warunki badawcze są zgodne z wymogami OECD, różnice w składzie medium, zwłaszcza te dotyczące stężenia jonów i przewodności, mogą potencjalnie wpływać na parametry toksyczności. Wskazuje to, że spełnienie podstawowych wymagań OECD oraz standardowy zakres charakterystyki medium mogą nie wystarczać do wyjaśnienia zmienności wyników uzyskanych w różnych badaniach. W związku z tym podczas porównywania danych ekotoksykologicznych pochodzących z różnych badań należy wziąć pod uwagę skład medium hodowlanego oraz jego parametry fizykochemiczne w całym okresie ekspozycji. Ma to szczególne znaczenie w przypadku farmaceutyków ulegających jonizacji, gdzie nawet niewielkie różnice w warunkach środowiskowych mogą wpływać na udział poszczególnych form jonowych, a także na biodostępność leku. Uwzględnienie szczegółowej charakterystyki medium ekspozycyjnego w ramach oceny ryzyka środowiskowego mogłoby zwiększyć wiarygodność oraz ułatwić interpretację i porównywanie wyników ekotoksykologicznych uzyskanych zgodnie z wytycznymi OECD.

6.3. Wpływ źródła i historii hodowlanej organizmów na wyniki testów ekotoksykologicznych

Daphnia magna w badaniach ekotoksykologicznych jest traktowana jako modelowy organizm testowy. Wyniki otrzymane w efekcie testów ekotoksykologicznych z wykorzystaniem tego organizmu powinny być porównywalne. Po zebraniu literatury dostępnej w tym temacie, okazuje się, że rozrzut wyników toksyczności DCF względem *D. magna* jest szeroki (**P5**; **Tabela S1**). Jednak nie we wszystkich publikacjach podano, z jakiego źródła pochodzą i jak były przygotowane organizmy wykorzystane w badaniach. Jak wykazano w eksperymencie opisanym w pracy **P5**, źródło i sposób przygotowania osobników testowych *D. magna* były istotnie związane ze zmiennością wartości EC_{50} (Two-way ANOVA $F(1, 8) = 24,08$; $p = 0,001$; partial $\eta^2 = 0,75$). Organizmy wykorzystywane w eksperymentach mogą różnić się historią rozwoju i hodowli, a w konsekwencji kondycją fizjologiczną. OECD podaje wiek młodych *D. magna*, którego należy przestrzegać, rozpoczynając eksperyment. Wytyczne

wymagają również podania źródła i gatunku organizmów testowych, opisu warunków ich hodowli (w tym karmienia), a także raportowania warunków prowadzenia testu. Nie wymagają jednak przedstawienia szczegółowej historii rozwoju organizmów, w tym sposobu uzyskania osobników testowych (wyklucia z efipiów lub pozyskania z hodowli partenogenetycznej), ani historii reprodukcji populacji rodzicielskiej. Informacje raportowane zgodnie z wytycznymi ułatwiają identyfikację potencjalnych źródeł zmienności danych ekotoksykologicznych, ale nie eliminują ich wpływu na wyniki. Postanowiono więc przyrzeć się temu zagadnieniu.

Udokumentowano, że *D. magna* uzyskane bezpośrednio z efipiów (powstające w wyniku rozmnażania płciowego) wykazywały większą wrażliwość na DCF w ostrym teście ekotoksykologicznym prowadzonym zgodnie z OECD 202 niż osobniki pochodzące z hodowli laboratoryjnej, rozmnażające się partenogenetycznie [P5]. Chociaż OECD 202 zaleca użycie osobników nie pochodzących z pierwszego miotu, zgodnie z deklaracją dystrybutora zestawu firmy Tigret sp. z o.o., wykorzystanie efipiów jest zgodne z OECD 202. Różnice fizjologiczne pomiędzy organizmami wykorzystanymi bezpośrednio po wykluciu z efipiów pochodzących z zestawu testowego a organizmami pochodzącymi z hodowli mogą mieć znaczenie pod względem odporności na stres. Już sam fakt powstawania efipiów w warunkach stresowych (Pietrzak, 2006), takich jak skrócenie fotoperiodu, zbyt wysoka gęstość populacji czy obniżenie temperatury, może potencjalnie przyczyniać się do zmienności wartości EC_{50} . Dodatkowo, jak opisano w pracy P5, w obu grupach testowych wykorzystano osobniki w wieku nieprzekraczającym 24 godzin, jednak ich rozwój embrionalny przebiegał w odmiennych warunkach, w zależności od sposobu rozmnażania (odpowiednio w efipium lub w komorze lęgowej). Dlatego obserwowane różnice między EE-Neo a P-Neo należy interpretować jako różnice związane ze źródłem i sposobem przygotowania organizmów, a nie jako izolowany efekt ich pochodzenia reprodukcyjnego.

Dokładny opis historii hodowli jest niezbędny również ze względu na zdolność *Daphnia* spp. do reagowania na obecność zanieczyszczeń oraz odmienną tolerancję na ich działanie. W literaturze opisano aklimatyzację fenotypową *D. magna*, efekty matczyne oraz potencjalne efekty międzypokoleniowe, które mogą przyczyniać się do zmian cech fenotypowych w kolejnych pokoleniach (Agrelius & Dudycha, 2025; Radersma et al., 2018). Ponadto obserwacja populacji *D. pulex* w osadnikach oczyszczalni ścieków, w których wykryto obecność farmaceutyków, wskazuje na możliwość funkcjonowania tych organizmów w warunkach stresowych i zdolności

adaptacyjne [P2]. W konsekwencji różnice w historii środowiskowej mogą przyczyniać się do zmienności wrażliwości na zanieczyszczenia w porównaniu z organizmami pochodzącymi z hodowli niepoddanej ekspozycji. Istnieją również dowody, że *D. magna* jako organizm referencyjny wykazywał niższą wrażliwość na zanieczyszczenia metalami ciężkimi i związkami organicznymi niż inni przedstawiciele Cladocera (*D. pulex* oraz *Simocephalus vetulus*) jako organizmy rodzime (Santos-Medrano & Rico-Martínez, 2019). Wyniki publikacji P5 rozszerzyły dotychczasową wiedzę dotyczącą wrażliwości toksykologicznej *D. magna* i wskazały, że może być modulowana nie tylko poprzez różnice pomiędzy gatunkami należącymi do Cladocera, ale także w zależności od źródła i sposobu przygotowania osobników testowych oraz ich historii hodowlanej. Podsumowując, pomimo przestrzegania znormalizowanych wytycznych OECD 202, organizmy referencyjne uzyskane z różnych źródeł oraz charakteryzujące się odmienną historią rozwoju oraz przygotowania mogą wykazywać różnice w wrażliwości na badane zanieczyszczenia. Brak istotnej interakcji między rodzajem medium hodowlanego a źródłem i sposobem przygotowania organizmów testowych [P5] wskazuje, że w przeprowadzonym badaniu nie wykazano, aby wpływ jednego z tych czynników na wartość EC_{50} zależał od drugiego. Jednocześnie oba czynniki istotnie wpływały na parametr EC_{50} , stanowiąc odrębne źródła jego zmienności w badanym układzie eksperymentalnym. Wynik ten przyczynia się do bardziej kompleksowej interpretacji danych ekotoksykologicznych i może częściowo wyjaśniać ich rozbieżność odnotowaną w literaturze.

6.4. Ocena bioakumulacji w warunkach *in situ*

Badania laboratoryjne dotyczące toksyczności oraz bioakumulacji farmaceutyków w organizmach wodnych dostarczają cennych informacji na temat ich potencjału bioakumulacyjnego oraz mechanizmów oddziaływania na organizmy. Jednak badania prowadzone w warunkach *in situ* mają szczególne znaczenie. Realne warunki panujące w środowisku biorą uwzględniają długotrwałą ekspozycję organizmów wodnych na mieszaniny zanieczyszczeń, interakcje biologiczne (m.in. dostępność pokarmu czy obecność pasożytów), a także zmienne warunki fizykochemiczne. Postanowiono więc przeprowadzić badania oceniające bioakumulację leków w warunkach rzeczywistych, jednocześnie biorąc pod uwagę obecność organizmów należących do rodzaju *Daphnia*, wykorzystywanego w standardowych testach ekotoksykologicznych

[P2]. Jako miejsce charakteryzujące się stałym dopływem pozostałości farmaceutyków wybrano osadniki wtórne oczyszczalni ścieków w Swarzewie, w których w ciepłych miesiącach bytują rozwielitki w dużych ilościach. Zebrane organizmy zidentyfikowano jako *D. pulex*. Warunki panujące w osadnikach wtórnych różniły się od warunków typowych

dla naturalnych stawów, charakteryzując się niższym pH (zakres 6,61-7,17 w lipcu i sierpniu 2021 r.; wartość referencyjna dla osadników ok. 6,58), niższym stężeniem tlenu rozpuszczonego (1,8-3,1 mg/l) oraz wyższą przewodnością elektrolityczną (6,8-7,7 mS/cm). Temperatura wody wynosiła ok. 20 °C. Badania samych ścieków potwierdziły, że *D. pulex* była ekspozowana na mieszaninę NLPZ, których stężenie w ściekach oczyszczonych przedstawiono w **Tabeli 2**.

Tabela 2. Stężenie oznaczanych analitów w ściekach oczyszczonych Oczyszczalni Ścieków Swarzewo (n=2) pobranych w dwóch różnych terminach (P2)

Analit	Stężenie [µg/l] analizowanych związków	
	28.07.2021	4.08.2021
diklofenak	1,309 ± 0,493	1,126 ± 0,074
4-hydroksydiklofenak	1,288 ± 0,032	1,263 ± 0,185
ibuprofen	0,113 ± 0,028	0,027 ± 0,020
2-hydroksyibuprofen	0,474 ± 0,017	0,194 ± 0,000
naproksen	0,174 ± 0,005	0,166 ± 0,000

Z badanej grupy związków w organizmach *D. pulex* wykryto tylko DCF. Metabolit diklofenaku 4-OH DCF, mimo występowania w ściekach oczyszczonych w stężeniu zbliżonym do stężenia związku macierzystego, nie został wykryty w próbkach rozwielitek. Może to wskazywać na różnice w pobieraniu, przemianach lub eliminacji związku macierzystego i jego metabolitu w rzeczywistych warunkach środowiskowych. DCF, prócz obecności w ściekach oczyszczonych oraz w organizmach rozwielitek, wykryto również w cząstkach osadu czynnego, które stanowią naturalne źródło pokarmu dla tych organizmów. Obliczone współczynniki podziału wskazały, że DCF charakteryzował się około 2,2-krotnie wyższym powinowactwem do organizmów rozwielitek niż do cząstek zawieszonych. Przewód pokarmowy *D. pulex* wypełniony treścią pokarmową przedstawiono na **Rysunku 2** w publikacji **P2**. W związku z tym

można przypuszczać, że cząstki zawieszone jako element diety *D. pulex* mogły stanowić dla nich źródło DCF. Przed analizą nie zastosowano etapu oczyszczania z treści pokarmowej, dlatego niemożliwe jest określenie udziału tej drogi narażenia ani odróżnienie DCF pobranego do tkanek *D. pulex* od związku obecnego w treści pokarmowej. Wyniki te wskazują, że badania *in situ* stanowią cenne uzupełnienie eksperymentów laboratoryjnych, pozwalając na ocenę rzeczywistych interakcji pomiędzy organizmami a zanieczyszczeniami w warunkach środowiskowych. Mimo wykrycia DCF w próbkach organizmów *D. pulex*, obliczony dla niego BAF był niski i wynosił 18-36 l/kg m.m. Według przywołanych wytycznych substancję uznaje się za bioakumulującą, jeżeli BCF przekracza 1000 l/kg m.m. (US EPA, 1996) lub 2000 l/kg m.m. (EMA, 2015). Należy podkreślić, że kryteria te odnoszą się do BCF, dlatego ich porównanie z BAF wyznaczonym w niniejszej pracy ma jedynie charakter orientacyjny. Uzyskana wartość BAF była jednak znacznie niższa od obu przywołanych progów, co wskazuje na niewielką zdolność DCF do bioakumulacji w *D. pulex* w badanych warunkach. Dla porównania, współczynniki biokoncentracji DCF określone po 48 godzinach narażenia *D. magna* wyniosły 70,94 i 8,02 dla nominalnych stężeń narażenia 5 i 100 µg/l (Nkoom et al., 2019). Wyniki te wskazują na zależność wartości BCF od stężenia ekspozycyjnego farmaceutyku, jednak na ich podstawie nie można jednoznacznie określić mechanizmu odpowiedzialnego za zaobserwowaną różnicę. Obecność licznej populacji *D. pulex* w osadnikach oczyszczalni ścieków świadczy o możliwości funkcjonowania tych organizmów w warunkach przewlekłego narażenia na mieszaninę zanieczyszczeń. Nie jest jednak bezpośrednim dowodem na wystąpienie mechanizmów adaptacyjnych, których potwierdzenie wymagałoby przeprowadzenia odrębnych badań. W przyszłości warto byłoby podjąć takie badania, by móc ocenić, czy zmiany warunków środowiskowych związanych ze zmianą klimatu wpływają na zdolność adaptacji rozwielitek do przewlekłego narażenia na zanieczyszczenia, w tym także na farmaceutyki.

7. Podsumowanie i wnioski

Głównym celem niniejszej rozprawy doktorskiej była ocena wpływu zmian pH, źródła i sposobu przygotowania organizmów testowych oraz składu medium hodowlanego na wyniki testów ekotoksykologicznych opartych na wytycznych OECD w kontekście oceny toksyczności farmaceutyków w warunkach zmieniającego się klimatu. Ponadto oceniono przydatność i ograniczenia metodyk opartych na wytycznych OECD w badaniach toksyczności farmaceutyków jonizowalnych. Eksperymenty laboratoryjne uzupełniono badaniami *in situ* dotyczącymi bioakumulacji diklofenaku w *Daphnia pulex* zasiedlających osadniki oczyszczalni ścieków. Wszystkie założone cele rozprawy doktorskiej zostały zrealizowane. Wpływ pH na toksyczność DCF i SMX, reprezentujących różne grupy farmaceutyków, oceniono z użyciem dwóch organizmów referencyjnych: skorupiaka planktonowego *D. magna* oraz rośliny wodnej *L. minor*. Wyniki wykazały, że zakwaszenie zwiększało negatywne skutki obecności badanych farmaceutyków. W przypadku SMX wykazano, że obniżenie pH zwiększało jego toksyczność poprzez zmianę stopnia zjonizowania, a w konsekwencji biodostępność. Natomiast zwiększonej toksyczności DCF wobec *D. magna* nie można było wyjaśnić zmianami stopnia jego jonizacji, ponieważ związek ten pozostawał w roztworze głównie w formie anionowej w obu testowanych wariantach pH. Sugeruje to, że wpływ zakwaszenia na toksyczność DCF może wykraczać poza prostą zmianę biodostępności badanego związku. Równie ważny jest wpływ pH na fizjologię rozwielitek, który może modyfikować ich wrażliwość na zanieczyszczenia.

Wykazano również istotne różnice w wartościach EC_{50} związane ze źródłem i sposobem przygotowania organizmów testowych. Rozwielitki *D. magna* EE-Neo wykazały większą wrażliwość na DCF niż rozwielitki P-Neo. Obserwowane różnice należy wiązać ze źródłem i sposobem przygotowania młodocianych osobników, ponieważ obie grupy testowe różniły się również historią rozwoju oraz warunkami poprzedzającymi rozpoczęcie badań. Ponadto rodzaj medium istotnie wpływał na wynik testu. W naturalnej wodzie stawowej *D. magna* cechowała się istotnie wyższą wartością EC_{50} , a tym samym mniejszą podatnością na immobilizację niż w medium M4, co wskazuje, że skład medium mógł modyfikować obserwowaną toksyczność DCF. Nie wykazano natomiast, aby wpływ źródła i sposobu przygotowania organizmów testowych na wartość EC_{50} różnił się w zależności od rodzaju medium. Oznacza to, że w przeprowadzonym eksperymencie nie wykazano, by wpływ jednego

z tych czynników na wartość EC_{50} zależał od drugiego. Jednocześnie oba czynniki istotnie wpływały na wartość EC_{50} , stanowiąc odrębne źródła jej zmienności w badanym układzie eksperymentalnym. Badania bioakumulacji *in situ* przeprowadzone z udziałem *D. pulex* zasiedlającej osadniki oczyszczalni ścieków dostarczyły cennych informacji dotyczących bioakumulacji DCF w warunkach rzeczywistej ekspozycji. Wyniki wykazały również możliwość funkcjonowania populacji tych organizmów w warunkach przewlekłego narażenia na mieszaninę zanieczyszczeń i podkreśliły znaczenie uzupełniania badań laboratoryjnych o dane uzyskane *in situ* oraz o ocenę losów farmaceutyków w środowisku.

Wyniki niniejszej rozprawy doktorskiej podkreślają znaczenie monitorowania oraz raportowania pH w trakcie testów ekotoksykologicznych. Parametr ten może modyfikować toksyczność farmaceutyków jonizowalnych zarówno poprzez wpływ na ich stopień dysocjacji i biodostępność, jak i na fizjologię badanych organizmów. Wyniki wskazują również na potrzebę wprowadzenia strategii pozwalających stabilizować pH podczas badań ekotoksykologicznych bez negatywnego wpływu na organizmy referencyjne. Podejście to wzmocniłoby wiarygodność oceny toksyczności w warunkach symulujących zakwaszenie środowiska wodnego.

Przeprowadzone badania wykazują, że nawet przy uwzględnieniu podstawowych wymagań OECD różnice w składzie jonowym medium oraz historii hodowli organizmów mogą wpływać na wyniki testów ekotoksykologicznych. Różnice te mogą prowadzić do statystycznie istotnych różnic w uzyskanych wartościach EC_{50} , mimo zastosowania metodyki opartej na tych samych wytycznych OECD. Podkreśla to znaczenie szczegółowego raportowania źródła i sposobu przygotowania organizmów testowych, w tym ich historii hodowlanej i rozwojowej, a także składu jonowego medium, co ułatwia właściwą interpretację i porównanie uzyskanych wyników.

Podsumowując, pH stanowi kluczowy czynnik wpływający na toksyczność jonizowalnych związków chemicznych, dlatego też powinno być systematycznie monitorowane oraz raportowane nie tylko na początku i na końcu eksperymentu, ale również podczas jego trwania, o ile to możliwe. Ponadto wyniki wskazują, że skład chemiczny wody oraz źródło i sposób przygotowania organizmów testowych mogą stanowić dodatkowe czynniki wpływające na zmienność wyników w znormalizowanych testach OECD. Łącznie wyniki te wskazują, że toksyczność farmaceutyków jonizowalnych jest wypadkową właściwości badanego związku, warunków środowiskowych, sposobu prowadzenia eksperymentu oraz cech biologicznych

organizmów testowych. Standaryzacja testów OECD nie jest więc równoważna z porównywalnością uzyskanych wyników, jeżeli w interpretacji danych nie uwzględnia się biologicznej i środowiskowej zmienności warunków prowadzenia eksperymentu. Uwzględnienie tych czynników poprawiłoby powtarzalność, porównywalność oraz istotność środowiskową badań ekotoksykologicznych przeprowadzanych zgodnie z wytycznymi OECD. Dlatego też pH oraz inne opisane zmienne powinny być uwzględnione jako stałe elementy przyszłych strategii analizy ryzyka farmaceutyków w środowisku wodnym.

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9. Wykaz tabel i rysunków

Tabele:

Tabela 1. Pobieranie sulfametoksazolu (SMX) przez *Lemna minor*, w przeliczeniu na powierzchnię członów pędowych, przy różnych stężeniach SMX i dwóch wartościach pH medium

Tabela 2. Stężenie oznaczanych analitów w ściekach oczyszczonych Oczyszczalni Ścieków Swarzewo (n=2) w dwóch różnych datach w 2021 roku

Rysunki:

Rysunek 1. Zdjęcie płytki testowej *Lemna minor*, przedstawiające zahamowanie wzrostu rośliny w obecności malejących stężeń sulfametoksazolu, począwszy od górnego lewego dołka

Rysunek 2. Formy kwasowo-zasadowe sulfametoksazolu (A) oraz diklofenaku (B)

Rysunek 3. Zależność zahamowania wzrostu *Lemna minor* od stężenia sulfametoksazolu (SMX) oraz wartości EC_{50} [mg/l] wyznaczone przy pH 5,5 (A) i pH 5,0 (B) za pomocą czteroparametrowego modelu nieliniowego log-logistycznego (LL.4). Poszczególne kolory przedstawiają wyniki trzech niezależnych powtórzeń testu, a pionowe linie - odpowiadające im wartości EC_{50} (n = 3; P4); wykresy wygenerowano za pomocą programu statystycznego R (wersja 4.3.2; R Core Team, 2023)

Rysunek 4. Wpływ stężenia diklofenaku (DCF) i pH medium na średnią liczbę żywych młodocianych rozwielitek przypadających na jedną samicę *Daphnia magna* podczas 21-dniowego testu przewlekłego prowadzonego w warunkach standardowych (pH 8,7) oraz obniżonego pH (pH 7,0). Statystycznie istotne różnice ($p < 0,05$) pomiędzy poszczególnymi stężeniami DCF a kontrolą oznaczono różnymi wielkimi literami dla warunków standardowych oraz różnymi małymi literami dla warunków obniżonego pH. Różnice pomiędzy wariantami pH przy tym samym stężeniu DCF oznaczono gwiazdką (*). Symbol † oznacza wynik zbliżony do progu istotności statystycznej ($0,05 < p < 0,10$) [P3]

Rysunek 5. Obrazy mikroskopowe *Daphnia magna* z różnych mediów hodowlanych

10. Oświadczenia autorów

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Oświadczenie

Oświadczam, iż w poniżej wymienionej pracy:

P1 Bethke Katarzyna, Kropidłowska Klaudia, Stepnowski Piotr, Caban Magda*, Review of warming and acidification effects to the ecotoxicity of pharmaceuticals on aquatic organisms in the era of climate change, Science of the Total Environment, 2023, vol. 877, s.1-24, Numer artykułu:162829. DOI:10.1016/j.scitotenv.2023.162829, 200 punktów, IF₂₀₂₃ 8,2 (Q1)

mój udział obejmował nadzór merytoryczny nad przygotowaniem pracy przeglądowej oraz opracowanie i krytyczną weryfikację manuskryptu.

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P1 Bethke Katarzyna, Kropidłowska Klaudia, Stepnowski Piotr, Caban Magda*, Review of warming and acidification effects to the ecotoxicity of pharmaceuticals on aquatic organisms in the era of climate change, Science of the Total Environment, 2023, vol. 877, s.1-24, Numer artykułu:162829. DOI:10.1016/j.scitotenv.2023.162829, 200 punktów, IF₂₀₂₃ 8,2 (Q1)

P2 Bethke Katarzyna, Kwidzińska Klaudia, Caban Magda*, Investigation of pharmaceutical bioaccumulation in *Daphnia* sp. living in a wastewater treatment plant, Science of the Total Environment, 2024. vol. 950, s.1-8, Numer artykułu:174915. DOI:10.1016/j.scitotenv.2024.174915, 200 punktów, IF₂₀₂₄ 8,0 (Q1)

P3 Bethke Katarzyna*, Caban Magda, Effect of acidification on the chronic toxicity of diclofenac to *Daphnia magna*, Aquatic Toxicology, 2025, vol. 287, s.1-7, Numer artykułu:107497. DOI:10.1016/j.aquatox.2025.107497, 140 punktów, IF₂₀₂₅ 4,6 (Q1)

P4 Bethke Katarzyna*, Caban Magda, Exploring the impact of pH on the toxicological response of *Lemna minor* to pharmaceuticals - insights from sulfamethoxazole exposure, Environmental Toxicology and Chemistry, 2026, vol.45, s.2610-2621, DOI: 10.1093/etoxjnl/vgag136, 100 punktów, IF₂₀₂₅ 3,6 (Q2)

P5 Bethke Katarzyna, Caban Magda*, Context-Dependent Toxicity of Diclofenac in *Daphnia magna*: Roles of Water Chemistry, Neonate Source and Preparation Procedure, Aquatic Toxicology, 2026, Numer artykułu: 108029. DOI: 10.1016/j.aquatox.2026.108029, 140 punktów, IF₂₀₂₅ 4,6 (Q1)

mój udział polegał na konceptualizacji, opiece merytorycznej, nadzorowaniu badań oraz współuczestniczeniu nad pisanem manuskryptów.


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P1 Bethke Katarzyna, Kropidłowska Klaudia, Stepnowski Piotr, Caban Magda*, Review of warming and acidification effects to the ecotoxicity of pharmaceuticals on aquatic organisms in the era of climate change, Science of the Total Environment, 2023, vol. 877, s.1-24, Numer artykułu:162829. DOI:10.1016/j.scitotenv.2023.162829, 200 punktów, IF₂₀₂₃ 8,2 (Q1)

P2 Bethke Katarzyna, Kwidzińska Klaudia, Caban Magda*, Investigation of pharmaceutical bioaccumulation in Daphnia sp. living in a wastewater treatment plant, Science of the Total Environment, 2024. vol. 950, s.1-8, Numer artykułu:174915. DOI:10.1016/j.scitotenv.2024.174915, 200 punktów, IF₂₀₂₄ 8,0 (Q1)

mój udział obejmował realizację części prac eksperymentalnych (ekstrakcja SPE, SLE, analizy LC-MS/MS) oraz współudział w opracowaniu manuskryptów.

mgr Klaudia Kwidzińska



11. Dorobek naukowy

DANE LICZBOWE

Sumaryczny IF¹ wszystkich publikacji	35,7
Sumaryczna liczba cytowań² wszystkich publikacji	48
Sumaryczna punktacja ministerialna MEiN	880
Indeks Hirscha³	3

¹Wartości IF według *Journal Citation Reports*, prezentowane w Bazie Wiedzy Uniwersytetu Gdańskiego, właściwe dla roku opublikowania poszczególnych prac.

²Sumaryczna liczba cytowań (bez autocytowań) według *Web of Science Core Collection*.

³Indeks Hirscha według *Web of Science Core Collection*.

*Dane bibliometryczne według stanu na 21.09.2026 r., z wyłączeniem publikacji w trakcie recenzji.

Publikacje naukowe niezaliczane do cyklu rozprawy doktorskiej, stanowiące dodatkowy dorobek naukowy o odmiennym zakresie tematycznym:

1. Lis Hanna, Kwidzińska Klaudia, Bethke Katarzyna, Caban Magda*, Cyanobacteria as generators of reactive oxygen species – A review of analysis methods and case studies, *Talanta*, 2026, vol. 297, Numer artykułu: 128648. DOI:10.1016/j.talanta.2025.128648, 100 punktów, IF₂₀₂₅ 6,7
2. Kwidzińska Klaudia, Bethke Katarzyna, Caban Magda*, Growth Inhibition and Beyond: Hormetic and Environmentally Relevant Effect of Pharmaceuticals on Cosmopolitan Cyanobacteria (*w trakcie recenzji; Aquatic Toxicology*), IF₂₀₂₅ 4,6 (Q1)

Staż naukowy:

Uniwersytet Techniczny w Dreźnie, Instytut Chemii Wody; 7.11 – 1.12.20223

Tytuł stażu: Ecotoxicity tests on water organisms

- przeprowadzanie testów ekotoksykologicznych zgodnych z OECD
- analiza bioakumulacji diklofenaku w *Daphnia magna*
- analiza wybranych związków za pomocą LC-MS/MS

Konferencje krajowe

- *Prezentacje ustne:*

Katarzyna Bethke, Klaudia Kwidzińska, Magda Caban „Zakwaszenie wód zwiększa toksyczność diklofenaku wobec organizmów wodnych”, XX Ogólnopolskie Seminarium Doktorantów i Studentów "Na pograniczu Chemii, Biologii i Farmacji", Jarnołtówek, Polska, 26-29.05.2024

Katarzyna Bethke, Weronika Koczur, Magda Caban, Piotr Stepnowski „Ostre i chroniczne testy ekotoksykologiczne w ocenie zmiany toksyczności sulfametoksazolu pod wpływem zmian klimatu”, Chemia, geochemia i ochrona środowiska morskiego, Gdynia 20.10.2023

Katarzyna Bethke, Magda Caban, Piotr Stepnowski "Wpływ wzrostu temperatury i obniżenia pH na toksyczność ostrą diklofenaku wobec *Daphnia magna*", XIX Ogólnopolskie Seminarium Doktorantów i Studentów "Na pograniczu Chemii i Biologii", Trzebiezowice, 4-7.06.2023

Katarzyna Bethke, Klaudia Kropidłowska, Magda Caban, Piotr Stepnowski „Analiza bioakumulacji wybranych farmaceutyków w zooplanktonie żyjącym w osadniku wtórnym oczyszczalni ścieków z wykorzystaniem techniki LC-MS”, XI Polska Konferencja Chemii Analitycznej „POKOCHA”, Łódź, 19-23.06.2022

Magda Caban, Klaudia Kropidłowska, **Katarzyna Bethke**, Piotr Stepnowski „Analiza lotnych związków produkowanych przez sinice techniką mikroekstrakcji do fazy stałej wspomaganą próżniowo”, XI Polska Konferencja Chemii Analitycznej „POKOCHA”, Łódź, 19-23.06.2022

Klaudia Kropidłowska, **Katarzyna Bethke** Piotr Stepnowski, Magda Caban „Wykorzystanie metod chromatograficznych i spektrofotometrii do analiz pigmentów fotosyntetycznych sinic *Synechocystis salina*”, XI Polska Konferencja Chemii Analitycznej „POKOCHA”, Łódź, 19-23.06.2022

- **Postery:**

Simona Kołek, **Katarzyna Bethke**, Magda Caban „Wpływ ocieplenia klimatu i zakwaszenia środowiska wodnego na toksyczność diklofenaku względem skorupiaków *Daphnia magna*”, IV Ogólnopolska Konferencja "ChemBiŚ", Gdańsk, 4.07.2022

Katarzyna Bethke, Magda Caban, Piotr Stepnowski „Toksyczność karbamazepiny w środowisku a globalne ocieplenie”, III Pomorskie Studenckie Sympozjum Chemiczne, konferencja online, Gdańsk, 25.08.2021

Simona Kołek, **Katarzyna Bethke**, Magda Caban „Wpływ wybranych farmaceutyków na środowisko wodne”, III Ogólnopolska Konferencja "ChemBiŚ", Gdańsk, 26-27.06.2021

Katarzyna Bethke, Magda Caban, Piotr Stepnowski „*Daphnia magna* as versatile test organism in ecotoxicological studies” National Scientific Conference e-Factory of Science, konferencja online, 10.04.2021

Katarzyna Bethke, Karolina Laszczuk, Kamila Bucholc, Hanna Lis, Magda Caban „Kalibracja dozymetrów typu PASSIL w celu analizy farmaceutyków w różnych matrycach wodnych”, II Ogólnopolska Konferencja "ChemBiŚ", Gdańsk, 28-29.06.2019

Karolina Laszczuk, **Katarzyna Bethke**, Mada Caban, Hanna Lis "Kalibracja dozymetrów PASSIL i POCIS metodą przepływową w matrycy wody morskiej i wodociągowej", II Ogólnopolska Konferencja "ChemBiŚ", Gdańsk, 28-29.06.2019

Konferencje międzynarodowe

- **Prezentacje ustne:**

Katarzyna Bethke, Klaudia Kwidzińska, Weronika Koczur, Anna Białk-Bielińska, Magda Caban „Acidification increases the toxicity of diclofenac to aquatic organisms” SETAC Europe 34th Annual Meeting, Sevilla, Spain 5-10.05.2024

Katarzyna Bethke, Klaudia Kropidłowska, Weronika Koczur, Magda Caban, Piotr Stepnowski "Effect of lowering pH and increasing temperature on toxicity of sulfamethoxazole to *Daphnia magna*", SETAC Europe 33rd Annual Meeting, Dublin, Ireland, 30.04-04.05.2023

- **Postery:**

Katarzyna Bethke, Klaudia Kwidzińska, Weronika Koczur, Magda Caban „Effect of pH change on chronic toxicity of diclofenac to *Daphnia magna*”, BEING SEA-EU, multidisciplinary conference University of Malta, 10-12.06.2024

Klaudia Kwidzińska, **Katarzyna Bethke**, Magda Caban „Miniaturization of ecotoxicological tests and their application to assess the toxicity of pharmaceuticals towards baltic cyanobacteria” BEING SEA-EU, Malta, 10-12.06. 2024

Magda Caban, Martyna Zalewska, Anna Aksmann, Justyna Kobos, Hanna Mazur-Marzec, **Katarzyna Bethke** and Klaudia Kropidłowska "Resistance of cyanobacterium *Microcystis aeruginosa* to diclofenac measured by various marker", SETAC Europe 33rd Annual Meeting, Dublin, Ireland, 30.04-04.05.2023

Klaudia Kropidłowska, **Katarzyna Bethke**, Magda Caban "Effect of naproxen, ibuprofen and diclofenac on cyanobacterium *Synechocystis Salina* with relation to salinity of medium", SETAC Europe 33rd Annual Meeting, Dublin, Ireland, 30.04-04.05.2023

Kierownik projektów BMN (Badania Młodych Naukowców, finansowanie przez Uniwersytet Gdański):

1. “Rozpuszczalność w wodzie i toksyczność cieczy jonowych dicyjanamidu triheksylo(tetradecylo)fosfoniowego i dekanianu triheksylo(tetradecylo)fosfoniowego dla organizmów wodnych” - 2021
2. “Wpływ wzrostu temperatury i spadku pH wód na toksyczność sulfametoksazol i sulfapirydyny dla *Daphnia magna*” - 2022
3. „Wpływ podwyższenia temperatury oraz spadku pH wód na toksyczność diklofenaku względem *Daphnia magna*” - 2023
4. „Wpływ podwyższenia temperatury oraz spadku pH wód na toksyczność atenololu względem *Daphnia magna*” – 2024

**12. Prace opublikowane wchodzące w skład rozprawy
doktorskiej P1-P5**



Review of warming and acidification effects to the ecotoxicity of pharmaceuticals on aquatic organisms in the era of climate change

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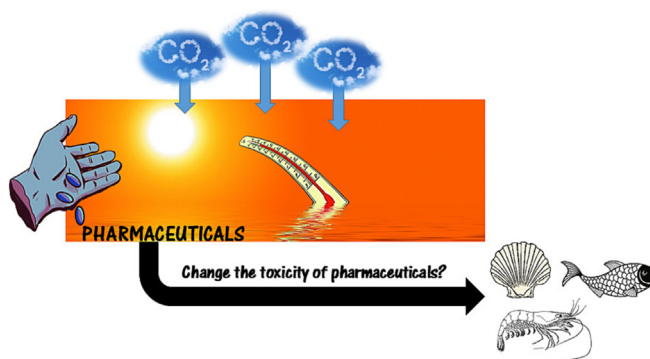
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HIGHLIGHTS

- Acidification and warming modulates the ecotoxicity of pharmaceuticals.
- Biochemical, cellular and behavioral biomarkers show a response.
- Trends of change in acute and chronic toxicity were drug dependent.
- Acidification modified the toxicity of selected ionizable pharmaceuticals.
- Bioaccumulation was modified by target effects of global warming.

GRAPHICAL ABSTRACT



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ABSTRACT

An increase in the temperature and the acidification of the aquatic environment are among the many consequences of global warming. Climate change can also negatively affect aquatic organisms indirectly, by altering the toxicity of pollutants. Models of climate change impacts on the distribution, fate and ecotoxicity of persistent pollutants are now available. For pharmaceuticals, however, as new environmental pollutants, there are no predictions on this issue. Therefore, this paper organizes the existing knowledge on the effects of temperature, pH and both stressors combined on the toxicity of pharmaceuticals on aquatic organisms. Besides lethal toxicity, the molecular, physiological and behavioral biomarkers of sub-lethal stress were also assessed. Both acute and chronic toxicity, as well as bioaccumulation, were found to be affected. The direction and magnitude of these changes depend on the specific pharmaceutical, as well as the organism and conditions involved. Unfortunately, the response of organisms was enhanced by combined stressors. We compare the findings with those known for persistent organic pollutants, for which the pH has a relatively low effect on toxicity. The acid-base constant of molecules, as assumed, have an effect on the toxicity change with pH modulation. Studies with bivalves have been overrepresented, while too little attention was paid to producers. Furthermore, the limited number of pharmaceuticals have been tested, and metabolites skipped altogether. Generally, the effects of warming and acidification were rather indicated than explored, and much more attention needs to be given to the ecotoxicology of pharmaceuticals in climate change conditions.

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1. Introduction

According to the Intergovernmental Panel on Climate Change (IPCC) Special Report, the Earth's temperature has risen by about 1 °C since the industrial revolution, and it is extremely likely that the temperature will rise by 1.5 °C by 2052 (Hoegh-Guldberg et al., 2018). Greenhouse gases are causing progressive environmental change not only on land and in the air, but also in the water environment (Harley et al., 2006). As much as 30 % of the CO₂ emitted in recent decades has been absorbed by the ocean (Feely et al., 2004). Before the industrial era, the global average pH at the ocean surface was 8.2, while today it is 8.1 and will continue to fall (EPA, 2022a). Currently, the average rate of global ocean acidification has been set at 0.002 pH units per year (Häder and Gao, 2019). Seemingly, this is a small difference in the average pH of the ocean, but acidity increases tenfold when the pH decreases by one degree. Since the beginning of the 21st century, ocean acidity has increased by 25–30 %, what is the fastest rate of change in the Earth's geological history according to the United States Environmental Protection Agency's (EPA's) estimation. The predicted decrease in pH in the oceans is 0.3 to 0.5 units over the next 100 years, causing acidity to double (Caldeira and Wickett, 2005). Reduced pH in the ocean environment directly affects organisms whose shells are composed of calcium carbonate – causing an inhibition of the calcification and growth (Feely et al., 2004; Doney et al., 2009). Additional studies confirmed the impact of ocean acidification on changes in the DNA methylation profile of aquatic organisms (Lim et al., 2021). Changes in gene expression occur also in organisms exposed to elevated thermal conditions (Somero, 2002). For many organisms, an increase in temperature means exceeding the upper limit of their thermal tolerance. The acclimatization to new thermal conditions carries an energy cost. In response to the temperature shock, organisms consume more energy. Peptide bonds break down, forcing the body to produce new proteins. The energy is also used to renew the structure of the damaged cell. Both climate change parameters – acidification and temperature rise, have an effect on water organisms, and probably most of these effects are still unexplored, as they cannot yet be observed on a large scale.

In 2009, Noyes et al. (2009a) published one of the first articles about the potential interactions between a global warming and ecotoxicology. In 2010, the journal Nature published an article noting that the toxicity of pollutants may be enhanced by the global warming effects (Lovett, 2010). A number of experiments have been conducted which have confirmed an

increase in the toxicity of pesticides (Freitas et al., 2017a, 2017b), heavy metals (Park et al., 2020; Belivermiş et al., 2020) and persistent organic pollutants (POPs) (Noyes et al., 2009a; UNEP/AMAP, 2011) on aquatic organisms under the influence of warming or a acidification. It is predicted that climate change will affect the partitioning, reaction rates, and the temporal and spatial distribution of pollutants (Noyes et al., 2009a). The lower pH of the waters alters the bioavailability and bioaccumulation of the chemicals (Belivermiş et al., 2020). The alteration of whole ecosystems by POPs is predicted with global warming (UNEP/AMAP, 2011).

However, there is an insufficient number of data about the impact of the chemical, physical and biological effects of global warming on altering the toxicity of the new emerging pollutants, such as pharmaceuticals (Tili and Mouneyrac, 2021). The bioavailability of these compounds strictly depends on the temperature and pH (Puckowski et al., 2016). Nevertheless, most studies to date have focused on the ecotoxicity and bioaccumulation of pharmaceuticals under conditions corresponding to the standard protocols of ecotoxicology studies (Świacka et al., 2019). The highly possible altering of pharmaceuticals' toxicity in the environment is an issue of great concern. (Nichols et al., 2015). Despite increased awareness of the origin and environmental fate of pharmaceuticals, there is still too little conscious action to minimize their risks to the environment, and their concentrations will likely increase in future (Caban and Stepnowski, 2021). The need to implement risk-based measures for pharmaceuticals is confirmed by their EU Water Framework watch lists (Water Europe, 2020), which include an increasing number of pharmaceuticals under surveillance, such as selected antibiotics and antidepressants (Aydın et al., 2021; Acuña et al., 2020).

To date, there is no review paper that systematizes the knowledge on the effects of increasing temperature and decreasing pH on the toxicity of pharmaceuticals on aquatic organisms, and therefore we decided to highlight the relevant and current literature addressing this issue. The aim of this review is also to answer the following questions: How does warming and acidification affect pharmaceutical ecotoxicity? Is it dependent on the specific organism they affect and does the synergistic effect of the aforementioned two factors enhance the toxic effect of pharmaceuticals? The obtained data for pharmaceuticals were compared to much more well explored POPs. The results section, and presented current reports, was divided into chronic and acute effects for separately for warming and acidification. Due to the fact that the research in the topic of the review is in the initial phase, we also point out the current shortcomings and suggest the necessary directions for further research.

2. Methodology of the literature review

The review paper was written using the search engines Pubmed, Google Scholar and Science Direct. The keywords and compilations such as: toxicity of pharmaceuticals/drugs, effects of increasing temperature, effect of decreasing pH on pharmaceutical/drugs toxicity, acidification pharmaceuticals toxicity, toxicity pharmaceuticals/drugs global warming, pharmaceuticals/drugs ecotoxicology of climate change, were used. The numerous scores found were filtered to take into account those that presented an acute or chronic effect of the pharmaceuticals on aquatic organisms, with finally <30 in the scope of the review. The experimental papers used to verify the questions asked were from the years 2008–2022. The general problem in the literature search was the different keywords used for the same type of experiment, with the use of the words drug, pharmaceutical, and specific names of pharmaceuticals used alternatively. Furthermore, several publications in which the effects of pH and temperature were tested did not mention that these factors related to a change of the climate. For example, Sun et al. (2020) performed test to check the pH effect pharmaceuticals had toward freshwater invertebrates but with no aim to test climate change conditions. Such a situation is reasonable while the same organisms can live in an environment characterized by different pH conditions and temperatures. This means that despite the general aim of this review to mark the effect of temperature rise and acidification of pharmaceutical toxicity as the effect of global warming, what we present is the general overview on this issue. Nevertheless, the trends reported are valid for changes observed in the environment.

The previous reviews of the ecotoxicology of climate change are presented in Table 1. None of them were focused on pharmaceuticals' toxicity, but they present, rather, the emerging need for the study of the problem not only of POPs, but also new-emerging pollutants.

3. Results

3.1. The effect of warming

3.1.1. The effects of temperature on acute toxicity toward marine species

Table 2 presents current reports on the effect of water warming on the acute toxicity of pharmaceuticals on marine and freshwater organisms. Only two papers were found for marine species. A temperature range of 15 °C to 33 °C and two representatives of antibiotics - oxytetracycline (tetracycline), cefalexin (beta-lactam) were tested. The marine organisms that were toxicologically tested were the stringfish *Pomatoschistus microps* and the protozoa *Euplotes crassus*. Lethal toxicity was tested by determining

Table 1

The selected up-to-day (January 2023) reviews about the problem of ecotoxicology on climate change with chronological order.

Title of review paper	Pollutants of interest	Ref.
The toxicology of climate change: Environmental contaminants in a warming world	Air pollutants, POPs, pesticides	(Noyes et al., 2009a)
Interactions between chemical and climate stressors: a role for mechanistic toxicology in assessing climate change risks	PAHs, POPs, pesticides, metals, endocrine disrupting chemicals (EDCs)	(Hooper et al., 2013)
Global warming and environmental contaminants in aquatic organisms: The need of the etho-toxicology approach	POPs, metals	(Manciocco et al., 2014)
Impacts of climate change on the ecotoxicology of chemical contaminants in estuarine organisms	Metals, pesticides, petroleum	(Delorenzo, 2015)
New challenges of marine ecotoxicology in a global change context	Specific groups not specified	(Tili and Mouneyrac, 2021)
The influence of global climate change on accumulation and toxicity of persistent organic pollutants and chemicals of emerging concern in Arctic food webs	POPs	(Borgå et al., 2022)

the LC₅₀ parameter (the concentration causing death in 50 % of the tested organisms), as well as sublethal toxicity by determining the EC₅₀ parameter (the concentration causing a toxic effect in 50 % of the tested organisms). These parameters were determined based on the OECD's (Organization for Economic Cooperation and Development) or the EPA's (U.S. Environmental Protection Agency) guidelines.

Specifically, an increase in toxicity (expressed as EC₅₀ predatory performance) was noted for cefalexin on juveniles of the common goby (*P. microps*) (Fonte et al., 2016). Interestingly, this effect was even enhanced when cefalexin (10 mg L⁻¹) was mixed with microplastics, appearing as increased acetylcholinesterase (AChE) activity (by 14 %) and lipid peroxidation (LPO) levels (by 72 %).

An inverse relationship of toxicity change with warming was noted for oxytetracycline (OTC) (Gomiero and Viarengo, 2014). The toxicity of this antibiotic toward the protozoa *E. crassus* decreases with warming, as shown by the values of the lethal - LC₅₀, and sublethal - EC₂₅ concentrations for 25 °C and 33 °C (Table 2). The percentage rate of endocytosis in the cell increases with warming, indicating an increase in food uptake. The stability of the lysosomal membrane of organisms increases with warming as well. The decrease in OTC toxicity with warming may be due to the decomposition of the compound at an elevated temperature and alkaline pH (experiments were conducted at pH 8) (Taylor et al., 2011), confirmed by the mass spectrometry analysis (Gomiero and Viarengo, 2014).

3.1.2. Effects of temperature on acute toxicity for freshwater species

The effects of temperature (from 15 °C to 25 °C) on acute toxicity (following EPA guidelines) on freshwater organisms (crustaceans *Daphnia magna* and *Atyaephyra desmarestii*) were reported in two papers (Table 2). The following representatives of antibiotics: enrofloxacin, chlortetracycline, sulfathiazole; NSAIDs (non-steroidal anti-inflammatory drugs) - diclofenac, ibuprofen; an analgesic and antipyretic pharmaceutical - acetaminophen; and a psychotropic pharmaceutical - carbamazepine, were tested.

The effect of elevated temperature on pharmaceuticals' toxicity on *D. magna* was studied by Kim et al. (2010), who conducted a 48-h acute toxicity test at 15 °C, 21 °C, and 25 °C. Pharmaceuticals such as acetaminophen (paracetamol), enrofloxacin, chlortetracycline and sulfathiazole, among others, showed an increase in toxicity (EC₅₀) with increasing temperature (Table 2), but for sulfathiazole the change appeared to be insignificant. The increased toxicity in the enhanced temperature can be an effect of the altered metabolism and enhanced biotransformation of the pollutants (Noyes et al., 2009b). Thus, it is possible that *D. magna* produced more toxic metabolites of acetaminophen when exposed to increased temperature, which is responsible for accelerated toxicity (8.3 times) when the temperature changes from 15 to 25 °C (Kim et al., 2010).

Despite the fact of the general observation that enhanced temperature accelerated the toxic effect of pharmaceuticals on toward water organisms, the effect depends on the molecules concerned. Nieto et al. (2016) conducted an acute toxicity test by investigating the effect of temperature (from 20 °C to 25 °C) on the toxicity of ibuprofen (IBF), diclofenac (DCF) and carbamazepine (CBZ) on freshwater shrimp *A. desmarestii* (Table 2). Warming significantly affected carbamazepine toxicity, decreasing the LC₅₀ by almost 1.5 times. The values of LC₅₀ for IBF in 20 °C was 13.3 mg L⁻¹ and decreased 1.32 times at elevated temperatures. The toxicity of DCF was not altered, despite some similarities in the mode of action of the NSAIDs (IBF and DCF).

3.1.3. Chronic toxicity effects of temperature rise for marine species

Table 3 presents current reports on the effect of increasing water temperatures on the chronic toxicity of selected pharmaceuticals on marine and freshwater organisms (5 and 3 papers, respectively). The tested pharmaceuticals included antibiotics (oxytetracycline), NSAIDs (diclofenac and salicylic acid), a psychotropic pharmaceutical (carbamazepine) an antihistamine (cetirizine), and synthetic oestrogen - 17 α-ethinylestradiol (EE2). The tested marine organisms were mussels: *Mytilus galloprovincialis*; clams: *Ruditapes philippinarum*; and the protozoa *Euplotes crassus* (Table 3). The classical biochemical markers were a main focus in the chronic tests.

Table 2

Reports presenting effects of increasing temperature into acute toxicity of selected pharmaceuticals on marine and freshwater aquatic species (EC_{25,50} - the pharmaceutical concentration causing a toxic effect in 25 %/50 % of the tested organisms; LC₅₀ - the pharmaceutical concentration causing death in 50 % of the tested organisms).

Species (reference method)	Temperatures tested/test duration	Pharmaceutical	Acute effect	Ref.
Marine species				
Juveniles of fish	20 °C, 25 °C/96 h	Cefalexin	Predatory performance of fish (EC ₅₀): - 20 °C → 8.4 mg L ⁻¹ - 25 °C → 3.8 mg L ⁻¹	(Fonte et al., 2016)
<i>Pomatoschistus microps</i> (OECD 203)				
Ciliated protozoa	25 °C, 27 °C, 29 °C, 31 °C, 33 °C/8 h or 3 h	Oxytetracycline	Cell viability (8 h, LC ₅₀): - 25 °C → 1.1 mM - 33 °C → 2.2 mM Lysosomal membrane stability (3 h, EC ₂₅): - 25 °C → 73 µM - 33 °C → 121 µM	(Gomiero and Viarengo, 2014)
<i>Euplotes crassus</i>				
Freshwater species				
Crustacean	15 °C, 21 °C, 25 °C/48 h	Acetaminophen	EC ₅₀ : - 15 °C → 39.7 mg L ⁻¹ - 25 °C → 4.8 mg L ⁻¹	(Kim et al., 2010)
<i>Daphnia magna</i> (US EPA 2002 EPA- 821-R-02-012)		Enrofloxacin	EC ₅₀ : - 15 °C → 58.5 mg L ⁻¹ - 25 °C → 20.1 mg L ⁻¹	
		Chlortetracycline	EC ₅₀ : - 15 °C → 288.0 mg L ⁻¹ - 25 °C → 111.2 mg L ⁻¹	
		Sulfathiazole	EC ₅₀ : - 15 °C → 142.4 mg L ⁻¹ - 25 °C → 116.4 mg L ⁻¹	
Prawn	20 °C, 25 °C/96 h	Ibuprofen	LC ₅₀ : - 20 °C → 11.3 mg L ⁻¹ - 25 °C → 10.1 mg L ⁻¹	(Nieto et al., 2016)
<i>Atyaephyra desmarestii</i> (US EPA Penaeid Acute Toxicity Test OPPTS 850.1045)		Diclofenac	LC ₅₀ : - 20 °C → 6.3 mg L ⁻¹ - 25 °C → 6.4 mg L ⁻¹	
		Carbamazepine	LC ₅₀ : - 20 °C → 94.3 mg L ⁻¹ - 25 °C → 66.4 mg L ⁻¹	

In addition, indicators of cell damage, indicators of energy metabolism and the integrated biomarker response (IBR), summarizes the biomarker responses and simplifies their interpretation. The analysis of biochemical markers is needed in advanced ecotoxicity studies, because they can show the chronic effects and response of an organism before any visible effects. The molecular biomarkers, such as the receptor interaction, gene upregulation/downregulation, protein and lipid oxidation, are the initiating toxicity events (Hooper et al., 2013). With the acceleration of the toxic effect the following organism response can obtain, for example lethality, hormonal system dysfunction, chronic diseases, and other. Finally, the population response can occur, with extinction as the worst-case scenario. The simultaneous observation of the molecular, cellular and organ response would help assess the mechanistic toxicity pathway.

The bivalves are one of the most frequently tested organisms in chronic tests, for which several well-understood biomarkers are available. As an example, Freitas et al. (2020) examined the effect of increasing temperature from 17 °C to 21 °C on the toxicity of DCF (2019b) and salicylic acid against *M. galloprovincialis*. The rates of energy metabolism (ETS - electron transport system), antioxidant protection, and indicators of cell damage were examined (Table 3). It was observed that, in the case of DCF, both temperature elevation and DCF exposure individually altered the ETS, GLY and PROT in *M. galloprovincialis*, but no enhanced toxicity was observed when the mussels were exposed simultaneously to both stressors (Freitas et al., 2019b). The mussels exposed to DCF strived to eliminate the excessive amount of oxygen free radicals formed as a result of the presence of the pharmaceutical - and thus, they increased the activity of antioxidant enzymes such as SOD and CAT. DCF induces oxidative stress in mussels regardless of the temperature. Also, the increase in GSTs at both temperatures in mussels exposed to the DCF indicates impaired cell metabolism and the initiation of DCF detoxification processes. The involvement of the GST enzyme in DCF detoxification has also been experimentally confirmed by other researchers (Schmidt et al., 2011; Mezzelani et al., 2018),

and the lack of increased activity of this enzyme under warming suggests that increasing temperature does not potentiate the toxicity of DCF against *M. galloprovincialis*. The effect of increased temperature on mussels was noted by an increase in LPO levels (Freitas et al., 2019b). This probably indicates that despite the active defense of SOD, CAT and GST, the exposure of the mussels to elevated temperature prevented the efficient neutralization of oxygen free radicals formed by the DCF. The studies of several biochemical markers of stress can help understand the effect of warming on the toxicity of pollutants, as Freitas et al. showed.

M. galloprovincialis responded differently to warming, with simultaneous exposure to salicylic (4 mg L⁻¹) (Freitas et al., 2020) acid compared to DCF (1 µg L⁻¹) (Freitas et al., 2019b), which can be an effect of the applied concentrations. The additive negative effect of both stressors was found using the integrated biomarker response (IBR). The metabolic capacity was weakened, and consequently, the mussel body increased lipid reserves (Table 3). The comparatively low ETS activity in mussels exposed to both salicylic acid + warming compared to ETS activity under control conditions indicates that these organisms were no longer able to increase their metabolism under both stressors, and, as a consequence, their antioxidant defense was impaired. It is significant that metabolism was increased under the elevated temperature or exposure to salicylic acid. Nevertheless, not all biomarkers changed significantly during the 28 day-long exposure (as show by arrows in Table 3, GLY and PROT content was not altered).

Other experiments using *M. galloprovincialis* were also carried out by J. Lopes et al. (2022), investigating the effect of elevated temperature (17 °C as the control and 21 °C as the predicted climate warming conditions) on the toxicity of the synthetic hormone 17 α-ethinylestradiol (EE2). The test lasted 28 days, and the mussels were exposed to concentrations of EE2 from 5 ng L⁻¹ to 625 ng L⁻¹, to show the effect in a more or less polluted environment. The mussels' metabolism, oxidative stress, neurotoxicity and histopathological changes were controlled during the experiment. It was shown that the biochemical response of the mussels was temperature-

Table 3

Reports presenting effects of increasing temperature into chronic toxicity of selected pharmaceuticals on marine and freshwater aquatic species (AChE - acetylcholinesterase activity; CAT - catalase activity; ChE - cholinesterase activity; CtDe - additional lysosomal cathepsin D activity; CtDL - lysosomal cathepsin D activity; EC_{20,25,50} - the pharmaceutical concentration causing a toxic effect in 20 %/25 %/50 % of the tested organisms; ETS - electron transport activity; GLY - glycogen content; GPx - glutathione peroxidase activity; GSH/GSSG - the ratio of reduced to oxidized form of glutathione; GSTs - glutathione S-transferases activity; LIP - lipids reserves; LPO - lipid peroxidation levels; MTS - thiol-bound metallothioneins concentration; PC - protein carbonylation value; PROT - protein content; SOD - superoxide dismutase activity; TBARS - thiobarbituric acid-reactive substances production).

Species (reference method)	Temperatures tested/test duration	Pharmaceuticals (concentration)	Chronic effect (↑ or ↓ if warming increase or reduce response)	Ref.
Marine species				
Ciliated protozoa	25 °C, 27 °C, 29 °C, 31 °C,	Oxytetracycline	Replication rate ↑ (EC ₂₅ at 25 °C → 56 µM, EC ₂₅ at	(Gomiero and Viarengo, 2014)
<i>Euplotes crassus</i>	33 °C/24 h	(5–500 µM)	33 °C → 99 µM)	
Mussel	17 °C, 21 °C/28 days	Diclofenac	Indicators of energy metabolism:	
<i>Mytilus galloprovincialis</i>		(1 µg L ⁻¹)	• ETS • GLY • PROT Indicators of oxidative stress: • SOD • CAT • GSTs Indicators of cell damage and redox balance: • LPO levels ↑ • PC value ↓ • GSH/GSSG	(Freitas et al., 2019a, 2019b)
Clam	17 °C, 21 °C/28 days	Carbamazepine	Indicators of energy metabolism:	(Almeida et al., 2021)
<i>Ruditapes philippinarum</i>		(1 µg L ⁻¹)	• ETS activity ↓ • GLY • PROT content ↓ Indicators of cell damage: • LPO content ↓ • PC • GSH/GSSG Indicators of oxidative stress: • SOD • GPx • GSTs • AChE	
		Cetirizine	Indicators of energy metabolism:	
		(0.61 µg L ⁻¹)	• ETS activity ↓ • GLY • PROT Indicators of cell damage: • LPO content ↓ • PC • GSH/GSSG Indicators of oxidative stress: • SOD activity ↑ • GPx • GSTs • AChE	
		Mixture:	Indicators of energy metabolism:	
		Carbamazepine	• ETS • GLY content ↓ • PROT content ↓ Indicators of cell damage and redox balance: • LPO content ↓ • PC • GSH/GSSG ratio ↑	
		(1 µg L ⁻¹)		
		Cetirizine		
		(0.61 µg L ⁻¹)		

(continued on next page)

Table 3 (continued)

Species (reference method)	Temperatures tested/test duration	Pharmaceuticals (concentration)	Chronic effect (↑ or ↓ if warming increase or reduce response)	Ref.
Mussel <i>Mytilus galloprovincialis</i>	17 °C, 21 °C/28 days	Salicylic acid (4.0 mg L ⁻¹)	Indicators of oxidative stress: • SOD activity ↑ • GPx • GSTs activity ↓ • AChE Metabolic capacity and energy reserves:	(Freitas et al., 2020)
			• LIP content ↑ • ETS activity ↓ • GLY • PROT Indicators of oxidative stress: • SOD activity ↑ • CAT • GPx Indicators of cell damage and redox balance: • LPO • GSH/GSSG ratio ↑ IBR - Integrated biomarker response ↑ Metabolic capacity and energy reserves:	
Mussel <i>Mytilus galloprovincialis</i>	17 °C, 21 °C/28 days	17- α -ethinylestradiol (5.0; 25.0; 125.0 and 625.0 ng L ⁻¹)	• ETS - ↓ activity at 25.0 ng L ⁻¹ - ↑ activity at 125.0 ng L ⁻¹ and 625.0 ng L ⁻¹ • GLY - ↓ content at 625.0 ng L ⁻¹ - ↑ content at 125.0 ng L ⁻¹ • PROT - ↓ content at 5.0 ng L ⁻¹ - ↑ content at 125.0 ng L ⁻¹ Indicators of oxidative stress: • SOD - ↓ activity at 25.0 ng L ⁻¹ , 125.0 ng L ⁻¹ , 625.0 ng L ⁻¹ • CAT - ↓ activity at 5.0 ng L ⁻¹ , 25.0 ng L ⁻¹ , 125.0 ng L ⁻¹ , 625.0 ng L ⁻¹ • GPx - ↓ activity at 25.0 ng L ⁻¹ • GSTs - ↓ activity at 5.0 ng L ⁻¹ , 25.0 ng L ⁻¹ , 125.0 ng L ⁻¹ , 625.0 ng L ⁻¹ • AChE - ↓ activity at 25.0 ng L ⁻¹ , 125.0 ng L ⁻¹ , 625.0 ng L ⁻¹ Oxidative damage and redox balance: • GSH/GSSG • LPO levels - ↑ level at 125.0 ng L ⁻¹ , 625.0 ng L ⁻¹	(Lopes et al., 2022)
Freshwater species Prawn <i>Atyaephyra desmarestii</i> (US EPA Peneaeid Acute Toxicity Test OPPTS 850.1045)	20 °C, 25 °C/7 days	Diclofenac (10 μ g L ⁻¹) Ibuprofen (70 μ g L ⁻¹)	Response to oxidative stress: • osmoregulatory capacity • ingestion rates • ↓ respiration rates and oxygen independence Response to oxidative stress: • osmoregulatory capacity • ingestion rates • respiration rates • oxygen independence	(Nieto et al., 2016)

Table 3 (continued)

Species (reference method)	Temperatures tested/test duration	Pharmaceuticals (concentration)	Chronic effect (↑ or ↓ if warming increase or reduce response)	Ref.
Crustacean <i>Daphnia magna</i> (OECD 211)	20 °C, 25 °C/21 days	Carbamazepine (10 µg L ⁻¹)	Response to oxidative stress: • osmoregulatory capacity • ingestion rates • ↓ respiration rates and oxygen independence	(Martins et al., 2013)
		Florfenicol (0.8–50.0 mg L ⁻¹)	Total number of living juveniles (EC ₅₀) ↓ The growth rate ↓ Mortality of parent ↑ Total broods ↑ Concentration at which juvenile death was observed ↓ The highest concentration at which breeding was still recorded ↓	
Clam <i>Unio tumidus</i>	18 °C, 25 °C/14 days	Mixture: Diclofenac (2 nM), Nifedipine (2 nM), Glyphosate herbicide (79 nM)	Indicators of oxidative stress: • SOD activity ↑ • GSTs Indicators of cell damage: • TBARS • PC Biomarkers of toxicity: • CtDL activity ↑ • Caspase 3 activity ↑ • CtDe activity ↑ • ChE activity ↑ • GSH/GSSG • RI-GSH • MTSH • Lysosomal membrane stability Accumulation and distribution of metals in the digestive gland: • Concentration of Cu ↑ • Concentration of Cu bound to MTSH ↓ • Concentration of Zn ↓ • Concentration of Zn bound to MTSH • Concentration of Cd ↑ • Concentration of Cd bound to MTSH ↓ • Zn/Cu molar ratio in the tissue ↓	(Khoma et al., 2021)

dependent, and that there is correlation between the EE2 concentration and the temperature against all the parameters tested, excluding AChE. Notably, the greatest toxic effect was observed when both stressors – the EE2 and elevated temperature – acted simultaneously (specific changes in various biomarkers were noted in Table 3). It was also shown that the EE2 induced biochemical changes in *M. galloprovincialis* regardless of the temperature tested. The highest bioaccumulation was noted while the bivalves were exposed to the highest EE2 concentrations. An increase in contaminant accumulation by bivalves with an increasing concentration gradient has been documented in previous studies (Cátia Velez et al., 2015; Velez et al., 2016b), and this is related to the lipophilic properties of EE2 (Caban et al., 2016). Consequently, EE2 in environmental waters has an affinity to accumulate in the suspended solid fraction, which is rich in organic matter, and is a food source for bivalves. Significantly, in a study by J. Lopes et al. (2022), the bioaccumulation was temperature-dependent and was lower at elevated temperatures (the bioconcentration factor - BCF at 17 °C was 39 L kg⁻¹, while BCF at 21 °C was 30 L kg⁻¹). At the control temperature, a higher biotransformation capacity (higher GSTs activity) and lower filtration and accumulation capacity were observed. *M. galloprovincialis* at 21 °C increased its metabolism. Although detoxification mechanisms were activated at 17 °C, the higher bioaccumulation of EE2 at this temperature may indicate insufficient activity to eliminate the pharmaceutical. Moreover, no increase in the activation of antioxidant defense mechanisms (SOD, CAT, GPx) was observed in mussels

exposed to EE2 at the control temperature. It is possible, therefore, that the concentrations tested in this experiment were not high enough to induce an enhanced antioxidant defense, or the activity of, for example, GSTs was considered by the body to be a sufficient defense. Similarly, it was noted in the experiment of Coppola et al. (2018) that, under the influence of arsenic and a control temperature in the organism of *M. galloprovincialis*, detoxification mechanisms – GSTs – were activated, but the activity of CATs was not increased. Despite the lower accumulated concentrations at 21 °C, a higher toxic effect on the mussels was noted, through the lack of CAT activation and inhibition of SOD and GST activity (Lopes et al., 2022). This is explained by the excessive stress levels that excessive ROS production initiates. At an elevated temperature, only GPx was activated in the presence of EE2. A slowdown in antioxidant defense was also noted in the studies of Pirone et al. (2019), Pes et al. (2021) and Morosetti et al. (2020). An increase in LPO levels was observed only in mussels exposed to EE2 at elevated temperatures (Lopes et al., 2022). At the control temperature, the increased detoxification defense was able to prevent cell damage. The cellular damage at 21 °C during exposure to the pharmaceutical was not the result of drug accumulation, but was probably related to the increased sensitivity of the organism to EE2 at the higher temperature. This results in impaired biotransformation and antioxidant defense, as previously observed by Freitas et al. (2017a, 2017b) and Coppola et al. (2018, 2017).

The effect of elevated temperature on the increased toxicity of EE2 against *M. galloprovincialis* was also noted by a slight reduction in AChE

activity in mussels exposed to both stressors compared to control mussels. The neurotoxic effect – the inhibition of AChE activity under elevated temperature in the presence of other contaminants was also observed by Coppola et al. (2018, 2017), Freitas et al. (2017a, 2017b) and Pirone et al. (2019). It is noteworthy that histopathological changes were noted in the gills and digestive glands of mussels subjected to simultaneous exposure to EE2 and elevated temperature (Lopes et al., 2022). The evaluation of these changes is important when assessing the effects of contaminants on mussels (Cuevas et al., 2015; Leite et al., 2020). The deposition of lipofuscin, an intracellular marker of aging, was observed in the gills, which Höhn and Grune (2013) explain as an effect of oxidative stress. The deposition of lipofuscin in *Mytilus trossulus* tissues exposed to DCF was also noted by Świacka et al. (2022).

Almeida et al. (2021) conducted an experiment in which they proved that elevated temperature had a positive effect on the oxidative status of clam *R. philippinarum* clams exposed to CBZ and cetrizine (CTZ) (higher GSH/GSSG levels, lower PC and LPO levels), especially when *R. philippinarum* were exposed to their mixture. This indicates the activation of a defense strategy by the bivalves under conditions of increased stress (e.g., a decrease in ETS activation, activation of SOD). However, the authors emphasize that the study used relatively low concentrations of the pharmaceutical, and therefore, given the increase in pharmaceutical pollution of the oceans and increasing heat waves, the biological response of the bivalves may change.

3.1.4. Effects of warming on chronic toxicity for freshwater species

Chronic tests with freshwater organisms were performed in temperature range of 18 °C to 25 °C (Table 3). The tested pharmaceuticals included: florfenicol, diclofenac, ibuprofen, carbamazepine. A mixture of diclofenac, nifedipine, and glyphosate herbicide was also tested (Khoma et al., 2021). The freshwater organisms tested were clams: *Unio tumidus*; crustaceans such as *Daphnia magna* (gillnets) and *Atyaephyra desmarestii* (armadillos).

Martins et al. (2013) investigated the effect of elevated temperature on florfenicol toxicity by conducting a chronic (21 days) toxicity test according to OECD 211 guidelines using *D. magna* organisms. Significantly, a negative effect of elevated temperature as a single stressor on *D. magna* was noted – the daphnia population increased with the increasing temperature, but declined sharply after a certain threshold. The effect (the total number of living juveniles produced) was experimentally confirmed by determining the EC₅₀ parameter, which was found to be 4 times lower at 25 °C than that determined at 20 °C (Table 3). The exposure of daphnia to florfenicol at 20 °C reduced the daphnia growth and reproduction compared to the control, but an additional temperature elevation resulted in an enhanced toxicity. Thus, both elevated temperature and florfenicol as single stressors were shown to have a negative effect on *D. magna*, while their combined effect proved to be the most toxic to the test organisms.

Examination of the effects of a mixture of contaminants on the functioning of the clam *U. tumidus* at elevated and standard temperatures was carried out by Khoma et al. (2021), proving that the IBR responses for single contaminants – DCF, the antihypertensive drug nifedipine and the herbicide glyphosate – were specific and different from the IBR responses in a mixture of these compounds. It was observed that DCF, which had the lowest IBR value among the tested compounds and thus the least toxicity to *U. tumidus*, affected the toxicity of nifedipine and glyphosate in a mixture, suppressing the characteristic changes in the mussel's body. Significantly, despite the similar profile of biochemical marker responses to mixtures of contaminants at both temperatures, specific features were noted at elevated temperatures: excessive activation of SOD, additional lysosomal cathepsin D activity (CtDe), lysosomal cathepsin D activity (CtDL) and ChE, and greater differences in the values of GST (down-regulation) and caspase 3 (up-regulation) relative to the control. Simultaneous activation of caspase 3, CtDe and CtDL can lead to the activation of apoptotic pathways in the cell (Conus et al., 2008), which means that an increase in temperature can increase the toxicity of the contaminant mixture against *U. tumidus* (Khoma et al., 2021).

3.2. The effects of acidification

3.2.1. Effects of lowered pH on acute toxicity for marine species

To our knowledge, to date only Freitas et al. (2015, 2016) has conducted an acute toxicity test of a pharmaceutical against a marine organism under acidification (Table 4). Freitas et al. evaluated the biochemical changes (energy metabolism and cellular damage) induced by the combination of two stressors: CBZ and reduced pH against *S. plana*; these tests were performed during acute – 96-h (2015) and chronic – 28-day (2016) toxicity experiments. The acute toxicity test was performed against *S. plana* collected from uncontaminated areas and mercury-contaminated areas (Table 4), thus checking whether the place of collection of individuals of the same species affects their defense response against stressors (Freitas et al., 2015). It appears that after the 96-h test, the combined effect of CBZ and lowered pH affected the physiological and biochemical performance of *S. plana*. Nevertheless, higher mortality was observed when exposed to single stressors than to a combination of stressors. Additionally, it was studied that mussels collected from the contaminated area showed enhanced stress responses. Thus, it is important to be mindful of where organisms are collected for toxicological testing, even within the same species.

3.2.2. Effects of lowered pH on acute toxicity for freshwater species

The overview of scientific reports presenting the effect of the changing of water's pH on the toxicity of selected pharmaceuticals for freshwater organisms is presented in Table 4. The pH range tested was 5.5–9.2. The tested pharmaceuticals included a representative of antibiotics (enrofloxacin, sulfadiazine), diclofenac, acetaminophen, chloroquine, antidepressants (sertraline and fluoxetine), and β -blockers (metoprolol, propranolol, atenolol and labetalol). The freshwater organisms tested were crustaceans: *Daphnia magna*, *Gammarus pulex*; fish: *Danio rerio*, *Pimephales promelas*, *Japanese medaka*; freshwater snails: *Physella acuta*; and insects: *Cloeon dipterum*. Lethal toxicity was investigated by determining the LC₅₀, as well as sublethal toxicity by determining the EC₅₀ and LOEC (lowest observed effect concentration).

Anskjær et al. (2013) conducted an acute toxicity test of sulfadiazine on *D. magna* (Table 3). The test was conducted at three pH levels: 6.0, 7.5, and 8.5. They observed a significant increase in sulfadiazine toxicity with acidification. The researchers also noted a significant increase in BCF of the pharmaceutical with decreasing pH (from 36 to 50 L kg⁻¹ with pH change from 8.5 to 6.0, respectively). Significantly, the fraction of undissociated sulfadiazine (α) was highest at the lowest pH (α = 0.76 at pH 6.0; α >> 0.01 at pH 8.5). It is possible that the toxicity of sulfadiazine increased at pH 6.0 because the increased amount of the undissociated fraction of the pharmaceutical resulted in increased diffusion through the cell membranes of the test organisms. Anskjær et al. (2013) explain the increased toxicity of SDZ at pH 6 by the ionization of sulfadiazine and retention inside the cells of *D. magna*. This effect is called ion-trapping, and it is caused by a higher pH inside the organism compared to the surrounding water (Neuwoehner and Escher, 2011; Rendal et al., 2011; Fahl et al., 1995). Undoubtedly, the toxicity of sulfadiazine and other sulfonamides is dependent on changes in the pH. This is also confirmed by the experiment of Kim et al. (2010), where the acute toxicity of sulfathiazole was tested at three different values of pH against *D. magna* (Table 4). For the acute toxicity test, the concentration of pollutant is in mg L⁻¹ concentration, and thus much enhanced compared to real environmental concentrations. In contrast, during the chronic toxicity experiments of sulfonamides against *D. magna* (Wollenberger et al., 2000) and other test organisms, the concentrations causing the toxic effect are similar to those found in the environment, and thus acidification may be a real problem causing an increase in sulfonamide toxicity.

Sun et al. also noted a relationship between the presence of pharmaceuticals in neutral form and toxicity for aquatic organisms (Sun et al., 2020). They noted that the toxicity of enrofloxacin increased along with acidification (Table 3). The EC₅₀ values after a 96-h acute toxicity test were 2 times lower when crustacean *G. pulex* organisms were exposed to pH 6.5, compared to pH 8.0; similarly, for the insect *C. dipterum* they were 1.4 times

Table 4

Reports presenting effects of decreasing pH into acute toxicity of selected pharmaceuticals on marine and freshwater aquatic organisms

(CAT - catalase activity; EC₅₀ - the pharmaceutical concentration causing a toxic effect in 50 % of the tested organisms; GLY - glycogen content; GSH/GSSG - the ratio of reduced to oxidized form of glutathione; GST_S - glutathione S-transferases activity; LC₅₀ - the pharmaceutical concentration causing death in 50 % of the tested organisms; LPO - lipid peroxidation levels; PROT - protein content, SOD - superoxide dismutase activity).

Species (reference method)	pH tested/test duration	Pharmaceuticals	Acute effect (↑ or ↓ if acidification increase or reduce response)	Ref.
Marine species				
Clam <i>Scrobicularia plana</i> collected from pristine location	7.8, 7.1/96 h	Carbamazepine at 3.0 µg L ⁻¹	Energy reserves: • GLY • PROT Indicators of cellular damage: • GSH/GSSG ratio ↑ • LPO Indicators of oxidative stress: • SOD activity ↓ • CAT Biotransformation enzymes: • GSTs	(Freitas et al., 2015)
Clam <i>Scrobicularia plana</i> collected from mercury contaminated area			Energy reserves: • GLY • PROT Indicators of cellular damage and redox balance: • GSH/GSSG ratio ↑ • LPO Indicators of oxidative stress: • SOD activity ↓ • CAT Biotransformation enzymes: • GSTs	
Freshwater species				
Crustacean <i>Daphnia magna</i> (ISO 6341)	8.5, 7.5, 6.0/48 h	Sulfadiazine	EC ₅₀ : - pH 8.5 → 310 mg L ⁻¹ - pH 7.5 → 188 mg L ⁻¹ - pH 6.0 → 27.2 mg L ⁻¹	(Anskjær et al., 2013)
Crustacean <i>Daphnia magna</i> (US EPA 2002 EPA- 821-R-02-012)	9.2, 8.3, 7.4/48 h	Acetaminophen (paracetamol)	EC ₅₀ : - pH 9.2 → 32.5 mg L ⁻¹ - pH 8.3 → 8.3 mg L ⁻¹ - pH 7.4 → 12.7 mg L ⁻¹	(Kim et al., 2010)
		Chlortetracycline	EC ₅₀ : - pH 9.2 → > 400 mg L ⁻¹ - pH 8.3 → > 400 mg L ⁻¹ - pH 7.4 → > 400 mg L ⁻¹	
		Ciprofloxacin	EC ₅₀ : - pH 9.2 → 1.3 mg L ⁻¹ - pH 8.3 → 1.2 mg L ⁻¹ - pH 7.4 → 1.1 mg L ⁻¹	
		Enrofloxacin	EC ₅₀ : - pH 9.2 → 75.8 mg L ⁻¹ - pH 8.3 → 53.3 mg L ⁻¹ - pH 7.4 → 45.8 mg L ⁻¹	
		Ibuprofen	EC ₅₀ : - pH 9.2 → > 45 mg L ⁻¹ - pH 8.3 → > 45 mg L ⁻¹ - pH 7.4 → 34.1 mg L ⁻¹	
		Lincomycin	EC ₅₀ : - pH 9.2 → > 500 mg L ⁻¹ - pH 8.3 → > 500 mg L ⁻¹ - pH 7.4 → > 500 mg L ⁻¹	
		Sulfathiazole	EC ₅₀ : - pH 9.2 → 71.4 mg L ⁻¹ - pH 8.3 → 53.2 mg L ⁻¹ - pH 7.4 → 23.0 mg L ⁻¹	

(continued on next page)

Table 4 (continued)

Species (reference method)	pH tested/test duration	Pharmaceuticals	Acute effect (↑ or ↓ if acidification increase or reduce response)	Ref.
Insect <i>Cloeon dipterium</i> (OECD 202)	8.0, 7.5, 7.0, 6.5/96 h	Enrofloxacin	EC ₅₀ (after 48 h): - pH 8.0 → 69.5 mg L ⁻¹ - pH 7.5 → 34.4 mg L ⁻¹ - pH 7.0 → 34.6 mg L ⁻¹ - pH 6.5 → 26.7 mg L ⁻¹ EC ₅₀ (after 96 h): - pH 8.0 → 29.2 mg L ⁻¹ - pH 7.5 → 26.8 mg L ⁻¹ - pH 7.0 → 26.9 mg L ⁻¹ - pH 6.5 → 21.4 mg L ⁻¹	(Sun et al., 2020)
Cochlea <i>Physella acuta</i> (OECD 202)	8.0, 7.5, 7.0, 6.5/96 h	Enrofloxacin	EC ₅₀ (after 48 h): - pH 8.0 → 206 mg L ⁻¹ - pH 7.5 → 192 mg L ⁻¹ - pH 7.0 → 133 mg L ⁻¹ - pH 6.5 → 115 mg L ⁻¹ EC ₅₀ (after 96 h): - pH 8.0 → 143 mg L ⁻¹ - pH 7.5 → 121 mg L ⁻¹ - pH 7.0 → 112 mg L ⁻¹ - pH 6.5 → 79.7 mg L ⁻¹	
Crustacean <i>Gammarus pulex</i> (OECD 202)	8.0, 7.5, 7.0, 6.5/96 h	Enrofloxacin	EC ₅₀ (after 48 h): - pH 8.0 → 58.2 mg L ⁻¹ - pH 7.5 → 55.1 mg L ⁻¹ - pH 7.0 → 42.1 mg L ⁻¹ - pH 6.5 → 35.5 mg L ⁻¹ EC ₅₀ (after 96 h): - pH 8.0 → 24.3 mg L ⁻¹ - pH 7.5 → 22.1 mg L ⁻¹ - pH 7.0 → 15.6 mg L ⁻¹ - pH 6.5 → 16.3 mg L ⁻¹	
Juvenile fish and larval stages <i>Oryzias latipes</i> Japanese medaka (OECD 107)	9.0, 8.0, 7.0/96 h	Fluoxetine	LC ₅₀ : - pH 9 → 0.2 +/− 0.02 mg L ⁻¹ - pH 8 → 1.3 +/− 0.2 mg L ⁻¹ - pH 7 → 5.5 +/− 1.3 mg L ⁻¹	(Nakamura et al., 2008)
Larval stages zebrafish <i>Danio rerio</i>	8.2, 7.0, 5.8/96 h	Sertraline	LC ₅₀ : - pH 8.2 → 0.67 μM - pH 7.0 → 0.78 μM - pH 5.8 → 2.75 μM	(Alsop and Wilson, 2019)
		Diclofenac	LC ₅₀ : - pH 8.2 → no mortality >35 μM (highest concentration tested) - pH 7.0 → no mortality >35 μM (highest concentration tested) - pH 5.8 → 15 μM	
		Fluoxetine	LC ₅₀ : - pH 8.2 → 1.18 μM - pH 7.0 → 3.54 μM - pH 5.8 → 8.59 μM	
		Acetaminophen (paracetamol)	No mortality in the studied range of pH	
Fish Fathead minnow <i>Pimephales promelas</i> (modified US EPA 2002 EPA-821-R-02-012)	8.5, 7.5, 6.5/48 h	Sertraline	EC ₅₀ : - pH 8.5 → 72 μg L ⁻¹ - pH 7.5 → 205 μg L ⁻¹ - pH 6.5 → 647 μg L ⁻¹	(Valenti et al., 2009)
Crustacean <i>Daphnia magna</i> (ISO 6341)	9.0, 8.0, 7.0/48 h	Chloroquine	EC ₅₀ : - pH 9.0 → 4 mg L ⁻¹ - pH 8.0 → 10 mg L ⁻¹ - pH 7.0 → 4 mg L ⁻¹	(Rendal et al., 2011)
Embryonal and larval stages Zebrafish <i>Danio rerio</i> (OECD 236)	8.6, 8.0, 7.0/96 h	Metoprolol	External effect concentration Lethality - LC ₅₀ : pH 8.6 → 0.054 mM L ⁻¹ pH 8.0 → 0.174 mM L ⁻¹ pH 7.0 → 1.91 mM L ⁻¹ Internal effect concentration Lethality - LC ₅₀ : pH 8.6 → 379 mM kg _{lipid} ⁻¹ pH 8.0 → 484 mM kg _{lipid} ⁻¹ pH 7.0 → 825 mM kg _{lipid} ⁻¹	(Bittner et al., 2018)
	8.0, 7.0, 5.5/96 h	Propranolol	External effect concentration Lethality - LC ₅₀ : pH 8.0 → 0.023 mM L ⁻¹ pH 7.0 → 0.128 mM L ⁻¹ pH 5.5 → 2.42 mM L ⁻¹ Internal effect concentration Lethality - LC ₅₀ : pH 8.6 → 858 mM kg _{lipid} ⁻¹ pH 8.0 → 664 mM kg _{lipid} ⁻¹ pH 7.0 → 989 mM kg _{lipid} ⁻¹	

Table 4 (continued)

Species (reference method)	pH tested/test duration	Pharmaceuticals	Acute effect ($\uparrow$ or $\downarrow$ if acidification increase or reduce response)	Ref.
		Atenolol Labetalol	Lethality – LC ₅₀ - no lethal effects in the studied concentration range Lethality – LC ₅₀ - no lethal effects in the studied concentration range	

lower, and for cochlea *P. acuta* 1.8 times lower. The enrofloxacin neutral form share was approximately 2-fold greater at pH 6.5, compared to the highest pH tested – 8.0. These results were also confirmed by Kim et al. (2010) – the toxicity of enrofloxacin to *D. magna* increased 1.7-fold when the pH decreased from 9.2 to 7.4, which the researchers also explained by the greater bioavailability of the non-ionized form of enrofloxacin at the lower pH. In most of the pharmaceuticals studied by Kim et al., the neutral form of the pharmaceuticals (%) - excluding IBU - increased with decreasing pH.

Nakamura et al. (2008), in their experiments, also proved that the ecotoxicity of fluoxetine (antidepressant) is closely related to water's pH. However, an inverse relationship was observed – the toxicity of fluoxetine for the fish *O. latipes* decreased with acidification. At the lowest tested pH of 7.1, the NOEC was 3.8 mg L⁻¹, whereas when the pH increased to 8.8, the lowest tested concentration (100 µg L⁻¹) caused the death of 3/10 individuals. The relation of fluoxetine toxicity with pH is associated with an increase in the bioaccumulation with increasing pH (weak base; pK_a = 10.1). At higher pH, fluoxetine was mostly present in the neutral form – with significantly higher hydrophobicity than its ionized form. The increased bioaccumulation of diphenhydramine (pK_a = 9.1) with the increase of pH from 6.7 to 8.7 was also noted in fish (Nichols et al., 2015).

The decrease in the toxicity of fluoxetine and another antidepressant pharmaceutical, sertraline (SSRI-selective serotonin reuptake inhibitor) with acidification was studied by Alsop and Wilson (2019). The study was conducted using *D. rerio* organisms during a 96-h acute toxicity test (Table 4). During an increase in pH from 5.8 to 7.0, the neutral fraction of fluoxetine increased from 0 % to 0.2 %, while toxicity increased 2.4-fold. Correspondingly for sertraline, the increase in the neutral fraction was from 0 % to 0.1 %, and the toxicity increased 3.5-fold. As the pH increased to 8.2, the pharmaceutical's neutral fraction continued to increase – for fluoxetine to 2.5 % (toxicity increased 3-fold) and for sertraline to 2 % (toxicity increased 1.2-fold). Similarly, the uptake of SSRIs (pmol larva⁻¹ h⁻¹) increased with increasing pH; for example, for sertraline it was 5.4 times higher at pH 8.2, compared to pH 5.8. A similar relationship was observed by Valenti et al. (2009) who observed higher sertraline toxicity at higher pH for both acute (Table 4) and chronic toxicity tests (Table 5). The researchers emphasize that the differences in the survival of *P. promelas* are not the result of stress associated with pH changes, but are the result of changes in the physicochemical properties of sertraline under the influence of the pH. The researchers also highlight the importance of including seasonal changes in surface water pH and the associated change in ionization in the toxicological risk assessment of pharmaceuticals.

The lowering of the toxicity of weak bases with acidification is also confirmed by the results of Rendal et al. (2011), who investigated the toxicity of chloroquine on *D. magna* (Table 4). The toxicity of the pharmaceutical increased with increasing pH, which is consistent with the higher lipophilicity of the weak base molecule under these conditions and thus its greater affinity for diffusion through the cell membranes of the organisms. The “trapping” of chloroquine in vacuoles, where the pH is low, causing its ionization - hinders the escape of the pharmaceutical from the cell.

For DCF, a weak acid with a pK_a of 4.2, the pH dependence of the toxicity was the opposite of that for the weak bases: fluoxetine and sertraline (Alsop and Wilson, 2019; Valenti et al., 2009) or chloroquine (Rendal et al., 2011). At pH 7.0 and 8.2, DCF produced no toxic effects against *D. rerio* at the highest concentration tested (35 µM), but when the pH dropped to 5.7, the death of half of the organisms was recorded with exposure to 15 µM DCF (Alsop and Wilson, 2019). Moreover, the percentage of the neutral fraction of DCF increased with decreasing pH (0 % at pH 8.2; 2.2 % at pH 5.8). EE2 with a pK_a of 10.3 was predominantly present in

the non-ionized form in all the pH ranges tested, resulting in no change in its toxicity and uptake (Alsop and Wilson, 2019).

A finding of interest is that of Bittner et al. (2018) studying the effect of pH on the uptake and toxicity of β -blockers to *D. rerio* embryos, not only relative to external concentrations of pharmaceuticals in water, but also relative to internal concentrations in embryos (Table 4). The researchers in their study demonstrated that the toxicity (expressed in lethality, heard rate, behaviour, phenotype) associated with external concentrations is pH-dependent and can be explained by toxicokinetics, as described earlier in this chapter (ionizability of ionic compounds, higher affinity of the hydrophobic neutral fraction of the molecule for diffusion through the membrane), but the effects associated with internal concentrations (in mM kg_{lipid}⁻¹) are not dependent on the external pH. For example, the LOEC for heart rate increases with lowering of the pH when external concentration of metoprolol and propranolol were used, but there was no direct relation when the internal concentration is used. They noted increased bioaccumulation potential for both metoprolol and propranolol as the neutral fraction of the pharmaceutical increased. The apparent BCF [L kg⁻¹] for metoprolol at pH 7.0 was 1.96 ± 0.04, while at pH 8.6 the BCF was 32.0 ± 1.51; for propranolol at pH 5.5 it was 1.86 ± 0.09, while at pH 8.0 the BCF was 169.0 ± 51.5.

3.2.3. Effects of lowered pH on chronic toxicity for marine and freshwater species

Table 4 shows the effect of acidification on the chronic toxicity of selected pharmaceuticals on marine organisms and for one freshwater organism – *P. promelas* (Valenti et al., 2009). The pH range tested was 7.1–8.1. The tested pharmaceuticals included diclofenac, carbamazepine and cetirizine. The marine organisms tested were mussels: *Mytilus galloprovincialis*; clams: *Scrobicularia plana*, *Ruditapes philippinarum*; urchins: *Heliocidaris crassispina*; gastropods: *Gibbula umbilicalis*. Various biochemical markers were tested: immune response, activity of antioxidant enzymes and total antioxidant capacity, genotoxic damage and transcriptomic response, indicators of energy metabolism, indicators of cellular damage, haemocyte parameters, and respiration rate.

The toxicity of DCF at concentrations of 0.05 and 0.5 µg L⁻¹ was investigated during a chronic toxicity test against two bivalves, the mussel *M. galloprovincialis* and the clam *R. philippinarum* by Munari et al. (2018). *R. philippinarum* appears to be less resistant to acidification as a single stressor. It is noteworthy that the combination of both stressors caused changes in the mussel DNA – both in the gills and digestive gland, although the changes were more severe in the gills. Genotoxic changes due to the presence of DCF were less drastic during the first week of the study, whereas on subsequent days these changes increased in both tissues under the highest acidification. Interestingly, there was a reduction in DNA damage in the gills over time. The researchers suggest that despite the lack of changes in CAT and SOD activity, other antioxidant defense mechanisms, unmeasured during the experiment, may have acted to offset the oxidative stress. Additionally, for both the mussels and clams during exposure to both stressors, the levels of LPO, which is one indicator of cell damage, increased.

During the long-term exposure of *S. plana* there was a higher mortality arising from the accumulated effect of CBZ and acidification than with a single stressor (Freitas et al., 2016). It was noted that the bivalves were able to adapt to the drop in pH. Interestingly, mortality was not recorded until 96 h into the test. The researchers explain that the clams during the initial period of exposure to the stressors may have activated defense mechanisms that are not sufficient to counteract long-term stress. This also confirms the utilization of energy reserves (decrease in glycogen content) during the prolonged exposure to reduced pH and CBZ. Significantly, the short-term exposure of *R. philippinarum* and *R. decussatus* to CBZ – examined

Table 5

Reports presenting effects of decreasing pH into chronic toxicity of selected pharmaceuticals on marine and freshwater aquatic species

(AChE - acetylcholinesterase activity; CAT - catalase activity; COX - cyclooxygenase; ETS - electron transport activity; GLY - glycogen content; GPx - glutathione peroxidase activity; GR - glutathione reductase; GSH/GSSG - the ratio of reduced to oxidized form of glutathione; GST_s - glutathione S-transferases activity; LOAEC - lowest-observable-adverse-effect concentrations; LPO - lipid peroxidation levels; NOAEC - no-observable-adverse-effect concentrations; PROT - protein content; Se-dep. GPx - Se-dependent glutathione peroxidases; TBARS - thiobarbituric acid-reactive substances production; TGSH - total glutathione).

Species (reference method)	pH tested/test duration	Pharmaceutical (concentration)	Chronic effect (↑ or ↓ if acidification increase or reduce response)	Ref.
Marine species Mussel <i>Mytilus galloprovincialis</i>	8.1, 7.6/28 days	Carbamazepine (1 µg L ⁻¹)	Immunological responses: • lysosomal membrane stability • phagocytosis rate • granulocytes/hyalinocytes ratio Indicators of oxidative stress: • GST_s ↓ • CAT • Se-dep. GPx • total GPx • GR • TGSH • AChE in hemocytes ↓ Total antioxidant capacity: • toward peroxyl radical ROO• • hydroxyl radical HO• • peroxynitrite ONOO- Genotoxic damage: • DNA fragmentation • micronuclei frequency Lipid peroxidation products: • malondialdehyde MDA in digestive gland ↑ The content of lipofuscin and neutral lipids (in a cross-section of the digestive glands) ↑ HQ – hazard quotient ↑	(Mezzelani et al., 2021)
Clam <i>Ruditapes philippinarum</i>	7.8, 7.5/28 days	Carbamazepine (1 µg L⁻¹) Cetirizine (0.6 µg L⁻¹)	Transcriptomic responses of genes: • metabolism and neurotransmission - Cyp 3 - GABA • inflammatory and immune response - MyD88 ↓ - TNF ↓ - Ikappa B - Cathepsin • biomineralization - Perlucin - SLC4 Metabolic capacity: • ETS Indicator of cell damage: • LPO Antioxidant protection: • GPx Transcriptomic responses: • metabolism and neurotransmission - GABA ↑ - Cyp 3 • inflammatory and immune response - MyD88 ↓ - Cathepsin ↑ - Ikappa B - TNF	(Almeida et al., 2018)

Table 5 (continued)

Species (reference method)	pH tested/test duration	Pharmaceutical (concentration)	Chronic effect (↑ or ↓ if acidification increase or reduce response)	Ref.
			• biomineralization - Perlucin ↑ - SLC4 - Metabolic capacity: • ETS - Indicator of cell damage: • LPO - Indicator of oxidative stress:	
Clam <i>Scrobicularia plana</i>	7.8, 7.1/28 days	Carbamazepine (3.00 µg L ⁻¹)	• GPX - Metabolic capacity and energy reserves: • GLY content ↓ • PROT • ETS - Indicators of cellular damage: • LPO - Elements quantification:	(Freitas et al., 2016)
Mussel <i>Mytilus galloprovincialis</i>	8.1, 7.7, 7.4/21 days	Diclofenac (0.05; 0.50 µg L ⁻¹)	• total concentrations of potassium ↓ - total concentrations of sodium - Total haemocyte count and lysozyme activity in HL protein depending of test duration, concentration and pH - Haemocyte volume, haemocyte diameter, Neutral Red uptake, haemocyte proliferation, lysozyme activity in CFHL protein - CAT and COX activity, LPO level and DNA damage in digestive gland and in the gills depending of test duration, concentration and pH	(Munari et al., 2019, Munari et al., 2018)
Clam <i>Ruditapes Phillipinarum</i>			- Total haemocyte count, haemocyte volume, haemocyte diameter, and haemocyte proliferation, Neutral Red uptake - no statistical important differences between pH values - Lysozyme activity in HL protein and CFHL protein, depending of test duration, concentration and pH - CAT and COX activity, LPO level and DNA damage in digestive gland and in the gills depending of test duration, concentration and pH - Indicators of energy metabolism:	
Clam <i>Ruditapes Phillipinarum</i>	8.0, 7.6/28 days	Cetirizine (0.6 µg L ⁻¹)	• ETS activity ↑ • GLY content ↓ • PROT - Indicators of cell damage and redox balance: • LPO • PC • GSH/GSSG - Indicators of oxidative stress: • SOD • CAT • GSTs activity ↑ - Indicators of energy metabolism: • ETS activity ↑ • GLY • PROT - Indicators of cell damage and redox balance: • LPO • PC content ↑ • GSH/GSSG - Indicators of oxidative stress: • SOD activity ↑ • CAT activity ↑ • GSTs	(Almeida et al., 2022)
		Carbamazepine (1 µg L ⁻¹)		

(continued on next page)

Table 5 (continued)

Species (reference method)	pH tested/test duration	Pharmaceutical (concentration)	Chronic effect ($\uparrow$ or $\downarrow$ if acidification increase or reduce response)	Ref.
		Mixture: Carbamazepine (1 $\mu\text{g L}^{-1}$) Cetirizine (0.6 $\mu\text{g L}^{-1}$)	Indicators of energy metabolism: • ETS activity $\uparrow$ • GLY • PROT Indicators of cell damage and redox balance: • LPO content $\uparrow$ • PC • GSH/GSSG Indicators of oxidative stress: • SOD activity $\uparrow$ • CAT • GSTs activity $\uparrow$	
Urchins <i>Heliocidaris crassispina</i>	8.0, 7.7/7 days	Fluoxetine (10; 100 ng L^{-1})	Mortality Embryonic development $\downarrow$ (c = 10 ng L^{-1}) Larval growth (total body length, growth rate, asymmetry) Respiration rate DNA integrity $\downarrow$	(Lo et al., 2021)
Gastropod <i>Gibbula umbilicalis</i>	8.2, 7.9, 7.6/28 days	Salicylic acid (5; 25; 125 $\mu\text{g L}^{-1}$)	Anti-inflammatory activity (125 $\mu\text{g L}^{-1}$) • COX activity $\uparrow\downarrow$ (depending of pH) Indicators of oxidative stress (25 $\mu\text{g L}^{-1}$) • CAT • GSTs activity $\uparrow\downarrow$ (depending of pH) Neurotoxicity (5 $\mu\text{g L}^{-1}$): • AChE $\uparrow\downarrow$ (depending of pH) Lipid peroxidation: • TBARS Shell hardness	(Dionísio et al., 2020)
Freshwater species Fish Fathead minnow <i>Pimephales promelas</i> (modified US EPA protocol 2002 EPA-821-R-02-013)	8.5, 7.5, 6.5/7 days	Sertraline (30–1000 $\mu\text{g L}^{-1}$)	Growth $\uparrow$ (EC ₁₀ at pH 8.5 $\rightarrow$ 30.3 $\mu\text{g L}^{-1}$, at pH 8.5 $\rightarrow$ 30.3 $\mu\text{g L}^{-1}$, at pH 7.5 $\rightarrow$ 118.7 $\mu\text{g L}^{-1}$, at pH 6.5 $\gg$ 469 $\mu\text{g L}^{-1}$) Feeding $\uparrow$ (EC ₁₀ at pH 8.5 $\rightarrow$ 8.7 $\mu\text{g L}^{-1}$, at pH 7.5 $\rightarrow$ 65.6 $\mu\text{g L}^{-1}$, pH 6.5 $\rightarrow$ 69.6 $\mu\text{g L}^{-1}$)	(Valenti et al., 2009)

by Almeida et al. (2014) resulted in an increase in glycogen content in the bivalves - meaning that during short-term exposure to stress, the bivalves are able to activate defense mechanisms. The enhanced stress caused by pH reduction and CBZ exposure is also confirmed by the increase in LPO levels (Freitas et al., 2016).

Almeida et al. (2018) conducted a 28-day toxicity test of CBZ and CTZ with different pH conditions of 7.8 and 7.5 against the clam *R. philippinarum* (Table 4). The BCF in clam gills at pH 7.8 was 1.6 L kg^{-1} for CBZ, while for CTZ it was 5.4 L kg^{-1} . There was a 31 % decrease in CTZ accumulation at the lower pH, while CBZ accumulation was not significantly different, which is due to the relationship between pK_a and pH – carbamazepine will be present at both pH 7.8 and 7.5 in a neutral form. The same was observed for *M. galloprovincialis* (Mezzelani et al., 2021) and *S. plana* (Freitas et al., 2016). CTZ, on the other hand, occurs in zwitterionic and anionic forms in the pH range studied, thus its different accumulation at different pH levels may be related to the presence of a neutral charge and the possibility of conformer formation (Testa et al., 1997). In the study by Almeida et al. (2018) no significant effect of lowered pH on oxidative stress was found in contrast to Freitas et al. (2015). This may be due to the lower pH, different mussels species and the higher CBZ concentration in the study by Freitas et al. However, Almeida et al. (2018) found changes in the mRNA transcription under both stressors. Changes in gene expression and DNA damage were also recorded by Mezzelani et al. (2021) during a 28-day test where *M. galloprovincialis* organisms were subjected to simultaneous exposure to CBZ and acidification (pH 7.6) (Table 4).

Mezzelani et al., investigating the effect of pH on the toxicity of CBZ to *M. galloprovincialis*, also summarized the results of the biomarkers tested in each experiment, determining the hazard quotient (HQ). A simultaneous exposure of *M. galloprovincialis* to CBZ and reduced pH, compared to exposure to a single stressor, resulted in increased HQ (Mezzelani et al., 2021).

Dionísio et al. (2020) examined the toxicity of salicylic acid, at three environmentally relevant concentrations, on *Gibbula umbilicalis* under different pH conditions. A statistically significant effect of pH on COX, GST and AChE activity was noted during the chronic test. These changes were pH-dependent and salicylic acid concentration-dependent.

In a publication by Lo et al. (2021), the effect of acidification on fluoxetine was investigated against the sea urchin *Heliocidaris crassispina*. Among other things, the level of DNA damage was examined – it was lower when the organisms were exposed to two (both) stressors. Acidification in combination with fluoxetine negatively affected the embryonic development of sea urchins, but this was noted only for the lower concentration of the pharmaceutical tested (10 ng L^{-1}). The researchers stress that lowering the pH alone had a negative effect on the test organisms, as noted in the reduced motility of sea urchin larvae.

3.3. The effects of combined stressors: warming and acidification

3.3.1. Effect of combined stressors on acute toxicity for marine species

To our knowledge, to date only Costa et al. (2019) has conducted an acute toxicity test of a pharmaceutical against a marine organisms under

the conditions of acidification and warming. During toxicological studies the metabolic capacity and energy related biomarker, enzymatic oxidative stress biomarkers, markers of oxidative stress damage and marker of neurotoxicity such as AChE activity were determined.

Costa et al. (2019) conducted a 7-day test examining the effects of the combined stressors of increased temperature and reduced pH (21 °C, pH 7.7) on DCF toxicity for two species of clam, *R. philippinarum* and *R. decussatus*, compared to control conditions (17 °C, pH 8.1) (Table 2). The test was preceded by a 30-day acclimatization of the organisms under the predicted climate change conditions and under control conditions. The researchers examined mortality (%) in both clam species after a 30-day acclimation period under control conditions, climate change conditions and after 7-day exposure of the clams to DCF (1 µg L⁻¹). Interestingly, under climate change conditions, during the 30-day acclimatization, only *R. decussatus* experienced mortality (22.8 %), while during the 7-day exposure on DCF under control conditions, mortality was noted only for *R. philippinarum* (6.7 %). Species differences were also revealed in the biochemical responses to stressors. However, it was noted, jointly for both clam species, that the DCF exposure was not as important for the operation of energy pathways and reserves, as it affected the activation of antioxidant defenses. The metabolic capacity of both clam species was not altered by DCF exposure, considering ETS. Cruz et al. (2016), investigating the effect of caffeine on metabolic activity in the same clam species without considering climate change conditions, also reported no change in the ETS. However, for *R. philippinarum*, climate change conditions caused ETS activity in DCF-contaminated clams to increase (Costa et al., 2019). This suggests that increasing temperature and decreasing pH, with simultaneous exposure to DCF, are additional stresses for this clam species. *R. philippinarum* responds to increased stress with an increased respiration rate, which consequently leads to increased metabolism. For the clam *R. decussatus*, there was no effect of either pharmaceutical or climate change conditions on the respiration rate. Analyzing the decrease of energy reserves, a significant decrease of glycogen was recorded only for *R. philippinarum* under climate change conditions. The effect of DCF on the reduction of PROT protein content in this bivalve species was also noted, regardless of the climatic conditions, whereas for *R. decussatus*, the PROT protein content as well as lipid content decreased under warming and acidification, regardless of the DCF exposure. Interestingly, there was no change in LIP content for *R. philippinarum* under climate change conditions, while the researchers Velez et al. (2016a) demonstrated experimentally that a acidification caused a decrease in lipid content in the same bivalve.

Exposure of both clam species to DCF (1 µg L⁻¹) resulted in the enhanced activation of defense mechanisms, particularly in *R. decussatus* (Costa et al., 2019). However, enzyme activation occurred independently of the effects of warming and acidification. The higher activity of SOD in *R. philippinarum* (Munari et al., 2018), or GST in *Dreissena polymorpha* (Quinn et al., 2011) under the influence of DCF was already noticed in previous studies without considering climate change. Similarly, Almeida et al. (2017, 2015, 2014), studying the effects of CTZ and CBZ against *R. philippinarum* and *R. decussatus*, showed the enhanced activation of biotransformation and antioxidant enzymes in both clam species. Notably, later studies showed that pH change did not affect the increase in antioxidant defense (GPX) in these organisms (Almeida et al., 2018).

The simultaneous warming and acidification resulted in a decrease in the ability to remove reactive oxygen species in *R. decussatus*, measured by reduced the activity of CAT and SOD (Costa et al., 2019). In the bivalve mollusk *R. philippinarum* there was no decrease in the activity of these enzymes; on the contrary, in the case of CAT their activity increased under the conditions of climate change. Thus, it can be concluded that *R. decussatus* is a bivalve species that is more sensitive to temperature and acidification changes compared to *R. philippinarum*. However, although the activity of antioxidant enzymes in *R. decussatus* decreased under climate change conditions, it was still much higher than the activity of CAT, GPx or GST in *R. philippinarum*. Therefore, *R. decussatus*, despite its higher sensitivity to climate change conditions, was able to resist its negative effects. In the context of cell damage markers, the antioxidant defense was sufficient to

prevent excessive LPO formation in both species; moreover, LPO levels were significantly lower under climate change conditions for *R. decussatus* exposed to DCF, consistent with the higher antioxidant activity in this species. Munari et al. (2018), when exposing *M. galloprovincialis* and *R. philippinarum* to DCF under different pH conditions, also reported no significant changes in LPO levels in both organisms. What is important, the ratio of reduced to oxidized forms of glutathione GSH/GSSG decreased in clams that were exposed to DCF under control conditions. This underscores that the presence of DCF in clam tissues affects the antioxidant balance in these organisms and thus induces oxidative stress (Costa et al., 2019). This was also confirmed in other studies (Freitas et al., 2019a, 2019b). This decrease was not observed during warming and acidification, which is likely due to lower DCF accumulation under these conditions (DCF bioaccumulation under control conditions in *R. philippinarum* and *R. decussatus* tissues was 40 ± 10 ng g⁻¹ dry weight and 89 ± 5.3 ng g⁻¹ dry weight, respectively, whereas under warming and acidification it decreased to 24.3 ± 8.1 ng g⁻¹ dry weight and 60.3 ± 8.1 ng g⁻¹ dry weight, respectively) (Costa et al., 2019).

Costa et al. (2019) also examined AChE activity in two species of clams. The researchers reasoned that the increase in AChE activity was most likely caused by the change in climatic conditions rather than exposure to DCF. The dependence of this enzyme activity on climate change was also observed in sharks (Rosa et al., 2016) and fish (Durieux et al., 2011). The higher AChE value in *R. decussatus* could again be explained by species differences - including naturally higher antioxidant enzyme activity in *R. decussatus*, or higher ETS activity.

3.3.2. Effects of combined stressors on acute toxicity for marine and freshwater species

To our knowledge, to date only Maulvault et al. (2019, 2018a) has conducted a chronic toxicity test of a pharmaceutical against a marine organism under acidification and warming. Maulvault et al. (2018a) reported a number of biochemical responses in the fish *D. labrax* in response to stress caused by the presence of DCF (exposure to DCF from dietary sources 500 ± 36 ng kg⁻¹ dry weight) and warming and/or acidification. As a cumulative effect of the three stressors, changes occurred such as decreased red blood cell viability, decreased GST activity in the brain, decreased ubiquitin synthesis in muscle and liver, decreased inhibition of the antioxidant enzymes CAT and SOD, or decreased VTG synthesis. The marking of proteins by ubiquitin, which should undergo proteolysis, can be inhibited when the cell contains too little energy to synthesize ubiquitin (Gravel and Vijayan, 2007). Protein synthesis is very energy-intensive, and in fish requires the total consumption of more than half of their oxygen demand. The decline in ubiquitin synthesis in muscle and liver may have been inhibited due to insufficient energy, which was reduced by excessive stress - exposure to DCF and both abiotic stressors. Significantly, the synthesis of ubiquitin was not inhibited during exposure to DCF alone, or in uncontaminated fish tissues exposed to climate change (Maulvault et al., 2018a). Based on the hazard quotient, the researchers found that the combined effects of both stressors (higher temperature and pH reduction) elicited the most detrimental stress responses in the test organism *D. labrax*. Notably, this study highlighted how diverse the molecular response of the organism to DCF stress in combination with climate stressors can be, depending on the tissue. In addition to the biochemical markers, Maulvault et al. also examined the effects of stressors on fish morphometrics and condition. A statistically significant decrease in animal condition was also caused by the exposure of animals to DCF under warming conditions. Additional stressors at elevated temperatures can generate additional energy costs for the body (Pörtner and Peck, 2010) as well as disrupt the allocation of energy resources in the body (Maulvault et al., 2017; Anacleto et al., 2018; Pistevo et al., 2015). It is important to note that elevated temperature without additional stressors did not adversely affect the condition of *D. labrax* (Maulvault et al., 2018a). The opposite effect of *D. labrax* exposure to DCF on the hepatosomatic index was also observed, compared to previous studies, where the presence of contaminants caused liver hypertrophy (Diniz et al., 2009; Maulvault et al., 2017; Sadekarpawar and Parikh,

2013). Analogous results were described for *Hoplias malabaricus* during oral exposure to DCF (Guiloski et al., 2015).

The previously described reduced state of the animals' condition, induced by simultaneous exposure to DCF, warming and acidification, is indicative of negative changes in the animals' metabolism, and this may have affected the glycogen deposition in the liver and the stability of lipids or proteins that build the liver – thus affecting the organ weight (Anacleto et al., 2018; Triebkorn et al., 2004). Combining DCF exposure with two additional stressors also resulted in a statistically significant decrease in brain weight relative to the fish body weight – the BB ratio (1.05 ± 0.08 in the control, 0.86 ± 0.4 during DCF + warming and acidification exposure) (Maulvault et al., 2018a). The additional stress of altered environmental conditions may have affected brain cell death, resulting in cognitive impairment. Individual stressors acting alone or in the presence of DCF also reduced this parameter, but the greatest decrease was observed when fish were exposed to acidification (BB ratio 0.56 ± 0.05) or acidification combined with warming (without the presence of DCF) (BB ratio 0.59 ± 0.01). The researchers explain that due to excessive CO₂ levels in the fish's environment, osmoregulatory mechanisms may have been triggered and the acid-base balance of CO₂ may have been disturbed (Heuer et al., 2016; Nilsson et al., 2012).

Acidification and climate warming have been noted to affect the toxicity of DCF on fish, exerting negative effects on their immunity (Maulvault et al., 2018a). Nuclear abnormalities of erythrocytes were also noted when fish were exposed to DCF alone, while no effect of increasing temperature and decreasing pH on its toxicity was observed. In other studies that examined the effects of DCF on *Rhamdia quelen* fish, no negative effect of DCF on cell genotoxicity was noted (Guiloski et al., 2017), similar to the studies by Boisseaux et al. (2017) in *Lymnaea stagnalis* and Parolini et al. (2011) in *D. polymorpha*. However, the authors of the experiments did not examine the effects of acidification and warming. According to Boisseaux et al. (2017), one of the reasons for the lack of genotoxicity may be the too short time of the organisms' exposure to DCF (3 days in studies by Boisseaux et al. and 96 h in studies by Parolini et al. (2011)).

Maulvault et al. (2019) in a subsequent study, also noted the impairment of defense mechanisms in fish when organisms were simultaneously exposed to acidification, warming and VFX. The experiment was conducted on *A. regius* over 21 days and examined the effects of the stressors on various tissues of the organism. What is important, the researchers also compared the route of exposure of the organisms to VFX: through water or through food. As in previous studies by Maulvault et al. (2018a), due to the diverse structure of each tissue, their biochemical responses to response to the stressors were different. An enhanced antioxidant response (e.g., CAT) under the simultaneous exposure of fish to abiotic stressors and a pharmaceutical was noted in the gills of fish that were exposed to VFX through water (Maulvault et al., 2019). In the brain, CAT and SOD activities were elevated under these conditions in both fish exposed to VFX through food and water. An increase in GST activity was also noted in the muscle and brain of fish during two routes of exposure to VFX. This agrees with the results of Freitas et al. (2016) and Maulvault et al. (2018a, 2017), who also reported an increase in the activity of these enzymes under simultaneous exposure to variable environmental conditions (warming and/or acidification) and pollutants. As described by Heise et al. (2006) and Pörtner and Peck (2010) the increase in antioxidant defense activity is associated with an increased appearance of oxygen free radicals due to enhanced mitochondrial respiration in response to stress. In this situation, the aerobic scope (as reported by Pörtner and Peck et al. to be the difference between the lowest and highest rates of aerobic respiration) of the fish is also reduced and, consequently, the body tissues may be hypoxic. Not without importance for the cell is the time of exposure to stressors, on which depends the response of the organism (Heise et al., 2006; Pörtner, 2002). When an organism is exposed to long-term stress, it will seek to adapt to the prevailing conditions, that is, to achieve homeostasis (Madeira et al., 2018). This can be seen by the return of enzyme activity and other biomarkers to control conditions. Such a strategy by the fish organism can be seen in the study by Maulvault et al. (2019) of CAT and SOD activity in the liver

(excluding conditions when the organism was simultaneously exposed to acidification, warming, and VFX-water). It may also be that the body fails to cope with chronic stress, as a result of which the activity of enzymes responsible for protein synthesis is inhibited (Maulvault et al., 2018a; Madeira et al., 2016). Such a situation was observed in CAT activity in *A. regius* muscles under all conditions tested, excluding the combined abiotic and VFX-feed stressors, and in the liver, where GST activity was inhibited under all acidification conditions, excluding the combination of the three stressors (Maulvault et al., 2019). In the same study, it was noted that despite the activation of antioxidant defense mechanisms, LPO levels increased in all the conditions tested compared to the control conditions (excluding food VFX exposure). Thus, this indicates damage to fish cells, particularly in the gills, where the greatest increase in malondialdehyde levels was noted relative to controls. This demonstrates that the gills are most sensitive to the disruption of the earlier-described aerobic scope of fish. If the changes are irreversible, the body begins to synthesize ubiquitin to destroy damaged proteins. Protein synthesis, however, requires a large expenditure of energy, which, as described earlier, may be insufficient under stress conditions (Maulvault et al., 2018a). Maulvault et al. noted that subjecting organisms to VFX exposure via water carried worse consequences for organismal tissues than exposure via food without intervening climatic stressors. In reference to this, severe negative changes were reported in *A. regius* tissues when they were exposed to three stressors simultaneously, and exposure to VFX was via the water route (Maulvault et al., 2019).

It is important to note that climatic factors such as pH and temperature affect fish reproduction, influencing ovulation and spawning, among others. Both of these factors cause the over-activation of AChE and VTG under elevated temperature conditions, while acidification inhibits VTG, regardless of exposure to VFX (Maulvault et al., 2019). Additional exposure to VFX attenuates the negative effects of climate change on AChE and VTG, but only if the exposure is via food. This confirms the importance of the route of exposure of organisms to pharmaceuticals. Negative effects of climate change (pH and warming) on *A. regius* in the presence of VFX were noted by Maulvault et al. (2018b) in previous studies, but the effects on the biochemistry of the organisms were not examined, while sensory and behavioral changes in the fish were. Bioaccumulation of VFX was increased in the plasma of *A. regius* when combined with acidification and warming, and it was noted that abiotic stressors acting together reduced fish school cohesion and caused lateralization disorders. The increased AChE activity in the brain that was noted in *A. regius* under exposure to the three stressors (Maulvault et al., 2019) is therefore consistent with negative changes in fish behaviour (Maulvault et al., 2018b). Scientists say that this may be indicative of neurological disorders and even brain cell death (Maulvault et al., 2019).

In another study, Serra-Compte et al. (2018) investigated the effects of increasing water temperature (from 18 °C to 22 °C) and acidification (pH decrease from 8.0 to 7.6) on the metabolism, bioconcentration and excretion of pharmaceuticals such as sotalol, sulfamethoxazole, VFX, carbamazepine and citalopram, among others, on the bivalves *M. galloprovincialis*. The organisms were subjected to a mixture of pharmaceuticals along with methylparaben and triclosan at concentrations of up to 15.7 mg L⁻¹ for 20 days, with another 20 days of depuration. The results showed that increasing the temperature resulted in a significant increase in the BCF of sotalol and sulfonamides and a slight increase in the BCF for carbamazepine. Navarro et al. (2016) showed that higher water temperature stimulates the metabolism of aquatic organisms, so nutrient adsorption is increased and thus organisms can adsorb contaminants in excess (Heugens et al., 2003). However, the BCF of VFX and citalopram decreased after warming (in the control, BCF for VFX = 528.1 mg L⁻¹, for citalopram 2606.2 mg L⁻¹; at an elevated temperature, BCF for VFX < 500 L kg⁻¹, for citalopram < 2000 L kg⁻¹), suggesting that the relationship between temperature increase and the bioconcentration factor is not the same for all pharmaceuticals. In other studies, it has been shown that a water acidification, among other things, produced a slowing of mussel metabolism as a result of a higher energy requirement to maintain homeostasis (Kroeker et al.,

2014, 2010). This would result in a reduced uptake of, among other things, pharmaceuticals by mussels under acidified conditions. This assumption would be consistent with the results of Serra-Compte et al. (2018) where the BCF of citalopram and VFX significantly decreased under acidification and the BCF of carbamazepine decreased slightly. In addition, the researchers tried to correlate the changes in the log D_{ow} values (the partition coefficient of analyte molecules between the aqueous phase and octanol, which informs about the lipophilicity of the analyte molecule; the logD distribution coefficient is determined at current pH) of the compounds under the influence of pH changes. For VFX and citalopram, as the log D_{ow} value decreased, the BCF value also decreased; for carbamazepine, no change in either parameter was seen. However, the decrease in hydrophobicity of sotalol at lower pH did not result in a decrease in its bioconcentration; on the contrary, the BCF of sotalol increased at pH 7.6 ($>20 \text{ L kg}^{-1}$), indicating that other mechanisms may be responsible for the affinity of a compound to a given matrix, in this case body tissues (Huerta et al., 2016; Yamamoto et al., 2009). Both abiotic stressors acting simultaneously caused a decrease in the BCF values of VFX and citalopram (Serra-Compte et al., 2018). The researchers emphasize that acidification determined the change in BCF when both abiotic stressors acted simultaneously – the BCF of pharmaceuticals takes on similar values under acidification, as well as with the combined stressors. This agrees with other studies that have shown that acidification interacts more with changes in calcareous organisms than changes in temperature (Duarte et al., 2014; Findlay et al., 2010). The advantage of acidification over warming during simultaneous exposure has also been reported for the mussel *Mytilus chilensis* (Duarte et al., 2014). Moreover, it has been noted that climate change is not indifferent to the elimination of compounds in the mussel body (Serra-Compte et al., 2018). For example, the elimination of sotalol in the control was 50 %, while under acidification it increased to 60 % and under warming to 68 %. The opposite was shown for the metabolization of contaminants, e.g., VFX under acidification – at lower pH the ability of *M. galloprovincialis* to metabolize this pharmaceutical decreased. The decrease in VFX metabolite content under acidification is consistent with the decrease in VFX bioconcentration at lower pH. What is important, in humans, some of the metabolites of VFX are pharmacologically less active than VFX (Magalhães et al., 2014). Thus, it is possible that the slowing of VFX metabolism under acidification for less active metabolites will alter the toxicity of this pharmaceutical to mussels (Serra-Compte et al., 2018).

To our knowledge, to date only Cruzeiro et al. (2019) has conducted a chronic toxicity test of a pharmaceutical against a freshwater organism under conditions of simultaneous warming and acidification, taking progestin levonorgestrel to assess the genotoxic effect on hemocytes of the organism *Gammarus locusta*. The researchers showed no increase in DNA strand breaks in *G. locusta* hemocytes when they were simultaneously exposed to both abiotic stressors and contamination (10 ng L^{-1} and 1000 ng L^{-1} ; duration of the test: 21 days). C. Cruzeiro et al., explain the lack of increase in DNA damage under additional stressors by the excessive duration of the experiment. It is possible that the organism was able to adapt to the stressful conditions and proceeded to repair DNA strand breaks and renew immune cells (Ellis et al., 2011).

4. Discussion

Fig. 1 shows the pharmaceuticals that appeared in the referred reports and the organisms subjected to these tests. Pharmaceuticals are a very large group of bio-active substances which are internally differentiated in terms of their physicochemical properties. Depending on the structure and attached functional groups, the pharmaceuticals may be acidic, basic or amphoteric, and highly polar to nonpolar. These features modulate the a biotic and abiotic processes to which they are subjected once they enter the environment. Differentiation of the physicochemical properties can be observed even inside a single class of pharmaceuticals. For example, beta-blockers, quite similar in their functional characteristics, have a wide range of lipophilicity, which characterizes their ability to be dissolved in

the non-polar phase, and indirectly the potential to penetrate cell membranes and bioaccumulation (Maszkowska et al., 2014b, 2014a).

The increase in toxicity with increasing temperature could change the classification that the chemicals have according to the EU Directive 93/67/ECC (European Commission, 2003). In this document there are the following categories: substances very toxic ($\text{LC}_{50} \leq 1 \text{ mg L}^{-1}$), toxic ($1 \text{ mg L}^{-1} < \text{LC}_{50} \leq 10 \text{ mg L}^{-1}$), or harmful ($10 \text{ mg L}^{-1} < \text{LC}_{50} \leq 100 \text{ mg L}^{-1}$) to aquatic organisms. The EU Directive does not take into account environmental changes, such as temperature during the toxicological evaluation of the pharmaceuticals, however, which should be improved.

• Effect of warming on the toxicity of pharmaceuticals

The temperature range of 18 to 33 °C was tested in the presented reports, and the temperature rise was between 5 and 10 °C. This needs a comment in regard to the aim of the review – the change in toxicity with global warming, which is presenting a much lower rate of temperature rise, and furthermore on a larger time span (normally, decades). Nevertheless, the global warming increase of temperature presents a medium value, and some regions already suffer from extreme temperatures, such as heat and other abnormalities in short time events. Furthermore, the absorption of solar radiation by water bodies promotes diurnal changes in their temperature. Changes in water temperature are also recorded seasonally. In large and deep freshwater bodies, such as lakes, changes in water temperature in the vertical profile are observed with the changing seasons are observed, being inherent in the property of freshwater, which reaches its highest density at 4 °C. Fluctuations in water temperature depending on the season also occur in saline waters. Examples are the coastal sea around Japan, where horizontal and vertical temperature variations are observed, the Gulf of Osaka, where the amplitude of the mean seasonal variation of water depends on the areas of the bay that are unequal in salinity and depth (Yanagi, 1987), or the Baltic Sea (Kapustina et al., 2017).

However, the long-term temperature changes of water bodies, which are confirmed in the literature, are becoming alarming. For example, increases in the subantarctic water temperature ($0.29 \pm 0.9 \text{ °C}$ per 10 years), as well as the subsurface subpolar deep waters ($0.04 \pm 0.01 \text{ °C}$ per 10 years) have been documented in the Southern Ocean (Auger et al., 2021). Considering the large marine ecosystems, water warming proceeds fastest in semi-enclosed or completely land-locked seas such as the Baltic Sea, the Black Sea or the North Sea (Belkin, 2009). It has also been documented that, due to increasing temperatures in the Arctic, the thickness of sea ice has decreased (a decrease of 9.1 % over 10 years between 1976 and 2006) (Häder and Gao, 2019). Soil and water devoid of ice or snow cover can absorb much more light, which contributes to the further heating of reservoirs. Temperature increases in the Baltic Sea (Bothnia Bay) between 1970 and 2009, recorded in autumn and winter, range from 0.5 °C to 0.6 °C for every 10 years (BACC II Author Team, 2015). In contrast, the central and southern parts of the Baltic Sea recorded temperature increases of between 0.2 °C and 0.3 °C per 10 years. Scientists say the average annual temperature in the Baltic Sea rose by $>1 \text{ °C}$ in the 10 years after 1987 (Belkin, 2009; BACC II Author Team, 2015). For 30 years, according to the EPA, sea surface temperatures have been higher than at any time since measurements began in 1880 (EPA, 2022b).

The referred changes in temperature are smaller than tested rise of temperature (Table 2 and 3). Nevertheless, testing the effects of temperature change by 0.1 °C in laboratory conditions would be problematic due to the temperature accuracy measurement. Furthermore, it would be problematic to observe significant changes in biomarkers. On the other hand, the tested temperature rise reflects the daily change in temperature or vertical temperature of a relatively small water reservoir, and the heat period. Thanks to checking the higher span of temperature with sufficient measurement points, it is possible to observe trends, and to specify the quality and quantity of changes, and maybe breakpoints. All the tests were performed in laboratory conditions, in strictly controlled temperatures. The organisms were not subjected to a daily change of temperature such as occurs in real waters. In the environment, selected mobile water organisms have abilities



Fig. 1. A representatives of pharmaceuticals and aquatic organisms reported in the scientific papers aimed to check effect of pH and temperature increase on toxicity (up to 2022).

to transfer to water with lower temperature, for example closer to the reservoir bottom. Nevertheless, this is a general issue in laboratory conditions – testing an isolated factor (here, only warming) to get a straight answer.

Generally, the tested temperature rather refer to tropical waters. The temperatures tested for marine species were from 17 to 31 °C, while for freshwaters from 15 to 25 °C. In the polar regions, temperatures are much lower (close to 4–6 °C) (Borgå et al., 2022). The problem is the lack of reference organism for polar regions. As aforementioned in the introduction, the polar surface waters are subject to acidification at a faster rate than oceans located in warmer geographic regions. This means that there is an emerging need to perform tests using polar organisms.

There should be two general conceptions for the testing of warming effects. In the first one, for which we found reports, organism are conditioned in a normal or accelerated temperature, and then exposed to pollutants. It seems, however, that there should be a second type, where the temperature is increased during exposure, which reflects momentary warming. Such an increase reflects the warming event, observed more often globally (Coffel et al., 2018). In this kind of test the organisms are cultivated in the

normal/standards temperature, and the temperature shock is tested together with exposure to pharmaceuticals. Such study was performed by Nardi et al. (2022), who performed test of heatwaves effect to neuro-immune and oxidative tolerance toward carbamazepine in *Mytilus galloprovincialis*. Having in mind the rather short lifespan of test organisms (invertebrates in the majority of cases) the experience of heat and its negative effects are more likely to occur and be observed compared to the slow increase of a median temperature. This means, that there is a need for more studies of the effect of heat, which can be the extreme effect of global warming in some regions.

For the POPs the general founding is that with increased temperature both their uptake and elimination by aquatic organisms increased (Noyes et al., 2009a; UNEP/AMAP, 2011). For example, for the 72 congeners of polychlorinated biphenyls (PCBs) elimination from yellow perch (*Perca flavescens*) was found to occur during spring and summers (Paterson et al., 2007). The metabolism of PCBs were found to be enhanced by a rise of temperature, which caused the formation of bioactive hydroxylated metabolites in rainbow trout (Buckman et al., 2007). The increased toxicity

with temperature was observed for selected pyrethroid pesticides (for example for daphnia (Ratushnyak et al., 2005)). In the case of pharmaceuticals, a generally increased toxicity with warming was reported. There were exceptions, for example for OTC (Gomiero and Viarengo, 2014), which is reasonable knowing the increased biodegradation and lower stability of selected pharmaceuticals at higher temperatures. What is missing, is the investigation of qualitative and quantitative analysis.

- Effect of acidification on the toxicity of pharmaceuticals

The value of pH strictly modulated the ratio of the charged to neutral fraction of pharmaceuticals, as >80 % of them are ionizable (Manallack, 2007). Unfortunately, in most of the ecotoxicology tests with standard procedure, the real pH was not reported, as the standard test allowed a variation of the pH (Boström and Berglund, 2015) (for example, between 6 and 9 in OECD 2002). Thereby, modeling of toxicity using the reported data gives high uncertainty. The models of the toxicity base of pK_a and/or $\log K_{ow}$ (octanol-water partitioning coefficient) have been developed (Armitage et al., 2013; Baumer et al., 2017; Neuwoehner and Escher, 2011; Nieto et al., 2016), having the assumption that the neutral fraction is more toxic or even strictly responsible for the toxicity. These relatively simple models were found to not fit all representatives of pharmaceuticals, and toxicity cannot be solely determined knowing the uncharged fraction (Boström and Berglund, 2015). The risk assessment and calculation of hazard quotients (HQs) for natural water needs to take into account the obtained pH. It was found that the HQ is corrected with the pH of the European rivers pH, and that the toxicity of weak bases and weak acids was underestimated and overestimated, respectively (Boström and Berglund, 2015).

From our own experience, testing of the pH lowering is not an easy task, especially while experimental water changes the pH during testing (for example, it increases during cyanobacteria cultivation). The buffering it is not always possible while tested organism are in a special growing medium. In publications it was not mentioned whether the pH was tested at the start or at the end of the experiments (Boström and Berglund, 2015).

From a pH standpoint, the pharmaceuticals of most interest are those that have a pK_a most similar to the pH of natural waters. A change in the proportion of the ionized form may then occur. An example of such pharmaceuticals is sulfonamides, whose acid-base balance perfectly illustrates the relationship of pK_a to the solution pH (Męczykowska et al., 2018; Biłk-Bielińska et al., 2012). It has been shown that the higher the pH of the solution, the greater the prevalence of the negative charge of sulfonamides. Additionally, it has been studied that sulfonamides in the anionic form are less sensitive to hydrolysis than the cationic, or neutral forms (Biłk-Bielińska et al., 2012). Thus, given that the pH of natural waters is in the range of pH 6–8.5, the hydrolysis process of sulfonamides in the environment is slow. NSAIDs are mainly carboxylic acids, which is why in aqueous solutions they occur with a predominance of a negative or neutral charge (pK_a close to 4). Like sulfonamides, they have a higher negative charge of the molecule at a higher pH. Other more basic pharmaceuticals, such as β -blockers ($pK_a > 9$), may only be present in solution in a cationic or neutral form, which may also affect their sorption capacity. The sulfonamides, NSAIDs and β -blockers were under investigation (Fig. 1), and the effect of acidification on toxicity was noted, as accepted.

What is important, in order for pharmaceuticals to be effective, they are constructed in such a way that they penetrate cell membranes. In the context of the penetration of the pharmaceutical through the cell membranes, the hydrophobicity and acidity are important. It appears that the neutral form of pharmaceuticals, which is more lipophilic than dissociated forms, has a higher affinity to penetrate the cell membranes (Almeida et al., 2018; Kim et al., 2010; Nakamura et al., 2008; Sun et al., 2020). For weak bases, the neutral form portion increases with acidification, which increases toxicity (Alsop and Wilson, 2019; Rendal et al., 2011; Valenti et al., 2009), while the opposite is true for weak acids – the percentage of occurrence of their neutral form increases with acidification, resulting in their increased toxicity in more acidic environments (Alsop and Wilson, 2019).

Pharmaceuticals which come in ionized form to facilitate their passage through the membrane, use, for example, the ion-pair strategy, which results in neutralizing the charge (Morrow et al., 2007). The ionization form of pharmaceuticals was considered as a factor of toxicity increase, as presented in Table 4 and in Table 5. It is probable, that different pollutants but with a similar mode of action (for example, causing intensive oxidative stress) give an accumulated response when mixed. Thank to this information, testing with simultaneous exposure of pharmaceuticals can be planned.

- Bioaccumulation changes with acidification and warming

Bioaccumulation is a process which has a direct connection with toxicity. It has been noted that the BCF value does not always increase with the observed increasing toxicity of the pharmaceutical (Lopes et al., 2022). When the body is exposed to low stress and when the metabolic reactions of the body's cells are slowed down, this can cause the contaminant to accumulate in the body. On the other hand, an increase in toxicity can cause excessive activation of detoxification mechanisms and increase the biotransformation process (GST activation), thereby lowering the BCF. However, this is not true for all pharmaceuticals. For example, for sulfonamides this relationship was not observed – the BCF coefficient increased with increasing toxicity (Anskjær et al., 2013; Serra-Compte et al., 2018). An increase in the BCF of pharmaceuticals was also noted when the pH of the aquatic environment decreased – as acidification of the environment often results in slower metabolism (Kroeker et al., 2014, 2010). Scientists also pay close attention to long-term studies of the toxicity of pharmaceuticals – organisms exposed to pharmaceuticals in the short term are often able to overcome stress, activating defense mechanisms that are unable to counteract long-term stress (Freitas et al., 2016, 2015), which has its reflection in bioaccumulation. In the case of temperature the effect was not so straight. The enhanced temperature can induce the defense mechanism and lower bioaccumulation. A decrease in bioaccumulation with temperature was reported (VFX, citalopram Serra-Compte et al. (2018)), which could have resulted in a faster metabolism and the removal of metabolites. On the other hand, increased metabolism and nutrient consumption can stimulate the co-adsorption of pharmaceuticals (Heugens et al., 2003), and finally bioaccumulation observed for example for sulfonamides (Serra-Compte et al., 2018).

The bioaccumulation is also driven by ion-trapping. This is the effect of increased fraction of ionic molecules inside a cell compared to the outer environment, because of cytoplasmic pH, what was observed for example for sulfonylurea herbicides (Fahl et al., 1995) and pharmaceuticals (Baumer et al., 2017; Neuwoehner and Escher, 2011). The general observation from gardened scientific reports, with single expectations, was that basic pharmaceuticals, such as fluoxetine, decrease its bioaccumulation with acidification, while acidic pharmaceuticals increase it. This is connected with the percentage of neutral fraction, which changes with the pH, and the higher potential for membrane penetration of neutral molecules. For selected pharmaceuticals, like CBZ (Almeida et al., 2018) and EE2, the bioaccumulation was not impacted by the pH, as they stay non-ionic at all tested pH values.

Bioconcentration of pharmaceuticals are rather low compared to POPs (Puckowski et al., 2016). The biomagnification is then even less probable. This means that there is a low potential to transfer to higher levels in the food chain, which differentiated the pharmaceuticals from POPs.

- Representation of test species

Despite our best attempt in the literature search, we found only one report involving the phytoplankton species (Neuwoehner and Escher, 2011). Nevertheless, this work present a model of change in the toxicity of pharmaceuticals toward *Scenedesmus vacuolatus*. Knowing that depletion of the primary producers has an impact on the whole food chain, more studies need to be performed using green algae. The cyanobacteria species produced blooms should also be a focus in near future studies. The performance of tests in the mesocosm will take research closer to the real

environment. The lifespan of microalgae (green algae and cyanobacteria) is short, and generally the blooms are initiated by increasing temperature. Selected pharmaceuticals were found to be toxic for microalgae in the $\mu\text{g L}^{-1}$ concentration, while others are rather inert in mg L^{-1} (Miazek and Brozek-Pluska, 2019). As there are numerous ecotoxicology tests and bioremediation experiments have been performed for microalgae, the tracking of temperature and pH characteristics in reports (if given) would help to find the most fragile strains and the most toxic pharmaceuticals. Nevertheless, this is beyond out of the scope of this review.

The bivalves are the most tested species for chronic tests for various reasons. Firstly, they are a fine test organism (relatively known biochemistry, sequenced genome, easy to handle, sedentary, easy to acquire, they are cosmopolitan, and an ecologically important organism). Furthermore, their sedentary life in their mature state means that they are unable to escape from unfavorable conditions. They are already used in monitoring programs (Kimbrough et al., 2008) and proposed to be biosensors (de Vargas Guterres et al., 2020). The first impacted tissue of bivalves are the gills. The negative effect on mussel gills was observed for DCF (Munari et al., 2018) during chronic impact of $\mu\text{g L}^{-1}$ concentrations and a drop in pH (genotoxic effects, COX activity, LPO level). The fish gills were also stressed by VFX (Maulvault et al., 2019). The mussels can slow their metabolism due to acidification (Kroeker et al., 2014, 2010), which in consequence reduces the bioaccumulation (Serra-Compte et al., 2018). Depuration from pharmaceuticals can also be affected by acidification (Serra-Compte et al., 2018). It's worth mentioning that the bivalves can be impacted by pollutant indirectly, as the microflora of its gut has demonstrated sensitivity to pollutants (Tili and Mouneyrac, 2021). The gastrointestinal microbiota was found to be a site for the biotransformation of pharmaceuticals (Sousa et al., 2008). The species of bivalves should be taken into account during the toxicological evaluation, as their defense system may respond differently to stress. For example, *R. philippinarum* clams have been shown to be less resistant to the acidification of waters than *M. galloprovincialis* mussels (Munari et al., 2018).

There were no clear straight differences between marine and freshwater bivalves, but the number of studies is too small for a final statement. The same can be stated for the stage of life, sex, and place of origin of the test organisms. Surely, there is a need for the testing of species living in vulnerable environments, already close to the edge of homeostasis, as the small change in water parameters can alter pharmaceuticals toxicity more greatly than for relatively stable and resistant populations. From the point of the concentrations of pharmaceuticals in the aquatic environment, the freshwater species are subjected to higher levels of contamination. Furthermore, in shallow freshwater reservoirs quick and significant changes in temperature and pH can occur.

• Other findings and recommendations

CO_2 is more soluble in saline waters due to the fact that these waters already contain carbonate ions, with which carbon dioxide reacts to form bicarbonates (Spokes, 2003). Due to the higher solubility of carbon dioxide in cool waters, polar surface waters are subject to acidification at a faster rate than oceans located in warmer geographic regions. Furthermore, the polar regions are subjected to global warming at a higher speed than other regions (Borgå et al., 2022), thereby they are of with special attention to research. The POPs are the pollutants of interest, as they transfer to the polar region, are persistent, biomagnified and have a proven toxic effect on arctic organisms (UNEP/AMAP, 2011). The concentration of pharmaceuticals in the polar region are sub-trace level and the frequency of detection is low. Their transport over long distances is a rather low probability, and the local wastewater treatment plants are the primary sources (Kallenborn et al., 2018). Nevertheless, the arctic parameters (low temperature, low light intensity, slow microbial activity) prolong the biotic and abiotic degradation of pharmaceuticals. There was no single report presenting the change in toxicity of pharmaceuticals in low temperatures as obtained in polar regions. It can be assumed that the bioaccumulation factors of selected pharmaceuticals can be higher in low temperatures

compared to the temperatures applied in standard tests, because of the limited metabolism rate. This assumption needs verification, as well as the toxicity of pharmaceuticals in low temperatures. Unfortunately, none of the found reports refer to the temperature conditions typical for polar regions.

The estuaries are unique habitats, which provide a critical ecosystem service and they are buffers against the impact of storms on to coastal regions (Delorenzo, 2015). Estuaries are generally zones that experiences high changes in their psychochemical characteristics (fluctuation of temperature, oxygen concentration, pH, salinity) due to the variable impact of fresh and marine waters, but they are considered especially sensitive to climate change. When fresh water meets salt water, the salting-out effect can occur and the sediment is enriched with pollutants. The estuaries organisms are adapted to tolerate a change in water quality, but the predicted increase in nutrients and contamination (especially pesticides and pharmaceuticals) are potential stressor makers by increasing the energy demand. This can cause the selection of more tolerant species, among other changes. The organisms living in the estuaries should receive special monitoring.

There is a need to perform longer testing, because short responses and long-term responses have varied (Almeida et al., 2014; Freitas et al., 2016). The long term stress can reduce the glycogen level (Freitas et al., 2016), while short-term stress act as increases the glycogen level (Almeida et al., 2014). The level of proteins are quite a good marker of long term stress. Protein synthesis uses significant energy and oxygen (Gravel and Vijayan, 2007). The mussels can adapt to acidification (Bamber, 1990), as well as other organisms (Madeira et al., 2018). But when organisms are in poor condition and have a low level of stored energy, the prolonged stress caused by pollutants can be significant. The linearity of the biological effect and the time of exposure need to be researched further, especially in the case of semi-persistent pollutants such as pharmaceuticals. In a chronic test the concentration is often at one level during exposure, and the gradient of the biological response in the concentration cannot be determined. The performance of such experiments can help to estimate the threshold for risk alarm. There are still relatively few advanced studies that are able to confirm changes in the genome of organisms exposed to pharmaceuticals and projected climatic conditions. However, those that have emerged so far confirm a negative scenario of changes in DNA expression (Mezzelani et al., 2021; Munari et al., 2018), which, in the worst case scenario, could contribute to species extinction. In further study, despite the toxicity, there is a need for testing/reporting stability in changed conditions as well as the metabolism rate and quality/quantity of metabolites, distribution within tissues, and the dynamic of removal. In such a situation additional information can be obtained during one exposure of the living organism, which can supplement the risk assessment. More is known about the activation of enzymes in non-target organisms, for example by checking metabolites that are produced (Mulkiewicz et al., 2021). It has been documented that the effects of pharmaceuticals against non-target organisms are most often manifested by increased oxidative stress (Giuliani et al., 2013).

5. Conclusions

As expected, the effects of global warming are not irrelevant to the ecotoxicity of pharmaceuticals for aquatic organisms. The acute and chronic toxicity of many of these bioactive chemicals has changed under the influence of increasing water temperatures and acidification, resulting in alterations at the molecular, cellular, tissue and organism levels. Unfortunately, preliminary data has shown that the combined stressors increase response. However, the direction of this change could not be clearly determined for all pharmaceuticals, because of their different chemical properties and unexpected mode of non-target action. The absence of increased acute toxicity does not necessarily mean that organisms' homeostasis is not affected. The chronic sub-lethal exposure of aquatic organisms to pharmaceuticals could impair their function and viability (Nieto et al., 2016). In addition, it has been confirmed that long-term stress does not necessarily manifest itself as oxidative stress, but the changes already extend further into the cell functionalities – causing changes in the mRNA

(Almeida et al., 2018; Mezzelani et al., 2021; Munari et al., 2018). For the POPs it is predicted that the temperature rise will increase their uptake, metabolism, elimination and toxicity (Noyes et al., 2009a). In the case of pharmaceuticals, as this review shows, such a straightforward situation is not obtained, and is rather molecule-to-organism-to-conditions specific. Furthermore, the non-linearity of response with time and stressor values, as well as the simultaneous impact of both acidification and temperature rise, make predictions and estimation highly uncertain. The effect of increased bioconcentration with temperature could lower or enhance the toxicity of pollutants, depending of the competition of the bio-activation (for example, the production of active metabolites, activation of receptors) and detoxication (the production of inactive metabolites, following depuration) (Noyes et al., 2009a), what was noted in this review for pharmaceuticals. For the POPs, the prediction models for the changing of bioaccumulation, fates, and effect are more or less given (Gouin et al., 2013; Moe et al., 2013). For the pharmaceuticals, these models need adaptation, as the pH is the significant factor. The effects of change in pharmaceuticals's ecotoxicity on the level of populations are unrecognized.

This review shows insight into the change of pharmaceutical toxicity with global warming and acidification. After decades of research on climate change itself, the study of the change of ecotoxicity of new emerging pollutants are now highlighted. The ecotoxicology of climate change needs adaptation and more attention, which is a consensus of specialists (Boxall, 2014; Landis et al., 2014; Manciocco et al., 2014; Scheringer, 2017; Tlili and Mouneyrac, 2021). What's alarming, as the concentration of pharmaceuticals in environment is likely to increase (Boxall, 2014; Caban and Stepnowski, 2021), the advance in ecotoxicology of pharmaceuticals in climate change conditions is needed urgently.

CRedit authorship contribution statement

Katarzyna Bethke: Conceptualization, Visualization, Writing- Original draft preparation, Writing- Reviewing and Editing. Klaudia Kropidłowska: Writing- Original draft preparation. Piotr Stepnowski: Supervision. Magda Caban: Conceptualization, Writing- Original draft preparation, Writing- Reviewing and Editing, Supervision.

Data availability

No data was used for the research described in the article.

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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Investigation of pharmaceutical bioaccumulation in *Daphnia* sp. living in a wastewater treatment plant

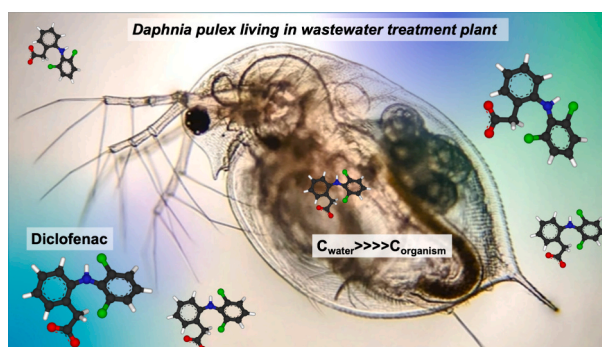
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HIGHLIGHTS

- *Daphnia* spending their lifetime in clarifiers of WWTPs were sampled.
- From tested NSAIDs, diclofenac was the only tested drug found in daphnia.
- The in-situ BAF of DCF was lower than the experimental values.
- Suspended particles can be a source of drug in daphnia.
- DCF was not bioaccumulate in wild-living daphnia.

GRAPHICAL ABSTRACT



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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used pharmaceuticals. Their presence in natural waters is due to the low removal efficiency in conventional wastewater treatment plants (WWTPs). Interestingly, certain zooplankton species can survive the mixture of pollution and abnormal water conditions in WWTPs. In our study, for the first time, we tested the in-situ bioaccumulation of NSAIDs and their metabolites in *Daphnia pulex*, which were obtained in high numbers in one WWTP during the summer. It was found that diclofenac (DCF) and 4-hydroxy DCF were present in the studied clarifiers and ponds. Among these chemicals, only DCF was detected in daphnia. The bioaccumulation factor of DCF in daphnia was below 36 L kg^{-1} and was lower than those obtained under experimental conditions for *Daphnia magna*. The tested daphnia adapted to chronic exposure to mixtures of drugs in $\mu\text{g L}^{-1}$ level and could be implemented in biobased WWTPs. According to our data, there is a need to supplement the risk assessment of anthropogenic pollutants with in-situ cases to demonstrate the adaptation possibilities of wild-living organisms.

1. Introduction

Cladocera is a cosmopolitan group of zooplankton that has become an important aspect of research over the last few decades due to its use in

reference tests for ecotoxicity (Siciliano et al., 2004). *Daphnia magna*, the prime species for such investigations (Tyagi et al., 2009), can also be used to test the toxicity of pharmaceuticals for mammals (among arthropods, daphnia gave the closest homology and best matches to

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human genes) and can eliminate pathogenic bacteria and viruses from water. They can be obtained in artificial reservoirs, such as ponds fed by wastewater, where they enhance organic matter removal (Kampf et al., 2004). On the other hand, selected species of Cladocera are used to test the toxicity of wastewaters, with *D. magna* again playing a leading role (Tyagi et al., 2009). However, the question remains: how is it possible that the same species can easily live in wastewaters, which could be toxic for them? This may be related to three factors: the species tested, wastewater characteristics, and adaptation possibilities.

In the final ponds found in wastewater treatment and stabilization ponds, ciliates, rotifers, Cladocera, and copepods can live (Harishkumar and Kiran, 2016; Nandini, 1999; Nandini et al., 2005, 2004). Among these groups, Cladocera is a major component in terms of biomass due to its large size and parthenogenetic reproduction. Reports have shown that native-living Cladocera can adapt to actual environmental conditions and are more resistant to environmental pollutants than exotic strains (Santos-Medrano and Rico-Martínez, 2019). Therefore, although the use of *D. magna* is recommended, some researchers propose the use of native species for toxicity tests.

In our previous work, we determined the level of pharmaceuticals in several wastewater treatment plants (WWTPs) in Poland (Kolecka et al., 2022, 2020, 2019). The targets were non-steroidal anti-inflammatory drugs (NSAIDs), in which diclofenac (DCF) was the emerging pollutant. We found that the reduction of the DCF concentration was low to negligible during conventional biological-chemical treatment, which was consistent with other reports (Muter et al., 2017; Pařga et al., 2019; Ślósarczyk et al., 2021). In one of the tested WWTPs (Swarzewo, North Poland), we observed a high number of zooplankton, mainly *Daphnia* sp., in clarifiers during the summer season. At the same time, the concentration of DCF was higher than $1 \mu\text{g L}^{-1}$, among other pharmaceuticals. This indicated that wastewater with such a concentration was not an obstacle to growing this type of zooplanktonic species. However, it was not determined if NSAIDs could accumulate and be metabolized by *Daphnia* sp. In the case of daphnia, testing of bioaccumulation is problematic due to the small size of a single organism, and, to date, only one report has described the bioaccumulation of NSAIDs in *D. magna* under experimental conditions (Nkoom et al., 2019). A comprehensive understanding of the bioaccumulation and depuration process of pharmaceuticals in wild-living organisms remains unexplored (Puckowski et al., 2016).

In the presented work, we examined the bioaccumulation of NSAIDs and selected metabolites by *Daphnia* sp. in real wastewater conditions. The daphnia were sampled, along with water and suspended solids, from a WWTP. To the best of our knowledge, such a study has never been conducted. Estimating environmental bioaccumulation can supplement the risk assessment of new emerging pollutants. Our work contributes to the research on the bioaccumulation of NSAIDs and their metabolites in the food web and chronic exposure studies at environmentally relevant concentrations (Lonappan et al., 2016).

2. Materials and methods

2.1. Chemicals

Analytical grade standards, including diclofenac (DCF) sodium salt, 4-hydroxydiclofenac (4OH-DCF), diclofenac methyl ester (M-DCF), ibuprofen (IBF), 2-hydroxyibuprofen (2OH-IBF), naproxen (NPX), clofibric acid (CFA), and diclofenac $^{13}\text{C}_6$ (IS1) were purchased from Sigma Aldrich. The organic solvents used in this study were methanol (MeOH, HPLC grade, VWR Chemicals), acetonitrile (ACN, HPLC gradient grade, POCH Poland), and hexane (99 %, POCH Poland). The salt and acid used for the mobile phase preparation were ammonium acetate (>99 %, HiPerSolv Chromanorm) and glacial acetic acid (100 %, AnalaR Normapur, VWR Chemicals).

2.2. Sampling

The Swarzewo WWTP is located in North Poland, near the coast of the Baltic Sea. The flow rate of the municipal wastewater in the summer season averaged approximately $14,000 \text{ m}^3 \text{ d}^{-1}$. Treatment was conducted in chambers with activated sludge using sequential batch reactors (SBRs). A more specific description of the treatment technology was previously reported (Kolecka et al., 2020). The conventional parameters of treated wastewater and removal levels were found to meet acceptable standards.

Sampling of water from clarifiers and ponds was conducted in two sets. The first set of sampling was conducted in 2019, where water from both clarifiers and ponds was collected for analysis. The sampling dates were July 24th, August 22nd, September 30th, and October 30th 2019. Fig. 1 shows the location of Swarzewo on a map of Poland (from <https://www.geoportal.gov.pl/>) as well as a satellite image (Google® company maps) of the WWTP Swarzewo with marked sampling points.

The clarifiers (C) were sampled at four locations labeled C1 to C4 to determine the mean level of pollution. Following the clarifiers, there are two sequential ponds designed for additional clarification of treated wastewater. Water samples from these two ponds were taken at the outflows.

In July and August 2021, the conditions in the clarifiers were as follows: temperature 20°C , pH 6.58–6.58, conductivity $6.8\text{--}7.7 \text{ mS cm}^{-1}$, and dissolved oxygen $1.8\text{--}3.1 \text{ mg L}^{-1}$. The values for these parameters from 2019 are presented in Table S1 (SI). We found that water parameters in the clarifiers differed from those in natural ponds, namely showing lower pH, dissolved oxygen concentration, and increased salinity.

The second sampling was performed in 2021 on two separate occasions: July 28th and August 4th. The water pH was between 6.61 and 7.17. During these dates, a bloom of daphnia was observed. In addition to water samples, daphnia were collected (zooplankton net, picture in Fig. S1, Supplementary Information). The daphnia created visibly deep red colonies, and it was possible to sample high numbers of organisms with few net immersions. While most of the sampled material consisted of daphnia, a few other zooplankton species were noted under the microscope. When possible (for example, in the case of bloodworm larvae), these other species were removed from the samples using metal tweezers. Daphnia with a low volume of water was placed in plastic jars. A microscopic picture of one of the sampled daphnia is presented in Fig. 2. Most organisms had eggs. The technicians at the WWTPs observed daphnia starting in spring, but a significant increase in presence was normally observed as temperatures rose. The excessive number of daphnia was favorable because they significantly increased the clarity of the water.

The water was sampled using a manual sampler. The treated wastewaters were placed in brown glass bottles together with suspended particles, which were a food source for daphnia. All the samples were stored in a portable fridge and transported to the laboratory on the same day.

The sample pretreatment procedure was as follows: water samples were filtered through a glass fiber under vacuum. The filters, along with the particles, were frozen (-20°C). The water sample was acidified to pH 3 using an HCl acid solution. The daphnia were separated from the water using medical gauze (picture in Fig. S2, Supplementary Information), and washed with deionized water to remove the freely attached particles. Then, the organisms were placed in plastic tubes and frozen. The particles stored on the filters and daphnia were lyophilized using a FreeZone 2.5 L (Labconco) device at -80°C for 24 h (picture in Fig. S3, Supplementary Information).

2.3. Extraction procedures

The extraction from water was performed using solid phase extraction (SPE) columns with Strata-X (100 mg of sorbent, 2 mL cartridge

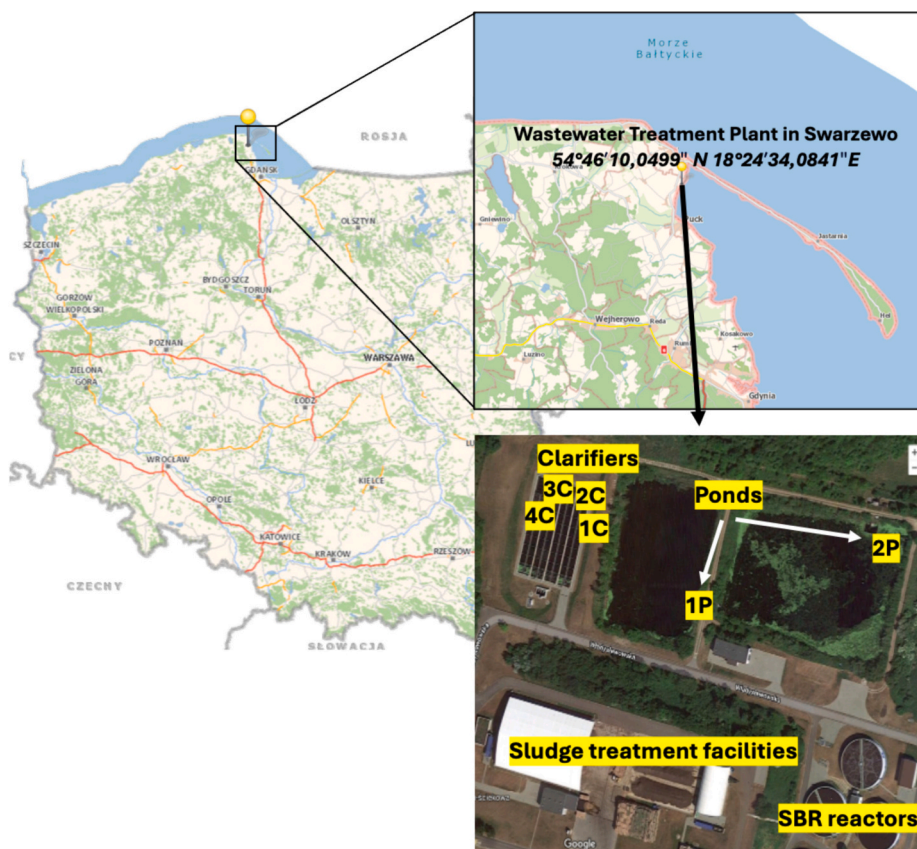


Fig. 1. Satellite picture (Google® company maps) of sampling points in tested municipal WWTPs.



Fig. 2. A picture of *Daphnia pulex* samples in clarifiers of tested municipal WWTPs.

volume, Phenomenex). The SPE process was as follows: sorbent conditioning with 3 mL of MeOH + 3 mL of water, application of 250 mL of filtrated treated wastewater (with internal standard (IS) spiking) using vacuum assistance, washing with 5 % aqueous MeOH, sorbent drying, second washing with 3 mL of hexane, and second drying. The elution was performed with 2×3 mL of MeOH. The organic extract was dried to remove the solvent using a nitrogen stream. The obtained residue was stored in a brown-glass chromatographic vial (1.5 mL) at -20°C before instrumental analysis.

The extraction of drugs from biological material and particle matter was performed using solid-liquid extraction (SLE) with purification of the extract by SPE. The mass of the lyophilized sample was measured using an analytical balance, ranging between 258 and 562 mg. In the

case of particle matter, the appropriate dry mass was obtained only for samples collected on July 28th 2021. For the extraction, a mixture of ACN and acetate buffer (5 mM, pH 4.1) was prepared in a volumetric proportion of 65:35. The volume of 25 mL of this extraction mixture was added for each sample placed in a polypropylene vial (50 mL total volume of the vial). Furthermore, the IS solution ($250\ \mu\text{L}$, $2\ \text{mg L}^{-1}$) was added to each sample before any processing. Then, 20 ceramic beds and 1 spoon of ceramic micro-beds (about 100 mg) were added to each vial. The extraction of analytes from solid samples to liquid was performed using bed homogenization (3 cycles, each 30 s, speed $4\ \text{m s}^{-1}$). Then, the solution was centrifuged (5000 rpm), and the liquid was dissolved in ultrapure water to obtain 5 % organic matter. Subsequently, the SPE was obtained using the protocol described above.

2.4. UHPLC-MS/MS analysis and calculations

Quantitative analysis was performed using ultra-high performance liquid chromatography coupled with tandem mass spectrometry (triple quadrupole analyzer) (UHPLC-MS/MS). The UHPLC-MS/MS system (Shimadzu) comprised a solvent delivery module LC-40B X3, an autosampler SIL-40C X3, a column oven CTO-40C, and a spectrometer LCMS-8050. The LC parameters were as follows: the column Eurospher II 100 3 C18H (50×3 mm, Knauer) with a precolumn, an autosampler temperature of 10°C , a column oven temperature of 35°C , a mobile phase flow rate of $0.3\ \text{mL min}^{-1}$, and an injection volume of $5\ \mu\text{L}$. The mobile phase A component was an acetate buffer (5 mM, pH 4.1), while component B was 100 % ACN. A gradient elution was employed starting from 5 % to 95 % of B for 5 min, then 3 min at 95 % B, and then returning to the initial conditions over 2 min. The ESI interface parameters were as follows: nebulizing gas flow rate of $3\ \text{L min}^{-1}$, a heating gas flow rate of $10\ \text{L min}^{-1}$, a drying gas flow rate of $10\ \text{L min}^{-1}$, an interface temperature of 300°C , a desolvation temperature of 520°C , DL temperature of

250 °C, and a heat block temperature of 400 °C.

Multi-reaction monitoring (MRM) mode was employed for registration, and it was automatically optimized by software. Both negative and positive modes of the ion registration were examined. Positive ions yielded a higher response for DCF, 4-OH DCF, and M-DCF, whereas the negative ions showed a higher response for IBF, 2-OH IBF, NPX, and CFA. The MRM fragmentation reaction and retention times were applied for qualitative analysis. Unfortunately, for selected analytes, only one MRM reaction gave a sufficiently high signal. The area ratio of the analyte toward the internal standard peak area was plotted against the ratio of the analyte and IS concentration. The parameters of the validated instrumental calibration curves (accuracy, precision, limit of detection, and quantification) are presented in Table S2 in Supplementary Information.

The recovery of the analyte using SPE-LC-MS/MS and SLE-SPE-LC-MS/MS was performed by spiking at a concentration level close to the mean of the calibration curve (nominal 100 ng per g of dry material). For SPE, the recovery of all relative analytes was between 98.4 %–105.1 % (mean 101.5 ± 3.4 %). The extraction of SLE + SPE gives a relative recovery between 61.8 % and 85.6 % (mean 73.4 ± 8.0 %). The bioaccumulation factor (BAF) was calculated using the following equation: concentration in solid sample [mg kg^{-1} d.m.]/concentration in water sample [mg L^{-1}]. The BAF was presented for dry mass and wet mass, using 90 % water content in the daphnia. The partitioning coefficient of DCF between suspended particles and water was calculated using the following equation: concentration in suspended particles [mg kg^{-1} d.m.]/concentration in water sample [mg L^{-1}]. The partitioning coefficient between daphnia and suspended particles was calculated using the

concentrations of DCF in daphnia and suspended particles in the same unit, resulting in a unitless coefficient.

3. Results

Preliminary monitoring of NSAIDs and metabolites conducted in 2019 detected DCF and 4OH-DCF in each water sample. Fig. 3 presents the concentrations of these pollutants in the pond and clarifier samples, using abbreviations of the sampling points described in Fig. 1. In the studied samples, IBF and NPX were also found (Fig. S4, Supplementary Information) with a concentration range of 0.03–1.56 and 0.01–0.70 $\mu\text{g L}^{-1}$, respectively.

It was observed that the concentration of the 4-hydroxy metabolite was similar to or higher than that of DCF, where the DCF concentration range was 0.75–3.32 (mean 2.21) $\mu\text{g L}^{-1}$, while the 4OH-DCF range was 0.20–4.44 (mean 2.68) $\mu\text{g L}^{-1}$. The concentration of DCF, with two exceptions, was similar in each sampling point. This was also the case for 4OH-DCF. Hence, the ponds in which plants, phyto- and zooplankton occur, had limited potential for the reduction of both chemicals. Furthermore, limited abiotic degradation occurred under the prevailing conditions.

In 2021, sampling was performed in clarifiers that contained a large number of daphnia. A composite sample was collected by taking equal volumes of water from points C1–C4. The daphnia were also collected in equal masses from sampling points C1 to C4. From the tested analytes, five were determined in water and one in daphnia. CFA and M-DCF were not detected in either water and daphnia samples. The concentrations of other drugs and metabolites in water, daphnia, and particles in 2021 are

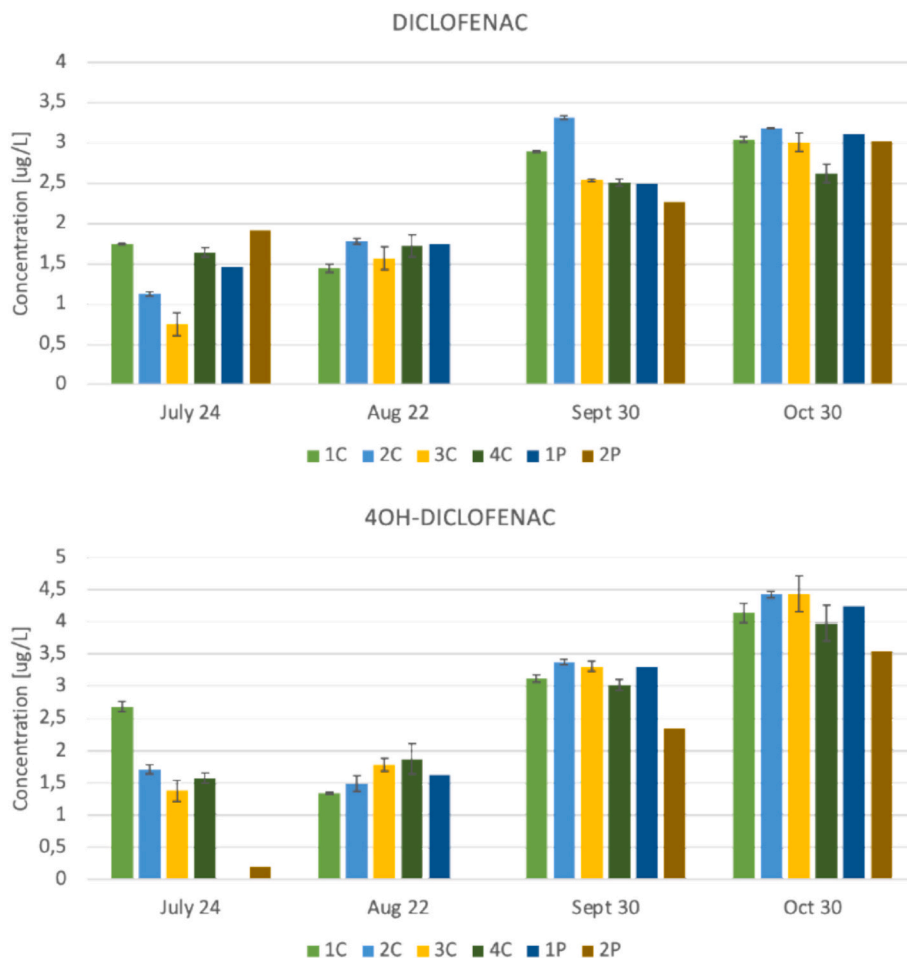


Fig. 3. Concentration of diclofenac and 4OH-diclofenac determined in clarifiers (C1 to C4) and ponds (1P and 2P) in 2019 in WWTP Swarzewo (North Poland) (momentary samples, three technical repetition per sampling point).

presented in Table 1.

The BAF values for DCF, which were calculated using the obtained data from Table 1, were determined as 363 L kg^{-1} (log BAF 2.56) and 206 L kg^{-1} (log BAF 2.31) using dry mass samples collected on July 28th and August 4th, respectively. The water content of the daphnia varied between 85 %–95 % (Bogut et al., 2010). Thus, considering the medium water content of 90 % in daphnia organisms, the recalculated BAF for wet mass was 36 L kg^{-1} (log BAF 1.56) and 18 L kg^{-1} (log BAF 1.25) for the samples taken on July 28th and August 4th, respectively. According to the European Medicines Agency (European Medicines Agency, 2015), a substance is considered to fulfill the bioaccumulation criterion when the bioconcentration factor in the aquatic species is higher than 2000. The US EPA (US EPA, 1996) states that a substance is considered not bioaccumulative when its BCF (Bioconcentration factor) is <1000 . Using both criteria, DCF does not meet the bioaccumulative threshold, noting that we determined the BAF factor instead of BCF. Furthermore, despite their presence in water, 4OH-DCF, NPX, IBF, and 2OH-IBF were not found in daphnia or suspended particles. Importantly, the DCF metabolite was not detected in the daphnia samples, despite the presence of a native drug. Similarly, DCF was found in the particles, but 4OH-DCF was not detected.

The DCF partitioning coefficient to the suspended particles was $195 \text{ L kg}_{\text{dw}}^{-1}$ (log 2.29), while BAF was 363 L kg^{-1} , both calculated for July 2021. This suggests that DCF is more accumulated in daphnia compared to suspended matter. The partitioning coefficient of DCF between daphnia and suspended matter was calculated for July 2021, and reached 2.16, again indicating a higher affinity of DCF for daphnia than for particles. This suggests that particles may serve as a source of DCF for daphnia, though further analysis needs to be performed to support this hypothesis. Furthermore, the DCF found in daphnia might have originated from the food stored in the digestive tract. This is confirmed by photos in Fig. 2, which show daphnia with digestive tracts full of food. The depuration from this content was not performed in the lab, because we were aware that the fast metabolism of daphnia could lower the DCF content in its tissue. Therefore, we performed the extraction as quickly as possible.

4. Discussion

The daphnia in the tested clarifiers were continuously exposed to NSAIDs and their metabolites from hatching to end of life. Furthermore, they were subjected to conditions different from natural reservoirs of water (lower oxygen level, lower pH, and higher salinity) and a mixture of other not-tested pollutants. Nevertheless, a large population of daphnia was present each year, supported by unlimited amounts of food. Meanwhile, nutrients in the wastewater were able to support the growth of both phyto- and zooplankton (Nandini et al., 2013). Daphnia can ingest particles sized 2–30 μm (Pau et al., 2013) and are efficient in eliminating such suspensions. The various daphnia species are

differentiated by the wastewater particles they use as a food source. Nandini et al. (2005) reported that when *Alona rectangula*, *Ceriodaphnia dubia*, *Moina macrocopa*, and *Daphnia pulex* were exposed to organic matter from several stages of wastewater treatment, they showed an increase in survival rate with the wastewater processing, with *D. pulex* surviving the longest. However, in another work by Nandini et al. (2004), the population growth patterns of four cladocerans species (*A. rectangula*, *C. dubia*, *M. macrocopa*, and *D. pulex*) in wastewater (WWTP in Mexico) were examined, with *D. pulex* exhibiting no growth in the tests, making it non-applicable for wastewater tertiary treatment. The combination of filtration by zooplankton with the nutrient removal capacity of bacterial + algal biofilm in a zooplankton-containing reactor provides a natural-based alternative for wastewater treatment (Pous et al., 2020). Therefore, cladoceran sensitivity/population characteristics in response to wastewater under real conditions must be investigated. In the sewage-fed tank in Karnataka (India), 6 species of cladocerans were detected, belonging to six families: Daphniidae, Moinidae, Bosminidae, Macrothricidae, Sididae, and Chydoridae (Harishkumar and Kiran, 2016). *Moina* was the most tolerant of wastewater.

The sampled daphnia were identified as *D. pulex* (by specialists from the Department of Hydrobiology, Warsaw University, Poland). According to an internal expertise performed in 2003 for WWTP Swarzewo, stabilization ponds had the highest amount of *D. longispora* (majority) and *D. magna* (minority). While these species might have lived in tested tanks, *D. pulex* constituted the majority of the representatives of daphnia among the samples in this study. The daphnia living in tested clarifiers could be implemented in bio-based wastewater treatment plants. In general, excessive numbers of daphnia are not beneficial in algae-based wastewater treatment plants - by feeding on algae, they can induce a reduction in algal biomass (Montemezzani et al., 2015). A solution to this is to keep the population of daphnia and other zooplankton representatives at a low, controlled level, allowing daphnia to clarify treated wastewater without losing algal biomass. Moreover, the removal of N and P by algae and the clarification of treated wastewater can be separated in space and time.

Cladocera can also be used to determine the effects of treatment methods on the toxicity of wastewater. For example, negligible toxic effects of treated effluents were observed on the *D. magna* and *T. platyurus*, whereas the secondary effluent was toxic to *D. pulex* (immobilization of daphnids reached up to 90 %) (Petala et al., 2006). Other reports have indicated that *D. magna* and *D. pulex* exhibit similar sensitivity to wastewater and xenobiotics (Cooman et al., 2003; Versteeg et al., 1997), and, according to some reference tests, they can be used alternatively (Siciliano et al., 2004).

An effective concentration of DCF in acute toxicity tests toward *D. magna* mortality/immobilization ($\text{EC}_{50-48\text{h}}$) was tested and ranged from 22.4 to 123.3 mg L^{-1} (reports presented in Table S3, Supplementary Information). The acute toxicity test applied a relatively high concentration to observe the mortality/immobilization as the toxic effect. In the experimental studies with much lower concentrations, NSAIDs provoked various responses in daphnia species (Parolini, 2020). For example, IBF at 4 $\mu\text{g L}^{-1}$ caused morphological deformation during chronic exposure to *D. magna* but had no significant effect on the molecular markers or the life history parameters (Grzesiuk et al., 2020). However, it caused lethal morphological deformities in the embryos and juvenile daphnids (lack of eyes, antennae deformation). The molecular response of daphnia to DCF in $\mu\text{g L}^{-1}$ demonstrated as changes in the enzymes connected to oxidative stress (Oliveira et al., 2015), reduced filtration, and food intake (Nkoom et al., 2019), as well as changes in gene expression (Liu et al., 2017). Currently, there are no reports related to the biological biomarkers of 4OH-DCF on *D. magna*. However, one report described the acute toxic effect of DCF compared to 4OH-DCF on various organisms (*D. magna*, *Vibrio fischeri*, *Lemna minor*, and *R. subcapitata*). The results showed a lower acute toxic effect of metabolites compared to native compounds (Grabarczyk et al., 2020). Furthermore, another report demonstrated the toxic effect of DCF and

Table 1

The concentration of target analytes in wastewater ($n = 2$), daphnia ($n = 2$), and particle matter sampled ($n = 1$) on two dates in 2021 in tested WWTP (nd – not detected, d.m. – dry mass).

Analyte	28th July 2021			4th August 2021	
	Wastewater [$\mu\text{g L}^{-1}$]	Daphnia [$\mu\text{g kg}^{-1}$ d.m.]	Particles [$\mu\text{g kg}^{-1}$ d.m.]	Wastewater [$\mu\text{g L}^{-1}$]	Daphnia [$\mu\text{g kg}^{-1}$ d.m.]
DCF	1.309 ± 0.493	483 ± 38	223 ± 27	1.126 ± 0.074	236 ± 30
4OH-DCF	1.288 ± 0.032	nd	nd	1.263 ± 0.185	nd
IBF	0.113 ± 0.028	nd	nd	0.027 ± 0.020	nd
2OH-IBF	0.474 ± 0.017	nd	nd	0.194 ± 0.000	nd
NPX	0.174 ± 0.005	nd	nd	0.166 ± 0.000	nd

4OH-DCF on mussels (Świacka et al., 2019).

The chronic impact of the real environmental concentrations of DCF on several generations of daphnia showed a discreet effect on physiology, morphology, biochemistry, and reproduction. $0.36 \mu\text{g L}^{-1}$ of DCF was applied by Dietrich et al. (2010) to determine the effect between the various generations of daphnia. Detectable effects caused by the pharmaceuticals occurred in the first generation, followed by an acclimation period. The same effect was observed for carbamazepine, metoprolol, and ethinyl-estradiol. At a steady state, none of the tested drugs influenced the reproduction process. The study detected the same resistance to DCF between generations, while daphnia was noted in the tested WWTP over several years. However, it was not possible to examine the biochemical and morphological impact of the chronic exposure to NSAIDs, because no negative control was present. The risk quotients (RQs) calculated using the standard procedure ($\text{RQ} = \text{EC}/\text{PNEC}$, where EC – the concentration found in the environment, PNEC - Predicted No-Effect Concentration), based on mean DCF concentrations found in our work, are presented in Section S1 (SI). When PNEC $8.72 \mu\text{g L}^{-1}$ (Drzymała and Kalka, 2020) for *D. magna* was applied, the values of RQs were between 0.13 and 0.38, indicating a medium risk for daphnias (according to the guidelines of the European Commission, 2003). The application of LOEC (Lowest Observed Effects Concentration) $2.96 \mu\text{g L}^{-1}$ (Russo et al., 2023) for *D. dubia* gave higher RQ values, indicating a high risk for daphnia. Nevertheless, the tested daphnia were capable of surviving and reproducing.

The situation in the tested clarifiers mirrors the performance of chronic exposure in sub-lethal concentration, providing insights regarding the detoxification process. *D. magna* has shown the possibility of detoxifying substances such as IBF and paracetamol during prolonged exposure (Dave and Herger, 2012). Detoxification-related genes in *D. magna* were inhibited or enhanced depending on the DCF concentration and exposure time, but reproduction was the most reliable marker of determining the effect of DCF (Liu et al., 2017). In our study, chronic exposure to low concentrations of DCF could cause permanent changes in the tested daphnia population, but the absence of a negative control makes it impossible to determine biomarkers.

The low bioaccumulation of NSAIDs in the daphnia was reflected by the low biomagnification in the food web. It was previously reported that the lack of biomagnification of DCF and IBF within the phytoplankton, zooplankton, zoobenthos, and fishes was found in Lake Taihu in China (Liu et al., 2015), as well as in Arctic food webs (Sokołowski et al., 2024). In our study, we tested the main metabolite of DCF to examine the detoxification process, but 4OH-DCF was absent in the daphnia at a concentration higher than the detection limit.

The ingestion of suspected particles represents a likely source of DCF for daphnia. It is important to note that pharmaceuticals, as ionic compounds, often carry a charge at the prevailing pH of wastewater (Stevens-Garmon et al., 2011). Positively charged compounds have been reported to exhibit the highest sorption potential. The tested NSAIDs exist as anions under wastewater pH conditions, which presumably lowers their sorption potential. The sorption into suspended matter can be obtained due to ionic or non-polar forces, and this process contributes to their removal by WWTP technology. According to the literature, the removal of diclofenac by activated sludge was estimated to be $<50\%$ when the concentration of DCF was $50 \mu\text{g L}^{-1}$. Furthermore, this efficiency decreased as the initial concentration of diclofenac increased (Nguyen et al., 2019). It has been determined that DCF is mainly removed by adsorption and, to a larger extent, by biodegradation (Aminot et al., 2018; Nguyen et al., 2019), which occurs through co-metabolic mechanisms. The work of Elshikh et al., (2022) confirmed the adsorption of DCF onto sewage sludge, with efficiency increasing as the initial concentration of DCF increases. Specifically, after 50 and 60 min, the adsorption efficiency reached as high as 98 %. It was also confirmed that acidic pH favored DCF adsorption processes (Elshikh et al., 2022; Yan et al., 2019). The efficiency of DCF and other NSAIDs' removal from wastewater by activated sludge may also depend on its

composition. The sorption of diclofenac was defined as second-order sorption, meaning it depends on the concentration of diclofenac in the solution as well as the number of active sites on the adsorbent (Ighalo and Adeniyi, 2020). In our work, the concentration of DCF in the suspected particles was $223 \mu\text{g kg}^{-1}$ d.m. (or 223 ng g^{-1} d.m.), which is much less than any adsorption capacity values of carbon-based adsorbents (7.3 mg g^{-1} for nanotubes to $>1000 \text{ mg g}^{-1}$ for activated carbon (Ighalo and Adeniyi, 2020)). The sorption capacity for DCF into suspended particles from wastewater was between 162 and $28,990 \mu\text{g g}^{-1}$ d.m. in one of the laboratory studies (Yan et al., 2019), which means that the concentration found in our work is rather low. Notably, the concentration of NSAIDs in solids from wastewater is not thoroughly reported in literature.

There is no data on NSAIDs' bioaccumulation in daphnia under environmental conditions, but an experimental study has been conducted (Nkoom et al., 2019). The bioconcentration factor (BCF) of DCF was determined after 48 h of aqueous exposure in *D. magna*, and was 70.94 and 8.02 (wet mass) for the nominal exposure concentrations of 5 and $100 \mu\text{g L}^{-1}$, respectively. In our work, BAF was between 18 and 36 (wet mass) after exposure to approx. $1 \mu\text{g L}^{-1}$. This suggested that the bioaccumulation of DCF depended on the exposed concentration, which was previously reported (Schwaiger et al., 2004). Another difference between our study and the cited work was the examination of BAF (the ratio of the contaminant in an organism to its concentration in the environment's steady state, where the organism can take in the contaminant through ingestion with its food as well as through direct contact). In contrast, the experimental work only focused on BCF (no dietary intake of contaminant). Still, DCF was not bioaccumulative according to official guidelines. However, the standard description of the bioaccumulation process was based on the behavior of non-ionic stable compounds, where pharmaceuticals could bio-transform, reducing their concentration in biota within a given period. The bioaccumulation of pharmaceutical metabolites remains a significant gap in understanding pharmaceutical ecotoxicity (Maculewicz et al., 2022), and requires a novel approach to the bioaccumulation process.

5. Conclusions

DCF and its hydroxy metabolite were consistently detected in the treated wastewater of the tested WWTP, while other target NSAIDs were less frequently observed. DCF was adsorbed in the suspended particles, which were a food source for daphnia. Among all compounds found in the tested water, only DCF was detected in daphnia tissues. Nevertheless, the DCF bioaccumulation factor was low, indicating the lack of bioaccumulation potential according to official guidelines. Our determined BAF factor ($36 \text{ L kg}_{\text{ww}}^{-1}$, $\log\text{BAF}$ 1.56) was lower compared to those reported in laboratory conditions. This was due to daphnia in the clarifiers being exposed to DCF over multiple generations, whereas under experimental conditions, the organisms were exposed for a specific period and life stage. The low bioaccumulation of DCF in their tissues indicates that the tested daphnia adapted to the conditions present in the clarifiers. Interestingly, the main metabolite, 4OH-DCF, was not detected in the daphnia tissues. The tested *D. pulex* can be implemented in natural wastewater plants for clarification from suspended particles. It is possible that other species of zooplankton can adapt to sub-lethal doses of pharmaceuticals upon exposure over multiple generations. Therefore, ecotoxicology studies should be supplemented by the analysis of bioaccumulation by native species to demonstrate their potential for adaptation in polluted environments.

CRedit authorship contribution statement

Katarzyna Bethke: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Klaudia Kwidzińska:** Writing – original draft, Investigation. **Magda Caban:** Writing – review & editing, Writing – original draft, Supervision, Methodology,

Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Magda Caban reports financial support was provided by National Science Centre Poland. If there are other authors, they 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2024.174915>.

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Supplementary Information
to
**Investigation of pharmaceutical bioaccumulation in *Daphnia* sp. Living
in a wastewater treatment plant**

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Section S1. The risk assessment by risk quotients calculation



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Figure S3. The solid material during the lyophilization step

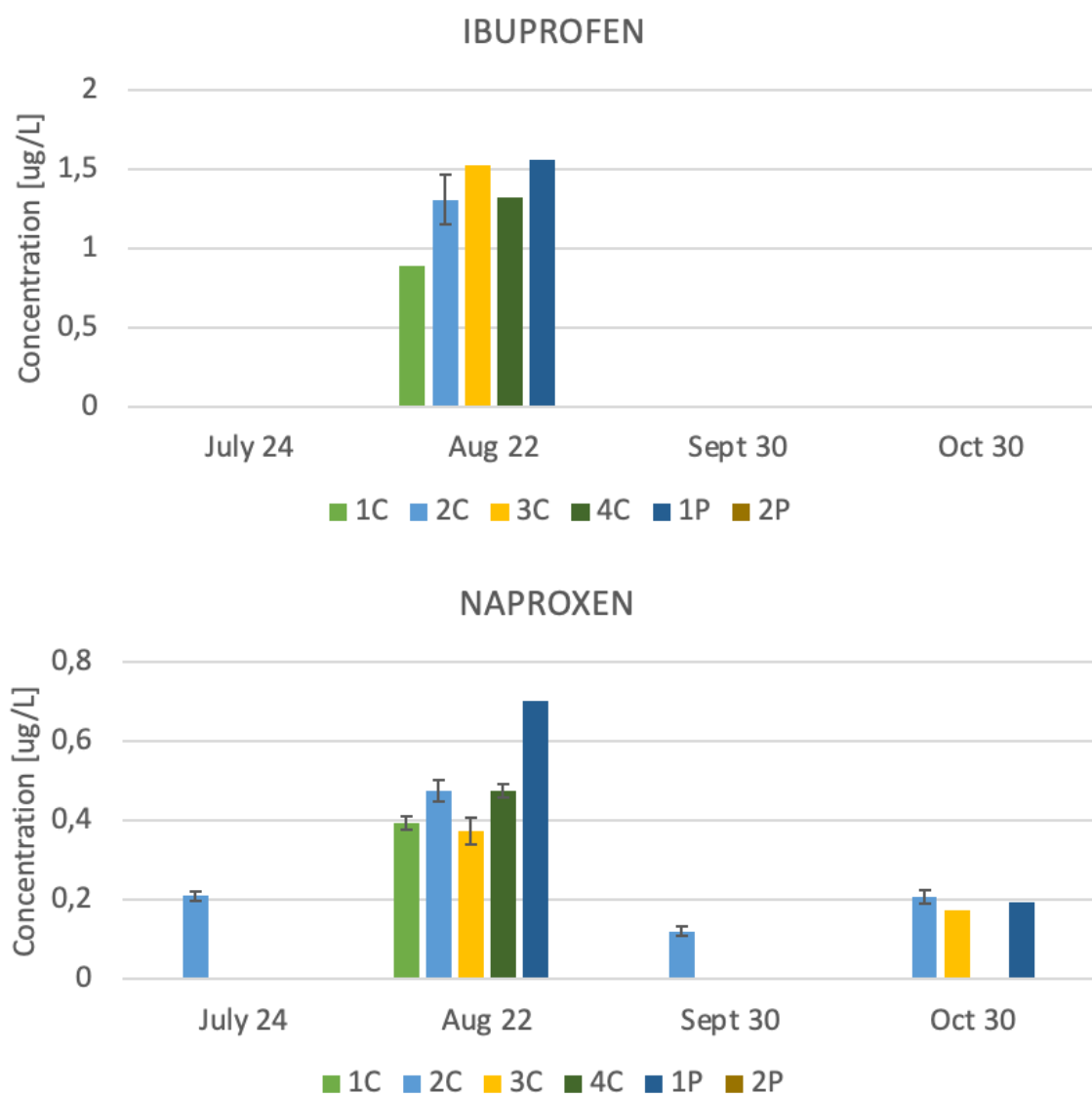


Figure S4. The concentrations of ibuprofen and naproxen determined in clarifiers (C1 to 4C) and ponds (1P and 2P) determined in 2019 in WWTP Swarzewo (North Poland)

Table S1. The temperature, oxygen content and conductivity of samples of treated wastewater and surface water from ponds sampled in four dates of 2019 (nt – not tested)

Parameter	Sampling side					
	1C	2C	3C	4C	1P	2P
July 24						
pH	6.62	6.61	6.61	6.58	6.8	7.28
mg O ₂ /L	2.8	1.8	2.1	3.1	2.9	3.9
Conductivity [mS]	6.8 (4.3 PSU)	7.2 (4.6 PSU)	7.5 (4.8 PSU)	6.7 (4.3 PSU)	5.5 (3.5 PSU)	7.0 (4.5 PSU)
Temperature	20.1°C					
August 22						
pH	6.72	6.66	6.73	6.55	6.8	6.9
mg O ₂ /L	2.9	3.4	4.0	3.9	5.2	7.0
Conductivity [mS]	nt	nt	nt	nt	nt	nt
Temperature	23.0 °C					
September 30						
pH	6.90	6.77	6.88	6.84	6.94	7.04
mg O ₂ /L	5.1	5.5	5.4	4.7	5.6	5.5
Conductivity [mS]	15.7 (10.0 PSU)	15.3 (9.8 PSU)	15.6 (10.0 PSU)	16.3 (10.4 PSU)	19.5 (12.5 PSU)	14.8 (9.5 PSU)
Temperature	15.8-16.8 °C					
October 30						
pH	7.04	7.07	7.05	7.07	7.00	7.41
mg O ₂ /L	5.7	2.7	4.2	3.0	4.1	5.4
Conductivity [mS]	12.0 (7.7 PSU)	9.3 (6.0 PSU)	14.27 (9.1 PSU)	12.55 (8.0 PSU)	16.33 (10.4 PSU)	16.78 (10.7 PSU)
Temperature	13.0°C					

Table S2. The parameters of validated instrumental calibration curves

Analyte Retention time [min]	Quantitative MRM	Reference MRM	Ratio of qualitative to reference MRMs [%]	ESI mode	Instrumental detection limit and instrumental quantification limit [ng/mL]	Precision [RSD, %]	Accuracy [%]	Recovery of SPE [%] Method quantification limit [ng/L]	Recovery of SLE+SPE [%] Method quantification limit [ng/g d.m.]
DCF 4.68	296 > 214	296 > 250	37	+	10 31	2.4-6.6	95.2-112.2	98.4-105.1 63 ng/L	61.8-85.6 36 ng/g
4-OH DCF 3.90	313 > 231	313 > 232	86	+	10 31	2.2-18.5	91.4-122.8	107.6-111.1 68 ng/L	62.3-111.4 28 ng/g
2-OH IBP 3.31	221 > 177	-	-	-	10 31	5.1-11.4	85.7-124.8	104.9-108.9 66 ng/L	55.6-109.1 28 ng/g
NPX 4.24	229 > 169	229 > 1185	22	-	10 31	0.4-26.0	91.4-119.1	111.0-125.3 73 ng/L	63.1-177.4 18 ng/g
IBF 4.97	205 > 162	-	-	-	10 31	2.0-13.0	91.9-114.7	97.4-102.5 62 ng/L	126.8-162.5 19 ng/g
M-DCF 5.90	310 > 214	310 > 215	72	+	10 31	2.5-12.0	90.7-114.5	29.5-31.0 200 ng/L	35.4-41.5 76 ng/g
CFA 3.67	213 > 127	-	-	-	10 31	1.2-20.1	84.5-128.7	168.5-179.1 35 ng/L	33.3-61.1 51 ng/g

Table S3. Ecotoxicity parameters by reference test for diclofenac toxicity toward *Daphnia sp.*

Species of crustacea	Endpoint value [mg/L]	Ref.
<i>D. magna</i>	EC ₅₀ 67.0 (48, mortality)	(Quinn et al., 2011)
<i>D. magna</i>	EC ₅₀ 39.9 (48, mortality)	(Haap et al., 2008)
<i>D. magna</i>	EC ₅₀ 22.4 (48, mortality)	(Ferrari et al., 2004)
<i>D. magna</i>	EC ₅₀ 68 (48, mortality)	(Cleuvers and Cleu, 2003)
<i>D. magna</i>	EC ₅₀ 80.1 (48, mortality)	(Han et al., 2006)
<i>D. magna</i>	EC ₅₀ 123.3 (48, mortality)	(De Oliveira et al., 2016)
<i>D. magna</i>	NOEC 0.36 (reproduction)	(Dietrich et al., 2010)
<i>D. dubia</i>	LC ₅₀ 12.71 (48, mortality)	(Russo et al., 2023)
<i>Ceriodaphnia dubia</i>	NOEC 1 (7 days, reproduction) PNEC 580 ug/L	(Ferrari et al., 2003)
<i>D. magna</i>	Lowest observed adverse effect level (LOAEL) 9.7 mg/L (48 h	(Gómez-Oliván et al., 2014)

Table S4. The molecular indicators of diclofenac (DCF) effect on *D. magna*

Test duration (concentration tested)	Molecular response	Reference
<i>D. magna</i> 21 day (50 µg L ⁻¹)	Induction of vitellogenin after 96 h exposure, for higher concentrations of change in gene expression after a shorter time, delay of egg release, but not their number, small effect on body length and growth rate	(Liu et al., 2017)
<i>D. magna</i> 48 hours	LOEC 30 mg L ⁻¹ using hsp70 level as a biomarker for proteotoxicity	(Haap et al., 2008)
<i>D. dubia</i>	Genotoxicity LOEC 2.96 µg L ⁻¹	(Russo et al., 2023)
<i>D. magna</i> 15 days (0.0005, 0.5 and 7.2 mg L ⁻¹)	Short term increase in AChE activity, followed by a decrease in this biomarker's activity to control levels by the end of an exposure period of 15 days, induction of oxidative stress in environmental relevant concentration	(Oliveira et al., 2015)
<i>D. magna</i> 48 hours bioconcentration test (5 and 100 µg L ⁻¹)	Decrease in filtration and food intake, oxidative stress, increase in glutathione reductase (detoxification process), but not glutathione s-transferase	(Nkoom et al., 2019)
<i>D. magna</i> 21 days test (2 to 486 mg L ⁻¹)	A significant decrease in the number of eggs and the frequency of their production in females, size reduction, when with mg L ⁻¹ of DCF	(Du et al., 2016)
<i>D. magna</i> 96 h	oxidative biomarkers were evaluated: lipid peroxidation; protein carbonyl content; activity of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase DNA damage observed after exposure to DCF	(Gómez-Oliván et al., 2014)

S1. The risk assessment by risk quotients calculation

The risk assessment was performed by calculation of risk quotients (RQs) using the following equation $RQ = EC/PNEC$, where EC – the concentration found in the environment, PNEC - Predicted No-Effect Concentration of LOEC – lowest observed effect concentration (European Commission, 2003). The risk was estimated using two water organism – *Daphnia magna* and *Daphnia dubia*. We have not found PNEC for *Daphnia pulex*, which was sampled in target WWTP. PNEC 8.72 ug/L (assessment factor 1000) (Drzymała and Kalka, 2020), while LOEC *D. dubia* 2.96 ug/L (Russo et al., 2023).

According to guidelines for risk assessment (European Commission, 2003) following criteria were applied: $0.01 \leq RQ < 0.1$ “low risk”, $0.1 \leq RQ < 1$ “medium risk”, $RQ > 1$ “high risk”.

PNEC of DCF for water organism was 0.05 ug/L was presented in report Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List EQS. Using this PNEC, the $RQ > 1$ obtained for all samples. Nevertheless, the water in clarifiers and ponds are treated as environmental waters. Moreover, this PNEC was estimated for single, most sensitive for DCF organism.

Table S5. Risk quotients values for DCF calculated using PNEC 8.72 ug L⁻¹ (Drzymała and Kalka, 2020) for *D. magna* and mean found concentration of DCF in tested tanks in various dates of sampling (nt – not tested)

Tank	July 24, 2019	Aug 22, 2019	Sept 30, 2019	Oct 30, 2019	July 28, 2021	August 4, 2021
1C	0.20	0.17	0.33	0.35	0.15	0.13
2C	0.13	0.20	0.38	0.36		
3C	0.09	0.18	0.29	0.35		
4C	0.19	0.20	0.29	0.30		
1P	0.17	0.20	0.29	0.36	nt	nt
2P	0.22	-	0.26	0.35	nt	nt

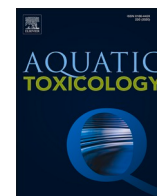
Table S6. Risk quotients values for DCF calculated using LOEC 2.96 ug L⁻¹ (Russo et al., 2023) for *D. dubia* and mean found concentration of DCF in tested tanks in various dates of sampling (nt – not tested)

Tank	July 24, 2019	Aug 22, 2019	Sept 30, 2019	Oct 30, 2019	July 28, 2021	August 4, 2021
1C	0.59	0.49	0.98	1.03	0.45	0.39
2C	0.38	0.60	1.12	1.08		
3C	0.25	0.53	0.86	1.02		
4C	0.56	0.58	0.85	0.89		
1P	0.49	0.59	0.84	1.05	nt	nt
2P	0.65	-	0.77	1.02	nt	nt

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Effect of acidification on the chronic toxicity of diclofenac to *Daphnia magna*

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ABSTRACT

Pharmaceuticals, as ionizable compounds, are a challenging group of pollutants to analyze because the pH of the environment can alter its ecotoxicological features. However, changes in the toxicity of pharmaceuticals toward aquatic organisms were observed even when there was no change in the ionization of the molecules with a pH shift. Therefore, we conducted a study that aimed to check how pH influences the chronic toxicity of diclofenac (DCF, $pK_a \approx 4.0$) toward *Daphnia magna* at two pH levels, 7.0 and 8.7, where DCF is obtained as relatively polar anion. The performance of the experiment with OECD 211 was found to be challenging because of the medium pH shift during the exposure test. Acidification increased the toxicity of DCF, reducing the number of neonates (at a concentration of 1.3 mg l^{-1}) and showing a tendency towards delayed hatching and reduced number of hatchings. In addition, the acidification itself also changes *D. magna* reproduction, affecting the number of hatchings and the first day of hatching. We conclude that the pH, as a factor of toxicity modulation for ionizable compounds, needs to be evaluated and presented in scientific protocols.

1. Introduction

Pharmaceuticals and their metabolites are one of the environmental threats with an unknown holistic effect on the environment. Despite being detected at trace and ultra-trace levels of $\mu\text{g l}^{-1}$ and ng l^{-1} (Bethke et al., 2024; Boxall et al., 2012; Nikolaou et al., 2007; Świacka et al., 2021) they can be hazardous to non-target aquatic organisms (Almeida et al., 2015; Nkoom et al., 2022). It has been documented that the ecotoxicity of selected pollutants can be altered by the effects of climate change (Lovett, n.d.). For example, persistent organic pollutants (POPs) uptake, metabolism, and toxicity are found to enhance with increasing temperature (Borgå et al., 2022; Noyes et al., 2009). However, the prediction of the change in ecotoxicity for most of the new emerging pollutants is challenging. The perfect example is pharmaceuticals, as confirmed by our latest literature review (Bethke et al., 2023). The main reason is this group's wide variety of physical and chemical properties. For ionizable compounds, such as pharmaceuticals, the change in water's pH affects the ionic forms' distribution, following lipophilicity (coefficient of distribution $\log D$), absorption, and toxicity. Generally, the nonionic form is better soluble in lipids and has a higher potential to penetrate organisms. Unfortunately, ecotoxicology tests are strict in terms of water pH and scarce data are obtained for the effect of

acidification on toxicity alteration. What is puzzling is that the change in toxicity was also observed for pharmaceuticals that have no potential to change in ionization form with a tested range of pH (Alsop and Wilson, 2019; Nieto et al., 2016). The probable reason is the change in the organism itself, mainly the structure of membranes and the shift in homeostasis.

One of the pharmaceuticals that does not change the ionic form in the pH of natural waters is diclofenac (DCF), a representative of non-steroidal anti-inflammatory drugs (NSAIDs). Despite the absence of this change, the scientific reports denote an increase in its toxicity toward bivalves (Costa et al., 2019; Munari et al., 2019; 2018) and fishes such as *Argyrosomus regius*, *Dicentrarchus labrax* (Maulvault et al., 2019; 2018) with acidification. The lowering of pH from 7.0 to 5.7 changes dramatically the toxicity of DCF toward *D. rerio* (Alsop and Wilson, 2019), despite only a small (2 %) fraction of the neutral form of DCF at pH 5.7. Unfortunately, no research was devoted to studying the effects on small organisms, such as zooplankton. Some papers report on the effects of DCF on *Daphnia magna* reproduction. Still, to date, to the best of our knowledge, there have been no tests investigating the effect of acidification. DCF is a pharmaceutical under special HELCOM supervision (HELCOM indicator report, 2018), being detected in surface waters at ng l^{-1} - $\mu\text{g l}^{-1}$ levels, making it a real threat to aquatic organisms

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(Sathishkumar et al., 2020). This chemical is also stable in natural waters and has a low removal rate in wastewater treatment plants - its presence in treated wastewater was also confirmed in our recent work (Bethke et al., 2024). *D. magna* is not only cosmopolitan crustacean with high environmental significance, but also versatile model system in ecotoxicology, ecology and evolution (Abdullahi et al., 2022; Ebert, 2022; Rajewicz et al., 2023). Furthermore, lowered pH may negatively affect the organism of *Daphnia* itself (Alibone and Fair, 1981; Weber and Pirow, 2009) making it even more susceptible to pollutants, including DCF.

Therefore, in the present work, we decided to evaluate whether the pH of the growth medium alters the chronic toxicity of DCF against *D. magna*. The testable hypothesis is that the effect of DCF on *D. magna* reproduction is enhanced with water acidification (as observed for fish and mussels). Another aim of the study is to check if such a study can be performed using the reference protocol OECD 211.

2. Materials and methods

2.1. Ecotoxicity tests performance

The chronic toxicity test (21 days long) was conducted according to OECD 211 guidelines. The *Daphnia* for the test were obtained from Tigret Ltd. *Daphnia* were hatched from ephippia according to OECD 202, for the chronic test young female *Daphnia* <24 h old, and that were not the first brood progeny were used. The culture was maintained in M4 medium under conditions corresponding to the test conditions (both pH 7.0 and 8.7). The OECD allows for *Daphnia* cultivation in pH from 6 to 9. Our tests therefore fall within the pH levels: tests were conducted at pH 8.7 (natural pH of M4 medium) and pH 7.0 (acidification). The acidification was performed by the addition of 1 % HCl aq. solution. The medium was aerated with air before transferring the daphnids to the test vessels. Chemicals used during the experiment are detailed in the Supplementary Information I (Section S1, SI).

The mothers of the test organisms were acclimated to acidification by breeding under pH 7.0 for one week. Neonates that were <24 h old were used for the test. In addition, they were not neonates from the first brood. Six replicates of each DCF concentration were performed and at the same time, a biotic control (*Daphnia* in M4 medium not exposed to DCF) was conducted in ten replicates. The volume of solutions in individual glass vessels was 50 mL with one organism per vessel. Five concentrations of DCF were selected for the test: 0.6, 1.3, 2.5, 5.0, and 10.0 mg l⁻¹. These concentrations were chosen based on NOEC parameters found in the literature for *D. magna* (Han et al., 2006; Lee et al., 2011), as well as based on previously conducted DCF acute toxicity tests against *D. magna* (unpublished data, Table S4). According to the OECD, the highest tested concentration should be high enough that the reproductive performance of *Daphnia* is significantly lower than that of the control. In addition, the highest tested concentration in the chronic test proposed by the OECD is 10 mg l⁻¹. In our test, this assumption was met. The test medium was exchanged about three times a week (corresponding to OECD 211). The specific days of test medium replacement are noted in the table in the raw data. The neonates were counted daily, and the pH, conductivity, and oxygen content of the test solutions were measured during the test (before and after changing the solutions). Exposure was conducted in an incubator (temperature 21 ± 1 °C) under day/night 16/8 lighting conditions with a light intensity of 1500 lx. *Daphnia* was fed daily with the green algae *Chlorella vulgaris* from our own culture at a rate of 1 mg organic C/*Daphnia*/day according to OECD 211. To calculate the organic carbon content of algal cells, TOC analyses were performed, and calibration curves were prepared showing (i) the number of algal cells per microliter (cells µL⁻¹) as a function of absorbance (Fig. S1, SI-I) and (ii) the dependence of organic carbon concentration in algal cells on absorbance (Fig. S2, SI-I). The stability of DCF in the test batch was confirmed using high-performance liquid chromatography (HPLC) with a diode-array detector (DAD) method (presented

in Section S2, SI-I). Mean values and standard deviations were calculated using Microsoft Excel, while other statistical analyses were performed using Statistica 13.1 software. All statistical calculations were carried out in accordance with OECD 211 guidelines. Due to the absence of a normal distribution in some study groups (Shapiro-Wilk test), non-parametric tests were conducted:

- Mann-Whitney U test – to assess the overall effect of pH on the number of alive neonates, number of hatchings, neonates per hatching, and the first day of hatching (comparing pH 8.7 vs. 7.0);
- Kruskal-Wallis test – to evaluate the effect of diclofenac on the aforementioned parameters at both pH levels.

Additionally, visualizations were created (Fig. S3, SI-I) using scatter plots to illustrate the relationship between the parameters and diclofenac concentration at pH 7.0 and 8.7. Non-parametric post-hoc analyses were performed using the Mann-Whitney U test (Supplementary Information II - SI-II):

- Statistical significance was assessed between all tested diclofenac concentrations, as well as between diclofenac concentrations and the control group, at a given pH (8.7 or 7.0), with Holm's correction applied.
- Statistical significance was compared between the same diclofenac concentrations and between the control groups across different pH levels.

3. Results

The validity of the test was confirmed by two factors required in the OECD protocol: parental *Daphnia* mortality not exceeding 20 %, and the average number of live neonates produced per parental *Daphnia* at each concentration was higher than sixty neonates. The raw data from the experiment were prepared in the form of a table (Raw Data, excel file) as proposed by the OECD. The hardness of the water was within the limits set by the OECD (required > 140 mg L⁻¹ CaCO₃): 203 mg L⁻¹ CaCO₃ at pH 7.0, and 211 mg L⁻¹ CaCO₃ at pH 8.7. Conductivity and dissolved oxygen content were also measured during the test, as described in Section S3, SI-I and the exact values are listed in Table S2, SI-I.

Examination of the pH of the test solutions during the test was essential to prove that in one set of experiments, the growth medium had a reduced pH. The exact values of pH can be found in Table S2, SI-I. The growth medium in test vessels was changed about three times a week. The mean pH measurements during the test under standard conditions for a DCF concentration of 10 mg l⁻¹ were 8.70 in the freshly prepared medium and 8.60 in the test vessel before replacement. The reduction of pH (acidification) was performed using an HCl solution addition to obtain a pH of 7.0 in the test medium. Unfortunately, after three days in the vessel, the pH shifted to 8.90. These were the effects of the buffering properties of the M4 growth medium (mainly carbonates) and air-derived CO₂ dissolution in the medium. The reduction of carbonate concentration in the medium was not possible because of the mentioned OECD requirements for test water parameters.

The main endpoint of OECD 211 is the number of neonates per mother *Daphnia*. Fig. 1A presents the values of these parameters for our test. The mean number of neonates at pH 8.7 in the control was 185 (110–215), at 0.6 mg l⁻¹ DCF was 172 (158–200), at 1.3 mg l⁻¹ DCF was 210 (175–231), at 2.5 mg l⁻¹ DCF was 190 (157–244), at 5.0 mg l⁻¹ DCF was 169 (149–187), at 10.0 mg l⁻¹ DCF was 128 (77–151). Accordingly, at pH 7.0 – it was 173 (154–191), 185 (169–222), 157 (122–184), 194 (171–231), 148 (119–165), 113 (96–122). In another test (Lee et al., 2011), the number of neonates per parental *Daphnia* was below 100 in the biotic control. The number of neonates in the biotic control in the paper by De Oliveira et al. (2016) was close to 140. Thus, it can be stated that in our study, both in the biotic control and exposed vessels, the

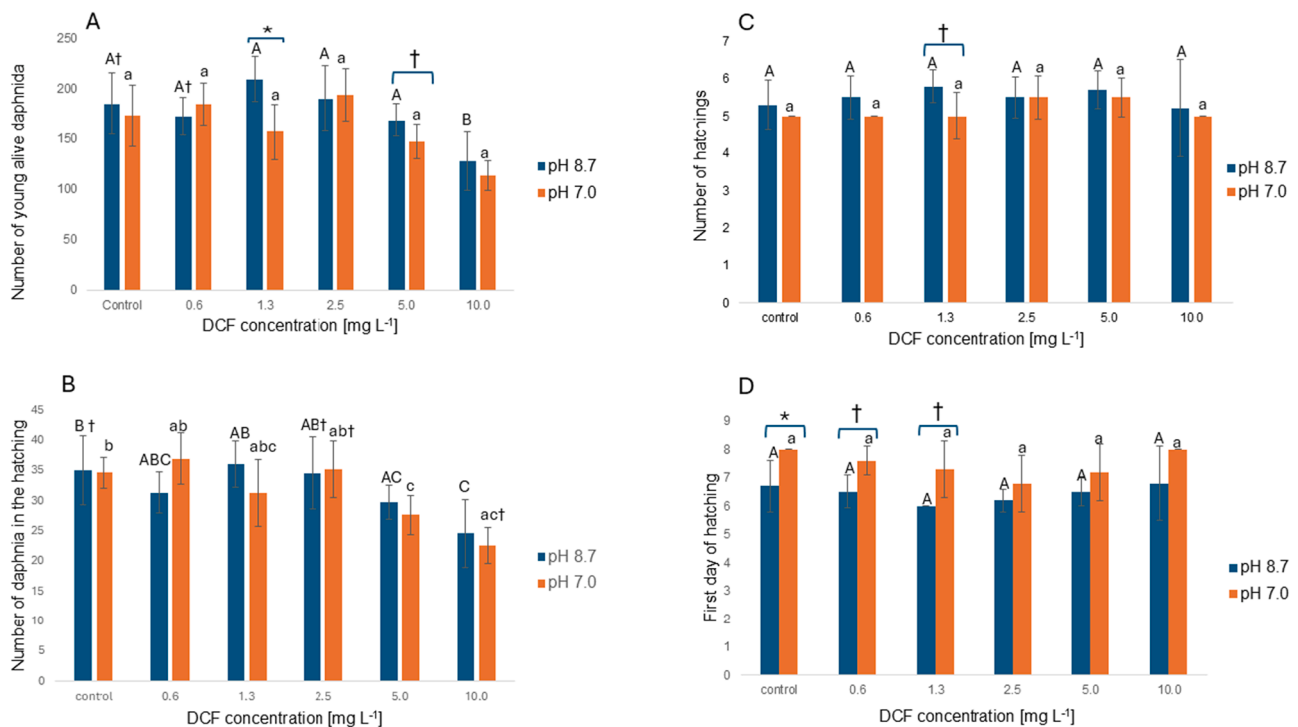


Fig. 1. The mean number of young alive daphnia neonates born during the test under standard conditions (pH 8.7) and under reduced pH conditions (pH 7.0) depending on the concentration of DCF (A); the number of daphnia neonates per hatchings (B); the mean number of hatchings per 1 parental daphnia (C); first day of hatching of daphnia (D); statistical differences ($p < 0.05$) compared between the concentration and control are marked with different uppercase letters (standard conditions) and different lowercase letters (reduced pH). Differences between concentrations under different pH conditions are marked with an asterisk; † = results near significance threshold ($p > 0.05$ but < 0.10).

number of neonates was at an elevated level.

The study showed a significant effect of DCF concentration on the number of *D. magna* hatched at both tested pH values (Kruskal-Wallis's test pH 8.7: $H(5) = 17.31$, $p = 0.004$; pH 7.0: $H(5) = 15.28$, $p = 0.009$). Despite the lack of statistical significance for pH influence ($p = 0.089$), graphical analysis (Fig. S3A, SI-I) revealed distinct pH-dependent responses to DCF: at pH 8.7, the number of neonates decreased markedly with increasing DCF concentration, while at pH 7.0 the declining trend was more moderate. Post-hoc analysis showed statistically significant differences between 10 mg l⁻¹ DCF and lower concentrations (1.3, 2.5, and 5.0 mg l⁻¹; $p < 0.05$), with marginally significant differences between 10 mg l⁻¹ and both 0.6 mg l⁻¹ and control ($p \approx 0.05$). These experimental results established the NOEC (No Observed Effect Concentration) as 5 mg l⁻¹ at pH 8.7. At pH 7.0, a non-monotonic response was observed, characterized by a transient increase in offspring at 2.5 mg l⁻¹ compared to control, flanked by stimulatory effects at 0.6 mg l⁻¹ and toxicity at ≥ 5.0 mg l⁻¹. Statistical analysis revealed that while the Mann-Whitney U test initially showed statistically significant differences between control and both 5.0 and 10 mg l⁻¹ DCF at pH 7.0 ($p = 0.025$ and 0.033), application of the Holm correction (which protects against overinterpretation but may mask biologically relevant effects) rendered these differences non-significant. This conservative statistical adjustment, while reducing test power, is necessary to maintain control over multiple comparison errors. Combined with the observed non-monotonic response (transient stimulation at 2.5 mg l⁻¹), this precluded reliable determination of NOEC at pH 7.0.

The marginal pH effect ($p = 0.089$) may suggest biological relevance, which is supported by: a statistically significant difference between pH 8.7 and 7.0 at 1.3 mg l⁻¹ DCF ($p = 0.028$), and a near-significant difference between pH conditions at 5.0 mg l⁻¹ DCF ($p = 0.066$). The presence of single dead neonates was observed during the test (Table S3, SI-I), however, no differences or correlations were noted between the pH values tested (a total of 12 at pH 8.7 and 10 at pH 7.0). It was noted that

the dead neonates were observed after day twelve of the 21-day exposure. Information on the percentage survival of parent daphnids during the test is also included in Table S3, SI-I. The data presented in Fig. 1B demonstrates the mean number of neonates per hatching across tested DCF concentrations at pH 8.7 and 7.0. Statistical analysis revealed no overall effect of pH (Mann-Whitney U test, $p = 0.723$), consistent with the absence of significant differences between corresponding DCF concentrations at both pH levels. However, diclofenac concentration significantly affected neonate numbers at each pH (Kruskal-Wallis test: $H(5) = 16.98$, $p = 0.005$ for pH 8.7; $H(5) = 17.16$, $p = 0.004$ for pH 7.0). At pH 8.7, post-hoc tests identified statistically significant differences between 1.3 and 10 mg l⁻¹ DCF, along with marginally significant effects between control and 5 mg l⁻¹ ($p = 0.061$), control and 10 mg l⁻¹ ($p = 0.051$), and 2.5 and 10 mg l⁻¹ ($p = 0.088$). The concentration-response curve exhibited a progressive decline in neonates with increasing DCF levels (Fig. S3B, SI-I). In contrast, pH 7.0 showed significant reductions in neonates between control and 5.0 mg l⁻¹, as well as between 0.6 and 5.0 mg l⁻¹. Marginally significant differences occurred between control and 10 mg l⁻¹ ($p > 0.050$) and between 2.5 and 5.0 mg l⁻¹ ($p = 0.053$). For pH 7.0, a steeper slope of the curve was observed (Fig. S3B, SI-I), suggesting a potential non-monotonic relationship between DCF concentration and *Daphnia* hatchling abundance. The toxic effect became pronounced only at higher concentrations, possibly indicating pH-dependent toxicity modulation. DCF also reduced neonates in the hatchings in the Lee et al. (2011) study (0.95–25 mg l⁻¹ DCF). However, the number of neonates per hatching was higher in our study - in biotic control, at pH 8.7 it was thirty-five neonates, while in the Lee et al. paper, it exceeded twenty.

Fig. 1C shows the mean number of hatchings per one parent *Daphnia* - a significant effect of pH on *Daphnia* hatching abundance was observed (Mann-Whitney U test, $p = 0.009$). In contrast, the Kruskal-Wallis test did not reveal any statistically significant effect of DCF concentration on hatching numbers at either pH level ($H(5) = 2.68$, $p = 0.749$ for pH 8.7;

$H(5) = 7.03$, $p = 0.218$ for pH 7.0). Visualization of the *Daphnia* hatching abundance versus diclofenac (Fig. S3C, SI-I) concentration confirmed that DCF had no significant impact on hatching success. However, the curve for pH 7.0 showed different slope characteristics compared to pH 8.7, indicating a potential pH $\times$ DCF interaction. The near-significant difference in hatching numbers between pH 8.7 and 7.0 at 1.3 mg L^{-1} ($p = 0.068$) further supports the hypothesis of pH-modulated effects.

Fig. 1D shows the first day of *Daphnia* hatching. A significant overall effect of pH was demonstrated (Mann-Whitney U test, $p < 0.001$), corroborated by a statistically significant difference in hatching speed between pH 8.7 and 7.0 in the biotic control (0 mg L^{-1} DCF; $p = 0.003$). Kruskal-Wallis tests revealed no significant effect of DCF on hatching day at pH 8.7 ($H(5) = 5.26$, $p = 0.385$) or pH 7.0, though the latter approached significance ($H(5) = 9.98$, $p = 0.075$).

A potential pH $\times$ DCF correlation is visible in Fig. S3D, SI-I: curves for pH 8.7 and 7.0 exhibit divergent trends. At pH 7.0, hatching was consistently delayed across all DCF concentrations (stable curve), while pH 8.7 showed a slight increasing trend. This pattern, combined with near-significant post hoc results (e.g., 1.3 mg L^{-1} , $p = 0.068$), suggests pH-dependent modulation of DCF effects.

In the chronic toxicity test of DCF against *D. magna* conducted by Lee et al. (2011) (pH 7.2–7.6, according to OECD guideline 202 - which is likely an editorial error, as the correct test number is 211 for a 21-day test), the first day of hatching of *Daphnia* occurred later at each tested DCF concentrations ($0, 0.9, 2.8, 8.3, 25 \text{ mg L}^{-1}$) than in our study. Reproduction occurred later than the 12th day of the test and, as in our study, no statistically significant differences in the first day of hatching were observed between the tested DCF concentrations. In the work of (Liu et al. (2017), delayed hatching of *D. magna* was noted at concentrations as low as 0.05 mg L^{-1} .

4. Discussion

According to the literature, non-ionized pharmaceutical molecules have a higher affinity for the cellular membranes of organisms and therefore can become more toxic (Anskjær et al., 2013; Sun et al., 2020). In the case of carboxylic acids, such as DCF, a greater proportion of non-ionic molecules is present at acidic pH. As pH decreases, the neutral form of DCF becomes dominant (Lara-Pérez et al., 2020). Consequently, a decrease in pH results in an increase in the distribution coefficient ($\log D$) for DCF (Kroll et al., 2024). Considering the pK_a of DCF ($3.99\text{--}4.15$, PubChem database) and the experimental pH range of 7.02 to 8.86 (Table S2), DCF does not change its ionized form under the test conditions - it remains in the anionic form as a conjugated base. Nevertheless, there was a statistically significant increase in the toxicity of DCF toward *D. magna* reproduction under pH 7.0 compared to pH 8.7 at a concentration of 1.3 mg L^{-1} DCF (Fig. 1A). Since DCF did not change its ionized form under the test pH conditions, there must be another reason for the drug's increased toxicity to *D. magna*. It appears that under reduced pH conditions, sodium and calcium uptake in *Daphnia* is impaired - at pH 4, sodium influx was virtually entirely inhibited compared to pH 8. Shadi Khudr et al. (2020) confirmed improved *Daphnia* condition under acidified and saline conditions compared to acidified conditions alone, due to the presence of salts that compensated for the loss of sodium ions, which is a consequence of reduced pH. Thus, lowered pH may negatively affect the *Daphnia* itself, making it even more susceptible to pollutants, including DCF.

It should be noted that, along with DCF, sodium cations were also introduced into the medium. However, the added mass of Na^+ was negligible in comparison to the overall salinity of the M4 medium. The mean values of conductivity under standard conditions ranged from 700.0 to 723.0 mS m^{-1} in the control, and from 659.2 to 720.5 mS m^{-1} at a concentration of 10 mg L^{-1} of DCF (Table S2, SI). In general, *D. magna* is capable of adapting to elevated salinity levels, even up to several grams per liter (Parlato and Kopp, 2020).

Acidification influences various biochemical pathways in *Daphnia*. For instance, it can inhibit respiration frequency, which in natural aquatic environments results from alterations in the CO_2 diffusion gradient due to acidified water (Alibone and Fair, 1981). The distribution of CO_2 species in water - present as dissolved gas, bicarbonate, and carbonate - is strongly dependent on pH. At a pH below 6, about 60 % of total CO_2 is in its free gaseous form, whereas at pH 4, nearly 100 % is present as dissolved CO_2 . The observed reduction in respiratory rate at lower pH levels may be linked to impaired ion metabolism. A decrease in respiration limits ATP production, and a resulting energy deficit can disturb ion regulation mechanisms within cells.

The effects of acidification on *Daphnia* were also studied by Weber and Pirow (2009). In daphnids of the species *D. pulex*, chronic exposure to acidification (pH 6.0 ± 0.1) resulted in changes in bicarbonate concentration in hemolymph, CO_2 partial pressure, extracellular pH, and energy metabolism. Physiologically, an impairment in the carapace structure of *Daphnia* was noted. Compensatory changes in response to stress were observed: extracellular buffering capacity of non-carbonates, which are essential in maintaining a stable pH in the body fluids of *Daphnia*, thus striving to maintain homeostasis, and increased tolerance of organisms to acidification stress. This indicates the activation of defense mechanisms in *Daphnia*. Similarly, this can be speculated in the case of our experiment, where a statistically significant difference in the number of live neonates depending on pH conditions occurred only at a concentration of 1.3 mg L^{-1} of DCF (Fig. 1A), and this difference was not observed at higher concentrations of the pharmaceutical (note, however, that the difference approaches statistical significance at 5.0 mg L^{-1} ($p = 0.066$)). Thus, it is possible that the DCF concentration of about 1.3 mg L^{-1} was not high enough to activate defense mechanisms in the *Daphnia*'s organism, but exposure to higher concentrations of this pharmaceutical may have triggered detoxification mechanisms. The activation or inhibition of defense mechanisms in the presence of cellular stress has already been documented in larger organisms, such as *Mytilus galloprovincialis* (Coppola et al., 2018; Lopes et al., 2022). In another chronic toxicity test, it was documented that *D. magna*, in response to heat stress, can alter the activity of enzymes such as CAT (catalase), SOD (superoxide dismutase), and GPX (glutathione peroxidase) to defend against oxidative stress, thereby losing energy to produce neonates (Im et al., 2019).

In our study, there were no toxic effects of acidification (pH 7.0) on the number of neonates, compared to pH 8.7 conditions in the biotic control (Fig. 1A), unlike in the study by Parra et al. (2016). However, in our study, the effect of pH was noted on the first day of hatching (Fig. 1D). This change, while it may be the result of stress and an associated change in energy allocation, may ensure the survival of parent daphnids (Parra et al., 2016). Adult *Daphnia* have an energy budget that allows them to grow and, most energy-intensively, produce young organisms (Nikolai N. Smirnov 2014). Under stressful conditions, however, the production of neonates is severely limited. It was found that DCF reduces the feeding rate of *D. magna* at concentrations from 5 to $100 \text{ } \mu\text{g L}^{-1}$ (Nkoom et al., 2019). If such an effect occurred, a lower number of neonates would be observed in the chronic exposure test, but in the case of our test this was observed only at a very high DCF concentration compared to the aforementioned work (10 mg L^{-1} concentration was statistically different from all tested concentrations at pH 8.7, but the control and lowest DCF concentration values showed a difference at the limit of significance).

To the best of our knowledge, no work has been published to date on the effect of pH on the chronic toxicity of DCF and other pharmaceuticals against *D. magna* (Bethke et al., 2023). Reports on the chronic effects of DCF under acidified conditions were published for *Mytilus galloprovincialis* and *Ruditapes phillipinarium* (Munari et al., 2019; 2018). The mollusks are more susceptible to the negative effects of reduced pH than to the effects of DCF alone or the interaction of the two stressors. There have been papers that describe the chronic effects of DCF on *D. magna* under standard pH conditions (De Oliveira et al., 2016; Han

et al., 2006; Lee et al., 2011). The literature NOEC parameters for DCF under pH 7.8 ± 0.2 were 10 mg l^{-1} (Han et al., 2006) and 8.3 mg l^{-1} (Lee et al., 2011), similar to 5 mg l^{-1} determined in our study. Results of a chronic test conducted by De Oliveira et al. (2016) showed no significant effect of DCF on *D. magna* at any of the concentrations evaluated ($29.5 - 72 \text{ mg l}^{-1}$). The researchers found this drug to be the least harmful among those evaluated (acetaminophen, chlorpromazine, propranolol). The same study also included an acute toxicity test of DCF, in which the EC_{50} was as high as 123.3 mg l^{-1} , indicating the lack of toxicity of this pharmaceutical to *Daphnia*. It is worth noting that the acute test of DCF on *D. magna* shows low reproducibility between reports, with EC_{50} values ranging from 22.4 mg l^{-1} (Ferrari et al., 2004), 39.9 mg l^{-1} (Haap et al., 2008), 80.1 mg l^{-1} (Han et al., 2006) to 123.3 mg l^{-1} (De Oliveira et al., 2016). It remains the open question whether the pH of the growth medium was a factor influencing the variability in EC_{50} , as the pH at the start and end of the experiment is rarely reported.

The fact of inconstant, naturally fluctuating daily or seasonal environmental water pH needs to be discussed. These changes depend on various natural factors such as solar radiation (Usui and Kasubuchi, 2013), salinity, and biological processes such as photosynthesis and upwelling (Wootton et al., 2008; Wootton and Pfister, 2012). pH values in natural water bodies also depend on the biogeochemical composition of water, as noted by (Flecha et al., 2015). Global pH change is also exacerbated by melting glaciers (Comiso, J. 2010) and increased water circulation on land due to greenhouse gas emissions (Levang and Schmitt, 2015). Acidification can contribute to the disruption of the homeostasis of individual organisms and, consequently, to changes in the entire trophic chains. Organism can respond to normal pH shifts, but the extent of today's changes is too rapid for adaptation. A perfect example is coral reefs (Anthony et al., 2008; Li et al., 2023). The study presented here shows that *Daphnia* reproduction is maintained under a pH shift from 8.7 to 7.0, and organisms are still under optimal conditions, provided that mother *Daphnia* were acclimated to the change. Furthermore, the pH shift during the test also does not affect the total number of neonates in biotic controls. This additionally proves that *D. magna* has an adaptive mechanism to pH change occurring in natural aquatic environments. Indeed, in one of the reports, the tolerable pH range for *D. magna* was found to be greater than 4.55 and <10.13 , with the optimum condition 7.9 and 8.3 (El-Deeb Ghazy et al., 2011). Unfortunately, we found a pitfall in this study, because the authors adjusted the pH of the growth medium using NaOH and HCl, but did not report the final pH, which was likely different from the initial one. Generally, the viable pH range for zooplankton is between 6 and 9, where basic physiological processes continue (Locke and Sprules, 2000).

The possibility of assessing acidification effects in a chronic toxicity test should be considered. As presented, in the acidified medium, an increase in pH was noted. The ability to control pH while strictly following the OECD 211 protocol is limited. The guideline allows pH adjustment using only HCl and NaOH, but as noted, the carbonate-based buffering capacity of the growth medium does not allow for a stable reduction of pH throughout the test duration. In our study, we tried not to introduce salts that would alter the buffer capacity of the growth medium because of potential unknown effects on organism condition and overall test performance. Let's review the pH correction methods in another study. The most effective is adjusting the CO_2 stream. For example, such a method was applied by (Freitas et al., 2015) to achieve pH 7.0 in artificial seawater in high-volume containers with clams under an acute toxicity test. In the case of OECD 211, due to the low volume of a single test vessel (50 mL), pH regulation using a CO_2 stream is not feasible. Another method is the introduction of additional buffering substances. For example, MES (4-morpholineethanesulfonic acid, 9.2 mM) and TRIS (2-amino-2-(hydroxymethyl)-1,3-propanediol, 3.3 mM and 2 mM), and the correction of pH by HCl and NaOH to obtain pH values 6.0, 7.5 and 8.5 was applied by Anskjær et al. (2013) for studying acute toxicity of sulfadiazine for *D. magna*. Rendal (2011) excluded NaCO_3 and phosphates from the standard ISO 6341 media, and used 5

mM TRIS instead, to buffer pH to the relevant levels. The pH levels of 7 and 8 were stable at 0.4 units, whereas pH 9 was stable at 0.5 units, which is, in our opinion, quite unsatisfactory stabilization. Kim et al. (2010) exposed *Daphnia* to pH conditions of 7.4, 8.3, and 9.2 and tested ibuprofen, acetaminophen, lincomycin, ciprofloxacin, enrofloxacin, chlortetracycline, and sulfathiazole as toxicants. *Daphnia* were also exposed to 0.75, 1.5, or 3 mM of TRIS-HCl per 96 h. The TRIS increased the mortality of *Daphnia* in short exposure, especially at a pH of 8.3. This means that the TRIS-based growth medium impacts the organism itself. The 3 mM of TRIS-HCl corresponds to a concentration of 363 mg l^{-1} . The EC_{50} of TRIS-HCl for *Daphnia* is $>117 \text{ mg l}^{-1}$ according to the datasheet (Merck company). In another datasheet for TRIS (Horiba Medical), $\text{EC}_{50} >980 \text{ mg l}^{-1}$. It seems that the TRIS can impact *Daphnia* in the concentrations that are used in the mentioned reports with pH stabilization. This needs investigation in future studies, especially in chronic exposure. The OECD guidelines assume toxicity testing with M4 and M7 types of growth medium with quite a high pH range of 6 to 9. This means that in some ecotoxic studies, the effect of pH could be potentially obtained but was not considered. It was noted that there are studies that do not record the starting and final pH values, which is one of the requirements of OECD 211. Finally, it needs to be concluded that despite the efforts, our study shows that testing of acidification with the standard OECD 211 protocols is problematic. The recommendation would be an elaboration of the new growth medium composition to stabilize the pH at the starting level with no effect on *Daphnia* vitality.

5. Conclusions

The change in pH from 8.7 to 7.0 caused a change in *Daphnia* reproduction - it delayed the day of *Daphnia* hatching. Under the tested pH 7.0 and 8.7, DCF was in an anion form, thereby having the same features of lipophilicity and membrane penetration. Nevertheless, the possible changes in *Daphnia* organisms (such as impaired ion metabolism, impaired ATP function, or weakened structure of the *Daphnia* carapace, as literature reports) with acidification may have contributed to the increased permeability of DCF and lower potential for detoxification. DCF at pH 7.0, compared to pH 8.7, increased its toxicity to *Daphnia* by reducing the number of neonates at 1.3 mg l^{-1} and showed a trend toward accelerating the start of hatching at 0.6 and 1.3 mg l^{-1} . This confirms our hypothesis that the change in the ionization form of the drug is not needed for the increased ecotoxicity of the drug with water acidification because the acidification changes the homeostasis of the *Daphnia* organism, which can make them more susceptible to toxicants. We recommend being very strict with the reporting of the starting and ending pH of the ecotoxicity tests, because of the high possibility that this factor impacts the resulting data. It should be emphasized that testing the effect of pH according to the OECD reference method is difficult, indicating the need to look for buffers that are not toxic to the test organisms. Considering the emerging trends indicated by borderline statistically significant results, further studies are needed to investigate the interactive effects of DCF and pH on *D. magna* toxicity.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to improve grammar and logical sequence in the article. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Katarzyna Bethke: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Magda Caban:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis,

Conceptualization.

Declaration of competing interest

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

Magda Caban reports financial support was provided by National Science Centre Poland. If there are other authors, they 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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.aquatox.2025.107497](https://doi.org/10.1016/j.aquatox.2025.107497).

Data availability

Data will be made available on request.

[Effect of acidification on the chronic toxicity of diclofenac to *Daphnia magna* \(Original data\)](#) (Most Wiedzy)

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Supplementary Information I

to

Effect of acidification on the chronic toxicity of diclofenac to *Daphnia magna*

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Figure S3. Effects of DCF concentration and pH on *Daphnia magna* reproductive endpoints: (A) number of young live daphnia neonates, (B) number of hatchings per 1 parental daphnia (C) first day of hatching of daphnia (D) first day of hatching of daphnia. Data are shown for pH 8.7 (blue curve) and pH 7.0 (orange curve)

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Table S4. Results of the EC_{50} [$mg\ L^{-1}$] parameters for acute toxicity tests of diclofenac against *Daphnia magna* conducted on individual days under different medium pH conditions: 8.0 ± 0.2 and 7.0 ± 0.2

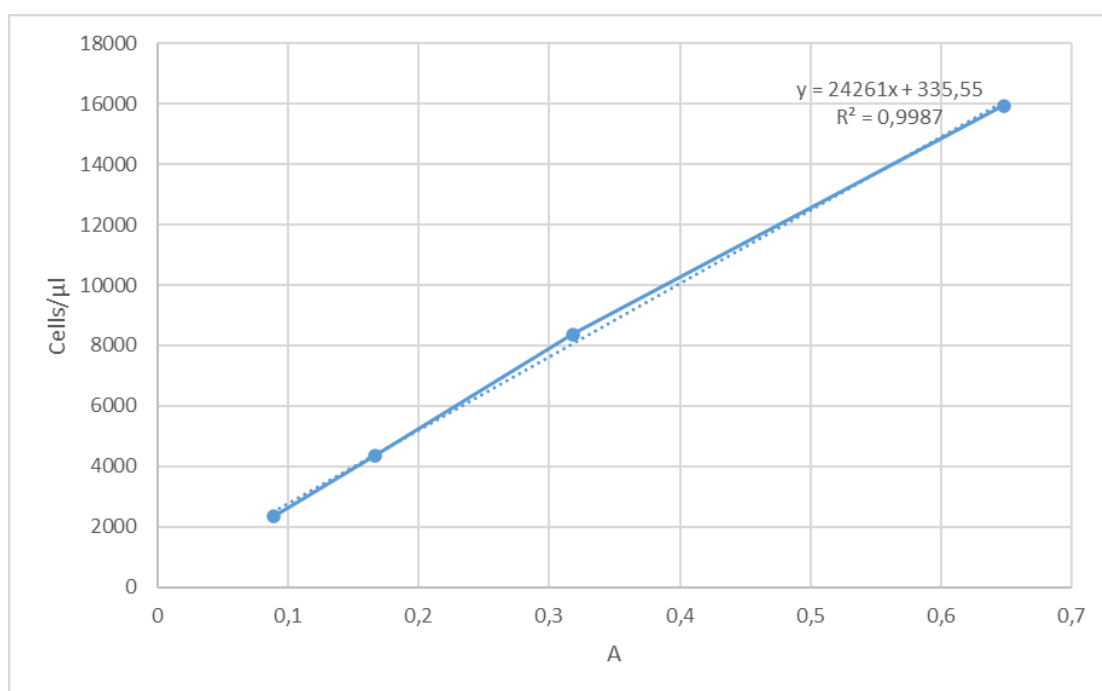


Figure S1. Dependence of the number of *Chlorella vulgaris* cells ($cell\ mL^{-1}$, counted by flow cytometry) on absorbance (680 nm) characteristic of chlorophyll

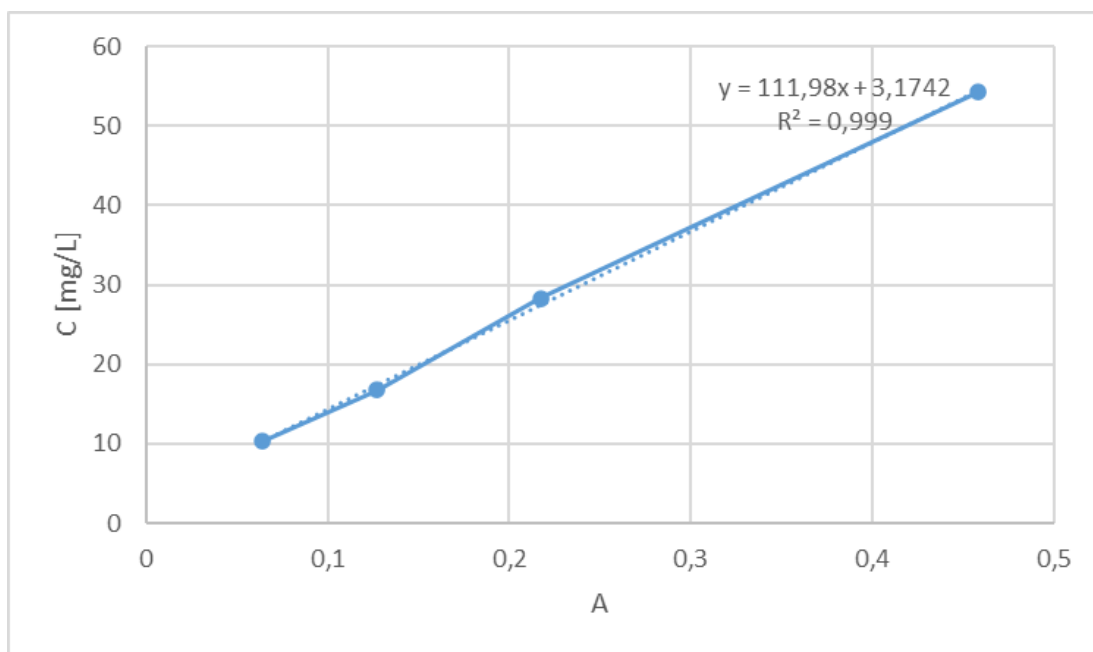


Figure S2. Dependence of organic carbon concentration [mg L⁻¹] on absorbance (680 nm) of green algae *Chlorella vulgaris* culture

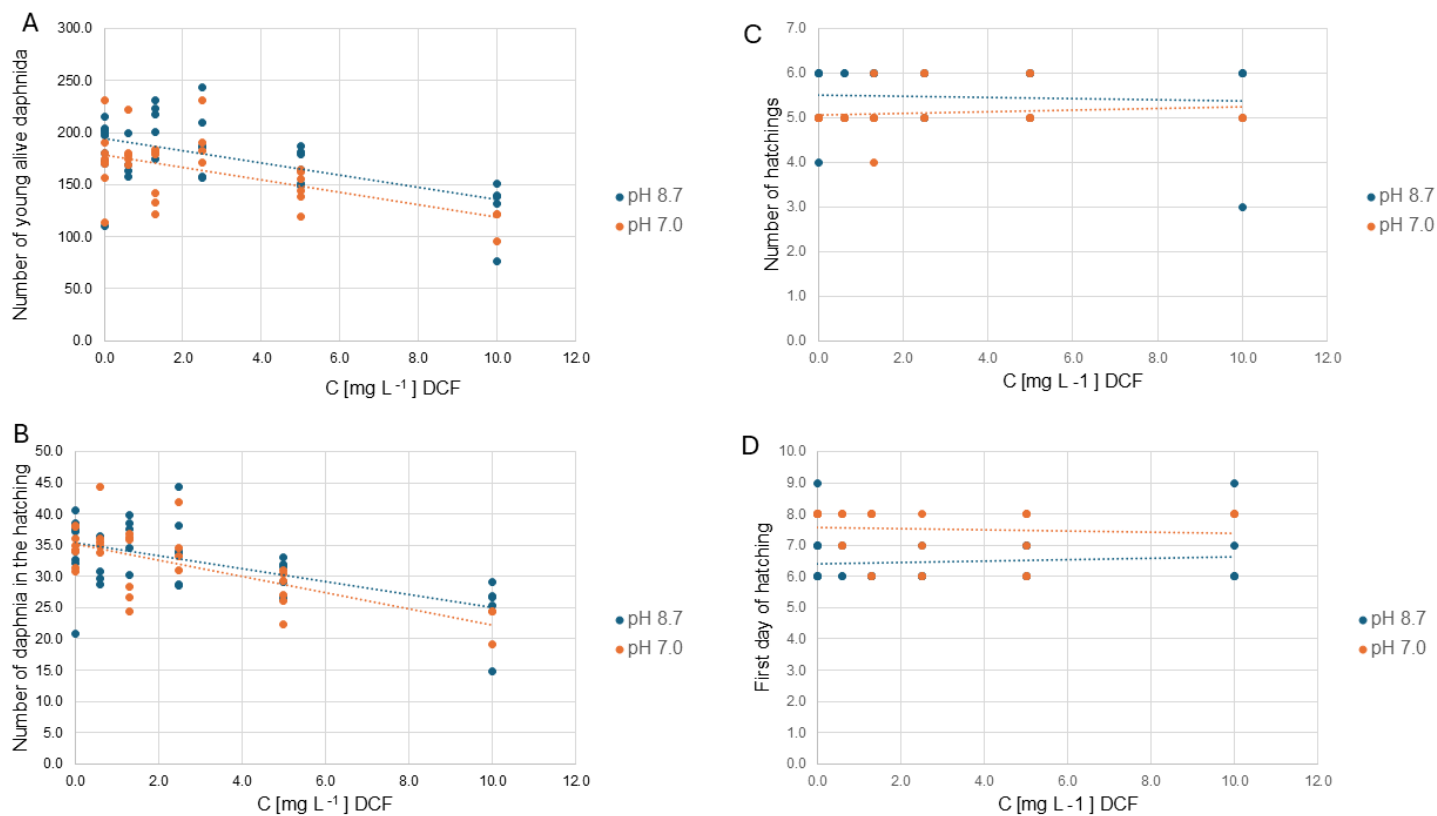


Figure S3. Effects of DCF concentration and pH on *Daphnia magna* reproductive endpoints: (A) number of young live daphnia neonates, (B) number of daphnia in the hatching, (C) number of hatchings, (D) first day of daphnia hatching. Data are shown for pH 8.7 (blue curve) and pH 7.0 (orange curve)

Section S1. Chemicals

The analytical standard of DCF sodium (DCF, CAS 15307-79-6) was purchased from Sigma-Aldrich (Germany). HPLC-grade acetonitrile (ACN) was purchased from POCH S.A. (Poland), and glacial acetic acid and ammonium acetate were purchased from VWR International (USA). HCl was purchased from Stanlab (Poland). Thiamine (Merck) and salts (POCH S.A.) were used to prepare the M4 medium, as described in OECD Guideline 211. Merck's Spectroquant (product number 1009610001) cuvette test was used to measure water hardness. The calibrants for the pH meter were purchased from Mettler Toledo.

Section S2. HPLC/DAD analysis and calculations

High-performance liquid chromatography (HPLC) with a diode-array detector (DAD) (Nexera XR, Shimadzu) was used as the DCF quantification technique, which made it possible to determine the actual concentrations assessed. The mobile phase water components were water buffered with ammonium acetate (5 mmol L⁻¹) and acetic acid (pH 4.1) with 5% CAN (phase B). The mobile phase organic component was ACN (HPLC grade) (phase A). Analysis occurred under isocratic conditions: 40% of phase B. The total analysis time was 6 min. The analytical wavelength for DCF was 272 nm, while the retention time was 3.2 min. The validation parameters of the method are presented in **Table 1**. Two calibration curves were made to maintain the best possible parameters of the method for a wide range of tested concentrations.

Section S3. Results of measurement of conductivity and dissolved oxygen content during the test

The mean conductivity values did not change drastically, under standard conditions varied from 700.0 - 723.0 mS m⁻¹ in the control, at a concentration of 10 mg L⁻¹ of DCF - 659.2 - 720.5 mS m⁻¹. Similarly, under the acidified condition: 800.0 - 773.0 mS m⁻¹, 707.0 - 744.0 mS m⁻¹. The mean values of dissolved oxygen were higher after several days of exposure: in the control under standard conditions, they ranged from 9.2 mg L⁻¹ on the day of replacement to 9.7 mg L⁻¹ before replacing the medium with a new one. At a concentration of 10 mg L⁻¹ DCF similarly: 8.7 - 9.8 mg L⁻¹. Under acidified conditions, a similar trend was noted: 8.9 - 10.1 mg L⁻¹ in control and 8.6 - 10.3 mg L⁻¹

Table S1. Validation parameters for HPLC/DAD method of diclofenac determination using two calibration curves for concentration ranges

Calibration curve number	Instrumental quantification limit MQL [$\mu\text{g mL}^{-1}$]	Instrumental detection limit MDL (MQL/3) [$\mu\text{g mL}^{-1}$]	Precision [RSD, %]	Accuracy [%]	Linearity range [$\mu\text{g mL}^{-1}$]	R ²
1	0.16	0.05	0.18-2.63	95.00-102.26	0.16-2.50	0.9999
2	-	-	0.06-0.88	90.24-102.64	1.25-20.00	0.9998

Table S2. Results of pH, conductivity, and dissolved oxygen measurements during the chronic test in fresh medium and medium before replacement for DCF concentration 10 mg L⁻¹ and control

Test conditions											
Standard						Acidification					
New, fresh medium			Medium before replacement			New, fresh medium			Medium before replacement		
pH	Conductivity [mS m ⁻¹]	Oxygen content [mg L ⁻¹]	pH	Conductivity [mS m ⁻¹]	Oxygen content [mg L ⁻¹]	pH	Conductivity [mS m ⁻¹]	Oxygen content [mg L ⁻¹]	pH	Conductivity [mS m ⁻¹]	Oxygen content [mg L ⁻¹]
10 mg L ⁻¹ diclofenac											
8.72	723	8.2	8.76	794	11.2	7.03	730	8.2	9.35	824	13.1
8.79	728	8.7	9.07	-	-	7.06	800	8.8	9.19	-	-
8.65	565	-	8.95	630	9.9	7.07	580	-	7.79	661	9.3
8.86	683	9.1	8.09	815	-	7.04	717	8.9	8.84	853	-
8.39	597	-	7.9	643	8.3	6.98	708	-	9.14	639	8.5
Mean value			Mean value			Mean value			Mean value		
8.68	659.2	8.7	8.55	720.5	9.8	7.04	707	8.6	8.86	744	10.3
Control											
8.73	722	8.4	8.95	745	11.4	7.04	744	8.2	9.09	761	11.8
8.65	767	8.9	8.92	-	-	6.98	799	8.1	8.44	-	-
8.68	-	-	8.69	688	11	7.07	-	-	7.89	789	8.8
8.86	743	10.3	7.8	806	-	6.99	771	10.4	8.84	916	-
8.65	569	-	7.59	653	6.6	7.0	889	-	7.51	629	9.6
Mean value			Mean value			Mean value			Mean value		
8.71	700	9.2	8.40	723	9.7	7.02	800	8.9	8.35	773	10.1

Table S3. The number of live neonates, death neonates and the number of parental daphnids that survived the entire test period - depending on diclofenac concentration and pH conditions

* For the calculations of newborn daphnids, parents were considered daphnids, who survived the whole test

	C [mg L ⁻¹]	Death, young daphnids	Lively, young daphnids	Parental daphnids that survived the whole test (%)
Standard conditions 21°C, pH 8.7	0	0.1 ± 0.3 (0-1)	185 ± 30.2 (110-215)	100
	0.6	1.8 ± 3.5 (0-7)	172.5 ± 18.9 (158-200)	80
	1.3	0.2 ± 0.4 (0-1)	209.6 ± 22.2 (175-231)	100
	2.5	0.3 ± 0.8 (0-2)	190.3 ± 33.0 (157-244)	100
	5.0	0.3 ± 0.9 (0-2)	168.7 ± 17.2 (149-187)	100
	10.0	0	127.8 ± 29.2 (77-132)	100
Acidification 21°C, pH 7.0	0	0.1 ± 0.3 (0-1)	173.2 ± 30.5 (114-231)	100
	0.6	0.6 ± 0.9 (0-2)	184.8 ± 21.2 (169-222)	100
	1.3	0	156.8 ± 27.6 (122-184)	100
	2.5	0.5 ± 1.0 (0-2)	193.8 ± 26.0 (171-231)	100
	5.0	0.8 ± 1.0 (0-2)	147.5 ± 17.2 (119-165)	100
	10.0	0.3 ± 0.6 (0-1)	113.3 ± 15.0 (96-122)	75

Table S4. Results of the EC_{50} [mg L⁻¹] parameters for acute toxicity tests of diclofenac against *Daphnia magna* conducted on individual days under different medium pH conditions: 8.0 ± 0.2 and 7.0 ± 0.2

Test dates	EC_{50} [mg L ⁻¹]	
	pH = 8.0 ± 0.2	pH = 7.0 ± 0.2
1. 12-14.03.2024	26.69	24.17
2. 19-21.03.2024	41.11	35.06
3. 09-11.04.2024	25.19	23.89



Exploring the impact of pH on the toxicological response of *Lemna minor* to pharmaceuticals—Insights from sulfamethoxazole exposure

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Abstract

Aquatic acidification driven by increasing atmospheric CO₂ is expected to modify the environmental fate and effects of ionizable pharmaceuticals; yet, pH-dependent toxicity is rarely addressed in standard ecotoxicological assessments. Over half of pharmaceuticals are ionizable within environmentally relevant pH ranges, meaning shifts in pH can affect their speciation, bioavailability, and ecotoxicity. Here, we investigated, for the first time, the influence of pH on sulfamethoxazole (SMX) toxicity toward the aquatic macrophyte *Lemna minor*, following Organisation for Economic Co-operation and Development (OECD) 221 guidelines. Experiments in Steinberg medium (pH 5.0–5.5) revealed increasing SMX toxicity with decreasing pH, consistent with a higher fraction of neutral SMX. Photolysis was a major degradation pathway, while interpretation of pH effects was complicated by pH increases during the test, associated with *L. minor* growth and medium alkalinization. Our findings demonstrate that the standard OECD Test Guideline 221 is not well-suited for assessing pH-dependent toxicity of ionizable pharmaceuticals without pH control. We highlight the need for buffering strategies and systematic pH monitoring in plant-based ecotoxicity tests, as accounting for pH is crucial for accurate environmental risk assessment of ionizable pharmaceuticals under ongoing aquatic acidification.

Keywords *Lemna minor* ecotoxicity, pH-dependent toxicity, sulfonamides ecotoxicity, climate change ecotoxicology

Introduction

Natural waters, especially oceans, are subjected to acidification as a result of anthropogenic increases in CO₂ levels in the atmosphere (Häder & Gao, 2019). Despite the known effects of acidification, such as impacts on shell-forming marine organisms and increased carbon fixation by phytoplankton (Doney et al., 2009), another effect urgently needs to be investigated—the change in the ecotoxicity of pollutants with pH shifts. This effect is observed especially for ionizable chemicals, whose absorption into organisms can be driven by their form (the non-ionic form is generally more lipophilic and bioavailable [Rendal et al., 2011]). More than 49% of compounds registered in REACH are ionizable in the pH range of 6–12 (Franco et al., 2010). For pharmaceuticals, more than 60% are ionizable within the pH 2–12 range. However, most natural freshwater systems typically fall within a narrower and ecologically more significant range with a pH of approximately 6.0–8.5 (Hem, 1985). Therefore, ecotoxicology assessment strategies need to include pH as an important modulating factor (Bethke et al., 2023; Boström & Berglund, 2015; Wilkinson et al., 2024), enabling identification of regions requiring special monitoring attention (Wilkinson et al., 2024).

Previously, the effect of pH on pharmaceuticals' toxicity to aquatic organisms was rarely investigated. The single reports presented changes (reduction or increase) in acute toxicity endpoints with water acidification. For example, the LC₅₀ (the concentration that causes 50% mortality in the test population) of fluoxetine (a basic pharmaceutical) for Japanese medaka fish was 5.5, 1.3, and 0.20 mg L⁻¹ at pH 7, 8, and 9, respectively (Nakamura et al., 2008). Similarly, the pH-dependent toxicity of basic pharmaceuticals in the green algae *Scenedesmus vacuolatus* was experimentally proven (Neuwoehner & Escher, 2011). Sertraline, another basic pharmaceutical, had average lethal concentration values toward *Pimephales promelas* of 647, 205, and 72 mg L⁻¹ at pH 6.5, 7.5, and 8.5, respectively (Valenti et al., 2009). The EC₅₀ of antibiotic sulfadiazine toward *Daphnia magna* was determined to be 27.2, 188, and 310 mg L⁻¹ at pH 6.0, 7.5, and 8.5, respectively, thus demonstrating a significant effect of acidification (Anskjær et al., 2013). The toxicity of weakly basic and acidic pharmaceuticals toward the crustacean *D. magna* changed in the pH range from 6 to 9 (Boström & Berglund, 2015). More examples can be found in a recent review (Bethke et al., 2023). Interestingly, none of the previous studies

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tested the effect of pH on the toxicity of pharmaceuticals toward *Lemna minor*. In this work, we decided to fill this gap.

In our recently published study, we found that the chronic toxicity of diclofenac, one of the most popularly used pharmaceuticals, toward zooplanktonic *D. magna* increased with acidification (from 8.7 to 7.1) (Bethke & Caban, 2025). This served as an impetus for the next study on pharmaceuticals, which are frequently found in the environment. The sulfonamides are antibiotics with high usage, low removal in wastewater treatment plants, and high frequency of detection in waters (Białk-Bielińska et al., 2012; Felis et al., 2024), capable of altering microbial communities (Kergoat et al., 2021) and mobile enough to reach groundwater (Jurado et al., 2020; Maszkowska et al., 2013). Sulfamethoxazole (SMX) exhibits high toxicity toward *L. minor*, with reported EC50 values ranging from 0.21 to 3.67 mg L⁻¹ (Aubakirova et al., 2017; Białk-Bielińska et al., 2018), affecting enzymes such as dihydropteroate synthase (DHPS), inhibiting folic acid synthesis, and reducing root growth and development (Brain et al., 2008; Cheong et al., 2020; Ma et al., 2025). Sulfonamides, as a group of chemical compounds, are diverse, and each may exhibit different mechanisms of toxic action associated with distinct transport mechanisms across the cell membranes of organisms (Białk-Bielińska et al., 2018). The transformation products of sulfonamides also show toxic effects toward plants (Białk-Bielińska et al., 2017). For this reason, we decided to select SMX as a representative target among the sulfonamides.

Standard ecotoxicity guidelines were not developed with ionizable chemicals in scope (Rendal et al., 2011, 2012). The Organisation for Economic Co-operation and Development (OECD) 221 protocol for *L. minor* does not regulate pH, only recommending measurement at the start and end of the test. According to OECD (2006): “Careful consideration should be given to the inclusion of a pH buffer in the test medium (4-morpholinepropane sulfonic acid [MOPS, CAS No: 1132-61-2] in *L. minor* medium and NaHCO₃ in *Lemna gibba* medium) when it is suspected that it might react with the test substance and influence the expression of its toxicity.” We therefore tested SMX toxicity without buffer addition, investigating pH stability in Steinberg medium and potential reasons for pH changes. Although the Steinberg medium typically used in this test has an initial pH of 5.5, this value is primarily due to the need to standardize and ensure the stability of the medium, rather than an attempt to reflect environmental conditions. While this initial pH is lower than what is typically observed in natural freshwater systems, it falls within the tolerance range of *L. minor*, which can thrive in slightly acidic environments (Landolt, 1986; Ullah et al., 2023). Importantly, during the experiment, *L. minor* can raise the pH of the culture medium to neutral or slightly alkaline levels, reflecting conditions more typical of natural freshwater ecosystems. The aim of this study was not to replicate real-world environmental conditions, but rather to identify trends in pH-dependent toxicity and to assess the sensitivity of toxic responses to pH changes under standardized OECD conditions. This preliminary study explores OECD 221 for pH-dependent toxicity, hypothesizing that exposure to SMX at different pH levels can alter toxicological results due to its amphoteric nature within environmental pH ranges.

Materials and methods

Chemicals

The analytical standard of SMX (CAS 723-46-6) was purchased from Sigma Aldrich (Germany). The following chemicals were obtained from various suppliers: 3,5-dichlorophenol (CAS 591-35-5) from Sigma-Aldrich (Germany), high-performance liquid chromatography (HPLC)-grade acetonitrile (ACN) and methanol from POCH S.A. (Poland), glacial acetic acid and ammonium acetate from VWR International (United States), and HCl from Stanlab (Poland). Salts (from POCH S.A.) were used to prepare the Steinberg medium, as described in OECD Guideline 221. pH buffer solutions used for calibration were purchased from Mettler Toledo.

Ecotoxicity tests performance

Acute toxicity tests were performed according to the OECD 221 guidelines (*Lemna* sp. Growth Inhibition Test) with a test duration of 7 days. The Steinberg growth medium's native pH was 5.5 (Steinberg, 1946). In order to confirm the validity of the test, an acute toxicity test was conducted on 3,5-dichlorophenol, a reference substance according to OECD 221. Tests were conducted under the standard pH of Steinberg medium (5.5) and under reduced pH conditions (5.0). To acidify the medium, 1% aqueous HCl was used. The pH was measured at the start and end of the tests. *Lemna minor* was cultured in Erlenmeyer flasks in autoclaved Steinberg medium. Culture was carried out in a phytotron at 25 °C, under 6,000 lx illumination and 60% humidity. Duckweed was acclimatized to the test conditions (for both standard and pH-reduced conditions) for 3 weeks, with a weekly transfer of three leaves to fresh medium under a laminar flow cabinet. One week before the test, eight healthy leaves were transferred to fresh medium. No differences in plant condition or growth were observed between cultures maintained at different pH levels. The test was carried out in six-well plastic plates (sterile six-well NEST plates, Genoplast; 10 ml of solution in each well). The parameter EC50, defined as the SMX concentration at which 50% growth inhibition of *L. minor* occurs, was calculated as the endpoint of the test. To determine the EC50 parameter, six concentrations were selected for the test: 0.31, 0.62, 1.25, 2.50, 5.00, and 10.00 mg L⁻¹. Concentrations were selected based on preliminary tests and literature (Aubakirova et al., 2017; Białk-Bielińska et al., 2011; Diogo et al., 2024; Grabarczyk et al., 2020). Sulfamethoxazole was dissolved directly in the growth medium, without additional solvents. The test was performed in duplicate for each tested pH, together with two biotic controls (duckweed without SMX) for each pH level. One healthy three-frond plant was transferred into each well using a sterilized inoculating loop. The tests were performed in three independent replicates, conducted at different times. The test was performed in static mode, without renewal of the test solutions, for 7 days. An abiotic control in the light (Steinberg medium with SMX and without duckweed, under the same conditions as the test plates) and an abiotic control in the dark (Steinberg medium with SMX under the same conditions as the test plates, but without access to light) were also performed (two replicates, SMX concentration of 20 mg L⁻¹). A photograph of the test plate

was taken using the software WinDIAS 3 on the first and last day of the test to calculate the growth inhibition of *L. minor*. Leaf area was calculated by including chlorotic leaves. Representative photographs of test plates under control conditions and during exposure to SMX at different pH values are presented in Figure S1 (see online supplementary material). Growth inhibition was determined according to OECD 221 by comparing the area of *L. minor* fronds exposed to SMX with the control fronds. The inhibition result was reported as the average specific growth rate (**Raw Data**, Excel file, available at <https://doi.org/10.71853/04g2-5280>). The growth rate was also calculated for biotic controls under both pH conditions. The pH in each well was measured at the end of the test. The SMX concentration was monitored using HPLC with a diode-array detector (DAD) on the first and last day of the test. The EC50 values were estimated using two methods, as recommended by OECD 221: Probit regression (GLM with a probit link) and a non-linear regression using the four-parameter log-logistic model (LL4), with all dose-response analyses conducted in the R environment (version 4.3.2; R Core Team, 2023). Statistical comparisons between tests conducted under two different pH conditions were performed using the Mann–Whitney *U* test in Statistica 13.1 without continuity correction.

The proportion of the undissociated form (HA) and the negatively charged form of SMX was calculated at the experimental pH values (5.5 and 5.0) using the fractional distribution equation (Henderson–Hasselbalch equation):

$$\alpha = \frac{HA}{[HA] + [A^-]} = \frac{1}{1 + 10^{[pH - pK_{a2}]}} \quad (1)$$

where: α , molar fraction of the undissociated form (HA) of SMX; HA, neutral (undissociated) form of SMX; A^- , anionic (dissociated) form of SMX; pK_{a2} , acid dissociation constant (for SMX, $pK_{a2} = 5.7$).

HPLC-DAD analysis

The HPLC-DAD system (Nexera XR, Shimadzu) was used for the SMX quantification with a Gemini NX (C18, 5 μ m, 110 Å, 150 $\times$ 4.6 mm, Phenomenex) column. The mobile Phase A component was water buffered with ammonium acetate (5 mmol L⁻¹) and acetic acid (pH 4.0) and contained 5% ACN. The mobile Phase B component was ACN (HPLC grade). Analysis was performed under isocratic conditions (75% Phase B). The total analysis time was 8 min. The analytical wavelength for SMX was 275 nm, while the retention time was 4.54 min. The standard SMX solution was prepared in methanol. The validation parameters of the method are presented in Table S1 (see online supplementary material). The blank was pure Steinberg medium.

Results

Validity of the test according to OECD 221

The validation test conducted with the reference substance 3,5-dichlorophenol and the standard pH of Steinberg medium (standard pH, $n = 4$, EC50 calculated by non-linear regression, LL4, in

R) yielded an EC50 (frond area) value of 2.99 (2.70–3.36) mg L⁻¹. The interlaboratory comparison EU Waste Ring Test 2006/2007 for 3,5-dichlorophenol in *L. minor* growth inhibition tests shows a range of EC50 values from 1.8 to 3.6 mg L⁻¹, with both frond area and frond number as endpoints (Collins et al., 2009). Thus, the EC50 determined in our study falls within the acceptable range. According to the International Organization for Standardization 20079, the EC50 of 3,5-dichlorophenol for *L. minor*, based on the frond number, is reported as 3.51 mg L⁻¹ (Park et al., 2021). Since our study used the frond area, which has been shown to be a more sensitive growth parameter (Xie et al., 2018), the lower EC50 value obtained here can be considered reliable and consistent with literature findings.

It was also confirmed that the doubling time of the number of leaves in control was less than 2.5 days, as required by OECD 221 (the mean values were 1.91 days at pH 5.5 and 1.95 days at pH 5.0; **Raw Data**, Excel file, available at <https://doi.org/10.71853/04g2-5280>).

Changes in pH values

The abiotic control in both light and dark conditions showed a mean pH change of 0.12 units (range: 0.03–0.18) between the start and end of the test for both initial pH values (5.0 and 5.5; see online supplementary material, Table S2). Changes in pH were noted in growth media with *L. minor*, both with SMX solutions and in the biotic controls (Table 1). The lower the SMX concentration, the greater the pH difference between the last and the first day of the test. The highest differences were noted in biotic controls ($\Delta 2.49$). This is related to the growth of *L. minor*, and the higher its growth, the greater the pH difference.

Calculated EC50 values

Growth inhibition curves of *L. minor* exposed to SMX are shown in Figure 1A and B (probit regression with log-transformed SMX

Table 1 pH values of the growth medium on day 1 and after a 7-day exposure of *Lemna minor* to sulfamethoxazole (SMX) for two experimental sets ($n = 3$).

	SMX (mg L ⁻¹)	Start pH	Final pH	Δ pH
Test set—pH 5.5	10.00	5.55 $\pm$ 0.13	6.97 $\pm$ 0.09	1.42
	5.00	5.51 $\pm$ 0.08	7.02 $\pm$ 0.13	1.51
	2.50	5.48 $\pm$ 0.05	7.21 $\pm$ 0.18	1.73
	1.30	5.46 $\pm$ 0.03	7.39 $\pm$ 0.17	1.93
	0.60	5.45 $\pm$ 0.02	7.57 $\pm$ 0.13	2.12
	0.30	5.46 $\pm$ 0.02	7.66 $\pm$ 0.04	2.21
	Biotic control	5.45 $\pm$ 0.01	7.94 $\pm$ 0.05	2.49
Test set—pH 5.0	10.00	4.97 $\pm$ 0.01	6.84 $\pm$ 0.12	1.87
	5.00	4.97 $\pm$ 0.01	6.76 $\pm$ 0.07	1.79
	2.50	4.98 $\pm$ 0.01	6.89 $\pm$ 0.14	1.91
	1.30	4.97 $\pm$ 0.01	7.26 $\pm$ 0.28	2.29
	0.60	4.97 $\pm$ 0.02	7.51 $\pm$ 0.12	2.54
	0.30	4.96 $\pm$ 0.02	7.67 $\pm$ 0.10	2.71
	Biotic control	4.98 $\pm$ 0.01	7.86 $\pm$ 0.06	2.88

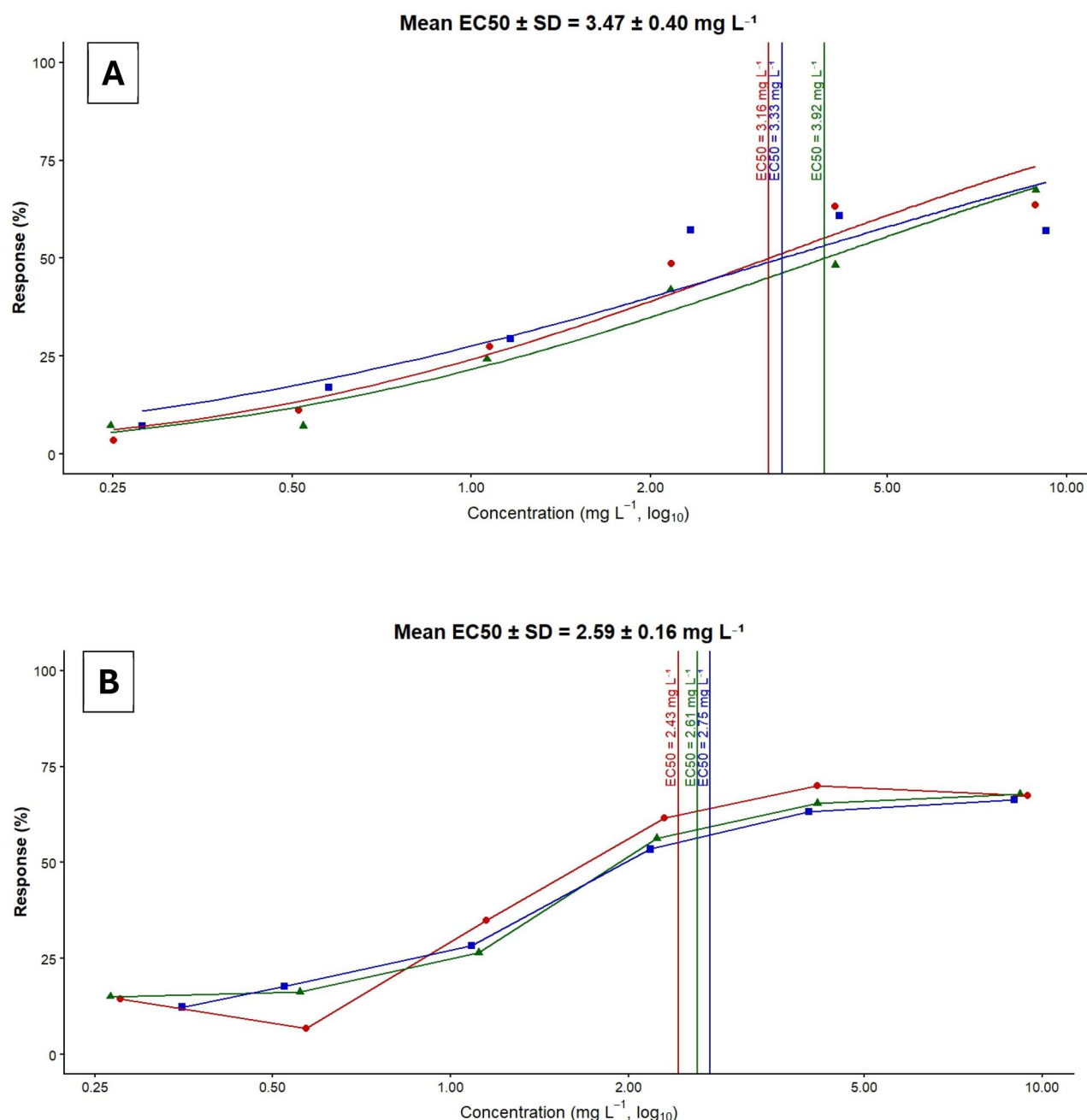


Figure 1 Growth inhibition curves of *Lemna minor* exposed to sulfamethoxazole (SMX), with EC₅₀ (concentration causing 50% growth inhibition) determined at pH 5.5 (A) and pH 5.0 (B) using probit regression with log-transformed SMX concentration ($n = 3$).

concentrations) and Figure 2A and B (non-linear regression). For each of the three tests performed under both pH conditions, mean EC₅₀ values were calculated from two technical replicates per two test repetitions. The mean EC₅₀ determined using the probit method was significantly higher at pH 5.5 than at pH 5.0 (3.47 ± 0.40 vs. 2.59 ± 0.16 mg L⁻¹; $p = .0495$). In contrast, the LL4 model did not reveal a significant difference between pH treatments (1.64 ± 0.59 vs. 1.43 ± 0.17 mg L⁻¹; $p = 0.827$; $p = 0.5637$ after removing a potential outlier, indicating that the result was robust to the exclusion of this point). To further illustrate the distribution of EC₅₀ values, a box-and-whisker plot was

generated (see online supplementary material, Figure S3A and B). In this plot, each box represents the interquartile range, which encompasses the middle 50% of the data. The horizontal line within the box indicates the median, the whiskers denote the range of non-outlier values, and the mean is marked with a cross. The EC₅₀ value was calculated using two approaches: Probit regression and the LL4 model (four-parameter logistic regression) to demonstrate potential differences depending on the analytical method. For comparison with literature data and to facilitate discussion, results obtained from the non-linear regression (LL4) were used.

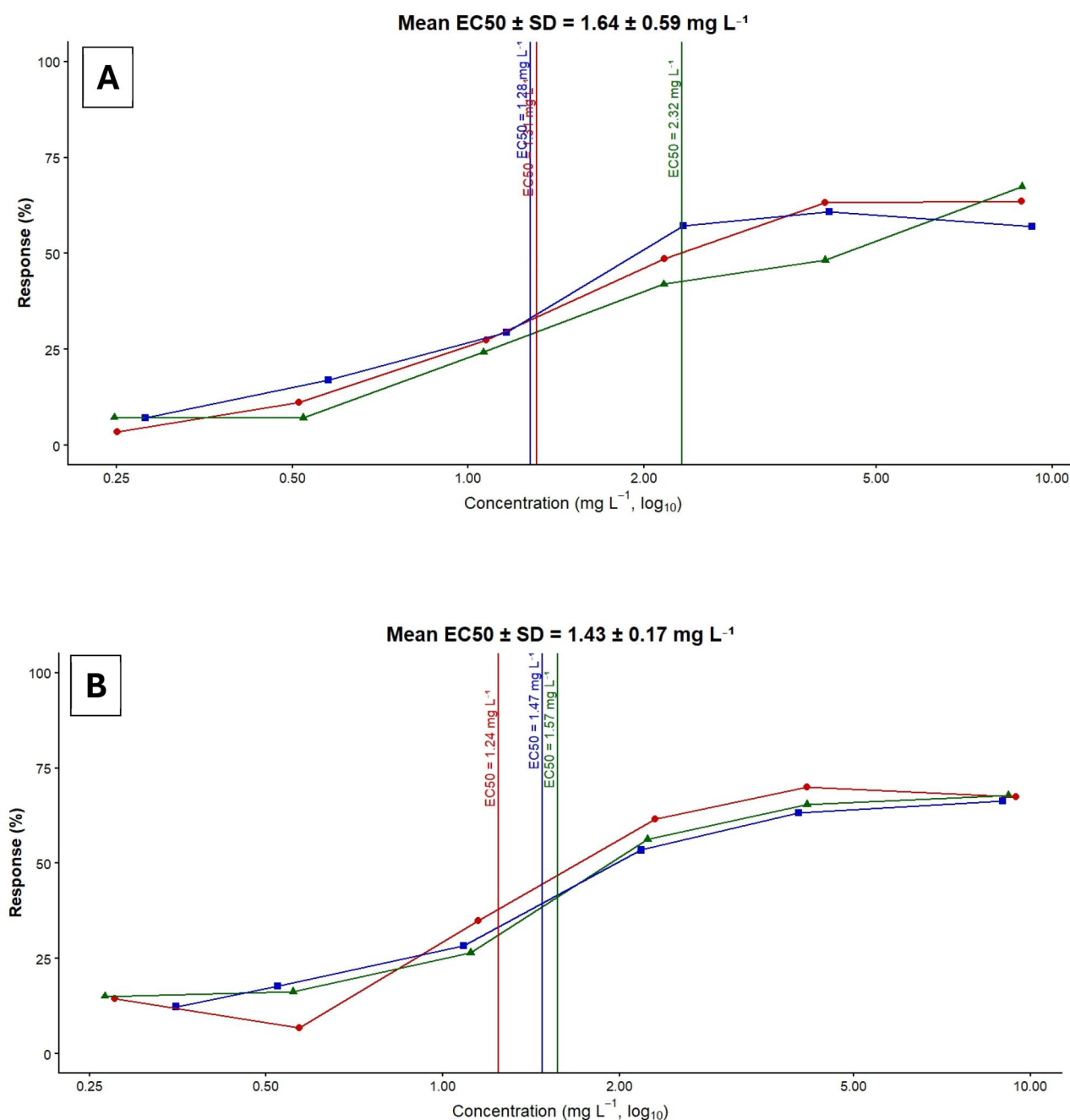


Figure 2 Growth inhibition curves of *Lemna minor* exposed to sulfamethoxazole (SMX), with EC₅₀ (concentration causing 50% growth inhibition) determined at pH 5.5 (A) and pH 5.0 (B) using a four-parameter logistic regression model (LL4; $n = 3$).

Stability of SMX

The stability of SMX during the test was assessed at the two investigated pH values, and the values reported represent the mean stability from three independent tests. The percentage recovery of SMX increased with higher initial concentrations, and the slopes of the trend lines at pH 5.5 and pH 5.0 were very similar. The effect of light (6,000 lx illumination) on SMX stability in the abiotic control was observed. Under light exposure, SMX stability was $89.90 \pm 2.79\%$ at pH 5.0 and $89.58 \pm 6.40\%$ at pH 5.5, based on the mean of two independent tests conducted at an

SMX concentration of 20 mg L^{-1} ; whereas in the dark, stability was close to 100%, reaching $104.73 \pm 0.06\%$ and $103.77 \pm 0.64\%$ at pH 5.0 and 5.5, respectively (see online [supplementary material, Table S3](#)). Under both pH conditions, light exposure resulted in additional signals in the chromatogram (retention times of approximately 2.5 and 3.8 min; see online [supplementary material, Figure S4A and B](#)), which were also observed on Day 7 in test samples where *L. minor* was exposed to SMX, at a concentration of 2.5 mg L^{-1} (see online [supplementary material, Figure S4C and D](#)). These signals were more pronounced at pH 5.5.

Discussion

Stability and uptake of SMX with pH and the effect of light

The abiotic control conducted in the dark showed SMX recoveries close to 100% at both tested pH values (see online [supplementary material, Table S3](#)), indicating that hydrolysis is negligible. From previous studies, it is known that acidification can enhance the hydrolysis of SMX (Białk-Bielińska et al., 2012). Although hydrolysis of sulfonamides increases with acidification and increasing temperature, for SMX, hydrolysis of <12% was reported after a 30-day degradation period, even under extreme pH and temperature conditions (pH 4.0, 70 °C; Białk-Bielińska et al., 2012).

The SMX recovery in the abiotic control under light conditions averaged $89.58 \pm 6.40\%$ at pH 5.5 and $89.90 \pm 2.79\%$ at pH 5.0 (see online [supplementary material, Table S3](#)), which suggests photodegradation. This is further confirmed by chromatograms from the seventh day of abiotic tests under both investigated pH conditions (see online [supplementary material, Figure S4A and B](#)). The signals appearing in the chromatograms (retention times of approximately 2.5 and 3.8 min) for samples exposed to light indicate the emergence of an additional compound during the test. Previously, two photodegradation products were identified for sulfonamides—sulfanilic acid and sulfanilamide (Białk-Bielińska et al., 2018). Chromatograms representing the analysis of test samples of *L. minor* exposed to SMX at 2.5 mg L^{-1} also demonstrate that SMX undergoes degradation during the experiment, regardless of pH (see online [supplementary material, Figure S4C and D](#)). Therefore, the process of photodegradation was not associated with the presence of plants. Peaks of degradation products, however, were more pronounced at pH 5.5. This observation is consistent with the SMX stability calculated for this concentration (2.5 mg L^{-1} SMX; see online [supplementary material, Figure S2](#))—stability at pH 5.5 was statistically lower (70.99%) than at pH 5.0 (75.81%; Mann–Whitney test, $p = 0.0209$). Similarly, a statistically significant difference was observed between different pH conditions for the 1.25 mg L^{-1} concentration ($p = 0.0433$), with 74.41% for pH 5.5 and 71.58% for pH 5.0. Thus, a reduction in pH may have contributed to the inhibition of SMX degradation. It is plausible that the parent compound is more toxic than the two transformation products (Białk-Bielińska et al., 2018). Consequently, slower degradation of SMX at lower pH results in a higher effective concentration, which may ultimately lead to higher toxicity to *L. minor* under lower pH conditions.

Importantly, in our study, EC50 values were determined based on the initial concentrations of SMX, which is a widely accepted practice in environmental toxicology. This approach facilitates direct comparison with literature values, but its limitation is that it does not fully reflect the dynamics of transformation processes. The results obtained should therefore be interpreted as a measure of the toxicity of the entire system, comprising both the parent compound and any transformation products formed during exposure. Consequently, differences in EC50 values between pH conditions (Figures 1 and 2) are attributed to changes in the composition of this mixture rather than solely to changes in SMX concentration.

No statistically significant differences were observed between the same SMX concentrations at the different tested pH values, except for the 2.5 and 1.25 mg L^{-1} concentrations. For these two concentrations, a slightly lower recovery at pH 5.5 compared to pH 5.0 suggests that a reduction in pH may have contributed to slower SMX photodegradation, potentially increasing toxicity to *L. minor*. In addition to pH effects, SMX recovery was observed to increase with higher initial concentrations, suggesting that other mechanisms, such as bioaccumulation by *L. minor*, may contribute to the observed decrease in SMX concentration. This raises the question of why the final recovery of SMX was lower in solutions with lower initial concentrations compared to those with higher drug concentrations. This effect can be explained by the greater efficiency of uptake by the plant at low concentrations, where transport is not yet limited by membrane saturation. At higher concentrations, the bioaccumulative capacity of *L. minor* may be partially restricted due to saturated uptake as well as potential toxic effects, which reduce the plant's ability to actively take up the compound. Representative images of the test plates with *L. minor* show chlorosis at higher pharmaceutical concentrations (see online [supplementary material, Figure S1C2 and D2](#)), whereas this toxic effect was not observed at lower SMX concentrations. Antibiotics can cause chlorosis in plants by disrupting metabolism and photosynthesis, as seen with tetracycline (Rydzynski et al., 2019). It should be noted that pharmaceuticals, as ionic compounds—including SMX—may exhibit different routes of absorption by plants, as described by Trapp et al. (2023). One observed mechanism is ion trapping, which depends on the pH of the environment. In the case of SMX, this mechanism may facilitate its absorption into *L. minor* and movement between the leaves of the plant (Trapp et al., 2023).

Proportionally, relative to the initial amount of the drug, more of the drug was “consumed” by the plant at lower concentrations of SMX. The absolute amount of substance absorbed can be calculated and is presented as μg of SMX per mm^2 of frond (Table 2). Uptake was higher at a more acidic pH, suggesting increased absorption of the compound in its acidic form (Table 2). For example, uptake at an exposure level of 10 mg L^{-1} was $0.161 \mu\text{g mm}^{-2}$ at pH 5.5 and $0.211 \mu\text{g mm}^{-2}$ at pH 5. Although the absolute quantity of SMX absorbed by the plant increases with increasing concentration, its proportional share relative to the initial amount in the medium is lower (Table 2). For example, $7.5\text{--}10 \mu\text{g}$ was absorbed by the plant at a concentration of 10 mg L^{-1} , whereas only $1.2\text{--}1.4 \mu\text{g}$ was absorbed at an SMX concentration of 0.31 mg L^{-1} . The accumulation of SMX by *L. minor* is also confirmed by Iatrou et al. (2015), who documented that uptake by the plant was the main mechanism for removing SMX from urine and treated wastewater, accompanied by simultaneous biodegradation.

Interestingly, at pH 5.5, the greatest depletion of SMX mass was not observed at the highest concentration, but rather at 5 mg L^{-1} . Since the rate of chemical reactions depends on concentration, it is possible that the proteins or cell membranes of *L. minor* were damaged more rapidly at the highest SMX concentration, preventing further accumulation by the plant. Previous studies have described the negative effect of nitric oxide (NO) on the cell membranes of *L. minor* under stress conditions (Krupka et al., 2021; Kummerová et al., 2016). Nitrates and

Table 2 The calculated uptake of sulfamethoxazole (SMX) from the test well with increasing SMX concentration at two tested pH levels of the growth medium.

SMX concentration (mg L ⁻¹)	SMX mass taken up by the plant					
	Test set—pH 5.5			Test set—pH 5.0		
	µg	%	µg SMX per mm ⁻² of frond	µg	%	µg SMX per mm ⁻² of frond
0.31	1.25	40.3	0.008	1.48	47.4	0.009
0.62	2.52	40.0	0.018	2.29	36.7	0.013
1.25	3.20	25.6	0.027	3.55	28.4	0.031
2.50	7.25	29.0	0.088	6.05	24.2	0.133
5.00	11.4	22.7	0.207	9.03	18.1	0.230
10.00	7.53	7.53	0.161	10.3	10.3	0.211

nitrites, which serve as NO precursors, accumulate excessively in cells, leading to an increase in NO synthesis. Under normal conditions, NO acts as a signaling molecule, regulating, among other processes, plant growth and ion metabolism. However, its excess can compromise membrane integrity and induce programmed cell death. Krupka et al. (2021) demonstrated an increase in nitrates and nitrites in *L. minor* treated with tetracycline at a concentration of 10 mM. Similarly, the accumulation of reactive nitrogen species was documented by Kummerová et al. (2016) in *L. minor* exposed to diclofenac and paracetamol. An increase in reactive nitrogen species and reactive oxygen species may cause increased lipid oxidation in *L. minor*, loss of cell membrane integrity, or damage to antioxidant systems. Sulfonamides, through the inhibition of DHPS, may also disrupt folate metabolism and contribute to oxidative stress (Brain et al., 2008; Xu et al., 2022).

Acute toxicity endpoints can be potentially affected by pH

The EC50 values of SMX determined in the performed test using the LL4 nonlinear regression model (1.64 ± 0.59 mg L⁻¹ at initial pH 5.5 and 1.43 ± 0.17 mg L⁻¹ at initial pH 5.0) were higher than the lowest values reported in the literature (Alfarsi et al., 2025; Białk-Bielińska et al., 2011), yet still within the overall range of previously documented values (Aubakirova et al., 2017; Diogo et al., 2024; Grabarczyk et al., 2020; Table 3). The EC50 for SMX, calculated based on the frond area in the study by Białk-Bielińska et al. (2011), was 0.21 mg L⁻¹, making it one of the most toxic sulfonamides (the EC50 range for other representatives of this group was 0.02–4.89 mg L⁻¹). The EC50 values calculated based on the wet weight of duckweed were even lower (0.081 mg L⁻¹ SMX; Brain et al., 2004), most likely due to the higher sensitivity of this parameter to early disturbances in plant metabolism (new fronds may still develop, but they can be thinner, contain less water, and therefore weigh less). Although these experiments were conducted on a different duckweed species, *L. gibba*, SMX was similarly found to be the most toxic sulfonamide, as previously reported for *L. minor* (Białk-Bielińska et al., 2011).

In our study, the pH-dependent differences in SMX toxicity, as reflected in EC50 values, appear to correlate with the degree of dissociation of the compound. According to the literature, the

non-ionized fraction of pharmaceuticals is generally more toxic to organisms than the dissociated fraction, as it penetrates cell membranes more readily (Białk-Bielińska et al., 2012). At pH 5.0, the proportion of non-ionized SMX was higher (83.37%) compared to pH 5.5 (61.31%), which may explain the lower EC50 observed at pH 5.0. Notably, these fractions were initially calculated based on the starting pH of the test. Considering the final pH values (7.30 ± 0.28 for the starting at pH 5.5 and 7.16 ± 0.38 for the starting at pH 5.0), the fraction of neutral SMX would be only 2.45% and 3.35%, respectively, suggesting that the actual toxicity of the compound may decrease during exposure. Although no statistically significant difference was observed in the LL4 analysis (see online supplementary material, Figure S3B), the probit model indicated a significant difference between pH treatments (see online supplementary material, Figure S3A). However, both approaches showed the same directional trend of increasing toxicity with decreasing pH, consistent with OECD 221 guidance, which allows both methods. The trend in which decreasing pH increases effectiveness (lowers EC50) is biologically plausible for many substances whose properties may change under more acidic conditions (Anskjær et al., 2013; Kim et al., 2010; Sun et al., 2020). In the case of sulfonamides, which are amphoteric in nature, pH changes are of key importance for toxicity. Although these compounds have two pKa values, from an environmental perspective, the second pKa is the most relevant, as it is close to the pH of natural water bodies. Therefore, with the acidification of waters, the speciation of SMX will change; more precisely, with decreasing pH, the neutral form of the sulfonamide will increase (Białk-Bielińska et al., 2012; Męczykowska et al., 2018).

Among the mechanisms considered, pH-dependent speciation combined with plant-driven pH drift appears to be the dominant factor influencing SMX toxicity, whereas photodegradation played a secondary role and did not fully explain differences in EC50 between treatments.

The pH drift driven by plants

In our experiment, a key objective was to investigate the impact of pH shifts on the toxicity of SMX toward *L. minor*. However, it is difficult to unambiguously attribute the observed effects solely to acidification, as pH underwent considerable fluctuations during the test (Table 1). The largest ΔpH values were observed in biotic controls at pH 5.5 (+2.49) and 5.0 (+2.88), with pH

Table 3 The up-to-date report on acute toxicity (EC50—sulfamethoxazole (SMX) concentration causing 50% growth inhibition of *Lemna minor*) of SMX toward *L. minor*, including selected basic methodological information.

Growth medium, endpoint measured	pH measurement	EC50 (mg L ⁻¹)	Model for determining EC50	Ref.
Swedish SIS medium frond number	Lack of pH measurement at the start and final	0.043	Nonlinear logistic four-parameter (LL4; R, drc package)	Alfarsi et al. (2025)
Steinberg medium frond area	Lack of pH measurement at the start and final	0.21	Dose–response regression model fitted to plant growth data; model details not provided in the publication.	Białk-Bielińska et al. (2011)
Steinberg medium biomass	Lack of pH measurement at the start and final	2.11	Nonlinear logistic 3-parameter (LL3; R, drc package).	Diogo et al. (2024)
Steinberg medium frond area	pH start 5.5 (±0.5), lack of final pH measurement	3.07	Linear logistic or logistic (best-fit model chosen; R, drfit package).	Grabarczyk et al. (2020)
Swedish SIS medium frond area	Lack of pH measurement at the start and final	3.67	Non-linear regression analysis.	Aubakirova et al. (2017)
Steinberg medium frond area	pH start 5.5 pH end 6.07–7.66	3.52 ± 0.41	Probit (for statistical comparison; R, glm package)	This work
		1.64 ± 0.59	Nonlinear logistic 4-parameter (LL4; R, drc package)	
	pH start 5.0 pH end 6.84–7.67	2.67 ± 0.11	Probit (for statistical comparison; R, glm package)	
		1.43 ± 0.17	Nonlinear logistic 4-parameter (LL4; R, drc package)	

Note: Each study was conducted according to Organisation for Economic Co-operation and Development Test Guideline 221.

increasing over time. Higher SMX concentrations reduced these shifts, corresponding to reduced plant growth; at 10 mg L⁻¹, ΔpH was 1.42 (pH 5.5) and 1.87 (pH 5.0). In abiotic controls (see online [supplementary material, Table S2](#)), no significant pH changes were observed throughout the experiment, clearly indicating the role of plants in driving pH shifts (differences between the first and the last day of the test ranged from 0.03 to 0.18 for pH 5.5 and from 0.09 to 0.17 for pH 5.0).

Several processes can affect pH during plant growth. Photosynthesis reduces CO₂, increasing alkalinity, while nutrient uptake can either raise or lower pH (e.g., Fe and Mg increase, Zn, Mn, and Ca decrease pH) (Yahya Jawad Sharba et al., 2024). The composition of Steinberg medium indicates that *L. minor* primarily assimilates nitrogen in the form of NO₃⁻ (derived from KNO₃ and Ca(NO₃)₂). In the Steinberg medium, nitrate assimilation by *L. minor* increases alkalinity via OH⁻ release and H⁺ co-absorption (Cedergreen & Madsen, 2002; Dijkhoorn, 1962; Hageman, 1984; Tischner, 2000; Tischner & Kaiser, 2007). Consequently, pH increases with biomass accumulation. *Lemna minor* can assimilate both NH₄⁺ and NO₃⁻, and pH changes related to nitrate uptake have been documented in physiological studies and phytoremediation systems (Devlamynck et al., 2020; Sigcau et al., 2022). Therefore, monitoring pH is crucial, and its variability should be regarded as an inherent part of the system's biological response. The decision was made to deliberately refrain from renewing the test medium during the experiment. In the OECD 221 guideline, it is specified that careful consideration should be given to the inclusion of a MOPS buffer in the *L. minor* test medium when it is suspected that it might react with the test substance and influence the expression of its

toxicity. Therefore, we decided not to supplement the growth medium with additional compounds because of possible interactions with SMX. In addition, the buffering capacity of MOPS is effective over the pH range of 6.5–7.9, which is outside the scope of our study. Buffers themselves may have a toxic effect or affect ion/metal speciation. Thus, an urgent need exists to test the applicability of buffers for *L. minor* tests. Such studies have been conducted for *D. magna* and *Staurosirella subcapitata* (De Schamphelaere et al., 2004; Rendal et al., 2012).

Importantly, the range of pH changes observed in this study (ΔpH up to ~3) indicates that exposure conditions in tests with *L. minor* may differ significantly from the initial conditions. Under such conditions, the system is not chemically stable, as both speciation and degradation processes change over time. Consequently, pH variation should be considered a primary driver of the observed toxicity of ionizable compounds such as SMX, rather than a secondary experimental parameter.

In previous studies, pH was found to impact the growth of *L. minor* and other aquatic plants. Ullah et al. (2023) observed that the growth rate of *L. minor* was highest (90 g m⁻² day⁻¹) at pH 7, while it was lowest (42 g m⁻² day⁻¹) at pH 4. In our study, in the control treatment, the growth rate reached 89 g m⁻² day⁻¹, which is comparable to that observed at pH 7. However, the referenced study used tap water adjusted with HCl and NaOH and did not report the pH values at the end of the experiment. The acidic pH (3.0) was found to reduce the growth rate of *Typha domingensis* because an increased passive influx of H⁺ decreases the electrochemical gradient across the plasma membrane, thus reducing the uptake of cations by plant roots (Dyhr Jnrnsen & Brix, 1996). Acidification may also promote *L.*

minor growth when ammonium nitrogen is present, as lower pH shifts the $\text{NH}_4^+/\text{NH}_3$ equilibrium toward NH_4^+ , whereas nitrate uptake leads to alkalization of the medium (Jones et al., 2023; Zhou et al., 2022). Despite the important role of pH in *L. minor* growth, this factor has not been considered in growth models (Van Dyck et al., 2021). On the other hand, pH control is necessary to maintain proper nitrate uptake by *L. minor* (Sigcau et al., 2022). Available literature does not provide clear evidence on the effect of pH on pharmaceutical toxicity to *L. minor*. However, this effect likely occurs when OECD 221 is applied, as the Steinberg medium “naturally” increases the pH up to 7.9 during a 7-day growth period of *L. minor*. The effect of pH has been tested for other photosynthetic organisms. For example, the toxicity of basic pharmaceuticals toward the green algae *S. vacuolatus* increased with rising pH (Neuwoehner & Escher, 2011). In the mentioned work, various buffers were applied to maintain pH from 6.5 to 10. According to OECD 221, it is necessary to report the pH at the start and end of the experiment. However, despite some authors (Table 3) specifying in the method section that they measured pH at the end of the experiment (for example, Aubakirova et al., 2017; Białk-Bielińska et al., 2011), none of them report the actual values (Aubakirova et al., 2017; Białk-Bielińska et al., 2011; Diogo et al., 2024; Grabarczyk et al., 2020).

Conclusions

Although no statistically significant differences were observed between the results obtained under different pH conditions (LL4 model), a consistent trend of increasing SMX toxicity with decreasing pH was evident. Different OECD-approved analytical models (probit vs. LL4) may yield different EC50 values due to distinct mathematical response functions; however, both showed the same direction of pH-dependent toxicity, supporting the biological relevance of the observed trend. This trend may be explained by the higher fraction of neutral SMX at lower pH. Growth rates in biotic controls were comparable across pH treatments, indicating that growth limitation did not drive the observed effects. Photolysis contributed to SMX degradation during the test, but it was not the primary driver of the observed toxicity differences. These findings highlight the importance of monitoring pH changes in *L. minor* toxicity tests, as speciation, degradation, and plant-chemical interactions jointly influence SMX toxicity.

The results of this study indicate that SMX toxicity is primarily governed by pH-dependent speciation, modulated by plant-driven pH changes. This highlights that exposure conditions in *L. minor* assays are inherently dynamic, which complicates the interpretation of toxicity data for ionizable compounds. Additionally, the pH-dependence of toxicity for ionizable pharmaceuticals should be considered in risk assessments (Boström & Berglund, 2015), including from a multigenerational perspective (Alfarsi et al., 2025).

Recent OECD 221 studies for pharmaceutical assessment often lack the presentation of pH at the start and end of the experiment and rarely consider pH as a modulating factor for ionizable pharmaceuticals. Our report demonstrated that, under standard OECD 221 conditions, tracking pH-dependent toxicity is limited. Therefore, the development and validation of appropriate buffering strategies for *L. minor* tests should be prioritized. Exemplary buffering strategies have been presented for *D.*

magna and *Pseudokirchneriella subcapitata* (Rendal et al., 2012), and similar approaches should be investigated for *L. minor* in pharmaceutical toxicity studies. This is particularly important when Steinberg medium is used, as its initial pH is not environmentally relevant.

Supplementary material

Supplementary material is available at *Environmental Toxicology and Chemistry* online.

Data availability

The data that support the findings of this study are openly available in repozytorium.bg.ug.edu.pl at doi: 10.71853/04g2-5280, reference number UOG7ee111817dd74c39898e247644a31a0e.

Author contributions

Katarzyna Bethke (Data curation, Formal analysis, Investigation, Methodology, Writing—original draft, Writing—review & editing) and Magda Caban (Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing—original draft, Writing—review & editing)

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Supplementary Information
to
**Exploring the impact of pH on the toxicological response of *Lemna minor*
to pharmaceuticals - insights from sulfamethoxazole exposure**

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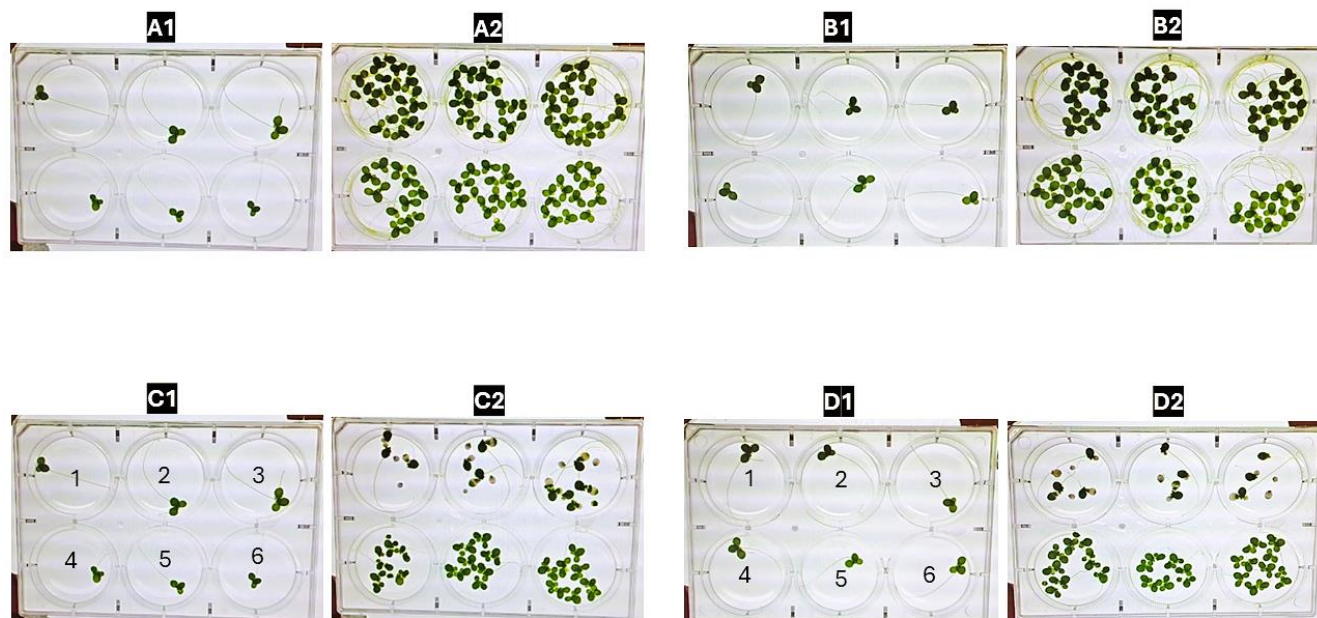


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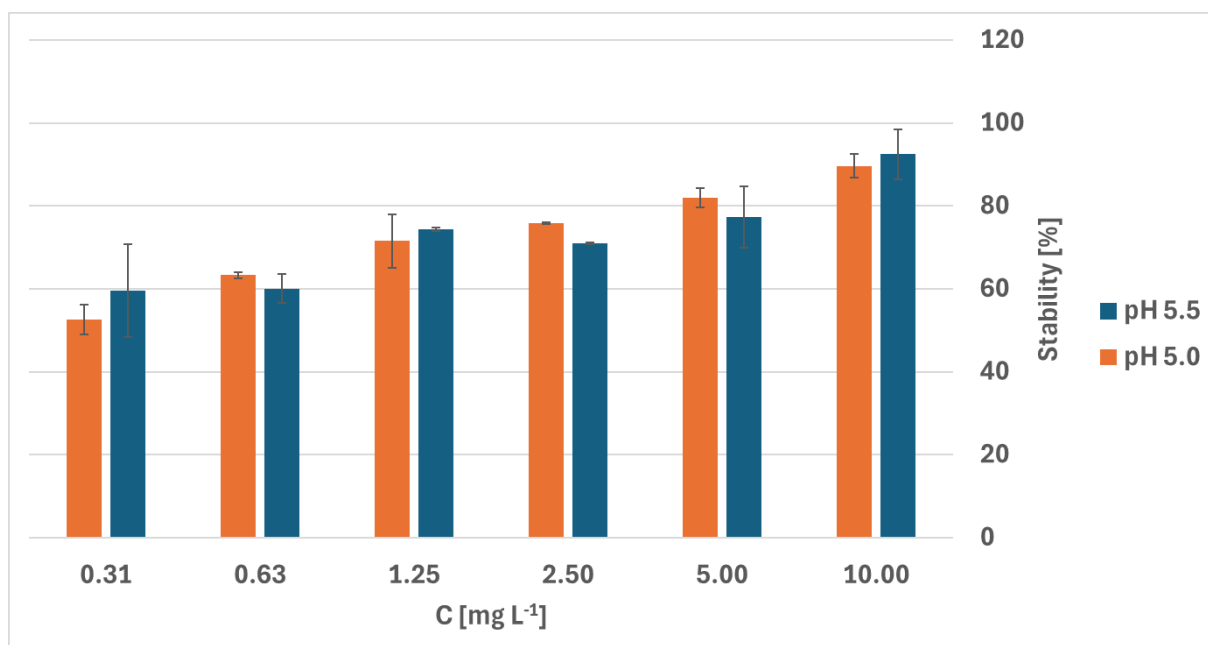


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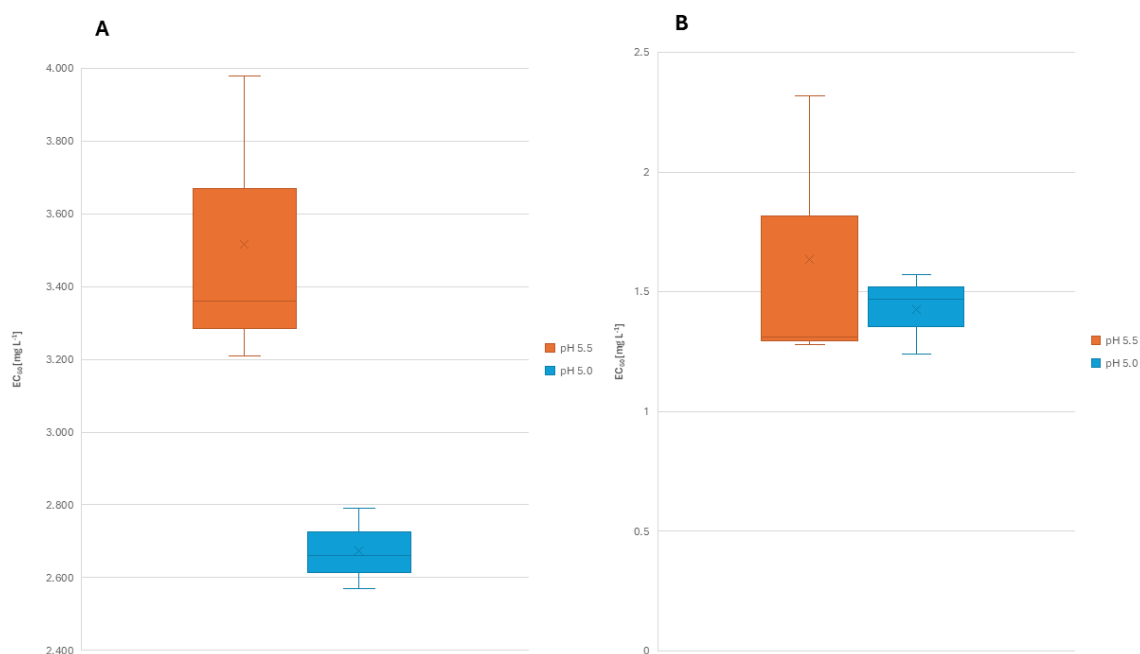


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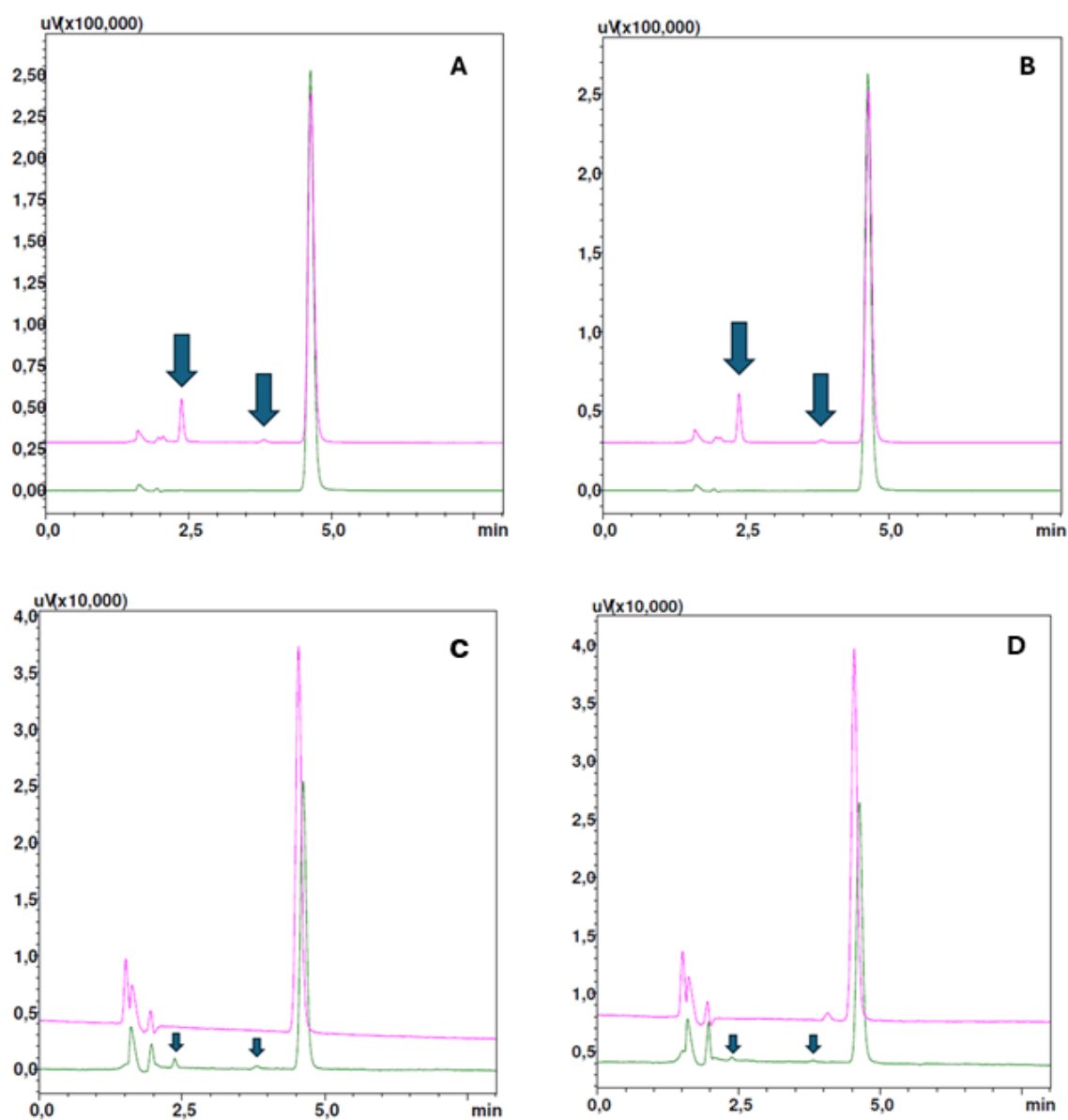


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Table S1. Validation parameters for HPLC/DAD method of sulfamethoxazole determination using two calibration curves for concentration ranges

Calibration curve number and linearity range [$\mu\text{g mL}^{-1}$]	Instrumental quantification limit MQL [$\mu\text{g mL}^{-1}$]	Instrumental detection limit MDL (MQL/3) [$\mu\text{g mL}^{-1}$]	Precision [RSD, %]	Accuracy [%]	R ²
1. 0.20-3.13	0.20	0.07	0.21-4.14	82.50-106.67	0.9995
2. 3.13-100.00	-	-	0.07-0.29	84.22-102.32	0.9998

Table S2. pH values of abiotic controls (sulfamethoxazole (SMX) solutions without *Lemna minor*) measured on day 0 and the final day under dark and light conditions

Test - pH 5.5		SMX [mg L^{-1}]	Start pH	pH after 7 days	pH difference between the last and first day of the test
	Abiotic control - light	20.00	5.61 ± 0.13	5.64 ± 0.19	0.03
	Abiotic control - dark	20.00	5.61 ± 0.13	5.43 ± 0.31	0.18
Test - pH 5.0	Abiotic control - light	20.00	4.93 ± 0.08	4.76 ± 0.09	0.17
	Abiotic control - dark	20.00	4.93 ± 0.07	4.84 ± 0.05	0.09

Table S3. Recovery of sulfamethoxazole (SMX) in abiotic controls under different pH and light conditions; recovery was calculated as the ratio of the measured concentration on day 7 to the concentration measured on day 1, expressed as a percentage; individual recovery values and mean $\pm$ standard deviation (SD) are shown

pH	Conditions	SMX stability (%) (mean + SD; individual)
5.0	Dark	104.73 $\pm$ 0.06 (104.69; 104.78)
5.0	Light	89.90 $\pm$ 2.79 (87.93; 91.88)
5.5	Dark	103.77 $\pm$ 0.64 (104.22; 103.32)
5.5	Light	89.58 $\pm$ 6.4 (85.05; 94.10)



Context-dependent toxicity of diclofenac in *Daphnia magna*: roles of water chemistry, neonate source and preparation procedure

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ABSTRACT

Daphnia magna is a well-established reference organism for ecotoxicological studies of pharmaceuticals. Standardized acute toxicity protocols specify the composition of test media, and any alternative water type must be fully characterized and reported. In the case of acute toxicity testing for *D. magna*, neonates are typically employed as test organisms. Despite the strict methodological framework, reported EC₅₀ values for diclofenac (DCF) in *D. magna* vary considerably, ranging from 22.4–123.3 mg L⁻¹, with test water being the most unreported factor. Therefore, in our study, we investigated the effect of exposure medium (M4 medium and the tested natural freshwater) and the source and preparation procedure of *D. magna* neonates on the toxicity of DCF. The results indicate that DCF toxicity toward *D. magna* may differ between the tested natural freshwater and M4 medium, with higher EC₅₀ values observed in the tested natural freshwater. Additionally, differences in sensitivity were observed between neonate groups under the tested conditions. These findings highlight the importance of considering the source and preparation history of test organisms and exposure medium when interpreting and comparing daphnid-based ecotoxicological data. Overall, the findings provide additional insight into the toxicological effects of this emerging contaminant on a model reference species under conditions that more closely reflect those of the tested natural freshwater than those prescribed in standard laboratory protocols.

1. Introduction

Diclofenac (DCF) is a non-steroidal anti-inflammatory drug (NSAID) widely detected in aquatic environments due to its low removal efficiency (in the majority of cases, lower than 50%) in conventional wastewater treatment systems (Alessandretti et al., 2021; Kolečka et al., 2020, 2022), and its presence in wastewater effluent (concentration range from 0.1 up to 5.0 mg L⁻¹) (Bethke et al., 2024; Cherik et al., 2015; Elshikh et al., 2022; Zhang et al., 2008) discharged into natural waters. Consequently, DCF persists in surface waters (with reported concentrations in European surface waters exceeding 50 ng L⁻¹) (Di Lorenzo et al., 2021) and can affect non-target organisms, with the catastrophic mortality of Gyps vultures representing an extreme example of its ecological impact (Oaks et al., 2004). The compound has been included on the EU Watch List and is classified as an emerging contaminant (Helcom, 2018). Its acute and chronic toxicity toward standard test organisms has been extensively investigated under standardized laboratory conditions (Leverett et al., 2021; Sathishkumar et al., 2020). Such ecotoxicity protocols, developed by international organizations,

aim to minimize experimental variability by controlling factors such as water composition, pH, temperature, photoperiod, and test duration. This standardization facilitates comparability among studies and supports the identification of sensitive species and hazardous substances. However, environmental conditions in natural waters often differ markedly from laboratory settings (Fischer et al., 2013). Therefore, toxicity estimates obtained under standardized conditions may not fully reflect responses observed under different environmental conditions (Franco, 2025). Moreover, emerging evidence suggests that environmental stressors such as water acidification and elevated temperatures may alter pharmaceutical toxicity (Bethke et al., 2023). Consequently, predicting chemical effects on ecosystems requires toxicity studies conducted under conditions that more closely resemble natural environments (Schneeweiss et al., 2023). Extrapolating laboratory observations of single compounds to real ecosystems - where multiple stressors interact within complex food webs - remains a major challenge (Nilsen et al., 2019). Integrating multiple-stressor approaches into ecotoxicological research has therefore become increasingly important (Gessner and Tlili, 2016). Therefore, current ecotoxicological research is increasingly

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moving toward more ecologically relevant experimentation and the generation of data that can be better extrapolated to responses in the environment (Franco, 2025).

Species of the genus *Daphnia* are cosmopolitan zooplankton organisms that inhabit environments with wide ranges of pH and temperature, including small ponds where physicochemical conditions fluctuate both seasonally and diurnally. Due to their ecological relevance and well-characterized biology, they are widely used as model organisms in ecotoxicology (Tkaczyk et al., 2021). Indeed, the ecology and biology of daphnids have been intensively studied for >250 years (Ebert, 2005). In a comprehensive review of NSAID toxicity toward aquatic invertebrates provided by Parolini (2020), DCF was found to be among the most toxic NSAIDs toward *D. magna* based on EC₅₀ (half maximal effective concentration) values. The acute toxicity of pharmaceuticals is commonly characterized using EC₅₀ values from laboratory tests. In the case of *D. magna*, the most common reference protocol is OECD 202 'Daphnia sp. Acute Immobilisation Test'. This protocol permits the use of natural water (surface or groundwater), reconstituted water, or dechlorinated tap water as test media, provided that daphnids survive without signs of stress during culturing, acclimation, and testing. The acceptable pH range is 6–9, and adjustments with NaOH or HCl are allowed. Physicochemical characteristics of test water should also be reported. However, a review of studies assessing DCF toxicity toward *D. magna* (Table S1, Supplementary Information 1, SI1) indicates that descriptions of test water are often incomplete. Most commonly, only a general reference to the use of reconstituted hard water is provided, without specifying the recipe for its preparation. Key parameters such as hardness, conductivity, and pH are also frequently missing. Reported EC₅₀ values vary widely (22.4–123.3 mg L⁻¹), suggesting that methodological (different protocols applied), biological (organism source), and physicochemical factors (for example test medium) may contribute to variability in acute DCF toxicity in *D. magna*.

One potential source of variability is the source and preparation procedure of test organisms. The OECD guideline recommends the use of daphnid neonates younger than 24 h and advises avoiding first-brood individuals to minimize variability. Neonates should originate from healthy laboratory cultures. However, in some studies - particularly those using commercial micro-bioassays - test organisms are obtained directly from ephippia (so-called culture-free tests) (for example, Baran et al., 2021; Białk-Bielińska et al., 2022; Eluk et al., 2021). The use of ephippia eliminates the need to maintain continuous cultures. Previous research has shown that ex-ephippial neonates (EE-Neo) and parthenogenetic neonates (P-Neo) may differ in their responses to chemicals (Moreira Dos Santos and Persoone, 2000). However, the reported EC₅₀ ratios for potassium dichromate, sodium pentachlorophenol, 2,4-dichlorophenoxyacetic acid, and malathion between 0.77 and 1.29 indicate relatively good agreement between both neonate types according to Moreira Dos Santos and Persoone (2000). Nevertheless, only four chemicals were tested, and none of the tested chemicals were pharmaceuticals. Therefore, a systematic investigation into the source of test organisms for determining the acute toxicity of pharmaceuticals should be undertaken. Under natural conditions, both types of neonates occur, but P-Neo predominate for most of the *D. magna* occurrence season in water bodies.

In the present study, we investigated two factors potentially affecting the DCF acute toxicity in *D. magna*. Firstly, we investigated the influence of test medium on DCF toxicity toward *D. magna* by comparing tests conducted in well-characterized natural freshwater (NF) (to increase ecological relevance) and M4 culture medium. The tested NF physicochemical characteristics fell within the OECD 202 recommendations for test water. Secondly, we examined whether the source and preparation procedure of neonates (EE-Neo versus P-Neo) were associated with differences in the observed toxicity. Finally, the effects of both factors and their interaction were evaluated. We hypothesized that DCF toxicity toward *D. magna* differs between the tested NF and M4

medium and between neonate source and preparation procedure (EE-Neo versus P-Neo). The overall objective was to evaluate DCF toxicity toward a representative cosmopolitan zooplankton species under conditions that more closely reflect those of the tested NF and to provide additional insight into the variability of acute toxicity estimates for this widely detected pharmaceutical.

2. Experimental

2.1. Chemicals

The sodium salt of DCF (C₁₄H₁₀Cl₂NNaO₂, CAS 15307-79-6, purity >98%, Pharmaceutical Secondary Standard) was purchased from Merck (Germany). Bold Modified Basal Freshwater (BMBF) solution was purchased from Merck as a concentrate. Deionized water (DI, 0.05 μS cm⁻¹) was supplied by a Hydrolab (Poland) system. Acetic acid and ammonium acetate (both HPLC grade) were purchased from VWR Chemicals. Artificial hard water (M4 growth medium) was prepared according to the OECD 211 guidelines using DI water and salts obtained from POCH (Poland).

2.2. Specification of natural water

The natural freshwater was collected from a small artificial pond located on the university campus (DD 54.39577, 18.57483). It is a shallow water reservoir (maximum depth of 2 m, approximately 25 m in width), inhabited by aquatic plants and fish. Zooplankton species were observed, as well as blooms of filamentous algae in summertime. Quality monitoring, including pH, oxygen content, temperature, conductivity, and basic ion concentrations, was carried out between March 2023 and November 2023 (with specific dates provided in Table S2, SI).

Ion chromatography (IC) was used for the determination of ion concentrations, employing a Metrohm Varian instrument. For cation analysis (Na⁺, K⁺, Ca²⁺, and Mg²⁺), the following parameters were applied: column Metrosep C4 100/0.4; mobile phase 1.7 mM nitric acid with 0.7 mM dipicolinic acid in DI water; column oven temperature 25 °C; mobile phase flow 0.8 mL min⁻¹. For anion analysis (F⁻, Cl⁻, PO₄²⁻, Br⁻, NO₃⁻, and SO₄²⁻) the following parameters were applied: column Metrosep a Supp 10 100/0.4; mobile phase 5 mM carbonate buffer in water, column oven 40 °C, mobile phase flow 1 mL min⁻¹. External calibration was performed using certified reference materials: for cations, a 6-component standard mixture, each 10 mg L⁻¹ (ref. E3A7.10.W.L05, CPACHEM), for anions, standard VII 9 100 mg L⁻¹ (ref. 110322.L1, CPACHEM). Validation parameters for calibration curves are presented in Table S3 (SI1). NF samples were filtered prior to analysis using syringe filters (0.2 μm, PET-20/15 MS, Chromafil).

2.3. Cultivation of *D. magna*

The stock culture of *D. magna* was derived from DAPHTOXKIT F ephippia (MicroBio Tests). This test kit is an ephippia-based system designed for acute immobilization testing of *D. magna* in accordance with ISO Standard 6341 and OECD Guideline 202, as stated by the manufacturer. The stock culture was over 3 years old, and therefore, numerous generations of daphnids had been produced before the test. The growth medium was regularly refreshed, and daphnids were fed with *Chlorella vulgaris* (from in-house cultivation) and sporadically with yeast (dried baker's yeast, food-grade). P-Neo were fed with *C. vulgaris* 2 h before the toxicity test, using the amount of algal carbon recommended by OECD 211. *C. vulgaris* was cultivated in glass bottles filled with BMBF solution under constant LED illumination at ambient temperature. Biomass was harvested by centrifugation and resuspended in M4 medium before feeding. One month before the experiment, an appropriate number of mature daphnids from this long-established stock culture were transferred to 1 L glass flasks. During this pre-test period, the daphnids

reproduced repeatedly, producing successive broods. The water in the flasks was aerated. The flasks were kept in a climate chamber at 23 °C with a 12 h light / 12 h dark LED light cycle. Every Monday, juvenile daphnids were removed from the flasks to ensure that neonates collected on Tuesday (P-Neo) were <24 h old and could be used in the tests. *D. magna* was cultured in M4 medium (as recommended by OECD 211) and NF. Observations indicated that *D. magna* thrived in the stock solution, with numerous neonates regularly observed. Microscopic images of *D. magna* are presented in **Figure S1** (SI1), showing numerous eggs visible under the carapace.

The DAPHTOXKIT F (MicroBio Tests) ephippia were also used to perform ecotoxicity tests with ex-ephippial neonates (EE-Neo). Hatching occurred within a maximum of three days in optimal conditions: light intensity 6000 lx and a temperature of 20–21 °C. The maximum number of EE-Neo was observed after 72–80 h. Hatching was carried out in either M4 medium or NF. According to the supplier's protocol, neonates were fed with lyophilized spirulina (provided with the kit) 2 h before the test began.

The analysis of carbon content in *C. vulgaris* was performed by photometric cell test (Spectroquant[®], Supeclo, 5.0–80.0 mg L⁻¹, ref. 1.14878.0001). Optical density (OD) was measured with a GENESYS 150 UV–Visible spectrophotometer (Thermo Scientific) at 680 nm (wavelength specific to chlorophyll), while cell counts were determined using a CytoFLEX 2L6C flow cytometer (Beckman Coulter). A graph illustrating the relationship between carbon content and culture absorbance is shown in **Figure S2** (SI1). Based on the measured absorbance of the culture, an appropriate volume of algal suspension was collected, centrifuged, and added to the daphnid culture flasks.

The suitability of the *D. magna* culture was confirmed based on its response to the reference substance, potassium dichromate (EC₅₀ = 1.11 mg L⁻¹), with the result falling within the expected quality control range for tests based on ISO 6341. Furthermore, mortality in the control group did not exceed 10%, in accordance with the validity criteria specified in OECD 202.

2.4. Acute toxicity test performance

The solution of DCF sodium salt was prepared directly before the experiment in aerated test water without any organic solvent addition. The minimum and maximum test concentrations of DCF were 1 mg L⁻¹ and 150 mg L⁻¹, respectively. The required test concentrations were prepared from DCF stock solutions either by serial dilution or by transferring appropriate volumes of the stock solution. The number and spacing of test concentrations varied between independent toxicity tests, and the specific concentrations used in each test are provided in the raw data. Conductivity, dissolved oxygen content, and pH were measured both before and after the test.

The Daphtoxkit F test was conducted using TOXKIT system plates, supplied by the manufacturer, consisting of 30 wells with a working volume of 10 mL per well and designed for standard immobilization tests on *D. magna*. Each well had dimensions of 3 × 3 × 4 cm (length x width x height). One row (6 wells) served as the biotic control group, without DCF. The remaining wells contained DCF solutions at various concentrations. Five neonates (either EE-Neo or P-Neo) were gently transferred into each well using a minimal volume of water to avoid dilution of the DCF solution. After preparation, the plates were sealed with parafilm and covered with a plastic lid. The plates were placed at 21 °C in the dark. After 24 and 48 h, the number of immobilized daphnids in each well was recorded. Each experimental combination was tested in three independent toxicity tests, each comprising five or six DCF concentrations, with four replicate wells per concentration and five neonates per well. Immobilization was recorded separately for each well. An EC₅₀ value was estimated from the concentration-response data obtained within each independent toxicity test. Thus, the four wells at each concentration represented within-test replicates and were

not treated as independent observations in the subsequent statistical analysis.

Table 1 presents an overview of the sets conducted in this study, indicating the test water used (NF or M4) and the source/preparation of neonates (EE-Neo or P-Neo).

The three independent toxicity tests were conducted on separate occasions, yielding three independent EC₅₀ estimates per experimental combination (n = 3).

DCF concentrations in the test solutions were measured at the beginning and after 48 h of exposure using HPLC-DAD, as described by Kropidłowska and Caban (2023) (method validation parameters are provided in **Table S4**, SI1). Changes in DCF concentration during the exposure period were assessed based on these measurements (**Table S6**; **Figure S5**, SI1). DCF concentrations were additionally measured in abiotic controls without test organisms (nominally 100 mg L⁻¹ DCF) (**Table S6**, SI1).

All dose-response analyses were conducted using R software (version 4.3.2; R Core Team, 2023). The data were fitted to both probit and logit models, and the probit model provided the best fit for most tests and was therefore selected for subsequent statistical analyses (Supplementary Information 2, SI2). EC₅₀ values were calculated using DCF concentrations measured at the beginning of the experiments. Geometric mean (GM) and geometric standard deviation (GSD) were calculated for each experimental group. Prior to statistical analysis, EC₅₀ values were ln-transformed to meet the assumptions of normality and homogeneity of variance. The Shapiro-Wilk test was used to confirm the normal distribution of the transformed data, and Levene's test was employed to verify the homogeneity of variances across groups. A two-way analysis of variance (ANOVA) was then conducted to evaluate the main effects of medium type and neonate source and preparation procedure and their interaction on the ln-transformed EC₅₀ values. Following ANOVA, Tukey's Honestly Significant Difference (HSD) post hoc test was used for multiple comparisons between individual groups. Differences were considered statistically significant at p < 0.05. All statistical analyses were performed using Statistica 13.1 software.

3. Results

3.1. The main differences between the natural water and the M4 growth medium

The results of NF temperature, pH, conductivity, and dissolved oxygen content are presented in **Table S2** (SI1). The maximum recorded temperature was 24 °C in June. The pH of the water ranged from 6.74 to 8.40 (median: 7.36), conductivity ranged from 457 to 901 µS cm⁻¹ (median: 594 µS cm⁻¹), and dissolved oxygen ranged from 5.0 to 8.1 mg L⁻¹ (median: 5.8 mg L⁻¹). The physicochemical properties of NF and M4 medium underwent only slight changes during the 48 h acute test (**Table S5**, SI1). In both systems, the pH and dissolved oxygen concentration remained essentially stable, while electrical conductivity showed moderate changes over the course of the experiment. Detailed ranges and median values of the physicochemical parameters for both

Table 1

Set of tests (number of independent experimental series) performed in this study, with test water (NF - natural freshwater or M4 growth medium) and neonate source and preparation procedure (EE-Neo - ex-ephippial neonates, P-Neo - neonates obtained from long-term laboratory culture maintained by parthenogenetic reproduction).

Test set name (number of independent repetitions)	Test water	Neonate source/preparation procedure
A (3)	M4	EE-Neo
B (3)	M4	P-Neo
C (3)	NF	EE-Neo
D (3)	NF	P-Neo

systems are presented in **Table S5** (SI1). The test vessels were not aerated during the exposure period. Overall, NF exhibited greater natural temporal variability in physicochemical parameters compared to the more stable M4 medium, which reflects the natural variability observed in the tested freshwater system.

Figure S3 (SI1) presents the concentrations of anions during the tested period in the natural freshwater, while **Table 2** shows the mean values. The F⁻ concentration in NF and M4 was 0.30 ± 0.12 mg L⁻¹ and 0.86 mg L⁻¹, respectively. The level of fluorine in natural freshwater typically ranges from 0.01 to 0.30 mg L⁻¹. Thus, the fluoride concentration was higher in M4 compared to NF. The Cl⁻ concentration in NF and M4 was 51.32 ± 4.18 mg L⁻¹ and 146.96 mg L⁻¹, respectively. This indicates that the synthetic medium contains much higher chloride levels compared to NF. For freshwater, chloride concentrations should not exceed 100 – 200 mg L⁻¹ (Hong et al., 2023; Nagpal and British Columbia. Water, 2003), meaning that both types of water have acceptable chloride levels.

The SO₄²⁻ concentration in NF and M4 was 22.48 ± 6.04 mg L⁻¹ and 52.16 mg L⁻¹, respectively. The sulfate level in the artificial growth medium is about twice as high as in NF. In Poland, the maximum acceptable sulfate concentration for first-class water is 80 mg L⁻¹ (Ministry of the Environment Poland, 2021).

The phosphates in M4 were below LOQ (0.25 mg L⁻¹), while in NF they were below LOD (0.02 mg L⁻¹), except for one sampling date with 0.37 mg L⁻¹. The nitrates were quantified only in one NF sample. Therefore, both M4 and NF can be characterized as oligotrophic.

Figure S4 (SI1) presents the concentrations of cations in M4 and NF on each sampling date. The concentrations of selected cations (mean values for the monitoring period in the case of NF) are presented in **Table 2**, together with values for M4 growth medium. The concentrations of sodium and potassium were lower in M4, while the calcium concentration was higher in M4 compared to NF. The magnesium concentration was similar in both waters. After the addition of DCF salt, sodium cation concentrations increased by 7.87 mg L⁻¹ in the case of a 100 mg L⁻¹ nominal DCF solution.

In conclusion, the pH, oxygen level, and conductivity were quite similar between NF and M4, with a higher variation of values observed for NF. The concentrations of fluorides, chlorides, sulfates, calcium, and magnesium were higher in the M4 medium. The concentrations of sodium and potassium cations were higher in NF water. The tested NF had physicochemical parameters within the ranges recommended by OECD 202 for pH, conductivity, and dissolved oxygen. However, other characteristics of NF, such as humic acids and other types of dissolved organic matter, were not characterized. These additional chemicals can potentially contribute to changes in DCF toxicity.

Table 2

The concentration of selected anions and cations in natural freshwater (NF) (mean from the period of monitoring, March to November 2023) and growth medium (M4) determined by ion chromatography.

Analyte	Natural water (NF)	M4 growth medium	NF/M4 ratio
Anions			
Fluorides	0.30 ± 0.12 mg L ⁻¹	0.86 mg L ⁻¹	0.35
Chlorides	51.32 ± 4.18 mg L ⁻¹	146.96 mg L ⁻¹	0.35
Sulfates	22.48 ± 6.04 mg L ⁻¹	52.16 mg L ⁻¹	0.43
Phosphates	<LOQ	<LOD (0.02 mg L ⁻¹)	-
Nitrates	Detected in one sample, 1.1 mg L ⁻¹	< LOD (0.62 mg L ⁻¹)	-
Cations			
Sodium	36.71 ± 5.22 mg L ⁻¹	14.94 mg L ⁻¹	2.46
Potassium	8.68 ± 0.76 mg L ⁻¹	3.00 mg L ⁻¹	2.80
Calcium	23.69 ± 2.13 mg L ⁻¹	49.73 mg L ⁻¹	0.47
Magnesium	7.74 ± 0.51 mg L ⁻¹	7.88 mg L ⁻¹	0.98

3.2. Acute toxicity of DCF toward *D. magna*

The percentage of DCF remaining after 48 h, relative to the initial measured concentration, ranged from 88.21% to 110.63% across the toxicity tests (**Table S6**; **Figure S5**, SI1). The smallest change in DCF concentration was observed in the abiotic control (nominal concentration 100 mg L⁻¹), where $100.81 \pm 0.92\%$ of the initial DCF concentration remained after 48 h.

Geometric mean EC₅₀ values and their natural ln-transformed values for all experimental groups are presented in **Table 3**. Before conducting the two-way ANOVA, the assumptions of normal distribution and homogeneity of variances were verified. The Shapiro-Wilk test did not reveal any significant deviation from a normal distribution - for all groups studied, $p > 0.05$ (SI2). Furthermore, Levene's test confirmed homogeneity of variance ($F(3, 8) = 1.911$; $p = 0.206$). Using two-way ANOVA, statistically significant main effects of medium ($F(1, 8) = 50.76$; $p = 0.001$; partial $\eta^2 = 0.86$) and neonate source and preparation procedure ($F(1, 8) = 24.08$; $p = 0.001$; partial $\eta^2 = 0.75$) on EC₅₀ were observed. No significant interaction between medium and neonate source and preparation procedure was found ($F(1, 8) = 0.15$; $p = 0.71$; partial $\eta^2 = 0.02$). Post-hoc comparisons using Tukey's HSD test (SI2) revealed that daphnids tested in NF had significantly higher EC₅₀ values than those in M4 medium, regardless of neonate source and preparation procedure (for EE-Neo: $p = 0.006$; for P-Neo: $p = 0.003$). Furthermore, in NF, daphnids from P-Neo exhibited significantly higher EC₅₀ values than those from EE-Neo ($p = 0.024$), while in M4 medium, this difference was not statistically significant ($p = 0.051$). The EC₅₀ values (mg L⁻¹) for each test group, along with an indication of statistical significance between the tests, are shown in **Fig. 1**.

4. Discussion

The literature reports a wide range of EC₅₀ values for DCF toward *D. magna*, ranging from 22.4 to 123.3 mg L⁻¹ (**Table S1**, SI1). This indicates that even when similar organisms and protocols are used, ecotoxicity results may vary by up to one order of magnitude. Comparable variability in GM EC₅₀ values was also observed in our test set (**Table 3**), where the obtained values ranged from 34.86 mg L⁻¹ (EE-Neo in M4 medium) to 90.52 mg L⁻¹ (P-Neo in NF). These results indicate that both medium and neonate source and preparation procedure had statistically significant effects on EC₅₀ values. The large partial η^2 values indicate substantial effects of both factors within the present factorial model. No statistically significant interaction was observed between medium and neonate source and preparation procedure. Thus, within the present dataset, there was no statistical evidence that the effect of one factor on EC₅₀ depended on the level of the other factor. However, the absence of a statistically significant interaction should not be interpreted as evidence that the two effects are intrinsically independent or stable across experimental conditions.

In general, the EC₅₀ values from different studies should not be directly compared because of the "laboratory-specific" sensitivity of daphnids strains (Persoone et al., 2009). For example, differences in chitin synthesis capacity were shown to cause varying sensitivities to diflubenzuron among three strains of *D. magna* (Kato et al., 2022). In

Table 3

Geometric mean EC₅₀ values (mg L⁻¹) with geometric standard deviations (GSD) and arithmetic mean ln(EC₅₀) $\pm$ standard deviation (SD) for *Daphnia magna* exposed to DCF in four experimental groups (A–D). GSD = $\exp(\text{SD of } \ln(\text{EC}_{50}))$.

Experimental groups	GM EC ₅₀ (mg L ⁻¹); GSD	mean ln(EC ₅₀) $\pm$ SD
(A) M4 EE-Neo	34.86; 1.15	3.55 $\pm$ 0.14
(B) M4 P-Neo	49.90; 1.26	3.91 $\pm$ 0.23
(C) NF EE-Neo	59.49; 1.07	4.09 $\pm$ 0.07
(D) NF P-Neo	90.52; 1.02	4.51 $\pm$ 0.02

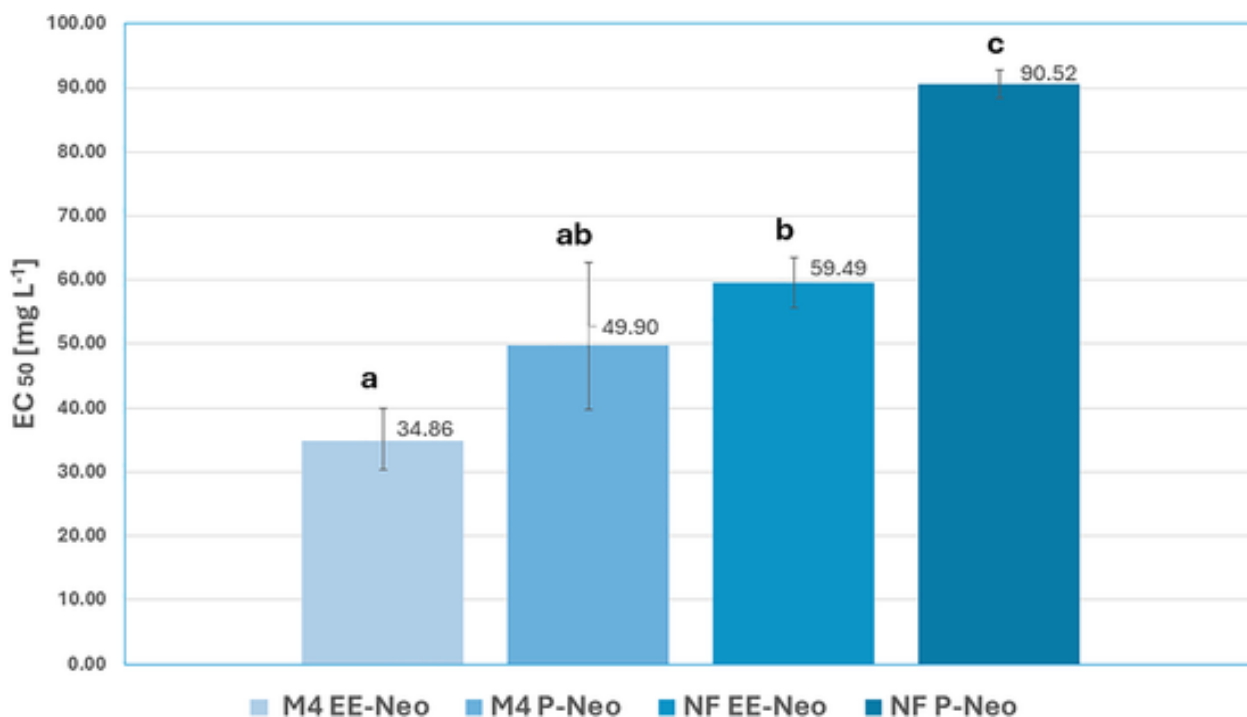


Fig. 1. EC₅₀ values [mg L⁻¹] for DCF in the experimental sets described in Table 1. Data are presented as geometric mean $\pm$ geometric standard deviation (GSD). Statistically significant differences (Tukey's post-hoc test) are indicated by lowercase letters.

the present study, the daphnids used to establish the P-Neo culture and the ephippia used to obtain EE-Neo originated from the same commercial supplier. Therefore, the genetic background of both groups was likely comparable, although their culture histories differed. Nevertheless, potential differences in test performance must also be considered. One factor is the variable quality of purchased ephippia, as batch consistency cannot be guaranteed. Moreover, the methods used by manufacturers to induce ephippia production are not always transparent. Ephippia formation is generally triggered by biotic or abiotic stressors (Pietrzak and Slusarczyk, 2006). In our experience, daphnids hatched from ephippia occasionally emerged later than expected, and sporadic mortality of EE-Neo individuals was observed in control groups. However, tests that did not meet validity criteria were excluded from further analysis. In contrast, daphnids maintained in laboratory cultures generally exhibited better fitness, with low mortality in control groups. This difference in overall fitness may have contributed to the differences in DCF sensitivity observed in this study.

Natural waters exhibit substantial variability in physicochemical properties, which may influence observed toxicity patterns (Pinheiro et al., 2021). The tested NF physicochemical parameters were within the recommended range for test water according to OECD 202. In addition to the recommended parameters, we tested the ionic composition over the monitoring period. Recent observations in a wastewater treatment plant in Swarzewo (Poland) revealed abundant populations of *Daphnia pulex* inhabiting clarifiers containing relatively high DCF concentrations (mean close to 2.5 $\mu\text{g L}^{-1}$), among other NSAIDs, under atypical physicochemical conditions including high salinity and low oxygen content (Bethke et al., 2024). Risk quotient estimates based on the DCF concentrations measured in the sampled waters indicated medium risk when the Predicted No-Effect Concentration (PNEC) for *D. magna* was applied and high risk when the Lowest Observed Effect Concentration (LOEC) for *Daphnia dubia* was used. Nevertheless, large populations of *D. pulex* were observed in the clarifiers under these conditions. These observations suggest that daphnids may occur and function under envi-

ronmental conditions that differ substantially from those used in standardized laboratory tests.

The differences between the target NF and M4 media also warrant closer examination. Species of the genus *Daphnia* inhabit a wide variety of freshwater environments, including temporary pools, and can tolerate substantial variation in water chemistry. Typically, daphnids thrive in waters with pH values between 6.5 and 9.5, with optimal conditions ranging from 7.2 to 8.5. Salinity is generally tolerated below 5‰ seawater (~1.5 g sea salt L⁻¹), although some species, including *D. magna*, can survive at salinities up to 20‰ seawater (Ebert, 2005). Both NF and M4 media used in this study fall within the optimal pH and salinity ranges for *D. magna* cultivation and testing. However, as shown in Table 2, the media differ primarily in their ionic composition. For compounds such as DCF, the ionic strength of the solution may play an important role because it can influence chemical speciation and thus bioavailability. Differences in sodium and potassium concentrations between the media may alter osmotic gradients, potentially affecting ion transport processes, chemical uptake, and physiological stress in aquatic crustaceans such as *D. magna* (as observed for other crustaceans (Henry et al., 2012)). Therefore, the observed differences in ionic composition between the media may have contributed to the variation in EC₅₀ values obtained in our experiments (Table 3). However, assessing this effect requires additional research focused directly on the relationship between salinity and DCF uptake. The organic matter present in NF was not characterized, and its potential contribution to the observed differences in DCF toxicity cannot be excluded. It was noted that DCF binds to dissolved organic matter (Rizzuto et al., 2021), which reduces its bioavailable fraction. In addition, natural organic matter (including humic acids) competes with pharmaceutical molecules in sorption processes (Chen et al., 2025), and can affect DCF uptake by *D. magna* (Kovacevic et al., 2018). Therefore, we recommend measuring the amount of humic acids when using test water from natural reservoirs.

Another potential stress factor for daphnids is food availability. According to OECD Guideline 211 ("*Daphnia magna* Reproduction Test"), the recommended algal food supply ranges between 0.1 and 0.2 mg C

per test organism per day. In the present study, the biomass of *C. vulgaris* supplied per individual was verified by correlating total organic carbon (TOC) measurements with the optical density (OD) of the *C. vulgaris* culture. Proper feeding is an important factor in successful cultivation, but also in acute test performance. Adequate feeding is essential because starvation can significantly increase the sensitivity of daphnids to pollutants, including metals, pesticides, and pharmaceuticals (Nielsen and Roslev, 2018; Pavlaki et al., 2014). This factor was not tested in our study. However, both neonate groups were fed before the test using the same feeding procedures.

The life cycle of Cladocera is well established and relevant for interpreting the observed differences. Ehippia (resting eggs) are typically produced through sexual reproduction, although exceptions have been reported (Ebert, 2005). Environmental triggers for sexual reproduction include shortened photoperiods, reduced temperature, high population density, and limited food availability. From these resting eggs, only females hatch, which subsequently reproduce asexually via parthenogenesis. The number of eggs per brood depends on the species and may exceed 100 in *D. magna*. Hatching occurs within approximately one day, after which embryos remain in the brood chamber for further development and are released as neonates after 2–3 days. Importantly, the brood chamber is in direct contact with the external environment (Morris and O'Donnell, 2019). Morphologically, neonates resemble mature individuals, except for the absence of a brood chamber. Consequently, in tests using P-Neo, the test organisms are approximately 3–4 days old, whereas EE-Neo individuals derived from ehippia are typically about 1 day old. Therefore, the observed differences between EE-Neo and P-Neo (Table 3) may partially reflect differences in developmental stage, which cannot be fully separated from other experimental factors in the present study. However, we could not identify definitive information on physiological or fitness differences between neonates originating from ehippia and those produced by parthenogenetic mothers. In this context, differences in sensitivity to DCF between the two study groups should be interpreted as resulting from a combination of factors, including differences in embryonic development, hatching physiology, general organismal condition, and the procedures used to prepare the organisms for the experiment. Consequently, this study should be regarded as a comparison of test organisms obtained from different sources and prepared differently for the test, rather than as an experimental separation of the effects of ehippia versus parthenogenetic origin.

Interestingly, several studies have proposed alternative ecotoxicological test designs using *D. magna* embryos within the brood chamber as rapid substitutes for conventional 21-day reproduction assays (Abe et al., 2001; Ohta et al., 1998; Sobral et al., 2001). In some cases, embryos were shown to be more sensitive than neonates. For example, Abe et al. (2001) reported higher embryonic sensitivity to aniline derivatives, while Pereira et al. (2020) demonstrated that iron oxide nanoparticles were non-toxic to adult daphnids but lethal to embryos. These reports are consistent with our observation that the two neonate groups differed in their susceptibility to DCF. However, the cited studies were conducted solely on parthenogenetic neonates.

The pathways by which chemicals enter daphnids - through absorption across external surfaces or via ingestion and the digestive tract - likely differ among compounds. For example, toxic metals such as cadmium, lead, and strontium accumulate primarily in the exoskeleton, whereas selenium accumulates in the cytoplasm (Smirnov, 1983). Much less is known about the bioaccumulation of organic contaminants in *D. magna*. In our recent study, the in-situ bioaccumulation of DCF in Cladocera was found to be relatively low ($BCF = 36 \text{ L kg}^{-1}$ wet mass), despite elevated concentrations in wastewater (Bethke et al., 2024). This suggests that daphnids may have limited accumulation of DCF, although the mechanisms responsible for this pattern are not clear. It is possible that the developmental stage of the daphnids also contributes to differences in bioaccumulation. However, testing this hypothesis is

beyond the scope of the present study. We did not observe statistically significant differences in the DCF decline between test water types (Figure S5, SI). The low number and biomass of the test organisms prevented us from performing a bioaccumulation analysis to examine differences in DCF sorption by neonates during the acute test. However, it is possible that test water composition affects DCF uptake and may contribute to the observed differences in acute toxicity. The body of cladocerans is enclosed within an uncalcified shell known as the carapace, which consists of a double wall through which hemolymph flows and which forms part of the body cavity (Ebert, 2005). The carapace is composed primarily of the polysaccharide chitin (Smirnov, 1983) and has relatively low permeability. Consequently, the uptake of DCF via ingestion and the digestive tract is likely more significant than passive diffusion across the carapace. To date, no studies have directly compared the two discussed pathways of pharmaceutical uptake in *D. magna*. For this crustacean, an additional uptake route should be considered. *D. magna* maintains a lower internal salt concentration than the surrounding water and therefore actively absorbs ions through chloride-absorbing glands and the nuchal organ (Morris and O'Donnell, 2019). Additional structures, such as shell glands and pores located mainly on the head, may participate in excretion and/or osmoregulation (Smirnov 2014). These organs could potentially represent sites of DCF interaction. However, we could not identify studies directly evaluating which pathway is responsible for DCF uptake in *D. magna*.

The mechanisms underlying DCF toxicity in *D. magna* have been investigated previously; however, mostly in chronic exposure. For example, increased malondialdehyde levels in DCF-exposed daphnids ($500 \mu\text{g L}^{-1}$) indicate overproduction of reactive oxygen species and the induction of oxidative stress (Nkoom et al., 2019). Exposure to $50 \mu\text{g L}^{-1}$ DCF has also been shown to induce the expression of detoxification-related genes in daphnids (Liu et al., 2017). Furthermore, DCF may act as an endocrine disruptor (Liu et al., 2017), which is consistent with reproductive effects observed in chronic toxicity studies (Lee et al., 2011; Nkoom et al., 2022). Also, our previous work has shown that prolonged exposure to DCF can negatively affect *D. magna*, particularly by disrupting reproductive processes (Bethke and Caban, 2025). As a non-steroidal anti-inflammatory drug, DCF may indirectly disrupt endocrine processes in daphnids by inhibiting prostaglandin synthesis, which is essential for the regulation of reproduction in young individuals. However, in the acute test with DCF, the mortality (immobilization) is probably due to a mechanism other than endocrine disruption. Having in mind the proportion of DCF and neonate mass in a single test well, the relatively high EC_{50} values obtained in the present study indicate that relatively high DCF concentrations were required to cause immobilization during short-term exposure. Chemicals may also affect organisms indirectly by altering their associated microbiota. However, because acute toxicity tests involve relatively short exposure periods, adverse effects mediated through microbiome alterations are unlikely to be observed. Therefore, the exact mechanism responsible for immobilization in our tests cannot be determined from the present study. Understanding this mechanism would help explain the change in DCF toxicity.

5. Conclusions

The results support the hypothesis that DCF toxicity toward *D. magna* differs between the tested natural freshwater and M4 medium and between neonate source and preparation procedure (EE-Neo versus P-Neo). The highest resistance (highest EC_{50}) was observed in P-Neo tested in NF ($GM = 90.52 \text{ mg L}^{-1}$, $GSD = 1.02$), while the lowest was observed in EE-Neo in M4 medium ($GM = 34.86 \text{ mg L}^{-1}$, $GSD = 1.15$). While the physicochemical characteristics of M4 and NF are well defined, no single chemical factor could fully explain the observed differences in EC_{50} . The differences in neonate age and conditions may contribute to the variation in sensitivity between EE-Neo and P-Neo. Over-

all, both biological and physicochemical factors may influence the response of *D. magna* to DCF.

Our findings highlight that variability in the source and preparation of the test daphnids and medium composition may contribute to differences in ecotoxicological endpoints obtained for the same chemical under different conditions. Consequently, greater attention to the standardization and reporting of culture conditions, neonate source, and exposure media may improve the comparability and reproducibility of *Daphnia*-based toxicity tests. The choice of test medium and the source and preparation procedure of the test neonates should therefore be considered when planning studies and comparing results between laboratories.

Uncited references

Gworek et al., 2019; Jonczyk and Gilron, 2005; Kikuchi et al., 2017; Sison-Mangus et al., 2015; Ulm et al., 2000.

CRedit authorship contribution statement

Katarzyna Bethke: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Magda Caban:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Magda Caban reports financial support was provided by National Science Centre Poland. If there are other authors, they 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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Data availability

The data that support the findings of this study are openly available in Repozytorium.bg.ug.edu.pl at doi 10.71853/gwj-nh56.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2026.108029.

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Supplementary Information

to

**Context-Dependent Toxicity of Diclofenac in *Daphnia magna*: Roles of Water Chemistry,
Neonate Source and Preparation Procedure**

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Table S1. A collection of scientific papers evaluating the toxicity of diclofenac to cladoceran species, including the reference protocols, origin of test organisms, endpoints assessed, and test media used in these studies

Species of crustacea	Reference protocol	Origin of daphnia	Test water	Endpoint value [mg L ⁻¹] (48 h)	Ref.
<i>D. magna</i>	ISO 6341:1996/Cor.1:1998: 'Water quality – Determination of the inhibition of the mobility of <i>D. magna</i> Straus (Cladocera, Crustacea)'	Not specified	Not specified	EC ₅₀ 67.0	(Quinn et al., 2011)
<i>D. magna</i>	OECD 202	Own culture	Reconstructed hard water, pH 7.8	EC ₅₀ 39.9	(Haap et al., 2008)
<i>D. magna</i>	French Standard Association. 1996. Water quality - Determination of the inhibition of the mobility of <i>Daphnia magna</i> Straus (Cladocera, Crustacea) - Acute toxicity test. T90-301. AFNOR, Paris, France.	Own culture	Reconstructed hard water pH not specied	EC ₅₀ 22.4	(Ferrari et al., 2003)
<i>D. magna</i>	Commission of the European Communities, 1992. Methods for determination of ecotoxicity; Annex V, C.2, <i>Daphnia</i> , acute toxicity to <i>Daphnia</i> . L 383A, 172–178. EEC Directive 92/69/EEC.	Own culture	ADaM, a culture medium imitating natural fresh water pH not specified	EC ₅₀ 68	(Cleuvers, 2003)
<i>D. magna</i>	U.S. Environmental Protection Agency. 1993. Method for measuring the acute toxicity of effluents and receiving waters to	Own culture	Reconstituted hard water, pH 7.8	EC ₅₀ 80.1	(Han et al., 2006)

	freshwater and marine organism. EPA/600/4-90/027F.				
<i>D. magna</i>	OECD 202	Own culture	Synthetic ASTM hard water medium	EC ₅₀ 123.3	(De Oliveira et al., 2016)
<i>D. magna</i>	OECD 202	Own culture	Not specified, 7.61 ± 0.35	LC ₅₀ 18.1 ± 1.26 mg/L	(Du et al., 2016)
<i>D. similis</i>	Not specified	Not specified	Not specified	EC ₅₀ 46 mg/L	(De Castro et al., 2014)
<i>D. magna</i>	Not specified	Own culture	Synthetic medium based on ultrapure water, trace elements, and phosphate buffer.	NOEC 0.36 (reproduction)	(Dietrich et al., 2010)
<i>D. dubia</i>	US EPA-600–4-90, 1993).	Not specified	Not specified	LC ₅₀ 12.71	(Russo et al., 2023)
<i>Ceriodaphnia dubia</i>	US EPA 600/4_90/027, 1991	Own culture	Moderately hard synthetic water MHW(hardness: 80 mg CaCO ₃ /L to 100 mg CaCO ₃ /L)	NOEC 1 (7 days, reproduction) PNEC 580 ug/L	(Ferrari et al., 2003)
<i>D. magna</i>	OECD 202	Own culture	The Daphnia medium consisted of CaCl ₂ , KCl, NaHCO ₃ and MgSO ₂ .	Lowest observed adverse effect level (LOAEL) 9.7 mg/L (48 h	(Gómez-Oliván et al., 2014)

Table S2. Results of measurements of air temperature, water temperature, pH, conductivity, and dissolved oxygen content in a natural pond on the university campus, recorded on specific sampling dates (dates in blues – cation and anions were measured)

Date	Air temperature [°C]	Water temperature [°C]	pH	Conductivity [μS]	Oxygenation [mg L⁻¹]
03.03.2023	6	3	8.25	805	-
10.03.2023	0	0	7.42	884	6.3
17.03.2023	4	3	7.89	755	6.6
24.03.2023	11	9	7.02	637	7.1
31.03.2023	11	8	6.76	651	7.0
14.04.2023	8	13	7.23	575	7.7
20.04.2023	10	11	8.07	603	8.1
27.04.2023	10	10	7.02	628	5.2
05.05.2023	10	14	6.88	585	5.8
12.05.2023	20	19	7.74	480	5.6
18.05.2023	15	13	6.74	548	5.2
25.05.2023	20	22	7.60	457	5.7
01.06.2023	20	22	7.31	548	5.1
07.06.2023	21	22	6.98	526	5.1
23.06.2023	21	23	6.88	525	5.0
26.06.2023	23	24	7.30	519	5.2
11.10.2023	16	11	8.19	901	6.2
20.10.2023	5	3	8.19	802	5.3
06.11.2023	4	8	8.40	612	8.1
16.11.2023	5	3	8.24	558	8.1

Table S3. Validation parameters for ion chromatography method of selected anions and cations

Ion	Concentration range [µg mL⁻¹]	Precision (RSD, %)	Accuracy [%]	Instrumental quantification limit MQL [mg L⁻¹]	Instrumental detection limit MDL (MQL/3) [mg L⁻¹]	R²
<i>Anions</i>						
F⁻	0.09- 3.00	0.02-0.05	97.00-111.10	0.19	0.06	1.00
Cl⁻	0.31-10.00	0.00-0.04	92.20-109.90	0.63	0.02	1.00
PO₄³⁻	0.63-20.00	0.00-0.03	81.80-99.30	0.63	0.02	1.00
Br⁻	0.63-20.000	0.00-0.04	96.30-100.70	0.63	0.02	1.00
NO₃⁻	0.93-30.00	0.00-0.39	91.80-100.70	1.88	0.63	1.00
SO₄²⁻	0.63-20.00	0.00-0.08	83.30-103.30	0.63	0.02	1.00
<i>Cations</i>						
Na⁺	0.02-100.00	0.00-0.04	98.00-106.30	12.50	4.17	1.00
K⁺		0.00-0.04	98.70-114.20	0.78	0.26	1.00
Ca²⁺		0.00-0.12	81.00-113.86	0.3900	0.1300	1.00
Mg⁺		0.00-0.76	97.60-115.30	0.7800	0.2600	1.00

Table S4. Validation parameters for HPLC/DAD method of diclofenac determination

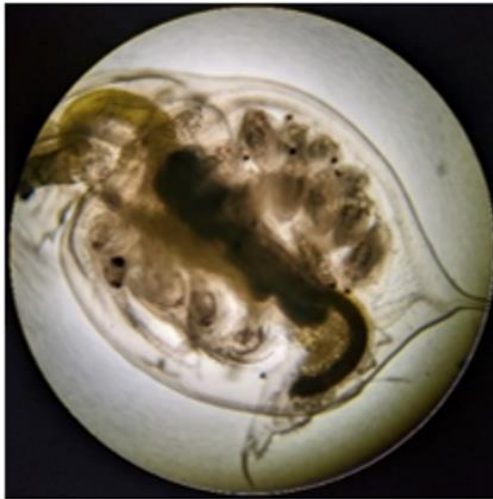
Instrumental quantification limit MQL [$\mu\text{g mL}^{-1}$]	Instrumental detection limit MDL (MQL/3) [$\mu\text{g mL}^{-1}$]	Precision [RSD, %]	Accuracy [%]	Linearity range [$\mu\text{g/mL}$]	R^2
0.024	0.008	0.02 - 0.29	75.00 – 106.00	0.024 – 150.00	0.9999

Table S5. Physicochemical parameters (pH, conductivity ($\mu\text{S cm}^{-1}$), and dissolved oxygen concentration (mg L^{-1}) recorded during acute toxicity tests on Day 1 and after 48 h for control and diclofenac (DCF) exposure groups in M4 medium and natural pond water (NF)

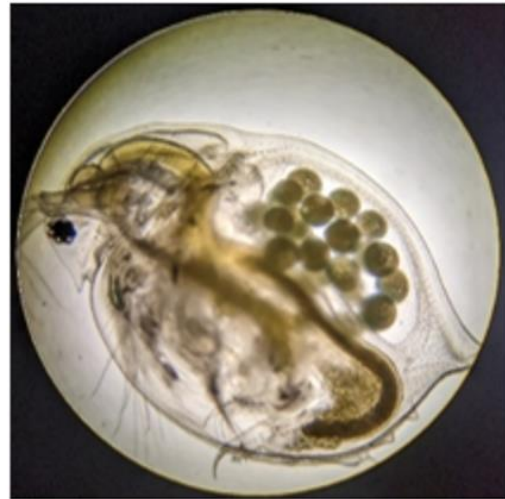
Medium	Group	Time	pH (median)	Conductivity ($\mu\text{S cm}^{-1}$) (median)	O ₂ (mg L^{-1}) (median)
M4	Control	0 h	7.98-8.03 (8.00)	578-601 (585)	7.1-8.2 (7.6)
M4	Control	48 h	7.76-7.92 (7.83)	386-607 (537)	7.2-7.2 (7.2)
M4	Exposure (DCF)	0 h	7.94-8.10 (7.99)	529-808 (593)	7.1-8.1 (7.6)
M4	Exposure (DCF)	48 h	7.58-8.05 (7.84)	374-723 (541)	7.1-7.8 (7.4)
NF	Control	0 h	7.98-8.04 (8.00)	339-702 (446)	7.1-9.2 (7.9)
NF	Control	48 h	8.21-8.28 (8.23)	250-517 (298)	7.1-7.8 (7.5)
NF	Exposure (DCF)	0 h	7.95-8.07 (8.03)	337-593 (401)	7.1-9.3 (7.9)
NF	Exposure (DCF)	48 h	8.16-8.43 (8.27)	205-467 (328)	7.1-8.0 (7.4)

Table S6. DCF concentration changes during the 48-h exposure under the four experimental conditions; values are presented as mean DCF remaining after 48 h (% of initial concentration) $\pm$ standard deviation (SD). n indicates the number of independent experiments for which measurements at both the beginning and after 48 h of exposure were available. Abiotic controls were prepared at a nominal DCF concentration of 100 mg L⁻¹

Medium and source/preparation	Nominal DCF concentration [mg L ⁻¹]	Mean DCF remaining after 48 h (% of initial concentration)	SD	n
M4 EE-Neo	1	88.21	12.12	3
	10	97.55	1.87	3
	25	95.26	3.37	3
	50	95.78	4.04	3
	100	95.73	2.44	3
NF EE-Neo	1	110.63	22.10	2
	10	91.63	5.99	3
	30	101.98	8.00	3
	50	97.78	3.56	3
	100	96.96	1.02	3
M4 P-Neo	1	93.05	1.27	3
	10	95.14	3.05	3
	30	97.10	0.13	2
	50	97.72	0.83	3
	100	98.35	0.53	3
NF P-Neo	50	88.68	4.19	3
	75	92.39	1.94	2
	100	96.08	1.79	3
	125	93.28	9.43	2
	150	99.45	5.40	2
Abiotic control (without <i>Daphnia magna</i>)	100	100.81	0.92	7



Medium M4



Natural water

Figure S1. Microscope images of *Daphnia magna* from different culture media

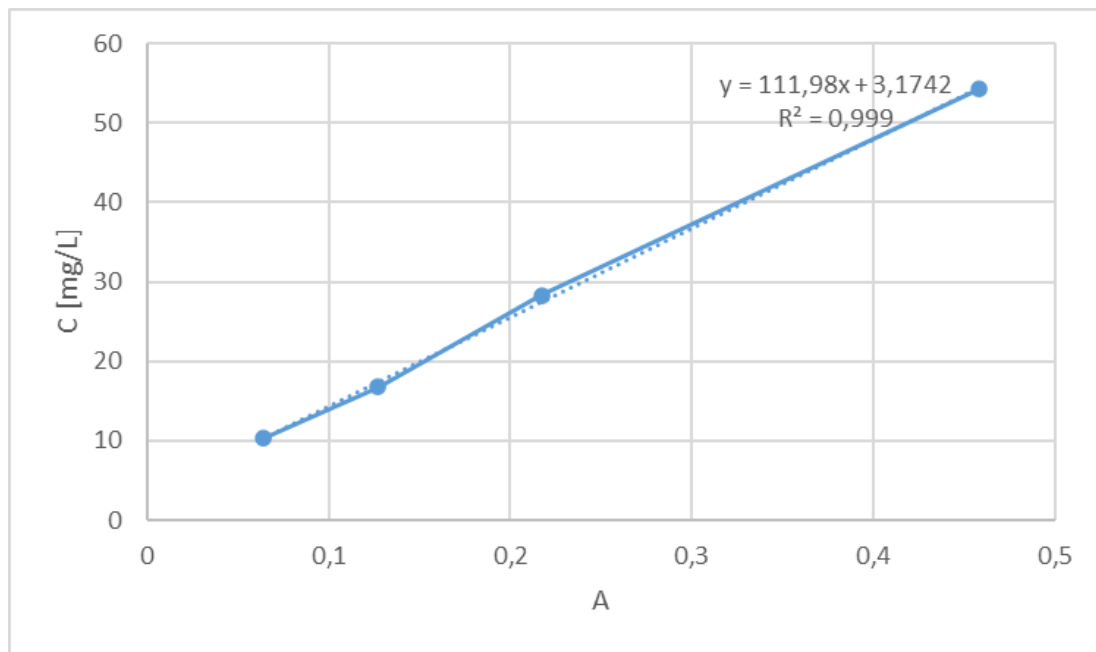


Figure S2. Graph showing the dependence of organic carbon concentration [mg L⁻¹] on the absorbance (A) of a *Chlorella Vulgaris* culture



Figure S3. Concentrations of selected anions in the studied freshwater pond during the monitoring period in comparison to M4 growth medium

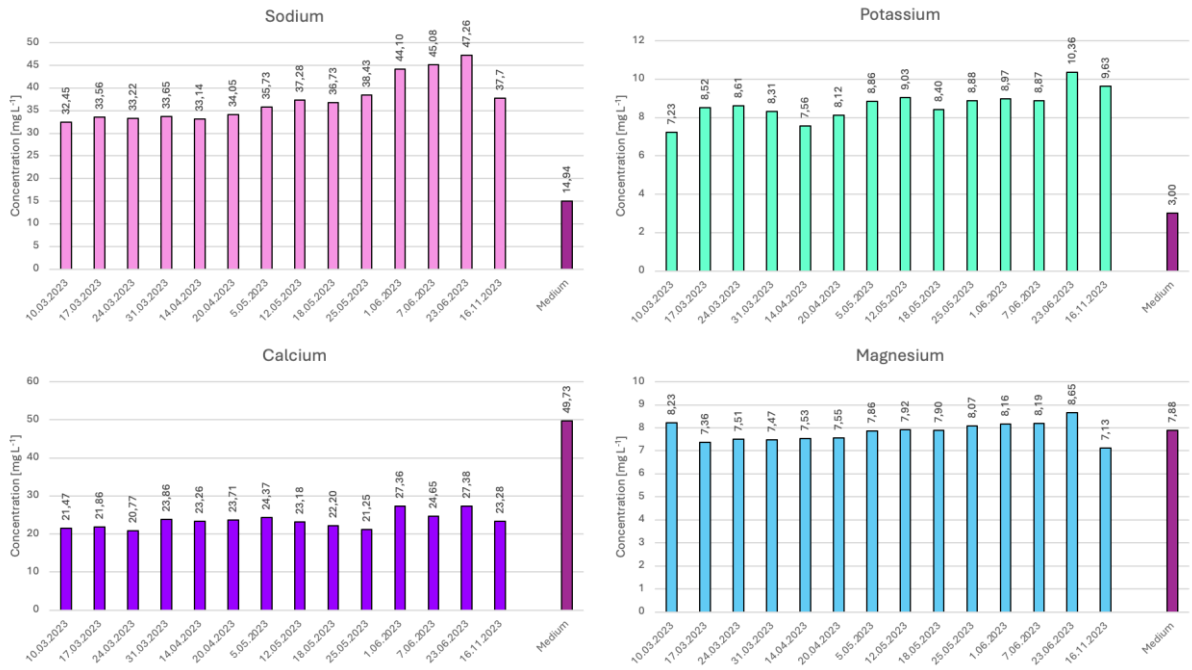


Figure S4. Concentrations of selected cations in the studied freshwater pond during the monitoring period in comparison to M4 growth medium

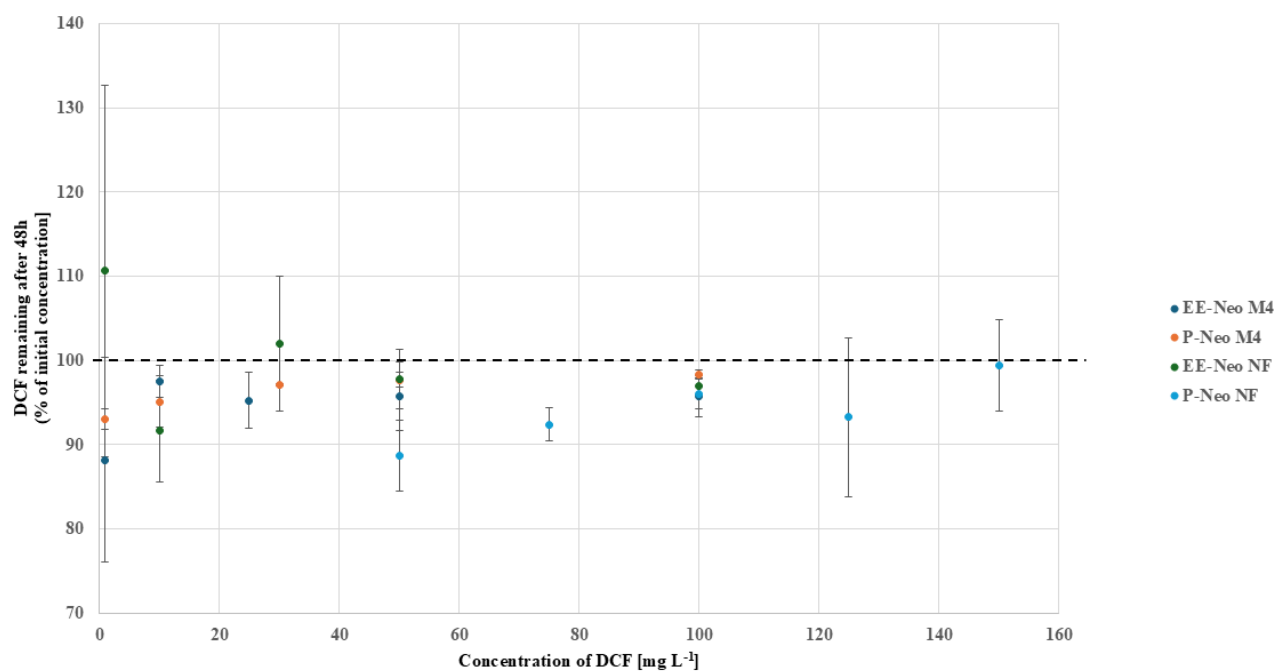


Figure S5. Diclofenac (DCF) remains after 48 h of exposure (% of initial concentration) under the four experimental conditions. Points represent mean values and error bars indicate standard deviation (SD); $n = 2-3$ independent experiments depending on DCF concentration. The dashed horizontal line at 100% indicates no change in DCF concentration during the 48 h exposure period

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