Identifying the responses of freshwater organisms to a range of pollutants and their mixtures

Allan McGivern BSc
School of Biotechnology, Dublin City University

Supervisors: Doctor Konstantinos Gkrintzalis, Professor Anne Parle-McDermott

To be examined for the award of MSc

January 2022

DCU Declaration for Plagiarism

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of MSc is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed:  ID No: 15512917 Date: 13/01/2022

Table of contents

Abstract	1
Acknowledgements	2
Introduction to ecotoxicology and risk assessment	3
Common pollutants, their sources and routes to the environment	4
Traditional approaches in pollution assessment and their shortcomings	5
Aquatic species as bioindicators	5
Daphniids in molecular ecology and ecotoxicology	6
Research focus and scientific aims	11
Assessment of individual and mixture of stressors	11
Materials and Methods	13
Culturing algae and daphniids	13
Acute exposure	13
Sample homogenization and analysis	14
Oxidative stress indices	14
Enzyme indices of physiology	16
Statistical analysis	18
Results	19
Exposure to individual stressors	19
Toxicity of metals	20
Toxicity of NSAIDs	22
Toxicity of pharmaceuticals with different mode of action	24
Toxicity of a variety of pollutants	26
Responses to mixture toxicology	28
Assessment of the impact of a composite mixture of chemicals	29
Assessment of toxicity of single, double, and triple mixtures	32
Discussion	37
Effects of metals on daphniids	37
Responses to active pharmaceutical compounds	39
Non-steroidal anti-inflammatory drugs (NSAIDs)	39
Common environmentally relevant pharmaceutical compounds	42
Responses to diverse environmental pollutants	45
Responses to mixtures	47
Responses to a composite 8 chemical mixture	47
Single, double, and triple mixtures of lithium, propranolol and nicotine	48
Final remarks, conclusions, and future work	49
Supplementary figures	51
References	57
Appendix A	61
Appendix B	62
Appendix C	63

Tables and figures

Table 1. Sources and routes of common examples of pollutants into the aquatic environment.	4
Figure 1. Anatomy of *Daphnia magna*	8
Figure 2. The sexual and the asexual (parthenogenetic) life cycle of a daphniids	9
Figure 3. Morphological characteristics of daphniids	10
Table 2. EC values (mg/L) for acute toxicity in 4-day old animals.	19
Figure 4. Cumene hydroperoxide standard curve	15
Figure 5. Glutathione standard curve	16
Figure 6. p-nitrophenol standard curve	17
Figure 7. Impact of metals on key enzyme activities	21
Figure 8. Impact of metals on GST, lipid hydroperoxides (LOOH), and reduced thiols.	22
Figure 9. Impact of NSAIDs on key enzyme activities	23
Figure 10. The impact of NSAIDs on GST, lipid hydroperoxides (LOOH), and reduced thiols.	24
Figure 11. Impact of pharmaceuticals on key enzyme activities	25
Figure 12. Impact of pharmaceuticals on GST, lipid hydroperoxides and reduced thiols.	26
Figure 13. Impact of pollutants on key enzyme activities	27
Figure 14. Impact of pollutants on GST, lipid hydroperoxides and reduced thiols	28
Figure 15. Acute toxicity curve (24 hour) for D4 daphniids exposed to a mixture of 8 chemicals.	29
Figure 16. Impact of a composite chemical mixture on key enzyme activities	31
Figure 17. Impact of a composite chemical mixture on GST, lipid hydroperoxides (LOOH) and reduced thiols	32
Figure 18. Acute toxicity curve (24 hour) for D4 daphniids exposed to mixtures of lithium, propranolol, and L-nicotine	33
Figure 19. Impact of single, double, and triple chemical mixtures on key enzyme activities.	35
Figure 20. Impact of single, double, and triple chemical mixtures on GST, lipid hydroperoxides and reduced thiols.	36
Figure 21. Schematic representation of pathways to discover toxicity mechanisms.	50
Supplementary figure 1	51
Supplementary figure 2	52
Supplementary figure 3	53
Supplementary figure 4	54

Abbreviations

ALP	Alkaline phosphatase
ACP	Acid phosphatase
βGal	Beta-galactosidase
LDH	Lactate dehydrogenase
GST	Glutathione-S-transferase
LOOH	Lipid peroxide
EC50	The concentration of a substance required to have a 50% mortality rate.
WWTP	Wastewater treatment plant
COD	Chemical oxygen demand
OECD	Organisation for economic cooperation and development
FA	First antennae
CE	Carapace edges
NSAID	Non-steroidal anti-inflammatory drug
CA	Concentration addition
IA	Independent action
CGM	*Chlamydomonas rheinhartii* growth media
CBB	Coomassie brilliant blue
DPS	2,2'-dipyridyldisulfide
PNPP	*p*-nitrophenyl phosphate
DMSO	Dimethyl sulfoxide
NADH	Nicotinamide adenine dinucleotide
EC_1	The concentration of a substance required to have a 1% mortality rate.
EC_5	The concentration of a substance required to have a 5% mortality rate.
EC_{10}	The concentration of a substance required to have a 10% mortality rate.
COX	Cyclooxygenase
SOD	Superoxide dismutase
CAT	Catalase
ChE	Cholinesterase
GPx	Glutathione peroxidase
GSH	Glutathione
DEHP	Diethylhexyl phthalate
DPB	Bibutyl phthalate
DEP	Diethyl phthalate
ROS	Reactive oxygen species
AOP	Adverse outcome pathway

Identifying the responses of freshwater organisms to a range of pollutants and their mixtures

Allan McGivern

Abstract

Freshwater organisms are exposed to a myriad of anthropogenic pollutants, and the physiological impacts of many of them remain understudied. Traditional endpoints such as mortality and reproduction are insufficient to determine the true risk level of pollutants, particularly where mixtures are concerned due to pollutant synergy and non-additive effects. Furthermore, these endpoints give little insight into the mechanisms of pollutants. This research evaluates the effects of a variety of chemicals on key enzymes and physiological markers in the cladoceran *Daphnia magna*, specifically alkaline phosphatase (ALP), acid phosphatase (ACP), β-galactosidase (βGal), lipase, lactate dehydrogenase (LDH), peptidase, glutathione-S-transferase (GST), lipid peroxides (LOOH), and reduced thiols, in order to assess the impact of a battery of pollutants. The tested pollutants were divided into 4 main categories: metals (aluminium, cadmium, cobalt, lithium, zinc, zirconium), non-steroidal anti-inflammatory drugs/NSAIDs (Acetylsalicylic acid, diclofenac, ibuprofen, indomethacin), pharmaceuticals (Diltiazem, metformin, chlorpromazine, mebendazole, propranolol), and general pollutants, a broader category (N-phosphonomethyl glycine, L-nicotine, 4-nitrophenol, phthalic acid). As aquatic pollution is rarely derived from a single chemical or pollutant, 2 mixture scenarios were also investigated. The first was a composite mixture of 8 chemicals taken from the previous 4 categories (lithium, aluminium, acetylsalicylic acid, propranolol, diltiazem, N-phosphonomethyl glycine, L-nicotine, metformin). Dose-response was investigated in this section using various concentrations of the composite mixture. The second scenario involved single, double, and triple mixtures of three selected chemicals (Lithium, propranolol, L-nicotine). EC_{50} concentrations for all listed chemicals and mixtures were derived from constructed toxicity curves. EC_{50} is defined here as the concentration of a substance required to have a 50% mortality rate in 4-day old *Daphnia magna*. Significant results were observed in all categories and mixtures, as well as non-additive toxicity effects in the mixture section. Novel biochemical data was produced for several understudied pollutants which can be found in the freshwater environment.

Acknowledgements

Over the last two years, through the research for, and writing of this thesis, I have been the happy recipient of strength, support, and assistance from many sources.

I would first like to acknowledge the support and funding of Science Foundation Ireland, which made this research possible. Secondly, I would like to thank my primary supervisor, Professor Konstantinos Gkrintzalis, for his time, assistance, and unwavering attention to detail and commitment to pursuing and encouraging excellence and precision. It is a trait I have come to admire and hope to emulate throughout my career. I would also like to thank him for his support of my decision to transfer from the PhD track to an MSc, a choice not made lightly. I would like to thank my secondary supervisor, Professor Anne Parle-McDermott for her support over the last two years.

To my colleagues and friends, Katie O' Rourke and Yongda Li: I'd like to think the word "colleagues" is a formality at this point. The last two years with you both have been an absolute pleasure, and your friendship has made many long days in the lab go by that much more quickly. Thank you for your assistance, your sound advice, and your company. I will greatly miss working with you both, and should I find people half as good in my future roles, I will consider myself extremely lucky.

To my parents, Robert and Dorcas: Thank you for your unwavering support, encouragement, and belief in me over all these years. I would never have made it even close to this far without your guidance and advice.

To my brother and sisters, David, Andrea, and Elaine : Thank you for always being there when needed, for your advice, and for helping me to gain clarity when I needed it most.

To Aislinn: Thank you for your constant support and companionship, and for helping me to keep a clear perspective on life.

Introduction to ecotoxicology and risk assessment

During the last decades, groundwater, surface water, and aquatic ecosystems in general, have been excessively utilised and polluted to the extent that they are in danger of becoming unsuitable for human (or any other) utilization. The deterioration of water quality is becoming a matter of grave concern, as a combination of the global climate change and the rapid population growth which together continue to exacerbate the imbalance between the supply and demand of fresh water. The condition of the aquatic ecosystem is of great importance, both in Ireland and worldwide. The presence of chemicals into surface waters is of particular concern, especially where pharmaceutical ingredients, metals, and microplastics are concerned. Increasing levels of plastic pollution have been flagged as a pressing issue by the United Nations [1]. The increased presence of metals such as lead and cadmium deriving from anthropogenic sources in the environment is also a recognised issue, as metals in aquatic systems are persistent and tend to accumulate in the tissues of aquatic organisms. Furthermore, some studies suggest a myriad of adverse effects on aquatic invertebrates, affecting their growth, fecundity, and overall biochemistry [2]. On the other hand, pharmaceuticals such as propranolol and fluoxetine are neuroactive human pharmaceutical ingredients, which are known to occur frequently in surface waters. They are typically excreted by humans to wastewater in trace amounts, either as the intact parent compound, or as bioactive metabolites. Both propranolol and fluoxetine have shown potential for bioconcentration and bioaccumulation in aquatic organisms. It seems likely that their acute toxic effects are limited, as their environmental concentrations are typically in the order of ng/L. However, there have been sublethal effects reported at the ng/L to µg/l range in several studies [3, 4]. The aforementioned pollutants are but a few of the many known anthropogenic chemicals which can be found at significant levels in the aquatic environment. In addition, many pollutants are still unregulated as yet, and hundreds of varieties of pollutants can be identified in wastewater effluents even after treatment. This is at least partially due to inadequate treatment in wastewater treatment plants (WWTPs) not designed to eliminate pollutants such as pharmaceutical compounds. Indeed, it has been reported that over 80% of the world's wastewater is released without proper treatment [5]. Many of these substances lack regulation or any toxicological data, such as unintended disinfection by-products which arise during wastewater treatment [6]. Thus, it is crucial that novel and sensitive methods are developed and utilised to detect and identify

pollutants in the aquatic ecosystem to preserve and protect the environment from any further damage and loss of biodiversity.

Common pollutants, their sources and routes to the environment

Some of the most common aquatic pollutants tend to be anthropogenic sources of pharmaceuticals and heavy metals, though new classes of pollutants are emerging. The entry of pharmaceuticals into the environment is primarily through human action. These may be either involuntary means, such as the excretion of medicines, or the washing of topical medicines down the drain, or purposeful methods, such as the disposal of unused/outdated medicines [7]. Anthropogenic sources of heavy metals include disposal of materials such as paints, batteries, and piping, as well as industrial activities such as mining and electricity generated through the burning of coal [8]. Table 1 presents several different classes of common pollutants and their detected environmental levels.

Table 1. Sources and routes of common examples of pollutants to the aquatic environment.

Chemical	**Type**	**Levels reported (ng per l)**	**Sampled location**	
Lithium chloride	Metal	0 - 97000	Surface water, County Carlow, Ireland	[9]
Cadmium chloride	Metal	0-94000	Hand pump water, Anpara, India	[10]
Aluminium sulfate	Metal	620000-2600000	Sinos river basin, Brazil	[11]
Zinc sulfate	Metal	25507244	Flanders surface water, Belgium	[12]
Cobalt	Metal	0-50000	Smeda river, Poland/Czech Republic	[13]
Diclofenac	NSAID	10-120	Höje river, Sweden	[14]
Acetylsalicylic acid	NSAID	<30-37.2 (±4.6)	Somes river, Romania	[14]
Ibuprofen	NSAID	1417	Pearl river delta, South China	[15]
Indomethacin	NSAID	0.1-100	Various river water	[16]
Propranolol	β-blocker	35-107	Tyne river, United Kingdom	[14]
1,1-dimethylbiguanide hydrochloride (metformin)	Type 2 diabetes drug	1820	Ootmaarsum STP effluent, Netherlands	[17]
Chlorpromazine	Phenothiazine antipsychotic	500	Hospital wastewater, Santa Maria, Brazil	[18]
Mebendazole	Anthelmintic microtubule depolymerisation drug	<1-4	Anil and Bacanga river sediments, Brazil	[19]
Diltiazem	Ca channel blocker	107	WWTP effluent, Ohio	[20]
N-phosphonomethyl glycine (glyphosate)	Herbicide (phosphonate)	144000-1e+6	Lake water, Brazil/Argentina	[21]
Nicotine	Stimulant	600-32000	Various	[22]
Phthalic acid	Plasticiser	0-554	Surface water, Spain	[23]

Due to the large variety of anthropogenic pollutants present in aquatic ecosystems, environmental monitoring is essential in order to ensure that polluted sites are remediated, and that sources of pollution are detected and dealt with before

irreversible harm can be caused. As such, this thesis focuses on the application of biochemical markers and whole-animal metabolic profile analysis using *Daphnia magna* as a model species for environmental monitoring. This approach provides the potential for novel insights into the toxicity mechanisms of many common pollutants, allowing a profound assessment of their effects on, and threat level to, freshwater species as a whole.

Traditional approaches in pollution assessment and their shortcomings

Traditional metrics and methods of assessing water pollution seem to be not sufficient to provide mechanistic insight or predict pollution. Traditional methods of water quality assessment include the measurement of basic physicochemical properties, such as pH, turbidity, and colour, as well as dissolved oxygen, salinity, nitrates, phosphates, and chemical oxygen demand (COD) [24]. Maximum permitted concentrations for a variety of organics, metals, and pollutants such as pesticides and pharmaceuticals are then generated as water quality standards. In such case, many chemicals which occur as pollutants are regulated using their maximum permitted concentration values, such as mineral oils, metals, and pesticides. However, with the advent of improved toxicological studies, many of these chemicals have been revealed to have more serious adverse effects than previous data suggested. Chemicals such as phthalates, steroids and alkyl phenols have since been proven to have strong endocrine disruption abilities in higher life forms, such as wildlife, fish, and even humans [6]. In light of these previously undetected ecotoxicological effects, it is clear that conventional chemical and physical characterisation is inadequate and fails to comprehensively evaluate the safety and quality of aquatic systems. Furthermore, these methods provide no mechanistic insight into the modes of toxicity exhibited by the multitude of known pollutants which remain understudied. Thus, they lack the capability to be employed in a predictive ecotoxicology context.

Aquatic species as bioindicators

As chemical and physical characterisation methods are not sufficient, the usage and assessment of an appropriate bioindicator can provide vital information on ecological health, as well as the levels and type of pollution. Bioindicators are defined as organisms or biological processes which can be exploited to detect or measure changes or alterations in environmental conditions. In an ecotoxicology context, the

usage and assessment of an appropriate bioindicator can provide vital information on the ecological health of an ecosystem, and the levels and type of pollution present within it. A suitable organism-indicator will undergo specific physiological or behavioural changes which in turn indicate changes or pollution in the ecosystem. Naturally, these specific changes vary greatly between organisms, so proper study is essential before an organism is selected as a bioindicator. Typically, an aquatic organism-indicator is exposed to a water sample taken from a test site for a set period of time. Following this exposure period, any observed behavioural or physiological change could lead to conclusive remarks concerning the effect of environmental pressures that the species is facing.

There are many well established and common classes of organisms of varying complexity which are used as aquatic bioindicators, including many species of microbes, phytoplankton, and macro-invertebrates. However, perhaps the best suited organisms for this application are zooplankton as they are subjected to both biotic (limited food, predator/prey interactions etc.) and abiotic (temperature, salinity, pollutants etc.) factors in their environment and are thus considered excellent bioindicators for the evaluation of aquatic systems.

Daphniids in molecular ecology and ecotoxicology

Daphnia magna are a species of small planktonic crustaceans of the order Cladocera and are found in fresh and brackish water throughout the northern hemisphere. *D. magna* are the largest of the cladocerans, with the females reaching sizes of up to 5 mm. Their ecology and physiology are well documented, as they are a widely known and utilised model organism within the field of ecotoxicology. As a result, they have been used in studies involving both acute and chronic toxicity testing in response to a variety of chemicals, and have established OECD guidelines for these procedures [25]. They are well suited to this role due to their high sensitivity to environmental change, high degree of phenotypic plasticity, and key position in the food chain of the aquatic environment, as well as their parthenogenic reproduction [26]. The genetic structure of *Daphnia* is also well studied, benefiting from a fully sequenced genome and a growing number of genes which are being annotated and characterised [25, 27, 28]. Moreover, our understanding of their physiology continues to grow with the introduction of powerful omics technology. Despite the well-studied ecology of *Daphnia*, data regarding the molecular basis of their responses to environmental

perturbations remains sparse, and traditional biological endpoints such as mortality and fecundity cannot uncover the modes of action and toxicity mechanisms responsible.

D. magna are enclosed within a carapace, composed primarily of the polysaccharide chitin. This carapace is double walled, allowing the haemolymph to flow between the walls, as *D. magna* utilise an open blood circulation system. They have 5 sets of limbs located on the trunk of their body (Figure 1). These limbs are a defining characteristic of the group Phyllopoda, and are generally described as “flattened, leaf-like legs”. These legs beat continuously, creating a water current which flows anterior to posterior through the organism, passing through a filtering mechanism along the way. Using this system, *Daphnia* are able to collect particles which are transferred into their food groove by specialised setae. This filtration system is *D. magna*’s feeding mechanism and is effective enough to filter bacteria from the water, though their main source of food is usually planktonic algae. They typically consume particles ranging in size from 1-50 µm, though particles of up to 70 µm have been found in particularly large individuals [29].

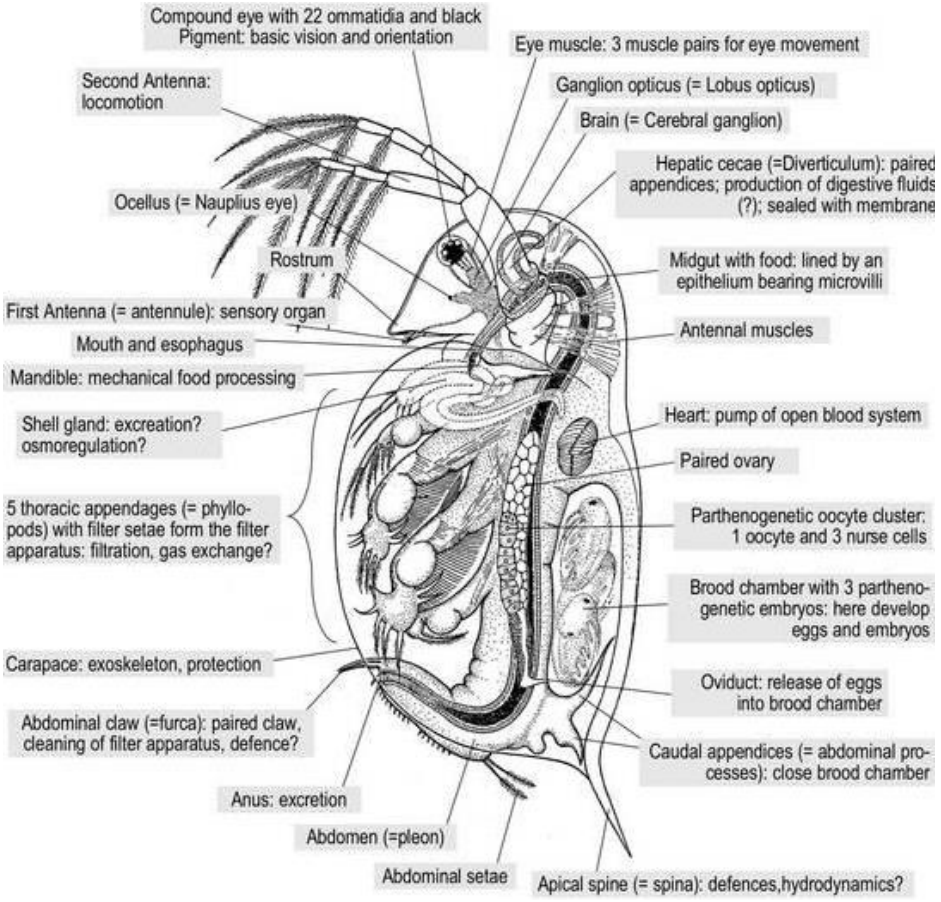


Figure 1. Anatomy of *Daphnia magna* [29]. Freely available at the online version of the book at https://www.ncbi.nlm.nih.gov/books/NBK2036/.

The life cycle of *D. magna* is characterised by their asexual parthenogenetic reproduction with the option for a sexual alternative approach (Figure 2). Under suitable feeding conditions, females of the species will produce a clutch of parthenogenic eggs after each adult moult. These eggs are placed into the brood chamber, enclosed within the carapace. The embryos typically hatch from these eggs after a day but remain within the brood chamber for a period of approximately 3 days to undergo further development prior to their release. New-born *Daphnia* look very similar to adults, with the exception of their undeveloped brood chamber. The first clutch of eggs is typically deposited into the brood chamber at 5-10 days old, though this may occur at a later age under suboptimal feeding conditions [29].

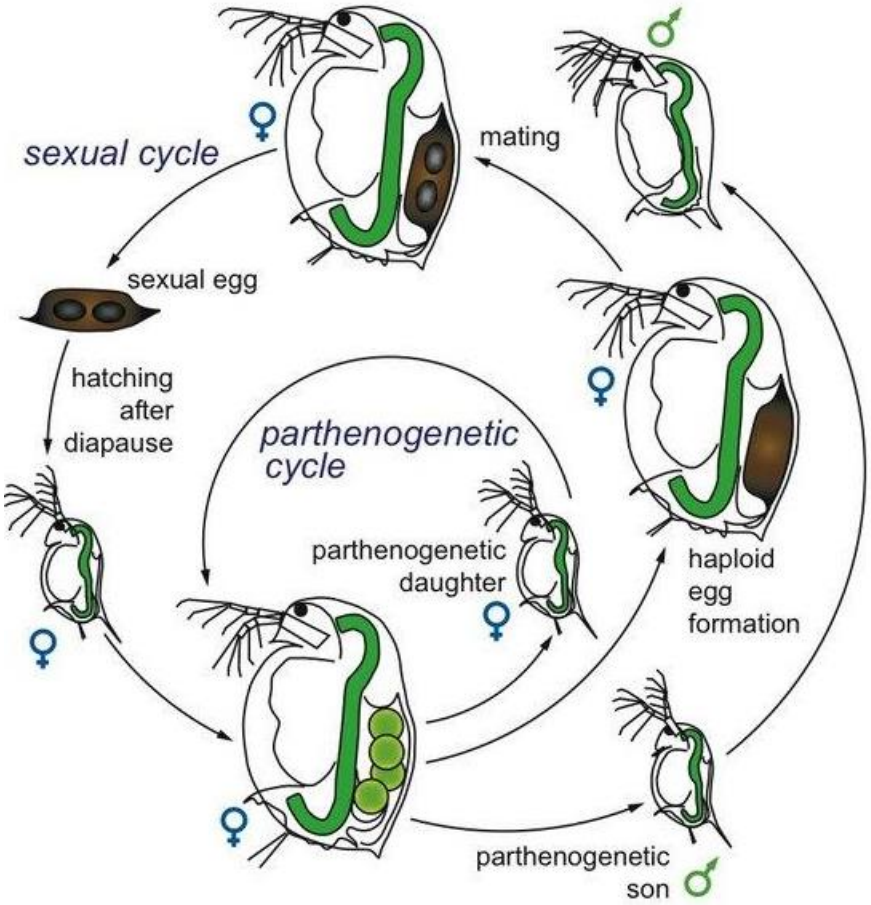


Figure 2. The sexual and the asexual (parthenogenetic) life cycle of a daphniids [29]. Freely available at the online version of the book at https://www.ncbi.nlm.nih.gov/books/NBK2036/.

As a primary consumer, and prey for many planktivorous species of fish, they are well regarded as a keystone species in many freshwater aquatic ecosystems. *D. magna* are also excellent candidates for model organisms in ecotoxicology research due to a number of factors. Their small size, rapid generation times and simplistic growth conditions make them an easy organism to work with. Each daphniid can produce dozens of eggs per clutch, meaning that a large number of test organisms can easily be obtained from a relatively small breeding culture. However, perhaps their most beneficial trait is their method of reproduction. *D. magna* are facultative parthenogens, and thus capable of producing and maintaining a clonal population (Figure 2). Under favourable (non-stressful) conditions, they will asexually produce diploid eggs, which will develop directly into female clones of the mother. They may also asexually produce diploid eggs which develop into sons, though this process is under environmental control. Typically, it will occur under unfavourable conditions, as a

method of population preservation. The utility of this in ecotoxicological research is that it allows the emphasis of the research to be placed on changing environmental conditions, as it minimizes genetic variation in the test subjects. Despite their genetic similarities, there remains some biological and phenotypical variance between individuals. This can be observed as varying levels of biomarkers such as enzyme activity between control groups.

It should be noted that *D. magna* are also capable of producing haploid resting eggs, which require fertilization by a male and will produce female offspring. These eggs must undergo a diapause before they will hatch. These eggs are enclosed in a protective layer (the ephippium) and are capable of enduring lengthy periods of adverse conditions until they are fertilised by a male. Production of resting eggs such as these will occur if they are exposed to unfavourable environmental conditions, so it is important that breeding cultures are kept properly fed and maintained at an appropriate temperature. Though they appear similar, *Daphnia* magna are a sexually dimorphic species, with a number of differentiating physiological characteristics between males and females (Figure 3).

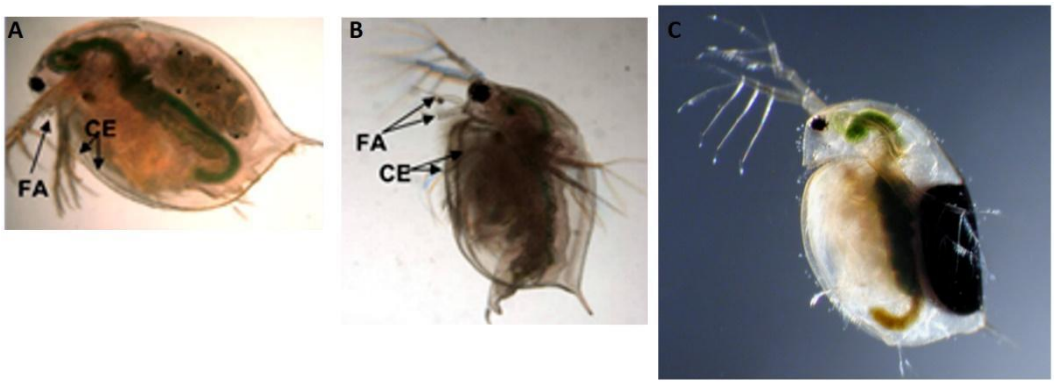


Figure 3. Morphological characteristics of daphniids. (A) Female and (B) male Daphnia magna. Differentiating sex characteristics include the pair of minute first antennae (FA) of the females, which are elongated in the males. The female-like diminutive FA is obscured by the male-like elongated FA in the micrograph. The bivalved-like carapace of the female has two uniform, symmetrical edges (CE, carapace edges). Both CEs of the male are asymmetrical and are edged by setae. (C) Resting egg at the back of *Daphnia magna*. Figure adopted from [29]. Freely available at the online version of the book at https://www.ncbi.nlm.nih.gov/books/NBK2036/.

Another key feature of *Daphnia* biology which makes them good model organisms is their feeding method. They are non-selective filter feeders, which may increase their

susceptibility to suspended or dissolved chemicals found in their environment or in a test sample. As *Daphnia* maintain a constant water current through their body for feeding purposes, suspended or dissolved chemicals may also be taken up via this route.

Under the “3Rs” (Replacement, Reduction, Refinement) principle, *Daphnia* are noted as a promising model organism for ecotoxicity experiments, as they are simple invertebrates and are not capable of experiencing suffering. The use of *Daphnia* in this context constitutes a partial replacement approach [30, 31].

Research focus and scientific aims

The general aim of this thesis is to explore the underlying mechanisms of toxicity and assess the impact of different pollutants and their mixtures through a battery of enzyme activity assays and physiological endpoints. The data produced could be used to guide legislation for the regulation of, pollutants for many of which are already in use with undocumented ecological effects or legislation. This work will be presented in distinct work packages as outlined below.

Assessment of individual and mixture of stressors

The first aim of this work was the investigation of a range of common pollutants such as metals and organics, as well as pesticides which have neurotoxic and endocrine disruption capabilities. These emerging contaminants were approached as four groups in different representative categories, ranging from common metals (Al, Cd, Co, Li, Zn, Zr), to pharmaceuticals of different (diltiazem, metformin, chlorpromazine, mebendazole, propranolol) or the same (NSAIDs) modes of action, and a diverse set of pollutants (nicotine, glyphosate, phthalate, nitrophenol). Phenotypic endpoints (such as lethality) coupled with biochemical oxidative stress indices (LOOH levels, GST activity, reduced thiol levels) and key enzymes (ACP,ALP, βGal, LDH, lipase, peptidase) were used to detect and confirm the impact of pollutants on the physiology of daphniids. ACP and ALP are involved in phosphate metabolism, while βGal is involved in energy production through the cleaving of lactose into galactose and glucose, ultimately fuelling glycolysis. LDH catalyses the reduction of pyruvate to lactate and is thus crucial in anaerobic metabolism. Lipase is essential for the digestion of fats, and peptidase (γ-glutamyl transferase) for the transfer of γ-glutamyl residues.

This set of enzymes was selected to give general insight into the widespread metabolic perturbations caused by the exposure of aquatic organisms to pollutants.

As a result of varying levels and sources of pollution over the years, it has become an unfortunate reality that aquatic organisms in the environment do not merely come into contact with the occasional, individual pollutant. Rather, it has been shown that these organisms encounter cocktails of pollutants. For example, one study detected between 20 and 64 chemicals at various sites along the Danube River [32]. As a result of the sheer diversity of pollutants which can be present in aquatic environments, traditional chemical analysis is insufficient for their detection, necessitating the use of complementary bioanalytical methods. Despite the environmental presence of such a variety of pollutants, many current water safety standards are based on risk analyses for individual chemicals. This fails to take into account the risks posed by complex chemical mixtures at low concentrations. Previously, there has been a misconception that chemical mixtures pose minimal risk, so long as each chemical constituent was below a level believed to be safe for that individual chemical. However, increasing evidence contradicts this theory, and supports the idea that chemicals may contribute to toxicity in a complex mixture even if they are present in a concentration that would have no measurable effect by itself [33, 34]. A complicating factor within the area of mixture toxicology is that, while the toxic effects of some mixtures can be predicted with reasonable accuracy by use of the reductionist principles of concentration addition (CA) and independent action (IA), other mixtures do not seem to behave in this manner. To the contrary, mixtures of certain chemicals seem to synergise, effectively amplifying their toxic effects, even at low concentrations [35]. This phase of the project focused on a series of combinations of stressors to elucidate mixture toxicity using biochemical endpoints.

Materials and Methods

Materials. All chemicals used in this study were of the highest analytical quality. Pollutants used were purchased from Sigma, ThermoFisher and AcrosOrganics. Diltiazem hydrochloride (CAS: 33286-22-5), N-phosphonomethylglycine (glyphosate) (CAS: 1071-83-6), and chlorpromazine hydrochloride (CAS: 69-09-0) were purchased from Sigma. Aluminium sulphate hexadecahydrate (CAS: 1628-11-8) and lithium chloride anhydrous (CAS: 7447-41-8) were purchased from ThermoFisher. Cadmium chloride hemipentahydrate (CAS: 7790-78-5), cobalt (III) nitrate (CAS: 10026-22-9), zinc sulphate heptahydrate (CAS: 7446-20-0), acetylsalicylic acid (CAS: 50-78-2), diclofenac sodium (CAS: 15307-79-6), (S)-(+)-ibuprofen (CAS: 51146-56-6), indomethacin (CAS: 53-86-1), 1,1-dimethylbiguanidine hydrochloride (metformin)(CAS: 1115-70-4), mebendazole (CAS: 31431-39-7), DL-propranolol hydrochloride (CAS: 318-98-9), L-nicotine (CAS: 54-11-5) and 4-nitrophenol (CAS: 100-02-7) were purchased from Acros Organics. Phthalic acid (CAS: 88-99-3) and zirconium (IV) chloride (CAS: 10025-11-6) were purchased from Alfa Aesar.

Culturing algae and daphniids. Algae of the species *Chlamydomonas rheinhartii* is the primary food for daphniids. Algal cultures were maintained in CGM media (for details refer to the appendix) under constant illumination and shaking (200 rpm) at room temperature. Algae were pelleted and re-suspended in ddH_2O (to a suspension of 7A at 440 nm). Daphnids were cultured in conformity with OECD guidelines in 4 litre 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) using a 16h:8h light:dark photoperiod at 21˚C and a density of 80 adults per 4 litres of media. Media was renewed every four days and cultures were fed daily with the algal suspension and dried baker's yeast (2 mL from 100 mg/l ddH_2O) and an organic seaweed (*Ascophyllum nodosum*) extract upon media renewal (for details refer to the appendix). Males and/or ephippia were removed if encountered and neonates from the first and second broods were discarded and not used for experiments. New cultures were always setup from third brood neonates (<24 hours).

Acute exposure. For toxicity curves 15 animals (4 days old) per replicate per tested concentration were exposed in 100 mL OECD media for 24 hours. A minimum of 4 replicates were prepared for each concentration.

Where possible, pollutants were dissolved directly in OECD media. Where a carrier solvent was necessary, the pollutants were dissolved in 100% DMSO and added to exposure vessels at a ratio of 50 µL DMSO/100 mL OECD.

Toxicity was assessed as immobilisation of animals, and toxicity curves were plotted using the GraphPad Prism software package and the EC_5, EC_{10}, EC_{20} and EC_{50} values were calculated. Animals were sampled for biochemical measurements. For single chemical experiments, animals were exposed to an EC_5 concentration. These concentrations were typically higher than environmental levels, but give some insight into the metabolic pathways which are affected by each pollutant. In the second mixture scenario, a 20% EC_5 concentration is used.

Sample homogenization and analysis. Animals were pooled together, snap frozen in liquid nitrogen and stored in Eppendorf tubes until analysis. For enzyme activity assays samples were homogenized in the appropriate buffer using an Eppendorf homogenizer pestle. The homogenates were cleared by centrifugation at 14,000 *g* at room temperature for 5 minutes and the clear supernatant was collected for the biochemical assays. The assayed enzymes were acid phosphatase (ACP), alkaline phosphatase (ALP), β-galactosidase (βGal), glutathione-S-transferase (GST), peptidase, lipase, and lactate dehydrogenase (LDH). Lipid peroxide (LOOH) and reduced thiol levels were also measured.

Oxidative stress indices.

Glutathione-S-transferase

Glutathione-S-transferase is generally found in the cytosol. The antioxidant enzyme activity of glutathione-S-transferases was measured by continuous kinetics of the product formed, S-2,4-dinitrophenyl-glutathione (S-DNP-GS). This was measured at 340 nm in 50 mM phosphate buffer pH 7.2. All enzyme activities were expressed as activity in units per individual [36, 37]. Rather than a standard curve, units per individual were calculated using a reaction coefficient.

Lipid hydroperoxides

Lipid hydroperoxides (LOOH) were extracted from 15 daphniids (D4) in 150 µl water, 150 µl methanol and 300 µl chloroform via vigorous vortexing, and separation of phases by centrifugation at 14,000 g at room temperature for 2 minutes. The

chloroform phase was isolated, evaporated using a speedvac and resuspended in 250 µl methanol. Samples of 200 µl were mixed with 25 µl xylenol orange (1 mM in 0.25 M H_2SO_4) and 25 µl ammonium iron (II) sulfate hexahydrate (8 mM in 5 mM H_2SO_4). Absorbance was measured at 560 nm and converted to concentration equivalents using the corresponding standard curve of cumene hydroperoxide (0.4-4 µM) [38].

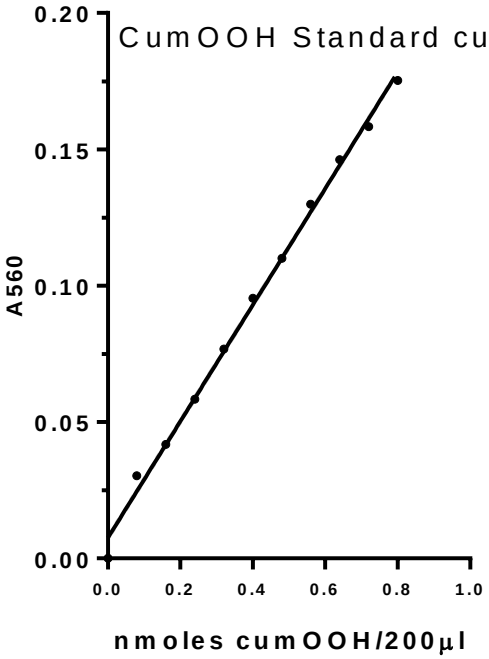


Figure 4: A cumene hydroperoxide (cumOOH) standard curve used in quantification of lipid peroxide levels.

Reduced thiols

To assess the thiol redox state, reduced thiol levels were quantified by reaction with 2,2'-dipyridyldisulfide (DPS) and the resulting formation of a 4-thiopyridine complex absorbing at 325 nm [39].

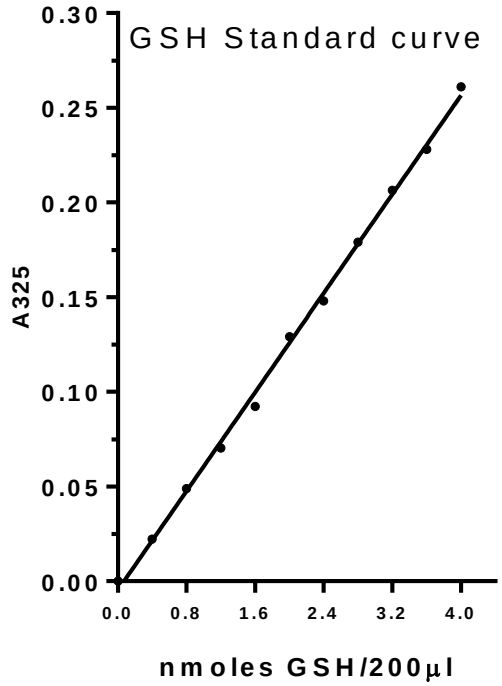


Figure 5. A glutathione (GSH) standard curve used to quantify reduced thiol levels.

Enzyme indices of physiology.

Acid and alkaline phosphatase

Acid phosphatase is typically found in the lysosomes, while alkaline phosphatases tend to be anchored on the extracellular surface of the cell membrane. Activities of alkaline and acid phosphatases was assessed by the release of *p*-nitrophenol (PNP) using *p*-nitrophenyl phosphate (pNPP) as substrate in 50 mM boric acid (pH 9.8) or 50 mM citric acid (pH 4.5), respectively. 200 µl of sample, appropriately diluted in either citric of boric acid buffer, was mixed with 50 µl of substrate in the buffer, at a concentration of 3 mg/mL, and incubated for 30 minutes at room temperature. After incubation, the reaction was halted by alkalisation using 50 µl of 4 M NAOH. The addition of NAOH converts *p*-nitrophenol to *p*-nitrophenolate, developing a strong yellow colour, while the concurrent change in pH halts enzyme activity. Absorbance was measured at 405 nm and converted to nmole *p*-nitrophenol using a *p*-nitrophenyl phosphate (pNPP) standard curve, and subsequently expressed as units/animal. A unit of enzyme activity is defined as the amount of enzyme activity which catalyses the conversion of 1 µmol of substrate into product in 1 minute [40].

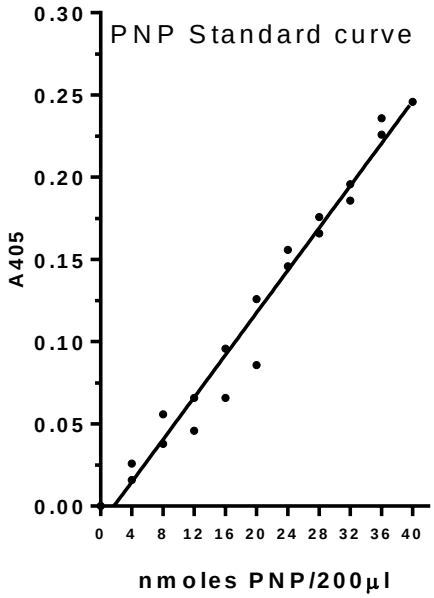


Figure 6. A PNP standard curve used to quantify the activity of ACP, ALP, and β-Gal

β-galactosidase

β-galactosidase is generally found in the lysosomes. The activity of β-galactosidase was quantified by the release of *o*-nitrophenol using *o*-nitrophenyl-β-galactoside as substrate. 200 µl of sample, appropriately diluted in 50 mM phosphate buffer (pH 7.2) was mixed with 50 µl of 8 mM substrate and incubated for 30 minutes at room temperature. Following this incubation period, the reaction was halted by alkalisation using 4 M NAOH. Absorbance was measured at 405 nm [41].

Lipase

The activity of lipase was quantified by the conversion of *p*-nitrophenyl butyrate to *p*-nitrophenol. 200 µl of sample, appropriately diluted in 50 mM phosphate buffer (pH 7.2) was mixed with 50 µl of 2.84 mM substrate and incubated for 5 minutes at room temperature. Following incubation, absorbance was read at 405 nm, converted to nmoles *p*-nitrophenol using a pNPP standard curve [42].

Peptidase

γ-glutamyl transferase is typically found in the cell membrane. The activity of peptidase (specifically γ-glutamyl transferase) was quantified using L-leucine-4-nitroanilide as a substrate. 200 µl of sample, appropriately diluted in 50 mM phosphate buffer (pH 7.2) was mixed with 50 µl of 8 mM substrate in 100% DMSO and the release of the product (*p*-nitroanilide) was measured by continuous kinetics at 418 nm [43].

Lactate dehydrogenase

Lactate dehydrogenase is typically found in the cytosol. The activity of lactate dehydrogenase was quantified by monitoring the decrease in absorbance at 340 nm caused by the oxidation of NADH. The substrate used was a 1:1 mixture of 40 mM pyruvate and 0.5 mM NADH. 200 µl of sample, appropriately diluted in 50 mM phosphate buffer (pH 7.2), was mixed with 50 µl of this substrate. The resulting reaction was measured by continuous kinetics at 340 nm [44].

All enzyme activities were expressed as activity in units per individual.

Statistical analysis. The data were analysed and plotted using the Excel and GraphPad Prism software packages. For biochemical and phenotypic analyses, the Student's unpaired *t*-test was performed for comparisons between exposed and controls or DMSO (as carrier solvent for chemicals dissolved in DMSO). For toxicity curves the dose response model was fit and EC values were calculated.

Results

Pollution assessment requires a deeper mechanistic understanding of the underlying toxicity mechanisms in freshwater species. As such, individuals and mixture of stressors were assessed for their impact in physiology of daphniids with biochemical and phenotypic endpoints.

Exposure to individual stressors

Acute toxicity was assessed to estimate the working concentrations for each chemical. Toxicity curves (Supplementary figures 1-4) carried out using 4-day old animals highlighted an EC_5 concentration as the optimal exposure concentration, in order to avoid high mortality rates due to excess stress, and additionally, because slight variations in concentration would result in higher shifts in mortality especially for chemicals with steep toxicity curves (Table 2).

Table 2. EC values (mg/L) for acute toxicity in 4-day old animals. Data represent results from toxicity curves (with N=4 replicates per concentration).

Type	Chemical	EC_{50}	EC_1	EC_5	EC_{10}
NSAIDs COX inhibitors	Acetylsalicylic acid	75.8	53.27	60.47	64.04
	Ibuprofen	35.15	20.12	24.58	26.92
	Diclofenac	84.86	35.61	48.65	56.03
	Indomethacin	34.92	16.94	21.97	24.71
Beta blocker	Propranolol	83.62	25.46	39.03	47.35
Oral antihyperglycemic	Metformin	145	89.55	106.47	115.15
Anthelmintic drug	Mebendazole	0.76	0.42	0.52	0.57
Calcium channel blocker	Diltiazem hydrochloride	80.82	61.04	67.52	70.67
Antipsychotic	Chloropromazine	12.51	5.35	7.26	8.33
Metal Ions	Aluminium sulfate hexadecahydrate	59.39	24.88	34.01	39.18
	Cadmium chloride	1.11	0.29	0.47	0.59
	Cobalt nitrate	90.53	41.11	54.55	62.03
	Lithium chloride	93.65	57.30	68.36	74.05
	Zinc sulfate heptahydrate	29.75	9.04	13.87	16.83
	Zirconium chloride	26.96	10.23	14.49	16.96
Herbicide (phosphonate)	N-phosphonomethyl glycine (glyphosate)	61.29	1.65	1.69	1.72
Stimulant	Nicotine	455	333.28	372.71	392.07
Phenolic compound	4-nitrophenol	8.609	6.65	7.29	7.61
Plasticizer	Phthalic acid	46.44	34.87	38.65	40.49

Mortality results were associated with the impact of chemicals in enzyme indices of physiology (such as phosphatases, galactosidase etc.) and oxidative stress indices (i.e. thiol redox status and antioxidant enzymes activity), as well as metabolic perturbations.

Toxicity of metals

The physiology responses of *Daphnia magna* to metal toxicity were evaluated with surrogate markers for the enzymatic activities of phosphatases, galactosidase, lipase and peptidase (Figure 7). Phosphatase activity tended to decrease following exposure to the tested metals. Aluminium, lithium, and zinc significantly decreased the activity of both phosphatases. Aluminium decreased ALP by 38% and ACP by 14%. Lithium decreased ALP by 68% and ACP by 59%. Zinc decreased ALP by 21% and ACP by 27%. Cadmium and zirconium only decreased the activity of acid phosphatase (-27% and -36% respectively), whereas cobalt only decreased alkaline phosphatase activity (-30%). In relevance to enzymes related to sugar and peptide degradation, there is a significant decrease in β-galactosidase activity for aluminium (-59%), cobalt (-59%), lithium (-72%), and zirconium (-56%). Only cadmium and zinc had no significant impact on the activity of β-galactosidase. Metal exposure resulted in a general decrease in lipase activity. The tested metals which caused this decrease were aluminium (-39%), lithium (-54%), and zirconium (-37%). Cadmium, cobalt, and zinc had no significant impact. Lactate dehydrogenase activity was greatly increased by all tested metals, with one exception. Aluminium exposure caused a 255% increase, cobalt a 239% increase, lithium 260%, zinc 76%, and zirconium 289%. Cadmium was the sole exception to this trend, causing a significant decrease (-63%) in LDH activity. A significant decrease in peptidase activity was also observed as a consequence of metal exposure. Aluminium (-49%), cobalt (-71%), lithium (-80%), and zirconium (-55%) caused significant decreases, while cadmium and zinc had no significant effects.

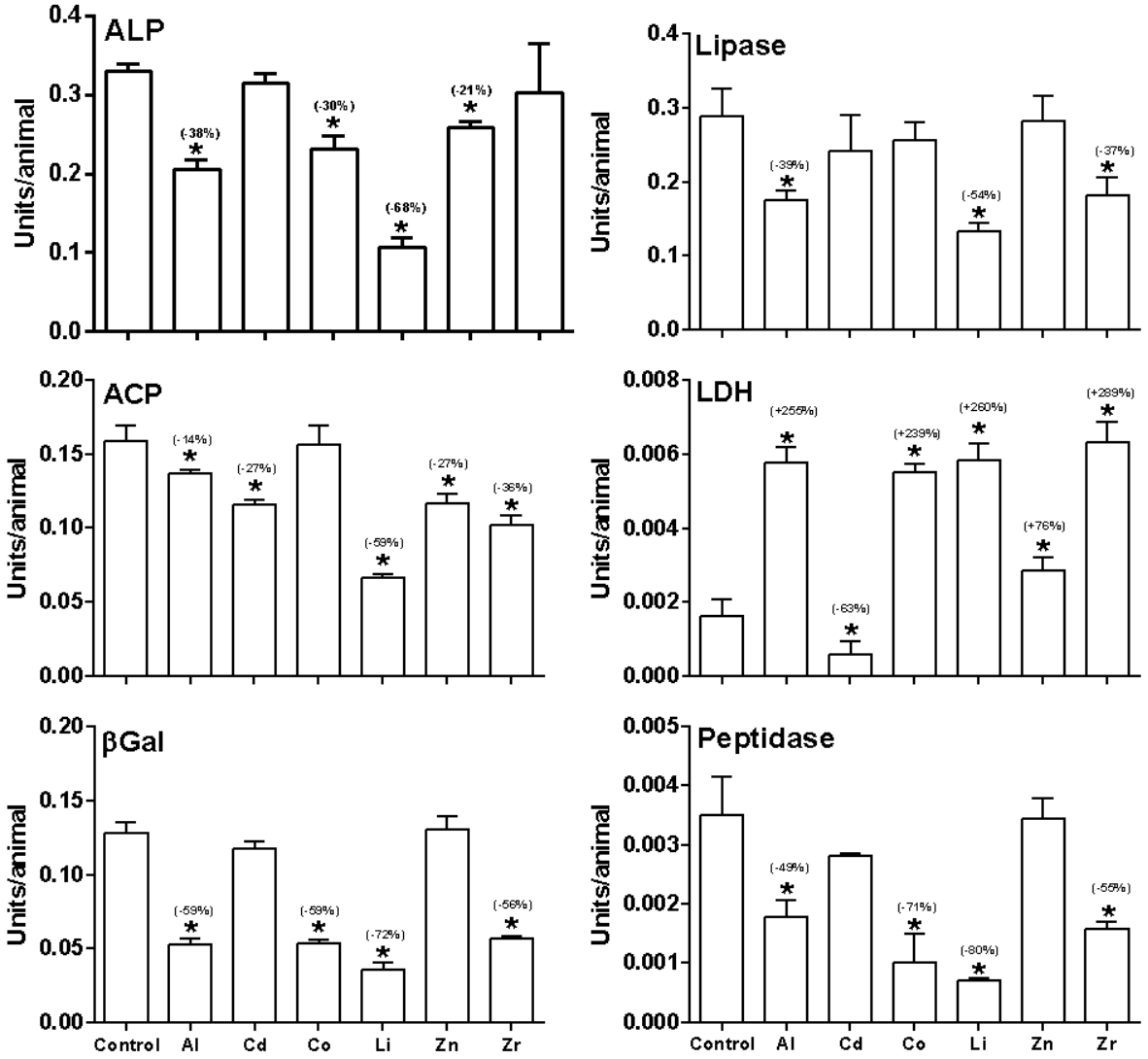


Figure 7. Impact of metals on key enzyme activities. Abbreviations: Con(Control), Al (Aluminium), Cd (Cadmium), Co (Cobalt), Li (Lithium), Zn (Zinc), Zr (Zirconium). Data represent average±SD (N=4) replicates for each condition. *Statistically significant by Student's *t*-test compared to control ($p<0.05$).

Regarding stress responses, GST, LOOH and reduced thiols were assessed (Figure 8). A significant increase in glutathione-S-transferase activity was recorded for all metals except for Zn. This increase varies greatly between metals. Lithium caused the greatest increase (+1100%),followed by aluminium (+560%), cobalt (+364%), zirconium (+103%), and cadmium (+68%). The tested metals also tended to increase lipid peroxide (LOOH) levels, though one condition caused a significant decrease. Lithium, zinc, and zirconium caused significant increases (+52%, +49%, +79% respectively), while cobalt caused a significant decrease (-32%), and aluminium and

cadmium had no significant effect. In terms of thiol redox state as portrayed through reduced thiols levels, the overall trend was a reduction in the levels of reduced thiols. Aluminium, cobalt, lithium, and zirconium decreased reduced thiol levels (-16%, -40%, -42% -13% respectively), while zinc broke this trend, and increased reduced thiol levels by 10%. Cadmium had no significant impact.

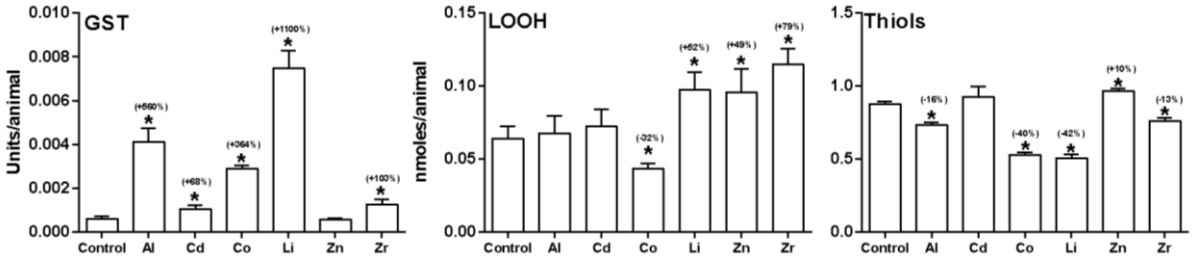


Figure 8. Impact of metals on GST, lipid hydroperoxides (LOOH), and reduced thiols. Abbreviations: Con(Control), Al (Aluminium), Cd (Cadmium), Co (Cobalt), Li (Lithium), Zn (Zinc), Zr (Zirconium). Data represent average±SD (N=4) replicates for each condition. *Statistically significant by Student's *t*-test compared to control ($p<0.05$).

Toxicity of NSAIDs

Upon exposure to various NSAIDs, a slight decrease in overall phosphatase activity was recorded (Figure 9). Acid phosphatase is more affected, showing a significant decrease for acetylsalicylic acid (-19%), ibuprofen (-16%), and diclofenac (-14%). Alkaline phosphatase activity was significantly decreased only by acetylsalicylic acid (-6%). Only acetylsalicylic acid significantly decreased the activity of both acid and alkaline phosphatase. Diclofenac and ibuprofen significantly decreased acid phosphatase levels only. The activity of β-galactosidase was not significantly affected by the majority of the tested NSAIDs, with the exception of acetylsalicylic acid, which caused a small increase of 8% in activity. NSAID exposure caused a general increase in lipase activity, with acetylsalicylic acid, diclofenac and indomethacin causing 30%, 40% and 22% increase, respectively. Only ibuprofen had no significant impact. For lactate dehydrogenase (LDH) activity diclofenac and ibuprofen caused a 38% and 31% increase, respectively, while acetylsalicylic acid and indomethacin had no significant impact. Peptidase was also affected in the opposite manner, with ibuprofen and indomethacin leading to a 44% and 34% decrease in activity, respectively. Acetylsalicylic acid and diclofenac had no significant impact.

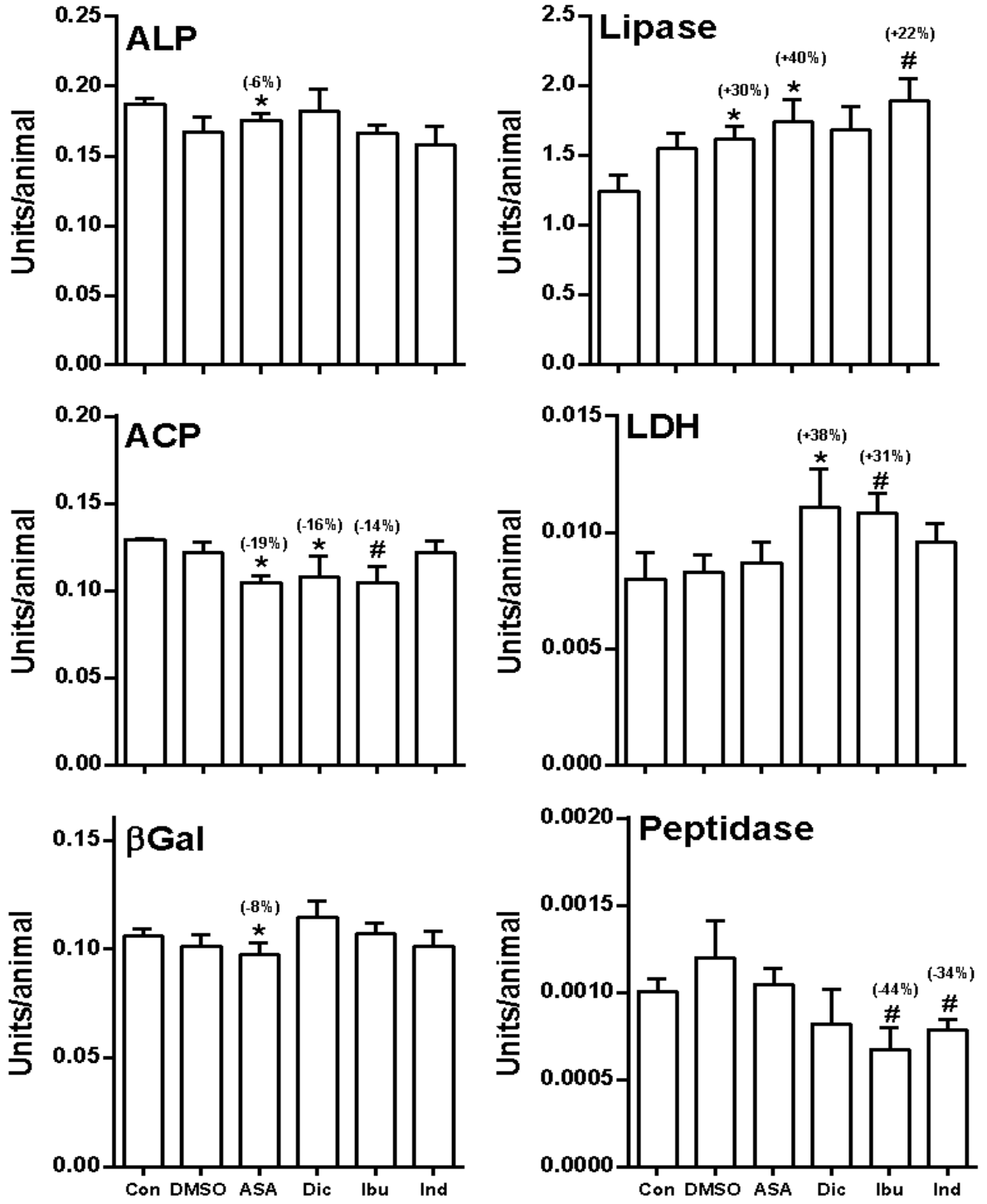


Figure 9. Impact of NSAIDs on key enzyme activities. Abbreviations: Con(Control), DMSO (dimethyl sulfoxide), ASA (Acetylsalicylic acid), Dic (Diclofenac), Ibu (Ibuprofen), Ind (Indomethacin). Data represent average±SD (N=4) replicates for each condition. Statistically significant by Student's *t*-test compared to control (*) or DMSO (#) for $p<0.05$.

Considering enzymes relevant to response to xenobiotics, the general trend was an increase in GST activity, though this was only statistically significant for acetylsalicylic acid (+81%) and diclofenac (+59%) (Figure 10). Of the tested NSAIDs, only diclofenac had a significant impact on lipid hydroperoxides (LOOH) levels, causing a 54% decrease, while acetylsalicylic acid, ibuprofen, and indomethacin had no significant impact. This could be also tied to small changes observed in reduced thiol levels, with only acetylsalicylic acid and indomethacin leading to 8% and 6% decrease, respectively.

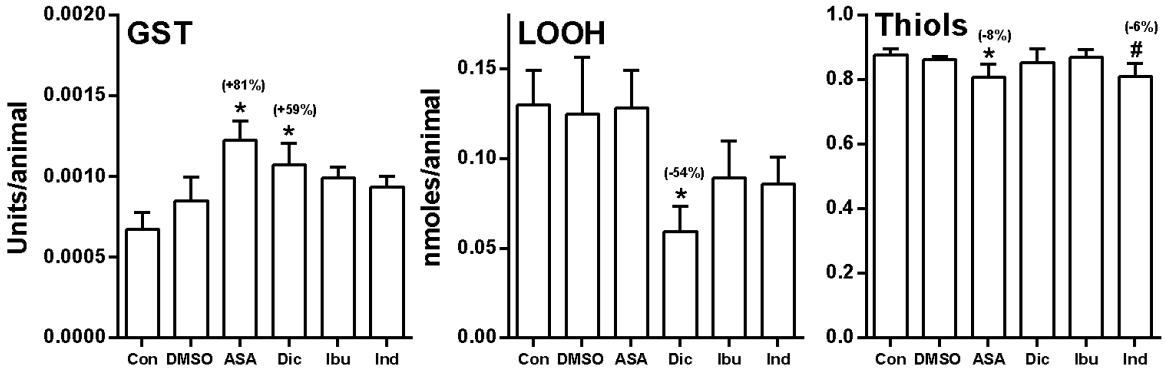


Figure 10. The impact of NSAIDs on GST, lipid hydroperoxides *LOOH) and reduced thiols. Abbreviations: Con (Control), DMSO (dimethyl sulfoxide), ASA (Acetylsalicylic acid), Dic (Diclofenac), Ibu (Ibuprofen), Ind (Indomethacin). Data represent average±SD (N=4) replicates for each condition. Statistically significant by Student's *t*-test compared to control (*) or DMSO (#) for $p<0.05$.

Toxicity of pharmaceuticals with different mode of action

Exposure to the tested pharmaceutical compounds tends to cause an increase in phosphatase activity (Figure 11). Diltiazem and propranolol significantly increased both acid and alkaline phosphatase activity. Diltiazem increased acid phosphatase activity by 47% and alkaline phosphatase activity by 47%, while propranolol increased acid phosphatase activity by 79% and alkaline phosphatase activity by 123%. Chlorpromazine and mebendazole increased only alkaline phosphatase activity, by 72% and 34% respectively. B-galactosidase levels were significantly decreased by metformin (-30%) and mebendazole (-40%). Diltiazem, chlorpromazine, and propranolol had no significant impact. Pharmaceutical exposure had no significant impact on lipase activity, whereas a minimal impact on LDH activity was observed for propranolol, with a 34% decrease. Exposure to the tested pharmaceuticals caused

significant decreases in peptidase activity, for metformin by 52%, chlorpromazine by 16%, mebendazole by 27% decrease, and propranolol by 22%.

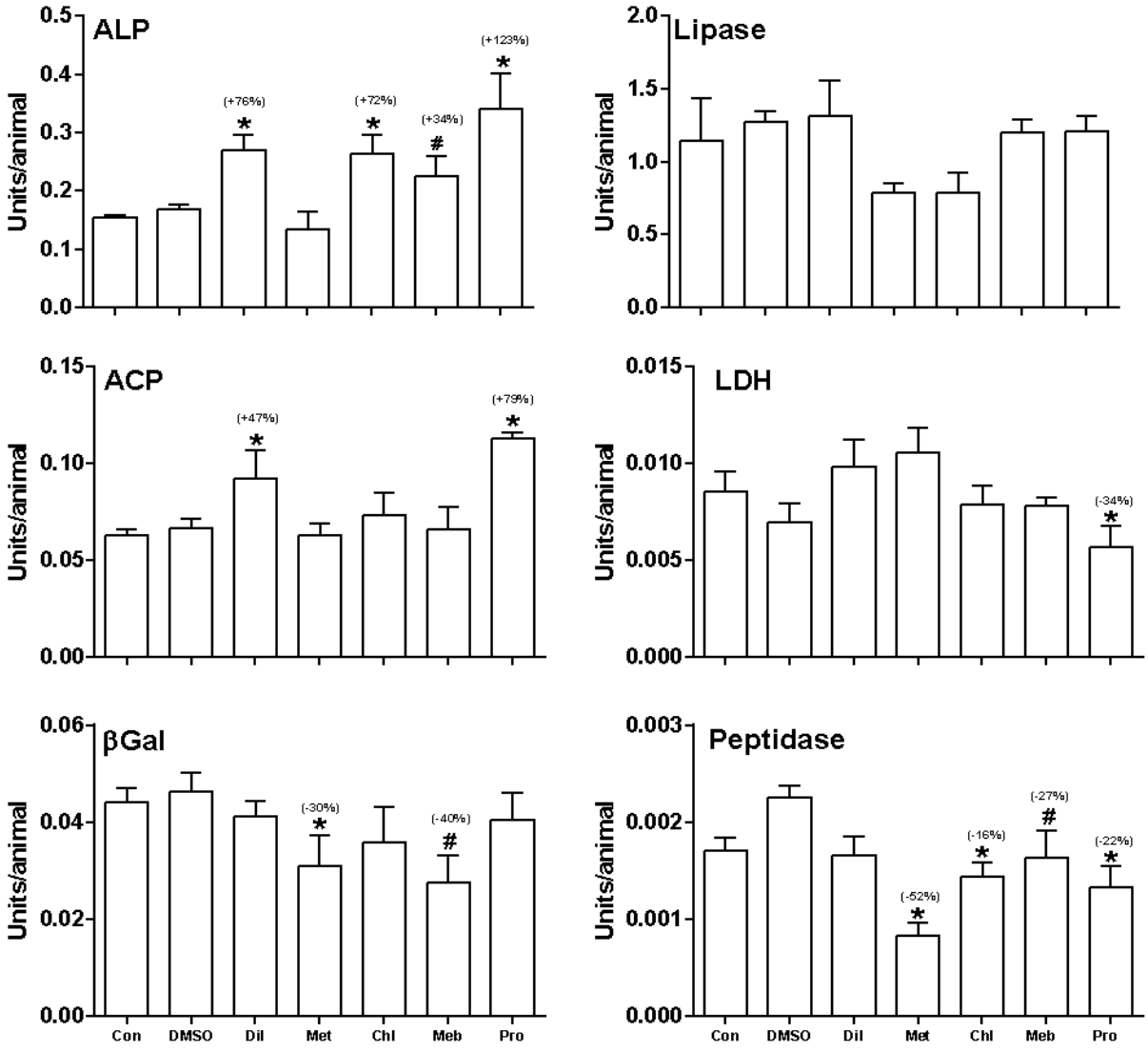


Figure 11. Impact of pharmaceuticals on key enzyme activities. Abbreviations: Con(Control), DMSO (dimethyl sulfoxide), Dil (Diltiazem), Met (Metformin), Chl (Chlorpromazine), Meb (Mebendazole), Pro (Propranolol). Data represent average±SD (N=4) replicates for each condition. Statistically significant by Student's *t*-test compared to control (*) or DMSO (#) for $p<0.05$.

In relevance to metabolizing enzymes such as GST, the aforementioned pharmaceutical compounds caused an increase in GST activity for diltiazem (+25%), metformin (+123%), mebendazole (+42%) and propranolol (+516%) (Figure 12). In parallel, LOOH were decreased for diltiazem by 20%, while increased form metformin

and propranolol increased by 18% and 15% respectively. Thiol redox state as it was described by reduced thiols were generally diminished by exposure to by all pharmaceuticals except metformin. Diltiazem reduced thiol levels by 16%, chlorpromazine by 32%, mebendazole by 26%, and propranolol by 17%.

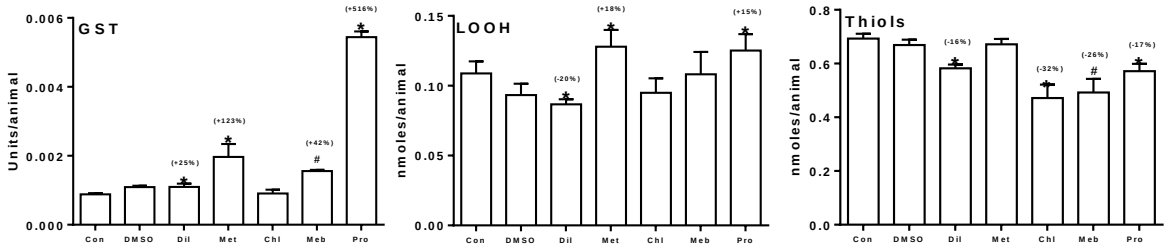


Figure 12. Impact of pharmaceuticals on GST, lipid hydroperoxides and reduced thiols. Abbreviations: Con(Control), DMSO (dimethyl sulfoxide), Dil (Diltiazem), Met (Metformin), Chl (Chlorpromazine), Meb (Mebendazole), Pro (Propranolol). Data represent average±SD (N=4) replicates for each condition. Statistically significant by Student's *t*-test compared to control (*) or DMSO (#) for $p<0.05$.

Toxicity of a variety of pollutants

Significant decreases on both acid and alkaline phosphatase for both N-phosphonomethyl glycine (glyphosate) and 4-nitrophenol were recorded (Figure 13). N-phosphonomethyl glycine decreased acid phosphatase activity by 17% and alkaline phosphatase activity by 49%, while 4-nitrophenol decreased acid phosphatase activity by 23% and alkaline phosphatase activity by 22%. L-nicotine and phthalic acid had no significant impact. A significant decrease in β-galactosidase activity was observed for L-nicotine and 4-nitrophenol. N-phosphonomethyl glycine and phthalic acid had no significant impact. Lipase activity was largely unaffected by exposure to the tested pollutants, showing no significant impacts. Exposure to pollutants had significant impacts on LDH activity levels. Both N-phosphonomethyl glycine (+38%) and phthalic acid (+136%) caused significant increases in LDH activity, while L-nicotine caused a significant decrease (-57%). 4-nitrophenol had no significant impact. The majority of tested pollutants had no significant impact on peptidase activity. 4-nitrophenol alone caused a significant decrease of 38% in peptidase activity.

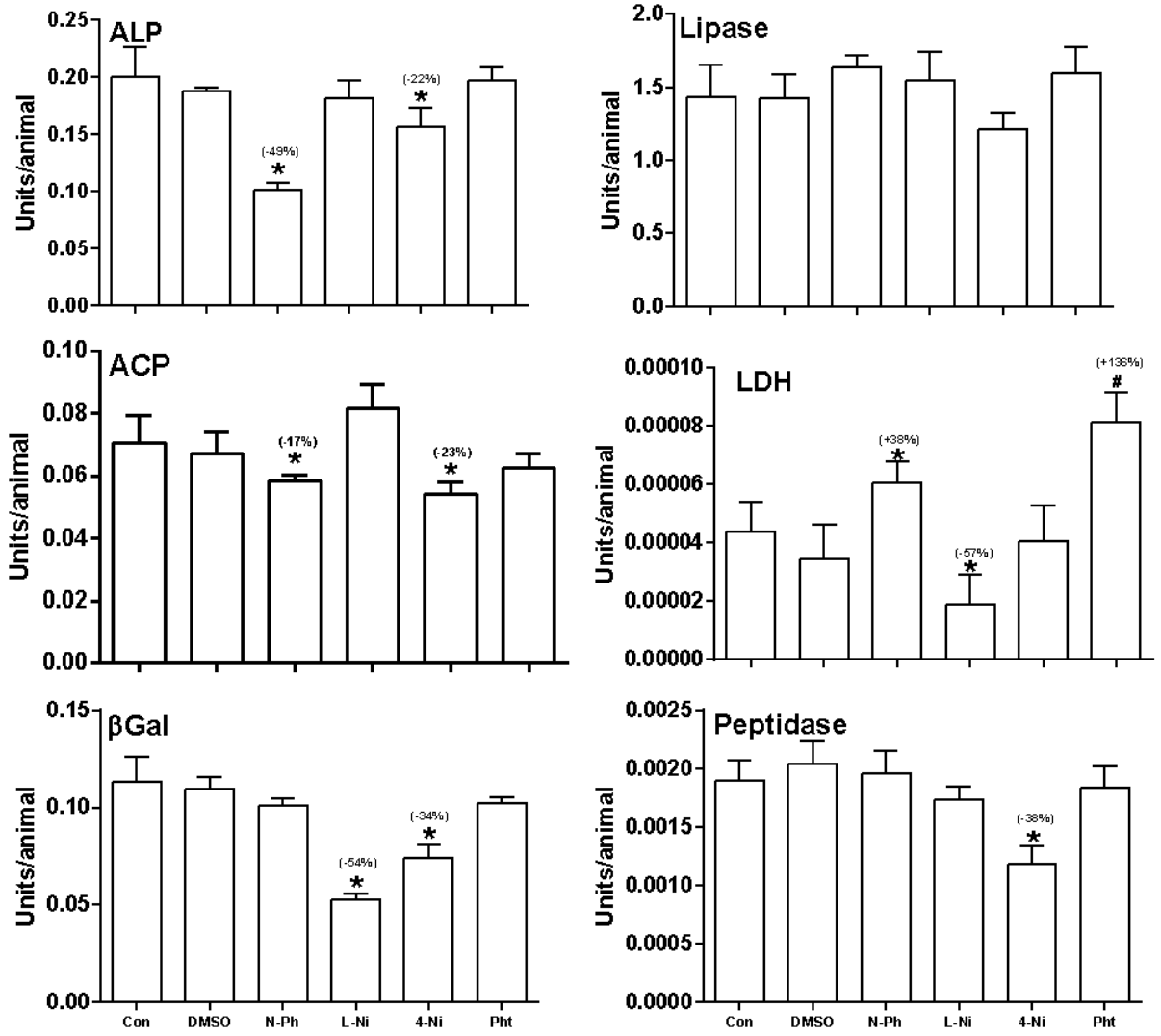


Figure 13. Impact of pollutants on key enzyme activities. Abbreviations: Con(Control), DMSO (dimethyl sulfoxide), N-Ph (N-phosphonomethyl glycine), L-ni (L-nicotine), 4-Ni (4-Nitrophenol), Pht (Phthalic acid). Data represent average±SD (N=4) replicates for each condition. Statistically significant by Student's *t*-test compared to control (*) or DMSO (#) for $p<0.05$.

Regarding oxidative stress responses, significant increases in GST activity were recorded for all four pollutants tested (Figure 14). All pollutants increased GST activity by a comparable amount. N-phosphonomethyl glycine was responsible for the largest increase (+23%), followed by phthalic acid (+21%), L-nicotine (+19%), and 4-nitrophenol (+18%). LOOH levels were significantly affected by two of the tested pollutants. N-phosphonomethyl glycine caused a 22% reduction in LOOH levels, while

phthalic acid caused a 35% increase. L-nicotine and 4-nitrophenol caused no significant impacts. The majority of tested pollutants caused a decrease in reduced thiol levels. L-nicotine caused a 36% decrease, 4-nitrophenol caused a 22% decrease, and phthalic acid caused a 16% decrease. N-phosphonomethyl glycine had no significant impact.

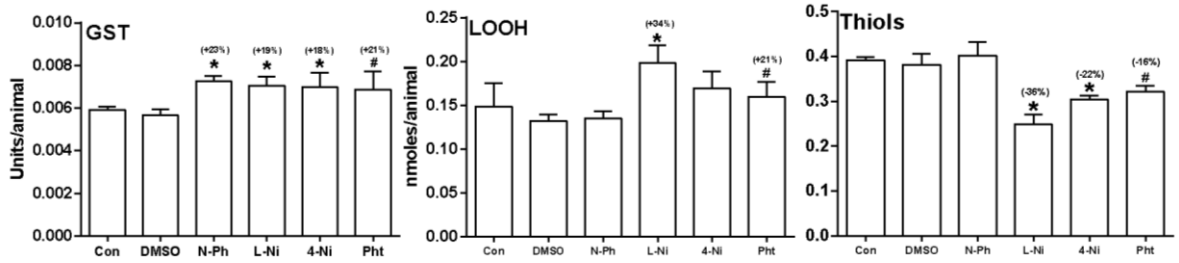


Figure 14. Impact of pollutants on GST, lipid hydroperoxides and reduced thiols. Abbreviations: Con(Control), DMSO (dimethyl sulfoxide), N-Ph (N-phosphonomethyl glycine), L-ni (L-nicotine), 4-Ni (4-Nitrophenol), Pht (Phthalic acid). Data represent average±SD (N=4) replicates for each condition. Statistically significant by Student's *t*-test compared to control (*) or DMSO (#) for $p<0.05$.

Responses to mixture toxicology

As environmental pollution is seldom caused by a single pollutant, two separate mixture scenarios were designed to reflect this. The first approach was a mixture of eight chemicals as a more complex mixture composed of chemicals of various types, while the second approach focused on three chemicals only and their possible combinations. For the first approach, the eight separate chemicals selected were lithium, aluminium, acetylsalicylic acid, propranolol, diltiazem, n-phosphonomethyl glycine, L-nicotine, and metformin. For the second approach, the three chemicals selected were lithium, propranolol, and L-nicotine. These were combined into 4 possible mixture combinations in the following manner: 3 double mixtures, consisting of lithium and propranolol, lithium and L-nicotine, and propranolol and L-nicotine, as well as a triple mixture containing all 3 chemicals.

Daphniids were exposed to each of the previously described mixtures for a period of 24 hours. Following this exposure period, whole animal homogenates were assayed to determine the impact of exposure on key enzymes, as well as oxidative stress indices. The ultimate goal of this thesis is to understand how the responses of *Daphnia*

magna are impacted by exposure to these pollutants, and how interactions between the components of the chemical mixtures employed change these responses.

Assessment of the impact of a composite mixture of chemicals

In the first approach a chemical mixture of eight environmentally relevant chemicals was explored. Lithium, aluminium, acetylsalicylic acid, propranolol, diltiazem, n-phosphonomethyl glycine, L-nicotine, and metformin were selected based on their results from the single chemical exposures. Each of these chemicals were combined at an EC_5 concentration, and from this mixture, a range of dilutions were used to construct a full toxicity curve (Figure 15). For this toxicity curve the dose-response relationship was plotted as $log_{10}EC_5$, and high (30% EC_5), medium (20% EC_5), and low (10% EC_5) concentrations were chosen for daphniid exposure.

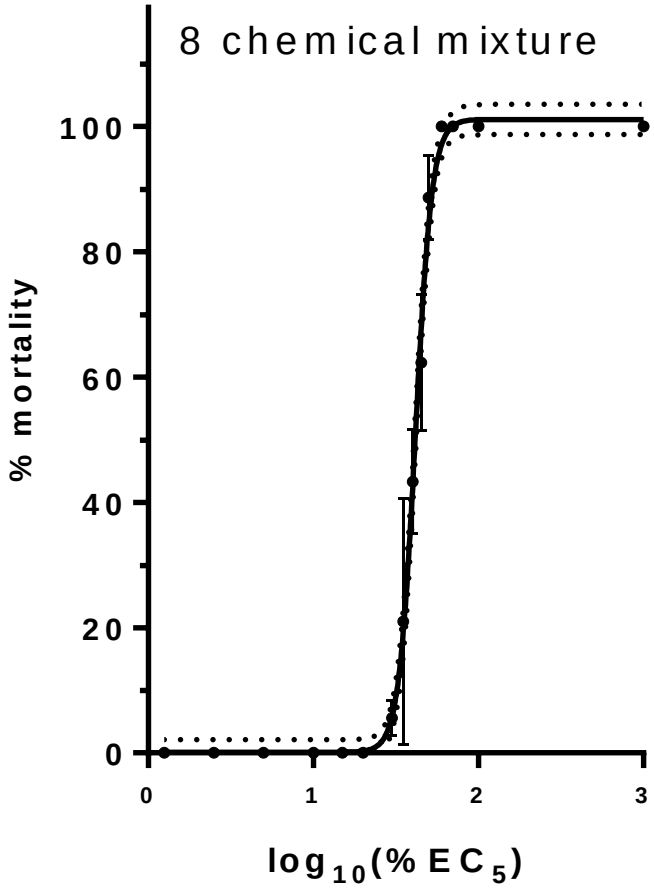


Figure 15. Acute toxicity curve (24 hour) for D4 daphniids exposed to a mixture of 8 chemicals. Data represent average±SD (N=6) replicates for each concentration. Y axis represents percentage of daphnia which died following 24 hour exposure, X axis

represents the $\log_{10}$ of the percentage of the EC_5 used for exposure. Solid line represents average mortality, dotted lines represent 95% confidence intervals.

Exposure to the 8 chemical mixture increased both acid (ACP) and alkaline (ALP) phosphatase activity in a dose-dependent manner (Figure 16). The 10% EC_5 mixture increased ACP by 20% and ALP by 44%, the 20% EC_5 mixture increased ACP by 44% and ALP by 54%, and the 30% EC_5 mixture increased ACP by 56% and ALP by 59%. It is worth noting that almost all assayed biomarkers for this mixture scenario followed this dose-dependent pattern. Exposure to the 8 chemical mixture showed an increasingly strong impact on β-galactosidase activity in a dose-dependent manner. Though all concentrations had statistically significant impacts, a clear trend is visible and higher concentrations of the mixture had greater impacts. 10% EC_5 mixture decreased β-galactosidase activity by 14%, 20% EC_5 mixture by 26%, and 30% EC_5 mixture by 38%. Exposure to the 8 chemical mixture caused an upward trend in lipase activity as concentration increased. The 10% EC_5 caused a 10% increase, 20% EC_5 caused a 34% increase, and 30% EC_5 caused a 68% increase. Exposure to the 8 chemical mixture had no significant impact on LDH levels until a concentration of 30% EC_5 was reached. At 30% EC_5, a 62% decrease in LDH activity was recorded. Exposure to the 8 chemical mixture had a significant impact on peptidase activity levels once a concentration of 20% EC_5 was reached. The 20% EC_5 mixture caused a decrease of 21% in peptidase activity levels, while the 30% EC_5 mixture caused a 13% decrease.

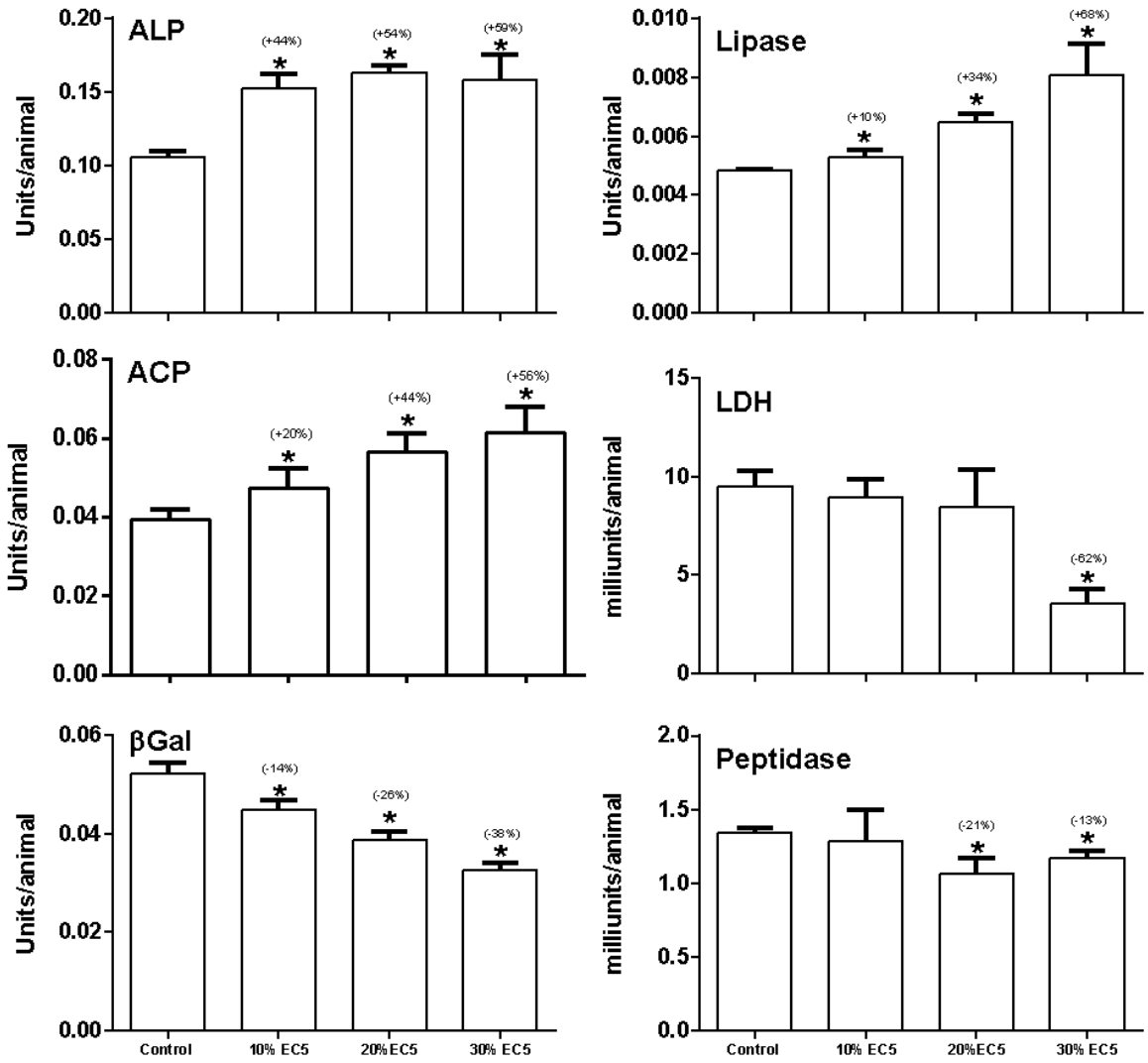


Figure 16. Impact of a composite chemical mixture on key enzyme activities. Data represent average±SD (N=4) replicates for each concentration. Statistically significant by Student's *t*-test compared to control (*) for $p<0.05$.

Exposure to the 8 chemical mixture resulted in a concentration dependent upward trend in GST activity (Figure 17). The 10% EC_5 mixture caused a 10% increase, 20% EC_5 caused a 34% increase, and 30% EC_5 caused a 77% increase. In parallel, a remarkably consistent increase in LOOH levels was observed for all tested concentrations. The 10% EC_5 mixture caused a 22% increase, 20% EC_5 caused a 25% increase, and 30% EC_5 caused a 24% increase. This was also correlated with a clear dose-dependent decrease in reduced thiols. The 10% EC_5 mixture caused a 24% decrease, the 20% EC_5 caused a 34% decrease, and the 30% EC_5 caused a 41% decrease, supporting a depletion of reduced thiols in response to pollution.

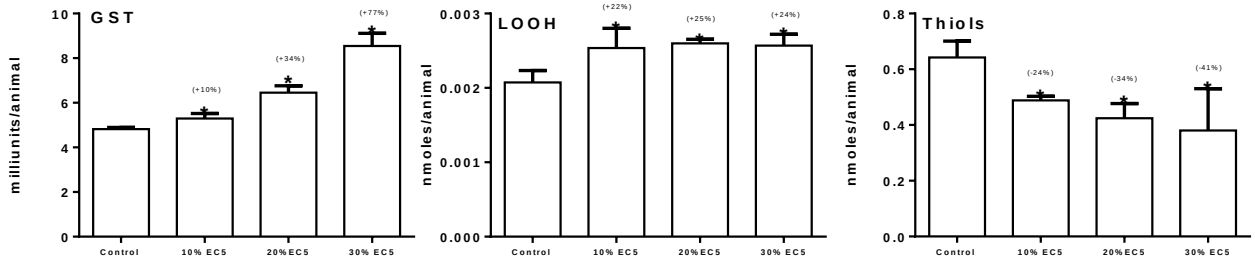


Figure 17. Impact of a composite chemical mixture on GST, lipid hydroperoxides (LOOH) and reduced thiols. Data represent average±SD (N=4) replicates for each concentration. Statistically significant by Student's *t*-test compared to control (*) for $p<0.05$.

Assessment of toxicity of single, double, and triple mixtures

The second scenario involved three chemicals and all possible mixture combinations. Lithium, propranolol, and L-nicotine were used in single, double, and triple mixtures. These chemicals were combined, at their EC_5 concentrations, in three double mixture combinations, each containing two of the three chemicals, and one triple mixture which contained all three of them. The EC_5 concentrations for each of these chemicals were derived from previously constructed toxicity curves. The decision to employ further dilutions of this EC_5 concentration was rooted in the idea that complex mixtures of chemicals could have potential synergistic toxic effects, and that this could cause high mortality among exposed daphniids, thus limiting the numbers which could be used for testing. Hence, sub-EC_5 concentrations were chosen to lessen this impact.

Full toxicity curves were generated for three double mixtures, consisting of lithium and propranolol, lithium and L-nicotine, and propranolol and L-nicotine, as well as a triple mixture containing all three chemicals (Figure 18). Based on these toxicity curves, a single concentration (at 20% EC_5) was chosen for daphniid exposure. This concentration was chosen so that a measurable impact would be present for each mixture, and so that mortality would not become excessively high. Also, as we would need to know the impact of each individual chemical as well, these were also included separately at a 20% EC_5 concentrations.

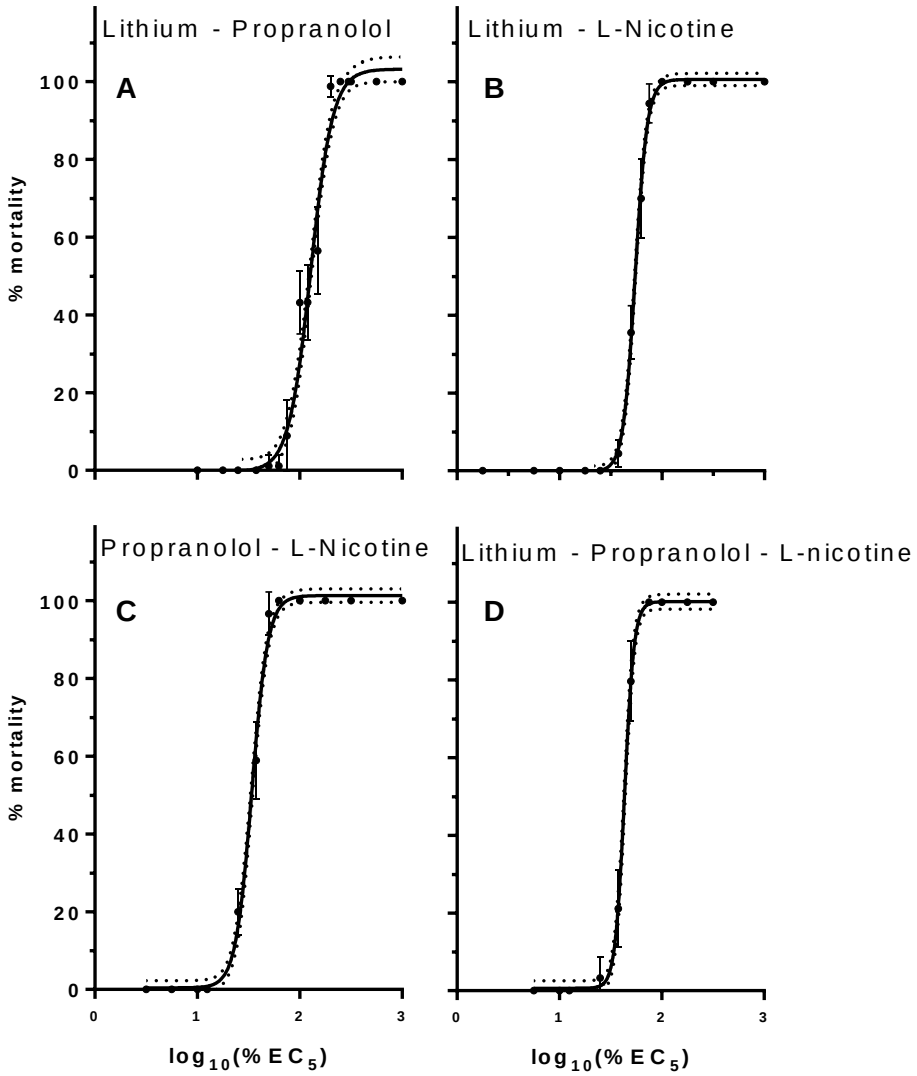


Figure 18. Acute toxicity curve (24 hour) for D4 daphniids exposed to mixtures of lithium, propranolol, and L-nicotine. Data represent average±SD (N=6) replicates for each concentration. Y axis represents percentage of daphnia which died following 24 hour exposure, X axis represents the $\log_{10}$ of the percentage of the EC_5 used for exposure. Solid line represents average mortality, dotted lines represent 95% confidence intervals.

The biochemical responses were assessed in single and mixture exposures (Figure 19). ALP was significantly impacted by all exposure conditions. Interestingly, only lithium caused a significant reduction (-56%), while all other conditions caused a significant increase. The other single chemicals, L-nicotine and propranolol, caused a 33% and a 28% increase respectively. Among the mixture conditions, lithium:propranolol caused a 35% increase, propranolol:nicotine a 101% increase, and lithium:propranolol:nicotine an 84% increase. ACP was significantly impacted by only

2 of the exposure conditions. Lithium resulted in a 47% decrease, while the propranolol:nicotine mixture caused a 40% increase. B-Gal activity was significantly increased by 2 of the mixture exposure conditions. The propranolol: nicotine mixture caused a 33% increase, while the lithium: propranolol: nicotine mixture resulted in a 17% increase. The general trend was an increase in lipase activity following exposure. Each of the single chemicals (lithium, L-nicotine, propranolol) significantly increased lipase activity, by 43%, 49%, and 23% respectively. However, of the double and triple mixtures, only the propranolol:L-nicotine double mixture caused a significant increase (+24%). The lack of impact by the other mixtures indicates a potential masking effect caused by interactions between the constituent chemicals. None of the exposure conditions tested had any significant impact on LDH activity. Exposure to single, double, and triple chemical mixtures had mixed impacts on peptidase activity. None of the single chemicals had a significant impact, nor did the triple mixture. However, the lithium:propranolol mixture and propranolol:L-nicotine mixture both had significant impacts, the former decreasing activity by 45%, and the latter increasing activity by 34%.

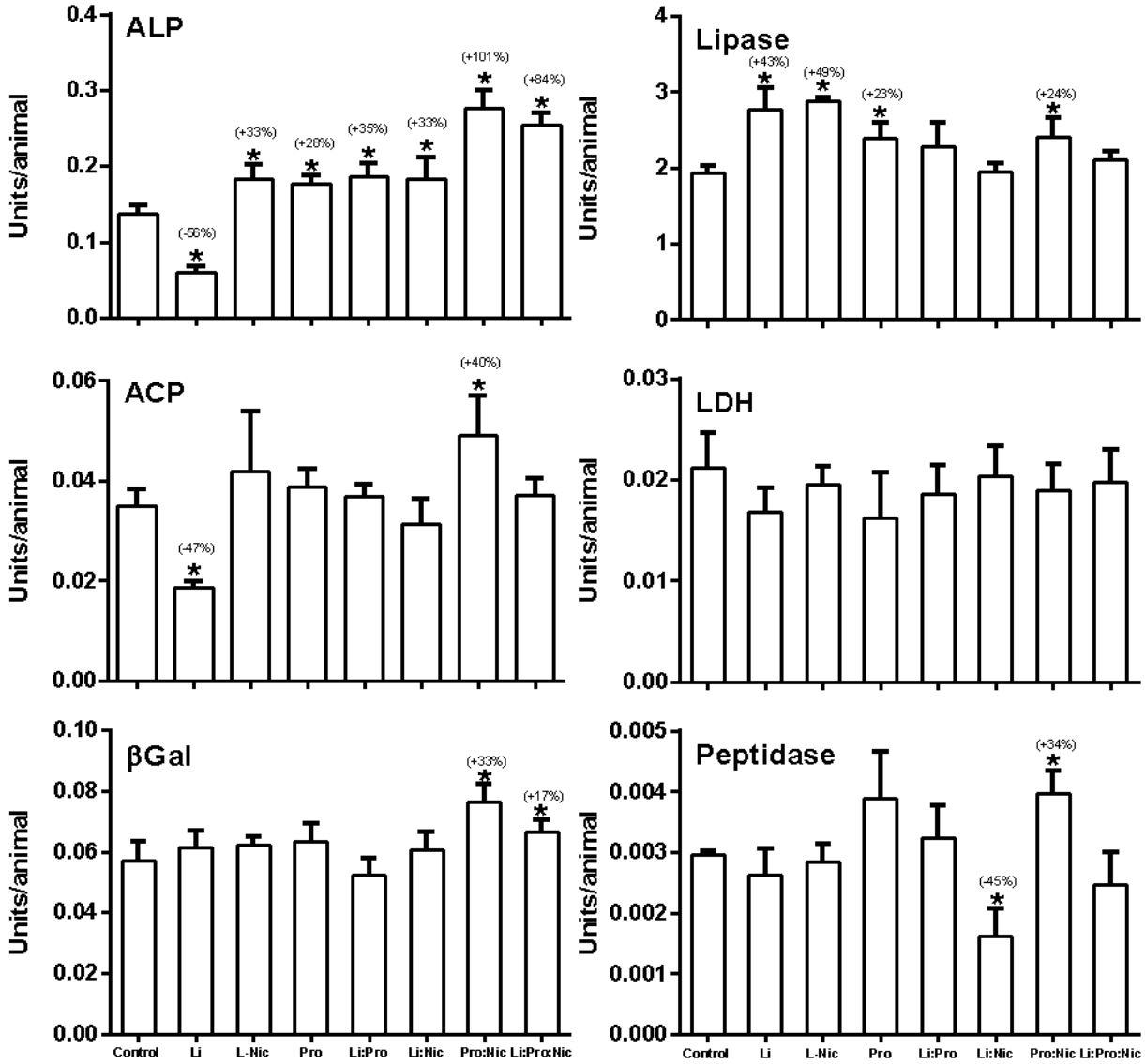


Figure 19. Impact of single, double, and triple chemical mixtures on key enzyme activities. Data represent average±SD (N=4) replicates for each condition. Statistically significant by Student's *t*-test compared to control (*) for $p<0.05$.

Considering the responses to oxidative stress (Figure 20), the activity of GST was significantly increased by several exposure conditions. Lithium caused a 22% increase, while the lithium: nicotine mixture caused a 26% increase, and the lithium: propranolol: nicotine mixture caused a 25% increase. The majority of exposure conditions had no significant impact on LOOH levels. Propranolol was the sole condition which had a significant effect, causing a 7% decrease. Reduced thiol levels were unaffected by most exposure conditions. Those conditions which had a significant impact were interesting, as they had converse effects. The

propranolol:nicotine double mixture caused an increase of 20%, while the lithium:propranolol:nicotine triple mixture caused a decrease of 21%.

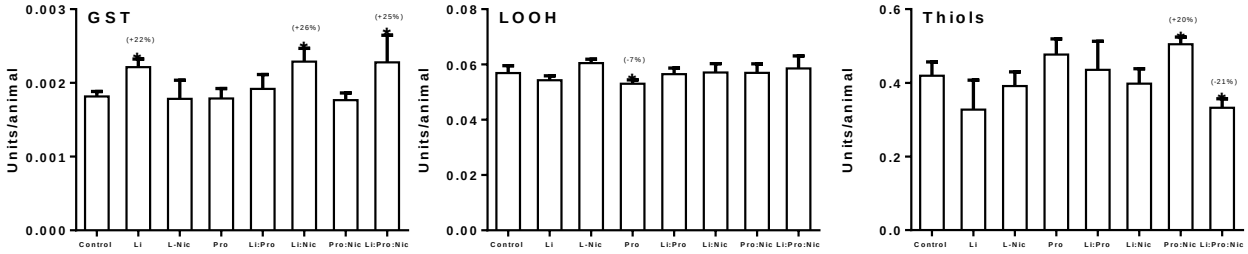


Figure 20. Impact of single, double, and triple chemical mixtures on GST, lipid hydroperoxides and reduced thiols. Data represent average±SD (N=4) replicates for each condition. Statistically significant by Student's *t*-test compared to control (*) for $p<0.05$.

Discussion

Despite the thousands of anthropogenic pollutants found in the environment, the ecotoxicity mechanisms of many remain a mystery, unstudied as of yet. This study explored the toxicity potential of a variety of pollutants in single and mixture scenarios. Characteristic responses were recorded and would be worthy of further in depth investigation in daphniids as they could pose key events for adverse outcome pathways.

Effects of metals on daphniids

Some initial steps have been taken in this area, for example copper is suspected to exert its toxic effects through disruption of the activity of Na+/K+ ATPase, resulting in issues in ionoregulation [45]. Many heavy metals are reported to cause oxidative stress and thus contribute to the generation of reactive oxygen species [46]. Toxicity mechanisms for other metals and metalloids, such as silver and arsenic are also suspected or assumed, but there remains a large knowledge gap in the understanding of the toxicity mechanisms of many common pollutants. Thousands of chemicals remain largely unscreened and unstudied in this regard, necessitating the development of a reliable holistic approach to screen them.

Aluminium has previously been shown to induce adverse effects on *Daphnia magna*, and one recent study reported a 44.27 mg/L EC_{50}, which is in a similar range to the reported EC_{50} of 59.39 mg/L in our study. Phenotypic and genomic impacts were shown, decreasing growth rates and causing a delay in the age of maturity from as low as 1.52 mg/L, as well as decreased moulting at 6.06 mg/L. Aluminium exposure also resulted in differential expression of 155 genes [47]. Aluminium toxicity is believed to be at least partly due to its capacity to strongly bind phosphorus, thus reducing its availability. Ionic aluminium can also inhibit extracellular phosphatases. Thus, the negative impacts of ionic aluminium on ACP, as well as on the entire phosphate metabolism of certain algal species have been suggested by many studies [48].This observation is in line with the decrease in phosphatase activity reported in our study, which was also the case for β-galactosidase, peptidase and lipase but not LDH.

Cadmium is a heavy metal of considerable toxicity in all species. In daphniids it has been shown to have ~80% mortality over a 21-day chronic exposure at a very low

concentration of 1.5 µg/L which also affected embryonic development and reproduction time [49]. Interestingly, daphniids appear to be capable of developing a level of tolerance to this metal through the synthesis of metallothionein-like protein which increase chelating of toxic metals [50]. Distinct responses were recorded in our study with the most prominent being a decrease in LDH which could be attributed to a metabolic shift and an impact on the activity of GST, which has also been observed in other organisms [51].

Cobalt appears to be understudied in the context of ecotoxicology. There is a notable lack of published studies on the impact of cobalt on freshwater organisms, particularly in the area of biochemical endpoints. One study which did investigate the effects of cobalt exposure on *Daphnia magna* (and other freshwater organisms) found an EC_{50} of 5.89 mg/L for neonatal daphnia [52]. This result is dramatically lower than our determined EC_{50} (90.53 mg/L), although it should be noted that our study used 4-day old daphniids as opposed to neonates, which may partially explain the difference in sensitivity. Despite its lack of prominence in the literature, cobalt was shown here to significantly impact the activities of ALP, βgal, lipase, LDH, peptidase, and GST, as well as levels of LOOH and reduced thiols. These results indicate a dramatic metabolic shift as a result of exposure and should warrant further study.

Lithium has been shown to exert toxic effects on various aquatic organisms and shown to alter gene expression in *Daphnia magna*. One study discovered a total of 143 genes which exhibited differential expression in response to lithium oxide exposure, some differentially expressed by over 3-fold. Protein folding was noted as the process most commonly altered in response to lithium exposure [53]. Additionally, lithium is known to interfere with the activity of Na+ in both humans and other vertebrates, as well as in several aquatic test species. Accordingly, toxicity to numerous aquatic species has been reported at concentrations of 0.15-0.5 mg/L [54]. Biochemical data on the responses of freshwater organisms to lithium exposure is quite limited, but our study observed significant impacts on all tested endpoints, suggesting a large-scale metabolic shift as a result. Marked decreases were seen in enzymes associated with energy production (ALP, ACP, βgal), as well as for lipase and peptidase, contrasting with a large increase in LDH activity. Remarkably large increases were recorded for the activity of the antioxidant enzyme GST, as well as

greatly increased lipid peroxidation levels, suggesting the induction of oxidative stress. Published data on this phenomenon appears to be slim.

Zinc is an essential metal at lower concentrations but can also have potentially toxic and disruptive effects. Zinc exposure has been shown to have impacts at the transcriptional level on *Daphnia magna*, modulating gene transcription differently in Zn exposed organisms compared to their non-exposed offspring. It has also been shown to cause growth reduction in 6-day old daphniids. Interestingly, in a multigenerational experiment, this reduction in growth was seen in the first and second generations, but not in the third, suggesting an adaptation to zinc exposure over a period of time. Further supporting this was an observed upregulation in the GST gene in the first generation, but not in the second [55]. Further reproductive effects have been shown, with dietary zinc intake decreasing reproduction by 40% [56]. Aside from these endpoints, little data is available on the enzymatic impacts of zinc exposure, though these impacts are certainly of concern. For example, phosphate metabolism is likely impacted, as we have recorded a significant decrease in the activities of both ALP and ACP, while an increase in LDH activity may suggest an increased energy demand. Additionally, the induction of oxidative stress seems likely, given the large observed increase in lipid peroxidation levels. GST activity appeared unaffected, though when coupled with the significant LOOH increase, this may be attributed to the overwhelming of the antioxidant defence system.

Zirconium appears to be another overlooked element in terms of freshwater toxicology. One study which attempted to record EC_{50} concentrations for 50 metals using *Daphnia magna* found that zirconium did not show an effect in lower than aqueous solubility, and thus did not determine an EC_{50} [57]. Conversely, our study determined an EC_{50} of 26.96 mg/L for zirconium, far below saturation point. Besides this study, there appears to be very little published data on the impacts of zirconium on freshwater organisms. Given the myriad significant impacts observed in our study, zirconium appears to be a likely candidate for further study in the freshwater environment.

Responses to active pharmaceutical compounds

Non-steroidal anti-inflammatory drugs (NSAIDs)

The main mechanism of NSAIDs is the inhibition of the cyclooxygenase enzyme (COX). Both isoforms of COX (COX-1 and COX-2) are membrane-bound glycoproteins, which catalyse the formation of prostanoids (prostaglandins, thromboxanes, prostacyclins) from arachidonic acid [58]. The method by which NSAIDs exert their toxic effects seems to occur simply as a result of excessive inhibition of COX-1, and a subsequent reduction in the synthesis of prostaglandins [59].

Acetylsalicylic acid is an API best known for its use in aspirin. It has been shown that acetylsalicylic acid exposure decreased survival rates, fecundity, and growth of *Daphnia magna* neonates [60]. The observed effect concentrations in this study (EC50 of 1293.05 mg/L) were far higher than both environmental levels and our determined EC50 (75.8 mg/L). Another study determined an EC50 more similar to ours (88.33 mg/L), although this was derived from a 48 hour exposure rather than a 24 hour. This study also found that ASA exposure significantly increased the level of lipid peroxidation (+10.15% after 48 hours) and oxidised protein content. Antioxidant enzymes were also found to be significantly different to controls after 48 hours, with the activity of SOD decreasing by 78.83%, and CAT activity increasing dramatically by 522.47%. this study also observed increased levels of DNA damage as a result of ASA exposure, and concluded that sublethal concentrations of ASA are capable of inducing both oxidative stress and oxidative DNA damage in *Daphnia magna* [61]. Our study also showed an increase in the antioxidant enzyme GST, but no impact in lipid peroxidation (LOOH) levels. This may indicate that the antioxidant defence system of *Daphnia magna* was capable of dealing with this level of ASA exposure in the short term without becoming overwhelmed. ASA has also been shown to have an effect on the differential expression of proteins, as one study assayed a set of 30 proteins, and found 3 significantly upregulated and 4 significantly downregulated in response to ASA exposure [62]. This is unsurprising, as ASA was shown in our study to have an impact on the activity of several classes of enzyme, including those involved in phosphate metabolism and energy production.

Diclofenac appears to affect a large number of enzymes in *Daphnia magna* as well as in other aquatic organisms. In daphniids, diclofenac exposure has been shown to cause a significant decrease in cholinesterase (ChE) activity. It has also been shown

to significantly decrease the activity of selenium dependent GPx at concentrations of 0.0005 and 0.5 mg/L [63]. Diclofenac exposure has also been shown to affect oxidative stress status in common carp (*Cyprinus carpio*), an effect which seems differential between organs. For example, diclofenac exposure was shown to reduce superoxide dismutase (SOD) activity in the liver (-74.2%), but to increase it in the gills (+203.7%). Similarly, it increased catalase activity in the brain (+299.1%) but decreased it in both the liver (-74.2%) and gills (-34.3%). Diclofenac exposure has also been shown to increase GPx activity in the gills (+294.1) [64]. Additionally it has been shown to cause significant induction of the GST gene in daphniids following acute exposure for 48 and 96 hours [65].This widespread impact on the oxidative stress status of *D. magna* is supported by our study, which showed significant impacts on both GST and LOOH levels. Curiously, while GST activity was significantly increased, LOOH levels were decreased. Also impacted were the activities of ACP, lipase and LDH, suggesting widespread metabolic perturbations as a result of diclofenac exposure.

Ibuprofen has been studied to a moderate degree in relation to freshwater organisms, though the studies often use traditional endpoints such as mortality and reproduction. However, the effects of ibuprofen on these endpoints are not negligible. For example, a 14 day chronic exposure with *D. magna* found that population growth rate was significantly reduced at all tested concentrations, with survival affected at 80000 µg/L. Furthermore, it was reported that reproduction was affected at 13.4 mg/L, and completely inhibited at 80000 µg/L [66]. In terms of oxidative stress and status, it has also been found that short term (6 hour) exposure to ibuprofen has the potential to cause induction of GST (2.75 fold at 50 µg/L), SOD, and CAT enzymes. Significant induction of the GST gene was also observed at 0.5 ug/L after 6h and 48h exposure, plus a significant induction at 50 µg/L. The total amount of eggs and brood per female, as well as body length, also significantly decreased under exposure to ibuprofen [67]. While these endpoints were not investigated here, such an impact on apical endpoints would likely be impossible without significant metabolic perturbations. In our study, ibuprofen exposure significantly decreased ACP and peptidase activity, while increasing the activity of LDH. GST activity and LOOH levels were also elevated, though not to a significant degree.

Indomethacin appears to be almost entirely elusive for its impact on freshwater organisms, although its presence in freshwater ecosystems has been detected (Table 1). In this study it showed significant impacts on the activities of both lipase and peptidase, as well as a minor impact on the redox state of *Daphnia magna* via reduced thiol levels and should thus be studied further to determine its true ecological impact.

Common environmentally relevant pharmaceutical compounds

Mebendazole is an anthelmintic microtubule depolymerisation drug**.** When used in its normal role, in the veterinary field, Mebendazole is designed to have a specific mechanism of action. This mechanism involves binding to beta-tubulin and inhibiting microtubule formation in the intestinal cells. This results in decreased uptake of glucose, and thus causes starvation of parasites [68]. However, as microtubules have a wide range of important roles in other, non-target organisms, it is important to understand the impact of this drug in the ecosystem. Despite these potential off-target effects, and the subsequent environmental concerns, little research has been published on the effects of mebendazole exposure on freshwater organisms. One study showed the potential of mebendazole to cause developmental toxicity in zebrafish through DNA damage [69]. This aligns somewhat with our results, as the activity of the antioxidant enzyme GST was increased significantly, although no change was seen in levels of lipid peroxidation. Regardless, mebendazole exerted a number of other significant impacts on *D. magna* reducing the activity of both βGal and peptidase, increasing activity of ALP, and diminishing the levels of reduced thiols present. These results imply the action of mebendazole on a large number of pathways in *D. magna*, from energy production to redox state, and thus warrant more thorough investigation to determine the risks it may pose as a pollutant in the aquatic environment.

Diltiazem is a non-dihydropyridine calcium channel blocker, which exerts its therapeutic effect through various mechanisms. Its primary mechanism is the inhibition of calcium influx into both cardiac and smooth muscles during depolarisation. Thus, it is most probable that toxic effects would occur through the disruption of calcium level regulation. Maintenance of appropriate calcium levels is involved in many key physiological processes in *Daphnia*, such as muscle contraction, peristalsis, carapace development, hormone release, and chemosensation. Accordingly, it has been

reported that a concentration of 500 ng/L caused an increase in the heart rate of *D. magna*, along with increased oxygen consumption. Furthermore, chronic exposure resulted in greatly decreased lipid reserves, and compromised reproduction in the form of fewer, larger neonates. It is likely that these results are the product of an energy imbalance caused by the increased heart rate and corresponding increased demand for energy [20]. Our study supports this theory of increased energy demand, as diltiazem increased the activity of several enzymes involved in energy production, namely ALP and ACP. The activity of the antioxidant enzyme GST was also increased, though interestingly lipid peroxidation levels were decreased. A significant decrease in reduced thiol levels was also found. A second study reported an EC_{50} of 165 mg/L, more than twice the concentration of our determined EC_{50} of 80.82 mg/L [70]. This is an unexpected finding, as this study used daphniid neonates rather than 4-day old daphniids, and intuitively we should expect them to be more sensitive rather than less.

Metformin is a type 2 diabetes drug, commonly found in aquatic ecosystems [17, 71]. Despite this, and despite concerns about its effects on the fecundity and reproductive status of some freshwater organisms [72, 73], little biochemical data is available. One study determined an EC_{50} of 64 mg/L for neonatal *Daphnia magna* after a 48 hour exposure period [74]. Our study showed an impact on enzymes involved in energy production, βgal, as well as protein metabolism. Additionally, oxidative stress status appears to be affected by metformin exposure, as increased activity levels of the antioxidant enzyme GST were reported, along with significantly increased levels of lipid peroxidation.

Chlorpromazine is a widely available antipsychotic, which exerts its effects primarily by blocking D2 dopamine receptors in the brain. Though developed for human therapeutic use, it has been shown to have effects on non-target organisms in the freshwater environment [75]. Many of these effects appear to result in reproductive difficulties, such as delays in time to first brood, decrease in total number of broods, and an impact on total number of neonates in the first brood, though there are conflicting reports on the latter, as both increases and decreases have been reported [76, 77]. A 48 hour EC_{50} of 1.81 mg/L has also been reported [77], significantly lower than our determined EC_{50} of 12.51 mg/L. However, this may be explicable, firstly due to the difference in exposure duration, and secondly due to the fact that this study

employed neonatal as opposed to 4-day old daphniids. Chlorpromazine has been shown to significantly increase catalase activity at a concentration of 0.001 mg/L. Chlorpromazine has been shown to inhibit enzymes such as cytochrome oxidase and monoamine oxidase, so the involvement of chlorpromazine in the oxidative pathway is a possibility in polluted environments [63]. Despite this, we saw no significant impact on either GST or LOOH, though an impact on the redox state of *D. magna* was detected by a decrease in reduced thiol levels. An impact on phosphate and protein metabolism was also seen through a marked decrease in ALP and peptidase activity.

Propranolol is a frequently prescribed β-blocker which can be found in the freshwater environment. Though designed with human therapeutic usage in mind, it can also exert differing effects on non-target organisms and is thus a pollutant of environmental concern. At a concentration of 28 µg/L, propranolol has been shown to bioaccumulate up to 1.6x its original concentration over the course of 10 generations of *D. magna* [78]. An EC_{50} of 7.5 mg/L was determined for neonatal daphnia following a 48 hour exposure [74]. This is significantly lower than our determined EC_{50} of 83.62 mg/L, although it should be considered that neonatal daphniids can be expected to be more sensitive than 4-day old daphniids, and the longer exposure period likely also played a role. Regarding mode of action, propranolol may exert the toxic effects seen in *Daphnia magna* in an organ specific manner, such as depression in heart rate [79]. This mechanism of action is quite intuitive, as it aligns with its therapeutic usage. It has been found to significantly reduce the heart rate of *Daphnia* at a concentration of 0.055 mg/L and can decrease the heart rate significantly in a dose-dependent manner. Less intuitively, it has also been shown to have an impact on the reproductive capabilities of daphniids, decreasing fecundity slightly at 0.22 mg/L, more so at 0.44 mg/L, and completely inhibiting it at 0.88 mg/L. Interestingly, a transgenerational experiment found a 2^{nd} generation of daphniids to be less sensitive to propranolol [80]. Propranolol has been shown to affect GSH metabolism, and induce an increase in GST activity at both 0.01 and 0.1 mg/L [63]. It has also been shown to significantly inhibit the activity of GPx, an enzyme belonging to the GSH family with a role in the removal of free radicals. Oliveira et al posit that their observed decrease in GPx activity may indicate that the antioxidant capabilities of GPx was overwhelmed by the volume of hydroperoxide products produced by lipid peroxidation [63]. These impacts on the antioxidant defence system of *D. magna* are supported by our own study, where we

saw a remarkable 5.16 fold increase in GST activity, coupled with a significant increase in lipid peroxidation levels.

Responses to diverse environmental pollutants

N-phosphonomethyl glycine (glyphosate) is a component of many commonly used herbicides and can be found in the environment as a consequence of agricultural or urban run-off, which result in leaching into local surface waters. There has long been concern over the implications of this, and its impact on both the environment and public health. These concerns appear to be well founded, as it has been shown that glyphosate can cause embryonic developmental problems, genotoxicity and DNA damage, and signalling interference at concentrations as low as 1 mg/L. The same study which reported these effects also reported impacts on carbon and fat metabolism as an effect of chronic exposure [81]. Additionally, glyphosate exposure has been shown to significantly impact the heart rate of *D. magna* at concentrations >20 mg/L [82]. Our own study supports these widespread metabolic impacts, as we found that phosphatase activity was significantly reduced, along with an increase in the activity of LDH and GST. The increase in the activity of the antioxidant enzyme GST particularly may be an indication of oxidative stress, resulting in ROS production and subsequent DNA damage. A further study determined an EC_{50} of 36.2 mg/L for *D. magna,* though the age of the test subjects is not specified in this study [83]. This EC_{50} is lower than our determined concentration of 61.29 mg/L, but this may be due to an unknown discrepancy in the age and sensitivity of the organisms used in the test.

Nicotine is a stimulatory substance which is widely consumed, and thus frequently found in surface water. Despite this, there is a lack of data on its impacts on freshwater organisms, with many existing studies focusing solely on mortality or heart-rate as endpoints, to the exclusion of other, less obvious investigative methods. One study also included reproduction as an endpoint, noting that a concentration of 100 µg/L negatively impacted reproduction, resulting in fewer neonates. It was also noted that concentrations of 10 µg/L were capable of triggering the production of male offspring. The 48 hour EC_{50} derived from this study was 0.789 mg/L [22]. Another study observed malformations in the antenna, carapace, and tail spine of *D. magna*, and recorded a 48 hour EC_{50} of 0.379 mg/L [84]. Although our derived EC_{50} was calculated after 24 rather than 48 hours, it is still significantly higher at 455 mg/L. Regardless, our study

revealed significant impacts on the key enzymes βGal and LDH, as well as an increase in activity of the antioxidant enzyme GST, coupled with an increase in lipid peroxidation (LOOH). Additionally, the redox state of *D. magna* was shown to be affected by a decrease in reduced thiols. With these effects in mind, and the frequency of nicotine's occurrence as a pollutant, further work should be carried out into its effects on freshwater organisms from a biochemical and mechanistic perspective.

4-nitrophenol is a phenolic compound with a nitro group at the opposite position of the hydroxyl group on the benzene ring. It is commonly encountered in the environment as it used in the manufacture of common drugs (e.g. acetaminophen) as well as leather treatment, dyes, and other industrial purposes [85]. Despite this, recent studies investigating its effects on freshwater ecosystems and organisms are almost non-existent. One study which exposed a synthetic ecosystem to 10 mg/L and 5 mg/L concentrations of 4-nitrophenol found that its presence severely disrupted algae and fauna populations, including cladocerans [86]. Our study showed a wide variety of impacts on *D. magna* , including alterations to phosphatase, βGal, peptidase and GST activity, as well as impacting the levels of reduced thiols. Given this myriad of physiological effects, coupled with the lack of available data on the environmental impacts of 4-nitrophenol pollution, this is a pollutant which warrants further in depth study to determine the risk it poses to the aquatic environment.

Phthalic acid is a representative of a diverse group of phthalates which are commonly used as plasticisers used to increase flexibility and durability in packaging materials, water bottles, toys, and medical devices, and subsequently are of great concern as an aquatic pollutant. Phthalates have significant toxic potential, and chronic exposure of *D. magna* to 3 phthalates, diethylhexyl phthalate (DEHP), bibutyl phthalate, and diethyl phthalate has been shown to have several negative impacts. All 3 reduced body size and caused metabolic perturbations resulting in increased lipid accumulation. DEP and DPB were found to significantly reduce lifespan [87]. Additionally, DPB has been found to increase SOD activity to a significant degree at a concentration of 0.5 mg/L, and to diminish it at higher concentrations. CAT and GST activity were also affected, decreasing significantly. These effects, along with an increase in detected levels of H_2O_2, suggest the induction of oxidative stress by phthalates. An EC_{50} of 3.48 mg/L for neonatal *D. magna* was reported [88]. Our determined EC_{50} was significantly higher

at 46.44 mg/L, although 4-day old daphniids were used to determine this rather than neonates. Our results support the induction of oxidative stress through phthalate exposure, as we report a significant increase in lipid peroxidation levels. Additionally, the antioxidant enzyme GST was impacted, though we saw an increase in its activity rather than a decrease. LDH activity was markedly increased, and a decreased in reduced thiols levels was also observed.

Responses to mixtures

As already mentioned, organisms in their natural environments are exposed to composite mixtures of several individual chemicals at very low concentrations. This is an issue, as chemical interactions within these mixtures can result in unintuitive results. This effect, wherein a mixture is more toxic than its constituents alone, is an exemplification of the adage “the whole is greater than the sum of its parts”. This is known as synergy, and it poses a major problem in the context of environmental risk assessments. To address the issue of pollutant synergy, it is necessary to understand the mode of action of each, how they may interact, and ultimately how these interactions may result in synergism or antagonism. Both of these effects are possible, and this can be seen in several studies, including our own. For example, one study which exposed common carp (*Cyprinus carpio*) to a 1:1 mixture of diclofenac and acetaminophen, as well as each individual chemical, seemingly unintuitive results have been observed. While SOD activity in the gill was unaffected by acetaminophen exposure, diclofenac exposure increased it by a dramatic 203.7%, and the mixture of the 2 chemicals increased it by only 60.47%. This is a clear indication of interaction between the two constituent chemicals. Synergy was also observed in this study, as diclofenac significantly decreased CAT activity in the gills (-34.3%), while the mixture of the 2 chemicals increased it by 182% [64]. These results are not easily predicted, so new methods are needed to understand the mechanisms and interactions within these mixtures. Compiling a library of biochemical responses to these mixtures is invaluable as a tool to assist in the bridging of these knowledge gaps.

Responses to a composite eight chemical mixture

Daphniids were exposed to low (10% EC_5), medium (20% EC_5) and high (30% EC_5) concentrations of an eight chemical mixture and dose-dependent responses were

determined in the absence of mortality. The majority of results showed either stepwise, dose-dependent increases or decreases. ***Phosphatases, lipase,*** and ***GST*** activities increase in a dose-dependent manner, while ***β-galactosidase*** activity and ***reduced thiol*** levels decreased dose-dependently. ***LDH*** and ***Peptidase*** activity were only impacted at the higher mixture concentration, indicating a response threshold somewhere between 20% and 30% of mixture EC_5. Finally, ***LOOH*** levels were uniquely affected, in that they increased approximately the same amount for all tested concentrations. This indicates that an induction of oxidative stress is at least part of the mechanism of action of this mixture.

Single, double, and triple mixtures of lithium, propranolol and nicotine

Three chemicals were selected to be diverse in their actions and used in single, double, and triple mixtures. Their responses on daphniids were assessed and showed different patterns. It is worth noting that in this phase, the single doses were not the same, but lower than the ones used in the individual-chemical phase.

Both ***phosphatases*** were responsive to the individual and mixtures of stressors. ***ALP*** activity was heavily altered. Significant impacts were recorded for all single chemicals and mixtures. Lithium alone caused a significant decrease, and this effect appeared to be overcome in the double and triple mixtures. The double mixture of L-nicotine and propranolol (both of which caused an increase in ALP activity) caused the greatest spike in activity, with the addition of lithium in the triple mixture decreasing activity slightly relative to this double mixture. ***ACP*** also exhibited decreased activity in response to lithium exposure. A similar pattern can be observed to ALP, wherein the propranolol:nicotine double mixture causes a significant increase in activity which seems to be muted upon the addition of lithium in the triple mixture.

In relation to enzymes relevant to metabolic physiology of daphniids, ***β-galactosidase*** also followed this trend, and although it was unaffected by the single chemicals, the same pattern is visible in response to the mixtures: a significant increase in activity in response to the propranolol:nicotine mixture, which is lessened upon the addition of lithium in the triple mixture. This is a clear example of pollutant synergy, as the effect of the propranolol:nicotine mixture was much greater than the impact of their individual doses would lead us to believe. On the other hand, ***lipase*** was affected differently, in that each of the single chemicals caused a significant increase, and these impacts were more significant than any effects caused by the mixtures. However, once again,

the propranolol:nicotine double mixture caused a significant increase which was not seen following the addition of lithium in the triple mixture. This is an excellent example of a non-additive effect in mixture toxicology, as intuitively we would likely expect a large increase in activity for the triple mixture based upon the results of the single chemical exposure. ***LDH*** activity was largely unaffected by any exposure conditions, and thus produced no observable trends. ***Peptidase*** activity was impacted in an interesting manner. Although none of the single chemicals had a significant impact, the lithium:nicotine double mixture resulted in a significant decrease, and the propranolol:nicotine double mixture a significant increase. Once again, this effect was muted by the addition of lithium in the triple mixture.

Considering oxidative stress related parameters, ***GST*** activity was increased by a similar margin by three exposure conditions; lithium, the lithium:nicotine double mixture, and the triple mixture. All of these exposure conditions contained lithium. Lithium was also shown to elicit an enormous increase in GST activity in the single chemicals section, so this is not altogether unexpected. Published data on the induction of oxidative stress by lithium exposure is scarce. ***LOOH*** levels were largely unaffected, being only slightly diminished by propranolol, and producing no observable trends. ***Reduced thiols*** were not significantly affected by any of the single chemicals. However, as seen for several of the enzyme assays, the propranolol:nicotine mixture caused a significant increase, contrasted against a relative decrease in response to the triple mixture.

Final remarks, conclusions, and future work

This research explored a significant number of pollutants in a well characterised freshwater system. Though a few of these pollutants have been well studied previously, many had little or no ecotoxicological impact data available beyond less powerful endpoints such as survival and fecundity. It was found that many lacked published data on the biochemical and enzymatic responses of freshwater organisms, despite having significant effects on the physiology of *Daphnia magna*. This study generated significant novel findings in this context and may be built upon in the future to establish novel adverse outcome pathways (AOPs) as more comprehensive biochemical data becomes available. In terms of mixture toxicology, clear interactions were shown between a range of different pollutants, though the exact manner and mechanism of these interactions remains unknown as of yet. More detailed analysis,

perhaps through the use of omics technology may prove instrumental in determining the precise mode of action of these pollutants and their mixtures, and the interactions which cause their mixtures to behave in unintuitive, non-additive manners. AOPs are likely to be crucial in this endeavour. AOPs are models which identify sequential pathways of molecular and cellular events which are required the produce an adverse outcome upon an organism's exposure to a pollutant. They can be used to organise information regarding toxicity mechanisms and biological interactions in a manner which can provide insight into the relationship between pollutant exposure and injury or physiological damage. Each of these pathways begins with a molecular initiating event (MIE) with a specific adverse outcome (Figure 21). If MIEs for pollutants such as these are identified, then these MIEs may be utilised as an early warning sign that a system is polluted or in need of remediation. An early warning system composed of an integrated framework of many identified AOPs could potentially detect the sublethal effects caused by low concentrations of pollutants long before serious and irreversible damage could be caused to the ecosystem [89]. For example, from the metals dataset, a common response mechanism identified was the induction of GST. This could represent an early MIE in a future formation of AOPs for a number of pollutants.

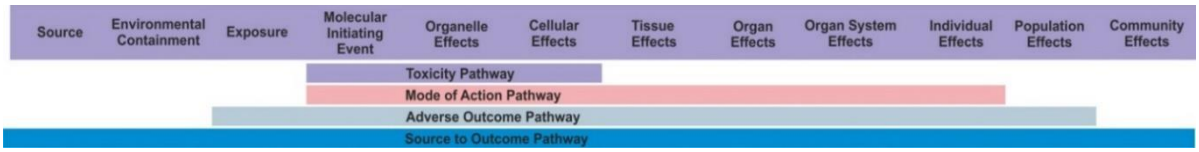
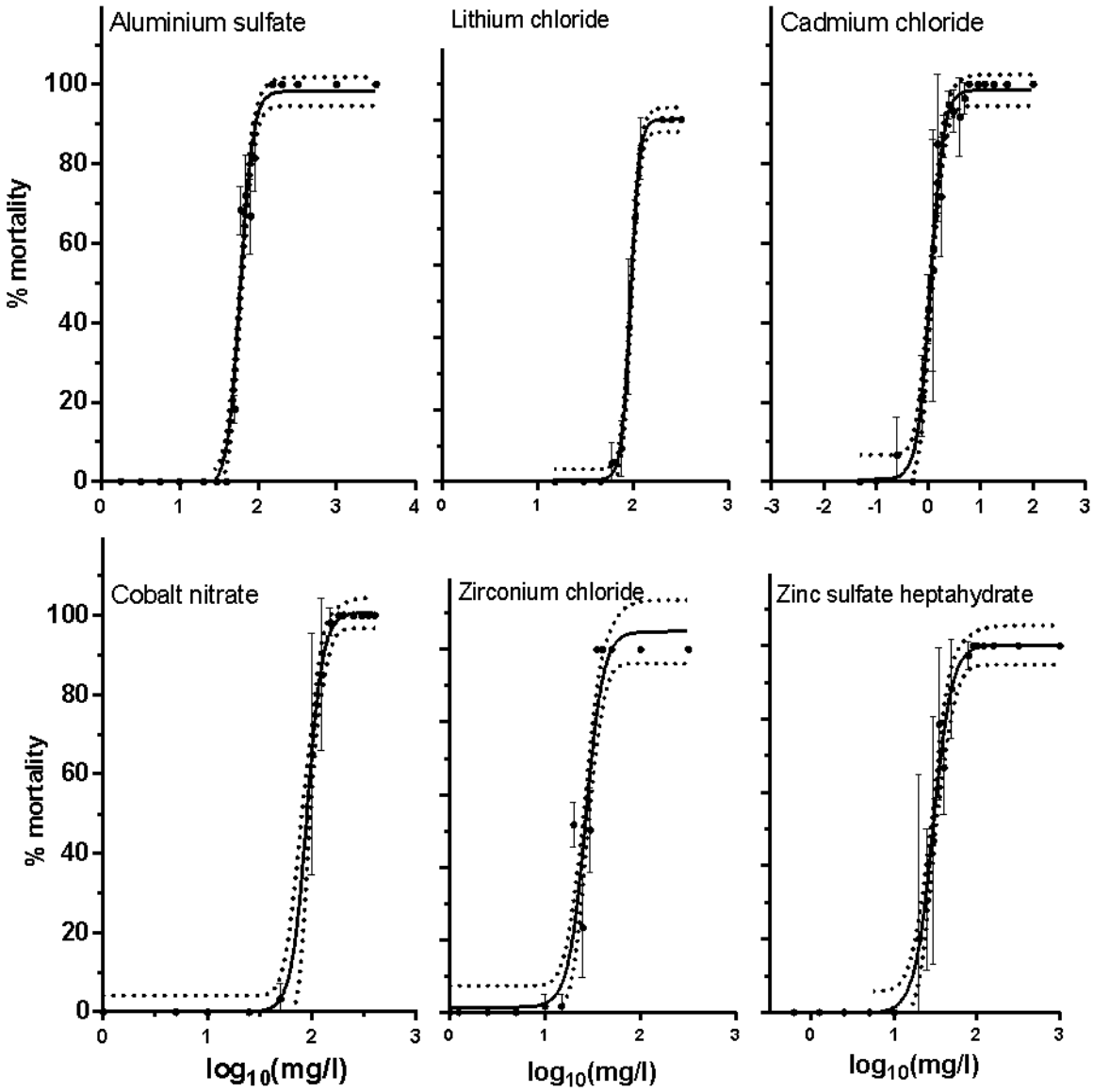


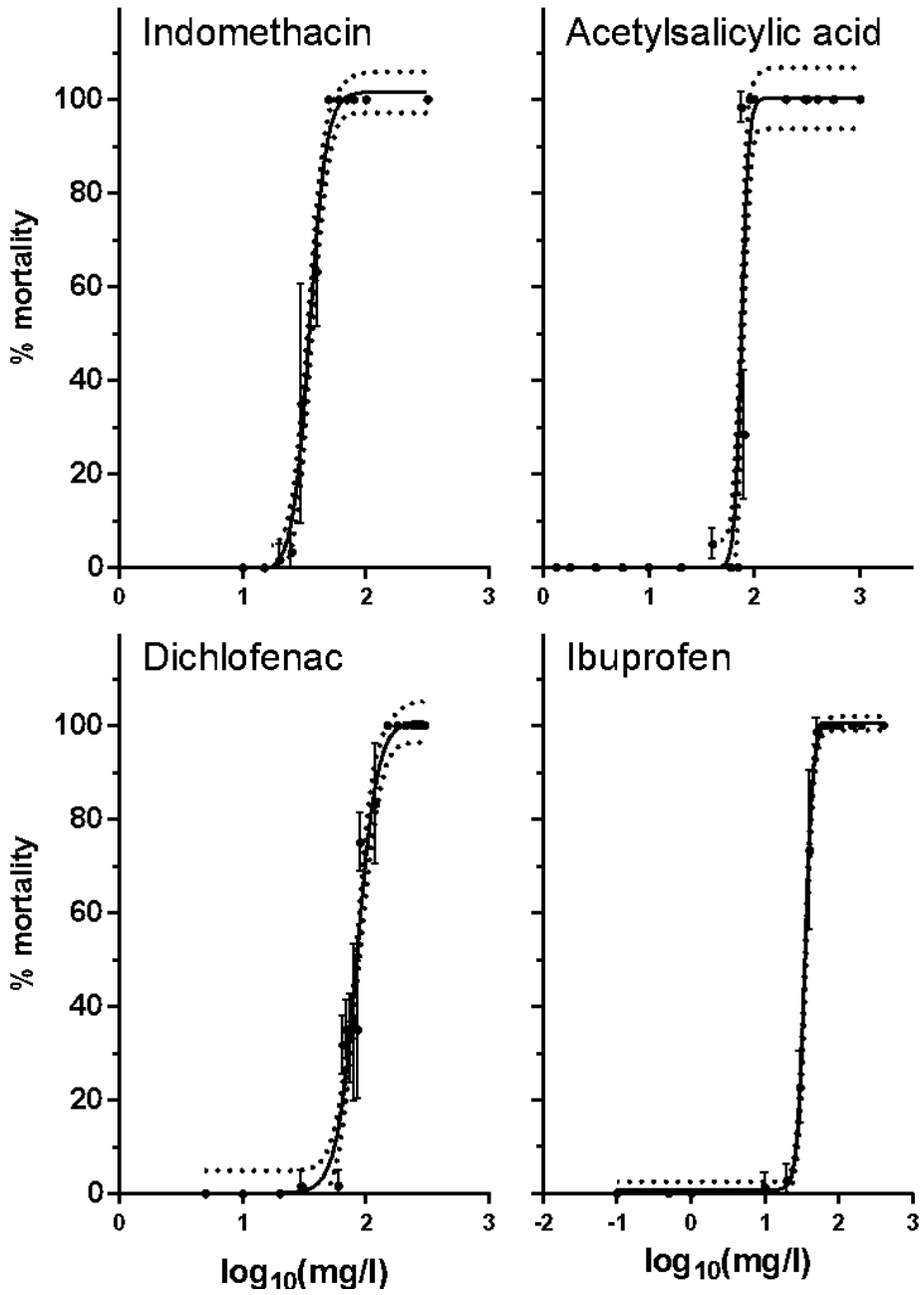
Figure 21. Schematic representation of pathways to discover toxicity mechanisms.

In conclusion, revolutionising risk assessment with novel biochemical indices of pollution assessment could prove transformative for our mindset and legislative framework, potentially moving from water quality standards to mechanistic endpoints in key species such as *Daphnia*. These molecular endpoints such as enzyme activity or physiological response could prove more sensitive and reliable as a pollution monitoring tool.

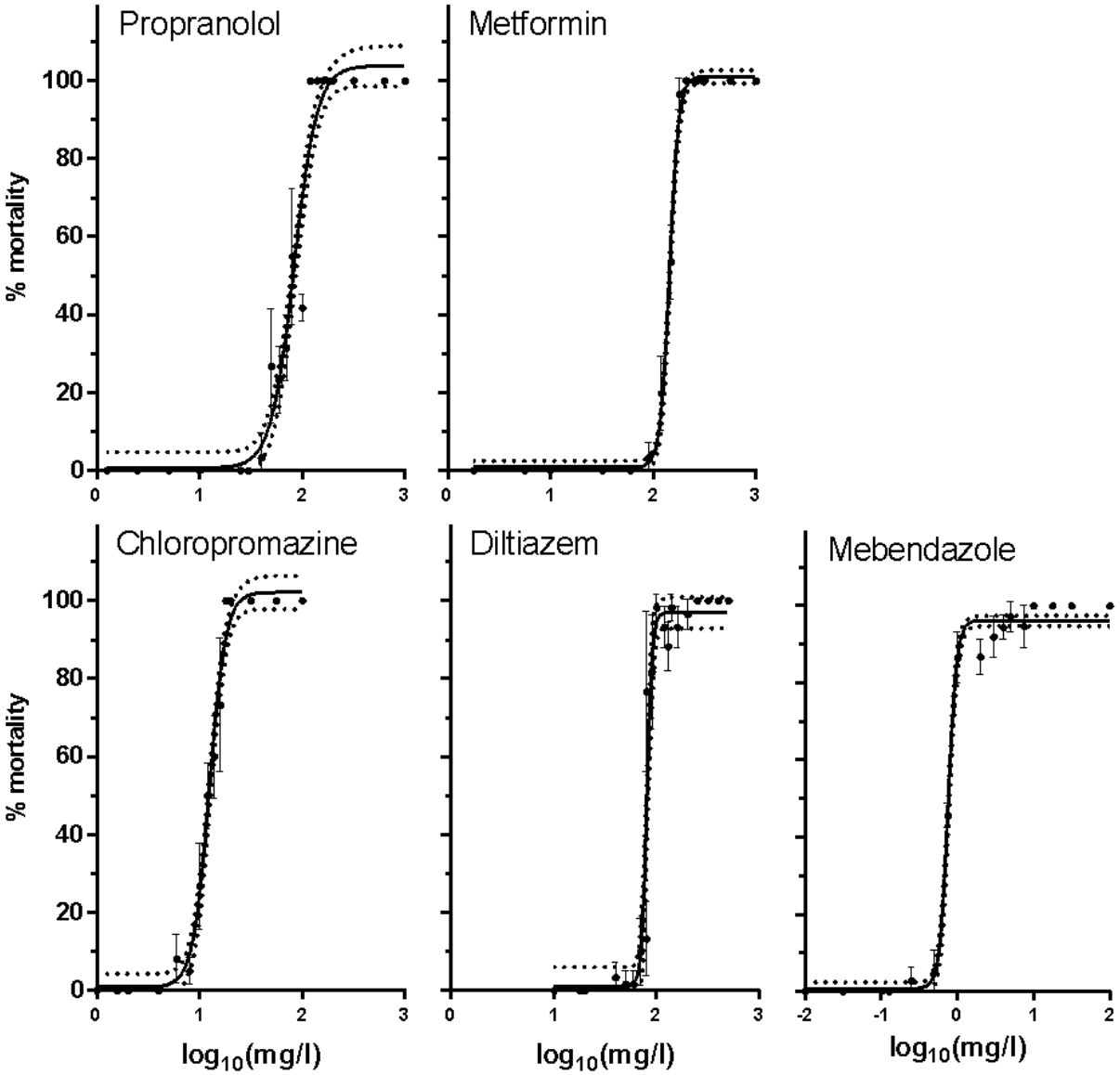
Supplementary figures



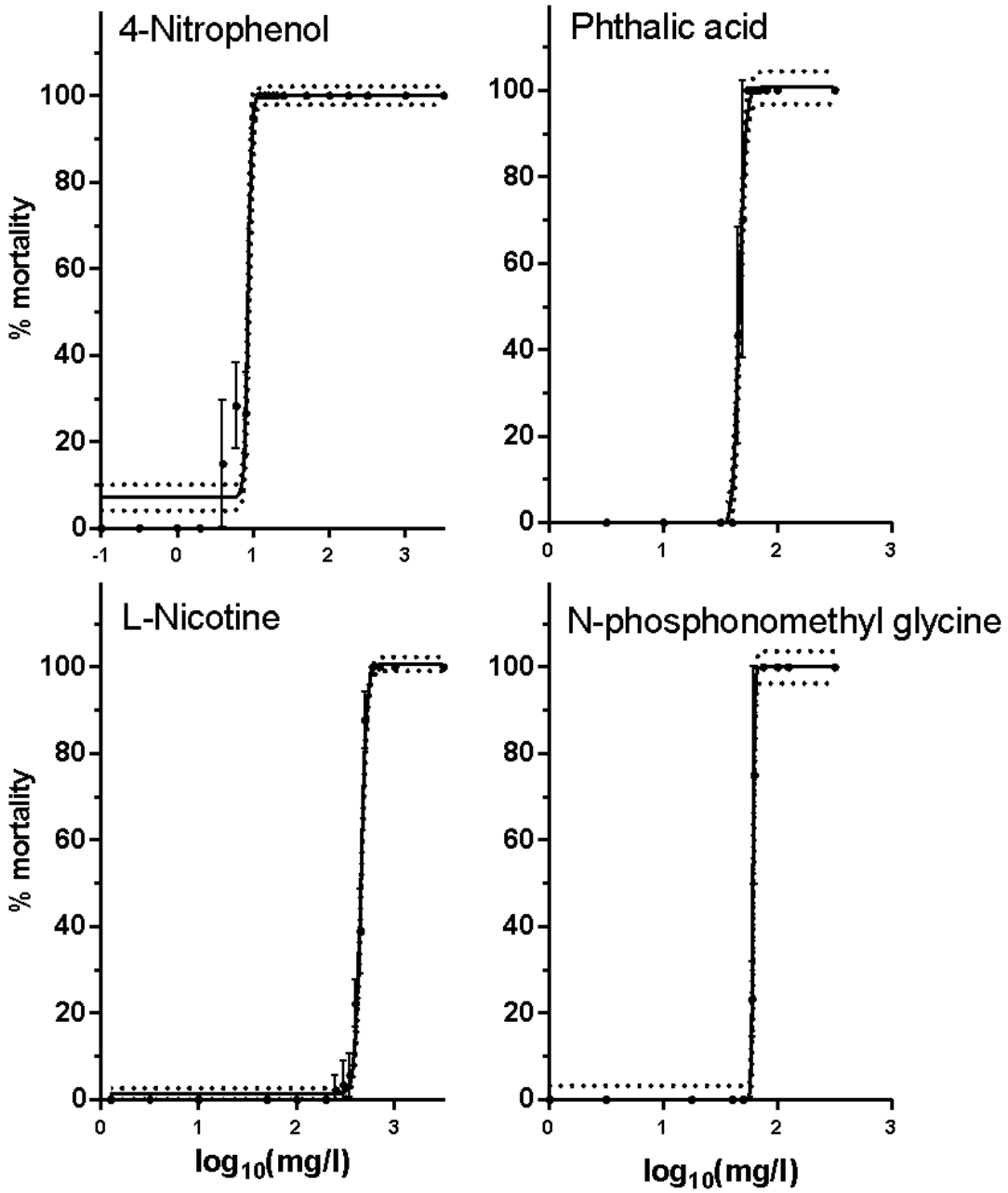
Supplementary Figure 1. Acute toxicity curves for D4 daphniids exposed to metals. Data represent average±SD (N=4) replicates for each concentration. Solid line represents average mortality, dotted lines represent 95% confidence intervals.



Supplementary Figure 2. Acute toxicity curves for D4 daphniids exposed to NSAIDs. Data represent average±SD (N=4) replicates for each concentration. Solid line represents average mortality, dotted lines represent 95% confidence intervals.



Supplementary Figure 3. Acute toxicity curves for D4 daphniids exposed to common pharmaceuticals. Data represent average±SD (N=4) replicates for each concentration. Solid line represents average mortality, dotted lines represent 95% confidence intervals.



Supplementary Figure 4. Acute toxicity curves for D4 daphniids exposed to common pollutants. Data represent average±SD (N=4) replicates for each concentration. Solid line represents average mortality, dotted lines represent 95% confidence intervals.

References

1. Sadler, D.E., F.S. Brunner, and S.J. Plaistow, *Temperature and clone-dependent effects of microplastics on immunity and life history in Daphnia magna.* Environ Pollut, 2019. **255**(Pt 1): p. 113178.
2. Kim, H., et al., *Response of antioxidant enzymes to Cd and Pb exposure in water flea Daphnia magna: Differential metal and age - Specific patterns.* Comp Biochem Physiol C Toxicol Pharmacol, 2018. **209**: p. 28-36.
3. Naja, G.M. and B. Volesky, *Toxicity and Sources of Pb, Cd, Hg, Cr, As, and Radionuclides in the Environment.* Heavy Metals in the Environment, 2009: p. 13-61.
4. Seebaugh, D.R., D. Goto, and W.G. Wallace, *Bioenhancement of cadmium transfer along a multi-level food chain.* Marine Environmental Research, 2005. **59**(5): p. 473-491.
5. Escudero, J., et al., *Antipsychotics as environmental pollutants: An underrated threat?* Sci Total Environ, 2021. **769**: p. 144634.
6. Xu, J., et al., *Bioassay: A useful tool for evaluating reclaimed water safety.* J Environ Sci (China), 2020. **88**: p. 165-176.
7. Daughton, C.G., *Drug Usage and Disposal: Overview of Environmental Stewardship and Pollution Prevention (with an emphasis on some activities in the Federal Government)*, in *Research Triangle Environmental Health Collaborative*. 2008: North Carolina Biotechnology Center, Research Triangle Park, North Carolina.
8. EPA, *Ireland's Environment 2016- An Assessment*, E.P. Agency, Editor. 2016.
9. Kavanagh, L., et al., *Lithium in the Natural Waters of the South East of Ireland.* Int J Environ Res Public Health, 2017. **14**(6).
10. Bhardwaj, S., et al., *Mercury, arsenic, lead and cadmium in waters of the Singrauli coal mining and power plants industrial zone, Central East India.* Environ Monit Assess, 2020. **192**(4): p. 251.
11. Bianchi, E., *Water quality monitoring of the Sinos River Basin, Southern Brazil, using physicochemical and microbiological analysis and biomarkers in laboratory-exposed fish.* Ecohydrology & Hydrobiology, 2019. **19**: p. 328-338.
12. Castaldo, G., et al., *Investigating the effects of a sub-lethal metal mixture of Cu, Zn and Cd on bioaccumulation and ionoregulation in common carp, Cyprinus carpio.* Aquat Toxicol, 2020. **218**: p. 105363.
13. Dvorak, P., et al., *Accumulation of selected metals pollution in aquatic ecosystems in the Smeda river (Czech republic).* Neuro Endocrinol Lett, 2018. **39**(5): p. 380-384.
14. Santos, L.H., et al., *Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment.* J Hazard Mater, 2010. **175**(1-3): p. 45-95.
15. Chopra, S. and D. Kumar, *Ibuprofen as an emerging organic contaminant in environment, distribution and remediation.* Heliyon, 2020. **6**(6): p. e04087.
16. Jimenez, J.J., et al., *Degradation of indomethacin in river water under stress and non-stress laboratory conditions: degradation products, long-term evolution and adsorption to sediment.* J Environ Sci (China), 2017. **51**: p. 13-20.
17. Oosterhuis, M., F. Sacher, and T.L. Ter Laak, *Prediction of concentration levels of metformin and other high consumption pharmaceuticals in wastewater and*

regional surface water based on sales data. Sci Total Environ, 2013. **442**: p. 380-8.
18. Reichert, J.F., D.M. Souza, and A.F. Martins, *Antipsychotic drugs in hospital wastewater and a preliminary risk assessment.* Ecotoxicol Environ Saf, 2019. **170**: p. 559-567.
19. Marisa de Jesus Silva Chaves, S.C.B., Maiara de Melo Malinowski, Duane Volpato,Ítalo Braga Castro, Teresa Cristina Rodrigues dos Santos Franco, Ednei Gilberto Primel, *Pharmaceuticals and personal care products in a Brazilian wetland of international importance: Occurrence and environmental risk assessment.* Science of the Total Environment, 2020.
20. Steinkey, D., et al., *The effects of diltiazem on growth, reproduction, energy reserves, and calcium-dependent physiology in Daphnia magna.* Chemosphere, 2019. **232**: p. 424-429.
21. Herek, J.S., et al., *Can environmental concentrations of glyphosate affect survival and cause malformation in amphibians? Effects from a glyphosate-based herbicide on Physalaemus cuvieri and P. gracilis (Anura: Leptodactylidae).* Environ Sci Pollut Res Int, 2020. **27**(18): p. 22619-22630.
22. Oropesa, A.L., A.M. Floro, and P. Palma, *Toxic potential of the emerging contaminant nicotine to the aquatic ecosystem.* Environ Sci Pollut Res Int, 2017. **24**(20): p. 16605-16616.
23. Dominguez-Morueco, N., S. Gonzalez-Alonso, and Y. Valcarcel, *Phthalate occurrence in rivers and tap water from central Spain.* Sci Total Environ, 2014. **500-501**: p. 139-46.
24. Government, Q. *Ecosystem health indicators*. 2018 03/09/2018 [cited 2020 08/05/2020]; Available from: https://environment.des.qld.gov.au/management/water/health-indicators.
25. Lee, B.Y., et al., *The genome of the freshwater water flea Daphnia magna: A potential use for freshwater molecular ecotoxicology.* Aquat Toxicol, 2019. **210**: p. 69-84.
26. Samiyappan, M., *Bioindicators in Aquatic Environment and their significance.* Journal of Aquaculture in the Tropics, 2019. **34**: p. 73-79.
27. Colbourne, J.K., et al., *The Ecoresponsive Genome of Daphnia pulex.* Science, 2011. **331**(6017): p. 555-561.
28. Ravindran, S.P., et al., *Daphnia stressor database: Taking advantage of a decade of Daphnia '-omics' data for gene annotation.* Sci Rep, 2019. **9**(1): p. 11135.
29. Ebert, D., *Ecology, Epidemiology and Evolution of Parasitism in Daphnia*, in *Ecology, Epidemiology and Evolution of Parasitism in Daphnia*. 2005.
30. Jéssica Cristina Izoton, T.R.M., Aline Lorenset, Muriel Pando Pereira, Sandra Elisa Haas, *The use of Daphnia magna for the toxicological evaluation of inorganic nanoparticles.* Current Topics in Toxicology, 2017(13): p. 113-125.
31. NC3Rs. *The 3Rs*. [cited 2020 07/05/2020]; Available from: https://www.nc3rs.org.uk/the-3rs.
32. Neale, P.A., et al., *Linking in Vitro Effects and Detected Organic Micropollutants in Surface Water Using Mixture-Toxicity Modeling.* Environmental Science & Technology, 2015. **49**(24): p. 14614-14624.
33. Escher, B.I., H.M. Stapleton, and E.L. Schymanski, *Tracking complex mixtures of chemicals in our changing environment.* Science, 2020. **367**(6476): p. 388-+.

34. Kortenkamp, A., et al., *Mixture risks threaten water quality: the European Collaborative Project SOLUTIONS recommends changes to the WFD and better coordination across all pieces of European chemicals legislation to improve protection from exposure of the aquatic environment to multiple pollutants.* Environmental Sciences Europe, 2019. **31**(1).
35. Altenburger, R., et al., *Simplifying complexity: Mixture toxicity assessment in the last 20 years.* Environmental Toxicology and Chemistry, 2013. **32**(8): p. 1685-1687.
36. Tang, S.S., C.C. Lin, and G.G. Chang, *Metal-catalyzed oxidation and cleavage of octopus glutathione transferase by the Cu(II)-ascorbate system.* Free Radic Biol Med, 1996. **21**(7): p. 955-64.
37. Mannervik, B. and C. Guthenberg, *Glutathione transferase (human placenta).* Methods Enzymol, 1981. **77**: p. 231-5.
38. Grintzalis, K., et al., *Method for the simultaneous determination of free/protein malondialdehyde and lipid/protein hydroperoxides.* Free Radic Biol Med, 2013. **59**: p. 27-35.
39. Grintzalis, K., et al., *Multiparametric protocol for the determination of thiol redox state in living matter.* Free Radic Biol Med, 2014. **74**: p. 85-98.
40. Worthington, K., and Worthington, V. *Phosphatase, Alkaline 06/12/2021<https://www.worthington-biochem.com/pap/default.html*>. [cited 2021 06/12/2021]; Available from: https://worthington-biochem.com/BAP/default.html.
41. Worthington, K., and Worthington, V. *Galactosidase, Beta*. [cited 2021 06/12/2021]; Available from: https://www.worthington-biochem.com/BG/.
42. Worthington, K., and Worthington, V. *Lipase*. [cited 2021 06/12/2021]; Available from: https://www.worthington-biochem.com/PL/.
43. Deniakina, E.K., et al., *[Production and properties of an immobilized enzyme preparation with peptidase activity].* Prikl Biokhim Mikrobiol, 1985. **21**(2): p. 177-83.
44. Worthington, K., and Worthington, V. *Lactate dehydrogenase*. [cited 2021 06/12/2021]; Available from: https://worthington-biochem.com/LDH/default.html.
45. Nagato, E.G., et al., *(1)H NMR-based metabolomics investigation of Daphnia magna responses to sub-lethal exposure to arsenic, copper and lithium.* Chemosphere, 2013. **93**(2): p. 331-7.
46. Hazrat Ali, E.K., Ikram Ilahi, *Environmental Chemistry and Ecotoxicology of Hazardous Heavy Metals: Environmental Persistence, Toxicity, and Bioaccumulation.* Journal of Chemistry, 2019. **2019**: p. 14.
47. Brun, N.R., et al., *Mixtures of Aluminum and Indium Induce More than Additive Phenotypic and Toxicogenomic Responses in Daphnia magna.* Environ Sci Technol, 2019. **53**(3): p. 1639-1649.
48. Jaroslav Vrba, J.K., Thomas Bittl, Jiří Nedoma, Alena Štrojsová, Linda Nedbalová, Leoš Kohout & Jan Fott *A key role of aluminium in phosphorus availability, food web structure, and plankton dynamics in strongly acidified lakes.* Biologia, 2006. **61**.
49. Perez, E. and T.C. Hoang, *Responses of Daphnia magna to chronic exposure of cadmium and nickel mixtures.* Chemosphere, 2018. **208**: p. 991-1001.
50. Tsui, M.T. and W.X. Wang, *Biokinetics and tolerance development of toxic metals in Daphnia magna.* Environ Toxicol Chem, 2007. **26**(5): p. 1023-32.

51. Herbert M. Espinoza, C.R.W., Evan P. Gallagher, *Effect of cadmium on glutathione S-transferase and metallothionein gene expression in coho salmon liver, gill and olfactory tissues.* Aquat Toxicol, 2012. **110-111**.
52. Stubblefield, W.A., et al., *Acute and Chronic Toxicity of Cobalt to Freshwater Organisms: Using a Species Sensitivity Distribution Approach to Establish International Water Quality Standards.* Environ Toxicol Chem, 2020. **39**(4): p. 799-811.
53. Hyo Jeong Kim, J.H.Y., Hyun Soo Kim, Yeo Jin Kim, Wonhee Jang & Young Rok Seo, *Exploring potential biomarker responses to lithium in Daphnia magna from the perspectives of function and signaling networks.* Mol Cell Toxicol, 2017.
54. Kszos, L.A., J.J. Beauchamp, and A.J. Stewart, *Toxicity of lithium to three freshwater organisms and the antagonistic effect of sodium.* Ecotoxicology, 2003. **12**(5): p. 427-437.
55. Vandegehuchte, M.B., et al., *Gene transcription and higher-level effects of multigenerational Zn exposure in Daphnia magna.* Chemosphere, 2010. **80**(9): p. 1014-20.
56. De Schamphelaere, K.A., et al., *Reproductive toxicity of dietary zinc to Daphnia magna.* Aquat Toxicol, 2004. **70**(3): p. 233-44.
57. Okamoto, A., M. Yamamuro, and N. Tatarazako, *Acute toxicity of 50 metals to Daphnia magna.* J Appl Toxicol, 2015. **35**(7): p. 824-30.
58. Osafo, N., Agyare, C., Obiri, D. D., Antwi, A.O., *Mechanism of Action of Nonsteroidal Anti-Inflammatory Drugs.* 2017.
59. Hunter, L.J., D.M. Wood, and P.I. Dargan, *The patterns of toxicity and management of acute nonsteroidal anti-inflammatory drug (NSAID) overdose.* Open Access Emerg Med, 2011. **3**: p. 39-48.
60. Marques, C.R., N. Abrantes, and F. Goncalves, *Life-history traits of standard and autochthonous cladocerans: I. Acute and chronic effects of acetylsalicylic acid.* Environ Toxicol, 2004. **19**(5): p. 518-26.
61. Gomez-Olivan, L.M., et al., *DNA damage and oxidative stress induced by acetylsalicylic acid in Daphnia magna.* Comp Biochem Physiol C Toxicol Pharmacol, 2014. **164**: p. 21-6.
62. Seung Hyuck Bang, N.-H.H., S.S.S. Ji-Young Ahn, and Y.-H.K.J. Min, *Proteomic Analysis of Daphnia magna Exposed to Caffeine, Ibuprofen, Aspirin and Tetracycline.* Toxicology and Environmental Health Sciences, 2015. **7**(2).
63. Oliveira, L.L.D., et al., *Evaluation of ecotoxicological effects of drugs on Daphnia magna using different enzymatic biomarkers.* Ecotoxicology and Environmental Safety, 2015. **119**: p. 123-131.
64. Nava-Alvarez, R., et al., *Oxidative Stress Induced by Mixture of Diclofenac and Acetaminophen on Common Carp (Cyprinus carpio).* Water Air and Soil Pollution, 2014. **225**(2).
65. Liu, Y., et al., *Toxic effects of diclofenac on life history parameters and the expression of detoxification-related genes in Daphnia magna.* Aquatic Toxicology, 2017. **183**: p. 104-113.
66. Heckmann, L.H., et al., *Chronic toxicity of ibuprofen to Daphnia magna: Effects on life history traits and population dynamics.* Toxicol Lett, 2007. **172**(3): p. 137-45.
67. Wang, L., et al., *Gene response of CYP360A, CYP314, and GST and whole-organism changes in Daphnia magna exposed to ibuprofen.* Comp Biochem Physiol C Toxicol Pharmacol, 2016. **179**: p. 49-56.

68. Wagil, M., et al., *Toxicity of anthelmintic drugs (fenbendazole and flubendazole) to aquatic organisms.* Environ Sci Pollut Res Int, 2015. **22**(4): p. 2566-73.
69. Sasagawa, S., et al., *DNA Damage Response Is Involved in the Developmental Toxicity of Mebendazole in Zebrafish Retina.* Front Pharmacol, 2016. **7**: p. 57.
70. Natalia Ros, L.L., Pilar Ribate, Estefania Zuriaga, Cristina B. García and Beatriz Giner, *Acute lethal and sublethal effects of diltiazem and doxepin for four aquatic environmental bioindicators covering the trophic chain.* Environmental Science, 2018.
71. Blair, B.D., et al., *Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern.* Chemosphere, 2013. **93**(9): p. 2116-23.
72. Niemuth, N.J., et al., *Metformin exposure at environmentally relevant concentrations causes potential endocrine disruption in adult male fish.* Environ Toxicol Chem, 2015. **34**(2): p. 291-6.
73. Niemuth, N.J. and R.D. Klaper, *Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish.* Chemosphere, 2015. **135**: p. 38-45.
74. Cleuvers, M., *Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects.* Toxicol Letters, 2003. **142**.
75. Li, T., et al., *Toxic effects of chlorpromazine on Carassius auratus and its oxidative stress.* J Environ Sci Health B, 2008. **43**(8): p. 638-43.
76. Alkimin, G.D., et al., *Daphnia magna responses to fish kairomone and chlorpromazine exposures.* Chem Biol Interact, 2020. **325**: p. 109123.
77. de Oliveira, L.L., et al., *Acute and chronic ecotoxicological effects of four pharmaceuticals drugs on cladoceran Daphnia magna.* Drug Chem Toxicol, 2016. **39**(1): p. 13-21.
78. Jeong, T.Y., T.H. Kim, and S.D. Kim, *Bioaccumulation and biotransformation of the beta-blocker propranolol in multigenerational exposure to Daphnia magna.* Environmental Pollution, 2016. **216**: p. 811-818.
79. Jeong, T.Y., et al., *Mode of action characterization for adverse effect of propranolol in Daphnia magna based on behavior and physiology monitoring and metabolite profiling.* Environmental Pollution, 2018. **233**: p. 99-108.
80. Dzialowski, E.M., P.K. Turner, and B.W. Brooks, *Physiological and reproductive effects of beta adrenergic receptor antagonists in Daphnia magna.* Arch Environ Contam Toxicol, 2006. **50**(4): p. 503-10.
81. Suppa, A., et al., *Roundup causes embryonic development failure and alters metabolic pathways and gut microbiota functionality in non-target species.* Microbiome, 2020. **8**(1).
82. Katherine Duan, A.K., Leanna Kish, Peter Faletra, Kelly Salmon, *The Impact of Glyphosate-Based Herbicides 1 and Their Components on Daphnia Magna.* bioRxiv, 2019.
83. Gustinasari, K., et al., *Acute toxicity and morphology alterations of glyphosate-based herbicides to Daphnia magna and Cyclops vicinus.* Toxicol Res, 2021. **37**(2): p. 197-207.
84. Kuei-Fang Chen, S.-Y.H., Yu-Ta Chung, Kai-Sung Wang, Chih-Kai Wang, and S.-H. Chang, *Detoxification of nicotine solution using Fe0-based processes: Toxicity*
evaluation by Daphnia magna neonate and embryo assays. Chemical Engineering Journal, 2018. **331**: p. 636-643.

85. Pangkita Deka, D.B., Pingal Sarmah, Ramesh C. Deka, and a.P. Bharali, *Catalytic Reduction of Water Contaminant ‘4-Nitrophenol’ over Manganese Oxide Supported Ni Nanoparticles*, in *Trends in Asian Water Environmental Science and Technology*. 2017.
86. Zieris, F.J., D. Feind, and W. Huber, *Long-Term Effects of 4-Nitrophenol in an Outdoor Synthetic Aquatic Ecosystem.* Archives of Environmental Contamination and Toxicology, 1988. **17**(2): p. 165-175.
87. Seyoum, A. and A. Pradhan, *Effect of phthalates on development, reproduction, fat metabolism and lifespan in Daphnia magna.* Sci Total Environ, 2019. **654**: p. 969-977.
88. Shen, C., et al., *Acute toxicity and responses of antioxidant systems to dibutyl phthalate in neonate and adult Daphnia magna.* PeerJ, 2019. **7**: p. e6584.
89. Lee, J.W., et al., *Significance of adverse outcome pathways in biomarker-based environmental risk assessment in aquatic organisms.* J Environ Sci (China), 2015. **35**: p. 115-127.

Appendices

Appendix A. *Daphnia magna* culturing protocols

Modified OECD standard media. OECD media stock solutions were prepared fresh and added according to the following order to a tank filled with 50 l ddH_2O. 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), and potassium chloride (0.3 g KCl in 0.5 l ddH_2O), 2.6 mL from a 40 µg sodium selenite/mL and 23 mL from 1M HCl were added. 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 after aeration was adjusted to 7.77.

Seaweed extract supplement. The standard organic seaweed (*Acophylum nodosum*) extract is added to the modified media on culture setup only at the day when media is renewed. The concentrated extract was diluted in ddH_2O to an absorbance of 8A at 400 nm.

Proposed plan for culturing daphniids. The following culturing scheme is followed only to generate clonal populations of mothers who will breed animals for experiments.

Daphniid 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 discard 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	Remove 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	

Appendix B. *Chlamydomonas reinhardtii* culturing

A semi-continuous stock culture of the algae in *Chlamydomonas* growth medium (CGM) was maintained for feeding. Algae were collected by centrifugation at 3,000 rpm for 10 minutes at room temperature and re-suspended in ddH_2O at a suspension of 7A at 440 nm.

Chlamydomonas growth medium (CGM)

Using a volumetric cylinder, the following volumes were added to 3.7 l ddH_2O under continuous stirring.

Stock	Concentration g/l	mL to add
NH_4Cl	20	100
$MgSO_4.7H_2O$	8	50
$CaCl_2.2H_2O$	4	50
K_2HPO_4	8.64	50
KH_2PO_4	8.4	25
EDTA/KOH	50/31	2
Acidified iron	4.98	4
Boric acid	11.42	4
$ZnSO_4.7H_2O$	14.12	2.5
$MnCl_2.4H_2O$	2.33	2.5
$CuSO_4.5H_2O$	2.54	2.5
$Co(NO_3)_2.6H_2O$	0.82	2.5
$Na_2MoO_4.4H_2O$	1.92	2.5
Acetic acid	100%	4

Following, 4.2 g MOPS buffer were added and pH was adjusted to 6.7. Media were sterilised by autoclaving for 15 minutes at 121˚C and stored in the cold room.

Appendix C. Dissemination works of this thesis

A list of publishable outcomes of this thesis are presented.

C.1 Oral presentations

- Allan McGivern, Konstantions Grintzalis. The impact of pharmaceutical compounds on *Daphnia magna.* BRS research day 2020- Dublin (Ireland)

C.2 Posters

- Allan Robert McGivern, Enya Kubitzky, Dimitrios Kakavas, Sinead Morgan, Grégory Genta-Jouve, Konstantinos Grintzalis. The impact of algal food on the physiology and resistance to metal toxicity in *Daphnia magna.* SETAC SciCon 2020- Dublin (Ireland)
- Dimitrios Kakavas, Enya Kubitzky, Allan Robert McGivern, Hannah Farrelly, Katie O'Rourke, Grégory Genta-Jouve, Konstantinos Grintzalis (2020). The impact of algal food on phenotypic endpoints of daphniids. Environ 2020- Dublin (Ireland)
- Allan McGivern, Konstantinos Grintzalis. The impact of pharmaceutical pollutants on key enzymes in Daphnia magna. JRC Summer School on Non-Animal Approaches in Science 2021- Online
- Allan McGivern, Konstantinos Grintzalis. The impact of a complex mixture of pollutants on key enzymes in Daphnia magna. OpenTox 2021- Online

C.3 Conference participation

- SETAC SciCon 2020- Dublin (Ireland)
- Environ 2020- Dublin (Ireland)
- JRC Summer School on Non-Animal Approaches in Science 2021- Online
- Opentox 2021- Online