Daphnia as the "canary in the coalmine"

–

Effect-based methods for pollution assessment

This thesis is presented for the degree of

Doctor of Philosophy

By

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List of Abbreviations

ACP	Acid phosphatase
AI	Artificial intelligence
ALP	Alkaline phosphatase
AMPA	Aminomethylphosphonic acid
AOP	Adverse outcome pathways
ASA	Acetylsalicylic acid
BMIM	1-butyl-3-methylimidazolium
BPA	Bisphenol A
BSA	Bovine serum albumin
CA	Concentration addition
CAT	Catalase
CBB	Coomassie brilliant blue
CDNB	1-chloro-2,4-dinitrobenzene
CHL	Chloride
COX	Cyclooxygenase
CROT	*o*-octanoyltransferase
DIMS	Direct infusion mass spectrometry
DMSO	Dimethylsulfoxide
DOM	Dissolved organic matter
EBMs	Effect-based methods
EC	Effective concentration
EE2	17α-ethinylestradiol
Enz	Enzyme activities
FAP	Tris(pentafluoroethyl)trifluorophosphate
Feed	Feeding
FIA	Flow injection analysis
GC	Gass chromatography
GPx	Glutathione peroxidase
GST	Glutathione-S-transferase
HCL	Hierarchical clustering
HF	Hydrofluoric acid
HFA	Hexafluoroantimonate
HFP	Hexafluorophosphate
HIF	Hypoxia-inducible factor
IA	Independent action
IL	Ionic liquid
LC	Liquid chromatography
LDH	Lactate dehydrogenase
LIP	Lipase
LOD	Limit of detection
Met	Metabolomics
Mort	Mortality
MS	Mass spectrometry
MSF	Methanesulfonate
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
NAMs	New approach metholodogies

NM	Nanomaterial
NMR	Nuclear magnetic resonance
NOM	Natural organic matter
NSAIDs	Non-steroidal anti-inflammatory drugs
OECD	Organisation for Economic Co-operation and Development
OMIM	1-methyl-3-octyl-imidazolium
PC	Principal component
PCA	Principal component analysis
PEP	Peptidase
PGs	Prostaglandins
qPCR	Quantitative PCR
Rep	Reproduction
ROS	Reactive oxygen species
RT	Reduced thiols
SAM	Significant analysis of microarrays
SD	Standard deviation
SOD	Superoxide dismutase
SPE	Solid-phase extraction
Surv	Survival
TFB	Tetrafluoroborate
ToF	Time of flight
VOCs	Volatile organic compounds
WHO	World Health Organization
βGAL	beta-galactosidase

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A. The Effects of Single and Combined Stressors on Daphnids-Enzyme Markers of Physiology and Metabolomics Validate the Impact of Pollution - DOI: 10.3390/toxics10100604
B. Novel Approaches Methodologies in ecotoxicology - Metabolism reveals transgenerational effects of pollutants on daphnids – (under review)

Chapter 3:

A. Toxicity of "green solvents" - The impact of butyl methylimidazolium ionic liquids on daphnids – DOI:10.1016/j.jil.2023.100059

Chapter 4:

A. Acute and Transgenerational Effects of Non-Steroidal Anti-Inflammatory Drugs on *Daphnia magna* – DOI:10.3390/toxics11040320
B. Exposure to chemical and commercial forms of NSAIDs at environmentally relevant concentrations exert transgenerational metabolic responses in daphnids - DOI: 10.1016/j.watbs.2025.100404

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Thesis abstract

Daphnia as the “canary in the coalmine” – Effect-based methods for pollution assessment

Anna Michalaki

The presence of pollutants in the aquatic environment poses a serious threat for key species in the ecosystem. Conventional approaches to assess the quality of water rely on spot or grab samples and the detection of chemical pollutants in the water. Furthermore, the presence of flora and fauna in water is characteristic of its condition. Nevertheless, these approaches do not reflect a realistic assessment of water quality since they are limited not only in sensitivity but also in their ability to detect and cover a wide range of pollutants. This lack of realism has redirected predictive ecotoxicology and risk assessment towards effect-based methods. Effect-based tools are modern approaches employed in predictive ecotoxicology and risk assessment. These approaches utilize *in silico* approaches and model species, such as daphnids among others, to observe the effects of different chemical compounds and provide mechanistic insight over their actions. From the main categories of emerging pollutants encountered in the ecosystem, for the context of this thesis emphasis will be given to ionic liquids, and pharmaceutical compounds, as well as complex chemical mixtures.

Thesis outline

This first chapter is an introduction to freshwater ecotoxicology, risk assessment, the biology of daphnids, the research chapters, the methodological approaches of this thesis and summary of published papers. Modern approaches in mechanistic ecotoxicology rely on phenotypic, biochemical and holistic techniques to identify key responses to these emerging pollutants. Therefore, these molecular fingerprints can be used to identify water pollution early.

Following the introduction, the manuscripts related to the second chapter are presented. The second chapter was divided into two parts: the first part assessed the effects of eight single stressors and their mixture on daphnids using acute exposures, while the second part addressed the impact of several concentrations of the mixture using chronic and transgenerational exposures. Metabolomic analysis confirmed the results in both parts.

The manuscript related to the third chapter focusing on ionic liquids, the effects of 1-butyl-3-methylimidazolium (BMIM) ionic liquids (ILs) were addressed. Acute and chronic exposures were assessed with a combination of phenotypic and biochemical endpoints to describe their toxicity potential.

The fourth chapter dealt with pharmaceutical pollution. Two manuscripts were published. Hence this chapter is divided into two parts. The first part assessed the impact of two non-steroidal anti-inflammatory drugs (NSAIDs) on daphnids using acute and chronic and transgenerational exposures, and the second part evaluated the impact of chemical and commercial forms of NSAIDs through chronic and transgenerational exposures.

The last chapter focused on the impact of a three-chemicals mixture, and it began with laboratory studies to translate these findings to the actual environment using two rivers as representative matrices. For this yet unpublished chapter, daphnids were exposed in acute and chronic and transgenerational scenarios to capture non-lethal changes.

Finally, this thesis closed with the discussion of all the individual studies and their respective manuscripts.

To provide an overview of the experimental designs followed in this thesis, a summary table (Table 1) and a schematic workflow (Figure 1) are presented. Table 1 summarizes the exposure types, generations and ages of daphnids used, along with the chemicals and their concentrations, solvents, media and endpoints assessed in each study. Figure 1 illustrates the experimental workflow of this thesis, starting with the exposure types (acute, chronic, transgenerational), followed by the endpoints evaluated. Initially, the toxicity of each

chemical was assessed using toxicity curves to determine mortality. Subsequently, phenotypic endpoints such as survival, reproduction and feeding assay were evaluated, followed by the assessment of enzymatic activities in daphnids. In some studies, metabolomic analyses were also performed.

The experiments conducted in this thesis can be categorized in laboratory and environmental. Laboratory experiments served as screening tests to evaluate toxicity of chemicals, and also to provide mechanistic insights, allowing the selection of appropriate working concentrations. Environmental studies, using natural water, aimed to show that daphnids can detect differences between artificial laboratory media and natural water, highlighting their potential as the canary in the coalmine in early-warning pollution assessment (Abdullahi et al., 2022).

Table 1. Overview of experimental designs used in this thesis.

Chapter	Study	Exposure type	Generations	*Daphnia* (age)	Chemicals & doses	Solvent	Media	Endpoints
2A	8 single stressors & mixtures	Acute	-	D4	8 single stressors[1], EC_5 & 10-30% of EC_5	OECD	OECD	Mort, Enz, Met
2B	8 stressors mixtures	Acute/Chronic	5	D1 & D21	8 single chemical mixture[1], 1 ng/l-1000 µg/l	OECD	OECD	Mort, Enz, Feed, Met
3	BMIM Ionic Liquids	Acute/Chronic	1	D1, D4, D7, D14 & D21	BMIM ILs[2], EC_5 (Acute) & 1 mg/l (Chronic)	OECD	OECD	Mort, Enz, Feed, Rep
4A	NSAIDs	Acute/Chronic	3	D1, D5, D7, D14 & D21	Indomethacin, ibuprofen, 1 mg/l	DMSO	OECD	Mort, Enz, Feed
4B	NSAIDs & commercial forms	Acute/Chronic	5	D1 & D14	Indomethacin, ibuprofen (chemical & commercial forms), 5 µg/l	DMSO	OECD	Mort, Enz, Surv, Met
5	River water study	Acute/Chronic	2	D1 & D21	Lithium, metformin, glyphosate, 0.01-10 mg/l	OECD	OECD & river water	Mort, Enz, Feed, Met

[1]Chemicals included: Aluminium sulfate, Lithium chloride, Acetylsalicylic acid, propranolol, metformin, diltiazem, glyphosate, nicotine

[2]BMIM ILs: BMIM Hexafluorophosphate, BMIM Chloride, BMIM Tetrafluoroborate, BMIM Hexafluoroantimonate, BMIM Methanesulfonate

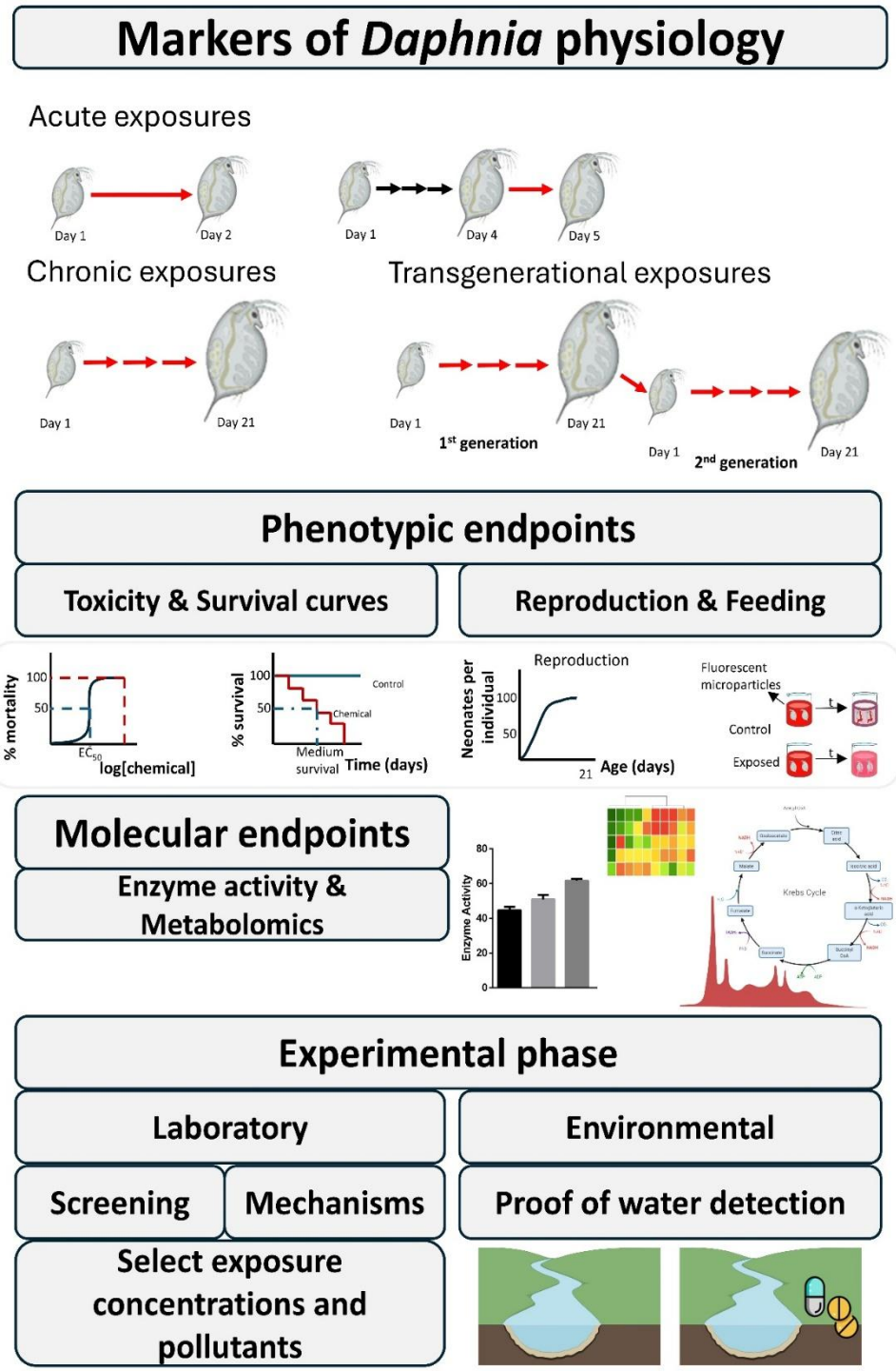

Figure 1. Graphical illustration of experimental design, followed in this research. The effects of pollutants on daphnids were assessed using following acute and chronic exposures. The acute exposure is divided into two parts. In the first part neonates were exposed directly to the chemical for 24h, while in the second part neonates were cultured up to day four and then were exposed for 24h. Regarding the chronic exposures, both single and multigenerational exposures were performed throughout this research. Following these exposures, phenotypic and molecular endpoints of daphnids physiology were performed for the evaluation of the pollutants' impact.

Chapter 1

Introduction

Background and Environmental Significance

Water pollution as a global issue

The increase of global population and the consumption of natural resources have led to a significant impact on the environment. As a result, the importance of safeguarding the terrestrial and aquatic environment has been highlighted as a priority globally (Ejiohuo et al., 2025). The concentrations of toxic chemicals, such as pharmaceutical compounds and novel materials such as ionic liquids among others in the aquatic environment are significantly increasing over the last decades. Until now, the quality of water in the aquatic environment is assessed by traditional methods, such as spot or grab samples, and comparisons with water quality standards (Escher et al., 2021). However, these methods have several weaknesses such as their cost for analytical instrumentation, their sensitivity limits, and the restriction that they can detect only a small number of possible pollutants and not the entire "iceberg" of the chemicals that could be present in the environment (Escher et al., 2021). Another approach to assess water quality focuses on the identification of flora and fauna present in the aquatic environment as indicators to assess its impact and health status (Escher et al., 2021). However, these approaches show a lack of realism because they are too late to provide early warnings before ecological damage is precarious. In addition, these approaches are not able to predict any future impact before the ecological damage has already occurred and is irreversible (Escher et al., 2021).

The resulting gap can be bridged and captured as a novel field; Systems Toxicology, which emerged in the interphase of analytical techniques and key species used as bioindicators to understand pollution (Escher et al., 2021, Sturla et al., 2014). This evolving field of research relies on key species and more sensitive molecular markers to identify the underlying biological processes responsible for toxicity mediated effects.

Complex chemical mixtures, novel materials such as ionic liquids, and pharmaceutical compounds are some of the chemicals that can easily be below the detection limits of traditional approaches and their responses from key species could play important means to assess pollution (Escher et al., 2021). Effectively the concept discussed in this thesis is the application of molecular and metabolic endpoints in freshwater species that can be used as early warning systems for pollution assessment.

Safeguard the aquatic environment – revolutionizing risk assessment

The assessment of the environmental exposure to toxic chemicals and their impact on biological systems is very important. Traditional pollution assessment approaches typically rely on chemical detection and analysis of the relationships between pollutant abundance and biodiversity, with results compared against established water quality standards (Ahmed et al., 2019). However, these methods possess several weaknesses (Madrid and Zayas, 2007). For example, a high cost for analytical quantification is a trade-off matched with the minimal detection of a limited number of possible toxic chemicals, as some chemicals may be present but not detected by a gap of a method or their concentration being lower than the detection limit. Additionally, these approaches fail to predict future impact before the ecological damage to the environment has occurred.

Systems Toxicology Approach is an innovative field of research which combines key species to gain biological information over the actions of pollutants (Escher et al., 2021, Sturla et al., 2014). This approach introduces more sensitive molecular markers of toxicity, markers of oxidative stress and holistic techniques. The evaluation of biological knowledge using genomic, metabolomic, transcriptomic and proteomic profiles and integrating holistic approaches is pivotal to describe the system studied in molecular detail. A System Biology approach of combining metabolomic with other omics data, to discover molecular information in toxicological key events would develop new adverse outcome pathways (AOPs). AOPs are useful to identify and characterize the environmental impacts of pollutants on organisms. AOPs represent a sequence of biological events leading to adverse effects with relevance to risk assessment (EPA, 2024) (Figure 2).

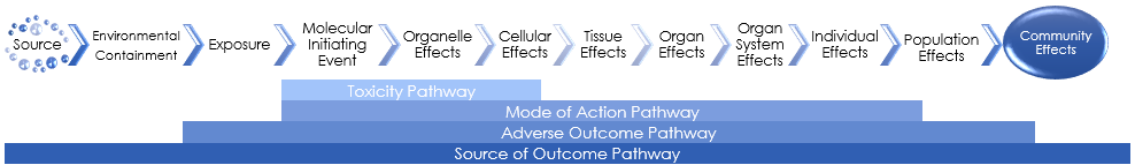

Figure 2. Schematic representation of adverse outcome pathways (AOPs) to discover toxicity mechanisms. AOPs link a chemical to a harmful effect by describing a sequence of biological events from the molecular to the organism level.

Effect-based methods (EBMs) and New Approach Methodologies (NAMs)

As ecotoxicology and risk assessment progress to handle more complex environmental challenges, new approaches reshape how we detect and deal with ecological risks. Among these, EBMs and NAMs are emerging as essential tools for understanding and safeguarding ecosystems (Brack et al., 2019, Cavoski et al., 2024). These methods provide greater

precision and depth than traditional approaches, delivering more precise, mechanism-based insights into the effects of pollutants on species and ecosystems.

EBMs evaluate biological responses to pollutants commonly using bioassays or biomarkers to detect a variety of harmful effects on organisms (Escher and Leusch, 2011). They are especially useful for evaluating environmental samples containing mixture of pollutants, as they can highlight cumulative or synergistic effects that traditional approaches (such as chemical analysis) may overlook (Brack et al., 2019). Additionally, EBMs aid in the early detection of ecological degradation, which promotes proactive environmental conservation (Pasanen-Kase et al., 2011).

On the other hand, NAMs represent a developing set of toxicology procedures invented to follow the 3Rs principle (Replacement, Reduction, and Refinement) with the goal of minimizing and eventually eliminating the use of animals in scientific research (Cavoski et al., 2024, Krewski et al., 2010, OECD, 2018). These approaches include in vitro procedures that assess specific biological processes using organs, tissues, cells, and subcellular systems (Krewski et al., 2010, OECD, 2018). *In silico* approaches, which use computational models, and machine learning to analyse data and predict chemical modes of action, provide a comprehensive risk assessment. Furthermore, omics technologies including genomics, transcriptomics, and metabolomics collect biomolecular information, offering a detailed understanding of the molecular effects of different compounds (Cavoski et al., 2024, Krewski et al., 2010).

Precision toxicology, along with NAMs, focuses on identifying precise chemical pathways that may contribute to detrimental health outcomes (Cavoski et al., 2024). Precision toxicology has three primary foundations: phylotoxicology, which employs non-mammalian species to study human-relevant toxicity pathways as an alternative to traditional animal testing; susceptibility variations, which analyse genetic diversity within populations to establish safe chemical exposure levels; and embedded translation, which integrates input from regulatory authorities, industry stakeholders, and civil society to align NAMs with practical regulations (Cavoski et al., 2024). NAMs and Precision Toxicology work together to provide a more humane, efficient, and long-term approach to toxicological research.

Daphnids: a key species in freshwater ecology and ecotoxicology

Detection of pollutants and evaluation of their effects on the environment and living systems have been used for freshwater pollution assessment. Daphnids are one of the most commonly used species in toxicity assessment and freshwater ecology. Daphnids (also known as water fleas), are freshwater planktonic crustaceans which belong in the order of *Cladocera* and

class *Branchiopoda*. There are more than 100 species, and they live as filter-feeding planktonic crustaceans filtering out bacteria, algae, cyanobacteria, protozoans and other small particles suspended in the water. Daphnids play a key role as primary consumers in the aquatic food chain of freshwater ecosystems. Daphnids have ten pairs of appendages including antennules, antennae, maxillae (Figure 3). At the end of the abdomen, there is a pair of abdominal claws. Daphnids are covered by a transparent chitinous carapace which protects thorax and abdomen. The transparent carapace allows to observe several pairs of limbs with setae (Ebert, 2005). The most characteristic feature of daphnids is a single large compound eye and two pairs of highly branched antennae which are used for locomotion (Figure 3). Daphnids have many advantages such as size of body, short life cycle, high fecundity, and parthenogenetic reproduction. It is one of the largest herbivorous cladoceran with a size of 0.5 mm to 6 mm, or even more. The size of an adult individual ranges from 5 mm to 6 mm. Males can be distinguished from females by their front legs which are equipped with clasping hooks, a modified abdomen, smaller size, and larger antennules (Figure 4). The size of the body is one of the most important factors, because it allows to use many animals simultaneously in one experimental plate. Furthermore, the short life cycle, high fecundity and parthenogenetic reproduction allow obtaining many organisms needed to test in a short period of time (Figure 5). Another advantage of these species is transparent body, which allows to measure many physiological parameters. Daphnids are a model organism with many practical advantages, such as simplicity of their culture under laboratory conditions, easy handling and low cost of maintenance, their geographical distribution, and their key role in freshwater food webs. Also, daphnids testing includes the principles of 3Rs. All these parameters highlight that daphnids are a simple alternative approach to toxicity testing. Among the different species, *Daphnia magna Straus* is a relatively large zooplankton, and it is commonly used in toxicity testing since 1940s (Tkaczyk et al., 2021). Together with *D. pulex*, they have been described as key species in toxicity research, however, upon a number of publications in literature *D. magna* has prevailed as the most preferable model (Tkaczyk et al., 2021).

Daphnia sp. has a significant role in genetic research due to their unique life cycle. Most of daphnids' species can reproduce sexually and asexually (Ebert, 2005). Under stressful conditions such as predation and competition for resources, the sexual reproduction is taking place. *Daphnia* produces diploid (2N) eggs which are enclosed in a protective black structure called an ephippium. Each ephippium contains 2 large eggs. Mating with a male individual is needed for the ephippium to be fertilized and the egg to be hatched. The eggs are being inert until the environmental conditions become suitable again. Then they hatch and young

females are released (Ebert, 2005). In favourable environmental conditions, daphnids can produce clonal females (Figure 4). Subsequently, this allows to have a specific genetic background.

D. magna is often described as the “canary in the coalmine”, referring to the historical use of canaries as early warning indicators of toxic gases in coalmines (Abdullahi et al., 2022). This characterisation relies on the idea that the sentinel species should be more sensitive to toxic chemicals than the majority of organisms, including humans, they should share the same environment as humans, and should generate adverse effects that can be easily detected and evaluated (Abdullahi et al., 2022). *D. magna* is known for its high sensitivity to chemicals making it an essential model in ecotoxicology. It effectively serves as a bioindicator for evaluating the adverse effects of various contaminants, including pharmaceuticals and household products. It has been observed that *D. magna* exhibits adverse effects following exposure to various doses of household products, as well as after multigenerational exposures to low concentrations of certain pesticides such as triadimefon (Hou et al., 2023, Tiwari et al., 2021). Additionally, comparative exposures of *D. magna* and the freshwater species Japanese medaka, to pharmaceuticals such as ibuprofen, acetaminophen and diclofenac revealed that *D. magna* is more sensitive that Japanese medaka (Oliveira Pereira et al., 2024b). Even acute exposures of daphnids to low concentration of pollutants in industrial effluent caused notable biochemical disruptions (Oliveira Pereira et al., 2024a). Finally, *D. magna* exhibits sensitive behavioural and physiological responses highlighting its utility for assessing the effects of pollutants in the aquatic environment and its organisms (Tkaczyk et al., 2021).

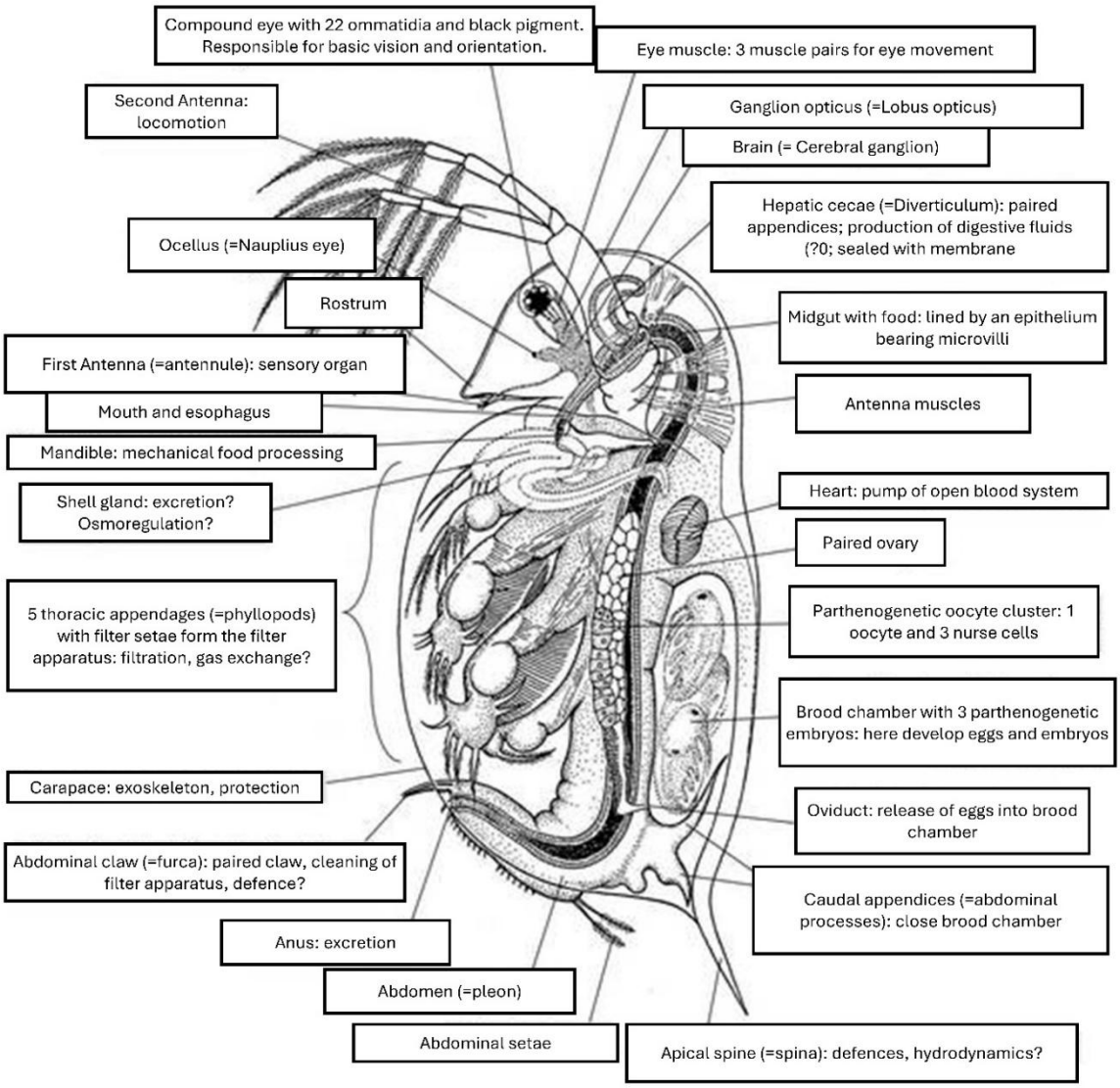

Figure 3. The anatomy of *D. magna*. This figure shows an adult female *daphnia* with embryos in her brood chamber. For better visualization, the carapace is transparent. Modified from Ebert (2005).

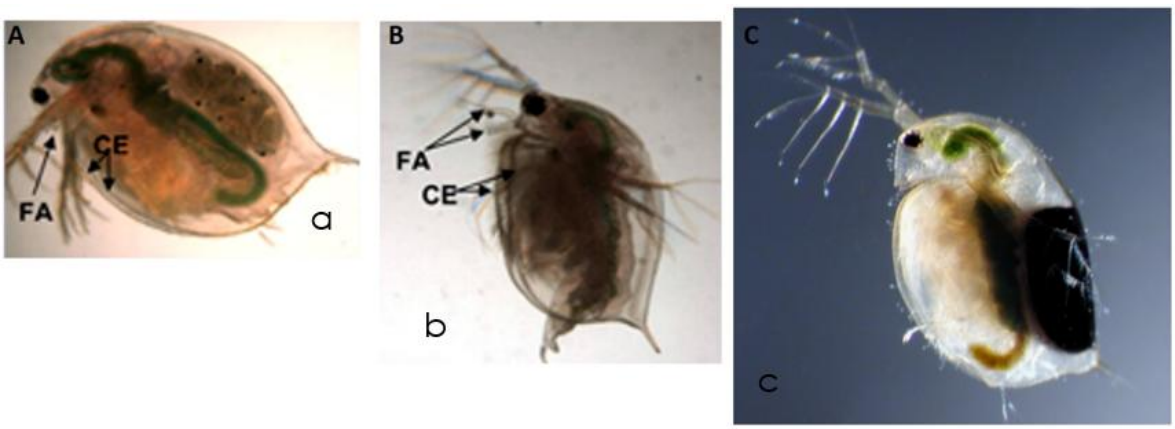

Figure 4. Morphological characteristics of daphnids. (a) Female and (b) male *D. magna*. Differentiating sex features such as pair of minute first antennae (FA) of the females, which are longer in the males, the bivalve-like carapace of the female with the symmetrical edges (CE). The males have asymmetrical CEs. (c) Female *D. magna* with ephippium egg at the back. Modified from Ebert (2005).

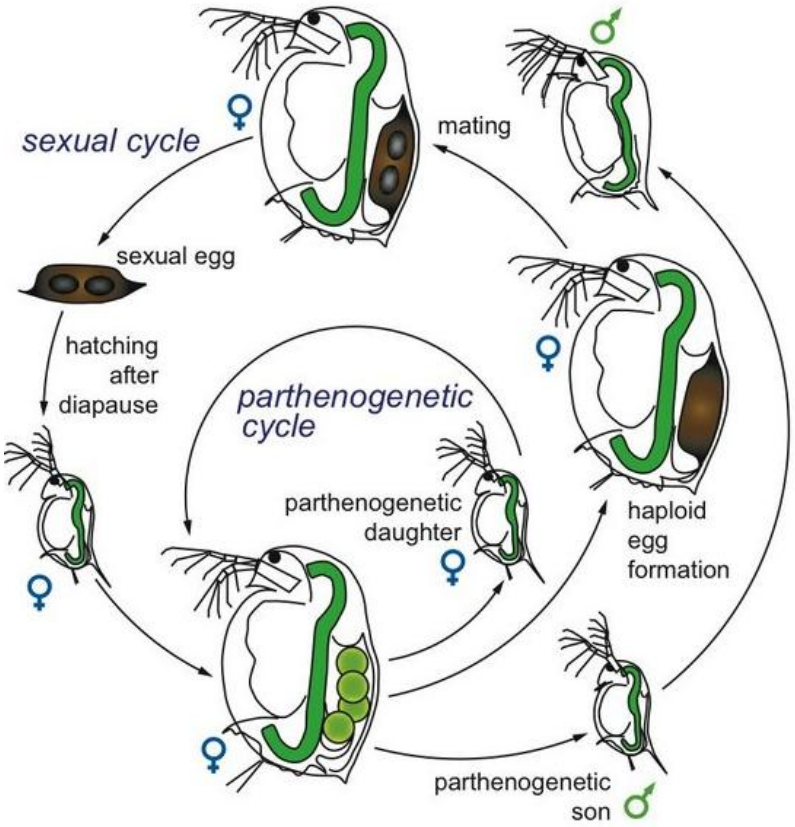

Figure 5. Life cycle of a parthenogenetic *D. magna*. The diagram represents the sexual and asexual (parthenogenetic) reproductive cycle of *D. magna*. During the asexual reproductive cycle female daphnids produce diploid eggs that develop into clonal females. Daphnids can also produce diploid eggs that develop into male daphnids. Under stressful conditions daphnids can produce haploid eggs that are enclosed into a protective structure (ephippium) and require male fertilization to be hatched. Non-modified from Ebert (2005).

Methodological approaches

Phenotypic and biochemical techniques to identify key responses to emerging pollutants

The body size of daphnids, their heart rate, respiration, swimming behaviour, and fecundity are some of the main parameters to assess their physiology with non-invasive approaches. Additionally, biochemical markers provide a more detailed view of metabolic changes (Tkaczyk et al., 2021). Some of these markers that can be used for the assessment of the toxicity responses of daphnids in toxic chemicals, are the activity of key enzymes such as antioxidant enzymes glutathione-S-transferase, glutathione reductase, catalase and lipid

peroxidase. In addition, phosphatases, lactate dehydrogenase, peptidase and lipase belong to the biochemical markers. All the markers that are mentioned above have the advantage of being inexpensive and time efficient methods of assessing the daphnids' responses to toxic chemicals.

In the following chapters a variety of phenotypic and molecular markers of physiology were used for the evaluation of the impact of the chosen chemicals on daphnids.

Mortality

The mortality was assessed with toxicity curves. Neonates and D4 daphnids were exposed for 24 h to several concentrations of the pollutants and their mixtures. Toxicity curves were plotted, and EC values were calculated. All plots were calculated using the Four parameter logistic (4PL) model, following the equation Span = Top − Bottom and Y = Bottom + (Top-Bottom)/(1 + 10^((LogIC50-X)*HillSlope)), using the GraphPad software. The parameters top and bottom were commonly fixed to 100 and 0, accordingly.

Feeding assay

The impact of chemicals on the feeding performance of the animals was assessed following acute or chronic (5-days) exposure of daphnids to the respective pollutants following the protocol of Giannouli et al. (2023), or a miniaturized version of it Rowan et al. (2024). The two protocols were used in different experimental sets, since the miniaturized version was later adopted to reduce the volume of test media, reagents and number of animals, while maintaining comparable sensitivity. According to (Giannouli et al., 2023) after the exposure, the animals (separated into four replicates per exposure condition) were brought in a 12-well plate containing 6 ml OECD and the latex beads, carboxylate-modified polystyrene, fluorescent red microparticles (at concentration of 13 mg/l). Daphnids were allowed to ingest the microparticles for 60 minutes and following media was collected every 10 minutes to estimate the removed microparticles by fluorescence at Ex/Em 560/590 nm. Furthermore, in some of the chapters, animals were homogenised in water, and the fluorescence was measured also in the homogenate representing the ingested microparticles. Fluorescence was expressed to amount of ingested microparticles using a standard curve. Similarly, in the miniaturized version of (Rowan et al., 2024) the exposed animals were transferred into a 96-well plate with 1 ml OECD media and the microplastic at a concentration of 26 mg/l. The media was collected every 10 min up to 40 min and the feeding rate was expressed as the slope of consumption of microparticles in 40 min.

Survival assay

For the survival assay, survival curves were constructed using the Kaplan-Meier model. Neonates (three replicates per condition) were exposed to several concentrations of the chosen chemicals for 28 days, during which the number of living daphnids were counted daily. Media and chemical were renewed twice a week and daphnids were fed daily.

Reproduction assay

The effects of chemicals to the fecundity of daphnids were evaluated with chronic exposures to the selected concentration of the chemical of preference. The reproduction of daphnids was assessed by the number of neonates released daily per individual. For this assay, neonates were exposed to a non-lethal concentration of a pollutant, or a mixture of them, for a 21-day period. Media and chemical were renewed daily and daphnids were fed every day.

Biochemical assays-Enzyme activities

Prior to the assessment of the enzyme activities of daphnids protein quantification is required. Total protein of the homogenates was quantified using an ultrasensitive method which is based on the electrostatic reaction of proteins with the Coomassie Brilliant Blue (CBB) G-250 reagent (Georgiou et al., 2008, Grintzalis et al., 2015). The CBB reagent (60 mg CBB reagent in 100 ml 2 M HCl cleared by filtration to remove undissolved dye particulates) was diluted 2-fold with 2 M HCl immediately before use. Bovine serum albumin (BSA) dilutions (2-20 μg/ml) for standard curve were prepared in ddH_2O. For the assay, 200 μl of the unknown samples (appropriately diluted in ddH_2O) or BSA standards were mixed with 50 μl of the CBB: 2 M HCl reagent. The reaction was incubated for 10 min at room temperature and absorbance was measured at 610 nm against reagent blanks (200 μl ddH_2O instead of standard or sample). The net absorbance of samples was converted to protein concentration equivalents using the corresponding BSA standard curve.

Phosphatase activity (acid and alkaline) was quantified from the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol. For the reaction, 200 μl sample appropriately diluted in buffer (100 mM acetic acid pH 4.5 for acid phosphatase and 100 mM boric acid pH 9.8 for alkaline phosphatase) was mixed with 50 μl 8 mM *p*-nitrophenyl phosphate (*p*NPP) (3 mg/ml buffer). The reaction was stopped after 30 min with the addition of 50 μl 4 M NaOH and absorbance was measured at 405 nm against reagent blanks (200 μl buffer instead of sample).

β-galactosidase activity was quantified from the conversion of *o*-nitrophenyl-phospho-galactoside (ONPG) to *o*-nitrophenol and galactose. The reaction was performed in phosphate buffer pH 7.2. Specifically, 200 μl appropriately diluted sample in phosphate buffer were mixed with 50 μl 8 mM ONPG in phosphate buffer (2.4 mg/ml buffer). The

reaction was stopped after 30 min with the addition of 50 µl 4 M NaOH. The absorbance was measured at 405 nm against reagent blanks (200 µl phosphate buffer and 50 µl 8 mM ONPG).

Activity of lipase was measured by the conversion of *p*-nitrophenyl butyrate to *p*-nitrophenol. The reaction took place by mixing 200 µl appropriately diluted sample in phosphate buffer pH 7.2 with 50 µl 2000x diluted stock of *p*-nitrophenyl butyrate (*p*NPB) in DMSO. The reaction was stopped after 30 min, but the absorbance can be measured earlier. The absorbance was measured at 405 nm against reagent blanks (200 µl phosphate buffer and 50 µl 2000x diluted stock of *p*NPB).

Aminopeptidase activity was quantified from the hydrolysis of L-leu-4-nitroanilide and the production of 4-nitroaniline. More specifically, 200 µl appropriately diluted sample in phosphate buffer pH 7.2 were mixed with 50 µl 8 mM L-leu-4-nitroanilide in DMSO (2 mg/ml DMSO). This is continuous kinetics as the production of 4-nitroaniline is measured continuously. The absorbance was measured at 418 nm against reagent blanks (200 µl phosphate buffer and 50 µl 8 mM L-leu-4-nitroanilide in DMSO).

The activity of the enzyme lactate dehydrogenase (LDH) was measured by continuous kinetics of the consumption of NAD(P)H (Worthington and Worthington, 2011). For this reaction, 200 µl appropriately diluted sample in phosphate buffer pH 7.2 were mixed with 50 µl NADH: 40 mM pyruvate in phosphate buffer pH 7.2 (4.4 mg pyruvate/ml buffer) (1:1). The absorbance was measured at 340 nm against reagent blanks (200 µl phosphate buffer and 50 µl NADH: 40 mM pyruvate in phosphate buffer pH 7.2 (1:1)).

Glutathione-S-transferase (GST) activity was quantified from the reaction of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) (Tang et al., 1996). 200 µl appropriately diluted sample in phosphate buffer pH 7.2 with 50 µl 2 mM CDNB: 6 mM GSH (1:1) (12.4 mg CDNB/ml methanol, 3.8 mg GSH/ml phosphate buffer). This solution of 2 mM CDNB: 6 mM GSH (1:1) needs to be prepared fresh as it is not stable for a long time. This is continuous kinetics as the production of the GSH-CDNB complex was measured continuously at 340 nm.

For the quantification of reduced thiols, 30 animals were homogenized directly in 500 µl 100 mM acetic acid pH 4.5 using an Eppendorf pestle homogenizer. Homogenates were cleared by centrifugation at 14000 rpm for 3 min and all the clear supernatant was isolated in small Eppendorf tubes. Reduced thiols were quantified by the reaction with aldrithiol (DPS). The absorbance was measured after 10 min at 405 nm against reagent blanks (200 µl 100 mM acetic acid pH 4.5 and 50 µl 0.75 mM DPS).

Environmental omics

In the field of toxicology, the fundamental target is to comprehend the effects of both single chemicals and mixture of chemicals on biological systems (Thomas et al., 2002). For this goal to be accomplished "omics" techniques are used. The term "omics" stands for "as a whole" and techniques such as (epi)genomics, transcriptomics, proteomics and metabolomics are included (Figure 6). These holistic approaches are different from the standard observation of phenotypes because of their ability to provide primarily mechanistic information and identification of the toxicity pathway (Fröhlich, 2017). Traditional toxicology models separate chemicals based on their pharmacokinetic and pharmacodynamics processes. These methods provide a descriptive observation and not a mechanistic understanding at the molecular level (Thomas et al., 2002). Even if the omics techniques provide more detailed and specific information, traditional methods are still valid and useful, as they provide a framework for understanding chemical toxicity (Thomas et al., 2002). Genomics investigates genes and their functions using recombinant DNA, DNA sequencing and bioinformatics to analyse function and structure of the genome. By definition, transcriptomics represents the entire set of transcripts or mRNAs present in a cell or an organism. Proteomics outlines the analysis of proteins which are functionally, structurally and anatomically related. Also, proteomics provides more direct information on cellular responses than gene regulation. Metabolomics identifies phenotypic changes that occurred in the presence of the toxicant, while transcriptomics and proteomics provide information of potential hazards. Metabolomics provides changes in the entire metabolome and usually is performed as footprint (extracellular metabolites analysis) or as fingerprint (intracellular metabolites analysis) (Fröhlich, 2017, Bernot et al., 2005).

Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS) are the two commonly used techniques for metabolomic analysis (Nguyen et al., 2024). NMR spectroscopy is known for its ability to analyse samples chemical profile with little preparation, retaining sample integrity while determining structures and concentrations (Pacholczyk-Sienicka et al., 2021).

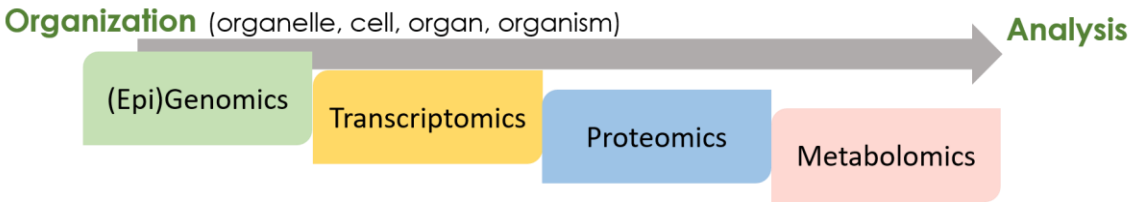

Figure 6. Holistic approaches for environmental monitoring. Progressing from genetic information to functional outcomes. (Epi)Genomics examines DNA-level changes,

transcriptomics focuses on gene expression, proteomics studies proteins, and metabolomics explores metabolic products.

Environmental metabolomics

Mass Spectrometry (MS) is crucial for detecting and quantifying a wide range of metabolites due to its sensitivity and specificity (Nguyen et al., 2024). It can function in several modes: Direct Infusion Mass Spectrometry (DIMS) analyses samples by injecting them directly into a mass analyser. This method is efficient, but susceptible to ion suppression in complex mixtures. Liquid Chromatography-MS (LC-MS) and Gas Chromatography-MS (GC-MS) separate the sample into its individual compounds prior to MS detection (Dunn et al., 2013). LC-MS is useful for evaluating non-volatile, polar molecules, whereas GC-MS is optimized for volatile organic substances (Nguyen et al., 2024). These techniques are beneficial for investigating the biochemical impacts of contaminants and tracking metabolic pathway changes caused by environmental exposures.

Metabolomic analysis can use either targeted or untargeted approaches. Targeted metabolomics focuses on quantifying a predetermined set of known metabolites, while untargeted metabolomics analyses as many metabolites as possible including unknown compounds (Nguyen et al., 2024, Patti et al., 2012).

In metabolomics, there are numerous approaches to properly identify molecules. Identification relies mainly on comparing experimental spectra to reference libraries, which is supported by advanced techniques such as fragmentation and derivatization (Dunn et al., 2013, Vinaixa et al., 2016). During fragmentation, a procedure commonly used with tandem mass spectrometry (MS/MS), molecules break down into smaller, known fragments under controlled conditions. As a result, this process yields structural information that aids in distinguishing between molecules of similar masses.

Derivatization, on the other hand, chemically alters substances to increase detectability, stability, or volatility, particularly in GC-MS. Polar metabolites, such as amino acids or sugars, are frequently derivatized via silylation with reagents such as N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), which replaces reactive hydrogen atoms with trimethylsilyl groups (Kind and Fiehn, 2007). Similarly, methylation or acylation can increase the volatility of analytes while preventing heat degradation during analysis. Derivatization is essential for detecting low-abundance or thermally sensitive compounds that would otherwise go undetected. Additionally, metabolites can be identified based on the natural abundance of isotopes ^{13}C, ^{15}N, and ^{18}O, using isotopic pattern matching (Dunn et al., 2012, Dunn et al., 2013). Other methods for compound identification include using the compound's retention time or experimental standards (Dunn et al., 2013, Vinaixa et al.,

2016). Retention time is the amount of time it takes for a substance to pass the chromatographic column, and it is unique for each molecule. The use of experimental standards is thought to be the most reliable way of compound identification in metabolomics. This approach involves analysing authentic standards of pure molecules under identical experimental settings and then comparing the resulting spectra and retention times with those of the unknown metabolites (Dunn et al., 2013). All the aforementioned methods are useful in their own way, however, to achieve accuracy in compound identification, combination of two or more of these methods is required (Dunn et al., 2013).

The efficacy of metabolomic analysis significantly relies on the extraction process, as it plays a crucial role in retrieving the metabolites from biological samples, while minimizing degradation and contamination. There are numerous extraction methods available, however, solvent-based extraction is one of the most frequently used. This method uses solvent mixtures, such as methanol-water, or chloroform to separate metabolites based on their polarity allowing the simultaneous extraction of both hydrophilic and hydrophobic molecules (Dunn et al., 2011). Another extraction technique is solid-phase extraction (SPE) in which the metabolites stay attached to the column, while the unwanted components are being removed. Thus, the resulted sample is more condensed and purer making the analysis more sensitive (Want et al., 2013). The extraction method used is crucial since it has a direct impact on subsequent analysis by producing various metabolite profiles depending on the sample type and biochemical properties (Lei et al., 2011). Therefore, choosing a proper extraction technique is critical for collecting reliable and high-quality metabolomic data.

Metabolites that were extracted from biological samples are being analysed. The result is a multi-dimensional dataset with many samples and many features; thus robust statistical approaches are required for data analysis and biological inference. The data analysis includes normalization and scaling to ensure all data are comparable (Broadhurst and Kell, 2006). Multivariate statistical approaches, such as principal component analysis (PCA) and partial least squares analysis (PLS), are usually employed for pattern prediction and identification, as well as classification of metabolomic profiles (Worley and Powers, 2013). Along with these analyses, further statistical analysis such as Student's *t*-Test and ANOVA shows the statistical significance among the altered metabolites. Additionally, pathway analysis aids in data visualization by connecting observed metabolic alterations to specific biochemical pathways (Tyanova et al., 2016). The selection of the appropriate statistical analysis is critical to ensure reliability and reproducibility of metabolomic analysis.

Metabolomic analysis

Following acute or chronic chemical exposures, daphnids were snap-frozen and stored at -80°C until metabolite extraction. The extraction process was conducted in the respective collaborating laboratories Metabolomics Core Technology Platform at the University of Heidelberg, Conway Institute of Biomolecular and Biomedical Science, and School of Agriculture and Food Science, University College Dublin.

For the manuscript titled "The Effects of Single and Combined Stressors on Daphnids—Enzyme Markers of Physiology and Metabolomics Validate the Impact of Pollution" untargeted metabolomic analysis was performed using GC-MS (GC-ToF) at the Metabolomics Core Technology Platform at the University of Heidelberg.

The analysis used in the manuscript titled "Exposure to chemical and commercial forms of NSAIDs at environmentally relevant concentrations exert transgenerational metabolic responses in daphnids" was a targeted LC-MS/MS and FIA-MS/MS which followed the Biocrates Life Sciences protocol (Innsbruck, Austria) at the Conway Institute of Biomolecular and Biomedical Science, and School of Agriculture and Food Science, University College Dublin.

Introduction to the Research Chapters

The effects of eight single stressors and different doses of their mixture

The majority of pollutants are present in the environment as mixtures and rarely as individuals. However, in the previous years, pollution assessment was conducted by assessing the effects of individual chemicals rather than mixtures on aquatic organisms (Backhaus and Faust, 2012). The effects of the mixtures can be either synergistic or antagonistic compared to the effects of the individual compounds (Cedergreen, 2014). As a result, chemical mixtures could be more toxic than the individual chemicals, even if they are present in low concentrations. Therefore, it is crucial to develop more effective methodologies for evaluating the impact of mixtures on aquatic organisms (Carvalho et al., 2014).

There are two models that predict mixture toxicity in Mixture Toxicology: Concentration Addition (CA) and Independent Action (IA) (BLISS, 1939). According to CA the chemicals in a mixture have the same mode of action, while based on the IA the chemicals have different mode of action. However, in realistic scenarios mixtures are usually consisted of chemicals with diverse mechanisms, making prediction more complicated (Altenburger et al., 2012).

As mentioned, *D. magna* is a model organism that is widely used for pollution assessment due to its numerous benefits, such as high sensitivity to chemicals, easy to culture under the laboratory conditions and its parthenogenetic cycle (OECD, 2004, Tiwari et al., 2021). Many studies have assessed the impact of complex mixtures of pollutants on daphnids, using phenotypic and biochemical endpoints, such as mortality, reproduction, growth, enzyme activities and feeding assay (Hecker and Hollert, 2009, Michalaki et al., 2022, Rowan et al., 2025).

Acute exposures of daphnids to different doses of an eight-chemical mixture showed a synergistic effect through biochemical markers and metabolomic analysis (Michalaki et al., 2022), while acute exposure to mixture of different pharmaceuticals revealed an antagonistic pattern of effect (Rowan et al., 2025). Additionally, chronic and transgenerational exposures to mixture of pharmaceuticals caused significant toxicity even at very low and environmentally relevant concentrations. These effects were transferred into the recovery generation where the animals were growing in the absence of pollutant, revealing the potential for inherited impairments across generations (Michalaki et al., 2025).

This chapter investigated the toxicity of eight single stressors: two metals (aluminium and lithium), four pharmaceuticals (acetylsalicylic acid, diltiazem, metformin and propranolol), an herbicide (glyphosate), and a stimulant (nicotine). Their effects both as individual compounds and as mixtures were assessed on *D. magna* through acute, chronic and transgenerational exposures.

Both metals are being widely used worldwide, and it is known that they are causing adverse effects on aquatic organisms. Specifically, aluminium affects the liver, kidneys, fertility and mortality, while it also causes protein and DNA damage (Botté et al., 2022, Closset et al., 2022). The toxicity of lithium is mostly depends on the concentration and the duration of the exposure (Zarse et al., 2011).

Acetylsalicylic acid (ASA) is a Non-Steroidal Anti-inflammatory Drug (NSAID) which is being used for over 90 years, due to its low cost and high efficiency. It has anti-inflammatory and analgesic properties, and it acts as an irreversible blocker of the enzyme cyclooxygenase (COX), which catalyses the conversion of arachidonic acid to prostaglandins. Its effects vary based on the organism, the dose and the duration of the exposure. Studies show that chronic exposures to lower concentrations can cause more significant impacts than the acute exposures to higher concentrations. Exposure of organisms to ASA can cause oxidative stress, changes in cell volume regulation, fertility and DNA damage (Gomes et al., 2019, Parolini, 2020). Propranolol is a drug prescribed for hypertension, and it belongs to the category of β-blockers. It affects fertility on aquatic organisms depending on the organism

and the dose, by increasing or decreasing it. Propranolol is a drug with critical bioaccumulation effects, and it is known for disrupting the metabolic profile of aquatic organisms (Damasceno de Oliveira et al., 2018, Michalaki et al., 2022). Diltiazem is a non-dihydropyridine calcium channel blocker highly present in the aquatic ecosystems due to its wide use for treating hypertension, angina and rhythm disorders (Steinkey et al., 2019). Additionally, its effects vary depending on the organism, the concentration of diltiazem, and the exposure period (Natalia et al., 2018). Chronic exposure of daphnids to diltiazem for 16-days revealed decreased reproduction while also energy imbalance (Steinkey et al., 2019). Metformin is a pharmaceutical administered to patients with diabetes type 2 (O'Rourke et al., 2023). Since it is not metabolized in the body, the administered dose is ended up in the aquatic environment having negative impact on aquatic organisms (Zheng et al., 2024). Additionally, metformin can cause cellular reaction and histopathological damage in gills (Barbieri et al., 2022), while it can affect growth and cause oxidative stress (Sibiya et al., 2023).

N-(phosphonomethyl) glycine, known as glyphosate, is an herbicide globally used in agriculture, often bleeding into the aquatic ecosystems significantly affecting aquatic organisms (Lares et al., 2022). Glyphosate is not a persistent chemical, and its presence can rapidly decrease from the environment (mean half-life of 20-30 days), yet its metabolite aminomethylphosphonic acid (AMPA) has been characterized as persistent and can move into the ecosystem, becoming a threat for living organisms (Lares et al., 2022). Glyphosate is known for affecting the morphology of zooplanktonic organisms and crustaceans and the heart rate of daphnids (Michalaki et al., 2022).

The main ingredient of tobacco, nicotine, is a stimulant widely consumed (Giannouli et al., 2023). According to literature, nicotine affects the reproduction of daphnids, by reducing the number of offspring released by females, and by producing male offspring (Giannouli et al., 2023, Vlasceanu et al., 2024). Acute exposure to several concentrations of nicotine ranging from 5 to 20 mg/l, showed decreased feeding rate. Heart rate and activities of key enzymes were also affected after exposure of daphnids to nicotine (Giannouli et al., 2023, Michalaki et al., 2022).

Ionic Liquids (ILs)

Ionic liquids (ILs) are mostly organic salts made of organic cations and organic/inorganic anions. ILs are a type of “green solvent”, and they are developed with the intention of replacing some of the most unsavoury volatile organic compounds (VOCs) used as solvents in industry. VOCs present a number of hazards, such as toxicity to both process operators

and the environment. Furthermore, their instability and tendency to produce flammable vapours presents a potential explosive hazard (Pham et al., 2010).

ILs are composed entirely of ions, which are typically bulky organic, asymmetric cations and weakly-coordinating, inorganic anions (Pham et al., 2010, Zhu et al., 2009). In comparison to a typical inorganic salt, ILs tend to have much less symmetry. The asymmetry of these compounds can achieve very low melting points (below 100°C) (Pham et al., 2010). The strong ionic interactions within ILs result in an appealing suite of characteristics. Among them are negligible vapour pressure, amphiphilic properties, rendering them soluble in both water and lipids, and exceptional thermal, mechanical, and electrochemical stability (Bubalo et al., 2017, Oskarsson and Wright, 2019, Zhu et al., 2009). One of the most interesting and exploitable aspect of ILs is their capability to be "designer solvents". By varying the cation/anion incorporated into the structure, as well as the length and branching of the alkyl chain, it is possible to create approximately 10^{18} ILs, each with differing physicochemical characteristics. Thus, it is theoretically possible to create "task-specific" ILs, with the optimal characteristics for a particular application (Bubalo et al., 2017, Pham et al., 2010). As a result, there are many potential applications for ILs, from organic synthesis, catalysis and biocatalysis to biofuel production, cellulose production, and even water purification (Bubalo et al., 2017, Oskarsson and Wright, 2019, Pham et al., 2010, Zhu et al., 2009) (Figure 7).

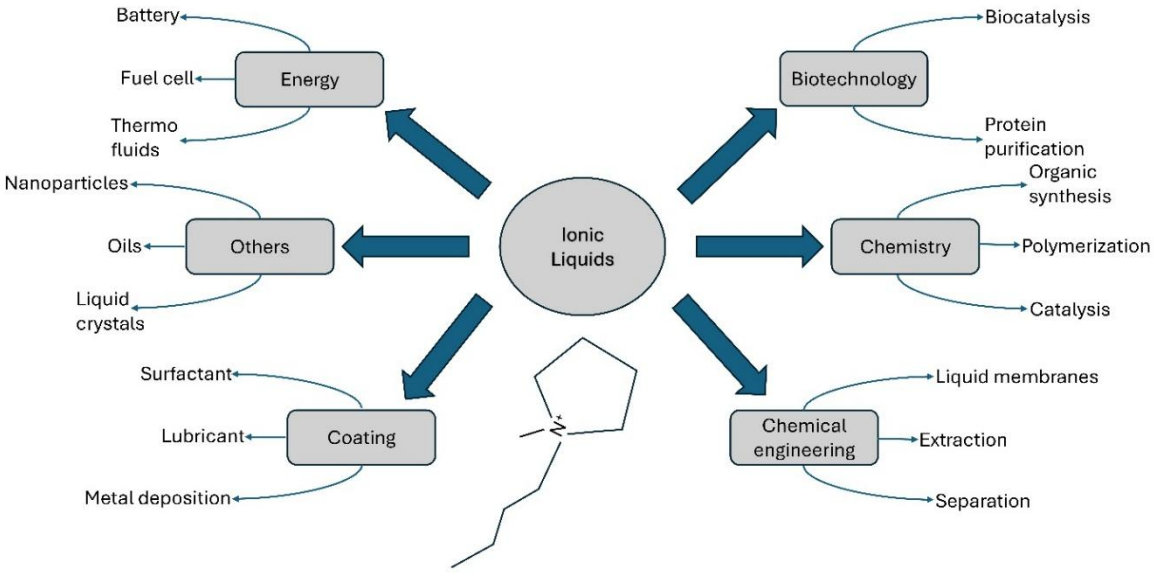

Figure 7. Applications of ILs. ILs can be used as reaction media in organic synthesis, catalysis and biocatalysis, as well as in membranes and energy production. Adapted from Pham et al. (2010).

ILs can enter the freshwater ecosystem mostly via water (e.g. spillage or discharge of industrial effluent) (Figure 8). Once in the environment, the properties which make them so desirable in an industrial context (thermal, mechanical, and electrochemical stability) have the potential to make them highly persistent aquatic pollutants (Pham et al., 2010, Zhang et al., 2017a).

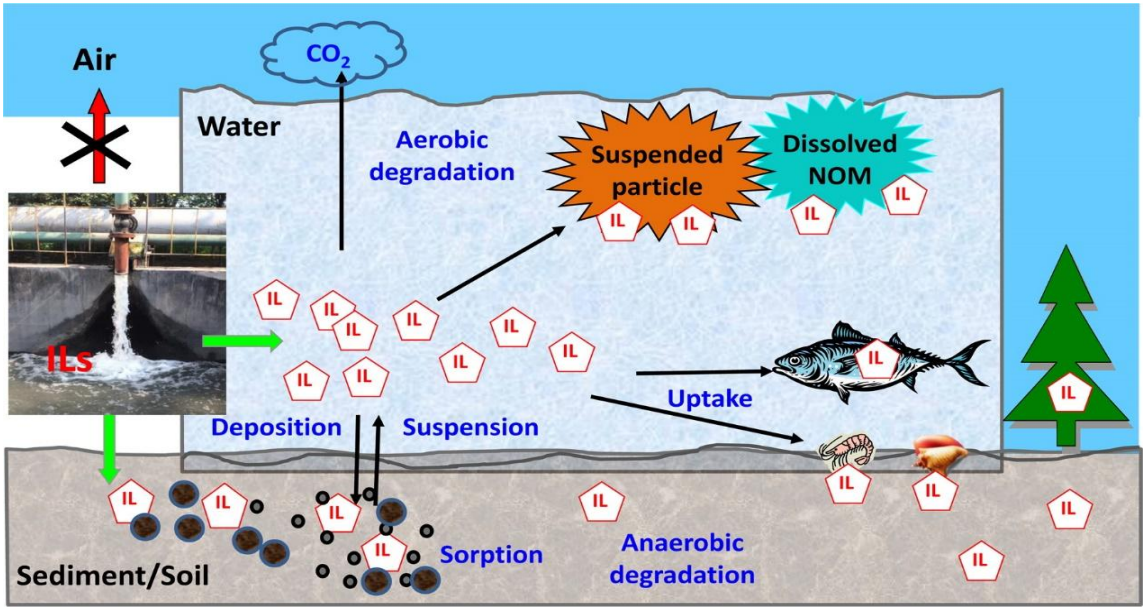

Figure 8. Transport and transformation of ILs in the environmental system. ILs can end up in the aquatic ecosystem through water and could pollute soils, sediments and surface or groundwater. Non-modified from Amde et al. (2015).

While ILs have been intensively studied under laboratory conditions, until now there is almost no data available on their occurrence in environmental water samples. Tris(pentafluoroethyl)trifluorophosphate (FAP) was detected in three connected rivers in Germany (Landgraben, Schwarzbach, Rhein), with peak concentrations of up to 3.4 µg/l. FAP is exclusively used as a constituent anion of ILs, usually employed in supercapacitors or as an extraction agent of polycyclic aromatic hydrocarbons. This is believed to be the first detection of FAP in environmental water samples (Neuwald et al., 2020).

Most research on ILs highlights that their toxic effects are directly related to the disruption of the cell membrane. The length of the alkyl chain of ILs bears a strong correlation with its toxicity which has been observed in several cell lines, as well as in unicellular and multicellular test organisms. A longer alkyl chain will confer greater toxicity, as it increases the lipophilicity of the IL and its ability to interact with both the phospholipid bilayer of the cell membrane as well as the hydrophobic domains of membrane proteins. These interactions can lead to the disruption of membrane physiological functions, resulting in leakage of cellular content and evidently cell death. The ability of ILs to enter cells through membrane

disruption means that they can also interact with various enzymes, altering their activity. Several examples of this have already been documented (Bubalo et al., 2017). For instance, 1-methyl-3-octyl-imidazolium (OMIM) ILs are expected to be more toxic than 1-butyl-3-methylimidazolium (BMIM) ILs due to their longer alkyl chain (Kuroda, 2022). The former are known for affecting several markers of physiology to frog and green algae (Leitch et al., 2020, Li et al., 2009, Liu et al., 2015, Tsarpali and Dailianis, 2015, Tsarpali et al., 2016). Additionally, it has been noted that toxicity of ILs is related with the presence or absence of aromatic ring (Kebaili et al., 2020, Ventura et al., 2013). ILs with aromatic cations (imidazolium and pyridinium) are more toxic than ILs with non-aromatic cations (pyrrolidinium and piperidinium) (Kebaili et al., 2020, Ventura et al., 2013). If molecules from ILs, having aromatic ring, reach the cytochrome P_{450} in the endoplasmatic reticulum, they have a great possibility of being oxidized (Jastorff et al., 2003). Consequently, all the naturally occurring metabolites could be further broken down into potentially hazardous fatty acids and imidazole (Bernot et al., 2005). On the other hand, BMIM ILs can cause adverse effects on marine species, such as mussels and rotifers, zebrafish, planarians, nematodes and *in vitro* systems (Piotrowska et al., 2018, Ranke et al., 2004, Swatloski et al., 2004, Tsarpali et al., 2015, Tsarpali and Dailianis, 2015, Zhang et al., 2016). ILs containing fluoride and fluoride-containing anion species have been identified as inhibitors of acetylcholinesterase (an enzyme involved in neural signalling in higher organisms) (Arning et al., 2008). N-methylimidazolium based ILs (with a variety of anions) have been shown to inhibit activity of LDH, with a stronger inhibitory effect shown by those ILs with longer alkyl chains (Dong et al., 2016). Cl^- and Br^- based ILs have been shown to inhibit lipase, again with a greater inhibitory effect shown by those with a longer alkyl chain (Fan et al., 2016).

Furthermore, oxidative stress which is the imbalance of antioxidant defences over the production of reactive oxygen species (ROS), has been described as part of the mechanism of toxicity in the algae *Ulva lactuca*, exposed to 1-dodecyl-3-methylimidazolium bromide. Significant levels of reactive oxygen species were detected in the cells (Kumar et al., 2011) and this has been the case in other organisms. Cell wall damage has also been reported as an aspect of toxicity mediated effect in algae, caused by exposure to phosphonium ILs (Petkovic et al., 2012).

Some studies into the acute toxic effects of ionic liquids on aquatic species such as *Danio rerio* (Zhang et al., 2017b), *D. magna*, and *Chlorella vulgaris* (Zhang et al., 2017a) showed their adverse effects. However, to date, no studies have examined their impact to enzymatic activities on aquatic organisms. Given the observed ecotoxic effects of ionic liquids, it is

crucial that their impact is estimated in greater detail to guide and put in place appropriate legislation for their usage before they become widespread enough to cause significant ecological damage.

In this chapter the effects of five BMIM ILs; hexafluorophosphate, chloride, methanesulfonate, tetrafluoroborate, hexafluoroantimonate, and their mixture were studied on daphnids following acute and chronic exposures.

Pharmaceutical compounds commonly encountered in the aquatic ecosystem

The worldwide production and use of pharmaceuticals is increasing over the years as a result of ageing of human population and an increase of drug administration in the developed countries. The large-scale production of pharmaceuticals and their widespread applications have led to their increasing entry into the aquatic environment. Pharmaceuticals have been described as emerging contaminants because of their presence in the aquatic ecosystems and their potential to act as bioactive compounds in the environment (Akpotu et al., 2019). As a result, the increasing concern about possible toxic effects of pharmaceuticals on humans and wildlife is observed (Tkaczyk et al., 2021). Consequently, it is necessary to draw our attention to understanding the pathways through which the pharmaceuticals end up in the environment and to realising their impact on the ecosystem (Ojemaye and Petrik, 2019).

Pharmaceuticals are natural inorganic or organic chemical compounds used to cure or prevent diseases. Because of their different and unique composition, structure and behaviour, their applications, metabolic pathways in animals and humans, as well as the ways with which they affect the environment vary. Although pharmaceuticals target humans and animals, there are concerns about their negative impact on non-targeted organisms. It is notable that more than 100 pharmaceutical compounds, from different classes, have been identified in drinking water, ground water, wastewater, marine organisms, sewage and surface water around the world (Ojemaye and Petrik, 2019).

As long as the use of pharmaceuticals from humans and animals is rising, followed by the incomplete absorption in the body, the presence of the parent drugs and their metabolites in aquatic environments will be continuously increasing (Akpotu et al., 2019, Ojemaye and Petrik, 2019).

There are many classes of pharmaceuticals based on their therapeutic uses. They are classified into anti-diabetics, beta-blockers, antibiotics, lipid regulators, anti-epileptic, tranquilizers, antimicrobials, antiulcer and antihistamine drugs, antianxiety or hypnotic agents, anti-inflammatories and analgesics, antidepressants, anticancer drugs, antipyretics and stimulants, oestrogens and hormonal compounds (FDA, 2015).

These pharmaceutical compounds are entering into the environment continuously through many pathways (Akpotu et al., 2019, Ojemaye and Petrik, 2019). Municipal sewage treatment plants play a significant role in the release of pharmaceuticals into the freshwater environment. Wastewater treatment plants (WWTPs) are not designed to decompose the majority of these compounds which are made to be stable and robust, polar and non-volatile in nature. As a result, they pass through the WWTPs and are released into the environment (Ojemaye and Petrik, 2019). Additionally, most individuals discard unused or expired drugs into sinks and toilets. Furthermore, due to incomplete absorption of the drugs, metabolites of pharmaceutical compounds are excreted through urine or faeces into the freshwater ecosystem (Ojemaye and Petrik, 2019). Drugs find their way into the aquatic environment via landfill sites, septic tanks, urban wastewater, showering and bathing, industrial effluent, and agricultural practices (Figure 9) (Akpotu et al., 2019, Ojemaye and Petrik, 2019). The release of pharmaceuticals into the environment can negatively affect water quality and domestic water supplies, human and animal health and at last the ecosystem balance (Akpotu et al., 2019). Hence, it is crucial to efficiently remove pharmaceuticals from the environment or at least detect their presence early.

NSAIDs are among the most widely prescribed pharmaceuticals globally (Kovacs et al., 2024). Despite their anti-pyretic, anti-inflammatory and analgesic properties, NSAIDs have been characterized as a major group of emerging contaminants because of their widespread use (Arfeen et al., 2024, Michalaki et al., 2025). They are easily found in aquatic ecosystems, since they represent more than the 15% of all pharmaceuticals that are present in the environment (Michalaki and Grintzalis, 2023). These drugs are inhibitors of COX-1 and COX-2, enzymes responsible for synthesis of prostaglandins (PGs). Thus, they can be categorized into two groups as per their COX selectivity: NSAIDs which block both COX isoforms-nonselective, and NSAIDs which inhibit only COX-2 (Arfeen et al., 2024, Michalaki and Grintzalis, 2023). Despite their analgesic properties though, these pharmaceuticals create ROS leading to oxidative stress, lipid peroxidation, protein carbonylation, and disrupted activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Michalaki et al., 2025). During the COVID-19 pandemic, NSAIDs were widely used as an alternative treatment, resulting in significant increase in the presence of their active ingredients in sewage treatment plants and the environment (Kovacs et al., 2024). NSAIDs represent a crucial threat, contributing to widespread contamination of soil and water (Kovacs et al., 2024). NSAIDs including indomethacin, ibuprofen and diclofenac are not chemically stable and can degrade through microbial activity. However, they are classified as emerging contaminants due to their pseudo-persistence, which derives

from their continuous discharge and incomplete removal (Liu et al., 2024, Michalaki et al., 2025). NSAIDs can be found at concentrations ranging from 1 ng/l to μg/l in WWTPs influx/outflow and in surface waters, respectively (Michalaki and Grintzalis, 2023). However, even though their concentrations in freshwater environments are low, their high biological activity might cause adverse effects to non-target organisms (Swiacka et al., 2020).

In this research, the effects of indomethacin, ibuprofen and their mixture (in both chemical and commercial forms) were evaluated on daphnids, using acute, chronic and transgenerational exposures. Their toxic effects on daphnids were investigated using phenotypic endpoints, biochemical assays and metabolomic analysis.

Indomethacin is one of the most potent nonselective NSAIDs available and among the first NSAID medications that used to treat headaches and migraines (Michalaki and Grintzalis, 2023). Indomethacin is an indole derivative and commonly found in the environment due to its widespread use (Liu et al., 2024, Michalaki and Grintzalis, 2023). Despite its therapeutic benefits, it is associated with adverse effects in 30-60% of patients receiving it. These effects extent to various systems, including cardiovascular, gastrointestinal, nervous, hematologic, hepatic, ocular and otic, renal and electrolyte, dermatologic, and reactions of hypersensitivity. Indomethacin can enter the environment through both direct and indirect pathways. It has been detected in wastewater samples from all over the world, and it has been found at concentrations range from 0.005 to 0.792 μg/l in surface waters in the UK and Ireland (Michalaki and Grintzalis, 2023, Michalaki et al., 2025).

Ibuprofen is the third most popular NSAID used globally (Osman et al., 2024). It inhibits both COX-1 and COX-2 enzymes and has a crucial role as an analgesic and anti-pyretic drug. It is a derivative of propanoic acid and can be found in several freshwater ecosystems (Osman et al., 2024). This is caused due to its overconsumption and the fact that a substantial portion (50-80%) of the administered dose (600-1200 mg/day) remains unmetabolized (Michalaki et al., 2025, Osman et al., 2024). Although it has significant analgesic and anti-pyretic benefits, excessive or improper use can negatively impact the gastrointestinal tract, kidneys, and coagulation system (Michalaki and Grintzalis, 2023).

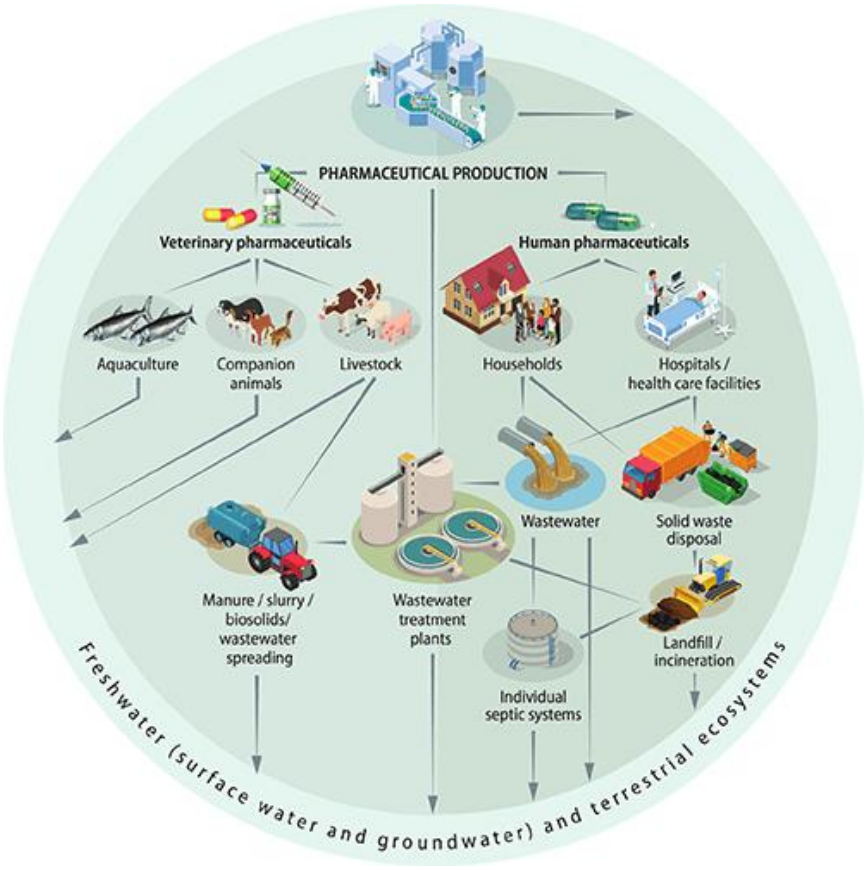

Figure 9. Major pathways of release of pharmaceuticals into the environment. Pharmaceutical compounds can end up in the environment via many routes. Some of them are through livestock or aquaculture and through households, hospitals, pharmaceutical industry and WWTPs. Non-modified from OECD (2019).

Environmental study

The toxicity of chemicals on aquatic organisms is usually assessed using a standardised media (OECD media), in laboratory conditions. Although these conditions ensure reproducibility and control, they only reflect the toxic effects of the individual chemicals, failing to capture the complexity of real aquatic ecosystems, in which other stressors will be present as well. River water is a chemically and biologically diverse matrix that contains dissolved organic matter, a variety of ion compositions, microbes, and a wide range of existing or recently added contaminants (Harwood et al., 2012). All these components might have a major impact on the toxicity of pollutants, either masking or enhancing their effects. Thus, testing compounds in river water sample is critical for evaluating the actual responses of bioindicators and increasing the ecological relevance of toxicity testing.

Studies with daphnids have shown that water matrices influence physiological, biochemical, and transcriptome responses. According to (Barata et al., 2007, Damásio et al., 2008) *in situ* bioassays using *D. magna* exposed to Mediterranean rivers showed significant changes in enzymatic activity, such as cholinesterase and antioxidant enzymes, in response to mixture

of pesticides and industrial effluent. These results highlighted that water matrices can change the effects of pollutants, in ways that possibly cannot be detected under laboratory circumstances. Such findings underline the limitations of synthetic media, which may fail to detect stress responses caused not only by the chemical in focus, but also by its interaction with the existing components in natural waters.

Further studies revealed that daphnids exposed to antiretroviral drugs, using river water as media, tried to respond to oxidative stress by increasing the activity of detoxification enzymes, emphasizing on the need to employ water samples to toxicity studies (Mahaye and Musee, 2022). Jankowski et al., found that it is possible for daphnids to express unique gene profiles based on different water sources, such as stormwater and wetland effluents, showing the impact of water chemistry to physiological responses of the animal at a molecular level (Jankowski et al., 2022). Ultimately, the aforementioned studies highlight the importance of including realistic scenarios into ecotoxicological assessments to improve ecological relevance. This will provide a further insight into the complex interactions between chemicals and the existing compounds in aquatic environments allowing us an earlier detection of pollution.

For this study, two rivers (Liffey, Kilcullen co. Kildare and Royal Canal way, Drumcondra Dublin) and a mixture consisting of three chemicals, lithium, metformin, and glyphosate, which were described previously, were selected. Acute, chronic and transgenerational exposures were performed in both laboratory media and river water, using multiple concentrations of the mixture. The toxicities were assessed using mortality, feeding assay, biochemical assays and metabolomic analysis.

Contribution of published manuscripts

This thesis compiles a number of studies focused on improving our understanding of how different pollutants affect *D. magna*, a bioindicator widely used for water pollution monitoring. Each publication contributed to a distinct area of environmental pollution, ranging from individual pollutants to mixtures, employing acute and transgenerational exposures. These studies illustrated the value of *D. magna* as an efficient and sensitive bioindicator for detecting the effects of stressors in the aquatic ecosystems.

Article No.1 - The Effects of Single and Combined Stressors on Daphnids-Enzyme Markers of Physiology and Metabolomics Validate the Impact of Pollution - DOI: 10.3390/toxics10100604

In this article, the effects of several categories of pollutants and their mixture were assessed on daphnids. The toxicity of these pollutants was evaluated using multiple endpoints of physiology of daphnids, such as mortality, feeding assay and biochemical assays. Finally, the results were further confirmed using metabolomic analysis. The results showed significant alterations on the enzymatic activity of daphnids, with the mixture being more toxic than the individual chemicals. This article improved our understanding of the different effects that complex mixtures can have on daphnids and further highlighted the importance of biochemical assays as a first step of pollution monitoring prior to metabolomic analysis.

Article No.2 (submitted – under review) - Novel Approaches Methodologies in ecotoxicology - Metabolism reveals transgenerational effects of pollutants on daphnids

This study stood as a continuation of the previous article, focusing on the effects of several environmentally relevant concentrations of the mixture on daphnids through transgenerational exposures. For this article, the same endpoints were used, and the results showed a dose-dependent effect with increasing concentration and generation, showing that the stress was accumulated and transferred to the subsequent generations.

Article No.3 - Toxicity of "green solvents" - The impact of butyl methylimidazolium ionic liquids on daphnids – DOI:10.1016/j.jil.2023.100059

This paper focused on the toxicity of 1-butyl-3-methylimidazolium (BMIM) ionic liquids (ILs) on daphnids. The study evaluated the acute and chronic toxicity of five BMIM ILs and their composite mixture on daphnids using mortality, feeding assay, biochemical assays, and reproduction. The findings showed the adverse effects of these "green solvents", implying that even if they have been described as environmentally friendly, these compounds are dangerous to aquatic species. The work supported the case for using *D. magna* as a bioindicator for water pollution monitoring.

Article No.4 - Acute and Transgenerational Effects of Non-Steroidal Anti-Inflammatory Drugs on *Daphnia magna* – DOI:10.3390/toxics11040320

This article examined the effects of two NSAIDs, indomethacin and ibuprofen on daphnids using acute, chronic and transgenerational exposures. Through both phenotypic and biochemical endpoints, it highlighted the effects of these NSAIDs and their mixture on the feeding performance as well as the enzymatic activity of daphnids. Despite their partial degradability, their ongoing release into the environment makes them pseudo-persistent pollutants. The results showed that both individual NSAIDs and their mixture caused

significant effects on the physiology of daphnids by affecting either the feeding performance or their enzymatic activities. Additionally, the results were more intense in the later generations. This research emphasized the ecotoxicological significance of pharmaceuticals constantly present in the aquatic ecosystems, as well as the importance of assessing chronic and low concentrations over multiple generations.

Article No.5 - Exposure to chemical and commercial forms of NSAIDs at environmentally relevant concentrations exert transgenerational metabolic responses in daphnids - DOI: 10.1016/j.watbs.2025.100404

This paper was a continuation study of the impact of NSAIDs on daphnids. In this study, the effects of not only chemical NSAIDs and their mixture, but also their commercial forms were assessed through transgenerational exposures up to five generations. Phenotypic endpoints, as well as biochemical assays, and metabolomic analysis were performed. The results initially revealed a distinct pattern of effect between chemical and commercial NSAIDs where the former were more toxic based on mortality and survival and the latter appeared to be more toxic based on biochemical assays and metabolomics. These results highlighted the need for using biochemical assays to assess toxicity of chemicals, along with phenotypic endpoints. Additionally, this study emphasized on the importance of using low and environmentally relevant concentrations and transgenerational exposures to mimic real case scenarios.

Article No.6 (drafted) – Molecular responses from water fleas serve as metrics for pollution – Moving from the lab samples to the river

The last article for this thesis investigated how actual river water can affect the toxicity of chemicals. River water from two sites were collected and used as exposure media instead of OECD media. Daphnids then were acutely and chronically exposed to a three-chemical mixture in different doses using these two river waters. The effects caused from exposure to the river waters as well as from the interaction between water-mixture were assessed with biochemical assays and metabolomic analysis. The results showed that water alone affects the toxicity of the pollutants implying the need to incorporate the use of actual water samples when assessing toxicity, as a more realistic scenario.

Overall, these studies together provide a vigorous and comprehensive assessment of how *D. magna* responds to environmentally relevant exposures to emerging pollutants. By merging acute, chronic and transgenerational exposures with multiple endpoints, such as biochemical assays and metabolomics, this thesis strengthened the value of *D. magna* as a sensitive and

adaptable model organism in ecotoxicology. Each study advocated for a more realistic and comprehensive approach to chemical risk assessment that combines chemical mixtures, transgenerational exposures and biologically significant enzymatic and metabolic perturbations that may not be detected by traditional endpoints.

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Chapter 2A

This chapter introduced the first published study of the thesis, in which the effects of individual stressors and their composite mixture were assessed on *D. magna*. This research compared the acute effects of eight single chemicals and their mixture, combining phenotypic, biochemical and metabolic markers of daphnids physiology to provide a mechanistic insight into the pollutants' adverse effects. A key finding was that the metabolomics validated the biochemical findings, reinforcing the use of biochemical assays as a first-step screening tool prior to omics techniques. These insights established both the methodological and conceptual basis for the following chapters that investigated more environmentally relevant exposure scenarios.

The Effects of Single and Combined Stressors on Daphnids—Enzyme Markers of Physiology and Metabolomics Validate the Impact of Pollution

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Abstract

The continuous global increase in population and consumption of resources due to human activities has had a significant impact on the environment. Therefore, assessment of environmental exposure to toxic chemicals as well as their impact on biological systems is of significant importance. Freshwater systems are currently under threat and monitored; however, current methods for pollution assessment can neither provide mechanistic insight nor predict adverse effects from complex pollution. Using daphnids as a bioindicator, we assessed the impact in acute exposures of eight individual chemicals and specifically two metals, four pharmaceuticals, a pesticide and a stimulant, and their composite mixture combining phenotypic, biochemical and metabolic markers of physiology. Toxicity levels were in the same order of magnitude and significantly enhanced in the composite mixture. Results from individual chemicals showed distinct biochemical responses for key enzyme activities such as phosphatases, LIP, PEP, βGAL and GST. Following this, a more realistic mixture scenario was assessed with the aforementioned enzyme markers and a metabolomic approach. A clear dose-dependent effect for the composite mixture was validated with enzyme markers of physiology, and the metabolomic analysis verified the effects observed, thus providing a sensitive metrics in metabolite perturbations. Our study highlights that sensitive enzyme markers can be used in advance on the design of metabolic and holistic assays to guide the selection of chemicals and the trajectory of the study, while providing mechanistic insight. In the future this could prove to become a useful tool for understanding and predicting freshwater pollution.

Keywords: *Daphnia magna*; mixture toxicology; combined stressors; mortality; biochemical markers; metabolomics

Introduction

Humans have a significant impact on the physical environment in many ways such as overpopulation, pollution, fossil fuels and deforestation, which are responsible for the observed climate change, increased pollution, and decrease in air, water and soil quality. Therefore, assessment of environmental exposure to toxic chemicals as well as their impact on biological systems is of significant importance. Until recently, most approaches in water monitoring were based on the detection of individual chemicals, the physicochemical and microbiological parameters in a typical water analysis (Ahmed et al., 2019) and the determination of abundance and diversity of fauna and flora, which were subsequently compared against water quality standards (Madrid and Zayas, 2007). However, these measurements have several weaknesses such as their cost for analytical quantification, the limited detection of a small number of possible contaminants in the environment and their detection limits, which cannot always cover the presence of low concentrations of pollutants. In addition, such approaches fail to produce a diagnostic insight concerning the type of stressor and at the same time they are unable to predict future impact early enough to avoid ecological damage.

More advanced approaches propose the use of models and well-characterized multi-response systems to assess the responses of pollutants based on an understanding of the underlying mechanisms moving towards effect-based methods (Brack et al., 2019), taking into account complex mixture interactions (Altenburger et al., 2019) and explaining toxicity effects via adverse outcome pathways (Groh et al., 2015). As a general approach, model species are exposed to single chemicals in laboratory studies to assess their individual mechanisms. However, since in the environment organisms are not just confronted with single pollutants but rather combinations of different stress factors in complex mixtures, their effects have been within the scope of current research, thereby identifying markers for pollution (Escher and Stapleton, 2020).

Focusing on the freshwater ecosystem, in this study, the impact of eight individual pollutants from diverse categories of commonly encountered pollutants were initially assessed individually on *Daphnia magna*. These chemicals represent four pharmaceuticals, two metals, one pesticide and a stimulant, which are commonly encountered threats for freshwater and marine species in the environment. Furthermore, for a realistic scenario, their composite mixture was assessed in non-lethal concentrations. Using lethality as a surrogate measure of toxicity and biochemical markers of physiology, individual toxicity and mixture effects for eight pollutants were assessed. To compare and validate how biochemical

markers could provide meaningful information for environmental complex contamination, an in-depth assessment of the impact of the composite mixture at three stress intensities was subsequently performed on the metabolic level.

Materials and Methods

Culturing of Daphnids and Toxicity Exposures

Daphnids were maintained in glass beakers in OECD media (final concentrations 0.29 g $CaCl_2.2H_2O$/l, 0.123 g $MgSO_4.7H_2O$/l, 0.065 g $NaHCO_3$/l, 0.0058 g KCl/l, 2 µg Na_2SeO_3/l, pH 7.7) at a density of 80 adults per 4 l of media and under a 16h:8h of light:dark photoperiod at 20°C (Grintzalis et al., 2017). For experiments, neonates (<24 h) were collected from the third brood of their mothers and cultured until four days old and then used for experiments. Typically, in experiments with daphnids, acute toxicity is performed with neonates (<24 h); however, in several cases this has proven to be not reproducible, mainly because of the time window of neonate selection which extends to up to 24 h, thus resulting in a less homogenous population for experiments affecting toxicity (Traudt et al., 2017). Furthermore, as acute exposures are performed in the absence of food, the animals experience an additional stress of starvation which we avoided by allowing them to grow over a period of four days prior to exposure to the chemicals. Based on the selection of 24 h exposure periods, the chemicals were only added once following the general outlined procedure of the OECD guidelines (OECD, 2004). The chemicals used in this study were aluminium (CAS 16828-11-8), lithium (CAS 7447-41-8), acetylsalicylic acid (CAS 50-78-2), diltiazem (CAS 33286-22-5), metformin (CAS 115-70-4), propranolol (CAS 318-98-9), glyphosate (CAS 1071-83-6) and nicotine (CAS 54-11-5). All chemicals were of highest purity >99.9%.

For exposures, fifteen four-day-old animals were exposed to each chemical separately in a final volume of 100 ml OECD media with four replicates per concentration tested. Toxicity curves were plotted for 24 h exposures and EC values were calculated. A mixture was constructed for all chemicals and further assessed for its toxicity (Figure 1). All plots were calculated using the Four parameter logistic (4PL) model, following the equation Span = Top − Bottom and Y = Bottom + (Top-Bottom)/(1 + 10^((LogIC50-X)*HillSlope)), using the GraphPad software. The parameters top and bottom were commonly fixed to 100 and 0, accordingly.

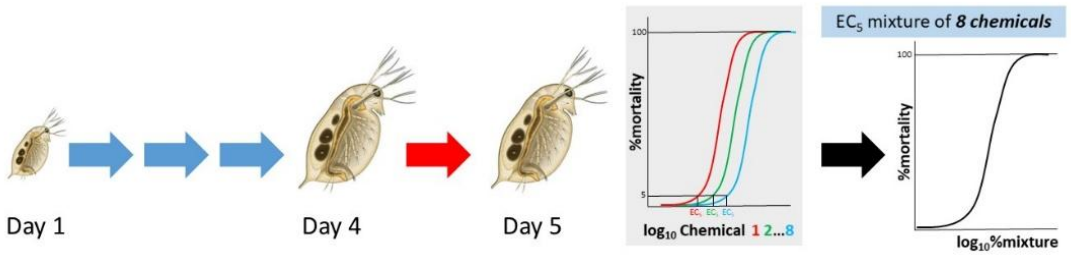

Figure 1. Experimental design. Four-day-old daphnids were exposed to eight chemicals individually for 24 h. Description of the combined mixture effect at EC_5 was used to assess its toxicity at 24 h.

Sample Homogenization and Biochemical Assays

Fifteen animals per biological replicate were pooled together and homogenized in 0.4 ml buffer using a pestle homogenizer. The homogenate was cleared by centrifugation (9000× *g* for 5 min at 5°C), and the clear supernatant was collected and assessed immediately for enzyme activity. Phosphatases were assayed in 100 mM acetic acid pH 4.5 (for acid; ACP) or 100 mM boric acid pH 9.8 (for alkaline; ALP) using the substrate *p*-nitrophenyl phosphate and monitoring the production of *p*-nitrophenol at 405 nm after its alkalinization. Similarly, the activities of β-galactosidase (βGAL) and lipase (LIP) were quantified by the generation of nitrophenol from the catalysis of *o*-nitrophenyl-β-galactoside or *p*-nitrophenyl butyrate, respectively, in phosphate buffer pH 7.2. The activity of peptidase (PEP) was quantified by the hydrolysis of L-leu-4-nitroanilide and the production of 4-nitroaniline (at 412 nm every five minutes for thirty minutes) in 100 mM phosphate buffer pH 7.2. Lactate dehydrogenase (LDH) activity was assessed from the consumption of NADH in a reaction with substrate of pyruvate (5 mM) at 340 nm (Worthington). Glutathione-S-transferase (GST) activity was measured by the formation of a complex between reduced glutathione with 1-chloro-2,4-dinitrobenzene in phosphate buffer pH 7.2 at 340 nm (Pacifici et al., 1981, Tang et al., 1996). For reduced thiols`, samples were homogenized in 100 mM acetic acid pH 4.5 and quantified following the protocol of Grintzalis et. al. (Grintzalis et al., 2014). Protein was quantified by a sensitive Bradford protocol (Grintzalis et al., 2015).

Metabolomic Analysis

Fifteen animals were snap frozen in liquid nitrogen and analysed in the Metabolomics Core Technology Platform at the University of Heidelberg. For metabolite extraction, the frozen sample material was ground with a micropestle in 190 µl 100% methanol and incubated for 15 min at 70°C with vigorous shaking. After the addition of 100 µl 100% chloroform, samples were shaken for 5 min at 37°C. To separate polar and organic phases, 200 µl HPLC-grade water was added and samples were centrifuged for 10 min at 11,000 *g*. While avoiding

the interphase containing cellular debris, 300 µl of the polar (upper) phase were transferred to a glass vial and dried using a vacuum concentrator (Eppendorf Concentrator Plus) without heating. Sequential on-line methoximation and silylation reactions were performed using an MPS autosampler (Gerstel, Mülheim Ruhr, Germany). Methoximation was performed by adding 20 µl 20 mg/ml methoxyamine hydrochloride (Sigma 226904) to pyridine (Sigma 270970) and incubation at 37°C for 90 min in an MPS Agitator Unit (250 rpm). For silylation reactions, 45 µl of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA; Sigma 69479) was added and samples were incubated at 37°C for 30 min with gentle shaking. Before injection, samples were incubated for 45 min at RT. For GC/MS analysis, a GC-ToF system was used consisting of an Agilent 7890 Gas Chromatograph (Agilent, Santa Clara) fitted with a Rxi-5Sil MS column (30 m × 0.25 mm × 0.25 µm; Restek) coupled to a Pegasus BT Mass Spectrometer (LECO). The GC was operated with an injection temperature of 250°C and a 1 µl sample was injected with a split ratio of 10. The GC temperature program started with a 1 min hold at 40°C followed by a 6°C/min ramp up to 210°C, a 20°C/min ramp up to 330°C and a bake-out at 330°C for 5 min using Helium as a carrier gas with constant linear velocity. The ToF mass spectrometer was operated with ion source and interface temperatures of 250°C, a solvent cut time of 9 min and a scan range (m/z) of 50–600 with an acquisition rate of 17 spectra/second. The ChromaTof v5.50 software (LECO Corporation, Saint Joseph, MI, USA) was used for data processing.

Statistical Analysis

The biochemical data were presented as mean ± standard deviation (SD) and were analysed and plotted with the GraphPad Prism software. For biochemical analysis, statistically significant differences were compared by Student's *t*-test over unexposed control with a *p*-value of 0.05 for single chemical exposures with a null hypothesis that differences would be observed due to chance. For the different concentrations of the mixture, One-Way ANOVA followed by comparisons with the control was performed and a test of a linear trend was validated. For metabolomic data (provided in Supplementary Materials), the values of peak area intensities were standardised by z scoring and then processed for multivariate statistical analysis with the freeware software Multi Experiment Viewer (Saeed et al., 2003) to perform principal component analysis (PCA) and hierarchical clustering with Pearson distance metrics. A significant analysis of microarrays (SAM) between each exposed group and control was performed to identify significant fold changes in metabolites.

Results

Toxicity of Individual Chemicals and Their Mixture

Acute exposure of daphnids to eight individual chemicals—Al, Li, acetylsalicylic acid, diltiazem, metformin, propranolol, glyphosate and nicotine—was assessed via toxicity curves (Figure 2) and the calculation of the effective concentration (EC) values (Table 1). As described, in this study we used older (four-day-old) daphnids and not the most sensitive neonates. The selection of this stage was made mainly because of the amount of tissue required but most importantly to avoid differences observed in some chemicals and their action because of the time window of the 0–24 h for collection of neonates. Specifically, it is well known that neonates have differences in toxicity responses as their collection could be from 0 to 24 h prior to the exposure, as shown for example for metals (Traudt et al., 2017). With this in mind and based on our previous experience, choosing a four-day stage as a starting point achieves better homogeneity allowing all individuals to grow to a level at which they will have a more unified response. Finally, in a freshwater population, all ages of daphnids are present, and therefore the neonate may serve as a more sensitive stage, but is not restrictive to the selection for this organism. As expected, the EC values recorded were in a similar order of magnitude but higher than reported EC_{50} values in the literature for neonates as neonates are more sensitive. In addition, a composite mixture in the ratio of the components' EC_5 (Table 1) was further explored for its toxicity in a range of dilutions to construct a full toxicity curve (Figure 3). For this toxicity curve the dose–response relationship was plotted as $\log_{10}EC_5$, and low (10% EC_5), medium (20% EC_5), and high (30% EC_5) concentrations were selected for the mixture exposures of daphnids for 24 h as non-lethal concentrations.

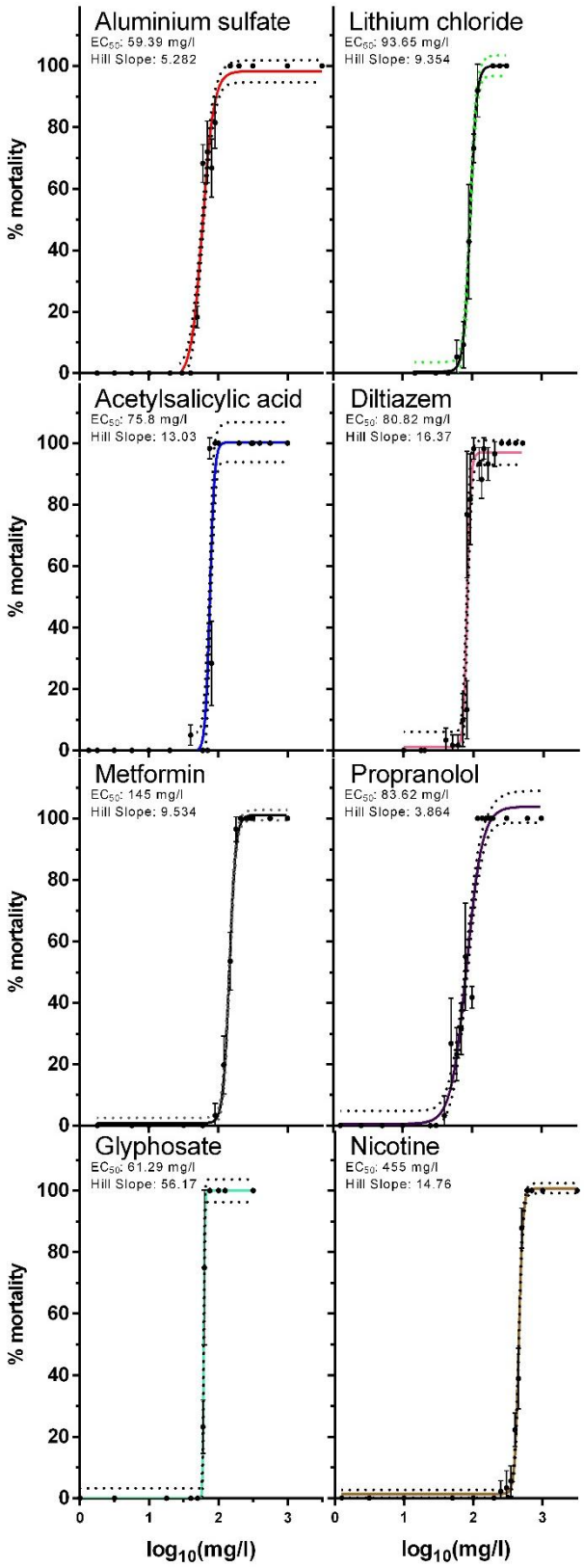

Figure 2. Acute toxicity curves for individual chemicals in this study. Data represent average ± standard deviation (N = 4 replicates).

Table 1. EC values (in mg/l) from toxicity curves and mixture ratio at EC_5 *.				
Chemical	**EC_{50}**	**Hill Slope**	**EC_5**	**% in Mixture**
Aluminium sulfate hexadecahydrate	59.4	5.282	34	4.53
Lithium chloride	93.7	9.354	68.4	9.11
Acetylsalicylic acid	75.8	13.03	60.5	8.06
Diltiazem hydrochloride	80.8	16.37	67.5	8.99
Metformin	145	9.534	106.5	14.19
Propranolol	83.6	3.864	39	5.19
Glyphosate	61.3	56.17	1.69	0.225
Nicotine	455	14.76	373	49.69

* presented precision does not signal significance but serves the purpose for reusability.

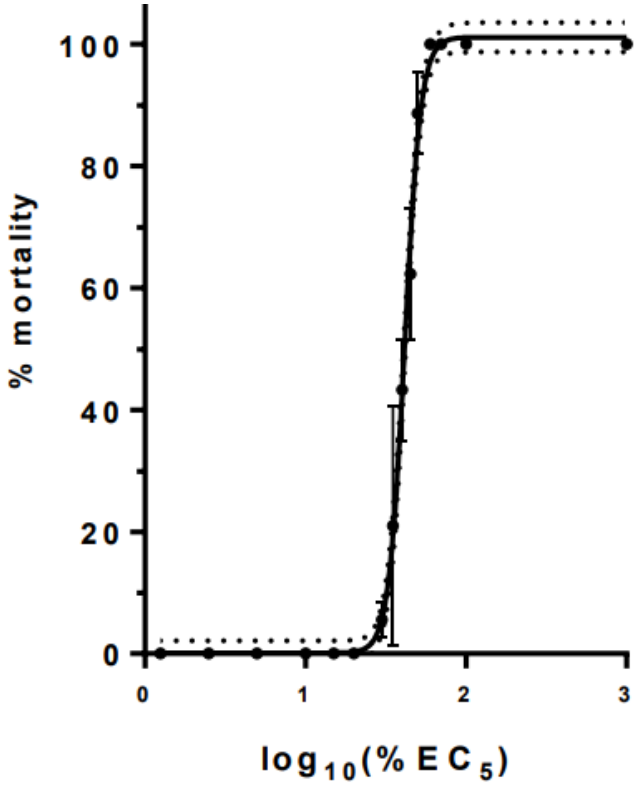

Figure 3. Acute toxicity curve of composite mixture of chemicals. Data represent average ± standard deviation (N = 6 replicates).

Enzyme Responses to Single Chemicals and Their Mixture

Exposure to individual stressors at EC_5 revealed distinct responses in the activity of enzymes among the different pollutants (Table 2). Acetylsalicylic acid, followed by nicotine, induced fewer changes in enzyme activities, while on the other hand, metals were the most impactful stress by decreasing all enzyme activities with the exception of GST which was increased by aluminium and not lithium. Interestingly, there is not a specific pattern on the responses triggered as for example, propranolol only resulted in increases in activities of both phosphatases and PEP. In relation to reduced thiols, four stressors (lithium, aluminium, nicotine and metformin) decreased their levels, while on the contrary, four chemicals

(acetylsalicylic acid, propranolol, diltiazem and glyphosate) increased the levels of reduced thiols.

Table 2. Biochemical markers of daphnid physiology upon exposure to a mixture of eight chemicals. Data represent mean ± standard deviation (N = 4) of enzyme activity. Enzyme activity was expressed as units/mg protein for LDH, LIP, βGAL and phosphatases, as munits/mg protein for GST, and for reduced thiols in nmoles/mg protein. Bold font indicates statistically significant difference by Student's *t*-test compared with the unexposed control.

	Control	**Li**	**Al**	**Acetyl Salicylic Acid**	**Propranolol**	**Diltiazem**	**Glyphosate**	**Nicotine**	**Metformin**
ALP	7.53 ± 0.88	**2.2 ± 0.21 (−71%)**	8.8 ± 0.31	8.13 ± 0.68	**13.98 ± 1.19 (+84%)**	**10.6 ± 0.34 (+41%)**	**5.42 ± 0.53 (−28%)**	7.77 ± 0.79	**4.7 ± 1.18 (−38%)**
ACP	5 ± 0.69	**1.8 ± 0.07 (−64%)**	**3.4 ± 0.13 (−32%)**	5.73 ± 0.09	**7.44 ± 0.46 (+49%)**	5.7 ± 0.24	5 ± 0.21	4.38 ± 0.3	**3.17 ± 0.71 (−37%)**
βGAL	11.63 ± 0.2	**1.85 ± 0.1 (−84%)**	**3.13 ± 0.05 (−73%)**	12.36 ± 0.87	9.96 ± 1.14	11.7 ± 1.02	11.7 ± 0.42	11.1 ± 1.25	**4.72 ± 0.71 (−59%)**
LIP	165 ± 10.1	**50.2 ± 2.3 (−70%)**	**104.6 ± 13.5 (−37%)**	181.64 ± 3.7	153 ± 16.4	**190 ± 12.5 (+15%)**	**187.8 ± 9.6 (+14%)**	169.4 ± 11.8	**84.2 ± 16.7 (−49%)**
PEP	286 ± 21.8	**53 ± 10.7 (−82%)**	**158 ± 11.8 (−45%)**	**240 ± 17.6 (−16%)**	**387 ± 41.5 (+35%)**	291 ± 41.8	**340 ± 8.9 (+19%)**	**217 ± 27.5 (−24%)**	**138 ± 27.8 (−52%)**
LDH	80.32 ± 6.52	**63.8 ± 5.33 (−21%)**	83 ± 5.22	84.6 ± 4.33	77.7 ± 2.51	86.42 ± 7.88	72.59 ± 6.85	**65.5 ± 3.15 (−18%)**	**67.4 ± 4.91 (−16%)**
GST	212 ± 21.7	**34.3 ± 19.7 (−84%)**	**274 ± 7.6 (+29%)**	198.6 ± 6.6	215.6 ± 24.5	**151.4 ± 6 (−29%)**	**134.7 ± 7.2 (−37%)**	**155.6 ± 4.8 (−27%)**	254.8 ± 9.2
Reduced thiols	64.9 ± 3.5	**36.7 ± 1.2 (−43%)**	**51.9 ± 1.7 (−20%)**	**79.9 ± 2 (+23%)**	**74.1 ± 2.5 (+14%)**	**70.5 ± 2.2 (+8.6%)**	**73.6 ± 3.9 (+13.5%)**	**50.7 ± 1.3 (−22%)**	**57.7 ± 3.2 (−11%)**

Exposure to the eight chemical mixtures resulted in dose-dependent changes for all enzyme biomarkers assessed (Table 3). Both ACP and ALP activity was increased in the ranges of 18–37% and 36–41%, respectively, in a concentration-dependent manner relative to the intensity of the mixture. Furthermore, GST activity showed a trend to increase between 29% and 52%. On the other hand, βGAL and LIP decreased between 12% and 42% dose-dependently with the stress intensity. PEP and lactate dehydrogenase, and reduced thiols, were also decreased in response to the concentration of the mixture, by 21% to 56%, respectively. The latter observed decrease in thiols could also be correlated with the increase in the activity of GST, which uses glutathione as a substrate to detoxify xenobiotics, and potentially other thiol-consuming enzymes.

Table 3. Biochemical markers of daphnid physiology upon exposure to a mixture of eight chemicals. Data represent mean ± standard deviation (N = 4) of enzyme activity. Enzyme activity was expressed as units/mg protein for LDH, LIP, βGAL and phosphatases, as munits/mg protein for GST, and for reduced thiols in nmoles/mg protein. Bold font indicates statistically significant difference by One-Way ANOVA followed by comparison with the unexposed control.

	Control	**10%**	**20%**	**30%**
ALP	8.28 ± 0.19	**11.67 ± 1.01 (+41%)**	**11.64 ± 0.32 (+41%)**	**11.22 ± 1.19 (+36%)**
ACP	3.08 ± 0.14	**3.62 ± 0.44 (+18%)**	**4.05 ± 0.27 (+31%)**	**4.29 ± 0.45 (+37%)**
βGAL	3.62 ± 0.06	**3.19 ± 0.09 (−12%)**	**2.6 ± 0.13 (−28%)**	**2.11 ± 0.09 (−42%)**
LIP	17.73 ± 0.59	**15.34 ± 1.31 (−13%)**	**10.43 ± 1.18 (−41%)**	**10.85 ± 1.24 (−39%)**
PEP	95.65 ± 4.44	93.09 ± 12.29	**73.8 ± 6.81 (−23%)**	**77.18 ± 6.58 (−20%)**
LDH	54.79 ± 2.32	50.44 ± 7.07	**32.9 ± 10.11 (−40%)**	**18.41 ± 4.3 (−67%)**
GST	149.52 ± 3.36	169.14 ± 13.02	**193.41 ± 7.56 (+29%)**	**227.61 ± 23.68 (+52%)**
Reduced thiols	201.44 ± 31.76	**158.82 ± 12.12 (−21%)**	**115.69 ± 17.18 (−43%)**	**88.69 ± 35.97 (−56%)**

The Metabolic Responses to Mixture Exposure

An untargeted metabolomic analysis revealed a significant number of changes in the metabolism of daphnids upon exposure to the different intensities of the combined stress, thus supporting the observations in enzyme activities. Principal component analysis (PCA) and hierarchical clustering (HCL) show a clear grouping and clustering, respectively, of the metabolic profiles based on the intensity of the combined mixture stress (Figure 4). There is a clear trend (Figure 4 green arrow) of increase in intensity towards PC1. This is also supported by the significance analysis of microarrays analysis (SAM) (Figure 5), which allows the identification of significant changes based on the differential expression between sets of samples. Although this analysis is used in microarrays, it is also applicable to metabolomic data (Diercks et al., 2016). Statistically, this analysis provided a number of significantly up- or down-regulated metabolites with increasing stress intensities. For example, even from the low stress, undecanol and β-alanine were down-regulated, and with the increase in the intensity of the mixture stress to 20%, citronellol and ethanoloamine were also decreased, and at 30%, methionine, phenylalanine and glycine were added to the list of significantly decreased metabolites. On the other hand, all stress intensities up-regulated threonine and putrescine, while other metabolites of the TCA cycle (citric acid, fumaric acid), the urea cycle (ornithine) and amino acids (proline, valine) appear increased only at

the middle (20%) and high (30%) intensity, thus indicating differences with the escalation of the stress effect.

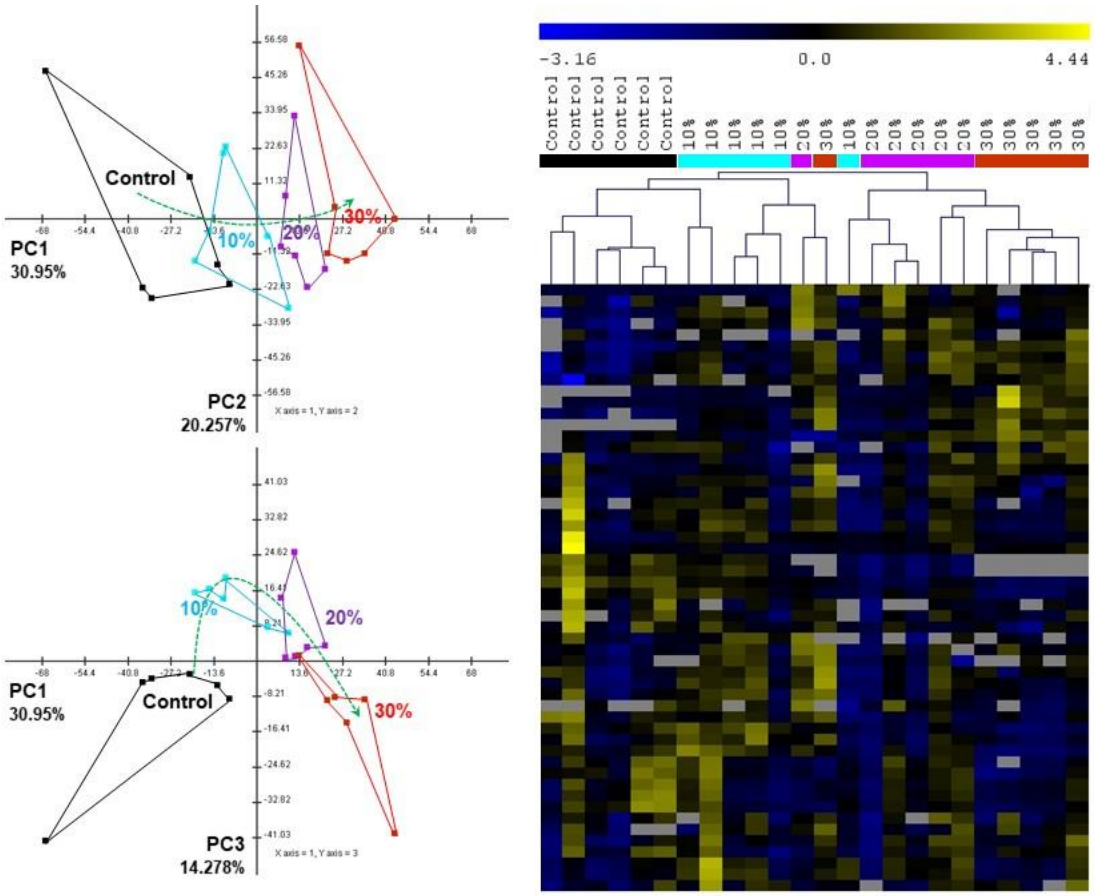

Figure 4. Multivariate statistical analysis of metabolomic data. PCA analysis and HCL shows the grouping and clustering of samples. The green arrow in PCA shows the gradual change in the metabolic profiles following the intensity of the mixture stress.

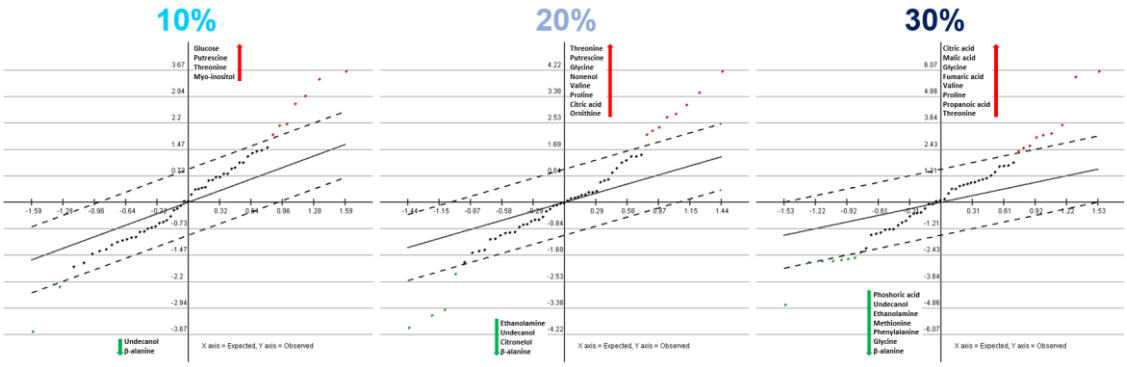

Figure 5. Significance Analysis of Microarrays (SAM) for each exposure compared with the unexposed control reveals the gradual change in the most significant metabolites.

Discussion

The Effects of Individual Stressors

There is a great number of chemicals simultaneously present in the environment, and a study of their effects separately is impossible in the actual environment. In most laboratory studies,

the individual effects of single stressors are assessed in controlled experiments to understand their underlying mechanisms of toxicity.

Metal contamination is a major concern in aquatic ecosystems and, therefore, it is important to find reliable indicators of metal stress on aquatic organisms. In this study, aluminium and lithium were selected as two metal stressors commonly present in freshwater ecosystems. The adverse effects of both these metals have been studied in a variety of aquatic organisms such as sea urchins, fishes and snails (Kszos et al., 2003), and in daphnids the reported EC_{50} for neonates is in a similar range to the EC_{50} reported in our study. In one study, aluminium exposure resulted in the differential expression of 155 genes (Brun et al., 2019) and its toxicity is believed to be mediated due to its capacity to strongly bind to phosphorus, thus reducing its availability. Ionic aluminium is able to inhibit extracellular phosphatases, and in our study this was the case for a decrease in the activity of acid phosphatase but not for alkaline phosphatase. Lithium has also been shown to exert toxic effects on aquatic organisms such as the fathead minnow and *Ceriodaphnia dubia*, implicating the role of other elements such as sodium decreasing lithium toxicity (Kszos et al., 2003). Furthermore, in relation to daphnids, lithium exposure resulted in 143 genes being differentially expressed, some even by over three-fold (Kim et al., 2017). In some studies, lithium led to significant metabolite variations, specifically in amino acids as well as uracil and the osmolyte glycerophosphocholine, thus revealing toxicity-mediated effects by impairing energy production and ionoregulation (Nagato et al., 2013). In the present study, both metals had a significant impact through decreasing most biochemical markers when applied independently. Furthermore, the observed increase in GST activity upon aluminium exposure has also been reported by others as a response to heavy metals in plants (Kumar and Trivedi, 2018) and to toxins in daphnids (Lyu et al., 2016).

With the demographic trend of an ageing of populations and the high accessibility of medication and drugs, pharmaceuticals have been highlighted as a class of emerging pollutants. This in turn is also amplified by their improper disposal and limited removal from waste water treatment plants. Non-steroidal anti-inflammatory drugs (NSAIDs) are probably the most widely used medication worldwide. In our study, acetylsalicylic acid, best known as aspirin, was studied for its impact on daphnids as a representative NSAID. Acetylsalicylic acid has been reported to decrease survival rates, fecundity and growth in daphnids (Marques et al., 2004), and the reported EC_{50} values are similar to the ones observed in this study. In daphnids, it has been shown that acetylsalicylic acid mediates its toxicity via an induction of oxidative stress with an increase in lipid and protein oxidation which is accompanied by DNA damage. In response to the aforementioned effects on oxidative stress, changes in

antioxidant enzyme activities such as superoxide dismutase and catalase have been recorded (Gómez-Oliván et al., 2014). A more holistic approach revealed that three genes are significantly up-regulated and four genes are significantly downregulated in response to acetylsalicylic acid (Bang et al., 2015). However, in our study, acetylsalicylic acid had a less significant impact, decreasing only the activity of PEP.

Diltiazem is a non-dihydropyridine calcium channel blocker prescribed to treat high blood pressure and to control angina (Crawford, 1985). Diltiazem inhibits calcium influx into both cardiac and smooth muscles during depolarisation, and as such, it is most probable that toxic effects would occur through the disruption of regulation of cellular calcium levels. Maintenance of appropriate calcium levels is important for many physiological processes in all organisms, including daphnids. At low concentrations of 500 ng/l, diltiazem has been shown to increase the heart rate of *Daphnia magna*, along with oxygen consumption, thus resulting in energy imbalance and a higher demand for energy (Steinkey et al., 2019). Although in our study we did not assess phenotypic endpoints, diltiazem decreased GST activity and increased alkaline phosphatase and LIP. The latter observed increase in LIP activity could be seen as consistent with the decrease in lipids reported in other studies (Steinkey et al., 2019).

Metformin is a medication used under many brand names and is the most common drug prescribed to treat type 2 diabetes and polycystic ovary syndrome. Because of its wide audience of patients, it is very much consumed and, thus, commonly present in the aquatic ecosystem (Oosterhuis et al., 2013). Metformin may interact with a variety of molecular targets across species (Ambrosio-Albuquerque et al., 2021). In relation to its actions on aquatic species, there is literature on its impact on fecundity and behaviour in fish (Alla et al., 2021, Niemuth et al., 2015) and therefore, metformin has been attributed as an endocrine disruptor (Elizalde-Velázquez and Gómez-Oliván, 2020). There are few biochemical data available for daphnids; however, the mechanism of action of metformin has been linked to the induction of the hypoxia-inducible factor (HIF) α and β genes (Sheng et al., 2012). In our study, metformin proved to be a strong stressor and decreased all enzyme activities simultaneously, thus showing a significant impact on daphnids.

Propranolol is a drug that belongs to the category of β-blockers, which are prescribed for the treatment of stable coronary ischaemic disease (Kloner et al., 1977). Propranolol has been detected in the freshwater environment in significant high levels, and although it is designed for human therapeutic usage, it exerts its effects on non-target organisms. As a drug with a significant bioaccumulation effect, propranolol at a very low concentration has been shown to bioaccumulate in daphnids at up to 1.6x its original concentration over 10 generations

(Jeong et al., 2016). Propranolol is toxic to neonates with an ambient EC_{50} of 7.5 mg/l (Cleuvers, 2003), which is significantly lower than the one determined in our study, which could be attributed to the higher sensitivity of neonates when compared with the four-day-old daphnids used here. Propranolol may exert its toxic effects in daphnids in an organ-specific manner, such as reducing the heart rate (Jeong et al., 2018), in addition to decreasing fecundity (at 0.22 to 0.44 mg/l) and completely inhibiting it (at 0.88 mg/l). It is worth noting that in transgenerational exposures, the second generation of daphnids was less sensitive to propranolol (Dzialowski et al., 2006) although in other studies even subtle environmentally relevant concentrations may induce physiological changes (Jeong et al., 2015). Propranolol has been reported to increase GST activity and inhibit glutathione peroxidase (GPx), an enzyme that removes peroxyl radicals and hydroperoxides (Oliveira et al., 2015), thus affecting the antioxidant defence system of daphnids. Interestingly, in our study, propranolol increased the activity of both phosphatases and PEP.

N-phosphonomethyl glycine is a well-known herbicide called glyphosate, which has been employed extensively in agriculture, and thus can be found in the environment as a consequence of agricultural or urban run-off and leaching into local surface waters (Noori et al., 2018). There have long been concerns over its implications in the environment and for public health. Glyphosate has been shown to cause morphological alterations in zooplanctonic organisms and crustaceans (Gustinasari et al., 2021), and to impact carbon and fat metabolism and the microbiome (Suppa et al., 2020) as well as the heart rate of daphnids (Duan et al., 2019). Our results support these findings, as an increase in LIP and PEP was observed along with a decrease in alkaline phosphatase and GST. The latter is in agreement with similar decreases in fish and could be explained as failure of detoxification processes and development of oxidative stress (Samanta et al., 2014). In addition, recent studies on daphnids showed that glyphosate exposure can modify the mRNA transcription and enzymatic activity of GST and lipid peroxidation (Zhang et al., 2020) and even exacerbate its toxicity in the presence of microplastics (Zocchi and Sommaruga, 2019).

The last chemical studied was a stimulant, nicotine, which is widely consumed within a number of products in daily life and is a key ingredient of tobacco. There is a general lack of data on the impact of nicotine on freshwater organisms, and many existing studies focus solely on mortality or heart-rate as physiology endpoints. Nicotine has been found to reduce fecundity in daphnids by decreasing the number of neonates released per individual. It also triggers the production of male offspring (Oropesa et al., 2017) and induces antenna, carapace and spine malformations (Chen et al., 2018) in daphnids. In our study, nicotine

decreased the activities of PEP, LDH and GST, and the latter could indicate a possible induction of stress in daphnids.

Mixture Effects and Omics in Toxicology

Organisms in their natural environments are exposed to composite mixtures of several individual chemicals at low concentrations. This is an issue, as chemical interactions within these mixtures can result in unintuitive results. The individual components were mixed at an EC_5 ratio; however, the mixture, as expected, was significantly more toxic than its constituents alone. This is known as synergy, meaning that the components act together, and this poses a major problem in the context of environmental risk assessments. These results are not easily predicted, so new methods are needed to understand the mechanisms and interactions within these mixtures. Effect-based methods gain more attention in the literature as complementary strategies to the chemical analytical characterisation of complex pollution patterns and they can provide new metrics for pollution assessment (Altenburger et al., 2018).

For mixture prediction, there exist two concepts—Concentration Addition (CA) and Independent Action (IA)—that allow the calculation of expectable combined effects based on individual components' bioactivities and mixture exposure knowledge. Both concepts are based on knowledge of the single-compound toxicities and the assumption of no interaction. CA assumes that the individual components behave as simple dilutions of one another, which is commonly interpreted as being the case for compounds of a mixture sharing a strictly similar mechanism of action. However, IA supports the completely independent action of chemicals, which is commonly interpreted as the compounds of a mixture having dissimilar mechanisms of action. In a recent study on daphnids where mortality was used as the only endpoint, four contaminants (sodium fluoride, boric acid, ammonium hydroxide and acetaminophen) were assessed in mixtures. Regardless of the assumption of dose- or response-additivity, independent action slightly outperformed concentration addition in most of the combinations of these multiple-class compounds (Silva et al., 2022). In this study, we considered that individual responses were low but we did not perform any analysis of non-interactive mixture modelling, but rather we chose to elaborate for observation of biochemical markers of enzyme activities, in order to understand the biological responses of daphnids when exposed to low (10% EC_5), medium (20% EC_5) and high (30% EC_5) concentrations of the composite chemical mixture. As it was hypothesised, a clear dose response in connection to the stress intensity was observed, which could be attributed to a synergy effect in the above meaning. However, a more in-depth level of knowledge would

require more sophisticated measurements. Holistic or omics methodologies are widely used in toxicological research and have a pivotal role in the understanding of mechanisms of toxicity (Harrill et al., 2021). These analytical approaches extend from the epigenome to the transcriptome, proteome and metabolome level. Metabolomics is the study of low-molecular-weight metabolites and metabolic pathways within living organisms, and is a fast-evolving research field with pioneering investigations in relation to the biochemical responses to toxicants. Metabolism is a decisive parameter for physiology and is of central importance for adaptation of all life forms. Therefore, the unique strength of metabolomics is that it measures the functional status of an organism as the alterations in metabolic levels are the primary adaptation mechanism in organisms (Roessner and Bowne, 2009). This is because by nature metabolism responds fast to environmental stimuli which allows the analysis and interpretation of the organism's response at a molecular pathway level. In our experiments, alterations in the TCA cycle, the urea cycle and metabolism of amino acids were highlighted as perturbed with intensity of stress. Although our analysis was a preliminary discovery analysis and not targeted to any pathway, targeted methods have identified perturbations in specific pathways (Labine and Simpson, 2021) or mapped these changes on specific tissues (Smith and Weber, 2022) in these crustaceans. Furthermore, this study focused on a composite mixture as a stress pool and not on a specific chemical; however, lithium, for example, has been recently assessed in its nanoparticle form to affect amino acid, starch and glucose metabolism, which could also be reflected in our study from changes in the relevant catabolic enzymes (Niemuth et al., 2021).

Metabolomic analysis in daphnids has been highlighted as a key mechanistic tool for environmental monitoring (Jeong and Simpson, 2019) and can provide valuable fitness information at the molecular level (Taylor et al., 2018). Our study verified that simple biochemical markers of enzyme activities show similar patterns in changes with sensitive holistic metabolomic analysis. In this context, simple enzyme activity endpoints can be used as a first step to evaluate and design metabolomic detailed studies safely. To our knowledge, this is the first study where a direct link was observed in daphnids between the activities of key enzymes relevant to the physiology of daphnids and metabolomic analysis, thus verifying the employment of the first as potent markers in toxicology assessment.

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KG being the guest editor of the special issue “Toxicity of Contaminants on Aquatic Organisms”.

Institutional Review Board Statement: Ethical review and approval were waived for this study, due to the fact that daphnids are regarded as “animals” in terms of being members of the kingdom Animalia, however, they are not “animals” as defined in regulation SI543 of 2012 on the protection of animals used for scientific purposes. Therefore, the study does not require authorization from the Health Products Regulatory Authority (HPRA), while is also in line with the aim of working under the 3Rs (reduce, refine, replacement) strategy, since daphnids are commonly used in ecology and ecotoxicology as replacements of more evolutionary advanced species (i.e. fishes), posing no ethical implications.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 2B

This chapter was a continuation of the preceding work by examining multigenerational exposure of *D. magna* to the eight-chemical mixture at environmentally relevant concentrations. For this study, daphnids were exposed for five generations and phenotypic and biochemical endpoints, as well as metabolomic analysis were used to evaluate the impact of the mixture. The results showed dose- and generation-dependent patterns of effects, which were further confirmed though metabolomic analysis, even at the lowest concentrations. This study highlighted the importance of multigenerational exposures for an in-depth understanding of the long-term cumulative effects.

Novel Approaches Methodologies in ecotoxicology - Metabolism reveals multigenerational effects of pollutants on daphnids

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Abstract

The field of ecotoxicology is advancing with the use of new approaches methodologies (NAMs) to scrutinize the effects of pollutants on aquatic ecosystems. This study employs cutting-edge methods to assess the impact of mixtures of eight pollutants on *Daphnia magna*, a keystone species in freshwater ecosystems. The levels of toxicity were in the same order of magnitude for the eight stressors, while the mixture was significantly more toxic. For the present study, daphnids were exposed for five consecutive generations to seven non-lethal and environmentally relevant concentrations ranging from 1 ng/l to 1000 μg/l. During the first generation, only the higher concentrations affected the enzymatic activities of daphnids, while after the third generation the effects were milder. In the fifth generation all concentrations, even the lowest, caused an impact on daphnids, while the animals exposed to the 1000 μg/l did not survive. Additionally, a feeding assay on D2 daphnids revealed a dose-dependent effect caused by the higher concentrations 500 and 1000 μg/l. Besides the biochemical markers, a targeted LC-MS/MS approach on the fifth generation daphnids confirmed the results from the former revealing a significant dose-dependent pattern even at the lower concentrations 1-100 ng/l. Our findings underscore the importance of not only acute or one generation chronic exposure, but multigenerational chronic exposures to investigate how the stress is carried out to the next generations.

Keywords: *Daphnia magna*, ecotoxicology assessment, metabolomics, multigenerational, mixture

Highlights:

- Higher exposure concentrations significantly decreased the feeding performance of daphnids
- Stress was more intense in later generations of daphnids
- Dose-dependent effects even at the nano-concentrations
- Metabolic changes in daphnids provide means for the early prediction of pollution

Introduction

Assessment of water quality is based mostly on the detection of pollutants and chemicals in the environment and comparison with water quality standards. These approaches are limited in sensitivity and cannot always provide the full cover of the spectrum of pollutants actually present. Furthermore, they provide no mechanistic insight for pollution assessment on aquatic species and cannot offer any prediction or early warning (Escher and Leusch, 2011). This is why in recent years, effect-based methods support the current water quality approaches with the aim to capture the impact of pollutants in the environment. Therefore, *in vitro* and *in vivo* systems offer alternatives which provide mechanistic insight into the action of pollutants and add up to our knowledge about contaminants. Effect-based methods are more sensitive tools, and they offer the potential for more accurate predictions of pollution assessment (Brack et al., 2019). Most chemicals in the environment occur as mixtures rather than as single substances. Despite that, pollution assessment is more focused on the effects of the chemicals individually on aquatic species (Backhaus and Faust, 2012). The impact of combined chemicals can have either synergistic or antagonistic properties, often making the mixture more toxic than the individual compounds, even at very low concentrations (Cedergreen, 2014). This highlights the importance of new improved methodologies to evaluate the effects of mixtures on aquatic life more accurately (Carvalho et al., 2014). According to the literature, there are two prediction models in Mixture Toxicology: Concentration Addition (CA) and Independent Action (IA). In the case of CA, the individual chemicals in a mixture have the same mode of action, while in IA the single compounds have different mechanism of action. In real-world conditions, chemical mixtures often comprise substances with varying mechanisms of action, which adds complexity to predicting their combined effects (Altenburger et al., 2012, BLISS, 1939).

Focusing on the freshwater ecosystem, crustaceans are among the key species in freshwater ecology and ecotoxicology. Daphnids are sentinel species used in aquatic toxicity testing mainly since they are sensitive and responsive to chemical and physical changes in the water environment (Abdullahi et al., 2022). Their small size allows to perform studies in limited space and consumption of resources, and this is further aided by their clonal reproduction which provides uniform responses among individuals. Daphnids are filter feeders and have been well established in the field of molecular ecology and ecotoxicology. Their responses to many categories of pollutants have been studied extensively and they provide meaningful information towards risk assessment.

Based on our previous study (Michalaki et al., 2022) a mixture of eight different chemicals (namely, aluminium sulfate, lithium chloride, acetylsalicylic acid, propranolol, diltiazem, metformin, glyphosate, and nicotine) at concentrations ranging from 1 ng/l to 1000 μg/l were selected for acute, chronic and multigenerational exposures of daphnids.

In this study, we assessed the impact of a mixture of eight commonly encountered pollutants from different categories such as pharmaceuticals, metals, stimulants, and pesticides. Using markers of physiology and specifically phenotypic endpoints, enzyme activities and metabolomics, the effect of the "pollutant cocktail" was assessed at environmentally low concentrations in multigenerational experiments. Aside from just acute experiments, this study focused on multigenerational experiments which realistically impact the real environment more, as it is seldom that pollutants are removed completely after short periods of being introduced to the aquatic ecosystem. Furthermore, the effects of contaminants may also change upon chronic and multigenerational exposures so it would be misleading to evaluate the impact of pollution solely based on acute experiments. The selection of the pollutants in this study was based on a previous acute study (Michalaki et al., 2022) as representatives of different categories. Composite mixtures of pollutants are a more realistic scenario and the aim here was to move towards very low and environmentally relevant exposure levels.

Materials and methods

Materials

All chemicals used in this study were of the highest purity >99.9% and quality. Aluminium sulfate (CAS 16828-11-8), lithium chloride (CAS 7447-41-8), acetylsalicylic acid (CAS 50-78-2), diltiazem (CAS 33286-22-5), metformin (CAS 115-70-4), propranolol (CAS 318-98-9), glyphosate (CAS 1071-83-6), nicotine (CAS 54-11-5), KCl (CAS 7447-40-7), Na_2SeO_3 (CAS 10102-18-8), bovine serum albumin (CAS 9048-46-8), Coomassie Brilliant Blue G (CAS 6104-58-1), *p*-nitrophenyl butyrate (CAS 2635-84-9), 2-nitrophenyl-B-D-galactopyranoside (CAS 369-07-3), 1-chloro-2,4-dinitrobenzene (CAS 97-00-7), L-glutathione reduced (CAS 70-18-8), sodium phosphate dibasic (CAS 7558-79-4), L-leu-4-nitroanilide (CAS 4178-93-2) were purchased from Sigma-Aldrich (St. Louis). $CaCl_2 \cdot 2H_2O$ (CAS 10035-04-8), $MgSO_4 \cdot 7H_2O$ (CAS 10034-99-8), $NaHCO_3$ (CAS 144-55-8), HCl (CAS 7647-01-0), *p*-nitrophenyl phosphate (CAS 4264-83-9), boric acid (CAS 10043-35-3), ammonium acetate (CAS 631-61-8), NaOH (CAS 1310-73-2), methanol (CAS 67-56-1), and DMSO (CAS 67-68-5) were purchased from ThermoFisher (Ireland).

Culturing of daphnids for exposures

Daphnids were maintained in glass beakers in OECD media and cultured as described previously described (Michalaki et al., 2022) under a 16h:8h of light:dark photoperiod at 20°C (OECD, 2012). Initially, fifteen neonates (<24 h) were collected from the third brood of their mothers and exposed for 24 hours to each pollutant (aluminium sulfate, lithium chloride, acetyl salicylic acid diltiazem, metformin, propranolol, glyphosate, nicotine) individually and to their mixture at equal concentrations in 50 ml volume. While the OECD guidelines suggest that at least twenty animals (preferably in four groups of five animals) should be used at each test concentration, in our study we increased this to sixty animals at each concentration tested, thus increasing our reproducibility even more (OECD, 2004). Toxicity curves were plotted using the four-parameter logistic Hill model, with the equations Span = Top Bottom and Y = Bottom + (TopBottom)/(1+10((LogLC50-X)*HillSlope)), and EC values were calculated using the GraphPad Prism programme. This allowed the estimation of mortality and the careful selection of the exposure concentrations from ng per l to extreme μg per l (Gomez-Olivan et al., 2014). For chemical exposures to the mixture of pollutants, thirty-six animals were cultured in 900 ml media as previously described (Michalaki and Grintzalis, 2023) (Figure 1). Individual stock solutions of the eight chemicals were prepared at 1000 mg/l in OECD media. A mixed stock solution (1.5 l) was prepared by adding 1.5 ml of each individual chemical and 1,488 ml of OECD, resulting in final concentration of 1 mg/l per compound. Serial dilutions were prepared with OECD media to obtain nominal concentrations of 100 μg/l, 10 μg/l, 1 μg/l, 100 ng/l, 10 ng/l, 1 ng/l. For clarity, all mixture concentrations reported throughout the study refer to dilutions of this stock solution and therefore represent nominal total mixture concentrations, with each compound present at equal proportions. Media and chemical mixture were renewed twice per week, and each generation was 21 days long. Daphnids were fed daily with fresh algae (*C. reinhardtii*, at 16.5 million cells per 900 ml of exposure), and a seaweed extract (*Ascophylum nodosum,* 1.5 ml/l of 5 g/l stock, thus 7.5 mg/l) which was only added on media changes. Animals were sacrificed at the first, third and fifth generation of exposure.

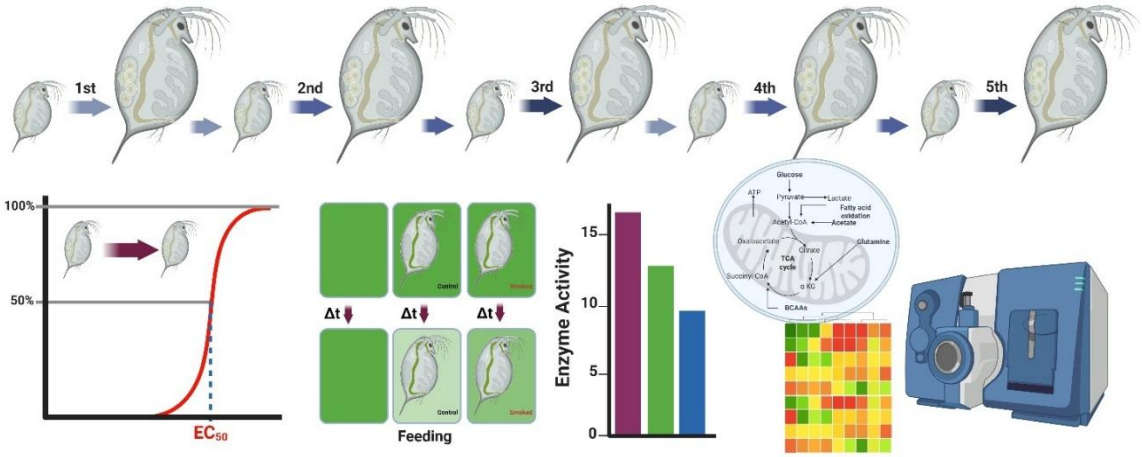


Figure 1. Experimental design. Acute and multigenerational exposures for up to five generations. Enzyme activities were used on the first, third and fifth generation, while metabolomic analysis was performed only on the fifth generation to capture the metabolic perturbations alongside the dose-dependent effect at such low concentrations. Feeding was assessed in neonates for acute toxicity. Figure created with Biorender.com.

Feeding experiments

Feeding was assessed with a modification of (Rowan et al., 2024) as described elsewhere (Michalaki and Grintzalis, 2023). Specifically, neonates were exposed to the eight concentrations of the mixture (1 ng/l to 1000 μg/l) for 24 h. After 24 h, twenty-five daphnids were transferred in a 12-well plate with 6 ml OECD containing the carboxylate-modified polystyrene, fluorescent red microparticles (2.0 μm mean particle size) at a concentration of 13 mg/l. The animals were exposed to microplastic for up to 50 min, and media was collected every 10 min to estimate the removed microparticles by fluorescence at Ex 560 Em 590 nm using a TECAN plate reader. The concentration of microparticles in the media was optimized to ensure an excess of microparticles for the accurate quantification of ingestion. Additionally, these particles have been extensively tested and demonstrate no toxicity to the daphnids for the short exposure periods used (Giannouli et al., 2023, Kakavas et al., 2023, Kakavas et al., 2024). Feeding rate was expressed as the slope for 50 min for the consumption of microplastic.

Enzyme markers of physiology

Daphnids from 21 days of exposures from the first, third and fifth generations were collected and immediately homogenized for the assessment of enzyme markers and protein content. Ten daphnids per biological replicate, were pooled together and homogenized in one ml of ddH_2O using a pestle homogenizer. The homogenates were cleared by centrifugation (15,000 *g* for 5 min at 4^oC) and the clear supernatant was collected and assessed immediately. The activities of all enzymes were quantified using a TECAN plate reader. With the suitable substrate, the activity of phosphatases, β-galactosidase (βGAL), and lipase

(LIP) were measured as released nitrophenol as described elsewhere (Michalaki et al., 2022). Phosphatase activity was measured where *p*-nitrophenyl phosphate was converted to *p*-nitrophenol by either an acid phosphatase (100 mM citric acid pH 4.5; ACP) or an alkaline phosphatase (100 mM boric acid pH 9.8; ALP) at 405 nm (Grintzalis et al., 2022). To measure the activity of LIP and βGAL, the release of nitrophenol from the catalysis of *p*-nitrophenyl butyrate or *o*-nitrophenyl-β-galactoside, respectively, in phosphate buffer pH 7.2, was used. Peptidase (PEP) was evaluated using continuous kinetics at 418 nm following the release of p-nitroaniline (from L-leucine-4-nitroanilide). Continuous kinetics were used to quantify the glutathione-S-transferase activity for the formation of the complex between glutathione and 1-chloro-2,4-dinitrobenzene at 340 nm (Tang et al., 1996). Finally, the activity of lactate dehydrogenase (LDH) was assessed from the consumption of NADH in a reaction with pyruvate as a substrate (5mM) at 340 nm (Worthington and Worthington, 2011). Using a sensitive assay (Grintzalis et al., 2015), all enzyme activity was normalized in units per protein.

Metabolomic analysis

Following exposure to the pollutant mixture, four daphnids were immediately snap frozen in liquid nitrogen per replicate and stored at -80°C until extraction of metabolites. For extraction, samples were homogenised in 100 µl Ethanol:PBS (85:15) and centrifuged at 24000 × *g* for 5 minutes at 4°C. The supernatant was collected and stored at -80°C until data acquisition. The AbsoluteIDQ® p180 assay (Biocrates Life Sciences, Innsbruck, Austria) was applied to acquire the metabolite data according to the manufacturers' manual. Briefly, 10 µl of supernatant was added to the 96-well plate and dried under a stream of nitrogen. Phenyl isothiocyanate (50 µl, 5%) was added to each well and incubated for 25 minutes at room temperature, and the plate was dried for 60 minutes under a continuous nitrogen stream. The extraction solvent (5 mM ammonium acetate in methanol, 300 µl) was added to each well and placed on a shaker for 30 minutes. The plate was centrifugated at 500 × *g* for 2 minutes and 150 µl of eluate was diluted with 150 µl of HPLC grade water for the liquid chromatography-tandem mass spectrometry (LC-MS/MS) run. A total of 50 µl of eluate was diluted with 450 µl mobile phase for the flow injection analysis-tandem mass spectrometry (FIA-MS/MS) run. The data were acquired on a SCIEX QTRAP 6500+ mass spectrometer coupled to SCIEX ExionLC™ Series UHPLC capability. The UHPLC column provided with AbsoluteIDQ® p180 assay was installed for the LC-MS/MS analysis, and the mobile phase A and B were water and acetonitrile (both with 0.2% formic acid), respectively. Amino acids and biogenic amines were identified and quantified in positive mode via LC-MS/MS

analysis. Acylcarnitines, lysophosphatidylcholines, phosphatidylcholines with acyl/alkyl and acyl/acyl side chains and sphingomyelins were identified and semi-quantified in positive mode via FIA-MS/MS. All metabolites were analysed by multiple reaction monitoring method.

Amino acids and biogenic amines were quantified based on isotopically labelled internal standards and 7-point calibration curves using AB Sciex Analyst® version 1.7.2 software. Other metabolites, such as acylcarnitines, lysophosphatidylcholines, phosphatidylcholines and sphingomyelins were semi-quantified by using 14 internal standards in the MetIDQ™ software (Biocrates Life Sciences). Data quality was evaluated by checking the accuracy and reproducibility of QC samples included in the p180 assay. The concentrations of metabolites were reported in µM. For further statistical analyses metabolites were included only when the concentrations of metabolites were above the limit of detection (LOD) in more than 80% of samples.

Statistical analysis

The feeding data were presented as average ± standard deviation while the biochemical data were presented as mean ± standard deviation. Both dataset were analysed and plotted with GraphPad Prism software (version 12) and statistically significant differences were identified with One-Way ANOVA corrected with post-hoc Dunnett's test. For metabolomic data, one sample was removed as outlier due to increased standard deviation from control, 10 ng/l and 100 ng/l. The final list of metabolites quantified was normalised over the control and processed for multivariate and univariate statistics using freeware software Multi Experiment Viewer (Saeed et al., 2003). Hierarchical Clustering (HCL) and Principal Component Analysis (PCA) were used to visualize the grouping of individual samples. One-Way ANOVA corrected with post-hoc Dunnett's test was used to identify the significantly affected metabolites. The metabolites that were significantly affected were additionally analysed using the pathway analysis from MetaboAnalystR (Pang et al., 2024).

Results and discussion

Mortality induced from individual pollutants and their mixture

Acute toxicity in neonates revealed significant differences in toxicity among the eight pollutants tested separately. Neonates exposed individually to the eight pollutants for 24 hours and EC values were calculated and compared with the reported concentrations in the environment (Table 1). Results showed that propranolol had the highest rate of mortality in neonates (EC_{50} of 14.24 mg/l), whereas nicotine produced the lowest toxicity rate (EC_{50} of

262.6 mg/l). These EC_{50} values and the mortality, in general, differ from the results that were generated on D4 daphnids, in our previous study. Specifically, toxicity curves on D4 daphnids showed that the most toxic chemical was aluminium sulfate (EC_{50} of 59.4 mg/l) and the least toxic chemical was nicotine (EC_{50} of 455 mg/l) (Michalaki et al., 2022). These results suggest that the younger the animals the more toxic impact the chemicals would have on them. A report showed that among fifty metals aluminium and lithium seemed to have medium to low toxicity on daphnids, following 48 h of exposure, with their EC_{50} ranging between 0.1-1 mg/l, and 1.01-100 mg/l, respectively (Okamoto et al., 2015). Exposure of daphnids to acetylsalicylic acid for 48h showed EC_{50} ranging from 88-1,293 mg/l (Parolini, 2020). The EC_{50} of propranolol to *D. magna* ranges between 5.53 and 9.06 mg/l (Damasceno de Oliveira et al., 2018). The toxicity of diltiazem varies depending on the organism. The EC_{50} values for *D. magna* are 165 mg/l after 24 h of exposure, 28 mg/l after 48h and 8.2 mg/l following 96h of exposure (Natalia et al., 2018). Acute exposure for 48h metformin led to an EC_{50} of 14.3 mg/l (Zheng et al., 2024). Regarding glyphosate *D. magna* appeared to be more sensitive compared to *Cyclops vicinus* with an EC_{50} of 76.67 mg/l (12h), 36.2 mg/l (24 h), and 21.34 mg/l (48 h) (Gustinasari et al., 2021). Finally, exposure of daphnids to nicotine for 48 h showed an extremely low EC_{50} concentrations of 0.789 mg/l (Oropesa et al., 2017). To represent pollutants in freshwater environments more accurately where chemicals do not exist individually but in composite mixtures, a “cocktail” of the eight pollutants in equal amount (in mg/l) was assessed for mortality. This “cocktail” was prepared using equal concentrations of each chemical, which results in unequal molar ratios. While this approach does not fully reflect the relative abundances of the individual chemicals in the environment, where each compound can be present at different concentrations, it provides a standardized framework for assessing combined effects under controlled conditions. While this design ensures that all components contribute equally to the mixture, future studies should consider adjusting the composition to better reflect realistic scenarios. Although a modelling for prediction of toxicity was not derived, as it was expected, the mixture of these pollutants resulted in the highest rate of mortality among neonates with an EC_{50} value of 6.395 mg/l, thus representing a potential synergistic effect among the individual constituents of the pollutant “cocktail”.

Table 1. EC values (mg/l) for acute toxicity in neonates. Values were calculated from toxicity curves (with N=4 replicates per concentration).

Chemical	**EC_{50}**	**Hill Slope**	**EC_1**	**Environmental concentration**
Aluminium sulfate hexadecahydrate	69.6	5.251	29	$85x10^{-6}$-3.7 (Botté et al., 2022, Sañudo-Wilhelmy et al., 2002, Takatsu et al., 2000)
Lithium chloride	56.6	4.367	19.7	0.17-10 (Gupta et al., 2024, Schrauzer, 2002)
Acetyl salicylic acid	62.8	20.52	16.4	$80.4x10^{-3}$ (Pagano et al., 2022, Stuer-Lauridsen et al., 2000)
Diltiazem hydrochloride	45	4.646	16.8	$41x10^{-6}$ (Natalia et al., 2018)
Metformin	86.1	9.163	52.2	$0.61x10^{-6}$-$325x10^{-3}$ (Ambrosio-Albuquerque et al., 2021, de Jesus Gaffney et al., 2017, Kong et al., 2015)
Propranolol hydrochloride	14.2	2.111	1.6	$3x10^{-7}$-$1.9x10^{-3}$ (Godoy et al., 2015)
Glyphosate	47.2	36.36	41.6	10^{-4}-105 (Brovini et al., 2021, Marques et al., 2021)
L-nicotine	262.6	3.609	73.5	$0.6x10^{-3}$-$32x10^{-3}$ (Oropesa et al., 2017)
Mixture of 8 pollutants	6.4	4.492	2.3	

Feeding rate

Feeding is a phenotypic endpoint used to evaluate the physiology of daphnids as a non-invasive test (Grintzalis et al., 2017). In this study, feeding rate was determined as the slope for 50 min (fluorescence was measured every 10 min) for the consumption of microplastic. Exposure of neonates to the mixture at concentrations from 1 ng/l to 100 μg/l did not significantly affect the feeding performance of the animals compared to the unexposed control. On the contrary, exposure to higher concentrations (500 μg/l and 1000 μg/l) resulted

in significant reductions in feeding rate by 30% and 50%, respectively, suggesting a potential dose-dependent effect (Figure 2).

Previous studies have shown that acute exposure of neonates to single compounds such as aluminium, lithium, acetylsalicylic acid, diltiazem, metformin, propranolol, and glyphosate, at concentrations corresponding to 5% and 10% of their EC_1, often led to an increase of the feeding performance. Particularly, lithium, diltiazem, metformin, and propranolol were shown to enhance the feeding activity, while aluminium and acetylsalicylic acid caused reductions (Rowan et al., 2024). However, in higher concentrations, 5 mg/l, 10 mg/l and 20 mg/l this effect was not observed. (Giannouli et al., 2023) reported that aluminium, diltiazem, metformin, propranolol and nicotine decreased the feeding capacity of daphnids and with lithium being the only compound associated with increased feeding. Collectively, these findings, along with the current results, underscore the complex and often unpredictable interactions of compounds in a mixture, emphasizing the necessity of mixture-specific risk assessments for aquatic ecosystems.

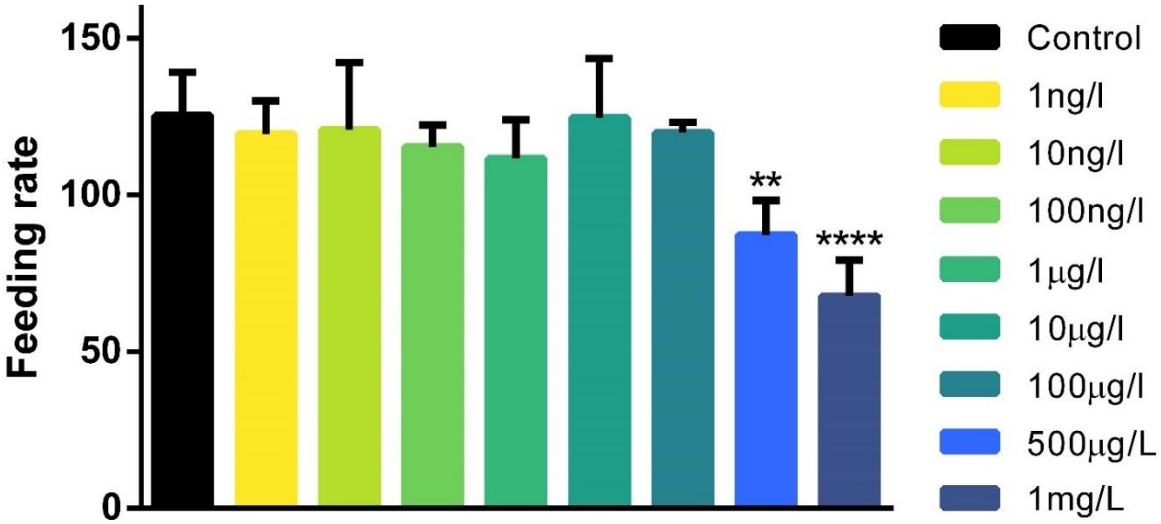


Figure 2. The impact of a complex mixture of pollutants on feeding rate to neonates. Feeding rate was quantified as the slope for 50 minutes for consumption of microplastic. Data represent average ± standard deviation (N=4 replicates). * Statistically significant by One-Way ANOVA corrected with post-hoc Dunnett's test against the unexposed control.

Environmentally relevant concentrations of a mixture of pollutants affect the physiology of daphnids over generations of exposure

To assess the impact of a complex mixture of pollutants, very low environmentally relevant concentrations from 1 ng/l were selected for multigenerational exposures. The impact of the composite pollutant mixture was assessed after 21 days of exposure for the first, third and fifth generations of exposure (Table 2). Although the concentrations of pollutants were not measured during their chronic and multigenerational experiments, their renewal twice a

week was sufficient to provide animals an exposure that would elaborate a more environmental approach, where animals are exposed to variations of concentrations of toxicants as the aquatic water column may be refreshed or changed during the lifetime of daphnids. In this study the aim was the establishment of thresholds of concentration where we can detect changes and compare the multigenerational imprinting of the stress in the physiology of daphnids.

During the first 21 days of exposure the enzymatic responses of daphnids were concentration-dependent, with more significant effects observed at medium to high concentrations. At the lowest exposure concentration of 1 ng/l, no significant effects were observed compared to the control group, while exposure to 10 ng/l decreased the activities of βGAL and LDH, by 26% and 12%, respectively. The 100 ng/l mixture caused an increase in the ACP activity by 23% and a decrease in the activity of LDH by 13%, while the 1 μg/l decreased its activity by 14% and increased the GST by 12%. With increasing concentrations, the impact became more pronounced. Exposure at 10 μg/l led to an increase in the activities of PEP (19%), GST (20%) and LDH (15%), while the 100 μg/l enhanced the activity of βGAL (14%) and reduce the activity of LIP (23%). At the highest tested concentration (1000 μg/l), the activities of ACP and LIP were significantly decreased by 15% and 32%, while the activities of ALP, βGAL, and GST were increased by 16%, 21%, and 18%, respectively.

In the third generation the observed impact is milder than the effects caused by the first generation. A plausible reason behind this observation could be the development of resistance in the exposed population. This resistance may occur either due to phenotypic acclimation, where individuals adjust physiologically to chronic exposure, or due to genetic adaptation, in which the population becomes less sensitive to the exposure chemical (Dietrich et al., 2010). The affected enzymes were ACP, LIP, GST and LDH. The first was decreased by 34% after exposure to the 1000 μg/l. The activity of LIP was affected and specifically decreased significantly after exposure to the three highest concentrations, 10 μg/l (30%), 100 μg/l (15%) and 1000 μg/l (52%). Additionally, the GST was mostly decreased by all concentrations except from the 1 μg/l. Finally, 1 μg/l, 10 μg/l and 100 μg/l increased the activity of LDH, while 1000 μg/l caused the opposite effects.

By the fifth generation, daphnids exposed to the highest concentration (1000 μg/l) did not survive. Among the surviving conditions, enzymatic alterations were still evident. The activity of ALP was reduced by 11%, 14%, and 13% at 1 ng/l, 10 ng/l, and 100 ng/l, respectively. βGAL activity was decreased at 10 ng/l and 100 μg/l, with the latter also reducing the activity of LIP. Exposure to all concentrations apart from 1 μg/l and 100 μg/l

increased the activity of PEP, while the 100 ng/l, 1 μg/l and 10 μg/l decreased the LDH activity.

The effects observed in the fifth generation are more intense compared to the results from the first generation, highlighting the importance of multigenerational exposures instead of one generation chronic exposure. In addition, there are several reports that point out the significance of multigenerational exposures. Chronic exposure of daphnids to metals for twenty-one days caused abnormalities in the body length of the second-generation animals, suggesting that longer exposure to toxic chemicals, even at minimal concentrations can have adverse effects on the following generations (Rodrigues et al., 2020). Furthermore, a study from Tsui and Wang showed that daphnids exposed to contaminants can transfer a trace of these chemicals to the subsequent generations (Tsui and Wang, 2004). It has been reported that DNA methylation in the first generation can lead to epigenetic changes in the future generations (Vandegehuchte et al., 2010). Moreover, a study in zebrafish revealed that contaminants can cause developmental defects in the following generations (Baker et al., 2014). Finally, one of our previous studies on the effects of chemical and commercial NSAIDs on daphnids, at extremely low and environmentally relevant concentration of 5 μg/l, showed pronounced effects on enzymatic activities and metabolic pathways of daphnids in the fourth generation of exposure (Michalaki et al., 2025).

Regarding the impact of the individual stressors on daphnids, this was not the focus of this study. Consequently, we are unable to evaluate the toxicity mechanism of the mixture. However, based on our previous study on D4 daphnids lithium and metformin affected the most the enzymatic activities of daphnids, while aluminium sulfate, propranolol, diltiazem, glyphosate and nicotine caused a medium effect. Lastly, acetylsalicylic acid was the least toxic. A more distinct dose-dependent effect was observed on acute exposure of D4 daphnids to three different doses of the chemicals' mixture. From these results, even though any analysis of non-interactive mixture modelling was performed in the previous study either, the dose-dependent effect of the mixture might be attributed to a synergy effect (Michalaki et al., 2022). In the present study, the dose-dependent effect might not be that clear mostly at the lower concentrations, yet it is more pronounced in the higher doses, especially in the fifth generation.

Table 2. The impact of the pollutant mixture on daphnids. Data represent average±standard deviation (N=4). Enzyme activity was expressed as units/mg protein for LIP, βGAL, ACP and ALP, and LDH as munits/mg protein for GST. Bold font indicates statistically significant difference by One-Way ANOVA corrected with post-hoc Dunnett's test against the unexposed control.

		Enzyme	Mixture of pollutants							
			0	1 ng/l	10 ng/l	100 ng/l	1 µg/l	10 µg/l	100 µg/l	1000 µg/l
Generation	1st	ACP	3.4±0.22	3.25±0.32	3.06±0.22	**4.19±0.13 (+23%)**	3.46±0.06	3.2±0.07	3.24±0.23	**2.9±0.13 (-15%)**
		ALP	4.25±0.27	3.99±0.31	4.36±0.26	4.18±0.27	4.55±0.35	4.53±0.43	4.35±0.15	**4.91±0.24 (+16%)**
		βGAL	2.66±0.07	2.47±0.2	**1.98±0.1 (-26%)**	2.61±0.13	2.71±0.14	2.63±0.15	**3.04±0.13 (+14%)**	**3.22±0.15 (+21%)**
		LIP	109.51±4.77	100.68±9.18	95.39±2.24	116.15±12.63	98±4.65	108.75±8.93	**84.33±3 (-23%)**	**73.96±9.33 (-32%)**
		PEP	2.08±0.07	2.1±0.13	2.04±0.13	2.12±0.17	2.18±0.09	**2.48±0.14 (+19%)**	2.11±0.09	2.1±0.1
		GST	152.2±5.4	155.4±6.3	155.5±3.9	159.6±5.5	**169.9±16.2 (+12%)**	**182.6±8.2 (+20%)**	168.6±1.8	**179.9±10.7 (+18%)**
		LDH	229.83±14.38	239.42±12.78	**202.06±9.84 (-12%)**	**201.06±11.65 (-13%)**	**197.26±10.39 (-14%)**	**263.78±17.83 (+15%)**	233.01±11.78	217.54±14.93
	3rd	ACP	5.03±0.46	5.2±0.67	5.39±0.54	5.49±0.59	4.96±0.59	5.68±0.72	4.68±0.69	**3.32±0.43 (-34%)**
		ALP	2.67±0.21	2.7±0.12	2.5±0.16	2.37±0.13	2.52±0.21	2.27±0.1	2.39±0.11	1.98±0.19
		βGAL	1.5±0.08	1.51±0.07	1.53±0.07	1.39±0.09	1.52±0.14	1.36±0.06	1.51±0.02	1.34±0.18
		LIP	102.63±7.42	101.77±4.08	106.63±7.26	99.48±8.81	95.51±4.59	**72.12±4.99 (-30%)**	**87.65±6.13 (-15%)**	**48.76±7.11 (-52%)**
		PEP	2±0.08	1.94±0.11	1.82±0.05	1.82±0.11	1.89±0.1	1.9±0.06	2.11±0.09	2.12±0.29
		GST	246.6±10.6	**219.7±0.5 (-11%)**	**210.6±12.7 (-15%)**	**198.3±14.2 (-20%)**	**278.8±8.2 (+13%)**	252.6±11.7	251.6±1.1	**168.1±10.2 (-32%)**
		LDH	139.65±8.66	137.51±12.68	123.41±8.82	134.18±5.74	**185±6.52 (+32%)**	**159.5±12.64 (+14%)**	**171.31±13.88 (+23%)**	**94.12±5.37 (-33%)**
	5th	ACP	6.19±0.48	7.24±1.05	5.63±0.2	6.49±0.22	7.43±1.71	6.31±0.49	4.45±0.07	
		ALP	4.45±0.19	**3.95±0.26 (-11%)**	**3.84±0.17 (-14%)**	**3.88±0.11 (-13%)**	4.28±0.24	4.08±0.15	4.1±0.13	
		βGAL	2.39±0.05	2.51±0.13	**2.1±0.14 (-12%)**	2.18±0.06	2.23±0.07	2.18±0.02	**2.16±0.11 (-10%)**	
		LIP	121.6±3.79	135.29±3.32	122.87±3.37	119.4±6.8	127.42±13.4	116.76±5.81	**93.73±6.79 (-23%)**	
		PEP	2.55±0.1	**3.06±0.14 (+20%)**	**2.8±0.13 (+10%)**	**2.77±0.02 (+9%)**	2.71±0.04	**2.91±0.05 (+14%)**	2.67±0.09	
		GST	136.3±9.2	123.3±3.3	136.3±5.3	133.2±7.4	134.5±12.4	143.6±2.1	151±6.9	
		LDH	136.04±13.55	128.24±3.49	130.88±7.24	**100.31±9.15 (-26%)**	**101.82±6.98 (-25%)**	**94.89±6.49 (-30%)**	121.31±15.95	

The impact of pollutants on metabolism of daphnids

Metabolomic analysis is known as a useful approach in ecotoxicology and environmental risk assessment, revealing how chemical stressors influence organisms at the molecular level. This approach is particularly valuable for detecting early metabolic alterations in bioindicator species, such as *D. magna*, even at low and possibly undetectable concentrations (Taylor et al., 2018, Viant, 2009). Understanding these changes is crucial for identifying potential ecological risks and improving pollution risk assessment. Moreover, some metabolic processes may be preserved across species, allowing evolutionary linkages to assist predict toxicological effects (Colbourne et al., 2022, Viant et al., 2019). A major advantage of metabolomics is its ability to reflect the organism's phenotypic and current physiological status, unlike other omics such as transcriptomics, which could be used for assessing the impact of pollutants, but they are unable to provide a "snapshot" of the animals' current active state (Fröhlich, 2017, Fuertes et al., 2019).

In the present study, a targeted LC-MS/MS metabolomic analysis was conducted on 21-days old daphnids from the fifth generation following chronic and multigenerational exposures to environmentally relevant concentrations of the mixtures (1 ng/l, 10 ng/l, and 100 ng/l). Data were normalised over the control condition and analysed using multivariate statistical approaches. Hierarchical clustering using Pearson correlation and Euclidean distance showed distinct clustering of conditions indicating dose-dependent effects even at such low concentrations (Figure 3A, 3B). Additionally, Principal Component Analysis (PCA) further confirmed this pattern, with the first three principal components explaining 62.5% of total variance (Figure 3C, 3D). Both PC1-2 and PC1-3 plots demonstrated a clear separation between exposed groups, where the 1 ng/l and 10 ng/l groups clustered closer to the control, while the highest concentration diverged considerably.

One-Way ANOVA followed by Dunnett's post-hoc test was performed to identify the statistically significantly affected metabolites for each exposure condition. Exposure of daphnids to 1 ng/l led to a statistically significant decrease in forty-eight metabolites, including glutamine (amino acid), carnosine (biogenic amine), C2 (acyl carnitine), forty-one phosphatidylcholines, two lysophosphatidylcholines (lysoPC a C17:0 and lysoPC a C20:4), and two sphingomyelins (SM 33:1 and SM 41:2). At 10 ng/l, three metabolites alpha-AAA, sarcosine and C3 were significantly increased, while twenty-seven metabolites were decreased comprising eleven amino acids, three biogenic amines, one acyl carnitine, nine phosphatidylcholines, and three lysophosphatidylcholines among them. The most pronounced impact was caused by the highest concentration of 100 ng/l which decreased the levels of a hundred and three metabolites, across the one hundred and seventeen metabolites analysed. These results indicate a broad suppression of metabolic activity.

In our previous study (Michalaki et al., 2022) daphnids were exposed for 24 h to three different doses of the same chemical mixture (10%, 20%, and 30% of the EC_5). Untargeted metabolomic analysis revealed significant metabolic disruptions that intensified with increasing dose. Specifically, the 10% of the mixture caused a down-regulation to undecanol and β-alanine, the 20% decreased the levels of citronellol and ethanoloamine, while the 30% decreased the methionine, phenylalanine and glycine. Additionally, threonine, putrescine increased at all doses, while metabolites of TCA cycle (citric acid, fumaric acid), urea cycle (ornithine), and amino acids (proline, valine) were elevated in response to the 20% and 30% of the mixture, highlighting the dose-dependent effect.

A Venn diagram was created to show the overlap of significantly changed metabolites in the three exposure concentrations (Figure 4). Ten of the total altered metabolites were altered by all concentrations, demonstrating a shared metabolic response regardless of dose. The

100 ng/l group had the most distinct modifications (forty), followed by 10 ng/l (three) and 1 ng/l (two), indicating a dose-dependent metabolic disturbance. Additionally, thirty-six altered metabolites were shared between 1 ng/l and 100 ng/l, with seventeen between 10 ng/l and 100 ng/l, highlighting the extensive impact of the greatest dose.

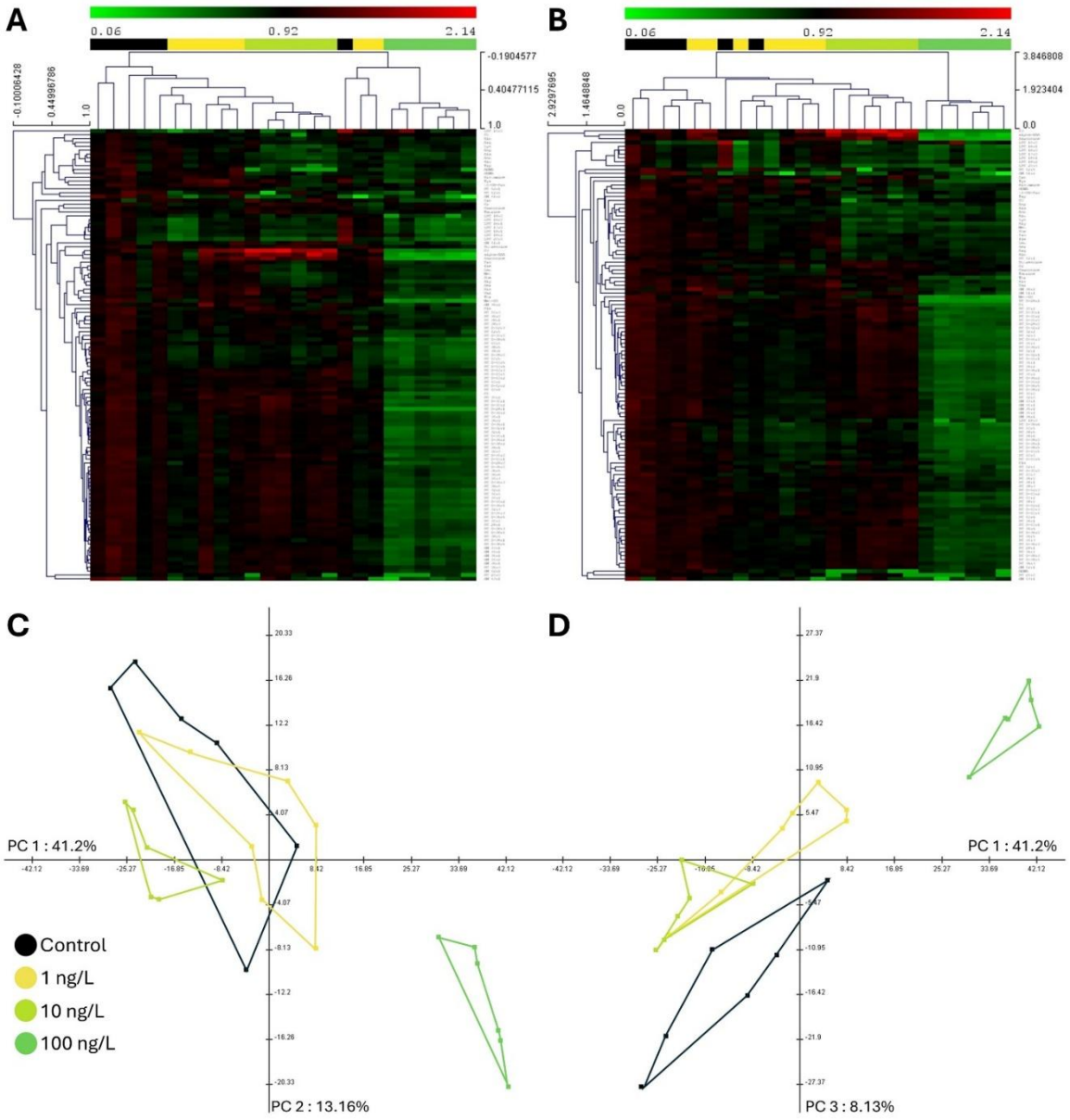
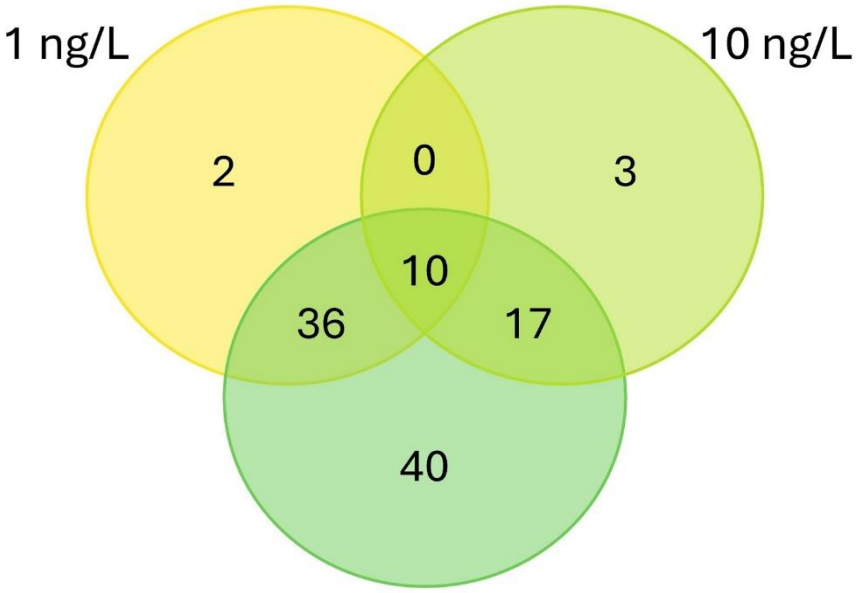
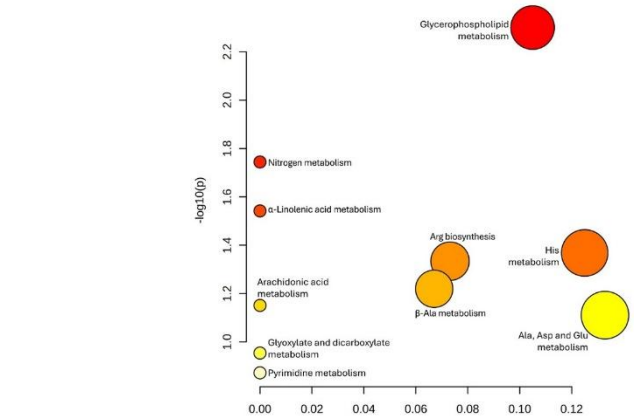
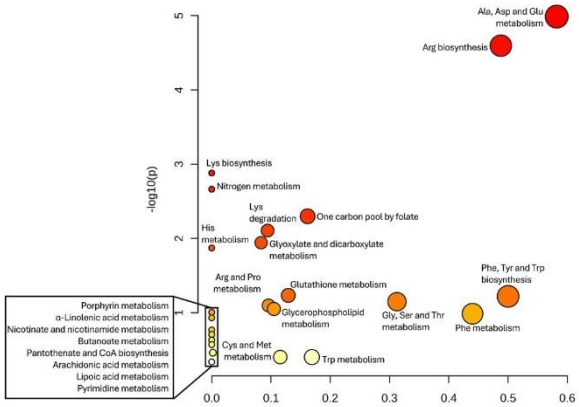


Figure 3. Multivariate statistical analysis of the impact of 1 ng/l, 10 ng/l and 100 ng/l of chemical mixture on daphnids from the fifth generation. Hierarchical clustering using Pearson correlation (A) and Euclidean distance (B) metrics and Principal component analysis (C: PC1,2, D: PC1,3) shows the grouping and clusters of samples, respectively (control: black, 1 ng/l: yellow, 10 ng/l: light green, 100 ng/l: dark green).

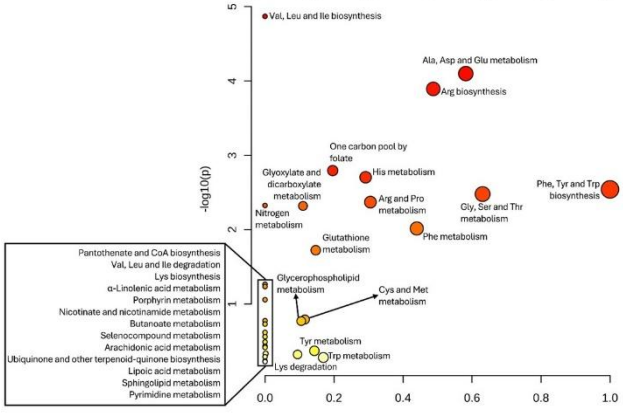




1 ng/l



10 ng/l



100 ng/l

Figure 4. Venn diagram and pathway analysis of metabolic perturbations. Venn diagram shows the number of common and unique metabolites that were significantly increased or decreased (compared to the unexposed control) among the three mixture concentrations. Pathway analysis of the significantly impacted metabolic pathways. Each graph shows the statistically significant and highly impacted pathways on the top right corner, and the less significant and low impacted pathways on the bottom left corner. Statistically significant difference was calculated with One-Way ANOVA corrected by Dunnett's post-hoc test. Graphs were created using the MetaboAnalystR platform (Pang et al., 2024).

Pathway analysis was performed using MetaboAnalystR, for the significantly impacted metabolites for the three exposure concentrations (1 ng/l to 100 ng/l), to identify the altered metabolic pathways. This method provides a comprehensive visualization of the metabolic perturbations and their potential biochemical implications (Figure 4). Exposure to the 1 ng/l of the chemical mixture induced subtle metabolic changes, primarily affecting glycerophospholipid metabolism, histidine metabolism, alanine-aspartate-glutamate metabolism, arginine biosynthesis and the metabolism of β-alanine. These pathways are related to membrane structure, neurotransmission and energy production, showing an early response to low-stress (Brosnan and Brosnan, 2020, Li et al., 2025, Reitzer, 2004). Arginine biosynthesis and the alanine-aspartate-glutamate pathway both participate in the TCA cycle, implying that energy balance may have been affected. Overall, these findings show that even exposure to low and environmentally relevant concentrations slightly impact energy production and homeostasis.

The medium concentration of 10 ng/l had an impact mostly on the amino acid metabolism, specifically alanine-aspartate-glutamate metabolism, arginine biosynthesis, phenylalanine-tyrosine-tryptophan biosynthesis, phenylalanine metabolism and glycine-serine-threonine metabolism. These pathways are essential for protein synthesis, neurotransmission, energy metabolism, and detoxification (Daniyal et al., 2020, Parthasarathy et al., 2018, Wu et al., 2021). The synchronized changes of several "amino-acid" related pathways indicate that daphnids were trying to cope with the stress, highlighting a state of metabolic stress that over time could affect physiological processes such as growth.

Exposure of daphnids to 100 ng/l had a more pronounced effect on metabolic pathways. All pathways that were previously impacted remained disturbed, with the addition of histidine and arginine-proline metabolism, indicating a cumulative dose-dependent effect (Dalangin et al., 2020). The affected metabolic pathways show more substantial impacts on energy metabolism, neurotransmission, growth and stress regulation. This systemic disruption suggests that chronic and multigenerational exposure to even low concentrations of pollutants may impair critical biological functions, such as energy production, growth,

nervous system operation, and stress tolerance, inhibiting survival and reproduction in the later generations.

Conclusions

In conclusion, this study evaluated the multigenerational effects of an eight-chemical mixture at several concentrations on *D. magna*, employing both biochemical markers and metabolomics. The findings showed a clear dose-dependent impact, with adverse effects becoming more intense at higher concentrations. Notably, daphnids exposed to 500 μg/l and 1000 μg/l exhibited reduced feeding performance compared to those under other conditions, a trend that persisted through chronic exposure, and was further supported by biochemical assays. Daphnids exposed to 1000 μg/l did not survive beyond the fourth generation, while concentrations of 10 μg/l and 100 μg/l had a more pronounced effect than lower doses. Metabolomic analysis revealed a cumulative dose-dependent effect even at the lowest concentrations (l-100 ng/l), highlighting the importance of multigenerational studies at low and environmentally relevant concentrations. These results underscore the complex, cumulative, and often unpredictable effects of pollutant mixtures across generations, emphasizing the need to evaluate chemical mixtures in environmental risk assessment.

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Chapter 3

This chapter studied the toxic effects of 1-butyl-3-methylimidazolium (BMIM) ionic liquids, a class of novel solvents characterized as "green solvents" on daphnids. Based on the previous studies and extended beyond the eight chemicals and their mixture, this study focused on a group of five emerging contaminants to evaluate their impact following acute and chronic exposure, using biochemical markers, feeding assay and reproduction assay. The findings showed that while reproduction was unaffected by the exposure, the feeding performance and the enzymatic activities of daphnids were significantly impacted, highlighting the toxic potential of these novel solvents. This work underscored the importance of *D. magna* as a sensitive early-warning bioindicator and broadened the thesis narrative by showing how novel classes of chemicals might trigger diverse impacts on freshwater ecosystems.

Toxicity of “green solvents” - The impact of butyl methylimidazolium ionic liquids on daphnids

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Abstract

Ionic liquids have been described as green solvents, however, their presence in the aquatic environment may indicate a threat for key species. Focusing on the freshwater ecosystem, five ionic liquids containing the 1-butyl-3-methylimidazolium (BMIM) cation were compared for their acute toxicity and chronic responses on daphnids. Biochemical markers of physiology including the activity of phosphatases, βGAL, PEP, LIP and GST were used to assess changes in physiology of daphnids. Feeding and reproduction were investigated as surrogate phenotypic measures. Feeding rate was decreased in all exposures, and severely impacted in BMIM hexafluorophosphate, chloride, and tetrafluoroborate, while reproduction was unaffected by all ionic liquids. A diverse set of responses were triggered from each BMIM ionic liquid in reference to enzyme activities providing insight for the toxicity impact of these emerging contaminants. Phosphatase activities were significantly decreased in all exposure scenarios to ionic liquids, but different patterns of biochemical responses were documented among acute and chronic exposures and different ionic liquids, indicating distinct mechanistic patterns. The aforementioned results highlight the toxic potential of ionic liquids which are characterised so far as green solvents.

Keywords: *Daphnia magna*, ionic liquids, toxicity, enzyme activities, feeding rate, reproduction

Introduction

Ionic liquids (ILs) are a class of salts that exist at temperatures lower than 100°C (Flieger and Flieger, 2020, Pham et al., 2010). These salts are entirely made up of ions, which include bulky organic, asymmetric cations and weakly-coordinating inorganic anions (Pham et al., 2010, Zhu et al., 2009). ILs are a type of "green solvent" that was developed to replace some of the most unsavory volatile organic compounds (VOCs) (Pham et al., 2010). ILs have been labelled "designer solvents" because the cation/anion combination can be changed to create approximately 10^{18} ILs (Bubalo et al., 2017, Pham et al., 2010). As a result, they can be used in a variety of fields such as chemistry (organic synthesis, polymerization), chemical engineering, and energy (battery) (Bubalo et al., 2017, Oskarsson and Wright, 2019, Pham et al., 2010, Zhu et al., 2009).

Besides the low melting points, ILs are characterized by their negligible vapor pressure, high thermal and chemical stability, and amphiphilic nature (Oskarsson and Wright, 2019). Given their negligible vapor pressure, it appears most likely that they would enter the environment via an aqueous medium, possibly through accidental spillage or discharge of industrial effluent. Once in the environment, the properties that make them so desirable in industrial settings (thermal, mechanical, and electrochemical stability) have the potential to make them highly persistent poorly or non-biodegradable aquatic pollutants (de Jesus and Maciel Filho, 2022, Pham et al., 2010, Zhang et al., 2017b).

As novel materials, ILs are on debate in reference to their toxicity potential. There are no reports in literature about the effects of BMIM ILs on the physiology of *D. magna* other than mortality, but it has been stated that BMIM and other ILs can harm marine species such as mussels and rotifers (Tsarpali et al., 2015, Tsarpali and Dailianis, 2015, Tsarpali et al., 2016), zebrafish (Piotrowska et al., 2018), planarians (Zhang et al., 2016), and even nematodes (Swatloski et al., 2004) and *in vitro* systems (Ranke et al., 2004). Furthermore, due to their aromatic ring, imidazolium-based ILs are more likely to be toxic to *D. magna* and related organisms. If molecules from these ILs reach the cytochrome P_{450} in the endoplasmatic reticulum, they have a high chance of being oxidized (Jastorff et al., 2003). As a result, the naturally occurring metabolites may be further decomposed into potentially hazardous fatty acids and imidazole (Bernot et al., 2005). Additionally, the toxicity of ILs depends on the length of the alkyl chain. ILs with longer alkyl chain are more toxic than ILs with shorter alkyl chain (Docherty and Kulpa, 2005, Frade and Afonso, 2010, Hernández-Fernández et al., 2022, Kuroda, 2022, Mena et al., 2020, Ventura et al., 2013). The toxicity mechanism

has been explained in the available literature: the alkyl chains of the cations penetrate into the cell membrane, rendering cell membrane maintenance impossible (Kuroda, 2022).

To improve our understanding over water pollution and especially focusing on novel materials for which research remains infant, emphasis is given to key species used as bioindicators. Combining phenotypic and sensitive molecular endpoints is a revolutionizing turn in ecotoxicology and risk assessment. Daphnids are key components of the freshwater ecosystem and are commonly employed in freshwater ecology and ecotoxicology mainly because of their geographical distribution, central role in freshwater food webs, their adaptation to a range of habitats and sensitivity to anthropogenic chemicals (Dodson and Hanazato, 1995, Lampert, 2011, Shaw et al., 2008). Daphnids are easy to culture in the lab and provide a useful model organism for molecular ecotoxicology and mechanistic insight for novel pollutants.

In this study, daphnids were exposed to five ILs of the cation 1-butyl-3-methylimidazolium coupled with differing anions; hexafluorophosphate (HFP), chloride (CHL), methanesulfonate (MSF), tetrafluoroborate (TFB), and hexafluoroantimonate (HFA) (Figure 1). Even though BMIM hexafluorophosphate, and BMIM hexafluoroantimonate, have been described as chemicals insoluble in water (Klahn et al., 2010), there have been reports which dissolve them in tap water or ddH_2O water to study their toxicity. Specifically, the toxic effects of BMIM hexafluorophosphate and BMIM hexafluoroantimonate to algae *Selenastrum capricornutum* were assessed by dissolving ILs in a sufficient amount of distilled water (Cho et al., 2008). More reports that dissolve these ILs in water or in an aqueous solution were reported by Zhang et. al. (Zhang et al., 2011) and Azimova et. al. (Azimova et al., 2009). BMIM hexafluorophosphate, in concentrations of 0.5%, 1%, or 2%, inhibited the growth of the bacterium *P. fluorescens* (Zhang et al., 2011), while it was also compared with three bacterial-based toxicity assays using bioluminescent *Pseudomonas* bacteria. Additionally, according to (Klahn et al., 2010) BMIM tetrafluoroborate becomes water-immiscible at 4°C. In studies, such as (Samori et al., 2010, Zhang et al., 2017a) BMIM tetrafluoroborate was dissolved directly in water to assess its toxicity in the liver cells of zebrafish (*Danio rerio*), daphnids and to *Vibrio fischeri*. Specifically, daphnids were exposed for 24 and 48h to BMIM tetrafluoroborate at concentrations ranging from 4 to 40 mg/l (Samori et al., 2010). Phenotypic, biochemical and metabolic endpoints were used to identify the impacts of ILs on the physiology of daphnids and provide mechanistic insight.

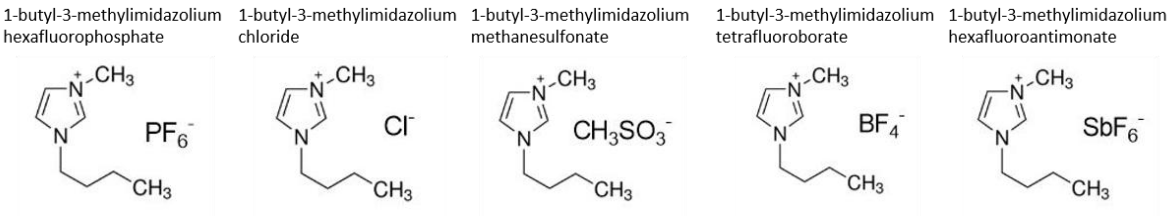

Figure 1. The 1-butyl-3-methylimidazolium ionic liquids used in this study.

Materials and methods

Culturing of daphnids and toxicity exposures

Daphnids were cultured in glass beakers in OECD media (final concentrations 0.29 g $CaCl_2.2H_2O$/l, 0.123 g $MgSO_4.7H_2O$/l, 0.065 g $NaHCO_3$/l, 0.0058 g KCl/l, 2 µg Na_2SeO_3/l, pH 7.7). Daphnids were maintained in breeding cultures constantly in a 16h:8h of light:dark photoperiod at 20°C, at a density of 80 adults per 4 liters of media. Media was renewed on the 6th and 13th day, cultures were fed daily with an algae (*Chlamydomonas rheinharti*) suspension and an organic seaweed extract (*Ascophylum nodosum*) only upon media renewal. For exposures, neonates (<24 hours) were collected from the third brood of their mothers and used for experiments. Neonates from the first two broods are discarded as not adequate for experiments according to OECD guidelines and only neonates from the third brood were used for experiments. For acute exposures, neonates were cultured until four days old (D4), and following, fifteen 4-days old daphnids were exposed to each ionic liquid in 100 ml OECD media, for 24 hours in the absence of food. Toxicity curves were plotted using the Four parameter logistic (4PL) model, following the equation Span = Top − Bottom and Y = Bottom + (Top-Bottom)/(1 + 10^((LogIC50-X)*HillSlope)), and the parameters top and bottom were commonly fixed to 100 and 0, accordingly, and the model defines in the dotted line the 95% confidence intervals. The EC_1, EC_5, EC_{10} and EC_{50} values were calculated. Based on the above, for acute experiments four days old (D4) daphnids were exposed to EC_5 in 100 ml as these animals are more resistant to toxicity in comparison to neonates. For chronic experiments, neonates (<24 hours) were exposed to BMIM ILs and toxicity curves were plotted. A concentration of 1 mg/l was selected for chronic experiments as a non-lethal and low concentration, which would be tolerable for the neonates through a 21 days chronic exposure. Twenty-eight neonates (<24 hours) were exposed from the first day to ILs at 1 mg/l in a 900 ml OECD media individually and in a mixture. Media and ionic liquid were renewed every 3 days and daphnids were fed daily with 2.75 ml algae/l OECD media. A seaweed extract (*Ascophylum nodosum*) was supplemented only at media change.

Daphnids were sampled at 7, 14 and 21 days for measurements of biochemical markers of physiology.

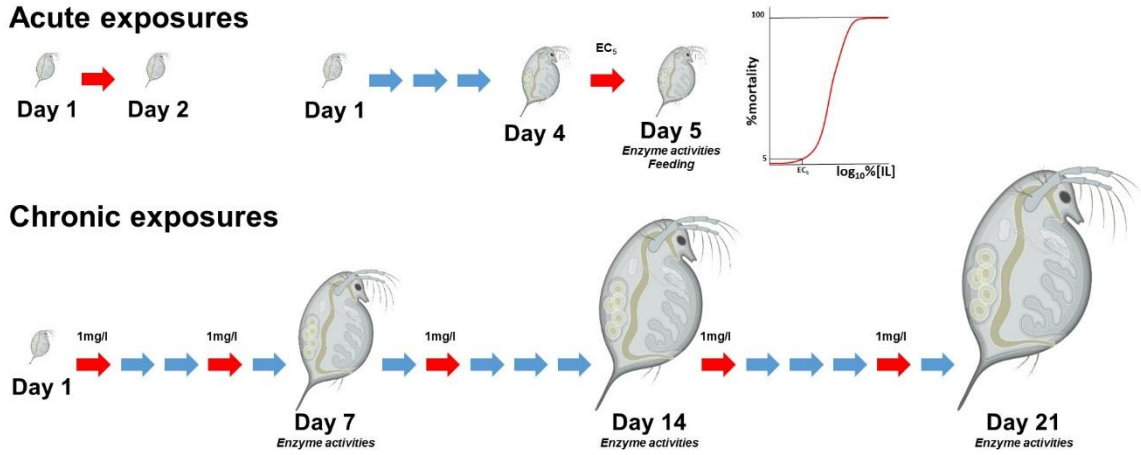

Figure 2. Experimental design for acute and chronic exposures.

Sample homogenization and biochemical assays

For acute exposures, fifteen animals were pooled together (per replicate), while for 7, 14 and 21 days old animals from chronic exposures 5 animals were pooled together. Animals were homogenized in the appropriate buffer using a pestle homogenizer, and the homogenate was centrifuged and the clear supernatant, was collected and assessed for protein and enzyme activity as described elsewhere (Michalaki et al., 2022). Activity of phosphatases was assayed in 100 mM acetic acid pH 4.5 (for acid; ACP) or 100 mM boric acid pH 9.8 (for alkaline; ALP) using the substrate *p*-nitrophenyl phosphate. The reaction was alkalined and absorbance of produced *p*-nitrophenol was measured at 405 nm and converted to units per protein. Similarly, the activities of β-galactosidase (βGAL) and lipase (LIP) were quantified by the generation of nitrophenol from the catalysis of *o*-nitrophenyl-β-galactoside or *p*-nitrophenyl butyrate, respectively, in phosphate buffer pH 7.2. The absorbance of nitrophenol was measured at 405 nm and converted to units per protein. The activity of peptidase (PEP) was quantified by the hydrolysis of L-leu-4-nitroanilide and the production of 4-nitroaniline (at 412 nm every five minutes for thirty minutes) in 100 mM phosphate buffer pH 7.2. Lactate dehydrogenase (LDH) activity was assessed from the consumption of NADH in a reaction with substrate of pyruvate (5 mM) at 340 nm(Worthington and Worthington, 2011). Glutathione-S-transferase (GST) activity was measured by the formation of a complex between reduced glutathione with 1-chloro-2,4-dinitrobenzene at 340 nm (Tang et al., 1996, Warholm et al., 1981). For reduced thiols, samples were homogenized in 100 mM acetic acid pH 4.5 and quantified following the protocol of

Grintzalis et. al. (Grintzalis et al., 2014). Protein was quantified using a sensitive Bradford method (Grintzalis et al., 2015) to normalize enzyme activity.

Feeding assay and imaging

For the feeding assay, D4 animals were exposed for 24 h to the five BMIM ILs. After the acute exposure, three animals were incubated in 6 ml OECD containing carboxylate-modified polystyrene latex fluorescent red microparticles (at a concentration of 13 mg/l) in 12 multi-well plates. Animals were allowed to ingest microparticles at a concentration of 13 mg/l for 60 minutes and following media was collected every 10 minutes to estimate the ingested microparticle by fluorescence at Ex/Em 560/590 nm using a TECAN plate reader. Furthermore, animals were homogenised in water and fluorescence was measured also in the homogenate representing the ingested microparticle to validate the result. The concentration of microparticles in the media was optimised (data not shown) to ensure excess of microparticles for the accurate quantification of ingestion. Fluorescence was expressed as the amount of ingested microparticle using a standard curve and normalised per protein. To confirm the ingestion of microparticles in the gut of daphnids, animals were imaged with a stereoscope and fluorescence microscopy using the TRITC filter for carboxyl functionalized particles.

Reproduction

Reproduction ability was assessed by the number of neonates released daily per daphnid. Neonates were exposed to 1 mg/l BMIM ILs and their mixture (one neonate in 50 ml OECD) for 21 days. Media and ILs were renewed daily and daphnids were fed every day with 3 ml fresh algae/l OECD and 1.5 ml seaweed extract (*Ascophyllum nodossum*)/l OECD.

Statistical analysis

The biochemical data were presented as mean±standard deviation (SD) and were analysed and plotted with the GraphPad Prism software. Statistically significant differences were identified by Student's *t*-test with a *P* value of 0.05 and One-Way ANOVA, followed by Dunnett's post hoc test, from the unexposed control.

Results

Toxicity of BMIM ILs

Acute exposures of neonates and four days old (D4) daphnids to BMIM ILs were assessed with full toxicity curves (Figure 3) and EC values were calculated (Table 1). The EC_{50} values for ILs were in the same order of magnitude and were between 34.5 to 40.8 mg/l for acute

exposure to neonates and 30.8 to 50.6 mg/l for acute exposure to D4 daphnids. To assure similar exposure levels, a low concentration of 1 mg/l was chosen to be comparable for all ILs independently and in their mixture for chronic exposure, and EC_5 was selected as the exposure concentration for acute experiments of D4 animals.

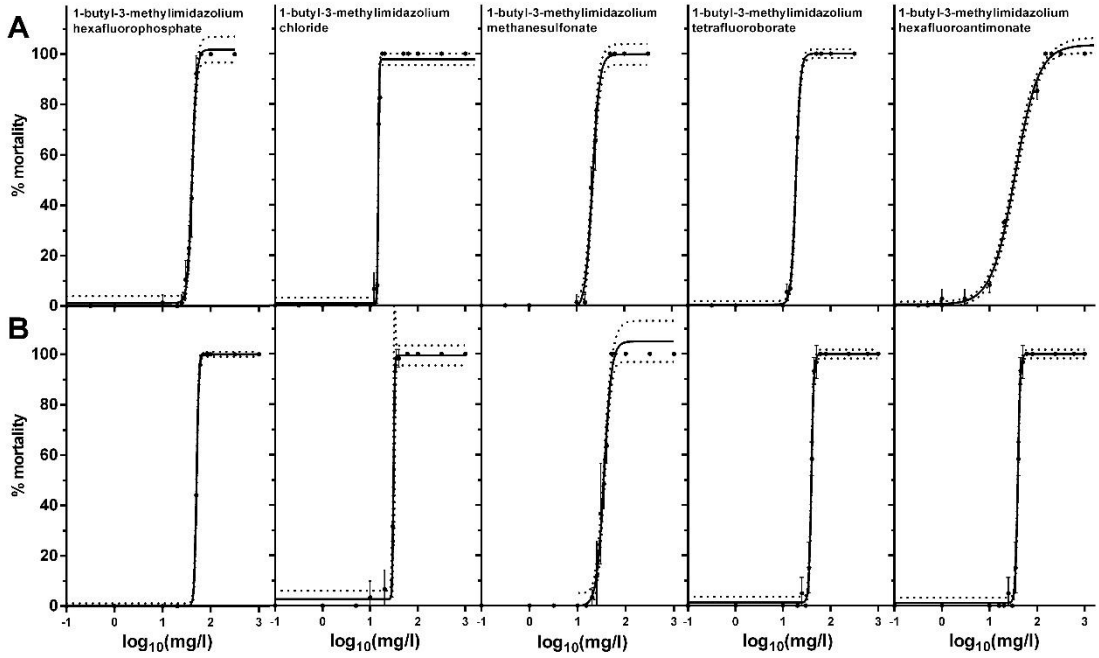

Figure 3. Toxicity curves for exposures of neonates (A) and four-days old daphnids (B) to BMIM ILs. Data represent mean ± standard deviation (N=4).

Table 1. EC values (in mg/l) of ionic liquids used in this study. For EC_{50} we provided the 95% confidence interval.

Ionic Liquid (BMIM)	Neonates (<24 hours)				Four days old daphnids (D4)			
	EC_1	EC_5	EC_{50} (min-max)		EC_1	EC_5	EC_{50} (min-max)	
Hexafluorophosphate	24.8	29.7	40.8	39.7-42	39.4	43.2	50.6	50.4-50.9
Chloride	13.2	13.7	14.7	14.6-14.8	26.9	28.2	30.8	29.7-31.8
Methanesulfonate	9.8	12.9	21.4	20.6-22.3	15.5	20.9	35.3	33.5-37.3
Tetrafluoroborate	11.12	13.4	18.5	18.2-18.8	29.9	33	39.2	38.8-39.6
Hexafluoroantimonate	2.5	6.5	34.4	30.7-38.5	29.9	32.9	39.2	38.8-39.6

Acute exposure to ILs

Daphnids exposed at four days old (D4) at EC_5 for 24 hours for BMIM ILs showed significant differences in their responses (Table 2). For all BMIM ILs, activity of ALP was decreased, with the greatest decrease observed for BMIM hexafluorophoshate, tetrafluoroborate and chloride, followed by hexafluoroantimonate and methanesulfonate. A similar trend was observed for the activities of ACP and βGAL. ACP and βGAL were decreased but not for BMIM hexafluoroantimonate, which was the only IL that increased the activity of LIP. Considering enzymes relevant to the core metabolism such as LDH, its activity was significantly increased in all ILs. Finally, oxidative stress was assessed by the enzyme activity of GST, as an enzyme, which uses reduced glutathione to detoxify xenobiotics. GST activity was decreased by BMIM methanesulfonate, tetrafluoroborate and hexafluoroantimonate. This could also be related to the decrease in reduced thiols for BMIM methanesulfonate, tetrafluoroborate and hexafluoroantimonate.

Table 2. Biochemical markers of responses from four days old (D4) daphnids following acute exposure to ionic liquids. Data represent mean ± standard deviation (N=4). Enzyme activity was expressed as units/protein. Bold font indicates statistically significant by Student's *t*-test from the unexposed control.

Enzyme	Control	BMIM hexafluorophosphate	BMIM chloride	BMIM methanesulfonate	BMIM tetrafluoroborate	BMIM hexafluoroantimonate
ALP	10.3±0.5	**3.3±0.6 (-68%)**	**3.8±0.4 (-63%)**	**8.7±0.5 (-16%)**	**3.5±0.2 (-66%)**	**8.4±0.7 (-18%)**
ACP	3.9±0.3	**2.3±0.2 (-41%)**	**2.4±0.2 (-38%)**	**4.9±0.5 (+26%)**	**2.6±0.1 (-33%)**	4.1±0.5
βGAL	9.1±0.7	**5.6±0.5 (-38%)**	**5.9±0.7 (-35%)**	**6.2±1.3 (-32%)**	**4.7±1 (-48%)**	9.1±0.4
LIP	69.8±9.9	64.8±10.9	72.6±11.3	65.8±11.03	53.3±13.1	**127.7±21.7 (+83%)**
PEP	6.1±0.5	**1.9±0.2 (-69%)**	**2.1±0.1 (-66%)**	5.7±0.5	**1.7±0.03 (-72%)**	**4.7±0.5 (-23%)**
GST	0.3±0.005	**0.37±0.01 (+23%)**	0.3±0.02	0.3±0.02	**0.39±0.06 (+30%)**	**0.27±0.01 (-10%)**
LDH	304.7±44.6	**531.6±35.9 (+74%)**	**512.1±16.8 (+68%)**	**517.4±60 (+70%)**	**553.4±3.1 (+82%)**	**535.9±40.4 (+76%)**
SH groups	117.9±6.7	107.2±3.4	111.5±3.9	**98.4±3.4 (-17%)**	**78.4±4.9 (-34%)**	**93.5±8.8 (-21%)**

Feeding is a phenotypic endpoint used in daphnids to assess their physiology(Grintzalis et al., 2017). The ingestion of fluorescent microparticles was used to quantify the feeding rate. Exposure to BMIM ILs decreased feeding rate, confirming an impact on animal physiology. Specifically, daphnids exposed to BMIM hexafluorophosphate, methanesulfonate, and hexafluoroantimonate showed significant decrease by 54%, 20.38%, 23.51%, respectively, compared to the unexposed control. On the other hand, daphnids exposed to BMIM chloride and tetrafluoroborate showed a significantly reduced feeding rate to 94.17% and 91.1% when compared to control (Figure 4). This was also visualised with stereoscopy and fluorescence microscopy (Figure 4), as their intestine was significantly lower stained by the microplastic.

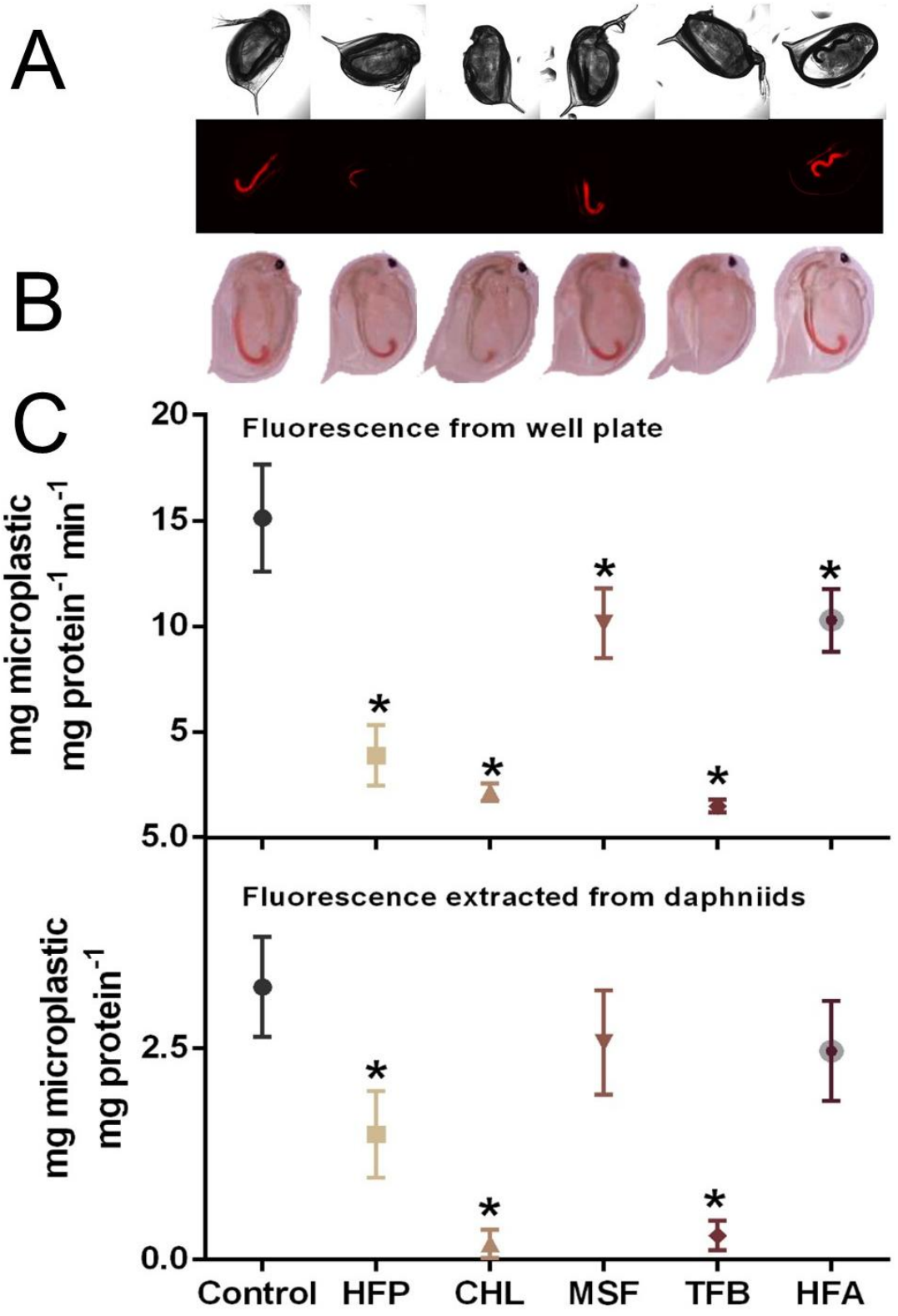

Figure 4. Ingestion of fluorescent microparticles for the assessment of feeding performance in four days old (D4) animals previously exposed to BMIM ILs at EC_5 concentration. The ingested microplastic was visualized by bright field and fluorescence microscopy (panel A) and optical stereoscopy (panel B). Feeding rate was quantified by the ingestion of microparticles based on their fluorescence in the incubation media or extracted from the ingested particles by the daphnids after 60 min (panel C). HFP-Hexafluorophosphate, CHL-chloride, MSF-methanesulfonate, TFB-tetrafluoroborate, HFA-hexafluoroantimonate. Data represent mean±standard deviation (N=6). The * symbol

indicates statistically significant by One-Way ANOVA, followed by Dunnett's post hoc test, from the unexposed control.

Chronic exposure to ILs

Chronic exposure to 1 mg/l for all ILs resulted in significant changes in the physiology of daphnids during their growth (Table 3). Following 7 days of exposure, ALP was increased BMIM methanesulfonate, hexafluoronatimonate and the mixture. ACP activity was increased for BMIM methanesulfonate, hexafluoronatimonate and the mixture but decreased for BMIM hexafluorophosphate. Activity of βGAL was increased in all ILs and their mixture exposure. LIP activity was decreased by BMIM hexafluorophosphate, chloride, and tetrafluoroborate, and increased BMIM hexafluoroantimonate. PEP activity was increased by BMIM methanesulfonate and hexafluoroantimonate. Interestingly, after 14 days of exposure less impact on enzyme activities and sometimes inversed as compared to the effects of 7 days exposures, were observed. ACP was decreased by BMIM hexafluoroantimonate, whereas βGAL was reduced by BMIM methanesulfonate. LIP activity was also reduced by BMIM hexafluorophosphate, chloride, and tetrafluoroborate. The activity of almost all enzymes was reduced following 21 days exposures. BMIM chloride, BMIM tetrafluoroborate, and their mixture, reduced the activity of ALP. Except for BMIM hexafluoroantimonate, all ILs and their mixture reduced ACP activity. The activity of βGAL decreased by BMIM hexafluorophosphate, chloride, and the mixture. BMIM hexafluorophosphate, tetrafluoroborate, hexafluoroantimonate, and their mixture, on the other hand, increased LIP activity. Finally, the activity of PEP was not affected by any of the ILs or their mixture.

Table 3. Enzyme activity responses of daphnids upon chronic exposure to ionic liquids. Data represent mean ± standard deviation (N=4). Enzyme activity was expressed as units/protein. Bold font indicates statistically significant by Student's *t*-test from the unexposed control.

Age (days)	Enzyme	Control	BMIM hexafluorophosphate	BMIM chloride	BMIM methanesulfonate	BMIM tetrafluoroborate	BMIM hexafluoroantimonate	Mixture
7	ALP	4.66±0.3	4.6±0.2	4.7±0.6	**7.96±0.9 (+71%)**	5.2±0.5	**6.5±0.9 (+39%)**	**6.3±0.2 (+35%)**
	ACP	3.1±0.1	**2.6±0.2 (-16%)**	3.1±0.4	**5.5±0.5 (+77%)**	3.4±0.6	**5.02±0.2 (+62%)**	**4.3±0.4 (+39%)**
	βGAL	1.2±0.03	**1.6±0.2 (+33%)**	**1.5±0.2 (+25%)**	**2.1±0.3 (+75%)**	**1.97±0.5 (+64%)**	**1.9±0.2 (+58%)**	**2.1±0.3 (+75%)**
	LIP	48.9±6.9	**37.2±1.1 (-24%)**	**25.8±4.1 (-47%)**	53.8±3.2	**26.5±3.3 (-46%)**	**64.9±5.9 (+33%)**	65.1±20.8
	PEP	1.9±0.1	1.95±0.1	1.8±0.2	**2.5±0.1 (+32%)**	2.01±0.4	**2.4±0.3 (+26%)**	1.98±0.2
14	ALP	4.97±0.4	**4.1±0.3 (-18%)**	4.1±0.6	**3.9±0.3 (-22%)**	**3.8±0.3 (-24%)**	4.3±0.4	4.7±0.3
	ACP	3.5±0.4	3.4±0.3	3.2±0.3	2.96±0.5	2.9±0.4	**2.8±0.2 (-20%)**	2.99±0.3
	βGAL	2.4±0.2	2.2±0.05	2.2±0.3	**2.04±0.1 (-15%)**	2.3±0.3	2.4±0.3	2.7±0.3
	LIP	54.5±5.5	**42.1±5.95 (-23%)**	**27.1±6.2 (-50%)**	**39.9±4.7 (-27%)**	**23.02±1.4 (-58%)**	59.6±5.7	54.7±3.2
	PEP	2.3±0.2	2.2±0.2	1.9±0.3	2.2±0.2	2.2±0.2	2.2±0.2	2.4±0.3
21	ALP	4.1±0.3	3.96±0.1	**3.2±0.2 (-22%)**	3.7±0.7	**3.3±0.3 (-21%)**	4.5±0.3	**3.8±0.2 (-7%)**
	ACP	3.99±0.3	**3.4±0.2 (-15%)**	**2.7±0.3 (-32%)**	**3.2±0.5 (-20%)**	**3.3±0.5 (-17%)**	3.7±0.4	**3.4±0.4 (-15%)**
	βGAL	2.5±0.1	**2.3±0.1 (-8%)**	**1.9±0.2 (-24%)**	1.9±0.4	2.3±0.3	2.7±0.3	**2.2±0.2 (-12%)**
	LIP	52.4±5.6	**71.2±6.9 (+36%)**	60.6±6.3	69.1±22.4	**67.2±7.2 (+28%)**	**84.1±13.01 (+60%)**	**67.5±3.1 (+29%)**
	PEP	2.5±0.3	2.4±0.1	2.2±0.04	2.6±0.7	2.2±0.2	2.4±0.3	2.1±0.2

Fecundity was assessed from the cumulative release of neonates per individual after exposure to BMIM ILs and their mixture for 21 days. There was no significant change observed in the reproduction between control and BMIM ILs exposures (Figures 5 and 6).

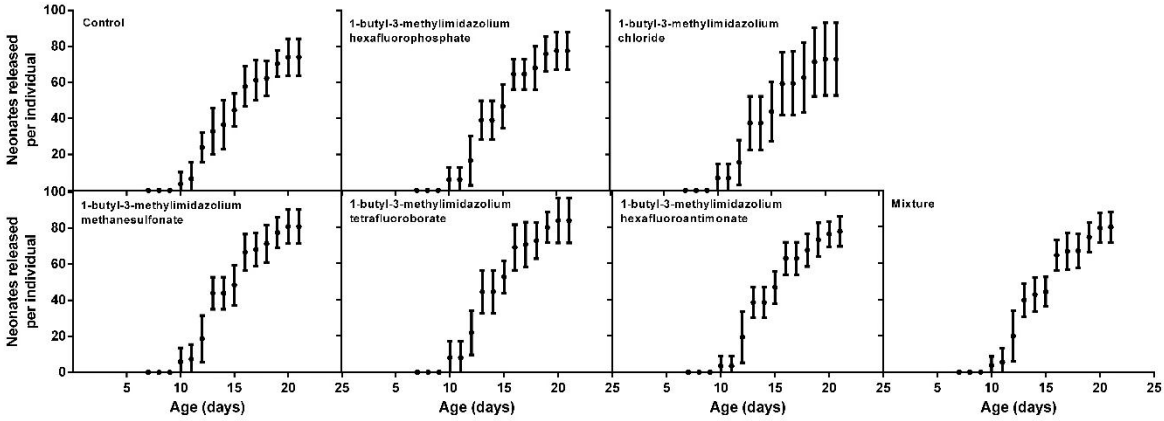

Figure 5. Reproduction of *D. magna* exposed to BMIM ILs and their mixture for 21 days. Data represent mean±standard deviation (N=12).

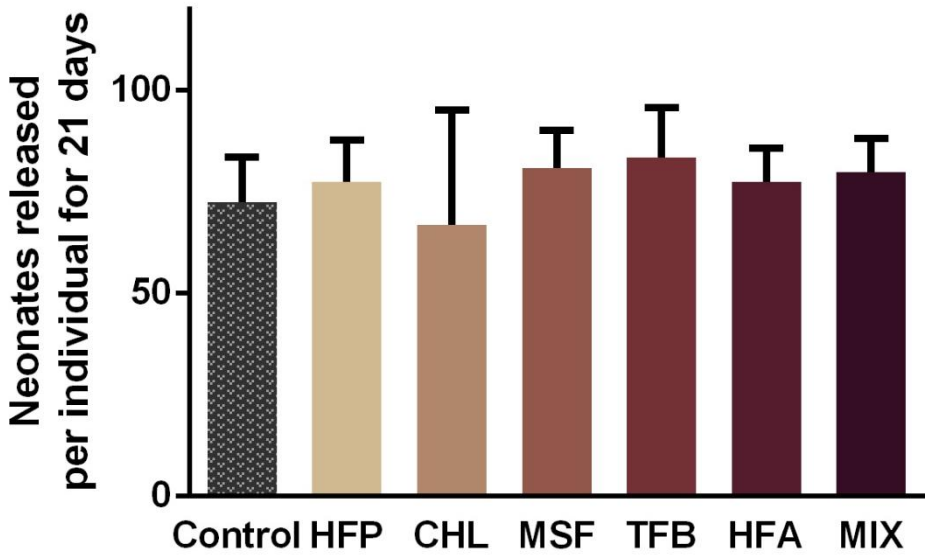

Figure 6. Neonates released per individual for 21 days. HFP-Hexafluorophosphate, CHL-chloride, MSF-methanesulfonate, TFB-tetrafluoroborate, HFA-hexafluoroantimonate. Data represent mean ± standard deviation (N=12).

Discussion

Ion dissociation can be affected by many parameters, including cation alkyl chain length, anion alkyl chain length, temperature, solvent and more. It has been reported that dissociation decreases with increasing the length of the cation alkyl chain because of the increased number of water molecules needed to solvate the cation. Furthermore, interactions between anion and cation can impact the ion dissociation. If the anion interacts significantly with the cation, dissociation is reduced. Additionally, ion dissociation can be connected with

anion polarity due to the higher electrostatic interactions of polar anions with cations (Nordness and Brennecke, 2020). Therefore, in aqueous conditions ion dissociation increases by reducing the anion polarity. In aqueous conditions, BMIM chloride and methanesulfonate are present as individual dissociated ions (Crundwell, 2019, Nordness and Brennecke, 2020). At room temperature BMIM tetrafluoroborate dissociates into hydrofluoric acid (HF) (Xue, 2019), while BMIM hexafluorophosphate and hexafluoroantimonate are sparingly soluble or insoluble in water (Klahn et al., 2010) and they tend to form a separate organic phase or undergo phase separation when added to water. However, according to (Camargo, 2003, Cho et al., 2008) hexafluorophosphate and hexafluoroantimonate ions are very unstable hydrolytically and in contact with moisture they form volatiles such as (HF). Hexafluorophosphate dissociates in phosphate ion (PO_4^{3-}) and (HF) with HNO_3 as a catalyst, while hexafluoroantimonate in HF and $[SbF_6\text{-}n(OH)n]^{-1}$ where $0<n<6$ (Xue, 2019). Fluoride ions (F^-) have been characterized as "enzymatic poison", as they inhibit enzyme activity (particularly phosphatases) and disrupt metabolic processes including glycolysis and synthesis of proteins. In invertebrates, the toxicity that fluoride can cause increases with increasing fluoride concentration, exposure time and water temperature (Camargo, 2003). Additionally, fluoride might have significant chronic effects on freshwater organisms (Metcalfe-Smith et al., 2003). Therefore, regardless of their solubility, these BMIM ILs have been extensively used in other studies where they were prepared directly in distilled water or in the culture media (Couling et al., 2006, Kebaili et al., 2020).

A combination of physiological indicators such as feeding and mortality, and enzyme activities was used to analyse the acute and chronic effects of BMIM ILs on daphnids. Most ILs are toxic to organisms because they are either poorly biodegradable or non-biodegradable (de Jesus and Maciel Filho, 2022). There are a few studies that have been published about the effects of certain BMIM ILs on *D. magna,* yet the majority of them rely solely on mortality as an endpoint to determine toxicity (Bernot et al., 2005, Samori et al., 2010). Extending to other aquatic species, it has been noted that imidazolium-based ILs can harm other marine organisms (Belavgeni and Dailianis, 2017, Deng et al., 2016, Leitch et al., 2020, Li et al., 2009, Liu et al., 2015, Nan et al., 2016, Tsarpali and Dailianis, 2018). Furthermore, other ILs such as 1-octyl-3-methylimidazolium tetrafluoroborate (OMIM tetrafluoroborate) can induce cytotoxic, oxidative, and genotoxic effects in mussel hemocytes (Belavgeni and Dailianis, 2017, Tsarpali and Dailianis, 2018). OMIM ILs are expected to be more toxic than BMIM ILs due to their longer alkyl chain (Kuroda, 2022). OMIM ILs have been also reported to be toxic to frogs (*R. nigromaculata*), on the early embryonic stage (Li et al., 2009, Leitch et al., 2020), to impact the membrane permeability,

cell morphology, and growth of green algae (*S. obliquus*) (Liu et al., 2015, Leitch et al., 2020, Tsarpali and Dailianis, 2015, Tsarpali et al., 2016). Furthermore, OMIM ILs can be toxic to other organisms such as the marine diatom (phytoplankton, *S. costatum*) by affecting the photosynthetic process and cell growth (Deng et al., 2016, Leitch et al., 2020), to the fish (*P. dabryanus*) and to zebrafish (*Danio rerio*) by causing oxidative stress and DNA damage (Leitch et al., 2020, Liu et al., 2016, Nan et al., 2016). The mechanisms behind their toxic effects on these species are still unknown, but there are reports in the literature that link the harmful effects of imidazolium-based ILs to their aromatic ring. Molecules from these ILs have a high chance of being oxidized if they reach the cytochrome P_{450} in the endoplasmatic reticulum (Jastorff et al., 2003). According to Ventura et al., 2013 ILs consisting of aromatic cations, such as imidazolium and pyridinium are always more harmful than ILs with non-aromatic cations, thus pyrrolidinium and piperidinium (Kebaili et al., 2020, Ventura et al., 2013). Therefore, naturally existing metabolites may be degraded further into potentially hazardous fatty acids and imidazole (Bernot et al., 2005). Overall, enzyme inhibition, membrane permeability disruption, and/or structural damage to DNA have been described as possible mechanisms of action of the ILs (Bernot et al., 2005, Costa et al., 2015).

Given the scarcity of studies specifically on the effects of BMIM ILs on freshwater organisms and especially daphnids, our study aimed to highlight their importance and also introduce additional markers of physiology than only mortality as a common endpoint used. We recently showed that markers relevant to the physiology and enzymatic activity are useful endpoints for pollution assessment along with metabolic perturbations (Michalaki et al., 2022). It is worth noting, that this is the first reference of ILs and daphnids using molecular markers and not only toxicity results.

The effects of acute and chronic exposures on *Daphnia magna* are not always the same. There have been cases where acute and chronic exposures did not impact the animals differently (Barmentlo et al., 2015, Walthall and Stark, 1999). However, studies have revealed that different exposure times might have varying effects (Sieratowicz et al., 2011, Wollenberger et al., 2000). Chronic experiments can reveal distinct mechanisms of actions of a pollutant that an acute exposure cannot (Rossetto et al., 2014). Similarly to the latter case scenarios, our study concluded that different exposure times of BMIM have various impacts on daphnids. According to our findings, BMIM ILs had an adverse effect on daphnids in both acute and chronic exposures, although in distinct manners.

It is worth noting that the solubility of the BMIM ILs employed in this study was not measured. However, regardless their solubility, these BMIM ILs have a noticeable impact on the physiology of daphnids based on the following results. In acute exposures, all BMIM

ILs affected the activity of nearly all enzymes showing a systemic effect and feeding was also impacted, confirming an impact on animal physiology. Increase of antioxidant enzymes has also been reported to be caused by the 1-alkyl-3-methylimidazolium bromide (Yu et al., 2009). Similarly, it has been observed that the activity of GST of zebrafish (*Danio rerio*) was increased after exposure to 1-octyl-3-methylimidazolium chloride and 1-octyl-3-methylimidazolium tetrafluoroborate (Liu et al., 2016). GST belongs to a group of enzymes involved in detoxification processes. In acute exposures, BMIM hexafluorophosphate and tetrafluoroborate significantly increased the activity of GST by 23% and 30%, respectively. These findings are consistent with those obtained by Galhano (Galhano et al., 2022) following acute exposure of *D. magna* to TiO_2NPs and AgNPs. According to the authors, daphnids may have used these detoxification processes as a way to adapt and survive through the chemical stress caused by BMIM ILs. Furthermore, in the same study activity of LDH was also significantly increased. LDH is essential for the anaerobic metabolism because it facilitates the conversion of lactate to pyruvate and the consumption of NADH produced by glycolysis, thus facilitating glycolysis to continue in the absence of oxygen. As a result, the activity of LDH is determined by the energy demands of the organism, and it is even more important if there are any additional needs, such as detoxification processes under stressful circumstances (Diamantino et al., 2001, Galhano et al., 2022). In our study, all BMIM ILs increased the activity of LDH, which implies the exposed daphnids have entered the anaerobic metabolism. Therefore, the increased activity of GST and LDH may have been used as a way of dealing with the increased energy demands imposed by BMIM ILs. Impact on feeding rate was assessed using a novel feeding assay. Advantages of the assay used include high reproducibility, less time consumed comparing to other feeding assays (Barata et al., 2008, Hite et al., 2020) and have no risk of reagent/pollutant degradation. Due to the stability of the microplastic this assay allows the quantification of ingested microplastic, which allows the accurate evaluation of the feeding rate. According to acute toxicity curves, EC_{50} values of the tested BMIM ILs were in the same order of magnitude, as expected, since the toxicity is mostly driven by the cation and not by the anion (Couling et al., 2006, Kebaili et al., 2020, Latała et al., 2009, Pretti et al., 2009, Ranke et al., 2004, Romero et al., 2008, Ruokonen et al., 2016, Salam et al., 2016). According to Garcia et al., 2005, the inorganic anion has little impact on the toxicity of BMIM ILs (Garcia et al., 2005). Nevertheless, it is established that as the length of the alkyl chain increases, so does the toxicity of the associated ILs (Jastorff et al., 2003, Kebaili et al., 2020). It has been reported a direct correlation between the toxicity of ILs and the nature of the cation. However, the anion might have a moderating effect on toxicity to certain and specific cases (Ventura et al., 2012).

Specific anions, particularly those with lipophilic characteristics or prone to hydrolysis may have more prominent effects. Notable inclusions are hexafluorophosphate, tetrafluoroborate and hexafluoroantimonate anions, which hydrolyse in the presence of water, releasing HF into the surrounding medium. Furthermore, interactions between anions and cations might result in synergistic toxicity effects, potentially modifying the toxicity hierarchy of different anion families. Therefore, it becomes challenging to isolate the individual contributions of anions and cations (Kebaili et al., 2020). Additionally, studies using *D. magna* and *Vibrio fisheri* have shown that ILs with longer alkyl chain are more toxic, with cationic properties having a stronger influence on toxicity than anionic properties. Although it is known that anions have a secondary role in toxicity, studies have shown that anions with positively charged atoms are more hazardous than those with one single negative charge (Couling et al., 2006). Additionally, the EC_{50} values of BMIM chloride and BMIM tetrafluoroborate are quite comparable to those previously reported by (Bernot et al., 2005) and (Samori et al., 2010). Interestingly, severe changes in the physiology of animals were observed, as BMIM chloride and tetrafluoroborate, almost completely abolished the feeding capacity of daphnids. It is noteworthy that BMIM ILs altered the physiology and metabolism of daphnids, while having no effect on reproduction. The latter is in contradiction with the 1-hexyl-3-methylimidazolium bromide, which, according to Yu et al*.,* 2020, has a considerable impact on development but also lowers reproductive activity (Yu et al., 2020). Additionally, daphnids exposed to 1-methyl-3-octylimidazolium bromide exhibit lower reproductive ability (Luo et al., 2008). The chronic exposures to individual ILs and their mixture at lower concentrations, showed multiple changes in enzyme activities. (Chen et al., 2005) observed that the activity of phosphatase decreased after 14 days of exposure of daphnids to high-dose microcystin. In our study, the activities of both phosphatases were increased the first 7 days of exposure, yet they gradually decreased after 14 days. BMIM ILs and their mixture increased or decreased nearly all enzyme activities of D7 daphnids. There is no data in literature regarding the impact of BMIM ILs on daphnids, however, there are reports of BMIM-induced toxicity to a small number of crustaceans used in ecotoxicology (Tsarpali et al., 2016). Additionally, BMIM ILs are known to cause oxidative stress and DNA damage to other aquatic organisms such as the zebrafish (*Danio rerio*) (Zhang et al., 2017a) and mussels (Tsarpali and Dailianis, 2015).

After 14 days of exposure, the impact of BMIM ILs was significantly reduced when compared to the observed impact on D7 daphnids. One possible scenario is the age difference between the two groups, since D14 daphnids are older and, therefore, possibly more resistant to the effects of pollutants. Another reason to explain this observation could be that D14

daphnids enter their reproductive stage preparing to release their first big brood of neonates, thus investing in their offspring (Ebert, 2005). On the other hand, enzyme activity in D21 daphnids were almost equally affected by BMIM ILs as in D7 daphnids. The lack of alterations in enzyme activities after 14 days of exposure to BMIM ILs was notable. While all BMIM ILs affected the activity of specific enzymes in D14 daphnids, their mixture showed no effect. BMIM ILs can obscure the harmful effects of their mixture on D14 daphnids, leading to skewed assumptions about their safety and classification as "green solvents". Furthermore, D7 and D21 daphnids were significantly affected by BMIM ILs mixture, while D14 daphnids were not impacted.

In this study, inhibition of phosphatases was observed during acute and chronic exposures. Specifically, BMIM hexafluorophosphate decreased the activity of both ACP and ALP during acute exposures and the ACP on D7, ALP on D14 and ACP on D21 daphnids. BMIM tetrafluoroborate reduced the activity of ALP, ACP on D4 daphnids, ALP on D14 and both the activities of ALP and ACP on D21 daphnids. BMIM hexafluoroantimonate decreased the activity of ALP on D4 daphnids and ACP on D14 daphnids. One plausible explanation of these results might be the presence of fluoride. Therefore, despite the immiscibility of these BMIM ILs, a noticeable impact on daphnids physiology was observed. Due to the adverse effects observed on daphnid physiology in this study, it is of imperative importance to further investigate ILs in relation to their characterisation as 'green solvents'.

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Declaration of Competing Interest

There are no conflicts of interest to declare.

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Chapter 4A

This chapter investigated the effects of two widely used non-steroidal anti-inflammatory drugs (NSAIDs), indomethacin and ibuprofen on *D. magna*. For this study, acute, chronic and transgenerational exposures were performed to evaluate the impact of NSAIDs on daphnids using phenotypic and biochemical markers, such as feeding assay and enzymatic activities. Results showed that while enzymatic changes happened in the first generation, these effects were transferred in the following generations, highlighting the cumulative impact of transgenerational exposures. Additionally, daphnids were able to recover from the stress in the recovery generation where the NSAIDs were removed from the media, underscoring the dynamic responses of aquatic organisms to environmental pharmaceuticals. This work advanced the concept by emphasizing the importance of transgenerational studies to deeper understand the ecological risk posed by chemicals, and by highlighting the value of integrating phenotypic and biochemical endpoints to detect subtle yet significant effects.

Acute and Transgenerational Effects of Non-Steroidal Anti-Inflammatory Drugs on *Daphnia magna*

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Abstract

Pharmaceuticals pose a great threat to organisms inhabiting the aquatic environment. Non-steroidal anti-inflammatory drugs (NSAIDs) are major pharmaceutical pollutants with a significant presence in freshwater ecosystems. In this study, the impact of indomethacin and ibuprofen, two of the most commonly prescribed NSAIDs, was assessed on *Daphnia magna*. Toxicity was assessed as the immobilization of animals and used to determine non-lethal exposure concentrations. Feeding was assessed as a phenotypic endpoint and key enzymes were used as molecular endpoints of physiology. Feeding was decreased in mixture exposures for five-day-old daphnids and neonates. Furthermore, animals were exposed to NSAIDs and their mixture in chronic and transgenerational scenarios revealing changes in key enzyme activities. ALP, ACP, LIP, PEP, βGAL, and GST were shown to have significant changes in the first generation at the first and third week of exposure, and these were enhanced in the second generation. On the other hand, the third recovery generation did not exhibit these changes, and animals were able to recover from the induced changes and revert back to the control levels. Overall, our study points towards transgenerational exposures as more impactful laboratory studies to understand pharmaceutical stressors with a combination of molecular and phenotypic markers of physiology.

Keywords: *Daphnia magna;* non-steroidal anti-inflammatory drugs; NSAIDs; indomethacin;
ibuprofen; toxicity; immobilization; enzyme activity; feeding; transgenerational

Introduction

Pharmaceutical compounds pose a greater risk to the aquatic environment and human health due to bioaccumulation and human activities (Luo et al., 2008, Zhang et al., 2017, Worthington and Worthington, 2011). Because of the global increase in human population and, as a result, the increase in elderly people, the use of pharmaceuticals is becoming highly significant. Pharmaceuticals end up in the environment as a result of their widespread consumption, where they are extremely difficult to biodegrade (Worthington and Worthington, 2011, Yu et al., 2009). Pharmaceutical compounds have been observed in potable and groundwater, surface water, sewage treatment plants, Waste Water Treatment Plants (WWTPs), soils, and sediments (Ranke et al., 2004, Liu et al., 2016). Pharmaceuticals are primarily derived from industrial or domestic wastewater, improper disposal of expired drugs, and products used in farming and aquaculture (Worthington and Worthington, 2011). Furthermore, pharmaceutical metabolites and their subsequent transformation products may enter the environment via the WWTP process (Zhang et al., 2017, Worthington and Worthington, 2011, Yu et al., 2009). Besides that, biotic or abiotic processes such as hydrolysis or photolysis can degrade pharmaceuticals further (Yu et al., 2009, Abdullahi et al., 2022). These procedures may result in the production of additional pharmaceutical products that are potentially more toxic than the parent compound (Yu et al., 2009, Adeel et al., 2017). As a result, it is critical to develop new, more efficient methods for removing pharmaceutical compounds from the environment (Zhang et al., 2017).

Non-steroidal anti-inflammatory drugs (NSAIDs) are a pharmaceutical class that is commonly found in aquatic ecosystems worldwide, accounting for more than 15% of all pharmaceuticals found in the environment (Alla et al., 2021, Swiacka et al., 2020). As an outcome, NSAIDs can be detected in concentrations greater than 1 μg/l in wastewater treatment plant (WWTP) influx and outflow and in lower concentrations (ng/l) in surface waters (Alla et al., 2021). Even though the concentrations of NSAIDs in freshwater ecosystems are low, their high biological activity may cause significant harm to non-target organisms (Alla et al., 2021, Swiacka et al., 2020). The constant inflow of NSAIDs from WWTPs poses a serious threat to the aquatic environment and its organisms, which are constantly exposed to these emerging contaminants (Swiacka et al., 2020). NSAIDs can be categorized into two groups based on their COX selectivity: nonselective NSAIDs, which block both COX isoforms (indomethacin and ibuprofen) and COX-2-selective inhibitors (Lucas, 2016).

Indomethacin is one of the most potent nonselective NSAIDs available, and it was among the first NSAID medications used to treat migraines and headaches (Lucas, 2016). It is an indole-derivative and one of the most commonly detected NSAIDs due to its widespread use (Bang et al., 2015). Additionally, indomethacin belongs to the class of intestinal toxicants that can cause gastrointestinal lesions through direct mucosal irritation and inhibition of prostaglandin synthesis (Tsarpali and Dailianis, 2018). Despite its therapeutic properties, indomethacin is known for its adverse effects in a percentage range of 30–60% of patients receiving it. These adverse effects can be classified as cardiovascular, gastrointestinal, nervous system, hematologic, ocular and otic, renal and electrolyte, dermatologic and sensitivity reaction, and hepatic (Lucas, 2016). Indomethacin can enter the ecosystem through both direct and indirect routes by being improperly disposed of in the toilet or as an excretion product. Furthermore, traces of indomethacin have been found in wastewater samples from Peterborough, Canada, as well as raw water samples from Bosnia and Herzegovina, Croatia, Serbia, and Romania (Worthington and Worthington, 2011, Crawford, 1985). It has been reported that indomethacin is one of the NSAIDs with the lowest detection levels (<2 μg/l) in Romanian WWTPs influents (Worthington and Worthington, 2011). This observation is in line with a previous publication, which states that indomethacin poses a less serious risk to the environment due to its low concentrations (de Oliveira et al., 2020).

Ibuprofen is the third most popular of the NSAIDs used globally and has been included in the WHO (World Health Organization) Essential Drug list 2021 (2013, Chopra and Kumar, 2020). Ibuprofen inhibits cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) and has a significant analgesic and antipyretic role (Diaz-Gonzalez and Sanchez-Madrid, 2015, Bushra and Aslam, 2010). Ibuprofen is a derivative of propanoic acid and can be detected in several freshwater ecosystems (Chopra and Kumar, 2020, Barreto et al., 2018, Bayda et al., 2019). Despite the fact that ibuprofen has significant analgesic and antipyretic properties, excessive or inappropriate use may have negative effects on the gastrointestinal tract, the kidney, and the coagulation system (Bushra and Aslam, 2010). Ibuprofen can enter freshwater ecosystems indirectly or directly (Barreto et al., 2018). Due to its inability to be fully metabolized by humans, it enters the ecosystem as an excretion product. The direct way for ibuprofen to end up in the environment is by being improperly disposed of in the toilet (Barreto et al., 2018). Ibuprofen has been detected at concentrations of 45 μg/l, 1.38 μg/l, and 5.78 μg/l in wastewater in Canada, South Africa, and Belgium, respectively. However, even concentrations of 1.673 mg/l and 1.417 mg/l have been detected in

wastewater in Pakistan and in surface waters in China (Oosterhuis et al., 2013, Chopra and Kumar, 2020).

To improve our understanding of water pollution, the use of key species as bioindicators is currently highlighted. The combination of phenotypic and sensitive molecular endpoints is a game changer in ecotoxicology and risk assessment. Such approaches fall under the category of New Approach Methodologies (NAMs), which can supplement current analytical tools with sophisticated molecular endpoints (El-Harbawi, 2014). Daphnids are important freshwater ecosystem components that are commonly used in freshwater ecology and ecotoxicology due to their geographical distribution, central role in freshwater food webs, adaptation to a variety of habitats, and, last but not least, sensitivity to anthropogenic chemicals. Daphnids are simple to culture in the laboratory and serve as a useful model organism for molecular ecotoxicology and mechanistic insight into novel pollutants (Li et al., 2009, Liu et al., 2015, Barata et al., 2008).

This study aimed to investigate the effects of indomethacin, ibuprofen, and their combined mixture on *D. magna*, specifically through chronic and transgenerational exposure at a non-lethal concentration that is not environmentally relevant, using feeding assay as a phenotypic endpoint and molecular markers of physiology. It should be noted that most studies usually focus on phenotypic endpoints such as mortality in daphnids and usually in acute or chronic exposures but only in one generation. This is the first study to look at the effects of indomethacin, ibuprofen, and their combined mixture on daphnids using molecular markers rather than toxicity or phenotypic markers alone and in-depth in more generations. Moving toward transgenerational studies provide more insight into the relation to imprinted stress and its mechanisms. In this study, the effects of two NSAIDs, indomethacin and ibuprofen, were assessed on *D. magna.* Furthermore, in the actual environment, chemicals are not encountered alone; therefore, for a realistic scenario, their 1:1 mixture was assessed in non-lethal concentrations. The toxicity of these pharmaceuticals and their mixture on daphnids was evaluated using acute toxicity curves. These toxicity curves were constructed based on the mortality of daphnids, which was assessed as immobilization (Ebert, 2005). Following this, their impact on a phenotypic endpoint, feeding rate, was evaluated with a novel approach based on the ingestion of fluorescent microparticles. The impact of indomethacin, ibuprofen, and their mixture on the physiology of daphnids was evaluated using biochemical markers in chronic exposures. The main goal of this study was to assess, even at higher than environmental concentrations, if daphnids are affected and to what extent and if they can actually recover from the stress of exposure when they are subsequently cultured in clean media.

Materials and Methods

Reagents

All chemicals used in this study were of the highest purity >99.9% and quality. Indomethacin, ibuprofen, KCl, Na_2SeO_3, latex beads, carboxylate-modified polystyrene, fluorescent red, bovine serum albumin, brilliant blue G, *p*-nitrophenyl butyrate, 2-nitrophenyl-B-D-galactopyranoside, 1-chloro-2,4-dinitrobenzene, L-glutathione reduced, sodium phosphate dibasic were purchased from Sigma-Aldrich. $CaCl_2 \cdot 2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$, HCl, *p*-nitrophenyl phosphate, boric acid, ammonium acetate, NaOH, methanol, and DMSO were purchased from Fisher Scientific.

Culturing of *Daphnids* and Acute Toxicity

Daphnids were cultured under a 16 h:8 h of light:dark photoperiod at 20°C in OECD media (final concentrations 0.29 g $CaCl_2.2H_2O$/l, 0.123 g $MgSO_4.7H_2O$/l, 0.065 g $NaHCO_3$/l, 0.0058 g KCl/l, 2 μg Na_2SeO_3/l, pH 7.7) (Michalaki et al., 2022). Indomethacin and ibuprofen were dissolved in DMSO, which had a final concentration of 0.005% v/v in the exposure, which is within the concentration range used in previous studies (Gómez-Oliván et al., 2014, Han et al., 2010, O'Rourke et al., 2023). Neonates (<24 h) were collected from the third brood of their mothers and used for experiments. To assess toxicity, fifteen neonates were exposed to each NSAID separately, and their 1:1 mixture in a final volume of 50 ml OECD media with four replicates per concentration tested. Based on similar studies (Heckmann et al., 2007, Heckmann et al., 2008, Grintzalis et al., 2017, Michalaki et al., 2022, O'Rourke et al., 2023, Tsarpali et al., 2015, Tsarpali and Dailianis, 2015), all assays were conducted with four (toxicity curves and biochemical assays) or five (feeding assay) replicates per condition tested. For toxicity, the OECD guidelines state that at least fourty animals (preferably in four groups of ten animals should be used at each test concentration); however, in our study, we exceeded that number to sixty animals. Toxicity curves were plotted for 24 h exposures, and EC values were calculated using the four-parameter logistic (4PL) model, following the equation Span = Top − Bottom and Y = Bottom + (Top − Bottom)/(1 + 10^((LogIC50-X) × HillSlope)), using the GraphPad software. The parameters top and bottom were commonly fixed to 100 and 0, accordingly. Mortality in daphnids was assessed as their immobilization (Gómez-Oliván et al., 2014).

Feeding Assay and Imaging

The feeding rate was assessed in neonates, which were exposed to NSAIDs at a non-lethal concentration of 1 mg/l for 24 h or cultured until five days old (Figure 1A). To assess the feeding rate of neonates, D1 daphnids were exposed for 24 h to indomethacin, ibuprofen,

and their mixture at 1 mg/l. After 24 h, twenty daphnids were transferred in a 12-well plate with 6 ml OECD containing the carboxylate-modified polystyrene, fluorescent red microparticles (2.0 μm mean particle size) at a concentration of 13 mg/l. The animals were exposed to the microplastic for up to 90 min, and media was collected every 15 min to estimate the removed microparticles by fluorescence at Ex 560 Em 590 nm using a TECAN plate reader. The concentration of microparticles in the media was optimized to ensure an excess of microparticles for the accurate quantification of ingestion. Furthermore, we have extensively tested these particles, which show no toxicity to the daphnids for the short exposure periods used. We performed control experiments with neonates, as they would be more sensitive, for even up to 10 h to microplastics at even higher concentrations (up to 52 mg/l), and all animals from all experiments following the feeding assay were alive and healthy. Fluorescence was expressed to the amount of ingested microparticles using a standard curve. To confirm the ingestion of microparticles in the gut of daphnids, animals were imaged with a stereoscope and fluorescence microscopy using the TRITC filter for carboxyl functionalized particles. For D5 daphnids, neonates were exposed for 5 days to indomethacin, ibuprofen, and their mixture at 1 mg/l in 600 ml OECD. The animals were fed daily with 2 ml of fresh algae (*Chlamydomonas reinhardtii*) with a concentration of 6 million cells/ml. On the 5th day of exposure, five daphnids were brought to a 12-well plate with 6 ml OECD containing the fluorescent red microparticles at a concentration of 13 mg/l. Daphnids were allowed to ingest the microparticles for 60 min, and then the same procedure as described above was followed (Figure 1A).

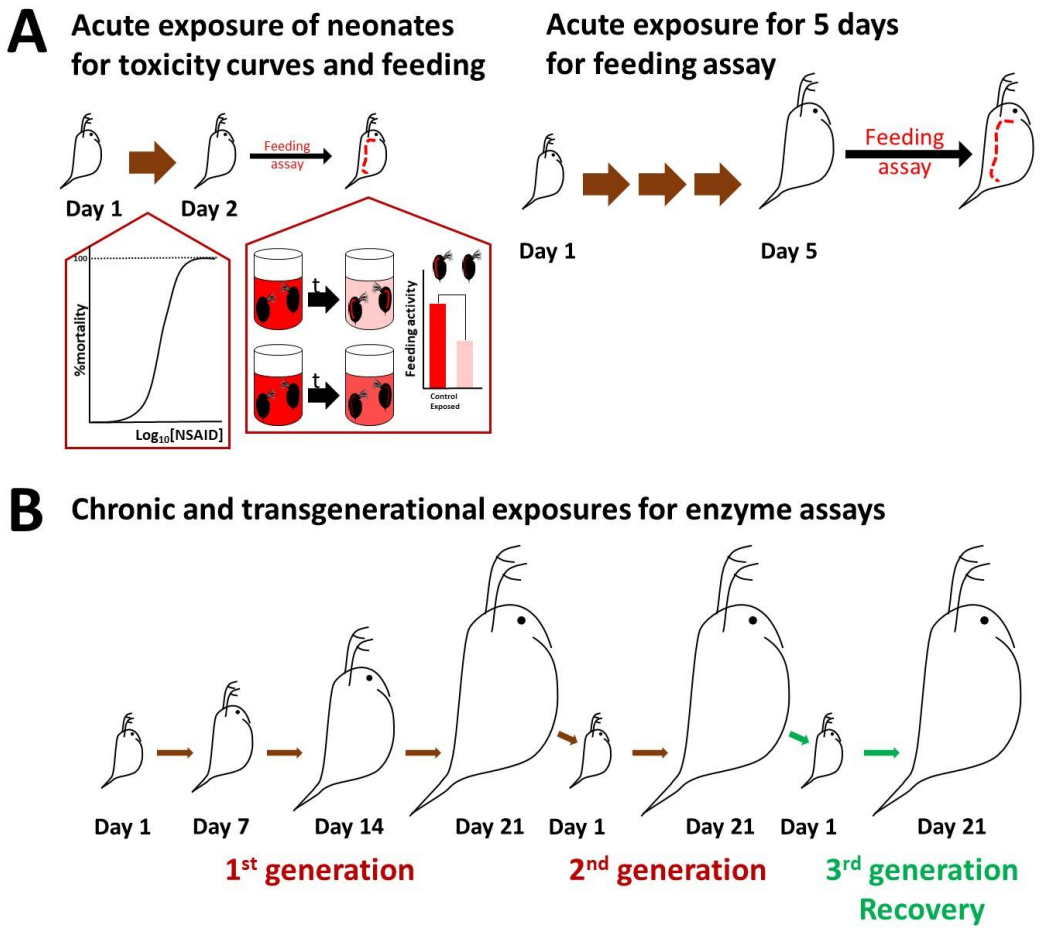

Figure 1. Experimental design. (A) Acute exposures in neonates and five-day-old daphnids to assess mortality and feeding. (B) Chronic and transgenerational exposures followed by a recovery generation for the quantification of enzyme activities. Figure created with BioRender.com. Brown arrows indicate exposure to NSAIDs and their combined mixture, while green arrows show recovery exposure in the absence of NSAIDs.

Biochemical Assays

Chronic exposure was performed at 1 mg/l of each NSAID and their 1:1 mixture separately, which was selected as a non-lethal concentration. For enzyme activities in chronic and transgenerational exposures, neonates were cultured for two consecutive generations and following a 21-day recovery period (Figure 1B). Twenty neonates (<24 h) were exposed for 7, 14, and 21 days to indomethacin, ibuprofen, and their mixture at 1 mg/l in 600 ml OECD media. Media and NSAIDs were renewed twice a week. Although the concentration of NSAIDs was not measured throughout the exposure, their renewal twice a week was sufficient to exert their action. Furthermore, indomethacin has been shown to be stable for up to 12 days at room temperature (Moudry et al., 2013, Walker et al., 1998). There are many reports stating that ibuprofen is stable for 15 days at room temperature (Walker et al., 1998, Volonté et al., 2005). Ibuprofen can maintain >90% average strength for 5 months, according to (Archibald and Brown, 2020), and half-life (t1/2) for more than a year (t1/2 >

1 year) at 25 °C (Tońki et al., 2019). Daphnids were fed daily with 2 ml fresh algae (*C. reinhardtii*) with a concentration of 6 million cells/mL, and a seaweed extract (*Ascophylum nodosum*) was supplemented only on the days of media change. Neonates from the D21 daphnids (of the first generation) were exposed to the same conditions for 21 days as the second-generation exposure. For recovery, neonates from the second generation were cultured only in OECD media in the absence of NSAIDs for 21 days as a recovery generation.

Five 7, 14, and 21-day-old animals were used per biological replicate and homogenized in 0.5 ml ddH_2O using a pestle homogenizer. The homogenate was cleared by centrifugation (12,000× g for 5 min at 5°C), and the clear supernatant was collected and used to assess the enzyme activity as described elsewhere (Michalaki et al., 2022, Worthington and Worthington, 2011). 200 µl appropriately diluted sample in buffer was assessed for the activity of phosphatases (in 100 mM acetic acid pH 4.5 for acid phosphatase; ACP or 100 mM boric acid pH 9.8 for alkaline phosphatase; ALP) using 50 µl of the substrate *p*-nitrophenyl phosphate (8 mM) and monitoring the production of *p*-nitrophenol at 405 nm after its alkalinization (with 50 µl 4M NaOH). In addition, the activities of β-galactosidase (βGAL) and lipase (LIP) were measured with the same experimental conditions by the generation of nitrophenol from the catalysis of *o*-nitrophenyl-β-galactoside or *p*-nitrophenyl butyrate, respectively, but in phosphate buffer pH 7.2. The activity of peptidase (PEP) was quantified by the hydrolysis of L-leu-4-nitroanilide and the production of 4-nitroaniline (at 412 nm every five minutes for thirty minutes) in 100 mM phosphate buffer pH 7.2. Glutathione-S-transferase (GST) activity was assessed by the reaction of reduced glutathione with 1-chloro-2,4-dinitrobenzene. 200 µl appropriately diluted sample in phosphate buffer pH 7.2 was mixed with 50 µl 2 mM CDNB, and 6 mM reduced glutathione, and the formation of the complex was measured continuously at 340 nm and converted to units of activity with the extinction coefficient (Tang et al., 1996, Warholm et al., 1981). Protein was quantified by an ultrasensitive Bradford protocol to normalize the results (Grintzalis et al., 2015).

Statistical Analysis

The biochemical data were presented as mean ± standard deviation and analysed with the GraphPad Prism software. Statistically significant differences were compared by Student's *t*-test over unexposed control with a P value of 0.05 for chemical exposures.

Results

Acute Toxicity of NSAIDs and Their Mixture

Acute exposures of neonates to NSAIDs, indomethacin, ibuprofen, and their mixture were assessed via toxicity curves (Figure 2), and the effective concentration (EC) values were calculated (Table 1). The EC_{50} values for indomethacin and ibuprofen were very close to each other, 17.46 mg/l and 17.80 mg/l, respectively. A 1:1 mixture for both NSAIDs was also tested for mortality and showed a lower EC_{50} of 8.304 mg/l, indicating a potential synergy of the two NSAIDs. A non-lethal concentration of 1 mg/l was chosen for both NSAIDs and their mixture for chronic exposures.

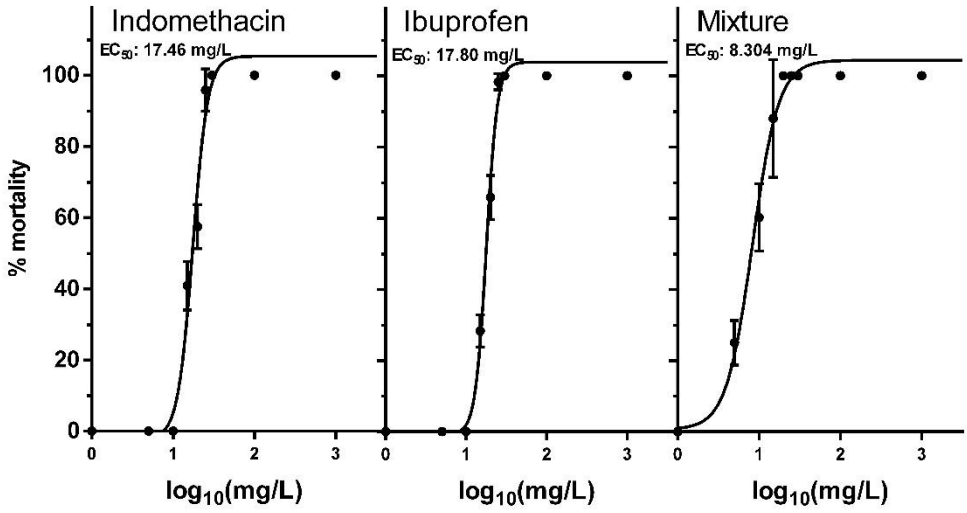

Figure 2. Acute toxicity curves in neonates for indomethacin, ibuprofen, and their mixture. Data represent average ± standard deviation (N = 4 replicates).

Table 1. EC values (in mg/l) from toxicity curves.

Chemical	EC_{50}	(Min-Max)	Hill Slope	EC_1	EC_5	EC_{10}	EC_{20}
Indomethacin	17.46	16.38–18.61	4.731	6.61	9.37	10.97	13.02
Ibuprofen	17.80	17.30–18.31	6.341	8.62	11.19	12.59	14.3
Mixture 1:1	8.304	7.48–9.21	2.696	1.51	2.78	3.67	4.97

Feeding Assay and Imaging

Feeding is a phenotypic endpoint used to evaluate the physiology of daphnids as a non-invasive test (Grintzalis et al., 2017). We used particles with a mean particle size of 2.0 µm because *D. magna* feeds non-selectively on a wide range of particles with sizes ranging from 1 to 50 µm (Eltemsah and Bøhn, 2019). These particles were selected as they would allow tracking with fluorescence microscopy and were not toxic to daphnids. The feeding rate was

determined by the ingestion of fluorescent microparticles in neonates and five-day-old daphnids (Figures 3 and 4). The ingestion of microplastics was confirmed using stereoscopy, bright field, and fluorescent microscopy. Exposure of neonates to indomethacin, ibuprofen, and their composite mixture reduced the feeding rate by 35%, 30%, and 49%, respectively, when compared to DMSO (Figure 3). However, when five-days-old animals were exposed, a different pattern was observed, and only their mixture decreased the feeding rate by 23% (Figure 4). This can be potentially explained as the animals are older and more resistant to the stress of NSAIDs when compared to neonates.

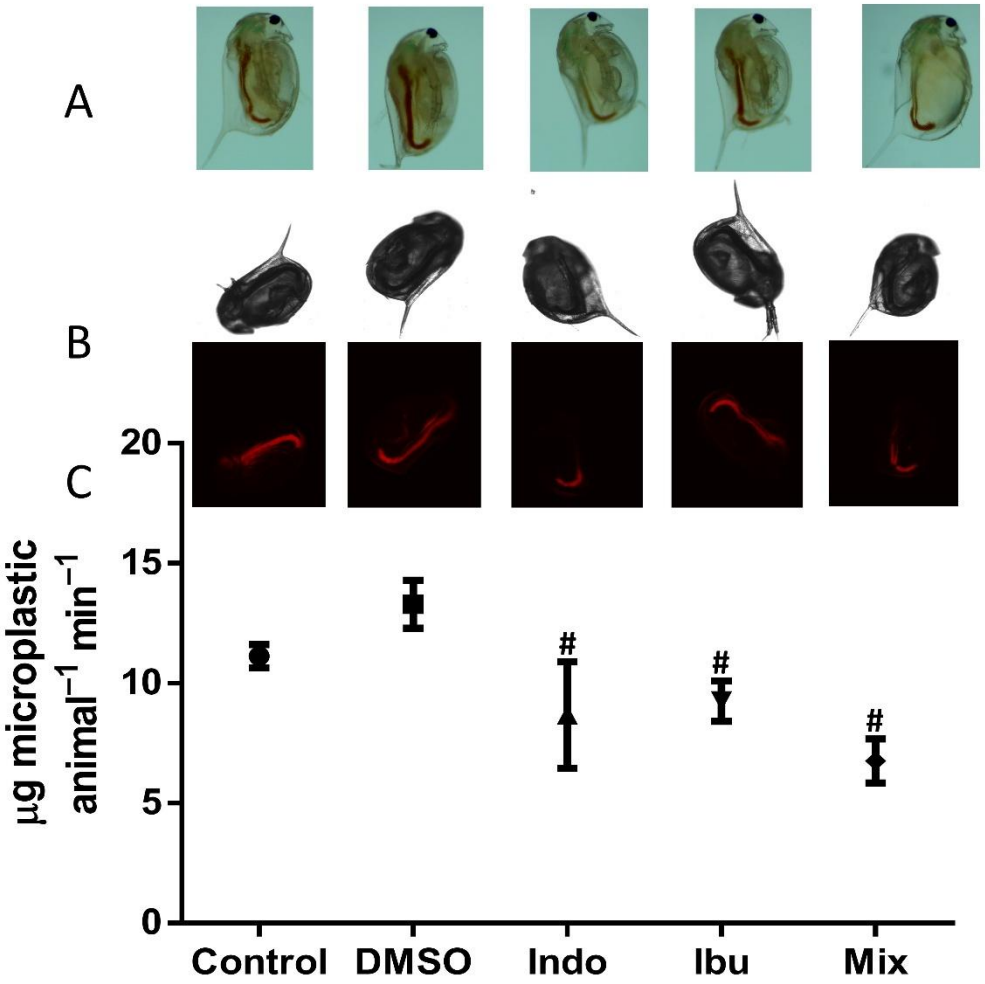

Figure 3. The impact of NSAIDs on feeding rate to neonates. The ingested microparticles were visualized by optical stereoscopy (**Panel A**), bright field, and fluorescence microscopy (**Panel B**). Feeding rate was quantified by the ingestion of microparticles based on their fluorescence in the incubation media (**Panel C**). Data represent average ± standard deviation (N = 5 replicates). # Statistically significant by Student's *t*-test denotes significant difference in comparison to the DMSO carrier solvent.

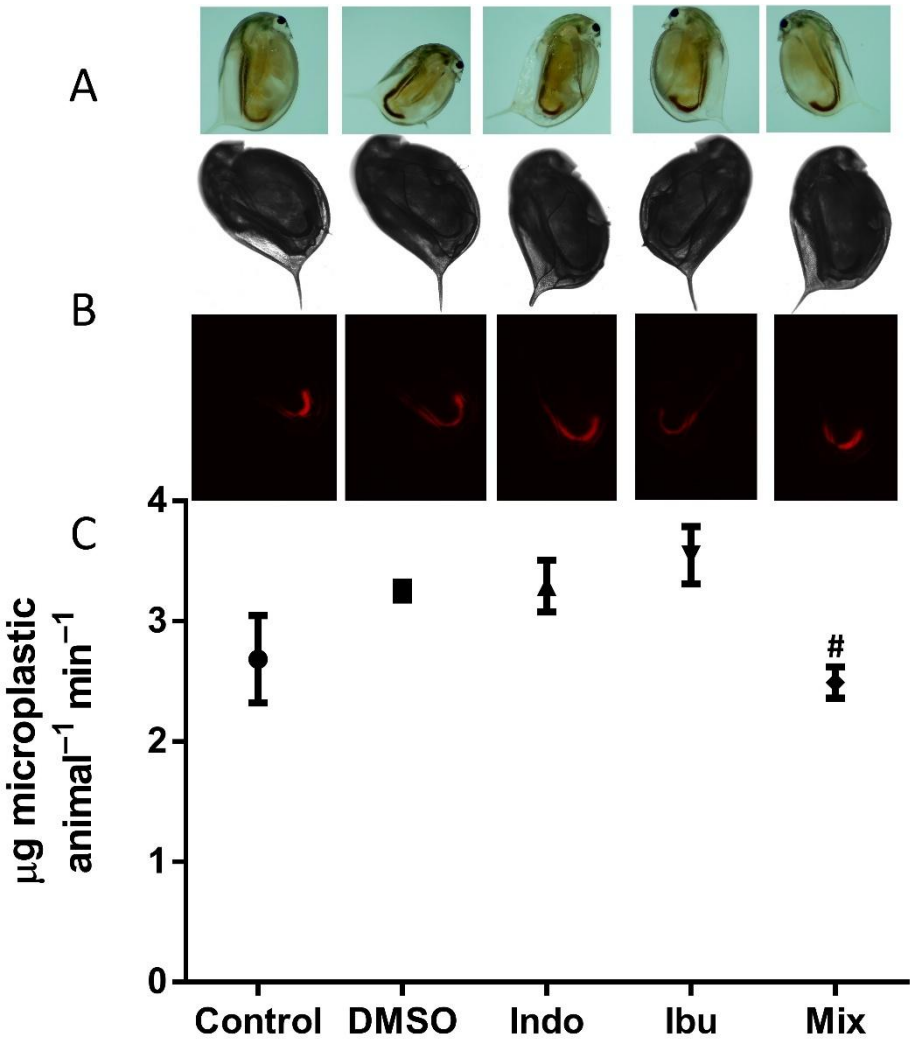

Figure 4. The impact of NSAIDs on feeding rate to D5 daphnids. The ingested microparticles were visualized by optical stereoscopy (**Panel A**), bright field, and fluorescence microscopy (**Panel B**). Feeding rate was quantified by the ingestion of microparticles based on their fluorescence in the incubation media (**Panel C**). Data represent average ± standard deviation (N = 5 replicates). # Statistically significant by Student's *t*-test denotes significant difference in comparison to the DMSO carrier solvent.

Enzymatic Activity following Chronic and Transgenerational Exposure of Daphnids to NSAIDs

Chronic exposure to 1 mg/l of indomethacin, ibuprofen, and their mixture resulted in significant changes in the physiology of daphnids during their growth (Table 2). Following the first 7 days of exposure, indomethacin significantly reduced the activities of ALP, βGAL, and LIP, while ibuprofen and their mixture decreased the activities of only the latter two. After 14 days of exposure, the only enzyme which showed a change in activity was βGAL by 28% in the NSAID mixture. Exposure for one more week resulted in a decrease in the activity of ACP for indomethacin and the NSAID mixture. In contrast, indomethacin increased the activities of βGAL and PEP, whereas ibuprofen and their mixture increased the activity of GST. The first generation of exposures was continued for additional 21 days in daphnids for the second generation. Indomethacin inhibited the activities of LIP, PEP, and GST, whilst ibuprofen inhibited the activities of ALP, βGAL, LIP, and PEP. Ibuprofen also increased the activity of GST. Apart from GST, the NSAID mixture had an impact on all enzymes. Except for ACP, the activities of ALP, βGAL, LIP, and PEP decreased. Finally, daphnids following their second generation of exposure were transferred for 21 days in OECD media as a third generation of a 21-day recovery. PEP and GST were the only enzymes with increased activity during the recovery period. Indomethacin and ibuprofen increased the activity of GST by 19% and 6%, respectively, and their mixture increased the activity of PEP by 16%, thus showing that the recovery period in clean media allowed the animals to return to a control condition for the majority of the enzymes. The general conclusion is that initial exposure had an impact on markers of physiology, while during the period the animals prepare for fertility (14 days), this was alleviated and followed more changes in the first generation over 21 days, which were even stronger in the second generation. Finally, for the recovery, it was observed a return to the control condition.

Table 2. Biochemical markers of daphnid physiology following chronic and transgenerational exposure to NSAIDs. Data represent mean ± standard deviation (N = 4 replicates) of enzyme activity. Enzyme activity was expressed as units/mg protein for phosphatases, βGAL, LIP, and PEP, and as munits/mg protein for GST. Symbols (#) and (*) indicate statistically significant differences by Student's *t*-test compared with DMSO and control, respectively.

Age (Days)		Control	DMSO	Indomethacin	Ibuprofen	Mixture
7	ALP	5.45 ± 0.34	7.16 ± 0.4 *	6.22 ± 0.21 #* (−13%)	6.55 ± 0.26 *	7.25 ± 0.34 *
	ACP	5.61 ± 0.44	7.74 ± 1.15	6.82 ± 0.51 *	8.14 ± 0.33 *	9.07 ± 0.72 *
	βGAL	1.66 ± 0.18	1.51 ± 0.04	0.91 ± 0.14 #* (−40%)	1.26 ± 0.06 #* (−17%)	1.41 ± 0.03 # (−7%)
	LIP	131.01 ± 19.67	137.72 ± 2.71	72.94 ± 11.09 #* (−47%)	123.76 ± 2.26 # (−10%)	93.14 ± 6.58 # (−32%)
	PEP	24.57 ± 1.04	23.62 ± 2.53	27.26 ± 5.01	29.54 ± 7.34	21.73 ± 4.54
	GST	91.2 ± 3	97.6 ± 12.3	107.9 ± 11.6	95.6 ± 10	76.7 ± 12.7
14	ALP	2.61 ± 0.02	3.24 ± 0.35	3.05 ± 0.29	2.63 ± 0.14	3.13 ± 0.16 *
	ACP	4.57 ± 0.28	5.35 ± 0.91	6.02 ± 0.71	4.55 ± 0.2	4.39 ± 0.19
	βGAL	2.08 ± 0.08	2.46 ± 0.19	2.23 ± 0.39	2.08 ± 0.75	1.77 ± 0.16 # (−28%)
	LIP	159.59 ± 16.43	141.47 ± 8.09	193.84 ± 25.81	148.12 ± 37.62	140.13 ± 10.69
	PEP	1.99 ± 0.1	2.32 ± 0.29	1.94 ± 0.06	1.93 ± 0.27	1.9 ± 0.12
	GST	132.3 ± 2.7	162.1 ± 12.4 *	144.7 ± 5.4 *	151.7 ± 11.7	151.3 ± 19.2
21	ALP	5.32 ± 0.37	4.86 ± 0.2	5.05 ± 0.17	4.89 ± 0.37	4.69 ± 0.18 *
	ACP	2.96 ± 0.07	3.17 ± 0.09 *	2.84 ± 0.13# (−10%)	3.09 ± 0.04	2.87 ± 0.11# (−9%)
	βGAL	6.04 ± 0.31	5.36 ± 0.24 *	5.93 ± 0.19# (+11%)	5.13 ± 0.25 *	5.05 ± 0.29 *
	LIP	95.93 ± 5.85	81.04 ± 7.29 *	90.86 ± 0.42	78.43 ± 4.27 *	77.29 ± 7.29 *
	PEP	11.41 ± 0.6	9.35 ± 0.43 *	10.82 ± 0.6 # (+16%)	10.52 ± 1.39	9.75 ± 0.59 *
	GST	44.2 ± 3.4	54.2 ± 3.7 *	52.3 ± 1.5 *	67.6 ± 4.4 #* (+25%)	67.7 ± 5.8 #* (+25%)
21 2nd generation	ALP	3.3 ± 0.26	3.64 ± 0.19	3.8 ± 0.18 *	3.27 ± 0.15 # (−10%)	3.09 ± 0.16 # (−15%)
	ACP	3.09 ± 0.09	2.75 ± 0.16 *	2.67 ± 0.15 *	2.78 ± 0.21	3.29 ± 0.19 # (+20%)
	βGAL	4.48 ± 0.24	4.76 ± 0.2	4.7 ± 0.23	3.93 ± 0.22 #* (−17%)	3.8 ± 0.09 #* (−20%)
	LIP	70.46 ± 3.87	66.3 ± 5.65	56.41 ± 3.09 #* (−15%)	47.53 ± 1.66 #* (−28%)	56.53 ± 4.31 #* (−15%)
	PEP	7.43 ± 0.6	7.89 ± 0.56	6.81 ± 0.26 # (−14%)	7.15 ± 0.13	6.44 ± 0.33 # (−18%)

	GST	98.4 ± 4.2	104.3 ± 0.6	94.6 ± 2.9 # (−9%)	123.5 ± 2.1 #* (+18%)	111 ± 5.8 *
21 3rd generation recovery	ALP	4.83 ± 0.36	4.2 ± 0.11 *	4.16 ± 0.24 *	4.55 ± 0.44	4.32 ± 0.28
	ACP	3.42 ± 0.28	2.83 ± 0.08 *	3.21 ± 0.28	3.24 ± 0.47	3.06 ± 0.14
	βGAL	6 ± 0.54	4.75 ± 0.17 *	5.03 ± 0.44 *	4.54 ± 0.24 *	4.97 ± 0.36 *
	LIP	55.14 ± 1.79	53.39 ± 2.08	51.19 ± 2.04 *	53.32 ± 6.51	53.56 ± 3.73
	PEP	6.21 ± 0.45	4.47 ± 0.27 *	5.33 ± 0.49	4.61 ± 0.35 *	5.18 ± 0.41 #* (+16%)
	GST	124.4 ± 11.8	130.3 ± 3.5	154.7 ± 4.2 #* (+19%)	138.3 ± 3.5 # (+6%)	141.8 ± 9.3

Discussion

The acute and chronic effects of indomethacin, ibuprofen, and their mixture were assessed using a combination of physiological indicators, such as mortality and feeding, as well as biochemical markers. As biochemical markers, we assessed the activity of phosphatases, βGAL, LIP, PEP, and GST. NSAIDs, such as ibuprofen, diclofenac, and naproxen, can form reactive oxygen species (ROS). The disruption of the balance between ROS and the antioxidant systems in the organism is referred to as oxidative stress. When oxidative stress occurs, it causes lipid and protein peroxidation, damage to DNA structure, as well as inhibition of digestive enzymes (trypsin, βGAL) (Gómez-Oliván et al., 2014, Lv et al., 2017). According to these reports, ibuprofen increases lipid peroxidation, protein carbonyl content (protein oxidation), and enzymes of antioxidant defense SOD and CAT. As a result, lipid peroxidation affects differently different types of enzymes, such as phosphatases and LIP. Additionally, the oxidation of proteins affects the activity of proteins such as PEP, βGAL, LIP, and phosphatases (Stadtman and Levine, 2000). GST belongs to a group of enzymes involved in the detoxification processes (Dasari et al., 2017). An increase in this enzyme might indicate that the daphnids use these detoxification processes in order to adapt and survive the stress that the chemicals cause (Galhano et al., 2022). Most NSAIDs are toxic to organisms due to their bioaccumulation in the ecosystems. There are a few studies that have been published about the impact of NSAIDs, such as indomethacin and ibuprofen, on *D. magna*, but the majority of them rely on mortality, growth, and reproduction rate to determine toxicity (Han et al., 2010, Hayashi et al., 2008, Heckmann et al., 2007, Heckmann et al., 2008, Kwak et al., 2018). Extending to other aquatic species, it has been noted that indomethacin and ibuprofen can harm other marine and freshwater organisms.

Indomethacin has been shown to significantly increase the biomass of *Chironomous riparius*, while it had no effect on the survival rate or biomass of *Physella acuta* (López-Doval et al., 2012). After 24 h of exposure, lethal concentrations for *Thamnocephalus platyurus*, *Oryzias latipes*, and *D. magna* were 16.14 mg/l, 81.92 mg/l, and 22.38 mg/l, respectively (Gheorghe et al., 2016, Kim et al., 2009). There are not sufficient data on indomethacin toxicity in daphnids, although the findings of the two aforementioned studies (Gheorghe et al., 2016, Kim et al., 2009) are very close to our lethal concentration. The toxicity of indomethacin in other species, such as male zebrafish (*Danio rerio*) for 96 h, was in a similar order of magnitude with an EC_{50} of 76.30 mg/l. However, indomethacin has been shown to cause significant changes in the transcriptome of zebrafish in marker genes such as superoxide dismutase 1, glutathione peroxidase 1, interleukin-1, tumor necrosis factor-alpha, and others (Ryu and Kim, 2018).

Ibuprofen has been described in the literature as a substance with high mobility in aquatic ecosystems. Despite the fact that up to 90% of ibuprofen can be removed from the environment efficiently, it is present in high concentrations in raw sewage (Grzesiuk et al., 2020, Heckmann et al., 2007). It has been reported that chronic exposure of neonates *D. magna* to concentrations of 20, 40, and 80 mg/l for 14 days affected somatic growth, reproduction, and survival (Heckmann et al., 2007). Somatic growth, in particular, increased with increasing concentrations. There was a significant delay in fecundity and a total decrease in reproduction in daphnids exposed to 40 mg/l and 80 mg/l, respectively. Finally, survival of daphnids exposed to 20 mg/l and 40 mg/l was unaffected, yet it was reduced by 19% in daphnids exposed to 80 mg/l (Heckmann et al., 2007). These findings are consistent with previous reports, which tested the same concentrations of ibuprofen to *D. magna* for a 10-day period (Hayashi et al., 2008). Ibuprofen had a similar dose-dependent effect on reproduction and survival (Hayashi et al., 2008). Exposure of adult daphnids (14 days old) for 8 days to 20, 40, and 80 mg/l ibuprofen showed a decrease in fecundity at concentrations greater than 20 mg/l. However, the brood release was not postponed. The broods produced after exposure to ibuprofen at 80 mg/l consisted of almost dead and/or undeveloped embryos (Heckmann et al., 2008). Grzesiuk exposed *D. magna* to ibuprofen over six generations and found that exposed daphnids had higher growth rates than unexposed daphnids in the 6th generation, which agrees with the findings of Heckmann and Hayashi. In addition, 20%, 70%, and 20% of the animals in the first generation of exposed daphnids produced abnormal offspring. These percentages increased significantly to 80%, 90%, and 50% of individuals in the fifth generation, respectively. They also discovered undeveloped embryos, neonates missing an eye, and daphnids with malformed antennules and carapace. All these

abnormalities are considered lethal, reinforcing the claim that even low concentrations of ibuprofen in the environment might have significant impacts on *D. magna* (Grzesiuk et al., 2020). The toxicity of ibuprofen has been studied in other crustaceans, including *T. platyurus*. *T. platyurus* was exposed for 24 h to a range of concentrations ranging from 0.1 to 66.7 mg/l. The lethal concentration after 24 h of exposure was calculated to be 19.59 mg/l. This EC closely matches our findings, while the EC for ibuprofen, according to our study, is 17.80 mg/l (Kim et al., 2009). It has also been reported in the literature that chronic exposure to ibuprofen at concentrations of 0.0001, 0.05, 1, 8, and 25 mg/l affects some phenotypic markers of zebrafish (Danio rerio), such as spontaneous movement, free swimming distance, duration and speed under dark condition, as well as enzymatic markers, such as the activity of GST (Bartoskova et al., 2013, Xia et al., 2017). Specifically, 28-day exposure of zebrafish to 25 mg/l ibuprofen caused an increase in the activity of GST (Bartoskova et al., 2013). Furthermore, ibuprofen caused toxic effects on the mussel *Mytilus galloprovincialis* (Gonzalez-Rey and Bebianno, 2011, Gonzalez-Rey and Bebianno, 2012), the crayfish *Procambarus clarkii* (Trombini et al., 2021), the marine clam *Ruditapes philippinarum* (Trombini et al., 2019), the clam *Corbicula fluminea* (Aguirre-Martínez et al., 2015) and to the mussel *Dreissena polymorpha* (Contardo-Jara et al., 2011) .

Given the scarcity of research on the effects of indomethacin and ibuprofen on freshwater organisms, particularly *D. magna*, our study aimed to highlight their significance while also introducing additional markers of physiology other than mortality, growth, and reproduction rate as common endpoints. We recently demonstrated that markers related to physiology and enzymatic activity, in addition to metabolic perturbations, are useful endpoints for pollution assessment (Michalaki et al., 2022, O'Rourke et al., 2023). It is worth noting that this is the first reference of indomethacin, ibuprofen, and daphnids using additional molecular markers rather than using only toxicity results.

According to our findings, both indomethacin and ibuprofen, as well as their mixture, were toxic to daphnids. Even though 1 mg/l is not an environmentally relevant concentration (ng/l to μg/l), there are many reports that tested concentrations of NSAIDs significantly higher than 1 mg/l (Bang et al., 2015, Gómez-Oliván et al., 2014, Han et al., 2010, Hayashi et al., 2008, Heckmann et al., 2007, Heckmann et al., 2008, Kwak et al., 2018, O'Rourke et al., 2023). In our setup, the concentration of NSAIDs was not measured during chronic exposures. Monitoring the concentration could provide valuable insight regarding their stability in water. Nevertheless, there are reports indicating that indomethacin and ibuprofen can be stable for 12 and 15 days at room temperature, respectively (Moudry et al., 2013, Volonté et al., 2005, Walker et al., 1998, Walker et al., 2011). A novel approach to assessing

the feeding rate was used as an alternative approach when compared to other feeding assays (Barata et al., 2008, Hite et al., 2020). To date, the feeding assays that are being used rely on algae counts, although these approaches employ large volumes of media or longer feeding periods (4 to 24 h) (Barata et al., 2008, Hite et al., 2020, Pablos et al., 2018, Pan et al., 2017). Microparticles, on the other hand, are uniformly defined by the manufacturer, and thus they offer flexibility in fluorescence, whereas algae are only fluorescent due to chlorophyll. Moreover, because microparticles differ from chlorophyll or other potentially fluorescent compounds, they can be easily identified and visualized inside the animals using microscopy. Therefore, this approach allows the measurement of the microparticles in the media missing and in the animal present. Another benefit of this test is that it is immediate and fast, allowing the process of a large number of samples in a high throughput manner. For these reasons, as well as the high reproducibility, our previously published method (Grintzalis et al., 2017) has been optimized to a microparticle approach. In terms of the toxicity of microparticles, while particles, in general, are toxic to daphnids, this is accurate for long periods of exposure and at higher concentrations (Fadare et al., 2019). The toxicity of the microplastics used in our study was assessed using a toxicity curve (part of another research paper), which revealed that these particles are toxic only at extremely high concentrations and over a longer exposure period. Polystyrene microplastics were toxic to *D. magna* at concentrations higher than 30 mg/l for neonates and 100 mg/l for adults, as well as during longer exposure periods (>96 h) (Eltemsah and Bøhn, 2019).

Indomethacin, ibuprofen, and their mixture appear to affect feeding rates differently in neonates and D5 daphnids. When compared to DMSO, the two NSAIDs and their mixture reduced the feeding rate in neonates by 35%, 30%, and 49%, respectively. Diclofenac is also known to reduce the feeding rate in neonates of *D. magna* (Alkimin et al., 2020, Nkoom et al., 2019). The assessment of the feeding rate in D5 daphnids, on the other hand, revealed that only the mixture affected, in particular, decreased the feeding rate by 23%. Exposure of D5 daphnids to ketoprofen did not affect the feeding (Alkimin et al., 2020). In chronic exposures of D7 daphnids, indomethacin, ibuprofen, and their combination reduced enzyme activities, although activities of all enzymes except βGAL were not affected after 14 days of exposure. One plausible explanation is that D14 daphnids are older and thus more resilient to indomethacin and ibuprofen. Another possibility is that D14 daphnids are entering their reproductive stage and preparing to release their first large brood (Ebert, 2005). After 21 days of exposure, indomethacin, ibuprofen, and their mixture impacted a number of key enzymes. It has been reported that ibuprofen caused an upregulation in the activity of triacylglycerol lipase (Heckmann et al., 2008). On the contrary, no increase in the activity

of LIP was observed in our study. Other NSAIDs, such as diclofenac or acetylsalicylic acid, did not affect the daphnids in the same way. For example, chronic exposure of daphnids to diclofenac reduced the activity of ALP and ACP, while increasing the activity of PEP (O'Rourke et al., 2023). Acute exposure of daphnids to acetylsalicylic acid only decreased the activity of PEP (Michalaki et al., 2022). In our study, we observed a reduction of ALP caused by indomethacin on D7 daphnids and by ibuprofen and their mixture on D21 daphnids of the second generation. The activity of ACP was decreased by indomethacin and their mixture on D21 daphnids. The activity of PEP was increased by indomethacin on D21 daphnids and decreased by indomethacin and their mixture on D21 daphnids of the second generation. Finally, the activity of PEP increased by their mixture on D21 daphnids of recovery generation. Ibuprofen and their mixture increased the activity of GST by 25% on D21 daphnids. These results are in line with the previous publication (Wang et al., 2016). However, it has been observed that exposure of *M. galloprovincialis* to ibuprofen for 14 days reduced the activity of GST (Almeida et al., 2020). Additionally, another NSAID, ketoprofen, increased the activity of GST in *D. magna* when exposed to intermediate concentrations (1.2 and 6 μg/l) (Alkimin et al., 2020). Daphnids may have used this detoxification enzyme as a way to survive the stress that the chemicals caused (Galhano et al., 2022). However, a more significant effect was observed in the second generation of D21 daphnids. This effect was mostly reversed by the third recovery generation in clean media, with the exception of GST activity. In a number of transgenerational studies in daphnids, stress has been shown to be more prominent with the increase of the generation applied, and this transgenerational inheritance has been shown to be environmentally induced by epigenetic marks (Feiner et al., 2022).

Conclusions

In conclusion, the acute, chronic, and transgenerational effects of indomethacin, ibuprofen, and their mixture were investigated on *D. magna* using a combination of physiological indicators and biochemical markers. The results revealed that these NSAIDs and their combined mixture impacted the feeding rate, as well as the activity of enzymes associated with digestion and detoxification processes in two generations of *D. magna*. Despite the fact that the chosen concentration of 1 mg/l was higher than environmentally relevant levels, the 14-day exposure of daphnids to these NSAIDs and their mixture did not impact the activity of several enzymes of metabolism in these animals. A plausible explanation for this outcome is that D14 daphnids are transitioning into their reproductive phase and preparing to release their first large brood (Ebert, 2005). As a result, the effect of these chemicals cannot be

detected on D14 daphnids even though the pollutants are still present. Furthermore, the third generation of daphnids was able to recover from the stress induced by these pharmaceuticals and their mixture in the previous generations. Therefore, even if the previous generations were exposed to the pollutants, the impact is not reflected in the recovery generation, despite the fact that the concentration used was greater than environmentally relevant. Consequently, our findings indicate that some pollutants may become undetectable even by bioindicator species once they enter the aquatic environment. Moreover, our research aimed to highlight the importance of species with plasticity and responsive mechanisms, with the aim to develop new metrics for pollution assessment using daphnids as an equivalent to the canary in the coal mine (Abdullahi et al., 2022). This study is a preliminary pilot work for a coming work on environmentally relevant levels but in the depth of exposures to more generations, thereby showing that we need to emphasize long-term exposures in this species.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, due to the fact that daphnids are regarded as "animals" in terms of being members of the kingdom Animalia, however, they are not "animals" as defined in regulation SI543 of 2012 on the protection of animals used for scientific purposes. Therefore, the study does not require authorization from the Health Products Regulatory Authority (HPRA), while is also in line with the aim of working under the 3Rs (reduce, refine, replacement) strategy, since daphnids are commonly used in ecology and ecotoxicology as replacements of more evolutionary advanced species (i.e. fishes), posing no ethical implications.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 4B

This chapter continued the investigation of the previous study by comparing chemical and commercial forms of the NSAIDs indomethacin, ibuprofen and their mixtures, over transgenerational exposures for five generations. Phenotypic and biochemical endpoints, as well as metabolomic analysis were used, and the findings revealed distinct patters of impact. The results emphasized the significance of not relying only on one or two endpoints but assessing several markers and environmentally relevant doses to detect subtle but significant impacts. Relying on the previous chapter, this work pointed out the crucial role of transgenerational studies in understanding how pharmaceutical pollutants affect aquatic organisms over time, as well as the value of integrating multiple endpoints for a more accurate ecotoxicological assessment.

Chemical and commercial forms of NSAIDs exert metabolic responses at environmentally relevant concentrations in transgenerational exposures in daphnids

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Abstract

Pharmaceuticals, including non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and ibuprofen, are prevalent pollutants in freshwater ecosystems, raising concerns about their ecological impact. While there is literature about pharmaceuticals in aquatic environments, the effects of both chemical and commercial forms of NSAIDs on aquatic organisms, particularly across generations remain insufficiently explored. This study aims to bridge this gap by assessing the impact of pure and commercial forms of indomethacin, ibuprofen and their mixture on *Daphnia magna,* a key freshwater species. Toxicity curves, enzyme activities and metabolomics were used as endpoints to assess physiological responses in chronic and transgenerational exposures at environmentally relevant concentration of 5 μg/l. Results showed changes in the activities of βGAL and LIP, while a targeted LC-MS/MS approach revealed distinct metabolic fingerprints as a result of exposure for four generations to the chemical and commercial indomethacin and ibuprofen. Notably, exposure to chemical mixture of the two NSAIDs increased amino acids and biogenic amines. A trend also observed with the commercial NSAIDs and their mixture, while the chemical NSAIDs did not have the same impact. This work emphasizes on the necessity of ecotoxicological studies with transgenerational exposures as an approach to comprehend the effect of pharmaceutical stressors at low exposure concentrations, using molecular responses in physiology. This approach contributes to the broader mechanistic understanding of the ecological implications of these pharmaceuticals in freshwater ecosystems.

Keywords

NSAIDs; indomethacin; ibuprofen; metabolomics; pharmaceuticals; toxicity; transgenerational; enzyme activities; commercial drugs; survival

Introduction

The increased use of pharmaceuticals over the last three decades has raised significant concerns with around 3,000 chemicals used annually, with annual production amounts exceeding hundreds of tons (Kümmerer, 2008, Ortúzar et al., 2022). Pharmaceuticals enter the environment through illicit disposal of expired drugs, industrial wastewater discharge, hospital effluent, and domestic wastewater treatment plants (WWTPs), as well as agricultural runoff and leaching from domestic septic tanks (Michalaki and Grintzalis, 2023, O'Flynn et al., 2021). Once released into the environment, these compounds can be found in surface waters, groundwater, WWTP effluents and influents, and sludge (Patel et al., 2019). Non-steroidal anti-inflammatory drugs (NSAIDs) known for their anti-inflammatory, antipyretic and analgesic properties, have been classified as a major group of emerging contaminants due to their extensive use which does not require any prescription (Brenner and Stevens, 2012, Lin et al., 2023). Emerging contaminants, as defined by the United States Environmental Protection Agency are "chemical substances or biological agents that are potentially hazardous or recently determined to be hazardous to humans and ecosystems and are often undetected due to their low concentrations and limited distribution (Antiñolo Bermúdez et al., 2023, Moreno Ríos et al., 2022). NSAIDs, such as indomethacin, ibuprofen and diclofenac are not chemically stable and can be broken down due to microbial action, however, they are emerging contaminants because of their pseudo-persistence in the environment due to their continuous discharge or their incomplete removal (Mussa et al., 2022, Takara et al., 2024). These pharmaceuticals generate reactive oxygen species (ROS) leading to oxidative stress, lipid peroxidation, protein carbonylation, and disrupted activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Gomez-Olivan et al., 2014, Lv et al., 2017, Stadtman and Levine, 2000). Despite minimal concentrations, their long-term ecological impacts highlight the need for advanced detection and removal techniques (Tyumina et al., 2020). Limited research on the effects of NSAIDs on aquatic species such as *Daphnia magna* underscores the importance to look beyond traditional endpoints to fully assess their toxicity in aquatic habitats (Han et al., 2010).

Indomethacin is one of the most efficient non-selective NSAID currently available for the treatment of pain and inflammation, including migraines and headaches. Notably, its availability for purchase over the counter contributes to its extensive use (Liu et al., 2022, Michalaki and Grintzalis, 2023). Consequently, substantial amounts of indomethacin are discharged into aquatic ecosystems, and its incomplete removal during WWT processes makes it one of the most persistent contaminants (Cruz-Carrillo et al., 2022, Liu et al., 2022).

It has been reported that around 0.005-0.792 µg/l indomethacin have been detected in surface water in the UK and Ireland (Liu et al., 2022).

Ibuprofen is ranked as the third most commonly prescribed NSAID globally and is widely available over the counter, extensively used to treat fever, muscle pain, and inflammation. Ibuprofen has been encountered in a variety of freshwater ecosystems and WWTPs due to its high consumption rate, coupled with the fact that a significant amount (50-80%) of the administered dose (600-1200 mg/d) remains unmetabolized (Chopra and Kumar, 2020, Das et al., 2022, Oba et al., 2021, Pounds et al., 2008, Rainsford, 2009). Consequently, in the excretion products, both ibuprofen and its metabolites are present, with the latter more toxic than the parent compound (Chopra and Kumar, 2020, Ikehata et al., 2006, Quero-Pastor et al., 2014).

Conventional water quality monitoring methods often fail to detect low levels of contamination that could exert adverse effects on aquatic organisms, making the evaluation of pharmaceuticals challenging (Escher et al., 2021). Effect-based methods using bioindicators such as *Daphnia magna* provide valuable insights by assessing physiological, behavioural, and reproductive responses to pollutants (Brack et al., 2019, Escher et al., 2021). Widely distributed and ecologically significant, *D. magna* is highly sensitive to pollutants, adaptable, and easily cultured, offering mechanistic insights into the effects of novel pollutants (Abdullahi et al., 2022, Ebert, 2005). Furthermore, transgenerational studies with daphnids offer deeper understanding of the long-term ecological impacts (Baker et al., 2014, Tsui and Wang, 2004, Vandegehuchte et al., 2010). Transgenerational studies reveal that even low concentrations of pollutants can induce morphological abnormalities and developmental defects in future generations of organisms, such as *D. magna* and zebrafish (Baker et al., 2014, Rodrigues et al., 2020). These effects are potentially driven by mechanisms such as the maternal transfer of contaminants to offspring and epigenetic alteration, such as DNA methylation (Tsui and Wang, 2004, Vandegehuchte et al., 2010). These findings highlight the importance of transgenerational approaches in uncovering the complex, lasting interactions between pollutants and aquatic organisms.

This study focused on the assessment of the impact of the chemical and commercial forms of indomethacin, ibuprofen, and their 1:1 mixture on daphnids. Transgenerational exposures were performed at a non-lethal environmentally relevant concentration of 5 µg/l, and the effects were assessed using phenotypic (toxicity), biochemical (enzyme activities) and metabolomic markers of the physiology of daphnids. It is crucial to study not only the active pharmaceutical ingredients but also their commercial forms, as these contain excipients and additives that can affect their behaviour and toxicity. Improperly disposed expired drugs, for

example, may leach into aquatic ecosystems, harming freshwater or marine organisms, such as molluscs (Politakis et al., 2018). These adverse effects underline the importance of rigorous study and control of both chemical and commercial compounds in order to safeguard the environment. It is worth noting that this is the first study to evaluate the effects of commercial indomethacin, ibuprofen, and their mixture on daphnids.

Materials and Methods

Reagents and chemicals

All chemicals included in this study were of the highest purity >99.9% and quality. Indomethacin (CAS 53-86-1), ibuprofen (CAS 51146-56-6), KCl (CAS 7447-40-7), Na_2SeO_3 (CAS 10102-18-8), bovine serum albumin (CAS 9048-46-8), Coomassie Brilliant Blue G (CAS 6104-58-1), *p*-nitrophenyl butyrate (CAS 2635-84-9), 2-nitrophenyl-B-D-galactopyranoside (CAS 369-07-3), 1-chloro-2,4-dinitrobenzene (CAS 97-00-7), L-glutathione reduced (CAS 70-18-8), sodium phosphate dibasic (CAS 7558-79-4), L-leu-4-nitroanilide (CAS 4178-93-2) were purchased from Sigma-Aldrich (St. Louis). $CaCl_2 \cdot 2H_2O$ (CAS 10035-04-8), $MgSO_4 \cdot 7H_2O$ (CAS 10034-99-8), $NaHCO_3$ (CAS 144-55-8), HCl (CAS 7647-01-0), *p*-nitrophenyl phosphate (CAS 4264-83-9), boric acid (CAS 10043-35-3), ammonium acetate (CAS 631-61-8), NaOH (CAS 1310-73-2), methanol (CAS 67-56-1), and DMSO (CAS 67-68-5) were purchased from ThermoFisher (Ireland). Commercial NSAIDs (indicated in the manuscript as pills; p) were Fortathrin 75 mg tablets for indomethacin, which was purchased from the pharmaceutical company GAP S.A. (Athens, Greece) and Brufen 600 mg tablets for ibuprofen, which was purchased from the pharmaceutical company Mylan N.V (Viatris, USA). The seaweed extract (*Ascophylum nodosum,* 500 g/l) was purchased from BioAtlantis (Ireland).

Culturing algae and daphnids and design of exposures

Daphnids were cultured under a 16:8 h of light:dark photoperiod at 20°C in OECD media and fed with algae (*Chlamydomonas reinhardtii*) (OECD, 2012). Chemical and commercial indomethacin and ibuprofen were dissolved in DMSO, which was kept minimum at a final concentration of 0.0025% v/v in the exposure media (Michalaki and Grintzalis, 2023). The commercial forms of indomethacin and ibuprofen were carefully prepared by removing the coating and using only the powdered content. This procedure ensured that the active pharmaceutical ingredients were directly comparable to the pure chemical forms. Neonates (<24 h) were collected from the third brood of their mothers and used for experiments. To assess toxicity, fifteen neonates were exposed to each NSAID individually, and their 1:1

mixture in a final volume of 50 ml OECD media, with four replicates per concentration tested. This approach was based on similar studies, where all assays were conducted with four replicates per condition tested (Heckmann et al., 2007, Heckmann et al., 2008, Pablos et al., 2015). For toxicity, while the OECD guidelines suggest that at least twenty animals (preferably in four groups of five animals should be used at each test concentration), in our study we increased this to sixty animals at each concentration tested, thus increasing our reproducibility even more (OECD, 2004). Toxicity curves were plotted following exposure for 24 hours, and EC values were calculated using the four-parameter logistic (4PL) model, following the equation Span = Top − Bottom and Y = Bottom + (Top − Bottom)/(1 + 10^((LogIC50 − X) × Hill Slope)), using the GraphPad software (version 12). The parameters top and bottom were commonly fixed to 100 and 0, accordingly. Mortality in daphnids was assessed as their immobilization (Gomez-Olivan et al., 2014).

Chronic exposures were performed at a non-lethal and environmentally relevant concentration of 5 µg/l for each NSAID in the chemical and its commercial form as well as their 1:1 mixture. Stock solutions for both forms of indomethacin and ibuprofen were prepared at 40 mg/ml, and serial dilutions were made to obtain 400 mg/l working solutions. To prepare the exposures of individual NSAIDs, a 200 mg/l solution was prepared from the 400 mg/l and diluted to the final concentration of the 5 µg/l. For the 1:1 mixture, equal volumes of the 400 mg/l stock solutions of indomethacin and ibuprofen were mixed to create a 200 mg/l solution, which was further diluted to the 5 µg/l. Based on our previous study for indomethacin and ibuprofen and the existing literature, both pharmaceuticals can be found in the environment at concentrations ranging from ng/l to even µg/l (Adamczuk, 2022, Huo et al., 2007, Michalaki and Grintzalis, 2023). Therefore, to achieve a more realistic scenario using an environmentally relevant dose, the concentration of 5 µg/l was selected for individual pharmaceuticals and their 1:1 mixture. Since the aim of the study is to show the impact on daphnids following exposure to the chemical and commercial NSAIDs, and based on the literature, a single exposure concentration was selected for the biochemical assays and metabolomics (Belavgeni and Dailianis, 2017, Gómez-Oliván et al., 2014, Grzesiuk et al., 2024, O'Rourke et al., 2023, Politakis et al., 2018, Ryu and Kim, 2018). For enzyme activities in transgenerational exposures, neonates were cultured for four consecutive generations, followed by a 14-day recovery period (Figure 1). Thirty-six neonates were exposed to the chemical and commercial NSAIDs (at 5 µg/l) in 900 ml OECD media. Media and pharmaceuticals were renewed three times per week to ensure that the concentrations of the NSAIDs were stable. In addition to the DMSO control which was used as a carrier solvent an unexposed control with only OECD media (without DMSO) was also included

(supplementary data). Daphnids were fed daily with fresh algae (*C. reinhardtii*, at 16.5 million cells per 900 ml of exposure), and a seaweed extract (*Ascophylum nodosum,* 1.5 ml/l of 5 g/l stock, thus 7.5 mg/l) which was added only on renewal of media and pharmaceutical.

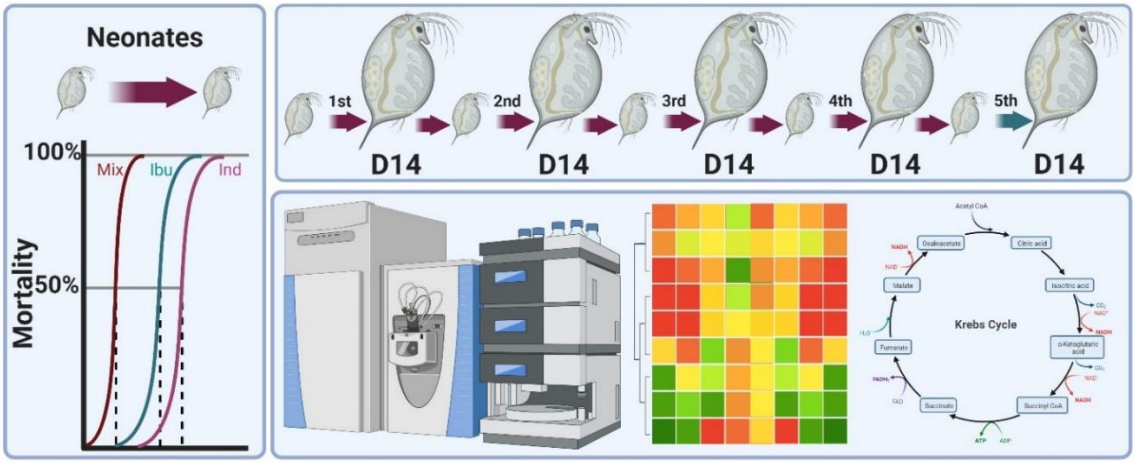

Figure 1. Experimental design. Acute (24 hours) exposures were performed in neonates to assess mortality. For transgenerational exposures for four generations (purple arrows) followed by a recovery generation (blue arrow) at environmentally relevant concentrations (5 μg/l) were selected. Enzyme activities and hyphenated techniques were used to capture metabolic perturbations. Figure created with Biorender https://www.biorender.com/ .

Biochemical markers of physiology

Ten 14-day old daphnids were pooled per replicate and homogenized in 1 ml enzyme assay buffer using a pestle homogenizer (product number BAF199230000, purchased from Sigma-Aldrich, St. Louis). The homogenate was cleared by centrifugation (12,000 × *g* for 10 min at 5°C), and the clear supernatant was collected and used to evaluate the enzyme activity as previously described (Grintzalis et al., 2022, Michalaki et al., 2023). For the activity of phosphatases, 200 μl appropriately diluted sample in buffer (100 mM acetic acid pH 4.5 for acid phosphatase; ACP or 100 mM boric acid pH 9.8 for alkaline phosphatase; ALP) was mixed with 50 μl of the substrate *p*-nitrophenyl phosphate (8 mM in buffer) and the production of *p*-nitrophenol was measured at 405 nm after alkalinization (with 50 μl 4M NaOH) (Grintzalis et al., 2022). Additionally, the activities of β-galactosidase (βGAL) and lipase (LIP) were quantified with the same experimental conditions by the generation of nitrophenol from the catalysis of *o*-nitrophenyl-β-galactoside or *p*-nitrophenyl butyrate, respectively, but in 100 mM phosphate buffer pH 7.2. The activity of peptidase (PEP) was quantified by the hydrolysis of L-leu-4-nitroanilide and the production of 4-nitroaniline (at 412 nm every five minutes for thirty minutes) in 100 mM phosphate buffer pH 7.2. The activity of glutathione-S-transferase (GST) was assessed by the reaction of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). Specifically, 200 μl

appropriately diluted sample in phosphate buffer pH 7.2 was mixed with 50 µl 2 mM CDNB, and 6 mM reduced glutathione, and the formation of the GSH-CDNB complex was measured continuously at 340 nm and converted to units of activity with the extinction coefficient (Tang et al., 1996, Warholm et al., 1981). Protein was quantified using an ultrasensitive Bradford protocol to normalize the results (Grintzalis et al., 2015).

Metabolomic analysis

For metabolomics analysis, five animals per replicate were immediately snap frozen in liquid nitrogen from the fourth generation. Samples were stored at -80°C until extraction in 100 µl of solvent (Ethanol:PBS, 85:15) and homogenised and cleared with centrifugation at 24,000 *g* for 5 min at 4°C following the manual and protocol of the manufacturer. Specifically, the supernatant was collected and the AbsoluteIDQ® p180 assay (Biocrates Life Sciences, Innsbruck, Austria) was applied to acquire the metabolite data according to the manufacturers' manual. Specifically, the supernatant (10 µl) was added to the 96-well plate and dried under a stream of nitrogen and phenyl isothiocyanate (50 µl, 5%) was added and incubated for 25 min at room temperature. The plate was then dried under nitrogen for 60 min and the extraction solvent (5 mM ammonium acetate in methanol, 300 µl) was added and placed on a shaker for 30 min. The plate was then centrifuged at 500 *g* for 2 min, and 150 µl of eluate was diluted with 150 µl of HPLC grade water for liquid chromatography-tandem mass spectrometry (LC-MS/MS) run. A total of 50 µl of eluate was diluted with 450 µl mobile phase for the flow injection analysis-tandem mass spectrometry (FIA-MS/MS) run. The data was acquired on a SCIEX QTRAP 6500plus mass spectrometer coupled to SCIEX ExionLC™ Series UHPLC. A UHPLC column provided with AbsoluteIDQ® p180 assay was installed for the LC-MS/MS analysis, and the mobile phase A and B were water and acetonitrile (both added 0.2% formic acid), respectively. Metabolites were identified using standards in line with recommended standards for metabolomics the Biocrates procedures. Amino acids and biogenic amines were identified and quantified in positive mode. For the FIA-MS/MS analysis, methanol was employed as the running solvent, and 40 acylcarnitines, 14 lysophosphatidylcholines (lysoPC), 38 acyl/acyl phosphatidylcholines (PC aa), 38 acyl/alkyl phosphatidylcholines (PC ae), 15 sphingomyelins (SMs), and the sum of hexoses (H1) were identified and semi-quantified in positive mode. All metabolites were quantified by multiple reaction monitoring (MRM) method. Amino acids and biogenic amines were quantified based on isotopically labelled internal standards and 7-point calibration curves using AB Sciex Analyst® version 1.7.2 software. Other metabolites, such as acylcarnitines, lysoPCs, PCs, SMs and hexose were semi-quantified by using 14 internal

standards in the MetIDQ™ software (Biocrates Life Sciences). Data quality was evaluated by checking the accuracy and reproducibility of QC samples included in the p180 assay. The concentrations of metabolites were reported in µM. For further statistical analyses, metabolites were included only when the concentrations of metabolites were above the limit of detection (LOD) in more than 50% of samples.

Survival assay

The effects of the NSAIDs and their mixture on the survival of daphnids were assessed with survival curves. For this assay, three replicates of ten neonates in each, were exposed to these NSAIDs and their mixture (1 mg/l, 10 mg/l, 20 mg/l, 50 mg/l and 80 mg/l), in 200 ml media for 28 days, during which the number of living daphnids was scored daily. Media and NSAIDs were renewed twice a week and daphnids were fed daily with fresh algae (*C. reinhardtii*, at 3.6 million cells per 200 ml of exposure) and the seaweed extract (*Ascophylum nodosum*, 7.5 mg/l) only on media changes.

Statistical analysis

The biochemical data were presented as mean ± standard error and analysed with the GraphPad Prism software (version 12). Statistically significant differences were compared by Student's *t*-test over DMSO (carrier solvent) with a *p* value of 0.05 for chemical exposures. For metabolomic data, the final list of metabolites quantified (measured in concentration according to manufacturer's manual) was normalized using the DMSO condition as reference (as the carrier solvent of all chemicals used in exposures) and processed for multivariate and univariate statistics using freeware software Multi Experiment Viewer (Saeed et al., 2003). Principal Component Analysis (PCA) was used to visualise the grouping of individual samples. Additionally, heatmaps of log2 fold changes (log2FC) were provided to illustrate changes in specific categories of metabolites. The metabolites that were statistically significant changed were analysed using the pathway analysis from MetaboAnalystR (Pang et al., 2024).

Results and discussion

Acute toxicity of chemical and commercial NSAIDs and their mixture

The impact of NSAIDs and their mixture on daphnids was assessed initially with toxicity curves, and the EC values were calculated (Table 1). The EC_{50} values for both chemical or commercial indomethacin and ibuprofen were similar to each other, while their 1:1 mixture appeared to be more toxic than the individual pharmaceuticals. This could be explained due to a synergistic effect between the two NSAIDs, since both pharmaceuticals are NSAIDs

and have the same mode of action. In contrast, in another study, exposure of daphnids to a mixture of NSAIDs (diclofenac, ibuprofen, naproxen, and acetylsalicylic acid) did not reveal a specific mode of action (Cleuvers, 2004). Additionally, this report agrees with the fact that pharmaceutical mixtures may exhibit unpredictable and more complicated effects compared to the individual drugs (Flaherty and Dodson, 2005). However, the EC_{50} values of commercial NSAIDs were significantly higher than the chemical NSAIDs, indicating that the commercial NSAIDs might be less toxic than the pure chemical forms.

Table 1. EC values (in mg/l) from toxicity curves (N=4). Commercial pharmaceutical compounds are indicated as p_.

Chemical	EC_{50}	EC_1	Hillslope
Indomethacin	17.46	6.6	4.731
Ibuprofen	17.80	8.6	6.341
1:1 Mixture	8.29	1.5	2.694
p_Indomethacin	53.98	24	5.660
p_Ibuprofen	43.14	37.7	33.87
p_1:1 Mixture	21.33	15.7	14.9

The concentrations at which the pharmaceuticals are present in the environment vary depending on the environmental sample (Table 2).

Table 2. Concentrations of indomethacin and ibuprofen in the environment.

NSAIDs	Environmental sample	Location	Detected concentration (µg/l)	
Indomethacin	WWTPs		0.01-1	(Topaç et al., 2018)
	River water		10^{-4}-0.1	
	Influent water		1.412	(Huo et al., 2007)
	Effluent water		0.871	
	Hospital		2.548	
Ibuprofen	WWTPs	Bosnia, China, Croatia, Greece, Herzegovina, Korea, Sweden, Switzerland, and the UK	4x10^{-3}-603	(Chopra and Kumar, 2020, Jan-Roblero and Cruz-Maya, 2023)
	Wastewater	Canada	45	
		South Africa	1.38	
		Belgium	5.78	
		Pakistan	703-1673	
	Surface waters	Canada	0.98	
		France	8	
		China	1.417	
		Greece	1-67	
		Korea	15-414	
		Taiwan	5-280	
	Influent wastewater	Girona, Spain	13.74	(Ferrando-Climent et al., 2012)
	Effluent wastewater		1.9	

This study comes as the natural continuation of our previous research on the effects of indomethacin, ibuprofen, and their mixture on daphnids (Michalaki and Grintzalis, 2023). A lower and more environmentally relevant concentration of 5 μg/l was chosen, along with the commercial versions of the two drugs (designated as p_indomethacin and p_ibuprofen). The primary goal of this study was to evaluate the impact of these NSAIDs and their mixtures on daphnids while also incorporating transgenerational exposures to comprehend the implications across multiple generations.

Impact of pharmaceuticals on enzyme activities

Based on our previous research, we used first key enzymes activities involved in phosphate metabolism, sugar, lipid, and protein catabolism, and the metabolism of xenobiotics as indices of physiology (Table 3). Enzymes such as ALP, ACP are great inexpensive markers for the evaluation of pollution (Saravanan et al., 2012).

Indomethacin

The chemical and commercial (p_) version of indomethacin affected differently the activities of enzymes in daphnids. Specifically, while the chemical indomethacin did not affect enzymes its commercial form decreased the activity of βGAL and increased the activity of LIP. However, during the recovery generation indomethacin increased the activities of βGAL and PEP, by 14% and 52%, respectively, while the animals exposed to the commercial form were able to fully recover after 14 days in the absence of the pollutant.

Ibuprofen

A distinct pattern of effect caused by the chemical and commercial forms of ibuprofen was observed in the fourth generation where the chemical ibuprofen decreased the activity of βGAL and PEP, while the commercial form impacted only the activity of LIP. During the recovery generation, the chemical ibuprofen continued to affect the activity of daphnids and specifically decreased the activity of ALP and increased the activity of βGAL. Conversely, the animals that were exposed to the commercial form of ibuprofen seemed to fully recover. A study revealed that ibuprofen altered the activity of antioxidant defence system enzymes GST, SOD, and CAT on daphnids. After 6 h of exposure, the activity of GST increased with increasing concentrations of ibuprofen. However, GST activity decreased during longer exposure periods, indicating that the activity of GST may be related to ibuprofen concentration, as well as the exposure period. Conversely, the activity of SOD increased with increasing both factors. While the activity of CAT showed a gradual increasing trend with increasing the concentration of ibuprofen, no significant difference in effect was detected when compared to the control (Wang et al., 2016). The effects of ibuprofen on the

enzymatic activity of various aquatic species have been studied, and it was discovered that following 7 days of exposure, ibuprofen induced lipid peroxidation and altered the enzymatic activities in zebra mussels (*Dreissena polymorpha*) (Jan-Roblero and Cruz-Maya, 2023). In addition, following exposure for 96 hour in zebra mussels at environmentally relevant concentration of ibuprofen (0.2, 2 and 8 mg/l induced genetic and cellular damage, as well as, cyto-genotoxicity and increased levels of CAT, SOD, glutathione peroxidase (GPx) and GST (Parolini et al., 2011, Sibiya et al., 2024). Chronic exposure to ibuprofen also resulted in genetic and cellular damage. Furthermore, exposure to ibuprofen caused cellular oxidative stress and alterations to enzymes CAT, SOD, and GST. Chronic exposure of zebrafish (*Danio rerio*) to ibuprofen at environmentally relevant concentrations ranging from 0.1 to 11 µg/l affected the activity of enzymes of the antioxidant defence system, liver protein carbonylation, and the activity of lactate dehydrogenase (LDH). Ibuprofen is also known to cause oxidative stress by generating ROS. This impact has been detected in zebrafish tissues following chronic exposure at environmentally relevant concentrations (Jan-Roblero and Cruz-Maya, 2023). Exposure of the clam *Ruditapes philippinarum* to ibuprofen impacted the expression of GST genes (Wang et al., 2016), whereas the activity of GST was altered after 96 h of exposure of a non-aquatic organism *Chironomus riparius* to 0.01, 1, and 100 µg/l ibuprofen. Exposure of freshwater mussel *Lamellidens marginalis* to 5 µg/l ibuprofen for 14 days increased significantly protein and lipid content, as well as ROS production (Sibiya et al., 2024).

Mixture

Highly unexpected results were observed when daphnids were exposed to the two distinct mixtures. Over the course of the fourth generation, the mixture of chemical indomethacin and ibuprofen had a noticeable impact on the activities of βGAL and LIP. On the contrary, the mixture of the commercial forms altered the activities of βGAL, LIP and GST. Intriguingly, during the recovery generation, the mixture of chemical forms of pharmaceuticals did not exhibit any effect on the enzymatic activities of daphnids. However, the animals exposed to the mixture of the commercial forms were unable to recover from the induced stress.

Table 3. Responses in enzymatic activities for daphnids exposed to chemical and commercial (p_) indomethacin, ibuprofen, and their mixture. Data represent average±standard error (N=4). Enzyme activities were expressed in Units/mg BSA for ACP, ALP, βGAL, LIP and PEP. Activity of GST were expressed in mUnits/mg BSA. #Statistically significant by Student's *t*-test denotes significant difference in comparison to DMSO as the carrier solvent.

Age Generation	Enzyme	DMSO	Indomethacin	Ibuprofen	1:1 Mixture	p_Indomethacin	p_Ibuprofen	p_1:1 Mixture
14 days at the fourth gen.	ALP	4.53±0.12	4.71±0.16	4.2±0.14	4.34±0.07	4.18±0.06	4.35±0.03	4.2±0.06
	ACP	1.42±0.11	1.54±0.08	1.34±0.14	1.58±0.02	1.32±0.1	1.42±0.03	1.32±0.02
	βGAL	4.12±0.08	3.89±0.17	3.72±0.09 # (-10%)	3.66±0.07 # (-11%)	3.77±0.06 # (-8.5%)	3.51±0.22	3.54±0.1 # (-14%)
	LIP	72.6±3.42	59.7±0.8	62.8±1.36	100.4±2.7 # (+38%)	97.3±4 # (+34%)	105.5±6.2 # (+45%)	94.8±4.8 # (+31%)
	PEP	11.3±0.16	10.9±0.71	8.4±0.42 # (-26%)	11.4±0.36	11.7±0.42	9.8±0.65	11.3±0.29
	GST	53.7±5.45	36.7±2.3	48.4±3.3	66±3.6	70.7±3.7	66.9±3.35	83.2±2.2 # (+55%)
14 days at the fifth gen. recovery	ALP	6.17±0.11	5.91±0.06	5.35±0.13 # (-13%)	5.88±0.23	6.05±0.28	5.82±0.09	5.9±0.08
	ACP	1.68±0.08	1.83±0.11	1.59±0.05	1.95±0.18	1.68±0.11	1.66±0.06	1.55±0.06
	βGAL	4.72±0.09	5.36±0.05 # (+14%)	5.36±0.07 # (+14%)	5.3±0.16	5.29±0.19	4.91±0.12	4.36±0.08
	LIP	121.1±3.47	127.5±5.94	115.5±1.18	126.6±5.6	120.9±9.14	102.6±4.54	97.1±2.95 # (-20%)
	PEP	14.6±0.36	22.2±0.69 # (+52%)	15±1.02	15.2±0.69	13.9±1.04	14.4±0.88	11.3±0.2 # (-22%)
	GST	62.5±2.4	63.3±3.2	67±1.4	64.8±2.55	70.3±2.75	69.8±1.25	81.7±0.8 # (+31%)

Metabolic perturbations induced by pharmaceuticals

Metabolomics has emerged as an essential tool in molecular ecotoxicology and risk assessment, advancing our understanding of how pollutants affect biological systems. This approach can detect subtle metabolic changes in daphnids and other organisms exposed to harmful chemicals, offering early warning signs of their adverse effects (Taylor et al., 2018, Viant, 2009). Recent research on the evaluation of metabolic responses from aquatic organisms to contaminants are crucial for the effective environmental monitoring and chemical risk assessment. The perturbation of metabolites after exposure to pollutants may indicate that phylogenetic origins can predict adverse effects (Viant et al., 2019) especially in conserved toxicological pathways (Colbourne et al., 2022). While there are other omics, such as transcriptomics, which could be used to assess the impact of chemicals on daphnids, it is known that metabolomics identifies phenotypic changes that occurred in the presence of a pollutant (Fröhlich, 2017, Fuertes et al., 2019). Additionally, metabolomics highlights alterations in the metabolic profile of the animals and could be characterised as a "snapshot" of the current active state of the animals (Fröhlich, 2017).

In a previous study we demonstrated how markers related to the physiology and enzymatic activity of daphnids can be paired with markers of metabolic perturbations to provide meaningful endpoints for pollution assessment (Michalaki et al., 2022). Exposure to NSAIDs such as ibuprofen and diclofenac has revealed a spectrum of complicated metabolic responses and biochemical alterations in a series of studies employing various aquatic organisms such as *D. magna*, zebrafish, and *Hyalella azteca* (Fu et al., 2021, Kovacevic et al., 2016, Song et al., 2018). Specifically, the effects of ibuprofen to daphnids showed a dose-dependent pattern, with lower and higher concentrations generating more pronounced metabolic responses than intermediate concentrations. Ibuprofen is known for inhibiting the synthesis of eicosanoids in mammals. There are studies that have shown that the system of eicosanoid is present in daphnids, and ibuprofen can cause a comparable impact to it, resulting in a decrease in reproduction and survival. Exposure of *D. magna* to acetaminophen and the NSAIDs diclofenac and ibuprofen altered the levels of glutamic acid, isoleucine, leucine, methionine, proline, threonine, tryptophan, alanine, glycine, histidine, lysine, phenylalanine, serine, arginine, glutamine, choline, carnitine, spermidine, ornithine, tyrosine, valine and putrescine (Oliveira Pereira et al., 2024b). Significant changes in amino acid profiles were also reported after exposure to ibuprofen. Glycine, tyrosine, asparagine, and serine showed significant declines following exposure to low concentrations of ibuprofen. Reductions in essential amino acids for crustaceans, such as leucine, arginine,

and lysine, can potentially disrupt key physiological processes in daphnids, such as growth, molting, and osmoregulation. Specifically, the importance of lysine is highlighted by its role as a precursor for carnitine, a chemical essential for fatty acid production and energy metabolism (Kovacevic et al., 2016). This can also affect the carnitine shuttle pathway, which is crucial for the transportation of long-chain fatty acids from the cytosol to the mitochondria matrix for further *β*-oxidation. This can be accomplished through the synthesis of acylcarnitine and then carrying it via carnitine transporters. Some transcriptomic studies revealed that ibuprofen represses genes related to energy metabolism (Adamczuk, 2022, Heckmann et al., 2008). Following exposure to diclofenac, another NSAID, it was reported that three out of four acylcarnitines were decreased, resulting in inhibition of the synthesis of fatty acid acylcarnitines. Carnitine acyltransferases, such as *o*-octanoyltransferase (CROT), catalyse the synthesis of carnitine conjugates of fatty acids. It has been reported that ibuprofen can alter the expression of CROT in mice, thus it can impact the fatty acid *β*-oxidation and lipid metabolism in general. Concluding, diclofenac and other NSAIDs can impact and modify the lipid metabolism and the fatty acid *β*-oxidation by affecting the carnitine shuttle pathway. This effect, along with the altered prostaglandin metabolism, was detected in *Hyalella azteca* following exposure to diclofenac (Fu et al., 2021). On the other hand, exposure of daphnids to higher concentrations of ibuprofen resulted in increased levels of alanine, tryptophan, isoleucine, and methionine, and decreased levels of threonine, indicating complicated alterations in the amino acid pool (Kovacevic et al., 2016). Similar changes in amino acid profiles were detected in zebrafish following exposure to ibuprofen at a concentration of 5 μg/l, implying possible effects on energy metabolism and oxidative stress. The levels of arginine, proline, and aromatic amino acids including phenylalanine, tyrosine, and tryptophane, all of which play critical roles in neurodevelopment and function of the nervous system were significantly affected. Ibuprofen also caused oxidative stress by impacting the levels of cysteine, methionine, histidine, and threonine. Changes in arginine, threonine, asparagine, glutamine, and glutamic acid, caused by ibuprofen, change the immune response, cell growth, gene expression, and energy metabolism (Song et al., 2018). Additionally, exposure of daphnids to industrial effluents reported significant perturbations in the levels of metabolites, such as alanine, arginine, aspartate, carnitine, choline, citric acid, cysteine, glutamate, glutamine, histamine, histidine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tyrosine, valine, indicating disruptions to energy metabolism and protein dysregulation (Labine et al., 2023).

In the present study, a targeted LC-MS/MS approach unveiled a unique pattern of impact exerted by both chemical and commercial NSAIDs. The Principal Component Analysis

(Figure 2), particularly PC1-2 and PC1-3, revealed that the metabolic profile underwent a shift upon exposure to commercial NSAIDs, in contrast to chemical NSAIDs. Notably, the chemical NSAIDs and their mixture formed groups that were more proximate to DMSO (the carrier solvent). This observation is in accordance with the results from biochemical markers, which indicated that the chemical NSAIDs did not induce a significant impact in the fourth generation of daphnids, unlike the commercial NSAIDs. Additionally, exposure to the chemical mixture, commercial NSAIDs, and their mixture led to an increase in the levels of several amino acids and biogenic amines, followed by a smaller number of acylcarnitines, phosphatidylcholines, and sphingolipids (Figure 3). Exposure to chemical indomethacin significantly affected only seven metabolites, ibuprofen affected ten, while their mixture impacted twelve metabolites. Conversely, exposure to commercial NSAIDs affected at least twenty metabolites, predominantly amino acids. Commercial indomethacin altered the regulation of thirty metabolites, ibuprofen affected twenty, and their mixture altered the levels of forty-four metabolites. Similar to the effect of diclofenac, both commercial NSAIDs impacted three out of four acylcarnitines, their mixture only two, while chemical NSAIDs impacted only one out of four.

Indomethacin

In the case of indomethacin, the chemical form increased the biogenic amines alpha-AAA, kynurenine, and the phosphatidylcholine PC ae C42:2. Conversely, it decreased the levels of biogenic amines histamine, t4-OH-Pro, the acylcarnitine C2 and the PC aa C40:6. Exposure to p_indomethacin led to an increase in the regulation of several amino acids, namely eighteen, the biogenic amines ADMA, kynurenine, Met-SO, SDMA, spermidine, and the acylcarnitine C0. However, it resulted in the reduction of the acylcarnitines C2, C3, the phosphatidylcholine PC aa C40:6, and the lysophosphatidylcholines lysoPC a C16:0, lysoPC a C16:1 and lysoPC a C18:1.

Ibuprofen

Regarding the effects of ibuprofen, the chemical form induced an increase of the amino acid ornithine, the biogenic amines Met-SO and taurine, the phosphatidylcholines PC aa C36:0, PC aa C38:6, PC ae C38:0 and the lysophosphatidylcholine lysoPC a C17:0. It also caused a decrease in tyrosine, sarcosine and the acylcarnitine C3. In contrast, exposure of daphnids to p_ibuprofen significantly affected a larger number of metabolites. Specifically, the commercial form increased the levels of seventeen amino acids, ADMA, kynurenine, SDMA, and the acylcarnitine C0, while it reduced only the C2 and C3.

Mixture

Exposure to the chemical mixture led to the increase of several amino acids, such as asparagine, glutamic acid, isoleucine, leucine, methionine, proline, threonine, tryptophan, and valine. Additionally, ADMA and Met-SO were increased, while only PC aa C40:6 was decreased. However, the majority of amino acids were increased after exposure to p_mixture, as well as ADMA, kynurenine, Met-SO, putrescine, SDMA, spermidine, and taurine, C0, and several phosphatidylcholines, while only the C2 and several lysophosphatidylcholines were decreased.

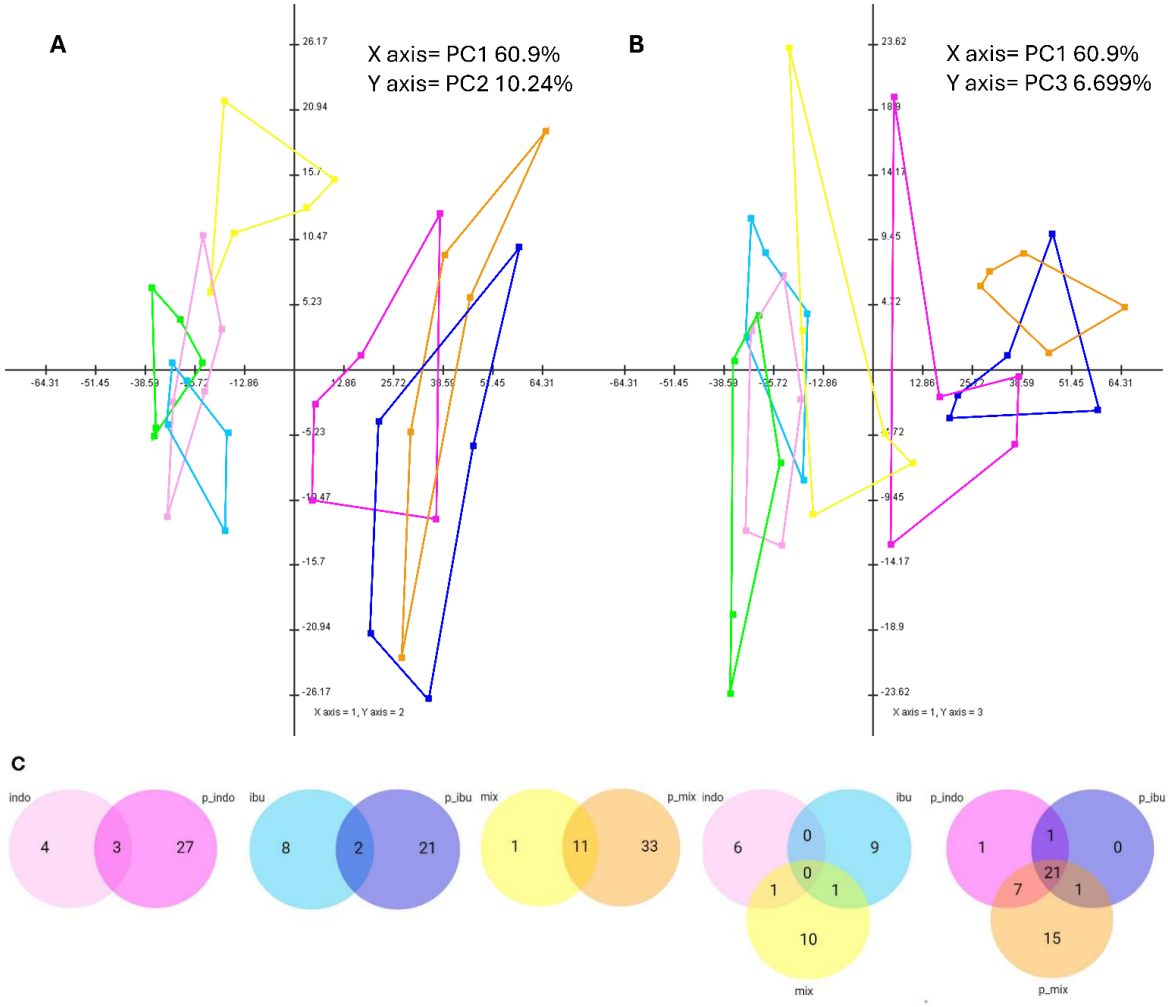

Figure 2. Multivariate statistical analysis of the impact of chemical and commercial (p_) pharmaceuticals on metabolic profiles of daphnids. Principal component analysis (A: PC1,2 and B: PC1,3) shows the grouping of samples (DMSO: green, indomethacin: light pink, ibuprofen: light blue, 1:1 mix: yellow, p_indomethacin: dark pink, p_ibuprofen: dark blue, p_1:1 mix: orange). Venn diagrams (C) show the number of increased or decreased metabolites among NSAIDs and 1:1 mixtures (left), or between the chemical and commercial form (right). Statistically significant changes were identified by One-Way ANOVA, followed by Dunnett's post hoc test shows significant difference against DMSO.

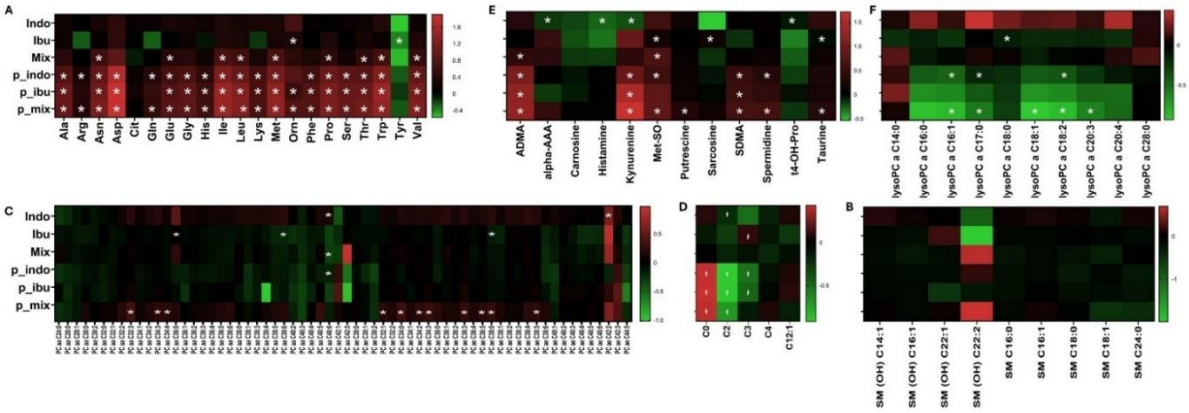

Figure 3. Heatmaps of metabolic perturbations expressed as Log2 fold change against DMSO (carrier solvent). *Statistically significant metabolites ($p<0.05$) over DMSO were identified with One-Way ANOVA followed by Dunnett's post hoc test. (A): amino acids, (B): biogenic amines, (C): phosphatidylcholines, (D): lysophosphatidylcholines, (E): sphingolipids, (F): acylcarnitines.

Pathway analysis, using MetaboAnalystR, was performed using the significantly impacted metabolites for each condition to identify the metabolic pathways disrupted. This approach offers a comprehensive visualization of the metabolic alterations and their possible biochemical implications (Figure 4). For the chemical forms of NSAIDs, indomethacin had an impact on lysine biosynthesis and degradation, histidine metabolism and α-linolenic acid metabolism, highlighting possible alterations in protein synthesis, energy production and inflammatory responses. In contrast, the commercial form, p_indomethacin, disrupted several amino acid-related pathways including valine, leucine, and isoleucine biosynthesis, alanine, aspartate, and glutamate metabolism, and arginine biosynthesis indicating changes in amino acid balance and energy production. Ibuprofen impacted glycerophospholipid metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis, and α-Linolenic acid metabolism, affecting cell membrane integrity, neurotransmitter production and inflammatory responses. However, p_ibuprofen altered valine, leucine, and isoleucine biosynthesis, glutathione metabolism, alanine, aspartate, and glutamate metabolism, arginine biosynthesis, and glycine, serine, and threonine metabolism. These effects highlight possible changes in protein synthesis, oxidative stress defence and growth. Exposure of daphnids to chemical mixture disrupted valine, leucine and isoleucine biosynthesis, and degradation, and alanine, aspartate and glutamate metabolism inducing changes in protein turnover and energy production. The p_mixture exhibited broader disruptions to mostly amino acid-related pathways such as glutathione metabolism, valine, leucine, and isoleucine biosynthesis, alanine, aspartate, and glutamate metabolism, and arginine and proline metabolism revealing possible alterations on oxidative stress regulation, growth and reproduction.

It has been reported that the metabolites which were significantly either increased or decreased could potentially disrupt the following metabolic pathways; glutathione metabolism, arginine and proline metabolism, aminoacyl-tRNA biosynthesis, histidine metabolism, cysteine and methionine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, arginine biosynthesis, valine, leucine and isoleucine biosynthesis, nitrogen metabolism, D-glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, and finally, purine metabolism (Oliveira Pereira et al., 2024a).

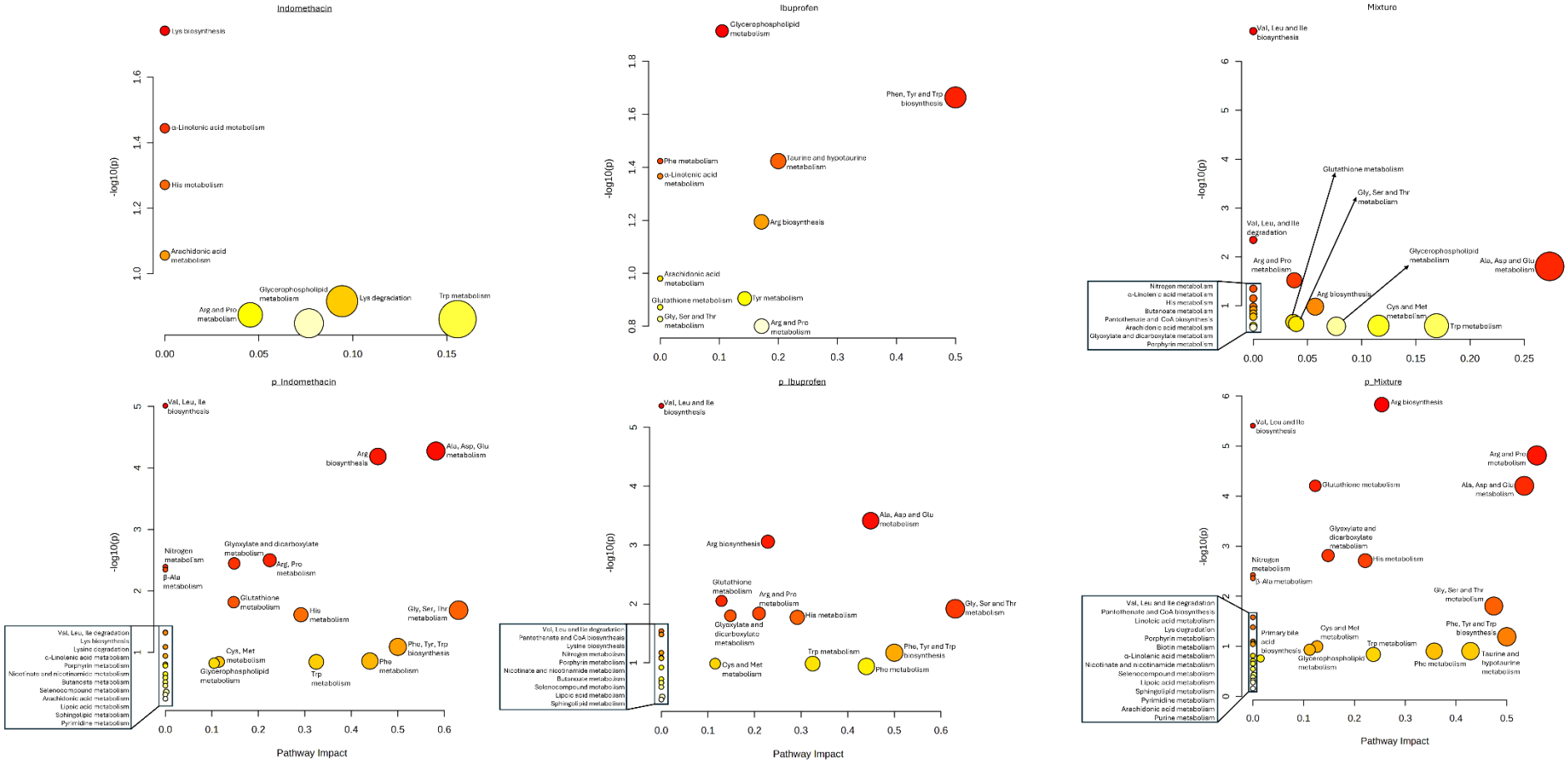

Figure 4. Pathway analysis of the significantly affected metabolic pathways. In each graph the pathways on the top-right corner are both statistically significant and highly impacted, while the pathways on the bottom-left corner have low significance and low impact. Graphs created using MetaboAnalystR (Pang et al., 2024).

The impact of chemical and commercial pharmaceuticals on survival of daphnids

For survival tests, neonates were exposed to selected NSAIDs and their mixtures for 28 days. Survival assays are used as additional mortality endpoint for the assessment of the impact of the chemicals at different ranges of concentrations for longer exposure periods. The EC values showed that chemical NSAIDs and their mixture are more hazardous than the commercial forms. This finding was corroborated by the survival curves, which revealed the toxic effects with increasing concentrations (Figure 5). Notably, both chemical and commercial NSAIDs and their mixtures had no effect on animal survival at 1 mg/l. At 10 mg/l, chemical and commercial ibuprofen, as well as their mixtures reduced survival in daphnids. However, it has been reported that exposing daphnids to another NSAID, diclofenac, at a concentration of 10 mg/l for seven days significantly affected survival (O'Rourke et al., 2023). At 20 mg/l, chemical indomethacin and their mixture had a stronger effect than ibuprofen. Both forms of ibuprofen produced comparable results, whereas the commercial mixture did not kill all exposed daphnids. These findings contradict another study where no impact on daphnids survival at ibuprofen concentrations up to 40 mg/l was observed (Heckmann et al., 2007). In our study, at 50 mg/l 100% mortality was observed for chemical NSAIDs, and both mixtures. At 80 mg/l chemical indomethacin, ibuprofen and the mixtures caused complete death, while the commercial forms of individual NSAIDs caused complete death after 10 days of exposure (Heckmann et al., 2007). These findings suggest that indomethacin (in both forms) is slightly more hazardous than ibuprofen, with the chemical form causing adverse effects at much lower concentrations than p_NSAIDs. In terms of mixtures, both forms were more harmful than their individual components, with chemical mixtures being significantly more toxic than commercial. Finally, the survival curves, combined with the toxicity curves, confirm that the concentration used for transgenerational exposures is non-lethal.

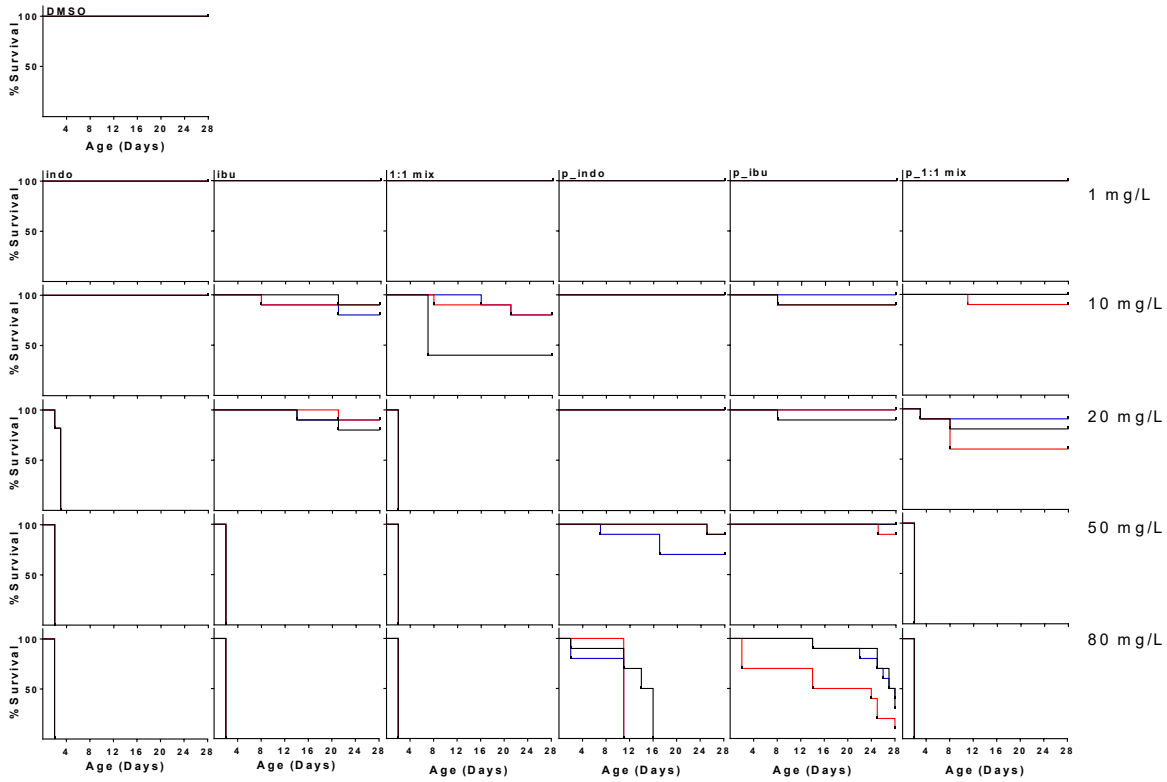

Figure 5. Survival curves of chemical and commercial NSAIDs. Data are presented from three independent experiments for each concentration.

Conclusions

In conclusion, the transgenerational effects of chemical and commercial NSAIDs and their mixtures were assessed on *D. magna* using a combination of biochemical markers and metabolomic analysis. The results unveiled that the effects of indomethacin and ibuprofen on daphnids varied depending on their form (chemical or commercial). The mixture of commercial NSAIDs had a greater impact on enzymatic activity and metabolic profiles compared to chemical NSAIDs. Additionally, transgenerational exposures to these NSAIDs showed that daphnids in higher generations were more susceptible to the adverse effects of the pollutants even at an environmentally relevant concentration of 5 µg/l. The results above support Adamczuk's argument that even very low concentration of pollutants, specifically ibuprofen, can have major effects on aquatic organisms (Adamczuk, 2022). However, during the recovery generation the effects were reversed and the daphnids exposed to commercial NSAIDs were completely recovered. Consequently, these observations underscore the complex interactions between pollutants and aquatic organisms and the importance of considering transgenerational effects in pollution assessment to detect adverse impact at environmentally relevant concentrations (Castro et al., 2018).

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Declaration of competing interest
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Chapter 5

This chapter studied how actual water samples can influence the toxicity of pollutants on *D. magna*, moving beyond the controlled laboratory conditions followed in the previous studies. Using water from River Liffey and Royal Canal as exposure media along with the artificial laboratory media (OECD), this chapter evaluated the impact of a three-chemical mixture on daphnids, following acute, chronic and transgenerational exposures. Results from phenotypic and biochemical endpoints demonstrated that the background composition of water can substantially change the toxicity and behaviour of the pollutants, revealing interactions between tested chemicals and naturally occurring compounds of the water. This study established the critical importance of incorporating real-world exposure conditions into ecotoxicological testing and reinforced how integrating biochemical and phenotypic endpoints provided more precise assessments of the effects of chemical mixtures in environmentally relevant conditions.

Molecular responses from water fleas serve as metrics for pollution – Moving from the lab samples to the river

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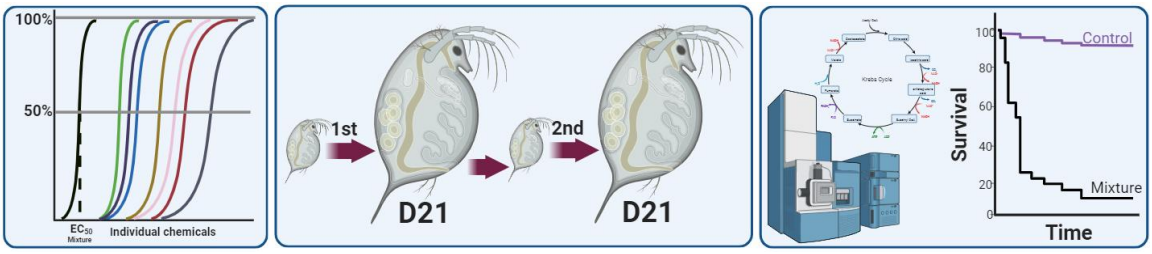
Abstract

Conventional methods for water pollution assessment rely on toxicity testing of one chemical at a time using artificial laboratory media. However, these approaches fail to capture the realism of aquatic environments, where animals are exposed to a combination of chemicals which interact with each other and with the physicochemical factors of the water matrix. This study aimed to mimic more realistic scenarios of exposure and to explore how different water matrices can affect the impact of a mixture of pollutants on *Daphnia magna*. The effects from acute, chronic and transgenerational exposures of daphnids to lithium, metformin, glyphosate and their 1:1:1 mixture, in artificial media and actual river water were assessed through a variety of endpoints. Water samples from river Liffey and Royal Canal were used as media. For the initial laboratory study, mortality, enzyme kinetics and feeding assays were performed, while for the field study enzyme kinetics were used to assess the impact of river water on the toxicity of the composite mixture. The biochemical results revealed that each river water affected differently the enzymatic responses of daphnids during the acute and chronic exposures, indicating that existed contaminants or background compounds of the water samples might interact with the introduced mixture altering its behaviour and toxicity on daphnids. Therefore, this study emphasizes on the importance of the incorporation of water samples for the evaluation of the effects of pollutants on aquatic organisms, since laboratory testing alone might fail to adequately mimic the effects of pollutants in real life.



Keywords: *Daphnia magna*, ecotoxicology assessment, transgenerational, river water

Highlights:

- Liffey river water significantly intensified the effects of the mixture in the acute exposure
- Royal Canal water increased the impact of the mixture during the chronic and transgenerational exposure
- Metabolic changes in daphnids provide means for early prediction of pollution

Introduction

Agricultural operations, industrial processes and domestic activities in urban areas are the principal contributors of anthropogenic pollution to aquatic ecosystems (Abdullahi et al., 2022, Bashir et al., 2020). Human interventions introduce a wide variety of pollutants through direct chemical use, unintentional spills during storage or transit, leaks from industrial sites, landfills and waste processing facilities, as well as through degradation of existing compounds (Akhtar et al., 2021). Traditional wastewater treatment plant facilities (WWTPs) frequently fail to adequately eliminate numerous pollutants, permitting their introduction and therefore persistence into natural water bodies. As a result, managing both the release of such compounds and their environmental repercussions remains a critical challenge in water quality monitoring and conservation (Bashir et al., 2020, Peñalver et al., 2021).

The overall quality of water ecosystems is shaped not only by these introduced pollutants but also by the presence of naturally occurring organic and inorganic compounds, which can become contaminants at high concentrations (Akhtar et al., 2021, Peñalver et al., 2021). Rivers are quite vulnerable to pollution since they pass through many landscapes and land uses, accumulating agricultural runoff, industrial discharges, and urban waste. Additionally, environmental factors such as soil composition, pH, salinity, and precipitation, as well as the chemical characteristics of pollutants, can affect their fate, and ecological impact (Ahmed, 2024, Akhtar et al., 2021, Moro et al., 2024). The river Liffey is classified as a nutrient-sensitive water body, particularly susceptible to pollution from groundwater, surface runoff, and site-specific sources within its catchment region (Peñalver et al., 2021).

Regardless the increasing concern regarding these pollutants, their impacts on aquatic organisms are primarily evaluated under regulated laboratory conditions using standardized artificial media, such as OECD media. While these conditions offer experimental reproducibility and monitoring, they fail to capture the complexity of natural aquatic ecosystems (Ahmed, 2024). Additionally, current approaches assess the effects of individual chemicals, while it is known that in real life scenarios hundreds of chemicals along with their degradation products and metabolites coexist in the water matrices interacting with each other (Abdullahi et al., 2022, Moro et al., 2024). In realistic scenarios, river water is a chemically and biologically complex matrix comprising dissolved organic matter, ionic compositions, microbial populations, and combinations of existing and new added pollutants (Harwood et al., 2012). These components can interact with the test chemicals in ways that either inhibit or enhance their toxic effects. Therefore, assessing the impact of pollutants

directly in actual water samples is vital for obtaining more realistic organism responses and improving ecological relevance of toxicity evaluations.

Within freshwater environments, crustaceans have a critical ecological role, and among them, *Daphnia magna* is globally used for water pollution assessments. Its remarkable sensitivity to anthropogenic chemicals and physical changes in aquatic ecosystems makes it suitable candidate for toxicity evaluation (Abdullahi et al., 2022). Due to their small size, *Daphnia* requires minimum space and resources for experimental setups. Moreover, their ability for clonal reproduction ensures genetic consistency among test organisms and multiple generations, thus minimizing variability and enhancing the reliability of results. As filter feeders, daphnids are well-established model organisms in the domains of molecular ecology and ecotoxicology (Moro et al., 2024). Their responses to a broad spectrum of contaminants have been extensively recorded, offering useful insights into environmental risk assessment (Ebert, 2005).

This study investigated how actual river water influences the biological effects of a 1:1:1 mixture, comprising lithium, metformin, and glyphosate at very low and environmentally relevant concentrations, on *D. magna*. The experimental design consisted of two parts: a controlled laboratory study where OECD was used as exposure media, and an environmentally relevant field-based study where actual river water was used as media. In the laboratory part, daphnids were exposed to the individual stressors at 1 mg/l and to their 1:1:1 mixture at concentrations of 0.1 mg/l, 1 mg/l, and 10 mg/l. Toxicity was assessed using mortality, and biochemical markers following acute exposures. The effects of the mixture at 0.01 mg/l, 0.1 mg/l and 1 mg/l were evaluated further through the feeding assay on D2 daphnids, and through enzymatic assays following chronic exposure for 21 days. For the field-based study, water samples were initially collected from six rivers across Dublin: River Liffey, Royal Canal Way, River Dodder, River Poddle, Santry River, and Tolka River. Neonates were exposed to these samples for up to 21 days as a preliminary screening test. However, daphnids exposed to water from the River Dodder, River Poddle, Santry River and Tolka River did not survive beyond approximately four days. Consequently, only River Liffey and Royal Canal way were selected for further experiments. Filtered river water samples from the River Liffey and Royal Canal Way were collected and used directly as exposure media (Moro et al., 2024). Acute exposures to the 1:1:1 mixture at 0.1 mg/l, 1 mg/l, and 10 mg/l were carried out to assess biochemical responses. Furthermore, transgenerational effects were evaluated by exposing daphnids for two consecutive generations (21 days each) to 0.1 mg/l and 1 mg/l of the 1:1:1 mixture in each river water,

followed by biochemical assays to detect potential chronic physiological impacts and how the water samples alter the effects of the mixture.

Materials and methods

Materials

All chemicals used in this study were of the highest analytical quality. Lithium chloride (CAS 7447-41-8), metformin (CAS 115-70-4), glyphosate (CAS 1071-83-6) KCl (CAS 7447-40-7), Na_2SeO_3 (CAS 10102-18-8), bovine serum albumin (CAS 9048-46-8), Coomassie Brilliant Blue G (CAS 6104-58-1), *p*-nitrophenyl butyrate (CAS 2635-84-9), 2-nitrophenyl-B-D-galactopyranoside (CAS 369-07-3), 1-chloro-2,4-dinitrobenzene (CAS 97-00-7), L-glutathione reduced (CAS 70-18-8), sodium phosphate dibasic (CAS 7558-79-4), L-leu-4-nitroanilide (CAS 4178-93-2) were purchased from Sigma-Aldrich. $CaCl_2 \cdot 2H_2O$ (CAS 10035-04-8), $MgSO_4 \cdot 7H_2O$ (CAS 10034-99-8), $NaHCO_3$ (CAS 144-55-8), HCl (CAS 7647-01-0), *p*-nitrophenyl phosphate (CAS 4264-83-9), boric acid (CAS 10043-35-3), ammonium acetate (CAS 631-61-8), NaOH (CAS 1310-73-2), methanol (CAS 67-56-1), and DMSO (CAS 67-68-5) were purchased from ThermoFisher (Ireland)

Water samples were collected from two sites from rivers across Dublin, Ireland, on the same day and stored at 4°C in a cold room after filtration. The rivers tested were the Royal Canal way in the city of Dublin and the river Liffey in Kilcullen, co. Kildare (Figure 1).

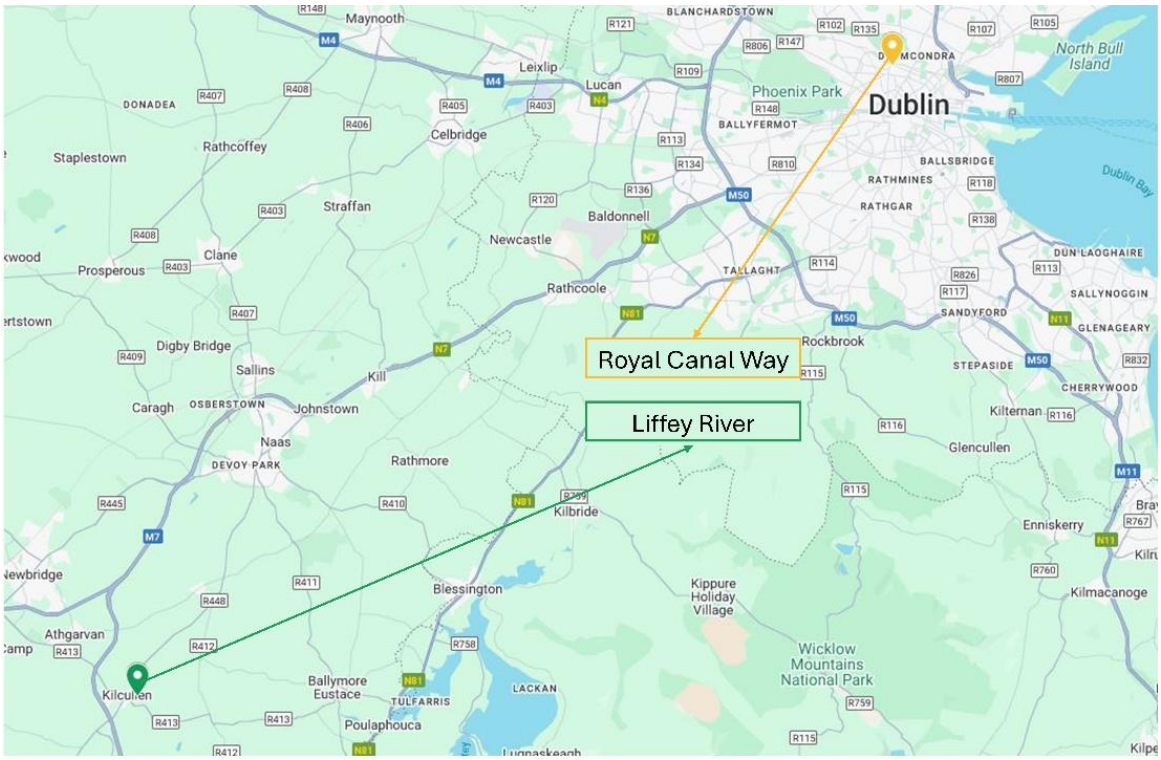


Figure 1. Map showing the two river sites used in this study. Coordinates for Liffey River 53.12972, -6.73933, and Royal Canal Way 53.36415, -6.27088.

Culturing of daphnids for exposures

Daphnids were maintained in glass beakers in OECD media and cultured as described previously described (Kakavas et al., 2024) under a 16h:8h of light:dark photoperiod at 20°C. Initially, fifteen neonates (<24h) were collected from the third brood of their mothers and exposed for 24 hours to each pollutant (lithium chloride, metformin, glyphosate) individually and to their mixture at equal concentrations in 50 ml volume. Toxicity curves were plotted using the four-parameter logistic Hill model, with the equations Span = Top Bottom and Y = Bottom + (TopBottom)/(1 + 10((LogIC50-X)*HillSlope)), and EC values were calculated using the GraphPad Prism programme. The parameters top and bottom were commonly fixed to 100 and 0, accordingly. Mortality in daphnids was assessed as their immobilization (Gomez-Olivan et al., 2014).

The experimental design of this study is divided into two parts. The first part involves exposures (both acute and chronic) conducted under laboratory conditions using OECD media. The second part focuses on environmental exposure, where daphnids were exposed to a mixture of Li, metformin, and glyphosate, in river water, rather than OECD (figure 2).

In the first part, both acute and chronic exposures were performed. For the acute experiments, neonates were exposed to individual stressors at a concentration of 1 mg/l, as well as to a 1:1:1 mixture of the pollutants at concentrations of 0.1 mg/l, 1 mg/l, and 10 mg/l for 24 h. The chronic exposure was performed using only the mixture at varying concentrations. Thirty-six neonates were exposed to the mixture for 21 days at concentrations of 0.01 mg/l, 0.1 mg/l, and 1 mg/l, in 900 ml media, as previously described (Michalaki and Grintzalis, 2023). Media and chemical mixture were renewed twice per week. Daphnids were fed daily with fresh algae (*C. reinhardtii*, at 16.5 million cells), and a seaweed extract (*Ascophylum nodosum*) which was only added on media changes.

For the environmental part, the same pattern was followed, however, the OECD media was replaced by river water collected by the two distinct sites: the Liffey River in Kilcullen co. Kildare and the Royal Canal Way in Drumcondra, Dublin. In the acute tests, neonates were exposed for 24 h to river water (control condition) as well as in river water spiked with a 1:1:1 mixture of those three pollutants at concentrations of 0.1 mg/l, 1 mg/l and 10 mg/l. This setup resulted in eight separate conditions: for each river, one control and three exposure concentrations. For the chronic experiments, neonates were exposed for 21-days across two generations using the same river water, but only two mixture concentrations (0.1 mg/l and 1 mg/l) were applied. Thus, for each river, the chronic exposure consisted of a control condition and two treatment conditions. The renewal of media and chemical was

every five days, while the feeding protocol was consistent with those employed in the OECD experiments.

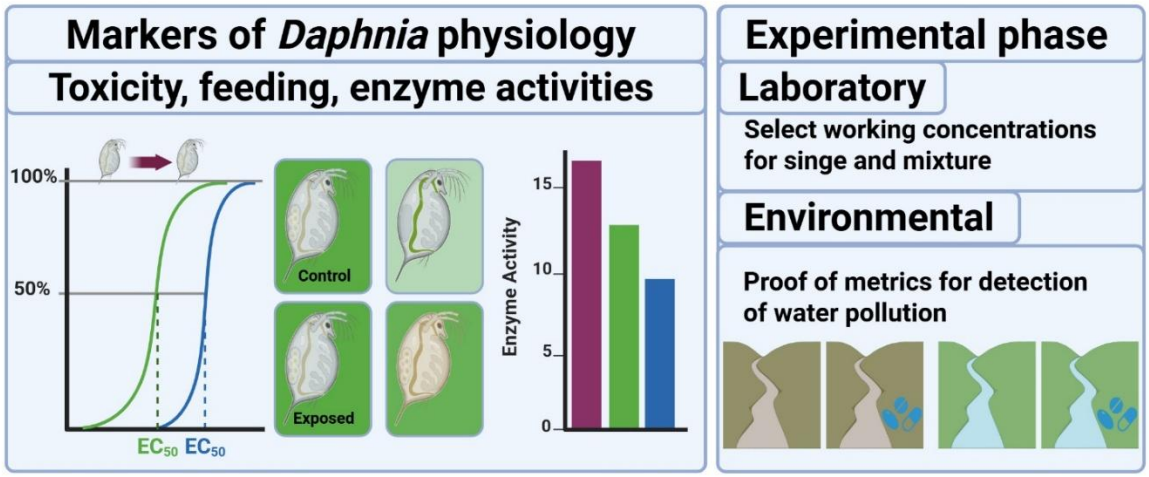


Figure 2. Experimental design of the study.

Feeding assay

Feeding was assessed following the protocol of Rowan et. al. (Rowan et al., 2024). Specifically, neonates were first exposed to 0.01 mg/l, 0.1 mg/l and 1 mg/l of the mixture for 24 h. Following exposure, five daphnids were transferred to a 96-deep well plate with 1 ml OECD media containing the carboxylate-modified polystyrene, fluorescent re microparticles (2.0 μm mean particle size) at a concentration of 26 mg/l. The animals were exposed to microplastic for up to 40 min, and media was collected every 10 min to estimate the consumed microparticles by fluorescence at Ex/Em 560/590 nm using a TECAN plate reader. The concentration of microparticles in the media was optimized to ensure an excess of microparticles for the accurate quantification of ingestion. Feeding rate was expressed as the slope for 40 min for the consumption of microplastic. Additionally, it is worth mentioning that these particles have been extensively tested and showed no toxicity on daphnids for the short exposure periods (Giannouli et al., 2023, Kakavas et al., 2024).

Biochemical assays

For the acute exposures, fifty animals were pooled together, per replicate, while for chronic exposures eight adult 21 days daphnids were collected and immediately homogenized in 1 ml of buffer using a pestle homogenizer for the assessment of enzyme markers and protein content. The homogenates were cleared by centrifugation (15,000 *g* for 5 min at 4ºC) and the clear supernatant was collected and assessed immediately. With the suitable substrate, the activity of phosphatases, β-galactosidase, and lipases were measured as released nitrophenol as described elsewhere (Michalaki et al., 2022). Phosphatase activity was measured where *p*-nitrophenyl phosphate was converted to *p*-nitrophenol by either an acid

phosphatase (100 mM citric acid pH 4.5; ACP) or an alkaline phosphatase (100 mM boric acid pH 9.8; ALP) at 405 nm (Grintzalis et al., 2022). To measure the activity of lipase (LIP) and β-galactosidase (βGAL), the release of nitrophenol from the catalysis of *p*-nitrophenyl butyrate or *o*-nitrophenyl-β-galactoside, respectively, in phosphate buffer pH 7.2, was used. Peptidase (PEP) was evaluated using continuous kinetics at 418 nm following the release of *p*-nitroanilide (from L-leucine-4-nitroanilide). Continuous kinetics were used to quantify the glutathione-S-transferase activity for the formation of the complex between glutathione and 1-chloro-2,4-dinitrobenzene at 340 nm (Tang et al., 1996). Finally, the activity of lactate dehydrogenase (LDH) was assessed from the consumption of NADH in a reaction with pyruvate as a substrate (5mM) at 340 nm (Worthington and Worthington, 2011). Using a sensitive assay (Grintzalis et al., 2015), all enzyme activity was normalized in units per protein.

Statistical analysis

In this study, Student's *t*-test, One-Way and Two-Way ANOVA were used to analyse the data. For the feeding assay, a One-Way ANOVA followed by post-hoc Dunnett's test was applied to show the impact of the mixture on the feeding performance of the animals compared to the unexposed control. For the biochemical assays, the data generated from the experiments performed in OECD were analysed with One-Way ANOVA with Dunnett's post-hoc test to compared treated groups with the control. However, in the environmental part, Student's *t*-test and Two-Way ANOVA were incorporated into the analysis. Student's *t*-test was used to demonstrate differences in enzymatic activity between the two control conditions across two generations. Finally, the effects of the combination of river source and dose on the activity of each enzyme on daphnids was highlighted with Two-Way ANOVA corrected with Tukeys's multiple comparisons test against the unexposed control.

Results and discussion

Laboratory study of single and mixture of chemicals

Mortality induced from pollutants and their mixture

Acute toxicity of neonates to Li, metformin, and glyphosate alone and in mixture was assessed with toxicity curves (Figure 3). Additionally, the effective concentration (EC) values were calculated (Table 1). The EC_{50} for Li, metformin, glyphosate was very similar to each other, 64.89 mg/l, 77.22 mg/l, 60.41 mg/l, respectively. The mortality of a 1:1:1 mixture for the three stressors was also tested and showed a lower EC_{50} 45.65 mg/l. For the acute exposures, a non-lethal concentration of 1 mg/l was selected for the individual chemicals, while 0.1 mg/l, 1 mg/l, and 10 mg/l were chosen for the mixture.

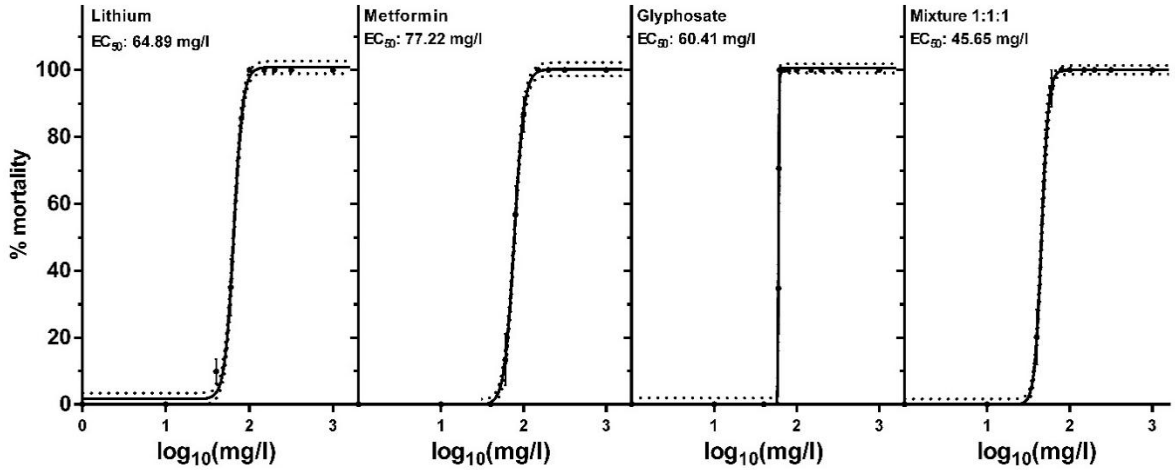


Figure 3. Acute toxicity curves in neonates of lithium, metformin, glyphosate and their 1:1 mixture. Data represent average ± standard deviation (N=4 replicates).

Table 1. EC values (mg/l) from toxicity curves.

Chemical	EC_{50}	(Min – Max)	Hill Slope	EC_1
Lithium chloride	64.89	(63.67-66.14)	8.061	36.7
Metformin	77.22	(75.76-78.71)	7.335	41.3
Glyphosate	60.41	(60.33-60.49)	105.3	57.8
Mixture 1:1:1	45.65	(44.62-46.69)	10.44	29.4

The impact of pollutants on ingestion rate

Feeding assay is used as an endpoint to evaluate the impact on the ingestion rate of daphnids following acute exposure to 0.01, 0.1 and 1 mg/l of the 1:1:1 mixture. Feeding rate was determined as the slope for 40 min (fluorescent was measured every 10 min) for the consumption of microplastic. Exposure of D1 daphnids to the mixture at concentrations of

0.01 and 0.1 mg/l did not affect the feeding activity, while exposure to 1 mg/l of the mixture significantly increased the feeding capacity of the animals by 66% (Figure 4).

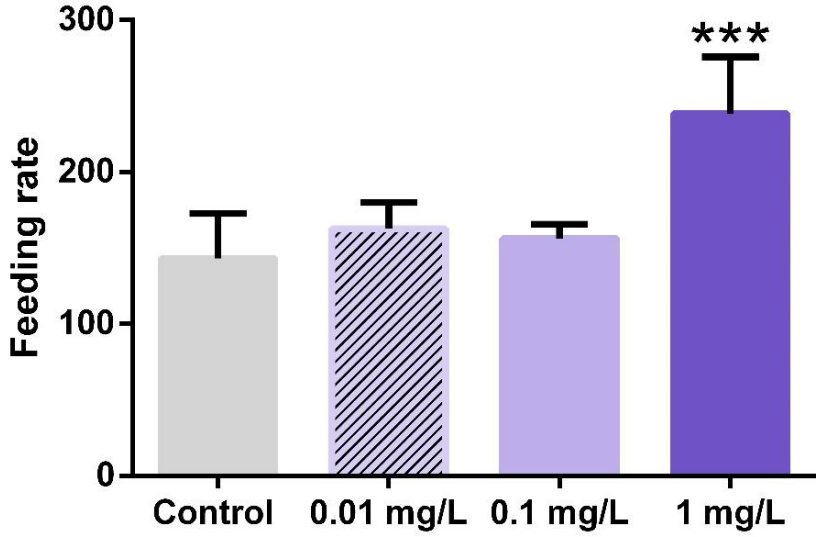


Figure 4. The impact of 1:1:1 mixture on feeding rate to neonates. Feeding rate was quantified as the slope for 40 min for consumption of microplastic. Data represent average ± standard deviation (N=4 replicates). *Statistically significant by One-Way ANOVA corrected with post-hoc Dunnett's test against the unexposed control with *p*-values of $p \leq 0.001$ (***).

Acute effects of single stressors and their mixture on daphnids

Acute exposure (for 24 h) of neonates to Li, metformin, glyphosate and the three different doses of their mixture caused significant alterations to the enzymatic activities of animals (Figure 5).

Acute effects of Li

Focusing on the individual stressors, Li increased the activity of ALP by 9%, while it decreased the activity of peptidase by 9%. Lithium has multiple applications in everyday life with the most well-known lithium batteries. However, as the consumption of lithium-based products increases, the concerns of the effects of this metal to aquatic environments increase as well (Duan et al., 2025). It has been reported that exposure of daphnids to Li in medium and high concentrations (83.44 μg/l and 834.4 μg/l) led to phenotypic and molecular responses. Particularly, it caused eye and tail deformities, and it affected body length, total neonates per female, and average neonate per time. In a molecular level though, Li disrupted the energy metabolism affecting swimming speed and range, while it also affected the dynamic balance of mitochondrial fission and regeneration, leading to ROS production and oxidative stress (Duan et al., 2025). However, these results were generated in monitored laboratory conditions, and its actual effects in the environment remain unknown. As all compounds found in actual water interact with each other, a study about the interactions between Li and sodium showed that the presence of sodium can decrease its toxic effects.

As a result, in natural waters where sodium exists, the toxic effects of Li might be eliminated (Kszos et al., 2003).

Acute effects of Metformin

Metformin increased both activities of ACP and ALP by 17% and 14% respectively. Metformin is a drug prescribed to patients with type 2 diabetes, polycystic ovary syndrome or cancer, and it is one of the most frequently detected drugs in aquatic environments (Ambrosio-Albuquerque et al., 2021). Specifically, metformin has been found in several aquatic ecosystems including wastewater, surface water, groundwater, and drinking water (Zheng et al., 2024). It has been reported that metformin is mostly released into the aquatic environments in its original form and in high concentrations, since approximately 70% of the administered dose is being excreted through urine and feces (Ambrosio-Albuquerque et al., 2021, Zheng et al., 2024). Recent studies have reported that exposure of fish to this drug can cause oxidative stress, genotoxicity, disruption of intestinal flora, morphological alterations and endocrine disruption (Ambrosio-Albuquerque et al., 2021, Zheng et al., 2024). Additionally, exposure of other aquatic organisms, such as bacteria, algae, cnidarians, crustaceans, and amphibians, to metformin can affect their morphology, growth, locomotor activity and their reproduction (Ambrosio-Albuquerque et al., 2021, Zheng et al., 2024).

Acute effects of Glyphosate

On the other hand, glyphosate at 1 mg/l did not affect at all the enzymatic activities of daphnids. Glyphosate, a commonly used herbicide globally, shows temperature-dependent toxicity. Exposure of *Lemna sp.* to different concentrations of glyphosate and at different temperatures resulted in a growth inhibition rate. Additionally, another marker, peroxidase activity was the most temperature-sensitive marker (Eck-Varanka et al., 2023). In realistic scenarios the physicochemical parameters of water have seasonal variations, making the chemicals in the water interact differently with each other. As a result, their effects are usually unpredictable and monitored laboratory exposure conditions cannot completely mimic these parameters. Therefore, it is important to develop methodologies that can adequately capture the realism of the aquatic environment, increasing the ecological relevance of toxicity assessment.

Acute effects of 1:1:1 mixture

However, a dose-dependent trend is observed to be caused by the three different doses of the mixture. Exposure of daphnids to 0.1 mg/l of the 1:1:1 mixture showed increased activities of ACP and ALP, while the 1 mg/l increased the activity of the first and decreased the activity of βGAL. The highest concentration of the mixture, 10 mg/l, affected almost all

enzymes except for ALP, GST and LDH. Specifically, the activities of βGAL, Lipase and Peptidase were decreased by 18%, 15%, and 16%, respectively, while the activity of ACP increased by 26%.

Acute exposure of D4 daphnids to Li at 68.4 mg/l for 24 h significantly decreased the activities of all enzymes as can be shown in our previous study (Michalaki et al., 2022). Additionally, exposure of D4 to metformin at 106.5 mg/l, following the same conditions, caused a decrease in all enzyme activities except from the GST, while exposure to 1.69 mg/l glyphosate affected several but not all enzymes. In the present study, the same results are not observed since the animals exposed are neonates and the selected exposure concentration is non-lethal and much lower than the previous study.

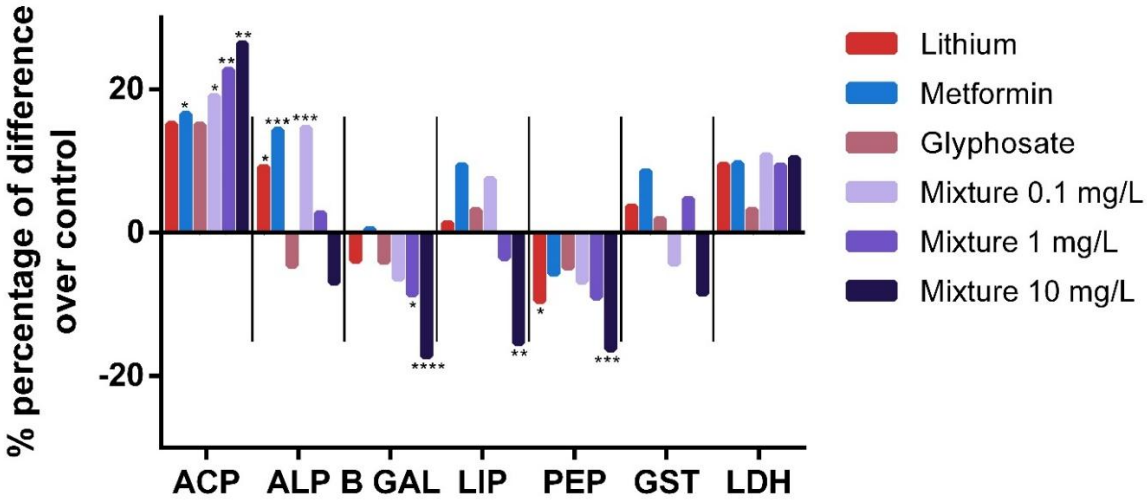


Figure 5. Biochemical markers of daphnid physiology upon exposure to Li, Metformin, Glyphosate and their mixture in three concentrations ranging from 0.1 to 10 mg/l. Data represent the % percentage of difference in the enzymatic activity over the unexposed control. Symbol * indicates statistically significant difference by One-Way ANOVA corrected with post-hoc Dunnett's test against the unexposed control with *p*-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

Chronic effects of 1:1:1 mixture on daphnids

Following 21 days of exposure to chemicals 1:1:1 mixture, at concentrations 0.01, 0.1 and 1 mg/l changed the enzymatic activities of ACP, βGAL, PEP, LDH and GST (Figure 6). More specifically, exposure of daphnids to the lowest mixture concentration decreased the activity of ACP by 17%. The medium mixture concentration decreased the activity of ACP by 19%, while it increased the activity of LDH by 42%. Finally, the highest concentration of the mixture, 1 mg/l, decreased the activities of βGAL and PEP by 8% and 11% respectively, and increased the activity of GST by 21%.

Although the concentrations were very low, at environmentally relevant levels, there is a dose-dependent effect with increasing concentrations. Additionally, while the 0.1 mg/l

increased the activity of ACP after 24h of exposure, it caused a decrease in this enzyme after 21 days indicating that the effects change with increasing exposure periods. The same cannot be said for the impact of 1 mg/l, as during the acute exposure decreased the activity of βGAL by 7%, and during the chronic exposure the activity decreased by 8%.

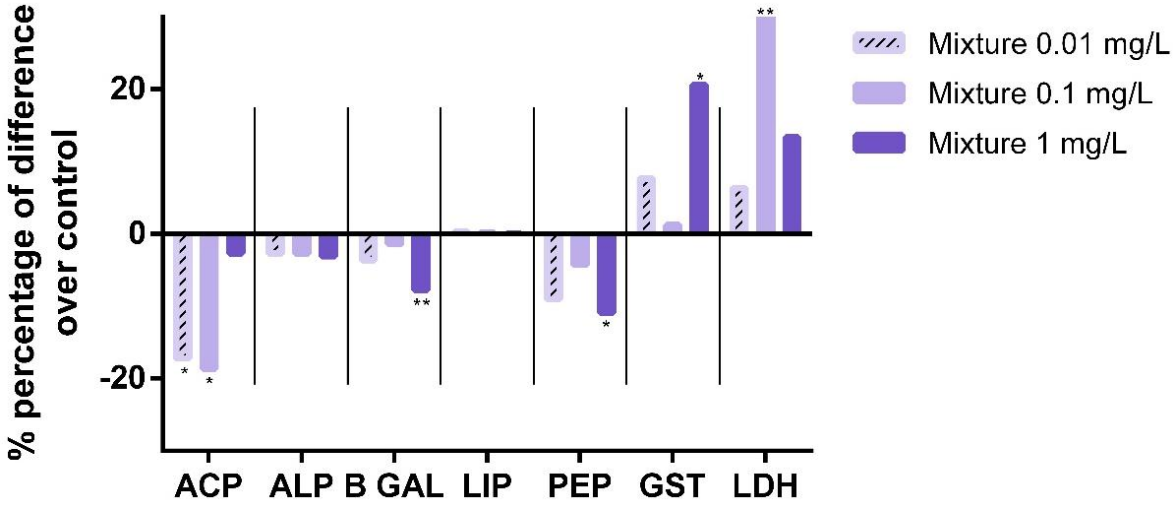


Figure 6. Biochemical markers of daphnid physiology upon chronic exposure to the chemicals mixture at concentrations 0.01 mg/l, 0.1 mg/l and 1 mg/l. Data represent the % percentage of difference in the enzymatic activity over the unexposed control. Symbol * indicates statistically significant difference by One-Way ANOVA corrected with post-hoc Dunnett's test against the unexposed control with *p*-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**).

Field study of mixture of chemicals

Acute effects of 1:1:1 mixture on daphnids in river water

Concentration-dependent effects within each river

Acute exposure to the three concentrations of the chemical mixture caused distinct dose-dependent responses in enzyme activities of daphnids exposed to Liffey, while such effect was not observed on Royal Canal (Figure 7). In the case where Liffey River water was used, increasing concentrations resulted in pronounced suppression of enzymatic activities such as ALP, BGAL, and PEP, with decreases of 31%, 48%, 42%, respectively, at 10 mg/l. In contrast, the activities of enzymes related to detoxification processes (GST) and energy metabolism (LDH) were significantly increased, by 18% and 16% respectively, at the higher concentration, suggesting that the animals were trying to cope with the stress (Dasari et al., 2017, Galhano et al., 2022). The activity of ACP was only affected by 1 mg/l, specifically increased by 33%.

On the other hand, exposure of neonates to the mixture using Royal Canal water as exposure media induced more moderate and inconsistent enzymatic changes, with no clear dose-response patter. Significant increases on the activities of ACP (+23%) and PEP (+5%) were caused by the medium concentration of 1 mg/l, while the lowest concentration of 0.1 mg/l

decreased the activity of LIP (-12%) and the 10 mg/l reduced the activity of ALP (-15%). These findings suggest that there was a limited or variable biological reactivity under these exposure conditions.

Water sources effects

A comparison between river water sources highlighted a greater impact from Liffey River, specifically at the highest concentration. Exposure to Liffey 10 mg/l induced significant enzymatic perturbations, including inhibition of digestive enzymes and activation of detoxifying GST, and LDH. Conversely, exposure to Royal Canal 0.1 mg/l, 1 mg/l and 10 mg/l elicited relatively milder responses, with smaller deviations from the control across all markers. The stronger effect observed in Liffey water could be attributed to differences in chemical composition, pointing to potential ecological risks specific to the water source (Figure 7).

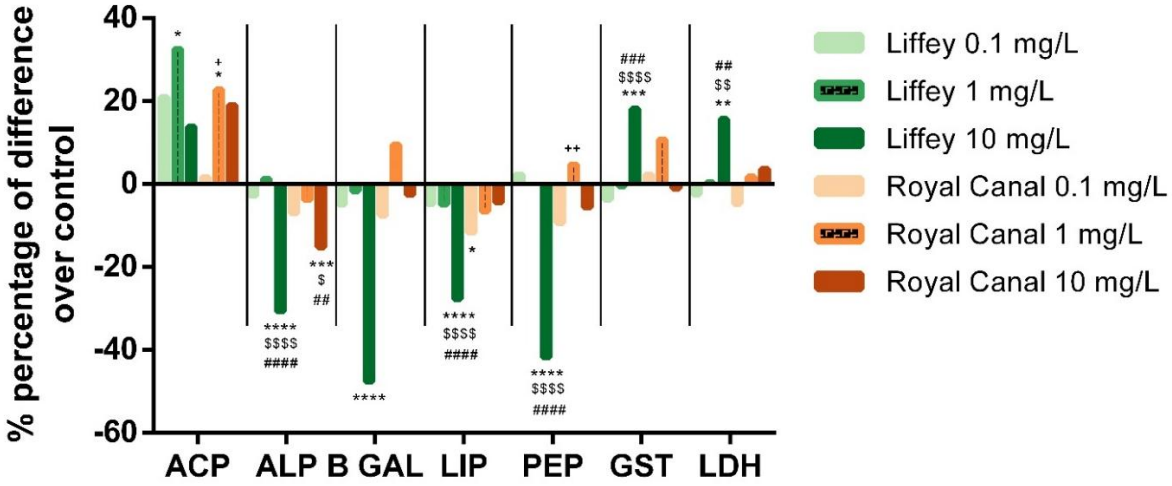


Figure 7. Biochemical markers of daphnid physiology upon acute exposure to the chemical's mixture at concentrations 0.1 mg/l, 1 mg/l and 10 mg/l. Data represent the % percentage of difference in the enzymatic activity over the unexposed control (river water). Symbols *, +, $, and # indicate statistically significant difference by Two-Way ANOVA corrected with Tukeys's multiple comparisons test against the unexposed control with *p*-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****), 0.1 mg/l vs 1 mg/l, 0.1 mg/l vs 10 mg/l and 1 mg/l vs 10 mg/l in each river, respectively. Similarly, the same applies for the other symbols (+, $, and #).

Chronic effects of 1:1:1 mixture on daphnids in river water

A distinct pattern of impact was observed to be caused by the two rivers, indicating a difference in their chemical composition (Figure 8). Specifically, during the first 21-day period, exposure of daphnids to water from the Royal Canal decreased the activity of ACP and LDH by 28% and 51%, respectively, while it increased the activity of PEP by 85% compared to the Liffey. During the 21-day period of the 2nd generation, water from the

Royal Canal decreased the activities of ACP, ALP, PEP and GST by 25%, 22%, 15% and 19%, respectively, compared to the Liffey River.

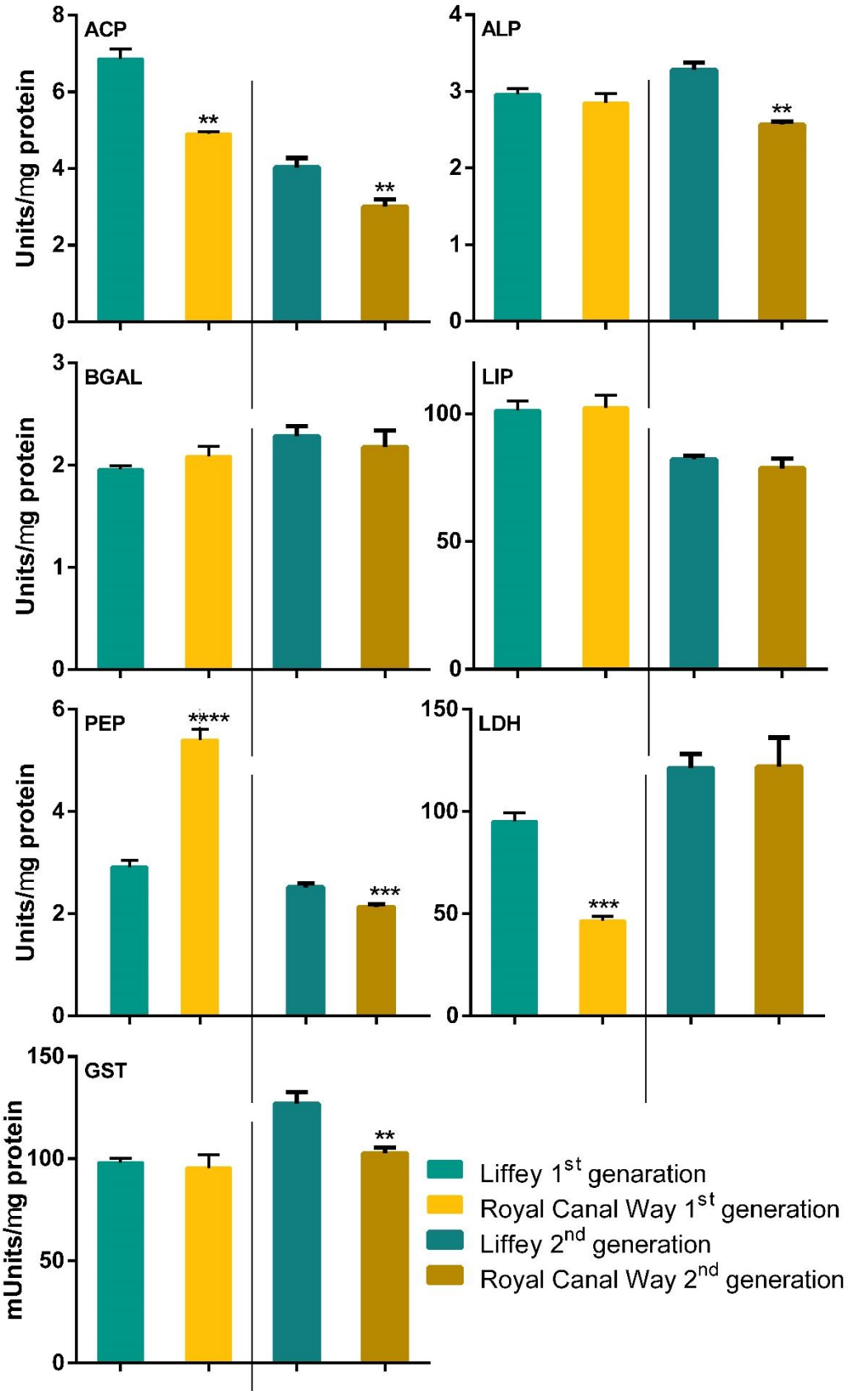


Figure 8. Biochemical markers of daphnid physiology upon chronic exposure for the 1st generation and 2nd generation to the river water. Data represent mean±standard deviation (N=4) of enzyme activity. Enzyme activity was expressed as units/mg protein for ACP, ALP, BGAL, LIP, PEP and LDH, and as munits/mg protein for GST. Statistically significant by Student's *t*-test denotes significant difference in comparison to Liffey (*) with *p*-values of $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

Concentration-dependent effect for each river

Chronic and transgenerational exposures of daphnids to 0.1 mg/l and 1 mg/l mixture, using Liffey River water as media, led to consistent and significant physiological disruptions (Figure 9). Specifically, in the first generation, both ACP and ALP were significantly decreased at 0.1 mg/l (-17%, -5%) and 1 mg/l (-9%, -7%), indicating suppression of key metabolic processes. The activity of GST was significantly increased at 1 mg/l by 7%, indicating induction of detoxification processes (Dasari et al., 2017). Moreover, the activity of LDH was inhibited at 0.1 mg/l suggesting potential disruptions of the energy metabolism (Galhano et al., 2022). During the second generation the effects were more enhanced. The activities of ACP, ALP and PEP were significantly decreased at both concentrations, suggesting potential cumulative effect. Specifically, exposure to 0.1 mg/l decreased ACP, ALP and PEP by 32%, 9% and 8%, respectively, while exposure to 1 mg/l decreased these enzymes by 16%, 13% and 13%, respectively. The effect on the activity of GST was the opposite compared to the first generation, decreased by 0.1 mg/l (-9%) and by 1 mg/l (-14%), possibly indicating potential regulatory collapse under prolonged stress. The activity of LDH exhibited biphasic response, with significant reduction at 0.1 mg/l (-18%) and increase at 1 mg/l (+3%), pointing to a dose-dependent metabolic shift. These findings support a pattern of progressive and concentration-dependent physiological stress in daphnids over subsequent generations.

In *Daphnia* exposed to Royal Canal water, statistically significant enzymatic changes were primarily evident at 1 mg/l, with more extensive disruption emerging in the second generation. In the first generation, ACP and GST were significantly increased at 1 mg/l, by 16% and 9%, respectively, suggesting initial activation of metabolic and detoxification processes (Dasari et al., 2017, Lv et al., 2017). In contrast, ALP was significantly decreased at 1 mg/l by 8%, and the activity of PEP was decreased at the lowest concentration by 9%. In the second generation, enzymatic responses intensified and diversified. ACP and GST remained significantly increased at 1 mg/l, by 23% and 33%, reinforcing the transgenerational persistence of stress-induced enzymatic activation. ALP and PEP were significantly increased by 13% and 15% at 0.1 mg/l, and by 12% and 13% at 1 mg/l, suggesting a rebound or compensatory metabolic activation following earlier inhibition.

Exposure to 1 mg/l significantly suppressed the activity of BGAL (-14%), reflecting continued disruption in carbohydrate metabolism (Nakrani et al., 2025). LIP activity displayed a dose-dependent shift, increasing at 0.1 mg/l (+12%) and decreasing at 1 mg/l (-14%). These findings show a pattern of amplified enzymatic disruption across generations, with Royal Canal water exposure eliciting a broader and more pronounced physiological response over time.

Water sources effects

When comparing the effects of chronic exposure across two river water sources, Royal Canal water elicited broader and more intensified enzymatic disruptions, especially in the second generation. While Liffey exposure led to consistent suppression of ACP, ALP, PEP and GST, its effects were mostly inhibitory and sustained across generations. In contrast, Royal Canal exposure resulted in a more dynamic enzymatic profile, including significant inductions (ACP, ALP, GST, PEP, LIP), and targeted inhibitions (BGAL, PEP in the first generation) indicating complex physiological adjustments. Notably, detoxification (GST) and metabolic enzymes showed intense increases in Royal Canal water, particularly at the highest concentration in the second generation, suggesting stronger or more toxic compounds in that matrix. The emergence of dose-dependent and transgenerational effects in Royal Canal water underscores its potential to result in additional physiological stress, likely affected by its unique chemical background (Figure 9).

While during the acute exposure scenario, Liffey water induced stronger immediate enzymatic responses than Royal Canal water, particularly at the highest concentrations (10 mg/l), this effect was not observed in the chronic exposures. Suppression of ALP, BGAL, LIP, PEP and induction of GST reflected an acute physiological stress response. In contrast, Royal Canal water caused only medium and less consistent changes acutely, with limited dose-dependence. However, this trend reversed in the chronic and transgenerational exposures. Over two generations, Royal Canal water caused broader and more significant enzymatic alterations, including both activation and suppression, particularly at 1 mg/l. Meanwhile, the effects from water of river Liffey remained inhibitory and consistent, with less variation in enzymatic responses. This contrast highlights a key ecological insight: Liffey water may exert stronger short-term toxicity, while Royal Canal water shows more pronounced long-term or cumulative impacts, potentially due to persistent contaminants or interacting background compounds that amplify over time.

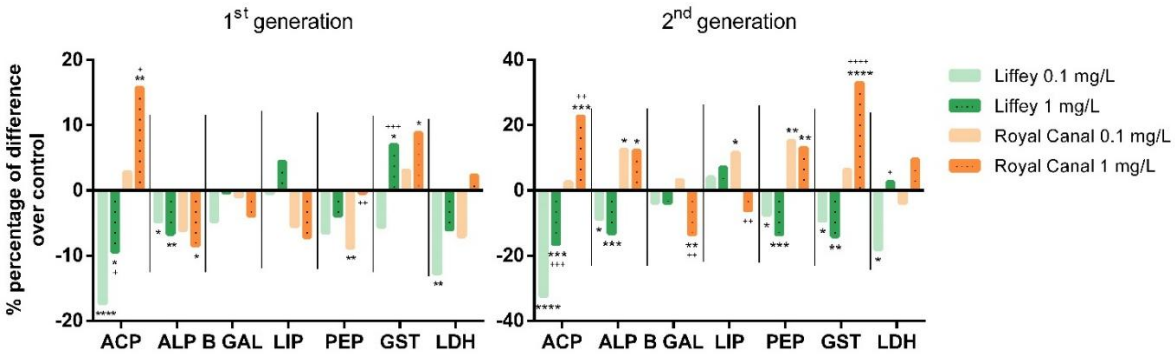


Figure 9. Biochemical markers of daphnid physiology upon chronic exposure, for two generations, to the mixture of chemicals at concentrations 0.1 mg/l and 1 mg/l. Data represent the % percentage of difference in the enzymatic activity over the unexposed control (river water). Symbols * and + indicate statistically significant difference by Two-Way ANOVA corrected with Tukeys's multiple comparisons test against the unexposed control with *p*-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****), and 1 mg/l against 0.1 mg/l in each river, respectively. Similarly, the same applies for the symbol +.

Toxicity evaluations in aquatic environments often depend on endpoints obtained from exposures in reconstituted artificial laboratory water. Nevertheless, these methods may fail to adequately reflect the actual toxicity of pollutants, as natural waters are chemically complex matrices comprising multiple variables that affect the bioavailability and behaviour of pollutants (Harwood et al., 2012). Research has shown that besides physicochemical factors such as pH, salinity, temperature, organic materials, including natural or dissolved organic matter (NOM and DOM) can alter the bioavailability of contaminants and hence their toxic behaviour towards aquatic organisms (Al-Reasi et al., 2012, De Schamphelaere et al., 2004, Wang et al., 2022). The toxicity of permethrin to *D. magna* exhibited significant variability among distinct water sources, impacting its toxicity. These data indicate that results derived only from laboratory exposures using artificial media might underestimate the actual effects of pollutants on aquatic organisms. Consequently, it is significant to develop more representative methodologies to improve ecological relevance of toxicity evaluations (Harwood et al., 2012).

NOM and DOM are essential regulators of the behaviour and toxicity of pollutants in aquatic ecosystems. Organic matter is categorized into two forms: autochthonous, which include organic compounds released or decomposed by aquatic organisms, and allochthonous compounds, primarily consisting of humic substances derived from terrestrial material (Al-Reasi et al., 2012, De Schamphelaere et al., 2004, Wang et al., 2022). Upon release into the environment, pollutants such as nanomaterials (NMs), undergo various alterations affected by parameters such as light, ionic strength, water hardness, pH, salinity, and particularly the presence of NOM. NOM may bond to the surface of nanoparticles, creating an "eco-corona"

that substantially modifies their behaviour, bioaccumulation and toxicity. In freshwater environments, NOM usually derives from soils and plants (Wang et al., 2022). Additionally, according to Kakavas et al., coronas affected differently the toxicity of silver nanoink of several physiological endpoints on *D. magna.* Specifically, while the conditioned "corona" media decreased the mortality caused by silver nanoink, it significantly altered its effects on biochemical markers and metabolomic pathways (Kakavas et al., 2025). Conversely, DOM has proven its ability to eliminate the bioavailability and toxicity of several metals, including copper, to freshwater organisms such as *D. magna*, mostly via complexation (Al-Reasi et al., 2012, De Schamphelaere et al., 2004). It has been reported that DOM can bond with metal ions, restricting their absorption and toxic impact. While copper is a crucial component for aquatic species, it can become toxic when it is present at high concentrations. Sodium concentration and the presence of DOM, considerably affects the harmful effects of copper on freshwater organisms such as daphnids (Al-Reasi et al., 2012, De Schamphelaere et al., 2004). These interactions emphasize the significance of evaluating the toxic effects of pollutants into real ecosystems, since factors such as NOM and DOM can significantly alter their behaviour and toxicity compared to the findings derived from controlled laboratory settings (Al-Reasi et al., 2012, De Schamphelaere et al., 2004, Wang et al., 2022).

Studies on daphnids have shown that water matrices can profoundly affect physiological, biochemical, and transcriptome responses (Barata et al., 2007, Damásio et al., 2008). These researchers documented that *D. magna* exposed *in situ* to Mediterranean river waters displayed significant modifications in enzymatic activities, specifically cholinesterase and antioxidant enzymes, due to exposure to pesticide and industrial effluents. The results showed that natural water matrices can alter the effects of pollutants in ways that may remain undetected in laboratory settings, underscoring the limitations of synthetic media in accurately reflecting the complex stress responses elicited by chemical interaction with natural water constituents. Further research indicated that daphnids exposed to antiretroviral medications in river water increased the activity of detoxifying enzymes to cope with oxidative stress, highlighting the need of incorporating real water samples in toxicity evaluations (Mahaye and Musee, 2022). Additionally, according to Jankowski et al., daphnids exposed to different water sources, such as runoff and wetland effluents, exhibited significant variation on gene expression based on the water source, underscoring the impact of water chemistry on molecular-level physiological responses in organisms (Jankowski et al., 2022).

These findings, together with the results of the present study, emphasize the necessity of including actual environmental scenarios into ecotoxicological testing to enhance the

ecological relevance of risk evaluations. This improves our comprehension of the intricate interactions between pollutants and natural compounds in aquatic environments, facilitating more efficient early detection and management of pollution effects.

Conclusions

The effects of three single stressors and their combined mixture were assessed on daphnids in laboratory and field settings. A combination of mortality, feeding assay, and biochemical assays after acute and chronic exposures revealed distinct toxicity patterns using artificial OECD media and river water from River Liffey and Royal Canal way. During the acute exposure to the mixture, River Liffey caused more intense effects on the enzymatic activities of daphnids, but these effects were less prominent following chronic and transgenerational exposures. Conversely, while Royal Canal way did not cause substantial effects in the acute exposure, it exhibited profound toxicity during the long-term exposure. These data indicate that physicochemical parameters and existing pollutants in natural waters interacted with the introduced mixture altering its behaviour and toxicity towards daphnids. Therefore, this study highlights the need to develop more realistic methodologies incorporating actual water samples when assessing the effects of pollutants, as laboratory testing alone may fail to accurately reflect the impact of contaminants on aquatic life.

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Conflicts of interest

There are no conflicts to declare.

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Chapter 6

Conclusions

The scope of this thesis was to evaluate the adverse effects of emerging contaminants on the aquatic model organism *D. magna*, with a focus on acute, chronic and transgenerational exposures. This research aimed to assess the efficacy of *D. magna* as a sensitive and ecologically relevant early-warning bioindicator in freshwater ecotoxicology. Various categories of pollutants were investigated in a number of studies, including chemical mixtures, novel solvents (ILs), and pharmaceuticals (specifically NSAIDs), all of which cause a growing ecological concern due to their widespread presence in aquatic ecosystems and their potential for triggering complex biological responses even at low concentrations. While these substances belong to different categories of chemicals with distinct modes of action, they challenge standard toxicological frameworks, which frequently rely on acute exposures of single compounds. Conventional methods for water pollution monitoring typically depend on short-period of exposure to single stressors using mostly phenotypic endpoints such as mortality, reproduction or survival (Ahmed, 2024, Connon et al., 2012, Fröhlich, 2017). Nonetheless, these techniques cannot sufficiently demonstrate the complexity of the chemical interactions in an aquatic ecosystem, where the organisms are exposed to complex mixtures for extended periods of time (Backhaus and Faust, 2012). Therefore, these individual studies aimed to not only examine the effects of each pollutant, but also to provide a more in depth understanding of how environmentally relevant concentrations of either single or combined chemicals can affect key physiological and biochemical endpoints of daphnids across generations. By integrating chronic and transgenerational exposures with molecular endpoints, such as enzymatic activities and metabolomics, this thesis provides a holistic perspective on the biological implications of pollution. Even though the pollutants studied had different characteristics and experimental setups, all of them offered an extensive overview of the risks that emerging contaminants pose to aquatic life.

Mechanistic insights into toxicity

The acute and chronic effects of pollutants, both individually and in mixtures, were examined in *D. magna* using a combination of phenotypic endpoints (mortality, reproduction, survival, feeding), simple biochemical assays for key enzyme activities

(kinetic determination of activity), and metabolomic analysis. Although phenotypic endpoints are established in ecotoxicology, they frequently overlook subtle, early, or reversible responses to stress (Fröhlich, 2017). Molecular markers, including enzyme activity and metabolomics elucidate the biochemical pathways and molecular disruptions induced by exposure to pollutants and provide essential insights that phenotypic data alone cannot offer. This study assessed biomarkers such as phosphatases, βGAL, LIP, PEP, GST and LDH. These enzymes participate in essential biological processes including digestion, detoxification, and energy metabolism (Briolay et al., 2021, Dasari et al., 2017, Galhano et al., 2022, Lv et al., 2017). Numerous contaminants from this study are known to cause oxidative stress by the generation of ROS, resulting in lipid and protein peroxidation, as well as DNA damage (Barr et al., 2007, Chang and Andreotti, 2023, Cui et al., 2019, Yuan et al., 2012, Zhao et al., 2020). As a result, lipid peroxidation affects in multiple ways different enzymes, including phosphatases and LIPs. Protein peroxidation impacts the activity of proteins such as PEPs, βGAL, LIPs and phosphatases. GST reflects detoxification responses, while LDH is indicative of shifts in energy metabolism (Michalaki et al., 2022). This evaluation of enzyme activities, conducted across acute, chronic and transgenerational exposures, demonstrated pollutant-specific and generation-dependent effects. Enzymatic perturbations were frequently observed even without apparent phenotypic alterations, underscoring their use as sensitive early markers of stress and toxicity.

Major findings

Transgenerational studies are becoming more prevalent in ecotoxicology, since they offer insights into the long-term effects of pollutant exposures which typical acute or one generation chronic exposure cannot capture (Padilla Suarez et al., 2023). (Bhandari et al., 2015) reported that exposure of medaka fish to bisphenol A (BPA) and 17α-ethinylestradiol (EE2) at concentrations of 100 µg/l and 0.05 µg/l, respectively, led to reproductive impairments, including reduced fertilization rates and increased embryo mortality. These effects appeared in the third generation and persisted through the fourth and fifth generations. Similarly, transgenerational exposures of rainbow trout to EE2 decreased male progeny survival in the second generation, but this impact was not detected in the third generation (Brown et al., 2009). In the case of invertebrate models, such as *D. magna*, there are reports showing the presence of transgenerational effects, but the literature remains limited. Exposure of daphnids to microplastic fragments, BP-3 leachates and a mixture of them for one generation, followed by three recovery generations, led to persistent effects. Aside of the mortality, which was recovered in the fourth generation, somatic growth and

reproduction remained decreased in the fourth generation compared to the control, highlighting that adverse effects could persist even after the removal of the stressor (Song et al., 2022). These transgenerational effects could result from pollutant-induced developmental changes, toxicant transfer from mothers to offspring, or the integration of multiple sublethal effects, or epigenetic alterations that persist across generations even after the pollutant has been removed (Castro et al., 2018, Michalaki et al., 2025).

One of the key findings across the studies was the confirmation of transgenerational effects caused by chronic exposures to pollutants. In the second chapter of chemical mixtures (Chapter 2B), daphnids exposed to several environmentally relevant concentrations of an eight-chemical mixture over five generations exhibited sustained changes in enzymatic activities and metabolic pathways, in the first and fifth generations. However, these toxic effects of the exposure were milder in the third generation. A plausible explanation for this non-linear pattern is the development of adaptive responses within the population. This resistance may occur either due to phenotypic acclimation, meaning that the animals adjust to chronic exposure to the specific pollutants, or due to genetic adaptation, in which the exposed population becomes less sensitive to the pollutants over generations (Dietrich et al., 2010). Despite this adaptation though, animals in the fifth generation exhibited significant dose-dependent alterations on the enzymatic activities and metabolic pathways. Similar transgenerational effects were observed following exposure to NSAIDs even at a low concentration of 5 μg/l (Michalaki and Grintzalis, 2023, Michalaki et al., 2025). In the fourth chapter regarding the impacts of NSAIDs (Chapter 4A), daphnids exposed to indomethacin, ibuprofen and their 1:1 mixture, at 1 mg/l, for two generations, followed by a recovery generation, showed more pronounced impacts in the second generation than in the first, with near-complete recovery by the third generation. In the follow-up study (Chapter 4B), chronic exposure to chemical and commercial NSAIDs at the environmentally relevant concentration (5 μg/l) across four generations, followed by a recovery (fifth) generation, revealed complex patterns of effect between chemical and commercial forms of the NSAIDs. The chemical forms did not induce substantial effects during the fourth generation, yet recovery was incomplete in the fifth. Conversely, the commercial NSAIDs exhibited stronger effects in the fourth generation, but animals recovered completely in the fifth. This divergence suggests that even subtle differences in chemical composition can lead to different transgenerational outcomes. Notably, only animals exposed to the chemical mixture showed signs of recovery, further underscoring the complexity of mixture toxicity. Finally, in the river water study (Chapter 5), *Daphnia* exposed to river water samples, spiked with a three-chemical mixture, exhibited enhanced adverse effects in the second generation

daphnids, indicating that pollutant-induced stress was transferred to subsequent generations even in more environmentally realistic exposure scenarios. Collectively, these findings emphasize the need to incorporate chronic and transgenerational exposures into ecotoxicological assessments. Traditional approaches relying on acute or single generation chronic exposures are likely to underestimate the real ecological risks posed by pollutants.

While prior research was focused on the transgenerational effects of single chemicals, this thesis advanced the field by systematically addressing the chronic and transgenerational effects of complex chemical mixtures at environmentally relevant concentrations on *D. magna* (Maggio and Jenkins, 2021, Wang et al., 2024). Unlike previous research, the individual studied of this thesis investigated the effects of up to five chronic generations using phenotypic endpoints, biochemical markers and metabolomic analysis.

Additionally, metabolomic analysis offered an enhanced, pathway-level explanation of the metabolic alterations, confirming the patterns observed in the biochemical assays. In the acute exposure (Chapter 2A), daphnids were subjected to three doses of an eight-chemical mixture, revealing significant dose-dependent effects, that aligned with biochemical data, indicating potential synergistic effect. In the chronic and transgenerational exposure of daphnids to the eight-chemical mixture (Chapter 2B), metabolomic analysis was conducted in the fifth generation. These results revealed dose-dependent effects even at the lower concentrations (1 ng/l, 10 ng/l, 100 ng/l), indicating that even environmentally relevant doses can induce adverse effects over time. Furthermore, the second NSAIDs study (Chapter 4B) demonstrated significant effects on the enzymatic activities caused mostly by the commercial NSAIDs, which were also confirmed through metabolomic analysis. The metabolomics data revealed a distinct pattern of impact with the commercial NSAIDs to have significantly affected more metabolic pathways than the chemical NSAIDs or their mixture, emphasizing on the different outcomes that distinct forms of pollutants can cause.

While metabolomic analysis was not conducted in recovery generations, it was used in key exposure periods, such as acute and later generations of chronic and transgenerational experiments. The findings offered comprehensive insights into disruptions in metabolic pathways and supported the patterns seen in biochemical assays. This combination of methods, when analysed alongside with chronic and transgenerational exposures, led to a more profound understanding of the progression of toxicity overtime, uncovering trends such as delayed effects, adaptation, and accumulating metabolic stress. Integrating chronic and transgenerational exposures scenarios with enzymatic and metabolomic analysis was essential for discovering complex, persistent, and action-specific effects of pollutants. This multifaceted approach improved our understanding of how contaminants interfere with

biological systems, providing a more comprehensive perspective on environmental toxicity than conventional endpoints alone.

Complexity in environmental exposure

The assessment of the adverse effects of chemicals on aquatic organisms has become significantly challenging due to their continuous exposure to a mixture of chemicals rather than single stressors (Backhaus and Faust, 2012). Particularly, in realistic scenarios, these organisms are subjected to prolonged exposure to complex mixtures, which may contain a variety of chemicals, such as metals, pharmaceuticals, herbicides, each having a distinct mode of action. In addition to this, the concentrations of the mixtures in the environment are not always steady (de Zwart et al., 2017).

Both studies regarding the effects of the eight-single stressors and their mixture revealed significant insights into mixture toxicity. Specifically, in the first study (Chapter 2A), three mixtures corresponding to 10%, 20% and the 30% of the eight chemicals' EC_5 showed a clear dose-dependent effect with increasing concentrations causing more pronounced responses than those caused by individual stressors (Michalaki et al., 2022). Additionally, to mimic environmentally relevant exposures (Chapter 2B), chronic and transgenerational exposure of daphnids to the eight-chemical mixture at concentrations ranging from 1 ng/l to 1000 μg/l, over five generations, revealed a dose-dependent pattern of effect, with 100% mortality observed at the highest concentration by the fourth generation. Even though prediction models for mixture toxicity (CA and IA) were not applied in both studies, the observed responses suggest potential synergistic interactions among the chemicals.

Ionic liquids, often described as "green solvents", exhibited notable adverse effects on daphnids following both acute and chronic exposures (7, 14 and 21 days) (Michalaki et al., 2023) (Chapter 3). The effects of the mixture were studied only during the chronic exposures, in which the enzymatic activities were evaluated at each time point. During the first seven days of exposure the mixture affected significantly most enzymes (ALP, ACP, βGAL), which were affected by almost all individual BMIM ILs. Following 14 days of exposure, the mixture did not affect any enzyme, despite moderate effects observed from individual ILs. A plausible explanation of this effect might be related to physiological changes during the reproductive phase, potentially increasing resilience to chemical stress (Ebert, 2005). After the 21-day exposure period, the mixture altered the activity of almost all enzymes, except peptidase. The overall effect of the mixture throughout the chronic exposure suggests complex interactions among the BMIM ILs. Even though no prediction model was applied,

the non-linear responses point to synergistic or antagonistic effects among the individual compounds, highlighting the complexity of mixture toxicity.

Pharmaceuticals, particularly indomethacin and ibuprofen when they enter the aquatic environment are not chemically stable and they can be broken down due to microbial action. However, because of their continuous introduction into the aquatic environment they have been described as pseudo-persistent emerging contaminants, even if they are present in very low concentrations (ng/l or μg/l) (Michalaki et al., 2025, Mussa et al., 2022). In the first NSAID study (Chapter 4A), the 1:1 mixture was more toxic compared to the individual NSAIDs, and that effect was observed in the toxicity curves and supported further by the feeding assay and the biochemical assays (Michalaki and Grintzalis, 2023). A similar pattern emerged in the second NSAID study (Chapter 4B), where the mixture induced pronounced effects even at much lower concentrations of 5 μg/l (Michalaki et al., 2025). Given that both compounds are NSAIDs with similar modes of action, this effect was expected and suggest a synergistic interaction between them. Beyond confirming mixture effects, the second study also underscored the importance of applying multiple endpoints to evaluate toxicity. Particularly, the toxicity curves and the survival assays, two phenotypic endpoints, revealed that the chemical form of NSAIDs and their mixture were more toxic than the commercial form. Conversely, the biochemical assays and metabolomic analysis showed that the commercial form of these drugs and their mixture was more toxic than the chemical form. These contradictory results highlight the limitations of relying solely on phenotypic endpoints and emphasize the need for integrated approaches that combine phenotypic, biochemical, and metabolomic data to achieve a more comprehensive understanding of toxicological effects.

Finally, in the environmental project of this thesis (Chapter 5), acute exposure of neonates to a three-chemical mixture at 0.1 mg/l, 1 mg/l and 10 mg/l induced adverse and dose-dependent effects on the enzymatic activities. Notably, the individual chemicals at 1 mg/l caused minimal to no effect after acute exposure, underlining a potential synergistic response. Similar effects were observed in the chronic single-generation exposure to the mixture at 0.01 mg/l, 0.1 mg/l and 1 mg/l, and in the feeding assay following acute exposure. These results showed that even mixtures of chemicals with limited toxicity as individuals can produce significant effects when combined.

These findings highlighted the complicated nature of environmental pollution, where organisms are being exposed to very low levels of complex mixtures rather than single chemicals in high doses (Heys et al., 2016). This research demonstrated that toxicity frequently occurs via interactions among many chemical components, each one of them

having different modes of action. The incorporation of diverse endpoints, including phenotypic, enzymatic and metabolomic approaches, was crucial in identifying critical biological disruptions that could otherwise go unnoticed. Additionally, inconsistencies among several endpoints, such as mortality, survival, and biochemical markers, underscore the challenges of depending on single, and most importantly, phenotypic approaches. Overall, these findings support the necessity for a more integrated approach in environmental risk assessment, which incorporates mixture effects and low-dose exposures. This change is necessary to better reflect real-world conditions and effectively safeguard aquatic ecosystems.

Refining ecotoxicological approaches for environmental relevance

Environmental realism and experimental design

Nowadays, it is crucial to adjust the old and develop new methodologies that would more effectively contribute to water pollution monitoring (Ahmed, 2024). For this to be done, ecological diversity and environmental realism should be reflective on these approaches. A significant finding emerging from the existing literature is the critical impact of test media, particularly the differences between standardized laboratory media and actual water samples (Barata et al., 2007, Damásio et al., 2008, Jankowski et al., 2022). Although artificial laboratory media provide experimental control and reproducibility, they do not reflect the physicochemical diversity of actual aquatic matrices, where salinity, pH, dissolved organic matter, suspended particles, and microbe populations significantly alter the effects of pollutants on aquatic organisms (Ahmed, 2024). Exposure of animals in actual water samples, spiked with pollutants, can more accurately reflect field exposures where the pollutants interact with the existing compounds in the water, affecting toxicity and transgenerational effects. The results indicated that exposure to the same chemicals can have varying physiological responses based on the exposure media, highlighting a significant oversight in current pollution assessment methods.

Incorporating transgenerational and molecular endpoints

In addition to this environmental realism, there must be a comprehensive approach to methodology. An essential implication of these findings is the necessity to incorporate transgenerational studies and molecular endpoints into water pollution monitoring (Michalaki et al., 2025). Traditional methodologies often underestimate delayed or inherited effects, which this thesis has demonstrated to occur even at low concentrations and persist across generations (Ahmed, 2024, Padilla Suarez et al., 2023). Significantly, perturbations

were detected at enzymatic activities and metabolomic pathways prior to any phenotypic changes, indicating biochemical and pathway-level alterations may act as early indicators of stress. These molecular markers provide predictive significance and precision that may enhance risk assessments (Escher et al., 2021, Tkaczyk et al., 2021). Therefore, authorities must extend their focus beyond phenotypic markers and implement transgenerational studies including biomarkers of oxidative stress, metabolic perturbations, and pathway alterations. This adjustment would enhance sensitivity and aid in early detection of pollution before the ecological damage has already occurred and is irreversible (Escher et al., 2021).

Addressing the complexity of mixture toxicity

Although single-chemical testing is more used in current methodologies, actual exposures consist of complex chemical mixtures, and this research shows that these mixtures might not always have additive effects. Organisms are exposed to persistent, low concentrations of mixtures of pollutants with different and often synergistic modes of action (Heys et al., 2016). Research studies regularly showed that exposure of organisms to mixtures resulted in synergistic effects, including increased alterations in enzymatic activities and transgenerational impairments, even though the individual chemicals appeared less toxic (Wagner et al., 2018). However, in realistic scenarios mixtures are usually consisted of chemicals with diverse mechanisms, making prediction more complicated (Altenburger et al., 2012). Consequently, traditional models such as CA and IA models would not be able to sufficiently capture the interactions that take place among these various types of chemicals (Heys et al., 2016). Furthermore, these impacts were more intense when actual water samples were used as media, where existing components of the water can affect the exposure. Additionally, the combination of complex media and chemical mixtures can cause unpredictable responses to aquatic organisms, emphasizing the need for mixture testing not only in standardized laboratory media, but also in actual water matrices as well.

Strengths and limitations of the present study

This research is characterized by multiple methodological features that improve its ecological relevance and mechanistic depth. The incorporation of chronic and transgenerational exposure scenarios allowed for the detection of delayed and cumulative effects, which are frequently overlooked by traditional acute or single generation chronic exposures. These techniques shed light on how chronic and transgenerational exposures to pollutants affects the physiology of animals across multiple generations, even without apparent phenotypic effects. The selection of *D. magna* increased the ecological significance and efficiency of this study. *D. magna*, a well-known model species in freshwater

ecotoxicology, presents numerous advantages. Some of the advantages are its short life cycle, easy to culture in the laboratory, and its parthenogenetic cycle, which minimizes genetic variability and offers controlled consistent cross-generational research (Tkaczyk et al., 2021). Additionally, the detailed evaluation of toxicity was performed using a multi-endpoint approach. This approach included traditional phenotypic endpoints, such as mortality, survival, reproduction, alongside the feeding assay, biochemical assays of key enzymes (such as phosphatases, βGAL, LIP, PEP, GST and LDH) and metabolomic analysis. Collectively, these endpoints offered a thorough comprehension of toxicological implications across physiological levels. This integrated methodology provided a systems-level perspective on pollutant stress that supports the argument for mechanistically based regulatory testing.

While this research presented significant advancements in ecotoxicological evaluation, several limitations must be recognized to put its findings into perspective and guide future research. The variation in experimental designs, including discrepancies in the mixture's composition, exposure length and timing of metabolomic sampling, may limit direct comparison among other studies (Kakavas et al., 2023, Kakavas et al., 2024). This shows the difficulty of assessing several kinds of chemicals under realistic conditions while maintaining experimental control. Furthermore, while the incorporation of actual water enhanced ecological relevance, the tests were performed under controlled laboratory conditions that cannot entirely mimic the dynamic characteristics of real ecosystems. Changes in temperature, light, pH, seasonal variations, and biotic interactions may significantly impact how the animals react and potentially modify toxicity effects. These interactions are challenging to quantify in laboratory studies. Subsequent research should thus explore mixed methodologies, such as mesocosm or semi-field studies, that combine environmental diversity with mechanistic precision. Standardized transgenerational and multiple exposure types, particularly under varying abiotic settings, such as temperature, light, pH, salinity, is crucial to enhance the ecological relevance and predictive accuracy of toxicological evaluations.

Future directions

This thesis enhanced the comprehension of acute, chronic and transgenerational effects of complex pollutant mixtures, different forms of chemicals and low environmentally relevant concentrations. Additionally, it identified critical topics for future study to further improve the ecotoxicology and environmental risk assessment. These future directions are essential

to bridge existing gaps, enhance mechanistic knowledge, and increase the ecological relevance of current testing frameworks.

Expanded multigenerational exposures

An important field of future research involves assessing the effects of pollutants on organisms beyond the fifth generation. The majority of recent research, including those presenting in this thesis, focus on a limited number of generations (Nigro et al., 2025, Vandegehuchte et al., 2010). However, persistent pollutants, especially those affecting epigenetic pathways may cause more significant effects in later generations (Padilla Suarez et al., 2023). Monitoring responses across multiple generations will enhance our understanding about whether these alterations are revolutionary changes, epigenetic reprogramming, or reversible physiological adaptation.

The integration of multi-omic approaches

Going forward a crucial step would be the integration of gene expression data into experimental designs (Padilla Suarez et al., 2023). Even though enzymatic biomarkers and metabolomics offered a mechanistic insight into this thesis, they show later reactions to the stress. Transcriptomic profiling, using quantitative PCR (qPCR), RNA sequencing, or microarray analysis, enable the identification of early regulatory alterations in stress-related pathways, such as oxidative stress response, xenobiotic metabolism and mitochondrial function (Ahmed, 2024, Aksakal et al., 2025). The identification of certain genes with affected expression over generations may function as early-warning biomarkers and as molecular markers linking chemical exposure to phenotypic results. Furthermore, epigenetic studies, such as analysis of DNA methylation and acetylation, may explain how pollutant exposures cause heritable alterations, clarifying the persistence of certain effects in recovery generations, where the chemical has been removed. Integrating multi-omics techniques such as transcriptomics, epigenomics, metabolomics, proteomics allow an extensive and system-level evaluation of the effect of chemicals in key species such as water fleas. These responses can be used as sensitive metrics for pollution monitoring, thus highlighting daphnids as equivalent to canaries in the coal mine for the early prediction of pollution (Gruszczynska et al., 2024).

Multispecies approach

The expansion towards several aquatic model organisms is critical for enhancing the environmental relevance in ecotoxicological pollution assessment (Heys et al., 2016). While *D. magna* serves as a fundamental, well-defined and sensitive model organism in aquatic toxicology, characterized by many benefits, it is crucial to note that its sensitivity

significantly varies among different categories of pollutants. This variability can have important consequences for risk assessment and therefore for the environmental protection (Abdullahi et al., 2022). Regarding the neonicotinoid insecticides, daphnids exhibit higher tolerance compared to other aquatic invertebrates, such as aquatic insects. Species from the orders *Ephemeroptera* and *Diptera* are more sensitive to neonicotinoids than daphnids which are among the least sensitive bioindicators (Morrissey et al., 2015). Therefore, risk assessments based only on *D. magna* or other bioindicators fail to protect the aquatic environment. On the contrary, *D. magna* demonstrates its value as bioindicator for broad-spectrum of contaminants due to its high sensitivity to chemicals such as metals, ionic liquids, and pharmaceuticals (Siciliano et al., 2015). The difference in sensitivity among bioindicator species highlights one of the limitations of the current study; while daphnids provide significant insight into the toxicity of mixtures, metals, ionic liquids and pharmaceuticals, relying solely on this species may not capture the full range of ecological risks, especially for pollutants where other taxonomic groups show higher sensitivity. Future research should focus on the integration of diverse bioindicators with varying physiology and habitats, to get a more in depth understanding of the pollution effects across various organisms, habitats and trophic levels. This multispecies approach is further supported by growing evidence from evolutionary biology and genomics (Colbourne et al., 2015, Colbourne et al., 2022). The field of Phylogenetic toxicology highlights the evolutionary conservation of genes and biological processes involved in reactions towards chemical stressors and supports the incorporation of numerous model organisms in ecotoxicology. More than 70% of gene families linked to human diseases are conserved across several animal species, emphasizing the importance of using vertebrates and invertebrates to assess the toxicity of pollutants. Even though invertebrates are evolutionary more distanced from humans, crustaceans share more ancestral gene families with humans than insects. This evolutionary relationship allows the use of *Daphnia sp.* and other model organisms in toxicological evaluations (Colbourne et al., 2015, Colbourne et al., 2022). This enables the investigation of modes of action for several chemicals as well as critical steps in toxicity pathways, which help improving both environmental and health risk assessments (Colbourne et al., 2015). Among aquatic invertebrates, species such as *Chironomus riparius* and *Lymnaea stagnalis* provide alternative perspectives. *C. riparius*, a benthic organism, is a key bioindicator for assessing pollutants that are found in sediments, and has been extensively used in studies on developmental, reproductive, and endocrine disruption. The larvae stage is highly vulnerable to environmental triggers, making it suitable for identifying subtle, sublethal impacts (Rigano et al., 2025). *L. stagnalis*, a freshwater snail, offers additional

diversity by its extended lifetime, and ability to exhibit neurotoxic and reproductive responses (Capela et al., 2024).

Bivalve mollusks, including *Dreissena polymorpha* (zebra mussel) and *Mytilus galloprovincialis* represent an additional category of key bioindicators of water pollution (Arrigo et al., 2024, Karatayev and Burlakova, 2025). Mussels, just like daphnids, are filter-feeders which continuously process substantial volumes of water, serve as effective indicators of waterborne pollutants, such as metals, pharmaceuticals, and endocrine-disrupting compounds (Dame, 1993). They also have the ability to accumulate toxins in tissues and that makes them suitable for evaluating bioaccumulation effects. Additionally, endpoints including filtration rate, lysosomal membrane stability, and biomarker responses (DNA damage, oxidative stress, and enzyme activity) can be used to evaluate the stress caused by pollutants (Belavgeni and Dailianis, 2017). Furthermore, these organisms have comparatively extended lifespan which makes them appropriate key species for chronic and transgenerational studies.

Additionally, vertebrate models such as zebrafish (*Danio rerio*) and Japanese medaka fish (*Oryzias latipes*) offer a better understanding on the effects of pollutants. Zebrafish has been extensively used since it has translucent body, fast development, and detailed annotated genome, benefits which make studies to focus on assessing its developmental behaviour, tissue specific toxicity and responses to oxidative stress and DNA damage (Diogo et al., 2025). Moreover, analysing and comprehending its behavioural characteristics provides essential insights into the underlying neural pathways, physiological biomarkers, and the genetic foundations of both normal and affected brain function (Kalueff et al., 2013). Studies on medaka fish improve research on zebrafish, specifically in the areas of reproduction, genotoxicity, and exposure to mildly saline habitats. The Japanese medaka fish is a well-established model organism in developmental and evolutionary biology, because of its well-characterised genome, ease of laboratory culture, and adaptability to environmental conditions (Dasmahapatra et al., 2023, Yao et al., 2010). Medaka is a small fish typically found in gently flowing rivers and waterways, with short reproductive cycle and adaptability to saltwater environments. These traits make medaka an ideal organism for evaluating the effects of pollutants through chronic and transgenerational exposures. Both species provide detailed evaluation of gene expression, chemical bioaccumulation in specific organs, and histopathological alterations (Dasmahapatra et al., 2023, Yao et al., 2010).

A comprehensive multispecies approach combining both invertebrates and vertebrates which occupy a variety of habitats can uncover a wide range of pollution effects, including those that may be missed by single species methodologies. This ecological diversity offers more

comprehensive and accurate risk assessments, increases regulatory significance and guides water quality monitoring practices more effectively.

Complex mixtures and realistic exposure patterns

Another interesting approach involves enhancing the complexity of mixtures and exposure designs. This thesis studied the effects of mixtures comprising various substances with known modes of actions, however, in realistic scenarios mixtures involve numerous compounds along with their transformation products making their effects even more complex and unpredictable (Heys et al., 2016). Moreover, organisms in natural ecosystems are rarely exposed to consistent conditions. Instead, they experience irregular and inconsistent exposure patterns caused by parameters such as precipitation, agricultural runoff, effluent release, and seasonal variations (Ahmed, 2024). Integrating exposure designs that mimic realistic conditions would improve the ecological accuracy of laboratory studies.

The use of AI in toxicology

However, studying the effects of mixtures consisted of hundreds of chemicals, or alternating parameters each time is not always feasible. To address this escalating complexity, artificial intelligence (AI) methods are gaining significance in toxicology (Kleinstreuer and Hartung, 2024). AI and machine learning methods can be trained on extensive ecotoxicity datasets to predict the effects of chemical mixtures, even in the absence of actual data (Ahmed, 2024). These models can detect potential synergistic or antagonistic effects, select combinations and formulate hypotheses for focused testing. AI allows the prediction of hazardous effects using minimum data, offering to the researchers the ability to evaluate hundreds of potential combinations more efficiently than conventional testing methods. When combined with molecular endpoints, AI can assist in identifying how exposure to mixtures impact particular biological pathways or life-history features (Hartung and Kleinstreuer, 2025, Kleinstreuer and Hartung, 2024). AI-assisted research can replicate multidimensional interactions, providing predictive insights that would be too complicated using experimental techniques alone. Even though there are still challenges when it comes to the use of AI in toxicology, it also offers valuable opportunities, such as following the 3R's principles (Hartung and Kleinstreuer, 2025, Kleinstreuer and Hartung, 2024). Collectively, these methodologies have the potential to transform mixture toxicology by integrating scientific knowledge with the reality of environmental exposure.

Field-based exposure studies

Regarding the study of the impact of river water, increasing the number and variety of sampling sites could provide a more accurate understanding of the environmental pollution that occurs across freshwater systems (Ahmed, 2024, Moro et al., 2024). The present study involved the collection of water samples from six different rivers across Dublin. Nevertheless, exposure of daphnids to water from four of these rivers resulted in 100% mortality within around four days. This might mean that either the selected organism cannot be used for this type of study, it is too sensitive and therefore should be replaced, or that some physicochemical characteristics, such as high salinity or pH, may have had an impact on the toxicity masking the effects of pollutants. To address this, future studies could consider two alternative approaches. One approach would be to dilute the river water sample with standards (bottled water or artificial laboratory media) which would minimize the concentration of these naturally occurring factors but also dilute the pollutants of interest. More targeted option would be to filter the river water, using solid-phase extraction (SPE) or passive filtering, to retain anthropogenic pollutants (metals, organic compounds, pharmaceuticals) in the filter while removing salts and other naturally occurring but hazardous ions (Cheng et al., 2024, Yu et al., 2024). Following the latter option the retained filter could be eluted and reconstituted in a controlled medium. This would ensure that the effect of the exposure primarily reflects on the presence of pollutants rather than the water chemistry of the river. By following this methodology, it would be possible to incorporate additional river sites into future tests, which would result in a more precise evaluation of the effects of pollutants under conditions that are both biologically and environmentally relevant.

Mesocosms

Another option for future study would be the use of mesocosm. The incorporation of mesocosm experiments into ecotoxicological studies is an important advancement in overcoming the limitations of conventional laboratory methods (Heys et al., 2016). Although laboratory conditions provide significant mechanistic insights into the effects of pollutants, ensure repeatability and control, they usually do not mimic the environmental complexity and ecological interactions found in real ecosystems (Macaulay et al., 2025). As a result, this may lead to an oversimplified comprehension of the effects of pollutants, as the broader ecological implications are not captured by single-species responses to individual stressors (Kattwinkel et al., 2016). (Li et al., 2025) reported that controlled laboratory studies often miss important aspects, including the variety of species, physicochemical properties, and

chronic exposure, all of which substantially affect toxicity results in real natural settings (Li et al., 2025).

Mesocosm experiments serve as an effective intermediate between laboratory and field studies. They can be either indoor or outdoor. Indoor mesocosms offer the advantage of the controlled conditions, while the outdoor mesocosms reflect better the realistic environmental conditions (Macaulay et al., 2025). This kind of exposures offer the ability to evaluate both direct and indirect the impact of pollutants on many biological systems, including individuals, populations, and communities. Additionally, they provide the ability to observe delayed, cumulative and persistent effects over extended periods of exposures. Raman et al., stated that aquatic habitats consist of complicated mixtures of chemicals and a basic evaluation of their effects as individuals on single organisms fails to accurately represent the reality of environmental pollution (Vasantha Raman et al., 2024). Conversely, mesocosms offer a realistic approach for assessing the toxicity of chemical mixtures under less artificial biological conditions.

Moreover, mesocosm studies incorporate chronic exposures for extended periods which together with the more realistic conditions offer robust datasets that represent actual ecosystem dynamics. These datasets substantially decrease the uncertainty linked to laboratory results, thus enhancing the environmental relevance of risk assessments. As a result, mesocosm exposures serve as a crucial approach for assessing the interactions between pollutants and environmental factors in a way that mimics realistic exposure conditions.

The combination of laboratory toxicity assessments and mesocosm exposures may provide a better understanding of environmental risks (Macaulay et al., 2025). This would not only improve the prediction of ecotoxicological data, but guide regulatory decisions based on knowledge that is experimentally robust and environmentally relevant.

Finally, future research should emphasize the standardization of transgenerational testing, especially for non-standard endpoints such as omics techniques. Standardizing exposure periods, media composition, and developmental stages of the animals would significantly enhance cross-study comparisons. Creating strong statistical models that incorporate multi-level data, including, phenotypic, enzymatic, omics techniques, will improve the prediction ability. Lastly, these adjustments will help to integrate mechanistic knowledge into environmental policy and increase efforts to protect aquatic biodiversity against increasing pollution.

Conclusions

The aim of this thesis was to highlight the importance of *D. magna* as an early-warning bioindicator in freshwater ecotoxicology. Focusing on its sensitivity to chemicals, broad ecological distribution and parthenogenetic cycle, this water-flea provides a reliable and responsive model to assess the complex effects of environmental pollutants.

This thesis showed the unique and crucial role of *D. magna* through a series of independent but interconnected studies investigating the impacts of chemical mixtures, ionic liquids, different forms of pharmaceuticals, as well as the impact of river water samples under acute, chronic and transgenerational exposures. Using phenotypic endpoints, biochemical assays and metabolomic analysis this work captured complex biological responses. These experiments revealed that *D. magna* can respond to adverse effects of pollutants at extremely low and environmentally relevant concentrations, with these responses frequently affected by duration of the exposure, concentration of the pollutant and developmental stage. These findings highlight the ecological significance of the organism and its role in detecting pollution that might be overlooked by conventional methods. Consequently, *D. magna* serves as the "canary in the coalmine" for freshwater ecotoxicology supporting its application in more realistic, and predictive water pollution monitoring approaches.

Following these approaches, it was observed that even at extremely low concentrations, mixtures of pollutants had significant effects on the physiology of daphnids. Similar patterns emerged in the study of the BMIM ILs, where age-specific responses were detected after 7, 14, and 21-days of exposure, despite the absence of transgenerational approach. These results highlight that even in a single generation, daphnids from different age groups could be affected differently by the same chemicals at the same concentration. Regarding the two NSAIDs studies, transgenerational effects were evident, with particularly notable outcomes in the second study, which revealed that different forms of the same chemicals at the same concentrations can cause significantly different biological effects. Finally, in the environmental project of this research, *D. magna* was able to successfully detect toxic effects from river water spiked with a three-chemical mixture at very low concentrations. This further confirmed its role as a sensitive tool for detecting environmentally relevant stress in realistic scenarios. Collectively, these findings highlight the importance of incorporating transgenerational exposure into ecotoxicology research. By using daphnids, heritable and adaptation effects were studied across multiple generations, an approach which is frequently overlooked in conventional methods of toxicity. Moreover, the consistent observation that mixtures often cause more significant or unpredictable effects compared to the individual compounds highlighted the necessity of shifting toward more realistic approaches.

In conclusion, this thesis shows that *D. magna* is a standard test organism. Specifically, it is a powerful model that can be used to understand and predict the complex impacts of chemical pollution on freshwater environments. The insights that were gained from this work not only contribute to the improvement of scientific understanding, but they also support the continued use and development of sensitive biological models to protect aquatic life by predicting ecological damage before it has already occurred and is irreversible.

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YU, X., WANG, Y., WATSON, P., YANG, X. & LIU, H. 2024. Application of passive sampling device for exploring the occurrence, distribution, and risk of pharmaceuticals and pesticides in surface water. *Science of The Total Environment,* 908**,** 168393.
YUAN, C. Y., LEE, Y. J. & HSU, G. S. 2012. Aluminum overload increases oxidative stress in four functional brain areas of neonatal rats. *J Biomed Sci,* 19**,** 51.
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Appendices

Appendix A

A.1 *Daphnia magna* culturing protocol

The culturing scheme on the table below is used to produce clonal populations of mothers who will breed animals for experiments. Set new breeder cultures every Wednesday, Thursday and Friday. Every Friday double-feed the cultures that are younger than 7 days old and the cultures that have new OECD media.

Daphnid age (days)	Procedure	OECD	Yeast	Fresh Algae	Seaweed Extract
1	**Setup new culture**	4 l	2 ml	4 ml	12 ml
2			2 ml	4 ml	
3			2 ml	4 ml	
4			2 ml	6 ml	
5			2 ml	6 ml	
6	Change media	4 l	2 ml	6 ml	12 ml
7	Remove neonates every day and throw them		2 ml	6 ml	
8			2 ml	8 ml	
9			2 ml	8 ml	
10			2 ml	8 ml	
11			2 ml	8 ml	
12			2 ml	8 ml	
13	Change media	4 l	2 ml	8 ml	16 ml
14	Collect and use the neonates every day for culturing or experiments		2 ml	8 ml	
15			2 ml	8 ml	
16			2 ml	8 ml	
17			2 ml	8 ml	
18			2 ml	8 ml	
19			2 ml	8 ml	
20			2 ml	8 ml	
21			2 ml	8 ml	

A.2 OECD standard media

OECD media stocks solutions were prepared fresh and added, according to the table, to a tank filled with 50 L ddH2O. The media were covered with cellophane membrane to prevent contamination and were aerated for at least 48 hours prior to use. The pH of the media was adjusted after the aeration to 7.77.

Chemicals	Quantity
Calcium Chloride	15.29 g $CaCl_2.2H_2O$ in 0.5 l ddH_2O
Magnesium sulphate	6.41 g $MgSO_4.7H_2O$ in 0.5 l ddH_2O
Sodium bicarbonate	3.37 g $NaHCO_3$ in 0.5 l ddH_2O
Potassium chloride	0.3 g KCl in 0.5 l ddH_2O
40 µg/ml Sodium selenite	2.6 ml
1 M HCl	23 ml

A.3 Seaweed extract supplement

As supplement the seaweed extract *Ascophylum nodosum* was added to the 4l of culture OECD media only during the media change (days 6 and 13). The extract (500 g/l) was diluted in ddH_2O to a final absorbance of 8A at 400 nm.

A.4 *Chlamydomonas reinhardtii* culturing

Algae (*Chlamydomonas reinhardtii*) are the primary food for daphnids. They were cultured in *C. reinhardtii* growth medium (CGM), collected by centrifugation at 3,000 rpm for 10 min at RT and re-suspended in ddH_2O to a 7A dilution at 440 nm (equals to 6.1M cells/ml).

A.5 Chlamydomonas reinhardtii growth medium (CGM)

Using a volumetric cylinder, add to a Duran bottle 5.25 l ddH_2O and a magnet. Under continuous stirring add the following volumes.

Stock	Concentration g/l	ml to add
NH_4Cl	20	140
$MgSO_4.7H_2O$	8	70
$CaCl_2.2H_2O$	4	70
K_2HPO_4	8.64	70
KH_2PO_4	8.4	35
EDTA/KOH	50/31	2.85
Acidified iron	4.98	5.7
Boric acid	11.42	5.7
$ZnSO_4.7H_2O$	14.12	3.56
$MnCl_2.4H_2O$	2.33	3.56
$CuSO_4.5H_2O$	2.54	3.56
$Co(NO_3)_2.6H_2O$	0.82	3.56
$Na_2MoO_4.4H_2O$	1.92	3.56
Acetic acid	100%	5.7

Appendix B

B.1 Papers as first author

Michalaki, A.; McGivern, A.R.; Poschet, G.; Büttner, M.; Altenburger, R.; Grintzalis, K. The Effects of Single and Combined Stressors on Daphnids-Enzyme Markers of Physiology and Metabolomics Validate the Impact of Pollution. Toxics **2022**, 10, 604.

Michalaki, A.; Grintzalis, K. Acute and Transgenerational Effects of Non-Steroidal Anti-Inflammatory Drugs on Daphnia magna. *Toxics* **2023**, *11*, 320.

Michalaki, A.; Grintzalis, K. A Multiparametric Protocol for the Detailed Phytochemical and Antioxidant Characterisation of Plant Extracts. *Methods and Protocols* **2023**, *6*, 40.

Michalaki, A.; Kakavas, D.; Giannouli, M.; Grintzalis, K. Toxicity of "green solvents" - The impact of butyl methylimidazolium ionic liquids on daphnids. *Journal of Ionic Liquids* **2023**, *3*, 100059, https://doi.org/10.1016/j.jil.2023.100059.

Michalaki, A.; Yin, X.; Brennan, L.; Grintzalis, K. Exposure to chemical and commercial forms of NSAIDs at environmentally relevant concentrations exert transgenerational metabolic responses in daphnids. *Water Biology and Security* **2025**, 100404, https://doi.org/10.1016/j.watbs.2025.100404.

B.2 Oral Presentations

Anna Michalaki, Xiaofei Yin, Lorraine Brennan, Konstantinos Grintzalis, (2025). The impact of chemical and commercial NSAIDs on *Daphnia magna*. BRS Research Day, DCU, Dublin.

Anna Michalaki, Allan Robert McGivern, Gernot Poschet, Michael Büttner, Rolf Altenburger, Konstantinos Grintzalis, (2023). Assessment of the impact of single and mixtures stressors on Daphnia magna: Using enzyme markers and metabolomics as endpoints of physiology. Setac, Dublin.

Anna Michalaki, Allan Robert McGivern, Gernot Poschet, Michael Büttner, Rolf Altenburger, Konstantinos Grintzalis, (2023). Assessment of the impact of single and mixtures stressors on Daphnia magna, using enzyme markers and metabolomics. BRS Research Day, DCU, Dublin.

B.3 Posters

Anna Michalaki, Dimitrios Kakavas, Maria Giannouli, Konstantinos Grintzalis, (2022). Toxicity response from acute exposure of daphnids to BMIM ionic liquids. BRS Research Day, DCU, Dublin.

Hannah Moynihan, Anna Michalaki, Konstantinos Grintzalis, (2022). The impact of indomethacin and ibuprofen on daphniids. Environ, Belfast.

Anna Michalaki, Dimitrios Kakavas, Evgenia-Maria Papadopoulou, Maria Giannouli, Konstantinos A Aliferis, Konstantinos Grintzalis, (2022). Toxicity responses from acute and chronic exposures of daphniids to 1-butyl-3-methylimidazolium ionic liquids. The biochemistry Global Summit, Lisbon.

Anna Michalaki, Dimitrios Kakavas, Maria Giannouli, Konstantinos Grintzalis, (2022). Toxicity responses from acute exposure of daphniids to BMIM ionic liquids. Annual Congress of British Toxicology Society, Newcastle.

Anna Michalaki, Dimitrios Kakavas, Panagiotis-Nikolaos Sarametidis, Panagiotis Katsoris, Keith D Rochfort, Konstantinos Grintzalis, (2021). *In vitro* toxicity of Helleborus extract. IC-3Rs Symposium.

Anna Michalaki, Dimitrios Kakavas, Maria Giannouli, Konstantinos Grintzalis, (2021). Toxicity responses of daphniids exposed to butyl methylimidazolium ionic liquids. OpenTox 2021.

Appendix C

C.1 Declaration of Authorship

Chapter 2A

Section 1: Candidate's Details	
Candidate's Name	Anna Michalaki
DCU Student Number	A21269604
School	School of Biotechnology
Principal Supervisor	Konstantinos Gkrintzalis
Title of PhD by Publication Thesis	*Daphnia* as the "canary in the coalmine" – Effect-based methods for pollution assessment
Section 2: Paper Details	
Title of co-authored paper included in the thesis under examination	The Effects of Single and Combined Stressors on Daphnids—Enzyme Markers of Physiology and Metabolomics Validate the Impact of Pollution
List of authors as in order given on accepted/published version	Anna Michalaki, Allan Robert McGivern, Gernot Poschet, Michael Büttner, Rolf Alternburger, Konstantinos Grintzalis
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	2022, Toxics, Issue 10, Volume 10, DOI: 10.3390/toxics10100604, https://www.mdpi.com/2305-6304/10/10/604
Publication Status (e.g. Published, Accepted)	Published
Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other,	This article was submitted for publication on the 19th of July 2022. There were two rounds of single blinded revisions, with two reviewers each time. The final revised manuscript was accepted on October 10th,

initial decision, revision timeline etc and any other details that speak to the rigour of the peer review process)	2022, and it was published on the 12th of October 2022.

This paper (Chapter 2-1st part) is one of 5 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination.

Section 3: Candidate's Contribution to the Paper

Briefly outline your contribution to the **conception** of the work described in this paper:

This was the first article published during my PhD; my supervisor came up with the idea as this article was the continuation of a previous student's study. I was 80% involved in the improvement of this idea.

Briefly outline your contribution to the **literature review** in this paper: I did extended research on the literature to be able to participate in the conceptualization and the methodology design. I got myself familiarized with background literature to ensure accurate performance of the laboratory work. I contributed 70% to this part.

Briefly outline your contribution to the **methodological design**: Followed the extended literature review I was able to contribute 80% to the methodology design. For this study, protocols that were already used in our lab were followed.

Briefly outline your contribution to **data collection**: contributed 85% to data collection by performing the majority of Daphnia-related experiments (all biochemical assays). Additionally, I provided all samples for metabolomic analysis and collected the data.

Briefly outline your contribution to **data analysis**: I analysed the data from biochemical assays, and I contributed to the interpretation of part of the metabolomics data. My contribution to data analysis was 85%.

Briefly outline your contribution to the **synthesis and summary of findings**: As this was the first article of PhD, I contributed 85% to the synthesis and summary of findings, under the guidance of my supervisor.

Finally, outline your contribution to the **writing** and **revision** of the paper: Overall, I contributed 85% to the writing and revision of the article. This article went to minor revision which me and the co-authors responded to all comments of the reviewers.

Section 4: Signature and Validation

I confirm that the following statements are true:

(a) the information I have provided in this form is correct
(b) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: Anna Michalaki **Date:** 16/06/2025

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Konstantinos Gkrintzalis **Date:** 18/6/2025

Chapter 2B

Section 1: Candidate's Details	
Candidate's Name	Anna Michalaki
DCU Student Number	A21269604
School	School of Biotechnology
Principal Supervisor	Konstantinos Gkrintzalis
Title of PhD by Publication Thesis	*Daphnia* as the "canary in the coalmine" – Effect-based methods for pollution assessment
Section 2: Paper Details	
Title of co-authored paper included in the thesis under examination	Novel Approaches Methodologies in ecotoxicology - Metabolism reveals transgenerational effects of pollutants on daphnids
List of authors as in order given on accepted/published version	Anna Michalaki, Anne Leung, Emma Rowan, Ya Gao, Claire Connolly, Lorraine Brennan, Konstantinos Grintzalis
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of	

Publication, DOI, ISSN, URL etc.)	
Publication Status (e.g. Published, Accepted)	Submitted to journal Aquatic Toxicology- Under review
Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc and any other details that speak to the rigour of the peer review process)	This article was submitted on the 18th of June, and it has a single blind peer review. It is currently in the 1st round of revisions.
This paper (Chapter 2-2nd part) is one of 5 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination.	

Section 3: Candidate's Contribution to the Paper

Briefly outline your contribution to the **conception** of the work described in this paper: After discussions with my supervisor, I proposed the idea of this paper as a continuation and in connection with my first article on the impact of eight single stressors and their mixture. My contribution in the design would be 80%.

Briefly outline your contribution to the **literature review** in this paper: This article is a follow up from my previous study and I was in very good command of the literature. I contributed to the literature review by 90% with relevant sources from the latest 5 years.

Briefly outline your contribution to the **methodological design**: The study builds on the previous one and shares similar protocols, many of which were developed in our group i.e. feeding assay. I contributed the 90% to the methodological design.

Briefly outline your contribution to **data collection**: I had the major contribution to the study in relation to data collection as 90%. I performed the feeding assay, all biochemical assays, and collected samples for metabolomic analysis which were analysed by the metabolomics facility; however, I processed all data.

Briefly outline your contribution to **data analysis**: My contribution in data analysis for this this paper is 95%. I analysed all data from feeding assay, biochemical assays and metabolomic analysis. I performed the multivariate statistical analysis of the metabolomic data obtained.

Briefly outline your contribution to the **synthesis and summary of findings**: contributed 95% to the synthesis and summary of findings.

Finally, outline your contribution to the **writing** and **revision** of the paper: I am the main author of this manuscript with 95% contribution to the writing of the original draft and the final review, and I received guidance from my supervisor.

Section 4: Signature and Validation

I confirm that the following statements are true:

(c) the information I have provided in this form is correct
(d) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: Anna Michalaki **Date:** 16/06/2025

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Konstantinos Gkrintzalis **Date:** 18/6/2025

Chapter 3

Section 1: Candidate's Details	
Candidate's Name	Anna Michalaki
DCU Student Number	A21269604
School	School of Biotechnology
Principal Supervisor	Konstantinos Gkrintzalis
Title of PhD by Publication Thesis	*Daphnia* as the "canary in the coalmine" – Effect-based methods for pollution assessment
Section 2: Paper Details	
Title of co-authored paper included in the thesis under examination	Toxicity of "green solvents" - The impact of butyl methylimidazolium ionic liquids on daphnids
List of authors as in order given on accepted/published version	Anna Michalaki, Dimitrios Kakavas, Maria Giannouli, Konstantinos Grintzalis
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	2023, Journal of Ionic Liquids, Issue 2, Volume 3, DOI: 10.1016/j.jil.2023.100059, https://www.sciencedirect.com/science/article/pii/S2772422023000113
Publication Status (e.g. Published, Accepted)	Published

Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc and any other details that speak to the rigour of the peer review process)	This article was submitted for publication on the 17th of October 2022. There were two rounds of single blinded revisions, with two reviewers each time. The final revised manuscript was submitted on the 14th of June 2023, and it was accepted on the 26th of June. Finally, it became available online on the 3rd of July 2023.

This paper (Chapter 3) is one of 5 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination.

Section 3: Candidate's Contribution to the Paper

Briefly outline your contribution to the **conception** of the work described in this paper:
This article was one of the first studies I started and after a discussion with my supervisor I came up with the idea of this study. My contribution in the design would be 80%.
Briefly outline your contribution to the **literature review** in this paper: Prior to methodology design I conducted and extended literature review to get myself familiar with the concept. I contributed 90% to this part.
Briefly outline your contribution to the **methodological design**: The methodology for this article was mostly based on protocols that were used before by our group; however, I contributed 90% by choosing and combining the protocols.
Briefly outline your contribution to **data collection**: I had major contribution to the data collected regarding this study. I contributed the 70% to the data collection. Specifically, I performed the feeding and reproduction assay, while I contributed the most to the biochemical assays.
Briefly outline your contribution to **data analysis**: Regarding the data analysis, I analysed the results from the biochemical assays (acute and chronic), feeding and reproduction assays. Therefore, I contributed 90%.
Briefly outline your contribution to the **synthesis and summary of findings**: As the main author, I contributed 100% to the synthesis and summary of findings.
Finally, outline your contribution to the **writing** and **revision** of the paper: I am the main author of this article; therefore, I contributed 100% to the writing and revision of the paper. Specifically, this article underwent minor revisions which I responded to all reviewers until it was successfully accepted and published.

Section 4: Signature and Validation

I confirm that the following statements are true:

(e) the information I have provided in this form is correct
(f) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: Anna Michalaki **Date:** 16/06/2025

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Konstantinos Gkrintzalis **Date:** 18/6/2025

Chapter 4A

Section 1: Candidate's Details	
Candidate's Name	Anna Michalaki
DCU Student Number	A21269604
School	School of Biotechnology
Principal Supervisor	Konstantinos Gkrintzalis
Title of PhD by Publication Thesis	*Daphnia* as the "canary in the coalmine" – Effect-based methods for pollution assessment
Section 2: Paper Details	
Title of co-authored paper included in the thesis under examination	Acute and Transgenerational Effects of Non-Steroidal Anti-Inflammatory Drugs on *Daphnia magna*
List of authors as in order given on accepted/published version	Anna Michalaki, Konstantinos Grintzalis
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	2023, Toxics, *11*(4), 320, DOI: 10.3390/toxics11040320, https://www.mdpi.com/2305-6304/11/4/320
Publication Status (e.g. Published, Accepted)	Published
Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc and any other details that	This paper underwent three rounds of single-blinded revision, with three reviewers. The article was initially submitted for publication on the 28th of January 2023, the final revisions were submitted on the 23rd of March and finally, on the 27th of March was accepted for publication. The article was published on the 29th of March 2023.

speak to the rigour of the peer review process)	

This paper (Chapter 4-1st part) is one of 5 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination.

Section 3: Candidate's Contribution to the Paper

Briefly outline your contribution to the **conception** of the work described in this paper: I suggested the idea of this article to my supervisor and together we came up with the details in conceptualization (95%).

Briefly outline your contribution to the **literature review** in this paper: I contributed 95% to the literature review. I did extended research to find the gaps in the literature regarding this article.

Briefly outline your contribution to the **methodological design**: This study followed methodologies that were already used by out group and some of them developed by our team (for the feeding assay). My contribution to this part was 90%, since I selected which protocols to use and came up with the specifics for each experiment.

Briefly outline your contribution to **data collection**: contributed 100% to the data collection. I performed all protocols for this article (mortality assay, feeding assay, all biochemical assays).

Briefly outline your contribution to **data analysis**: I analysed all data for this article (mortality assay, feeding assay, and all biochemical assays). Therefore, my contribution was 100%.

Briefly outline your contribution to the **synthesis and summary of findings**: Since I am the main author, I contributed to this part 95% and my supervisor helped me with the synthesis.

Finally, outline your contribution to the **writing** and **revision** of the paper: My contribution to the writing and revision of the paper was 95%. This manuscript went to minor revision which I responded to all the comments of the three reviewers until the success and publication. My supervisor helped me with the editing and responses to the reviewers.

Section 4: Signature and Validation

I confirm that the following statements are true:

(g) the information I have provided in this form is correct
(h) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: Anna Michalaki **Date:** 16/06/2025

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Konstantinos Gkrintzalis **Date:** 18/6/2025

Chapter 4B

Section 1: Candidate's Details	
Candidate's Name	Anna Michalaki
DCU Student Number	A21269604
School	School of Biotechnology
Principal Supervisor	Konstantinos Gkrintzalis
Title of PhD by Publication Thesis	*Daphnia* as the "canary in the coalmine" – Effect-based methods for pollution assessment
Section 2: Paper Details	
Title of co-authored paper included in the thesis under examination	Exposure to chemical and commercial forms of NSAIDs at environmentally relevant concentrations exert transgenerational metabolic responses in daphnids
List of authors as in order given on accepted/published version	Anna Michalaki, Xiaofei Yin, Lorraine Brennan, Konstantinos Grintzalis
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	2025, Water Biology and Security, DOI: 10.1016/j.watbs.2025.100404, https://www.sciencedirect.com/science/article/pii/S2772735125000472
Publication Status (e.g. Published, Accepted)	Published
Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc and any other details that speak to the rigour of the peer review process)	This paper underwent two rounds of single-blinded revision, with two reviewers. The article was initially submitted for publication on the 19th of September 2024, the first revisions were submitted on the 17th of December 2024, while the second round of revisions were submitted on the 17th of January. On the 26th of March 2025 was accepted for publication.

This paper (Chapter 4-2nd part) is one of 5 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination.

Section 3: Candidate's Contribution to the Paper

Briefly outline your contribution to the **conception** of the work described in this paper: This study was the natural continuation of my first article on NSAIDs. The conceptualization of this study came as a result of the reviewers' comments during the revisions of the first NSAIDs article. I contributed 95% to the conception of this article.

Briefly outline your contribution to the **literature review** in this paper: Since I was the main author on the first NSAIDs article, I had done an extended literature review while writing the first one. Therefore, my contribution to the literature review in this paper was 100%.

Briefly outline your contribution to the **methodological design**: This study is based on the previous one and some of the protocols are similar. However, based on the reviewers of the first-NSAIDs article, more protocols were included to make this study more environmentally relevant. My contribution was 90%.

Briefly outline your contribution to **data collection**: Except the metabolomics data, I performed all assays (mortality, biochemical assays, survival). I also provided the samples for metabolomic analysis and collected all data. My contribution to this part was 95%.

Briefly outline your contribution to **data analysis**: Regarding the data analysis, I analysed all data of this study (mortality, feeding, biochemical and survival assays). Additionally, I performed the multivariate statistical analysis for the metabolomics data. Therefore, my contribution was 100%.

Briefly outline your contribution to the **synthesis and summary of findings**: Since I am the main author, I contributed to this part 95%. Xiaofei Yin and Lorraine Brennan provided the section of the metabolomic analysis methodology.

Finally, outline your contribution to the **writing** and **revision** of the paper: My contribution to the writing and revision of the paper was 100%. This manuscript went to minor revision which I responded to all the comments of the reviewers until the success and publication.

Section 4: Signature and Validation

I confirm that the following statements are true:

(i) the information I have provided in this form is correct
(j) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: Anna Michalaki **Date:** 16/06/2025

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Konstantinos Gkrintzalis **Date:** 18/6/2025