The Integration of Holistic Techniques for the Detection of Pharmaceuticals in the Freshwater Environment

This thesis is presented for the degree of Doctor of Philosophy

By

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy 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.

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List of Abbreviations

ACP	Acid Phosphatase
ADMA	Asymmetric Dimethylarginine
ALP	Alkaline Phosphatase
AMP/AMPK	Activated Protein Kinase
AOPs	Adverse Outcome Pathways
APIs	Active Pharmaceutical Ingredients
ARGs	Antibiotics Resistance Genes
BM	Broadmeadow
CA	Concentration Addition
CBB	Coomassie Brilliant Blue
CBZ	Carbamazepine
CE	Symmetrical Edges
CHE	Cholinesterase
COX	Cyclooxygenase
CV	Cross-Validated Analysis of Variance
E1	Oestrone
E2	β-estradiol
E3	Oestriol
E4	Oestretrol
EBMs	Effect Based Methods
EC	Effective Concentration
EDCs	Endocrine Disrupting Compounds
EREs	(O)Estrogen Response Elements
ERs	(O)Estrogens Receptors
FA	First Antennae
GABA	γ-Aminobutyric acid
GC	Grand Canal
GO	Gene ontology
GPX	Glutathione Peroxidase
GST	Glutathione -S-Transferase
HCL	Hierarchical Clustering
HDLs	High Density Lipoproteins
HILIC	Hydrophilic Interaction Liquid Chromatography
K	Kettle’s
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KEs	Key Events
LCMS	Liquid Chromatography – Mass Spectrometry
LDH	Lactate Dehydrogenase
LIP	Lipase
LPL	Lipoprotein Lipase
MIEs	Molecular Initiated Events

NAMs	New Approach Methodologies
NMDA	N-Methyl-D-aspartate
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
PABA	Para-Aminobenzoic Acid
PCA	Principal Component Analysis
PEP	Peptidase
PLS-DA	Partial least square discriminant analysis
PNPB	p-Nitrophenyl Butyrate
PPARA	Peroxisome Proliferation Activated Receptor – α
QCs	Quality Controls
REACH	Registration, Evaluation, Authorisation and Restriction of Chemical substances
SAM	Significance Analysis of Microarrays
SDMA	Symmetric Dimethylarginine
SOD	Superoxide Dismutase
T	Tolka
TCA	Tricarboxylic Acid
WFD	Water Framework Directive
WHO	World Health Organisation
WWTPs	Wastewater Treatment Plants
B-GAL	Beta-galactosidase

Thesis Abstract

The Integration of Holistic Techniques for the Detection of Pharmaceuticals in the Freshwater Environment

Katie O'Rourke

Pharmaceutical compounds are emerging contaminants of concern, which can elicit sub-lethal effects to non-target organisms and present a human health risk via the contamination of drinking water. Exacerbated by their increased consumption and poor removal rates from wastewater treatment plants, pharmaceutical compounds have been classified as pseudo-persistent in the aquatic environment, and their monitoring is imperative. It is now widely accepted that conventional methods of monitoring are inadequate, and the implementation of novel holistic approaches which provide biological data, identify mechanisms of toxicity as well as predict pollution are essential to safeguard the environment from pollutants including pharmaceuticals.

Employing the sentinel species *Daphnia magna* and integrating holistic techniques such as metabolomics and biochemical markers, the molecular responses to individual pharmaceuticals or complex mixtures was explored in the concept of daphnids acting as the "canary in the coal mine" within the aquatic environment. Specifically, the effects of oestrogens, antibiotics, environmentally relevant drugs (anticonvulsants, NSAIDs, lipid regulators, antidiabetics) and a pharmaceutical mixture were assessed through acute, chronic and in some instances transgenerational exposures, endpoints ranged from phenotypic i.e. mortality, enzyme, reproduction, size to molecular i.e enzyme activity and metabolomics.

Exposure to oestrogens revealed impaired enzyme activity, distinct differences in the polar metabolic profile, as well as decreased protein content and size of daphnids, while antibiotic exposure showed altered enzyme activity, the upregulation of hydrophilic metabolites following acute exposure, and a trend to downregulate the metabolites of central metabolism following transgenerational exposure. This contrasts with the environmentally relevant drugs which mostly upregulated the intermediates of central metabolism and polar metabolites. To assess the synergistic effect of the pharmaceuticals, daphnids were exposed to a mixture as well as environmental samples, which revealed many changes in enzyme activities and the metabolome.

Chapter 1

Introduction to freshwater ecotoxicology and risk assessment

Abstract

Human actions have impaired the condition of the ecosystem and a major component of human presence is the extensive use of pharmaceuticals. Pharmaceuticals eventuate in the freshwater environment via several pathways, including wastewater effluent and their improper disposal. Attributed to their poor removal rates from wastewater treatment plants, and their stable nature, pharmaceuticals are often biologically active upon entry to the environment, where they can pose sub-lethal effects on non-target organisms. Exacerbated by the increased prevalence of age-related illness and chronic diseases such as diabetes, global pharmaceutical consumption has steadily increased over the decades and thus more pharmaceuticals have been ubiquitously detected in environmental matrices. However, as traditional methods of water monitoring provide no biological data, it is not yet fully understood the magnitude of the impact pharmaceuticals are posing to the freshwater environment. In recent years there has been a shift towards implementing New Approach Methodologies (NAMs), to deal with the challenge of pharmaceutical pollution. These approaches aim to unravel the underlying mechanisms of toxicity using lesser evolved organisms, thus, reducing *in vivo* testing and to develop novel and sensitive tools for the early prediction of pollution. Underpinned by NAMs are effect-based methods, which forms the basis of this project, by combining bioassays and metabolomics the effect of three diverse classes of pharmaceuticals (oestrogens, antibiotics and commonly detected drugs) was assessed on the ecotoxicological model *Daphnia magna*.

1.1 Introduction to ecotoxicology and risk assessment

Pharmaceuticals represent a diverse class of emerging contaminants which are ubiquitously detected in the aquatic ecosystem, their presence in freshwater is an issue which affects freshwater biota but also human health via the contamination of drinking water sources. Driven by increased anthropogenic activities such as industrialisation, urbanisation and agricultural practices, pharmaceutical pollution of aquatic ecosystems has escalated, and together with other pollutants like personal care products, heavy metals and agrichemicals, presents one of the most serious challenges of modern society (Ding et al., 2016). Advancements in chromatography and mass spectrometry techniques, has led to increased pharmaceutical detection within the environment (Kanda & Glendinning, 2011; Richardson & Kimura, 2020) and pharmaceutical compounds are therefore ubiquitously detected in the ng/L range of freshwater systems (Hughes et al., 2013; Ortúzar et al., 2022). However, in receiving waters of hospitals (A. Hartmann et al., 1999; Andreas Hartmann et al., 1998), and effluents of waste water (Lindqvist et al., 2005) and industrial plants (Larsson et al., 2007), pharmaceuticals have been detected in the µg/L order of magnitude. As a result of ageing populations, increased chronic disease prevalence, and trends to self-medicate, pharmaceutical consumption rates continue to soar (Flaherty & Dodson, 2005; OECD, 2019). Certainly, there has been an increase in pharmaceutical usage in OECD countries, for example the consumption of anti-hypertensive drugs increased by 65% from 2000- 2019, lipid regulators quadrupled, and antidiabetics doubled over the same period. Furthermore, there has also been a 35% increase of pharmaceutical intake in developing countries as well (Van Boeckel et al., 2014).

Pharmaceutical compounds eventuate in the freshwater environment via many routes, including household waste, agricultural run-off and improper disposal (Figure 1.1). Once consumed, the fate of pharmaceuticals and their transformation products is cryptic and can follow several pathways. The most consistent pathway however is through wastewater effluent, as depending on characteristics such as polarity, water solubility and persistence chemicals may be degraded, become associated with sewage sludge or released into surface waters following treatment processes and therefore removal efficiencies varies for each compound (Couto et al., 2019; Meredith-Williams et al., 2012). It is widely accepted that modern waste water treatment plants (WWTPs) do not efficiently remove pharmaceuticals from influent, this is a result of their design which was intended to remove larger particles and organic compounds in

the mg/L range (Patel et al., 2019). Even when advanced removal techniques are implemented e.g. granular activated carbon, membrane technologies, ozonation, and ultraviolet radiation, certain chemicals remain tolerant to such processes and can enter potable supplies (Boyd et al., 2003; Jones et al., 2005; Kolpin et al., 2002). In some cases, elimination rates can be as low as 10%, which is true for pharmaceuticals like carbamazepine and diclofenac (M. de Oliveira et al., 2020; Ternes et al., 2002). As a result, pharmaceuticals are now described as pseudo-persistent because of their ongoing release into the environment and their stability once they eventuate there (Gómez-Canela et al., 2019; Trombini et al., 2019).

Pharmaceuticals have been designed to exert their mechanisms of action on target organisms, however, pharmaceuticals that end up in surface waters can provoke sub-lethal effects on non-target organisms, even at low concentrations. Moreover, these sub-lethal effects are amplified in a realistic scenario resulting from the combination effects of several different pharmaceuticals and chemicals that act synergistically.

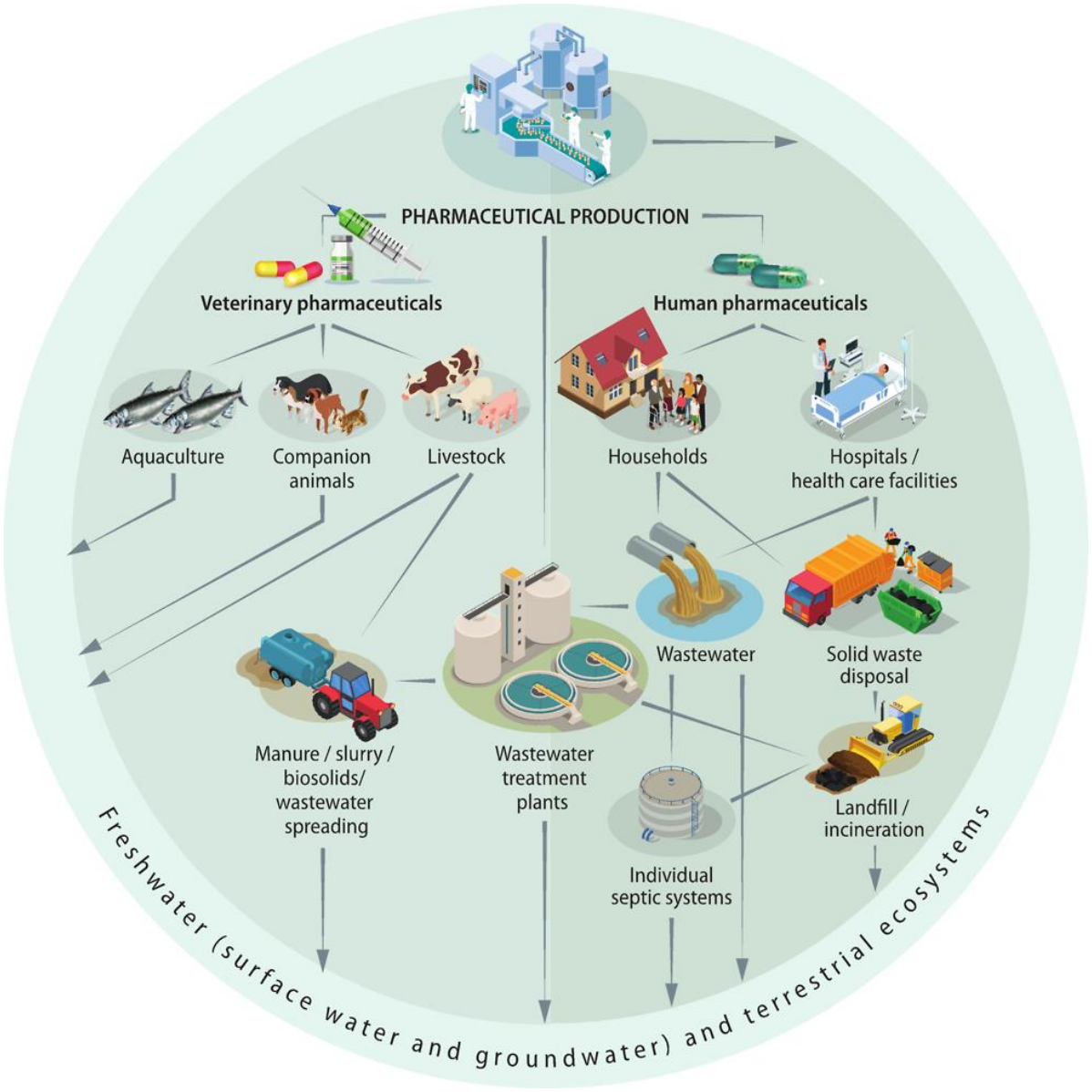

Figure 1.1. Environmental sources of pharmaceuticals (OECD, 2019).

1.2 Revolutionising ecotoxicology and risk assessment

There is significant concern regarding the impact of anthropogenic activities on the aquatic environment, specifically in relation to the contamination of surface waters from pollutants. Although Active Pharmaceutical Ingredients (APIs) have been widely detected in all environmental matrices (T. aus der Beek et al. 2016), there is limited information regarding their effect to wildlife, it is estimated that there is only 5% data of the 100’000 pharmaceuticals in existence on this subject. This uncertainty highlights the importance of monitoring and controlling the presence of APIs in the environment. Traditional methods for water quality monitoring generally involve the quantification of chemicals in a spot or grab sample by analytical techniques such as liquid or gas chromatography coupled with mass spectrometry, elemental analysis etc. Although

improvements in these approaches have led to broader chemical annotation capabilities, they are still limited in their sensitivity as they cannot detect unknown chemicals, transformation products or chemicals below detection limits. Additionally, conventional methods, do not produce biological data and therefore do not give insight on mechanisms of toxicity, and thus the comprehensive impact pharmaceuticals pose to the environment cannot be attained through existing methods of monitoring. For this reason, there has been a shift towards New Approach Methodologies to safeguard the environment from chemicals and to assist in regulatory decision making (Stucki et al., 2022). Such approaches aim to unveil mechanisms of toxicity, reduce *in vivo* testing and to predict pollution before it reaches precarious levels. Underpinned by these novel approaches are Effect Based Methods (EBM), which involves the employment of ecotoxicological organisms, following exposure to individual or mixture chemicals, multiple endpoints can be measured to identify mechanistic effects of chemicals (Brack et al., 2019). Within aquatic ecotoxicological studies, there are several key species predominately used as bioindicators, such as *Daphni*a, *Gammarus* and *Mytilus* species.

Pharmaceuticals are a large and diverse group of chemicals and exist in a number of therapeutic classes according to their mechanisms of action, chemical structure and physiological effect. For example, anti-microbial agents, anti-convulsants, pain relievers and hormonal drugs. Although pharmaceuticals were designed with specific molecular targets in human and animals, as we are phylogenetically related to organisms of the aquatic ecosystem, some of these targets are evolutionarily conserved and therefore there is potential for adverse effects on non-target organisms (Ding et al., 2016). Even though APIs may be present at low concentrations in the aquatic environment, they are in the inevitable presence of many other pollutants which can jointly produce a “cocktail” effect, that can lead to impaired development. and functioning (Novák et al., 2018). Perhaps one of the most well- known instances of deleterious effects of wildlife by pharmaceuticals is the rapid decline of *Gyps* vultures in South Asia by diclofenac present in the carcasses of livestock (Swan et al., 2006), this has led to a 95% reduction in the populations of 3 species, which are now deemed critically endangered (Green et al., 2004; Prakash et al., 2003). Contaminants can perturb normal biological functioning through several modes of actions and can inhibit and affect various different processes. For example, anti-anxiety drugs have been documented to alter behaviour in perch fish, β-blockers have demonstrated

endocrine disruption in fish and oestrogens have been reported to exhibit gene interference in goldfish (Brodin et al., 2013; Ding et al., 2016; Massarsky et al., 2011; Yan et al., 2013).

Due to the potential threat to human health and ecosystems from pharmaceuticals, there has been a number of legislative frameworks implemented globally for the monitoring and assessment of aquatic ecosystems (Vrana et al., 2005). Registration, Evaluation, Authorisation and Restriction of Chemical substances (REACH) and the Water Framework Directive (WFD) are two major pieces of legislation which govern the countries of the European Union (Connon et al., 2012). The WFD is the first legislation of its kind, in the sense it governs all water bodies, rivers, lakes, estuaries, coastal waters and ground waters and their corresponding wildlife and habitats and it aims to achieve “Good Water Status” of these water bodies by 2027, however some countries including Ireland are yet to fully adopt the required legislation and therefore have made unsatisfactory progress to reach this target. Furthermore, even with the existence of this legislation, as the monitoring programmes are based on conventional methods, the lack of mechanistic insight means we have not attained the full picture of water pollution across Europe. Therefore, the integration of bioassays, phenotypic endpoints and holistic techniques such as metabolomics, can be implemented to detect the mechanisms underlying the actions of toxicity and to revolutionize risk assessment by providing the necessary molecular tools for the early detection of active pharmaceutical ingredients.

1.3 Pharmaceuticals detected in the environment

The presence of pharmaceutical compounds is constantly increasing as a result of the overreliance and widespread use of pharmaceuticals. Over the last two decades the pharmaceutical industry has undergone significant growth and global revenues amounted to 1.48 trillion USD in 2022. Pharmaceutical consumption is continuously increasing to deal with the increased prevalence of age-related illnesses and chronic diseases, in consequence of increased life spans and risk factors like obesity. As a result, pharmaceuticals are now ubiquitously detected in surface waters globally and possess a broad geographical presence in the aquatic environment.

For this study a diverse range of pharmaceutical drugs were selected (Table 1.1), based on their different modes of action, their global prevalence in the aquatic environment, and their significance in human and veterinary health. The chosen drugs

range from antibiotics, oestrogen hormones, anti-convulsants, non-steroidal anti-inflammatory drugs (NSAID), fibrates, anti-hyperglycaemics and anti-depressants.

Table 1.1. Reported pharmaceutical contamination of the global aquatic environment. Source: (Patel et al., 2019) (David Azanu,2018)

Chemical	Type	Concentration in the environment	Sample Location
Erythromycin	Antibiotic, macrolide	292 ng/L	Serbia, Surface water
Trimethoprim	Antibiotic, antifolate	156 ng/L	Beijing, Changzhou, Shenzen, River water
Amoxicillin	Antibiotic, penicillin	2.7 ng/L	Kumasi, Ghana, river water
Sulfamethoxazole	Antibiotic, sulphanilamide	0.44−115.3 ng/L	Chongqing, China, River water
Estrone	Hormone	0.02−2.9 ng/L	Singapore, Mangrove water
β-estradiol	Hormone	0.0011−0.003 ng/L	Sweden, Dal River
17α-ethynylestradiol	Synthetic hormone	0.001−0.0016 ng/L	Sweden, Dal River
Carbamazepine	Anti-convulsant	0.51-38.24 ng/L	Beijing, China, Tap water
Gabapentin	Anti-convulsant	4.5 ng/L	Sweden, Dal River
Diclofenac	NSAID	0-324 ng/L	1010−10200 ng/L, South Africa
Gemfibrozil	Fibrate	0.77−17.7 ng/L	Singapore, Mangrove water
Metformin	Anti-hyperglycemic	8.4 ng/L	Sweden, Dal River

1.3.1 Antibiotics

Antibiotics and antimicrobials are chemical agents used to inhibit bacterial, fungal and protozoan growth in both human and veterinary medicine. Antibiotics are classified by their mode of action in distinct subclasses (Figure 1.2, Table 1.2).

Table 1.2. Classes and mechanisms of action of antibiotics.

Antibiotic	Class	Mode of Action	Use

Azithromycin, erythromycin, clarithromycin.	Macrolide	Inhibitor of bacterial protein biosynthesis	Respiratory and skin infections
Norfloxacin	Fluoroquinolone	Inhibitor of DNA synthesis	Urinary tract infections
Ciprofloxacin	Fluoroquinolone	Inhibitor of DNA synthesis	Bone/joint, abdominal, and skin infections
Chlortetracycline	Tetracycline	Inhibitor of bacterial protein biosynthesis	Skin infections (topical)
Benzylpenicillin	β-lactam	Bactericidal, inhibitor of peptidoglycan cell wall synthesis	Syphilis, strep throat, pneumonia.
Amoxicillin	β-lactam	Bactericidal, inhibitor of peptidoglycan cell wall synthesis	Ear infections, strep throat and urinary tract infections
Trimethoprim	Co-trimoxazole	Inhibitor of folic acid synthesis	Bladder and ear infection/diarrhoea
Sulfamethoxazole	Co-trimoxazole	Inhibitor of folic acid synthesis	Bronchitis, prostatitis and urinary tract infections

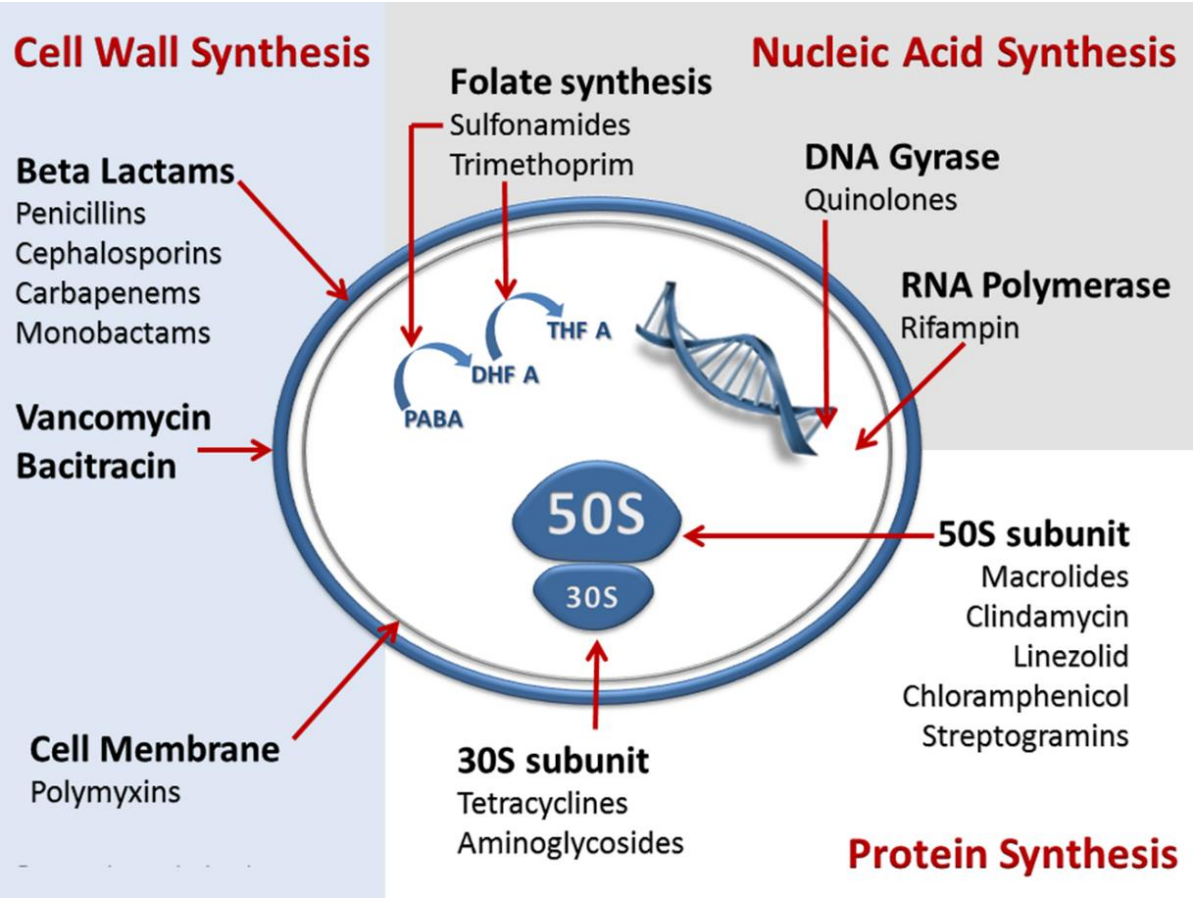

Figure 1.2. The different modes of action of antibiotic compounds (Upadhya R et al., 2018).

Azithromycin is an antibacterial compound with broad-spectrum application, which is frequently prescribed to treat respiratory, skin, eyes, ear and sexually transmitted infections. Azithromycin belongs to the macrolide class of antibiotics and can be characterised by a macrocyclic lactone ring of 12 or more elements. Macrolides are protein synthesis inhibitors, and their mode of action involves the inhibition of peptidyltransferase from adding the growing peptide attached to tRNA to the next amino acid as well as the prevention of RNA translation. Similarly, erythromycin and clarithromycin are also macrolides, which too are used to treat skin and respiratory infections. Ciprofloxacin is a fluoroquinolone antibiotic that targets bacterial infections mainly of the skin, bones/joints, urinary tract, sinus and respiratory tract. Fluoroquinolone's mode of action involves inhibition of DNA topoisomerases, two enzymes which are involved in bacterial DNA synthesis. Topoisomerases are essential for DNA replication and therefore enable these agents to be both specific and bactericidal. Trimethoprim is a human and veterinary prescribed anti-bacterial

drug, which is classified as a pyrimidine inhibitor of the enzyme dihydrofolate reductase. Primarily, it is used to treat urinary tract infections such as cystitis and is potentiated by sulphonamides, as when used together they inhibit successive steps of the folate synthesis pathway. Therefore, trimethoprim is most frequently sold in combination with the other antibiotic sulfamethoxazole which belongs to the class of antibiotics known as sulphanilamides. Specifically, sulfamethoxazole functions by competitively inhibiting (acting as a substance analogue) the enzymatic reaction involving para-aminobenzoic acid (PABA). Amoxicillin is a semi-synthetic form of penicillin with broad spectrum applications, it is a β-lactam antibiotic which has bactericidal action through the inhibition of peptidoglycan cell wall synthesis. It is frequently co-administered with clavulanic acid as “Augmentin Duo” or “Co-amoxiclav”, the addition of clavulanic acid inhibits β-lactamase enzymes produced by bacteria, preventing the breakdown of amoxicillin.

The prominent occurrence of antibiotics in surface and ground waters poses significant environmental and human health impacts, in a European context, the concentration of antibiotics in inland surface waters ranges from 0.0006-0.548 µg/L. However, clarithromycin and sulfamethoxazole tend to have higher average concentrations of 0.193 µg/L and 0.548 µg/L (Sanseverino Isabella et al., 2018). As a result of their importance in the treatment of infectious diseases, antimicrobial compounds have been heavily relied on since their initial discovery. Antibiotics are large contributors to water contamination due to high usage rates in human and veterinary care and low removal rates within wastewater treatment plants (WWTPs). Perhaps the largest contributor of water contamination by antibiotics is the use of them as growth promoters in the agricultural industry and the prophylactic use of antibiotics in aquaculture, it is reported that approximately 70% of all antibiotics sold in the USA is for agricultural use (Anomaly, 2019). Antibiotics are excreted through the urine of the farm animal and can directly run-off into surrounding surface waters, this issue is exacerbated by the land management practice known as slurry spreading, which involves the topical application of slurry (manure and urine) to the lands surface (Manyi-Loh et al., 2018). As a result of their continuous release into surface waters via improper disposal or WW effluent, antibiotics are ubiquitous and persistent in nature with high environmental degradation rates. Similarly, to other pharmaceuticals, antibiotics exert toxic effects to non-target organisms they encounter, however they also directly impact aquatic microbes, which are often key to environmental processes

e.g. denitrification. (Costanzo et al., 2005). The discernible presence of antibiotics in aqueous environments is concerning from an environmental and human health standpoint. Antibiotics compromise the quality status of drinking water and water used for artificial irrigation. Moreover, antibiotics which eventuate in aquatic ecosystems expedite the spread of antibiotic resistance genes, finally they can cause detrimental effects or death to integral aquatic microbes (Huerta et al., 2013). Depending on the antimicrobial compound, efficacy of wastewater treatment processes varies, several studies have alluded to the insufficient removal of certain antibiotics and their release into receiving waters in their active form, leading to the spread of antibiotic resistance, which was deemed by the World Health Organisation (WHO) as one of the three biggest threats current society faces (Rodriguez-Mozaz et al., 2015). Antibiotics resistance genes (ARGs) have been widely detected in a number of environmental matrices; however, their presence is exceptionally high in hospital effluent and WWTP influent, although studies have indicated their obvious presence even after treatment e.g. genes promoting resistance to β-lactams (*blacTEM*), resistant to macrolides (*ermB*), and resistance to tetracyclines tetW (Rodriguez-Mozaz et al., 2015). In the EU, it is reported that over 30’000 people die annually of antibiotic resistant-bacterial infections, 39% of which were treated using last line antibiotics like carbapenems and colistin (Cassini et al., 2019; Polianciuc et al., 2020). There are potential major implications of increasing concentrations of antibiotics in freshwater ecosystems i.e. prolonged or untreatable illness in humans, this highlights the importance of employing modern effect-based methods to monitor the aquatic environment closely.

1.3.2 Oestrogens

Oestrogens are a category of sex hormones comprised of 4 compounds: oestrone (E1), oestradiol (E2), oestriol (E3) and oestretrol (E4). Oestradiol is considered the major hormone of the reproductive lifespan, while oestrone is dominant post-menopause, while oestriol and oestretrol are produced during pregnancy (Fruzzetti et al., 2021). Oestrogens are primarily female sex hormones and are predominantly produced in the ovaries, corpus luteum, adrenal cortex and by non-gonadal organs such as the skin, liver and brain in smaller amounts (Cui, J. Shen, Y. and Li, 2013). Oestrogens play a vital role in the development of female sex characteristics as well as menstrual cycle regulation, but also have further roles in nervous system and cardiovascular function (Mauvais- Jarvis et al., 2013). Although oestrogens are

considered female hormones, they are also critical in normal male sexual function, estradiol in particular is produced in the testes in smaller amounts and modulates male libido, erectile function and spermatogenesis (Adeel et al., 2017; Schulster et al., 2016; Woniak et al., 2014). Oestrogens are steroidal sex hormones produced mainly from dietary cholesterol (low-density lipids) via steroidogenesis (Figure 1.3). The primary site of steroidogenesis are theca cells, where cholesterol is converted to androgens, following this androgens are converted to oestrogens in the granulosa cells (Fuentes et al., 2019). The classical mechanism of oestrogen signalling is known as direct genome signalling. This process involves the oestrogen receptors (ERα and Erβ) acting and transcriptional factors, once the oestrogen molecule binds to the receptor, it undergoes a conformational change, which activates the receptor. Subsequently, the oestrogen-oestrogen receptor complex is translocated to the nucleus, where it binds to specific DNA sequences called estrogen response elements (EREs), inducing transcription of proteins that mediate physiological effects of oestrogens (Dily, 2018; Fuentes et al., 2019; Klinge, 2001; Malley, 2005; Marino et al., 2006).

In menstruating women, oestrone is considered a minor oestrogen hormone and is approximately one third as potent as oestradiol. Although oestrone can bind to ERα and ERβ, it exhibits weak oestrogenic activity (Sato et al., 2016). However, oestrone is the major female hormone in post-menopausal women and is mainly produced from the conversion of the androgen androstenedione, and is lesser amounts by the ovaries and the adrenal glands (Eric C. Meyers, Bleyda R. Solorzano, Justin James, Patrick D. Ganzer, Elaine S., Robert L. Rennaker, 2018). Estradiol, specifically 17β-estradiol exhibits the strongest oestrogenic activity of all oestrogens and is the main circulatory hormone during the reproductive years. Due to its natural high potency, β-estradiol controls major endocrine processes throughout the human body, such as; puberty and the development of secondary female sex characteristics, regulation of menstrual cycle and ageing (Roby, 2019; Stillwell, 2016). Although, oestrogens are endogenous, they are also widely synthesized by pharmaceutical companies for therapeutic applications i.e the contraceptive pill and hormone replacement therapy. Commercially, oestradiol is manufactured as a hormone therapy drug, used to treat conditions associated with menopause and hypoestrogenism, it is also a component of certain oral contraceptive pills and patches, commonly sold under the trade name Zoely, Activella and Alora. 17α-ethinylestradiol (or EE2), is an oestrogenic synthetic hormone, which pertains high potency when administered orally. Similarly, to β-

estradiol, EE2 is an oestrogen receptor antagonist which binds to receptors and activates DNA transcription in the nucleus of the cell. EE2 is a common component of the oral contraceptive pill, specifically the combination pill, sold under the trade names Yasmin, Alesse and Triphasil.

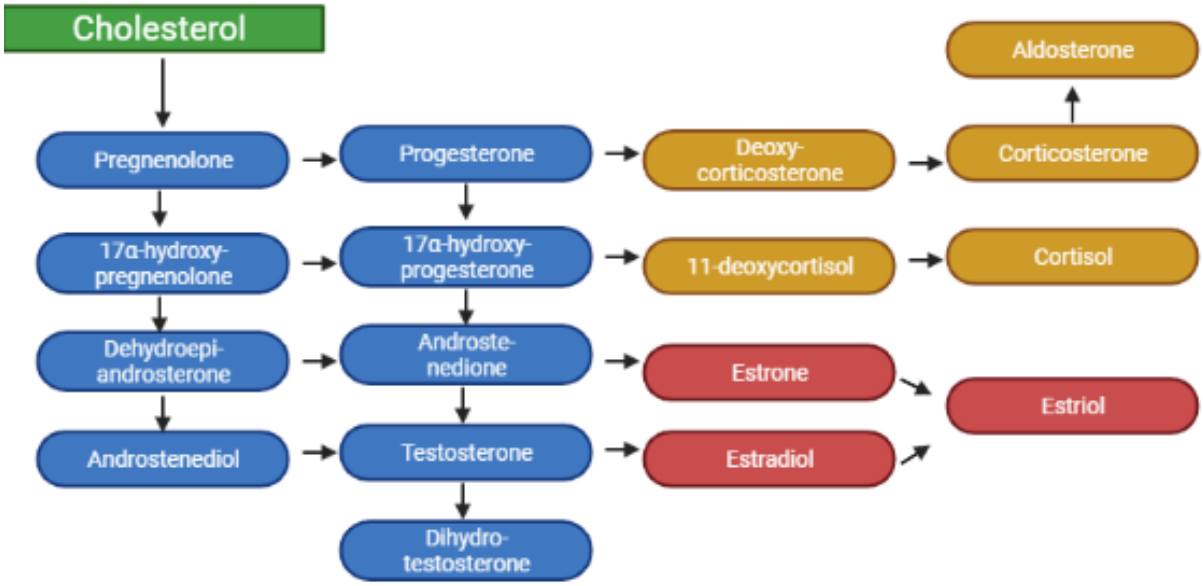

Figure 1.3. The biochemical pathways of steroidogenesis (Figure adapted from Waszut et al., 2017).

1.3.3 Environmentally relevant pharmaceuticals

Pharmaceuticals represent a diverse class of manufactured medicinal compounds which are used to cure, treat and prevent disease. Although the generation of this vast class of chemicals has certainly benefited society through the prolonging and saving of lives, it has now become an issue of major concern with severe environmental implications. Pharmaceuticals have major applications in both human and veterinary care, and therefore enter the environment continuously in low yet frequent amounts from many sources. As a result, pharmaceuticals have been widely detected in nearly all environmental matrices (surface waters, ground waters, seawater, sludge and soil) on almost every continent. In fact, pharmaceutical compounds have been detected in the polar regions of the globe, which are considered the most pristine areas on the planet (Patel et al., 2019). Moreover, improvements in analytical techniques such as liquid chromatography – mass spectrometry (LCMS) has also contributed to the increased detection of pharmaceutical compounds (Richardson & Ternes, 2020). In surface waters, pharmaceuticals are commonly recorded in the ng/L range (Hughes et al., 2013), although in the receiving water of effluents derived from hospitals

(Andreas Hartmann et al., 1998), wastewater treatment plants (Lindqvist et al., 2005) and industrial plants (Larsson et al., 2007), they have been documented in concentrations within the µg/L range. Pharmaceuticals can remain biologically active even after conventional treatment methods and can pose uncharacterized toxic effects to non-target organisms (Bungau et al., 2018; Rogowska & Zimmermann, 2022). Although the concentration of pharmaceuticals in aquatic ecosystems are low (ng/L - µg/L), the long-term effects associated with chronic exposure to these compounds could result in impaired reproduction and or alterations of biochemical processes in aquatic biota (H. Chen et al., 2019)

1.3.3.1 Anticonvulsants

Anticonvulsants or commonly referred as antiepileptic drugs are pharmaceutical agents with curative and prophylactic properties. As their name suggests, anticonvulsants are prescribed in the treatment of disorders that provoke seizures, such as epilepsy. Additionally, anticonvulsants can be taken to alleviate neuropathic pain and symptoms of borderline personality disorder (Dean, 2012; Y.-J. Wu, 2012). For example, carbamazepine (CBZ) (commonly known as Tegretol), is used in the treatment of epileptic seizures and neuropathic pain caused by trigeminal neuralgia and to treat bipolar and borderline personality disorder (Yongjun Zhang et al., 2008). The mechanism of action for carbamazepine's is not fully clear and is still debatable, however, it is widely thought that carbamazepine is a sodium channel blocker, meaning it inhibits sodium channel firing, which in turn prevents seizure activity. It is believed that carbamazepine exerts these effects by binding to voltage-gated channels in their inactive from, this prevents action potentials which would normally provoke nerve stimulatory effect (Kennebäck et al., 1995). Moreover, another drug, gabapentin is a widely used anti-convulsant originally designed for first line treatment of partial seizures, however, it's primary use currently is to treat chronic neuropathy pain, in particular pain associated with the shingles virus (Rullán et al., 2017; Viniol et al., 2019). Similar, to carbamazepine, gabapentin's mechanism of action is ambiguous and its full array of neurobiological effects has not been defined. Although it was designed as a γ-Aminobutyric acid (GABA) analogue, it does not bind to GABA receptors or affect the uptake or release of GABA in the central nervous system. It is thought that gabapentin's mode of action as an antiepileptic drug is the inhibition of the alpha 2-delta subunit of voltage-gated calcium channels (Hendrich et al., 2008).

Among the many pharmaceuticals recorded in surface and ground waters, carbamazepine remains one of the highest detected in treated and untreated water samples (Kovačević et al., 2017; Nkoom, Lu, Liu, Yang, et al., 2019a; P. Oliveira et al., 2017). Moreover, its urinary metabolite 0,11-dihydro-10,11-expoxycarbamazepine (CBZ-epoxide) is reported to be as pharmaceutically active as CBZ. Furthermore, another urinary metabolite of CBZ, trans-10,11-dihydro-10,11-dihydroxycarbamazepine (CBZ-diol) is thought to have the same prevalence within the environment as its parent compound (Yongjun Zhang et al., 2008).

1.3.3.2 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs are a long-established class of pharmaceuticals and are one of the most prescribed analgesics in clinical settings, their wide distribution and use is attributed to their three principal functions as pain relievers, anti-inflammatories and fever reducers (Alfaro & DD., 2022). Depending on the individual compound, NSAIDs can be used to treat acute conditions like headaches or administered to manage chronic conditions such as arthritis, they can be applied topically or ingested orally through tablet form. NSAIDs mechanism of action involves the inhibition of lipids known as prostaglandins which are responsible for inflammation, blood clotting and blood flow at the site of injury or infection. NSAIDs inhibit the enzymes responsible for the production of prostaglandin, cyclooxygenase-1 and –2 (COX-1 and COX-2). There are two major types of NSAIDs available, non-selective and COX-2 selective. Non-selective NSAIDs reduce platelet formation (aspirin) while reducing inflammation, however these drugs also increase the risk of gastrointestinal ulcers. COX-2 selective NSAIDs (rofecoxib and celecoxib) have less gastrointestinal risk associated with them however they do promote blood clot formation (Altman et al., 2015). Diclofenac is arguably the most well-known NSAID available on the market and is commercially sold under the trade name Voltaren and as a topical cream or oral tablets. Diclofenac has been considered as a contaminant of emerging concern for many years and is renowned for contributing to the near extinction of *Gyps* sp. vultures in subcontinent India (Swan et al., 2006). Notably, diclofenac was included on the primary EU Watch List of 2013 (2013/39/EU) this is attributed to substantial research which argues its safety for human health and aquatic ecosystems (Simon et al., 2022). Studies have elucidated to the acute and chronic toxic effects of diclofenac on aquatic invertebrates, such as observable effects of phenotypic endpoints (growth rate and fecundity) in *D.*

magna and disruption of metabolic pathways of *Mytilus galloprovincialis* (Bonnefille, Gomez, Alali, et al., 2018; Y. Liu et al., 2017a).

1.3.3.3 Medicinal compounds to treat metabolic disorders

Metabolic disorders manifest themselves when abnormal chemical reactions take place and inhibit normal metabolic functioning, it often results in a surplus or deficit of important substances that are necessary to remain healthy, examples of such disorders include diabetes and Graves' disease. Gemfibrozil is a valeric acid belonging to a class of lipid-regulating compounds known as fibrates, for this reason its principal use is in the treatment of dyslipidaemia disorders (Roy & Pahan, 2009). Gemfibrozil provokes an increase in high density lipoproteins (HDLs) and a decrease in plasma triglycerides (Saku et al., 1985). These effects are achieved by the activation of the peroxisome proliferation activated receptor – α (PPARα) and the upregulation of lipoprotein lipase (LPL). In addition to elevated HDLs, gemfibrozil inhibits apo β synthesis, peripheral lipolysis and the decreased removal of free fatty acids by the liver. Another pharmaceutical, metformin, is the most prescribed drug for the regulation of type II diabetes. Metformin is an anti-hyperglycaemic agent belonging to the biguanide class, which has inherited the name from its ability to lower blood glucose levels without inducing hypoglycaemia. The mechanism of action for metformin is unique when compared to other anti-hyperglycaemic drugs. Metformin reduces blood sugar levels through several mechanisms, by decreasing glucose absorption in the intestine, decreasing of hepatic gluconeogenesis and increasing the uptake and utilisation of glucose (Nasri & Rafieian-Kopaei, 2014). As metformin is the first line of treatment for diabetes type 2, it is one of the most heavily prescribed APIs globally, hence it is also one of the most detected in surface waters and wastewater influent. This is exacerbated by the fact metformin is excreted unmetabolized by the body, furthermore, its physio-chemical properties increase its persistence in the environment (Godoy et al., 2018). In addition, it is suspected that metformin can be regarded as an Endocrine Disrupting Compound (EDC), thus re-enforcing its potential threat to the aquatic environment (Briones et al., 2016). Similar to metformin, the consumption of the lipid regulator gemfibrozil has increased in recent years as a result of poorer diet and lifestyle habits. For this reason, it has been highly detected in many aquatic compartments, gemfibrozil has been documented in the 3.47-63.8 µg/L range for groundwater, waste water influent and effluent samples (Fang et al., 2012).

1.4 Daphnids as a key species for ecology and ecotoxicology

Assessment of freshwater pollution is based on the detection of pollutants but also the effects of these substances on flora and fauna. Among the aquatic organisms, daphnids are important components of the food web in freshwater environments and have acquired a central position in pollution monitoring. Daphnids or more commonly known as water fleas, are freshwater planktonic crustaceans of the class Branchiopoda and the order Cladocera. Branchiopods are characterised by flattened leaf like legs, that serve to produce a water current for their filtering apparatus. The bodies of daphnids are enclosed by an uncalcified shell known as a carapace, while haemolymph circulates through a double wall, situated between the carapace and the body cavity. Daphnids possess nine appendages, antennules, antenna used for swimming, maxillae and paired mandibles for filter feeding and five limbs attached to its trunk which facilitate feeding and respiration (Figure 1.4). Within the genus *Daphnia* there are over 100 species, which at the adult stage depending on the species, daphnids can range from <1mm – 5mm in size, males can be differentiated from females by their front legs which are equipped with clasping hooks, and a modified post abdomen (Figure 1.5). These small crustaceans are a well-established model species in toxicology and are frequently used in environmental monitoring. *Daphnia* have many characteristics which make them suitable and valuable in both biomonitoring and experimental studies. Their value is attributed to their natural high abundance in the environment, their integral role in the aquatic food web and their wide geographical distribution. Daphnids biology and functional morphology has been of central interest of scientists for many centuries, naturalists, taxonomists and experimental biologists have been conducting research studies on daphnids since the 17th century. As a result, there is a remarkable amount of information and resources available about daphnia (Shaw et al., 2008). *D. pulex*, a smaller species of *Daphnia* compared to its counterpart, *D. magna,* was the first crustacean to have its genome sequenced, and was deemed as “ecoresponsive” (Shaw et al., 2008). The revelation of the complete genome sequence of *D. pulex* is encouraging and facilitates a greater understanding of the interaction between genome structure, gene expression, individual fitness, and population-level responses to environmental change.

Daphnia sp. play a significant role in genetic research. They possess unique characteristics which allow scientific findings to be translated and connected across other disciplines on different biological scales. Most of their value is attributed to their

unique life cycle. Almost all species of daphnids are classified as cyclical parthenogens, meaning they can reproduce sexually and asexually (Figure 1.6). In natural circumstances, environmental stress factors influence which form of reproduction takes place, predation and competition for resources are examples of factors that trigger sexual reproduction. Therefore, in controlled laboratory conditions, Daphnia can be manipulated to solely produce clonal females, this allows for, a constant genetic background. This is an integral concept as by maintaining a constant genotype, it allows the comparison of various treatments during genetic experiments (Shaw et al., 2008). Moreover, daphnids inhabit a vast array of aquatic environments, ranging from oligotrophic to eutrophic waterbodies, hypersaline and freshwater systems and from coastal areas to high alpine lakes.

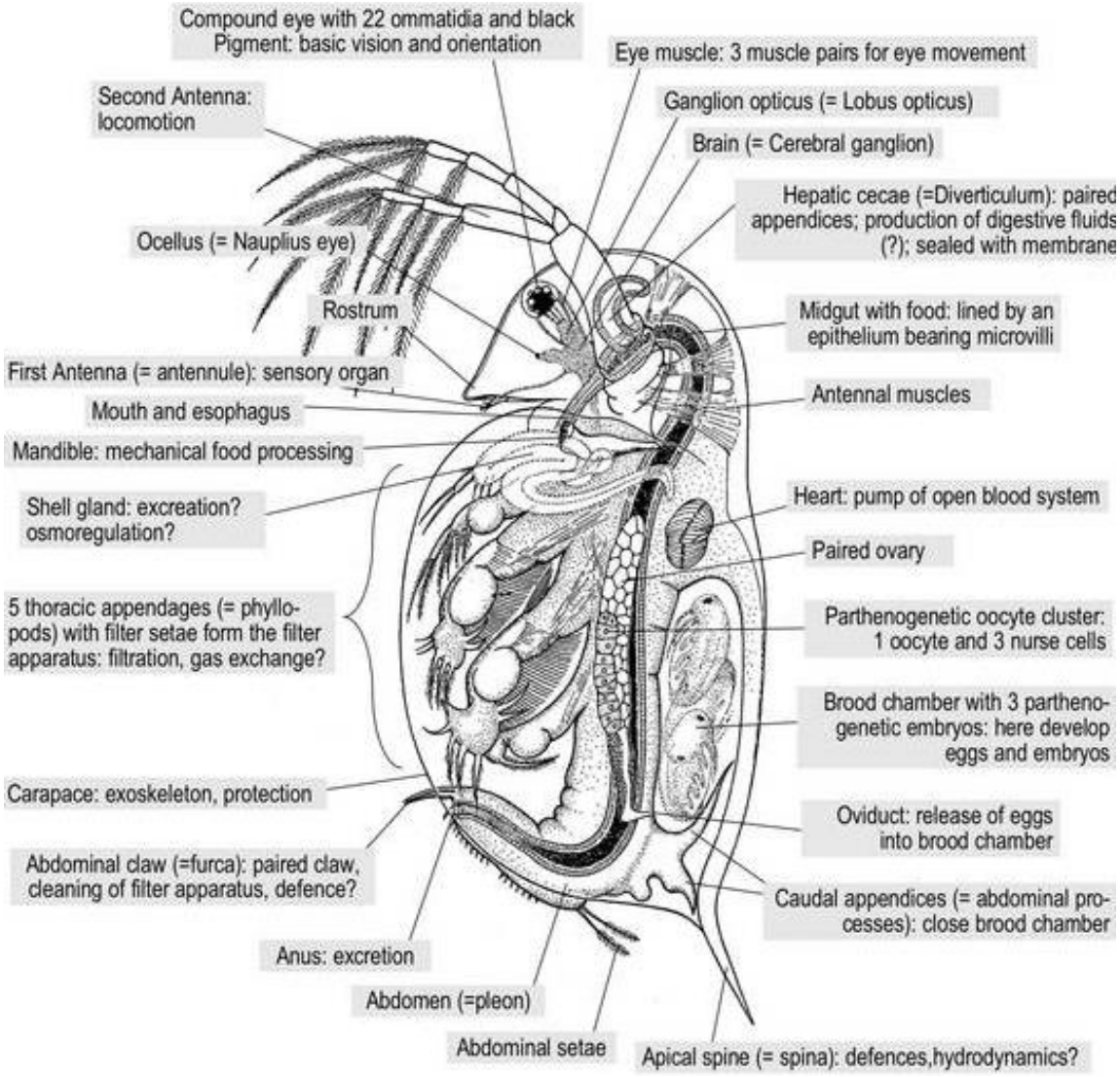

Figure 1.4. Anatomy and morphology of *Daphnia magna*. Figure adopted from (Ebert, 2005).

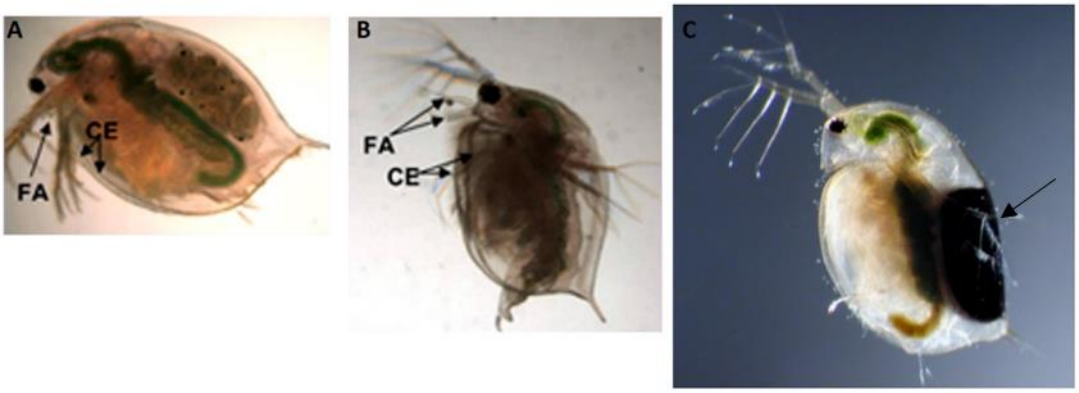

Figure 1.5. Morphological characteristics of daphnids. (A) Female and (B) male *Daphnia magna* (C) Ephippium formed in the dorsal area of female daphnid

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). Both CEs of the male are asymmetrical and are edged by setae. (C) Ephippia or resting egg case at the back of *Daphnia magna*. Figure adopted from (Ebert, 2005).

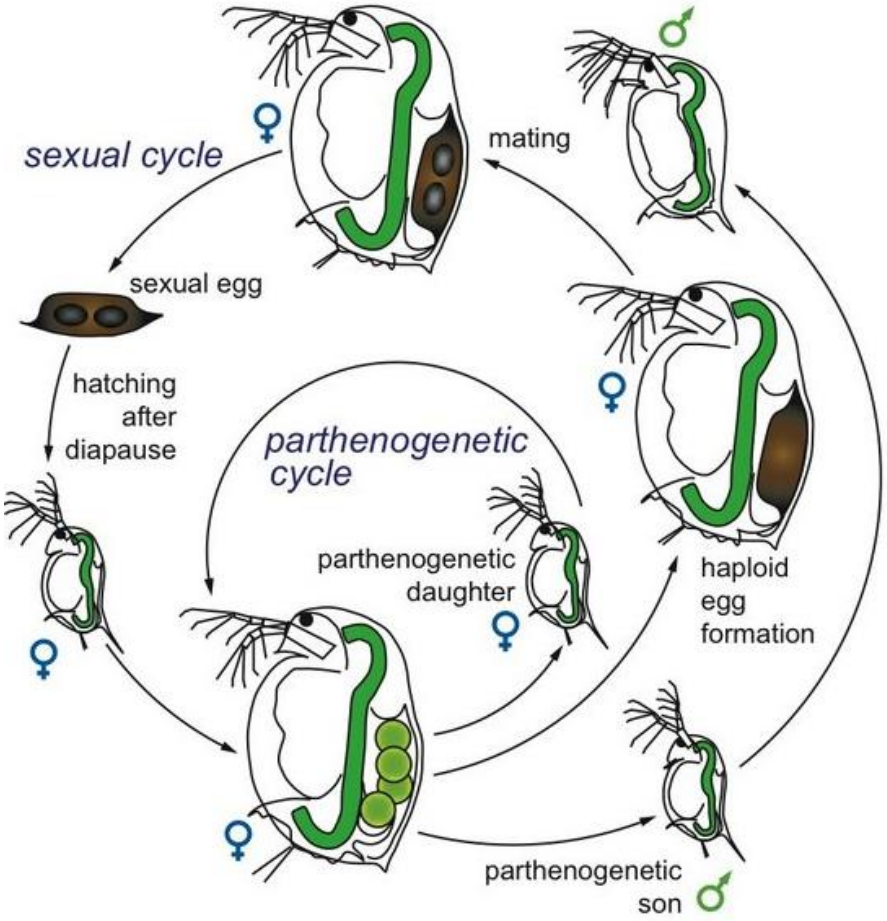

Figure 1.6. The life cycle of *Daphnia magna* (Ebert, 2005).

1.5 The effects of pharmaceutical compounds on daphnids

Active pharmaceuticals ingredients (APIs) and their metabolites eventuate in the aquatic ecosystem through several pathways and have been detected in surface waters around the world (Wilkinson et al., 2022). Ubiquitous and biologically active in nature, APIs can pose deleterious effects on non-target organisms even at low concentrations (Horký et al., 2021; Kidd et al., 2007). Traditional methods of water are based on the quantification of chemicals in a passive or grab sample, which does not accurately reflect an ecosystem's status and provides no biological data, thus there is a relatively low amount of publicly available data for APIs and their respective non-target effects. Since novel methods of water monitoring have been adopted, there has been an obvious increase in reported chemical effects on aquatic organisms. Effects

vary depending on individual chemical but can affect phenotypic endpoints such as size, reproduction and swimming behaviour and molecular endpoints.

1.5.1 Antibiotics

Although antibiotics are well-researched, information regarding their impact on ecological systems and their biological effect of aquatic organisms remains insufficient and sporadic (Richardson & Kimura, 2020; Semerjian et al., 2018). It is now widely accepted that the continuous influx of pharmaceuticals including antibiotics into surface and ground waters is a cause for concern, in particular for the exacerbation of antibiotic resistance (Akiba et al., 2016; Tello et al., 2012). In recent years, the impact of antibiotics on multiple endpoints of ecotoxicological models has been investigated (Nicolussi et al., 2022; Petersen et al., 2021), and have alluded to the potential harmful impact they pose to non-target organisms (Kümmerer, 2009; Q. Yang et al., 2021). Antibiotics have been reported to affect phenotypic endpoints such as swimming behaviour, in *Daphnia* (Bownik et al., 2019) and in higher organisms like zebrafish (Petersen et al., 2021). Moreover, exposure to norfloxacin has been reported to affect several other phenotypic traits, including feeding efficiency, heart rate and quiescence (Pan et al., 2017). The molecular effect of antibiotics on daphnids is not sufficiently understood. Although, chronic exposure to ciprofloxacin resulted in decreased lipid peroxidation and GST activity, while increased genetic damage was recorded (Nunes et al., 2018). However, the impact of antibiotic exposure on the gut microbiome and how it influences host fitness has been studied to a greater extent. Studies have reported decreased microbe abundance, lower diversity, and alterations in feeding and digestion following trimethoprim exposure (Gorokhova et al., 2015). Chronic exposure to ciprofloxacin also resulted in reduced microflora diversity, however in contrast to trimethoprim it demonstrated a stimulatory effect on growth and fecundity (Motiei et al., 2020). *Daphnia* magna which were chronically exposed to concentrations of erythromycin which greatly surpass those found in the environment (6-100µg/L) resulted in no detectable differences of survivorship, fecundity, sex ratio and morphology were recorded (Flaherty & Dodson, 2005; Meinertz & Schreier, Theresa M, Bernardy, 2011). However, when *D. magna* was exposed to a mixture which included erythromycin and other antibiotics trimethoprim and triclosan (30 µg/L) a significant measurable change in sex ratio was recorded. Moreover, when acutely exposed to 0.25 mg/L of trimethoprim, *D. magna* illustrated several changes in

phenotypic and genomic endpoints. After 24 hours exposure, the microbiome of daphnids had shifted significantly, reduced feeding and digestion were also recorded. In addition, there was a 21-fold decrease in 16S rRNA genes (Gorokhova et al., 2015b). Although it should be noted at lower concentrations e.g. 10 µg/L, trimethoprim or sulfamethoxazole exerted no significant effects on morphology, fecundity, ephippium formation, and sex ratio of *D. magna* (Flaherty & Dodson, 2005). Furthermore, when environmentally relevant concentrations of sulfamethoxazole (0.8-80 µg/L) were chronically exposed to D. magna over 6 generations, no apparent effects on reproduction or growth were observed (XiaoFan et al., 2013). There is a relatively low amount of literature regarding amoxicillin and its sub-lethal effects on aquatic organisms, specifically *Daphnia.* However, there is some research regarding amoxicillin's effect on biochemical endpoints of the bivalves *Ruditapes philippinarum* and the *Mytilus galloprovincialis.* Exposure to amoxicillin at concentrations of 100-400 µg/L for 1-7 days only elicited to slight changes in biochemical endpoints, namely; GST, CAT, LPO and PCC (Matozzo et al., 2016). An important factor of ecotoxicology research is the assessment of interactions between multiple chemicals and their combined synergistic impact on organisms. The "cocktail effect" of chemicals, which has been overlooked in the past, reflects realistic environmental conditions and lends itself to accurate assessment of water toxicology. The synergistic impact of phenicol antibiotic mixtures combined with suspended particles enhanced acute toxicity and oxidative stress responses in *Daphnia magna* (Yuxuan Zhang et al., 2021).

1.5.2 Oestrogens

Oestrogens have been widely detected in the aquatic ecosystem within the ng/L range, their apparent increased occurrence can be attributed to their broad application within healthcare and agricultural industries (Adeel et al., 2017). Natural and synthetic oestrogens are widely recognised as EDCs and there is growing evidence suggesting their potential threat to wildlife (Wojnarowski et al., 2021). As a result of extortionate discharge volumes of natural and synthetic oestrogens by humans and livestock, oestrogens are ubiquitously detected in the environment. Over 30'000 kg of natural oestrogens are excreted annually by humans, additionally 700 kg of EE2 (synthetic) is also excreted by humans per year. However, the release of oestrogens into the environment from livestock is considered the main source of oestrogen pollution currently, the US and EU national herds combined contribute an average of 83'000 kg

of oestrogens per year. This issue is exacerbated by land-management practices such as slurry spreading or the application of marinure as fertilizer on agricultural land. If these practices are followed by periods of high precipitation, surface water run off can occur, and surrounding surface waters can become contaminated. Furthermore, oestrogens can eventuate into groundwater through leachate derived from landfills, oestrone has been detected at 68.1 ng/L in groundwater (Kjaer et al., 2007). Depending on oestrogen type and metabolization, oestrogens half-lives can differ significantly, resulting in some compounds being very persistent in aqueous environments. Naturally occurring oestrogens excreted by humans and livestock generally have short half-lives in aquatic environments as they are characteristically hydrophobic, whereas synthetic oestrogens e.g., EE2 are considerably more persistent in nature. Furthermore, the oxidative state of the environment in which the oestrogens eventuate has a direct role in the degradation of these compounds, and anaerobic environments tend to substantially increase a compounds half-life (Ying et al., 2003). Through different mechanisms, such as feminization, disruption of normal physiological function and status, and disturbances in processes related to reproduction, oestrogens have been documented to negatively affect non-target organisms (Wojnarowski et al., 2021). Most notably, is the evidence of male fish feminization following oestrogen exposure (Seki et al., 2005), although there are also reports of oestrogens affecting several aquatic organisms, including *Hydra vulgaris, Gammarus pulex, Chironarus riparius, Hyalella Azteca,* and *Lymnaea stagnalis* (Segner et al., 2003). For many arthropods, hormones control behavioural and physiological processes such as moulting, movement, and development (Ishimoto & Kitamoto, 2010). It is suspected that vertebrate hormones are analogues of these arthropod hormones and via mimicking, oestrogens can cause biological disruption (Kashian & Dodson, 2004a). As a result of structural similarities of vertebrate oestrogens and the steroid ecdysone, it is reported that oestrogens and other xenobiotics exerting oestrogenicity can influence moulting in arthropods, including daphnids (Zou & Fingerman, 1997). Other reported phenotypic effects of oestrogens in daphnids include reduced moulting and fecundity when exposed to diethylstilbestrol (Baldwin et al., 1995; Kashian & Dodson, 2004a). However, this may not be true for every oestrogen as studies using β-estradiol do not support the same findings (Kashian & Dodson, 2004a). The most potent oestrogen, 17b-estradiol has been reported to cause transcriptomic alterations in *D. magna* at environmentally relevant

concentrations (10, 100 ng/L) when exposed for 3-12 hours. Upregulation and down regulation of genes were recorded (251-17’221 and 505-10’282 respectively), with immune response and cancer pathways being those most enriched (Zheng et al., 2020). Phenotypic alterations namely reduced heart rate, ephippia production and lethargy have also been observed. Moreover, exposure to ethinylestradiol, a component of the oral pill, resulted in reduced heart rate, ephippia production and immobilisation in *D. magna* after 72 hours of exposure (0.3 mg/L) (Walker et al., 1998).

1.5.3 Environmentally relevant pharmaceuticals

Carbamazepine is the most consumed anti-convulsant globally. Attributed to its consumption rates, persistence and low removal efficiency from WWPs it is also the most highly detected anti-convulsant in the environment. Carbamazepine has been reported to evoke a phenotypic, behavioural and molecular responses in invertebrates and vertebrates (Qiang et al., 2016). Exposure to 5-100 µg/L of carbamazepine, a decline in acetylcholinesterase activity was observed which highlights the potential neurotoxicity of carbamazepine. Similarly, inhibition of multiple enzymes of detoxification were recorded, namely, superoxide dismutase, catalase and glutathione reductase. In addition to biochemical alterations, phenotypic variations of feeding behaviour, digestion and ingestion also occurred (Nkoom, Lu, Liu, Yang, et al., 2019a). Availability of evidence for gabapentin’s sub-lethal effect on aquatic organisms specifically for cladocerans, with most studies using fish as their model. The exposure of the embryo of *Danio rerio* (zebra fish) to gabapentin at environmentally relevant concentrations (0.1 / 10 µg/L) evoked obvious changes within the transcriptome, and in some instance approximately 700 genes were differently expressed (He et al., 2019). It is widely accepted that non-steroidal anti-inflammatory drugs (NSAIDs) are the most highly consumed pharmaceuticals of all. Although NSAIDs are a significantly important class of pharmaceuticals, specific members of its class namely diclofenac are known to be emerging contaminants of concern. It is estimated that approximately 1443±58 tons of diclofenac is consumed annually (Acuña et al., 2015) and has been detected in multiple environmental matrices. *D. magna* chronically exposed to diclofenac (50 µg/L) elicited several changes to phenotypic endpoints such as delayed brooding, size and life history parameters (Y. Liu et al., 2017a). The blood lipid regulator, gemfibrozil has limited available information regarding its toxicity towards aquatic biota, existing research elicits mixed reviews. Some studies have shown that

at environmentally relevant concentrations, gemfibrozil actually had a positive impact on phenotypic endpoints, specifically size and neonate production (Steinkey et al., 2018). Whereas (Zurita et al., 2007) report *D. magna's* sensitivity to gemfibrozil during immobilisation tests. As a result of the global diabetes epidemic coupled with the fact metformin is currently the only treatment available for diabetes type 2, metformin has become one of the most consumed pharmaceuticals. Metformin is ubiquitous and persistent in nature as it is excreted unmetabolized from the body, for this reason metformin has been detected in raw sewage effluent at levels two times the order of magnitude compared to other pharmaceuticals. In an investigation of metformin's effect on multiple invertebrates and vertebrates, cladocerans *D. similis* proved to be the most sensitive (Godoy et al., 2018). There has been limited investigation into metformin's potential effect on aquatic biota. However, there is substantial evidence which illustrates metformin's oestrogenicity potential in fish models, specifically *Pimephales promelas* (fathead minnow). Research has indicated metformin's endocrine disrupting capabilities and its responsibility for intersex fish globally (Niemuth & Klaper, 2015).

1.6 Phenotypic and biochemical endpoints to identify the underlying toxicity mechanisms of pharmaceuticals in daphnids

Phenotypic endpoints such as heart rate, body size, and fecundity provide surrogate measurement of physiology and provide a rapid insight to general animal status. Phenotypic endpoints are extremely useful, as they are generally inexpensive to assess, yet still give genuine insight to the effect's pharmaceuticals pose on model species. Additionally, biomolecular markers are also useful endpoints of molecular physiology. Such examples are the measurement of biochemical endpoints namely, the activity of antioxidant enzymes catalase, glutathione-S-transferases, glutathione reductase, and lipid peroxidation. Moreover, biomolecular markers possess the advantage of being a relatively inexpensive and time efficient method of determining working concentrations of pharmaceuticals for future experiments. This proves beneficial as no effect concentrations and sub-lethal concentrations can be ascertained before using more expensive and specialised techniques such as omic (metabolomic, transcriptomic and proteomic) techniques are employed.

1.7 Environmental omics in risk assessment

In recent years there has been a shift from conventional methods of water monitoring, which typically involve chromatography techniques coupled with mass spectrometry to the implementation of Effect Based Methods (EBMs). The fundamental basis of EBMs is to measure the responses of sentinel species to an individual chemical or chemical mixture. The advantage of this approach is that it allows the timely prediction of pollution-hotspots in the environment before irreversible damage occurs (Brack et al., 2019; Könemann et al., 2018; Kruger et al., 2022). These enhanced modern practices are also referred to as “New Approach Methodologies” (NAMs), which describes the use of *in chemico, in vitro* and *in silico* approaches to reveal mechanisms of toxicity of chemical substances. When used in combination with analytical methods and novel tools, NAMs can assist in chemical risk assessment and safety regulation (Punt et al., 2020). The objective of toxicology is to understand the effect single or chemicals mixtures pose on an organism or a whole biosystem, omic techniques integrate classical models of toxicology with novel techniques to enhance the understanding of an organism’s response to chemical exposure and its pathway of toxicity at a molecular level (Thomas et al., 2002). Adverse Outcome Pathways (AOPs) more specifically can determine pathways of toxicity and use this information for the timely prediction of pollution in the environment. AOPs can be described as a “biological map” that determine the Molecular Initiated Events (MIEs) and Key Events (KEs) that occur to produce a toxic effect (adverse outcome) in an organism (J. Jeong & Choi, 2017). These adverse outcomes generally occur as a result of a series of changes in the organism usually at the molecular or biochemical level through subcellular, cellular tissue organ and individual levels. AOPs can be utilised in freshwater risk assessment as if a MIE for a toxicity pathway is identified, the MIE itself as opposed to the final AO can aid as an early warning sign of pollution. Therefore, an early warning system composed of an integrated framework of many identified AOPs could detect the sublethal effects caused by low concentrations of pollutants before a water source is subjected to serious or irreversible damage. The term “omics” refers to a suite of holistic approaches namely, (epi)genomics, transcriptomics, proteomics and metabolomics (Figure 1.7). Omic techniques allow the identification of molecular signatures of surrogate species, in response to chemical mixtures, to replace traditional animal testing models with evolutionarily diverse organisms on the tree of life which humans are phylogenetically related to (Colbourne et al., 2015). These

modern techniques of toxicology monitoring can be differentiated from their traditional method counterparts in that they provide information of underling mechanisms and pathways of toxicity (Fröhlich, 2017). Classical toxicology models separate chemicals according to their pharmacokinetic and pharmacodynamic processes and is generally a descriptive observation rather than mechanistic. Unlike outdated techniques which solely identify toxicity through phenotypic observation, omic approaches give a comprehensive overview of minute cellular responses to environmental stressors at low level toxicity (Fröhlich, 2017). It is important to note that although traditional descriptive methods to toxicology are lacking alone, it does provide a valuable framework when paired with omic techniques.

Genomics is the study of an organism's genome which is an organism's complete set of genetic instructions. Merging a combination of DNA, DNA sequencing and bioinformatics, genomics allows genetic material to be sequenced, assembled and its structure and functions examined (Spudich, n.d.). Contrary to conventional techniques, genomics acknowledges an organism's full complement of DNA as opposed to a single gene or gene product. Genomics play two distinct roles in environmental toxicology, firstly it can identify certain individuals as more sensitive to specific chemical toxicity by examining its genetic sequence, secondly it can flag any changes in the genotype as a result of chemical exposure (Thomas et al., 2002). By definition, proteomics is "the high throughput separation, display and identification of proteins" (Kennedy, 2002). In recent years, proteomics, specifically toxicoproteomics has emerged as an effective tool for chemical pathway identification and biomarkers which provide a risk assessment framework for premature detection of chemical exposure (Wilson-Frank, 2019). This is achieved through proteomics' unique speed and sensitivity in toxicology screening. The concept of proteomics is based upon the separation of proteins from complex matrices by mostly gel electrophoresis, namely 1-D, 2-D and 2-DGE, coupled with a mass spectrometry technique to identify the proteins. This advancement in technology has allowed researchers to identify quantitative and qualitative changes in several sample types such as plasma, blood and organs (kidney, liver and brain) (Wilson-Frank, 2019). Metabolomics is the study of lower molecular weight compounds (metabolites) which participate in metabolic reactions required for growth, maintenance and general function within cells, biofluids, tissues and organisms. Metabolomics has revolutionised biochemical research in the sense that it can analyse the global metabolome (opposed to selective metabolomes),

identify unknown effects of chemicals, and identifies targets of importance. Like the other omics techniques, metabolomics facilitates toxicology research by identifying alterations of the metabolome and toxicity pathways, leading to the discovery of biomarkers which are necessary as early detection systems of toxicity as well as assessing therapeutics effectiveness. Metabolomics possesses several advantages, firstly, large volumes of sample can be acquired non -invasively and quickly e.g., urine and blood. Secondly, large quantities of data can be acquired for large volumes of metabolites through a single measurement.

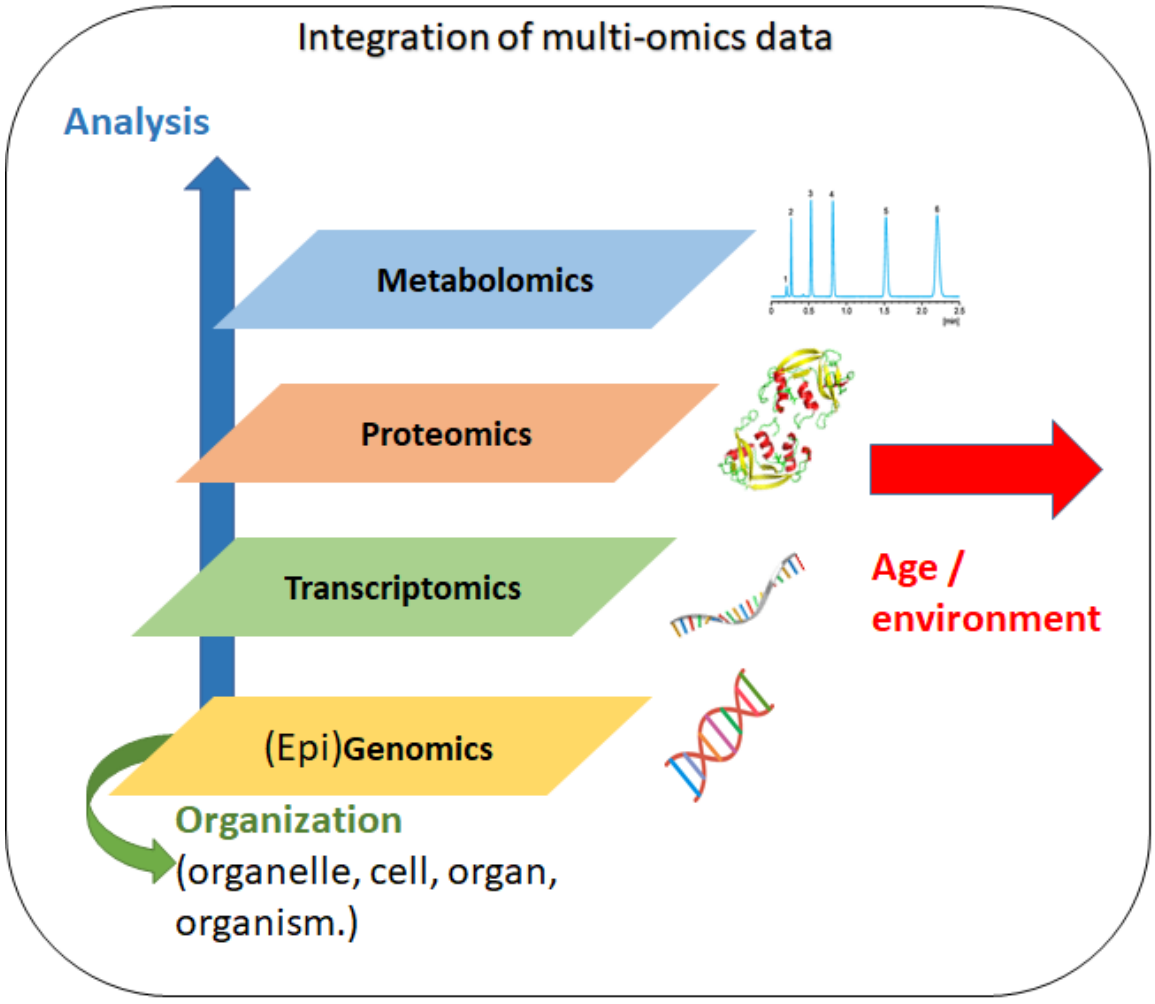

Figure 1.7. Integration of multi-omics data. Advanced high-throughput technologies have made it possible to assess effectively all molecular alterations across virtually all levels of an organism. Omics techniques enable the "simultaneous quantification" of the genome, transcriptome, proteome and the metabolome, and their integration provides a more comprehensive snapshot of the potential effects of chemicals on model organisms (Ebner, 2021).

1.8 Thesis outline

This chapter introduces the reader to the concept and background of this thesis in relevance to freshwater ecotoxicology, risk assessment, biology of daphnids, environmentally relevant pharmaceuticals and their effects on freshwater organisms, and the phenotypic, biochemical and omic techniques employed to quantify such effects. To assess the responses of daphnids to pharmaceuticals, several representative pharmaceuticals from different categories were selected. Chapter 2 focused on oestrogen hormones; oestrone, β-estradiol and ethinylestradiol, which were investigated for their acute and chronic effect on both phenotypic and biochemical endpoints. Molecular endpoints such as activity of antioxidant enzymes (glutathione-S-transferases)and biochemical markers related to physiology (alkaline and acid phosphatase, β-galactosidase, lipase) were combined with phenotypic endpoints such as growth (size), and fecundity. These findings were combined with metabolomic experiments in acute and chronic scenarios to identify metabolic perturbations related to the action of oestrogens. Similarly, in chapter 3, a range of antibiotics varying in mode of action i.e amoxicillin, trimethoprim, erythromycin and sulfamethoxazole were investigated for their potential acute, chronic and transgenerational toxicity to daphnids using the aforementioned biochemical assays and metabolomics. In Chapter 4 investigated the physiological and metabolic effects of a series of environmentally relevant drugs, commonly detected in the environment and specifically, diclofenac, metformin, gabapentin, carbamazepine and gemfibrozil are investigated. The cumulative results and findings derived from the individual chapters guided the selection of a composite pharmaceutical mixture which was investigated in chapter 5. Combining phenotypic endpoints, biochemical assays and metabolomics in a series of acute, chronic and transgenerational exposures, the responses of the “pharmaceutical cocktail” was studied in laboratory conditions. Finally, the mixture was then “spiked” in environmental samples collected from rivers in the area of Dublin, and the impact on daphnids was compared for exposed to spiked and unspiked lab media and the river waters used.

1.9 References

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Chapter 2

The molecular and phenotypic impact of oestrogens on daphnids

Abstract

It is widely accepted that endocrine-disrupting compounds such as natural and synthetic oestrogens can impede the growth and development of non-target organisms, however, their interaction with the endocrine system of invertebrates is poorly understood. Attributed to their widespread applications within human and veterinary medicine, oestrogens frequently enter the freshwater system via human and livestock excretions and through the wastewater effluent. Their increased detection in environmental matrices, coupled with their potential for biomagnification and bioaccumulation has exasperated the requirement to assess their toxicity potential. The present study evaluated the acute and chronic effects of the oestrogens namely; Oestrone (E1), β-estradiol (E2) and ethinylestradiol (EE2), as well as their combined mixture on the cladoceran *Daphnia magna.* By combining biochemical and phenotypic endpoints, as well as a targeted metabolomic analysis, their toxicity was investigated. *Daphnia* enzyme activity and metabolism proved more sensitive to oestrone and beta-estradiol exposure within the acute scenario, with particular impact on βGAL, PEP and LDH. The acute metabolomics study revealed the dysregulation of many amines and nucleobases, specifically adenosine, cadaverine and acetylcarnitine. Chronic exposures showed ethinylestradiol and the oestrogen mixture to be responsible for the most alteration of enzyme activities, metabolic impairment and of protein and size growth rate.

2.1 Introduction

This chapter focuses on the collection of molecular signatures of daphnids following a period of exposure to an individual or mixture of oestrogen compounds (Figure 2.1), specifically phenotypic and biochemical markers, combined with metabolomic analyses. The selection of these oestrogens is owed to their recognition as emerging contaminants, stemming from reports identifying their endocrine disrupting capabilities and molecular alterations in non-target organisms. Their inclusion on the primary surface water watchlist (WFD, EU Directive 2000/60/EC) also exacerbates the importance of assessing their impact in the context of ecotoxicology. Additionally, E1 is the primary circulating hormone in post-menopausal women, E2 is the most potent oestrogen and is highly abundant in menstruating women, and has many therapeutic applications in hormone replacement therapy, furthermore, E2 and EE2 are common components of the contraceptive pill which has been increasingly used in recent decades. Hence the above, E1, E2, and EE2 are the most highly detected oestrogens in environmental matrices, emphasising the requirement to assess their potential effects on aquatic biota. (Ciślak et al., 2023)

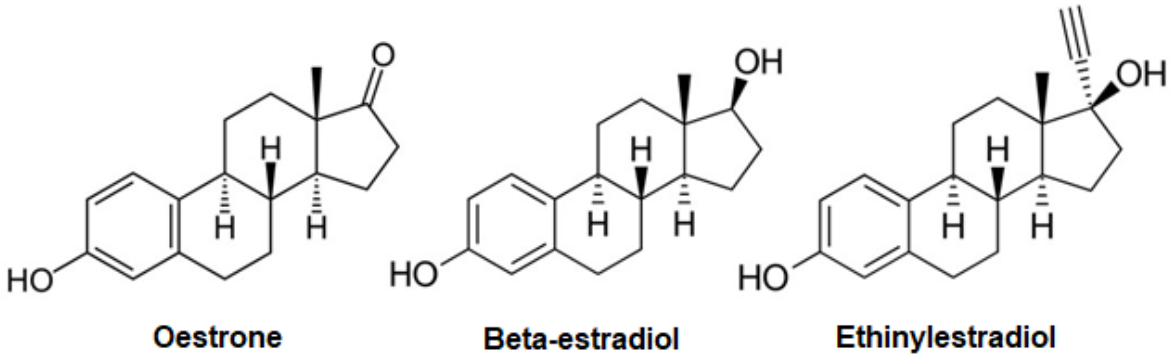

Figure 2.1. The oestrogenic compounds used in this chapter.

2.2 Materials and methods

2.2.1 Algae and daphnids culturing

The primary food for daphnids is algae (*Chlamydomonas rheinhartii*) which was cultured in a modified version of Sueoka (CGM) media (Schade et al., 2019) for details refer to the appendix). Algae were cultured in individual glass flasks under constant illumination and stirring at approximately 200 rpm on Thermo Scientific™ RT Basic Series Magnetic Stirrers, at room temperature. Algae were pelleted and re-suspended in ddH_2O to a 7 A suspension at 440 nm. Additional food supplements were added to

the cultures and specifically, a yeast stock (100 mg/L ddH_2O) was added daily and a seaweed extract (*Ascophyllum nodossum*, prepared in ddH_2O at an 8 A at 400 nm) was added at media renewal every five days (for details refer to the appendix). Daphnids were cultured in conformity with OECD guidelines in 4 liters 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 16h:8h of light: dark photoperiod at 20˚C and a density of 80 adults per 4 liters of media with media renew bi-weekly. 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).

2.2.2 Acute toxicity exposure

For toxicity curves 15 animals (4 days old) per replicate and per concentration were exposed in 100 ml OECD media for 24 hours (Figure 2.2). A minimum of 4 replicates were prepared for each concentration. Toxicity was assessed as immobilisation of animals, and toxicity curves were plotted, and EC values were calculated using the GraphPad Prism software package. Specifically, the four-parameter logistic model (4PL) was used, following the equation Span = Top – Bottom and Y = Bottom; + (Top – Bottom) / (1+10^ ((LogIC50 – X) X Hillslope)), available on the GraphPad software. The Top and Bottom parameters were fixed to 100 and 0 respectively. Animals were collected for biochemical measurements and metabolomics. Acute experiments, for biochemical and metabolomic endpoints were set up following the same design. On day 4, daphnids were exposed to chemical for 24 or 48 hours and then the required endpoints were measured.

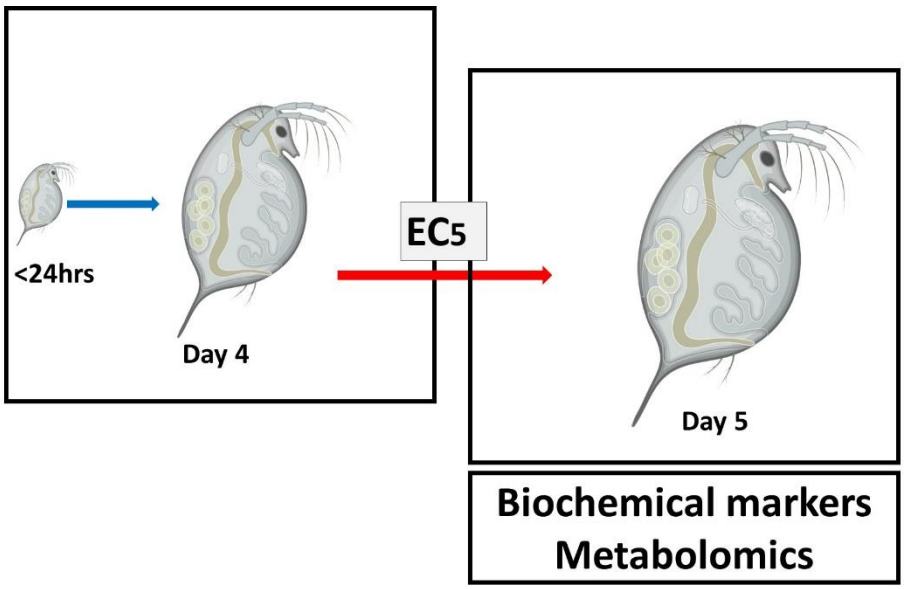

Figure 2.2. Basic experimental design of acute toxicity exposures.

2.2.3 Chronic toxicity exposures

Forty-five neonates (<24 hours) were cultured for 21 days in 1800 ml OECD media. Daphnids were fed daily with algae (5.5 ml) and a supplement of seaweed extract (2.7 ml) (Figure 2.3) at the renewal of media every three days. Chemicals were also refreshed with media change for the duration of the experiment. Newly released neonates were removed daily by filtration. The endpoints of growth (size and protein) were measured for 21 days at 7-day intervals and on the first day (neonates) as a baseline. Daphnids from each condition were collected on day 21 and 3 daphnids per replicate were snap-frozen for metabolomics and homogenized for biochemical assays.

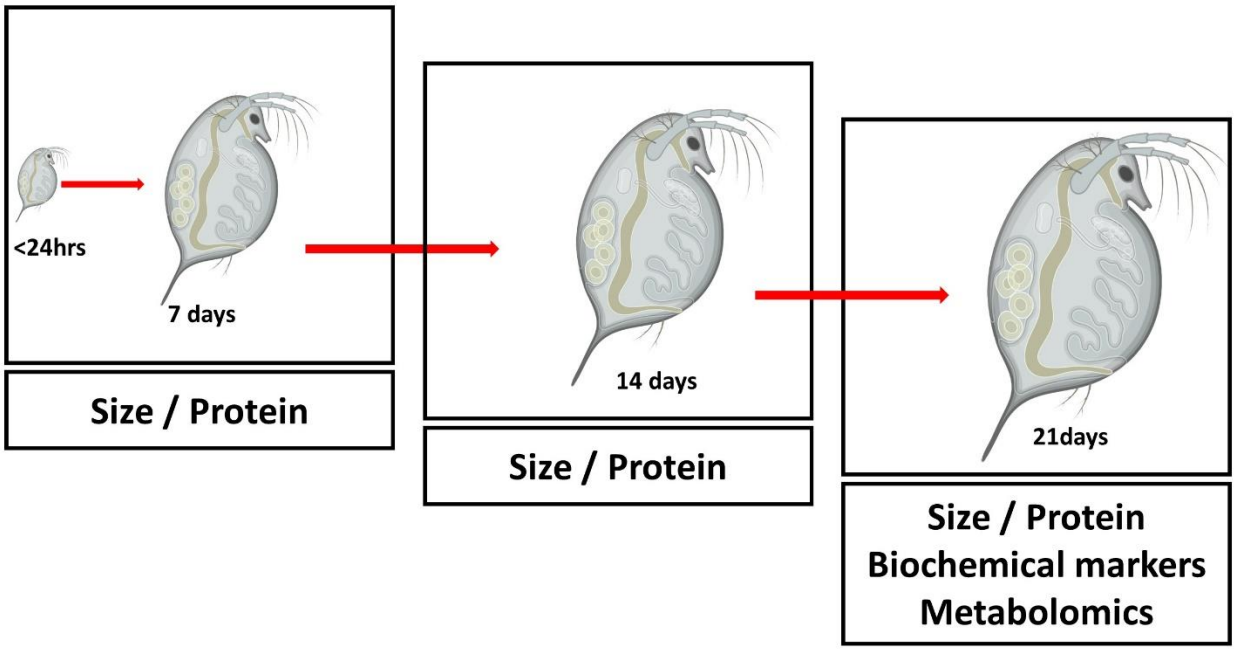

Figure 2.3. Experimental design of chronic exposure.

2.2.4 Phenotypic endpoints

Animals were collected for size and protein measurements to assess their growth every seven days. Body length was observed using the Image J software, by measuring the length from the top of the eye to the base of the caudal spine (Figure 2.4). Growth rates for size measurements were calculated from the equation: GR=[ln(size/protein) t-ln(size/protein)1]/t (DeMott et al., 1998)(Chopelet et al., 2008)

Figure 2.4. Body length (yellow line) was measured using the ImageJ software.1

2.2.5 Reproduction test

Fecundity was assessed by recording the number of neonates released per individual over the course of 21 days. One daphnid per replicate was cultured in 40 ml exposure media. The exposures were set up following the previous chronic protocol, as a scaled down version.

2.2.6 Sample homogenization for biochemical analysis

Samples were homogenized in ddH_2O or relative buffer solution using an Eppendorf homogenizer pestle. To collect clear supernatant for biochemical assays, homogenized samples were centrifuged at 6,000 *g* for 5 minutes.

2.2.7 Protein quantification

Protein was quantified using an ultrasensitive method based on the electrostatic reaction of proteins with the Coomassie Brilliant Blue (CBB) G-250 reagent (Georgiou et al., 2008) followed by a rapid and sensitive microplate method, total protein was quantified. CBB was prepared in 2M HCl (60 mg CBB in 100 ml 2M HCl, filtered to remove undissolved dye particulates) and diluted 2-fold with 2M HCl prior to use. BSA standards (2-20 µg/ml) were prepared in ddH_2O. The unknown samples and standards were assayed for protein concentration by mixing 200 µl sample/standard with 50 µl CBB:2M HCl. Absorbance was measured against reagent blanks (containing water instead of sample/standard) at 610 nm and the net absorbance of samples was converted to protein concentration equivalents using the corresponding standard curve.

2.2.8 Biochemical markers of oxidative stress and physiology

Activity of glutathione-S-transferases was measured by continuous kinetics of the product formed (S-DNP-GS) measured at 340 nm in 50 mM phosphate buffer pH 7.2 (Mannervik & Guthenberg, 1981) (Tang et al., 1996). Activities of alkaline and acid phosphatases were assessed by the release of *p*-nitrophenol using *p*-nitrophenol phosphate as substrate. Specifically, 200 µl appropriately diluted sample in buffer (100 mM boric acid pH 9.8 for alkaline phosphatase or 100 mM citric acid pH 4.5 for acid phosphatase) was mixed with 50 µl *p*-nitrophenyl phosphate in buffer (3 mg / ml buffer). The activity of β-galactosidase was quantified by the release of *o*-nitrophenol using *o*-nitrophenyl-β-galactosidase as substrate. The activity of the enzyme lipase was measured by the conversion of *p*-nitrophenyl butyrate (pNPB) to *p*-nitrophenol. Samples were appropriately diluted in phosphate buffer and pNPB was diluted in DMSO. Aminopeptidase activity was quantified from the hydrolysis of L-Leu-4-nitroanilide and the production of 4-nitroaniline measured at 418 nm. Activity of lactate dehydrogenase was quantified by continuous kinetics of the consumption of NAD(P)H

measured at 340 nm. Acute enzyme activities were expressed as units per individual and chronic enzyme activities were expressed as units per µg protein.

2.2.9 Statistical analysis for biochemical and phenotypic markers

The acute toxicity and biochemical activity data were analysed and plotted using Excel and GraphPad Prism software packages. Statistically significant comparisons were identified by Student's *t*-test.

2.2.10 Extraction of metabolites

Animals were pooled together, snap frozen using liquid nitrogen and stored in Eppendorf tubes at -20˚C until extraction and analysis. For acute experiments, fifteen (Day 4) animals were homogenized in 400 µl 80% HPLC-MS methanol 20% HPLC-MS water using an Eppendorf homogenizer pestle. For chronic experiments, three Day 21 animals were homogenised in 600 µl 80% methanol 20% water. The homogenates were cleared by centrifugation at 14,000 *g* for 5 minutes and the clear supernatant was collected and 100 µl were evaporated using a speedvac. Quality controls (QCs) and blanks were prepared as described in the appendix.

2.2.11 Metabolomic analyses

Metabolomic analysis of samples from acute exposures

A targeted metabolomic analysis of the polar metabolites was performed for the acute samples. This was achieved by a validated hydrophilic interaction liquid chromatography-tandem MS (HILIC LC-MS/MS) method, which is capable of detecting >100 endogenous metabolites (Virgiliou et al., 2015). The samples previously extracted, were reconstituted in 100 µL of 95%-5% acetonitrile-water. A quality control (QC) sample was prepared by pooling the samples extracts and the same procedure was followed. A blank sample without biological tissue following the extraction procedure was also prepared. Chromatographic separation was achieved by an ACQUITY UPLC H-Class system fitted with an Acquity UPLC BEH Amide column (Waters Ltd., Elstree, U.K.). Metabolites were annotated by MS, which was performed on a Xevo TQD mass spectrometer (Waters Corporation, Millford) using the polarity switching mode. The quaternary solvent system comprised of mobile phase (A) acetonitrile−water, 95:5 (v/v) and (B) acetonitrile−water, 30:70 (v/v), and both contained 10 mM ammonium formate. The samples were inserted in random

order sequentially, and a QC sample was analysed every 10 samples to ensure instrument stability and confirm data quality. To validate analyte concentrations, the individual metabolite results within the medium concentration QC were required to be within ±20% of the nominal value, while QC data must possess a CV < 20%. Partial least square discriminant analysis (PLS-DA) was performed using Simca-P v15 software in order to visualise differentiations and correlations between the studied groups. Validation of the constructed models was assessed by taking into account Cross-Validated Analysis of variance (CV ANOVA) p value (<0.05) from the Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) models.

Chronic metabolomics

Dried extracts of daphnids were resuspended in 50 µl 1:1 v/v methanol/water (LC-MS grade) pre-cooled to 4°C, vortexed for 10s, and cleared by centrifugation for 10 min, 16.000 xg at 4°C. The supernatants were transferred to LC-MS vials and directly used for analysis with a randomized injection sequence. Metabolomics was performed as previously described (Kamrad et al., 2020). Briefly, compounds were analyzed using an Agilent 1290 liquid chromatography, hyphenated to an Agilent 6470 triple quadrupole mass spectrometer. Metabolites were separated using hydrophilic interaction liquid chromatography (HILIC) with a Waters BEH amide 2.1x100mm, 1.7µm particle size column with acetonitrile (phase A) and eluted with an aqueous 100 mM ammonium carbonate buffer (phase B). The multi-step gradient started at 30% B (0 - 3 min), and then was then steadily increased to 60% B (3-7 min), kept at 60% B (7-8 min), and then equilibrated to starting conditions (8-10 min). The column compartment was kept at 35°C, the flow rate was 0.3 ml/min, and 1 µl was injected. The mass spectrometer was operating in dynamic MRM mode with polarity switching. Integrated peak areas were obtained using MassHunter Quantitative Analysis software and obtained responses were reported. Metabolites were identified by matching retention time and transitions obtained using analytical standards.

2.2.12 Reagents and chemicals

L-leucine-4-nitro-analide and beta-nicotinamide adenine dinucleotide reduced (NADH) were purchased from Alfa Aesar. Boric acid, citric acid, sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, 1-chloro-2,4,-dinitrobeneze (CDNB), *o*-nitrophenyl-butyrate (ONPG), L-glutathione reduced (GSH), *p*-nitrophenyl-

butyrate (*p*NPB), and bovine serum albumin (BSA) were purchased from Sigma Aldrich. Acetic acid, hydrochloric acid, dimethyl sulfoxide (DMSO) was purchased from ThermoFisher Ireland. Sodium pyruvate, magnesium sulfate heptahydrate, and sodium hydrogen carbonate were purchased from Fisher Scientific. Calcium chloride dihydrate and *p*-nitrophenyl phosphate, disodium salt, hexahydrate (*p*NPP) The ultrapure water was made with Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.3 Results

The acute and chronic effects of oestrogens were explored in a series of biochemical, phenotypic, and metabolic experiments in this chapter.

2.3.1 Acute exposures to oestrogen compounds

Four-days old daphnids were exposed to oestrone, β-estradiol and ethinylestradiol initially at a concentration of 50 mg/L for 24 hours to assess their toxicity potential and to determine the working concentrations. Results indicated that only ethinylestradiol induced mortality and, therefore, it was investigated further to generate a full toxicity curve and EC values were calculated; EC_1 - 3.19 mg/L, EC_5 - 4.79 mg/L, EC_{10} - 5.75 mg/L and EC_{50} - 9.862 mg/L (Figure 2.5).

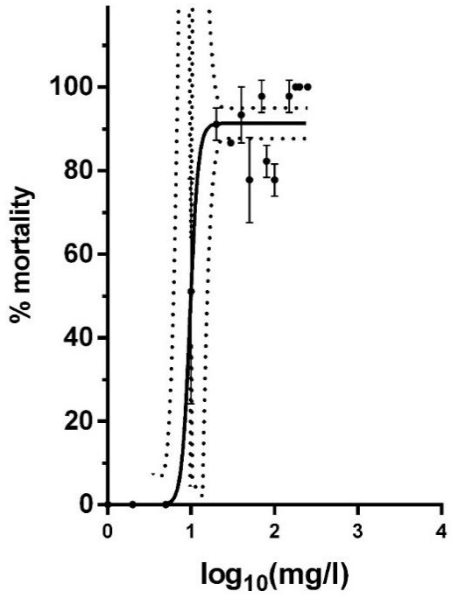

Figure 2.5. Acute toxicity curve for ethinylestradiol (EE2). Fifteen 4-days old daphnids per replicate of each concentration were exposed to EE2 for 24 hours. Data represents average ± SD (N=3).

2.3.1.1 Phenotypic and biochemical markers of physiology

A series of biochemical markers were performed for all oestrogens prior to omic analyses. Biochemical markers are extremely useful, possessing the advantage of being quick and inexpensive to execute. The markers used confirmed physiological alterations within the *Daphnia* in turn indicating which chemicals would be of importance within the omic segment of the project. Daphnids were exposed to each oestrogen compound at a concentration of 1 mg/L. This is a sublethal concentration of all 3 oestrogen compounds, and specifically it is lower than the EC_1 value of ethinylestradiol, which is close to EC_5 and EC_{10} concentration. Following 24 hours exposure, the protein content of daphnids was quantified, and enzyme activities for phosphatases (acid; ACP, and alkaline; ALP), β-galactosidase (β-gal), glutathione-S-transferase (GST), lipase (LIP) aminopeptidase (PEP), and lactate dehydrogenase (LDH) were assessed (Table 2.1). Significant changes in the activity of phosphatases were recorded for several conditions. Consequentially, changes in the activity of ACP were observed for oestrone and ethinylestradiol, decreases of -25.3% and -15.4% were recorded. It should be noted that β-estradiol had a trend to decrease ACP activity also. Regarding ALP activity, DMSO was responsible for an increase of 24.4% whereas β-estradiol generated the opposite effect, leading to a decrease of -18.1%. BGAL activity was impacted by DMSO, oestrone and β-estradiol, the carrier solvent caused a decrease of 14.6%, whereas the oestrogens oestrone and β-estradiol had the opposite effect and caused increases of 16.3% and 17.1% respectively. Twenty-four-hour exposure to oestrogen compounds did not have a significant impact on lipase activity, however peptidase activity was altered by DMSO (17.4%), oestrone (-18.5%), β-estradiol (-40.7%) and ethinylestradiol (-33.3%) respectively. Beta-estradiol was the only oestrogen compound to impact LDH activity (-44.3%) and no statistically significant changes were observed in GST activity. Overall β-estradiol was the oestrogen to impact the enzyme activities of daphnids.

Table 2.1. The acute effect of oestrogens on multiple enzyme markers of *Daphnia magna* (age day 4).

Data represents average ± SD (N=4) replicates for each condition. Statistically significant by Student's t-test compared to control (*) or DMSO ($) (p<0.05). ALP, ACP, BGAL, LIP, PEP, LDH, GR, GST are expressed as milliunits/animal.

Enzyme	Control	DMSO	Oestrone	β-estradiol	Ethinylestradiol
ALP	129±6.5	160±9***** **(24.4%)**	147±15	132±17.4**$** **(-18.1%)**	147±10.4
ACP	44±2.84	44±1.8	33±2.1**$** **(-25.3%)**	36±8.8	37±1.7**$** **(-15.4%)**
BGAL	73±4.45	62±4.3***** **(-14.6%)**	73±2.6**$** **(16.3%)**	73±5.5**$** **(17.1%)**	59±8
LIP	367±21.6	342±49.5	371±33.6	346±61.8	357±36.7
PEP	2.3±0.12	2.7±0.26***** **(17.4%)**	2.2±0.23**$** **(-18.5%)**	1.6±0.25**$** **(-40.7%)**	1.8±0.24**$** **(-33.3%)**
LDH	50±7	55±8	41±11	31±4**$** **(-44.3%)**	56±8
GST	6.6±0.61	6.8±0.52	6.3±0.54	7.3±0.28	7.4±0.7

2.3.1.2 The impact of acute oestrogen exposure on metabolism of daphnids

When compared to the DMSO control, the metabolic profiles of daphnids exposed to the two oestrogens were clearly disrupted. A clear separation between β-estradiol and oestrone samples from the control be seen in PLS-DA (Figure 2.6A) plots. Moreover, a distinct separation of β-estradiol and oestrone exposure samples from each other can also be observed, indicating that each oestrogen posed a unique effect to daphnid metabolism. Models constructed with daphnids exposed to E1 and E2 oestrogens against DMSO had significant acceptable CV ANOVA (Figure 2.6C) values. Regarding ethinylestradiol samples the constructed OPLS-DA model showed low predictability and the subsequent CV ANOVA p value>0.05 thus samples were not assessed further. In total 30 metabolites were found significantly affected in daphnids exposed to E1 and E2 oestrogens compare to control-DMSO. Log2FC heatmap plot (Figure 2.6B) show the compounds that were affected statistical significantly. B-estradiol resulted in increased levels of amino acids and amino acid derivatives (phenylalanine, proline and methionine) with an exception of asparagine, nucleosides (adenosine, inosine and uridine), vitamins (pantothenate and nicotinic acid) purine (hypoxanthine), acetylcarnitine, cadaverine and histamine. Oestrone decreased the levels of most metabolites detected in daphnids including amino acids and amino acid derivatives, nucleosides and nucleobases, purines and others with the exception of acetylcarnitine, adenosine, cadaverine and pantothenate.

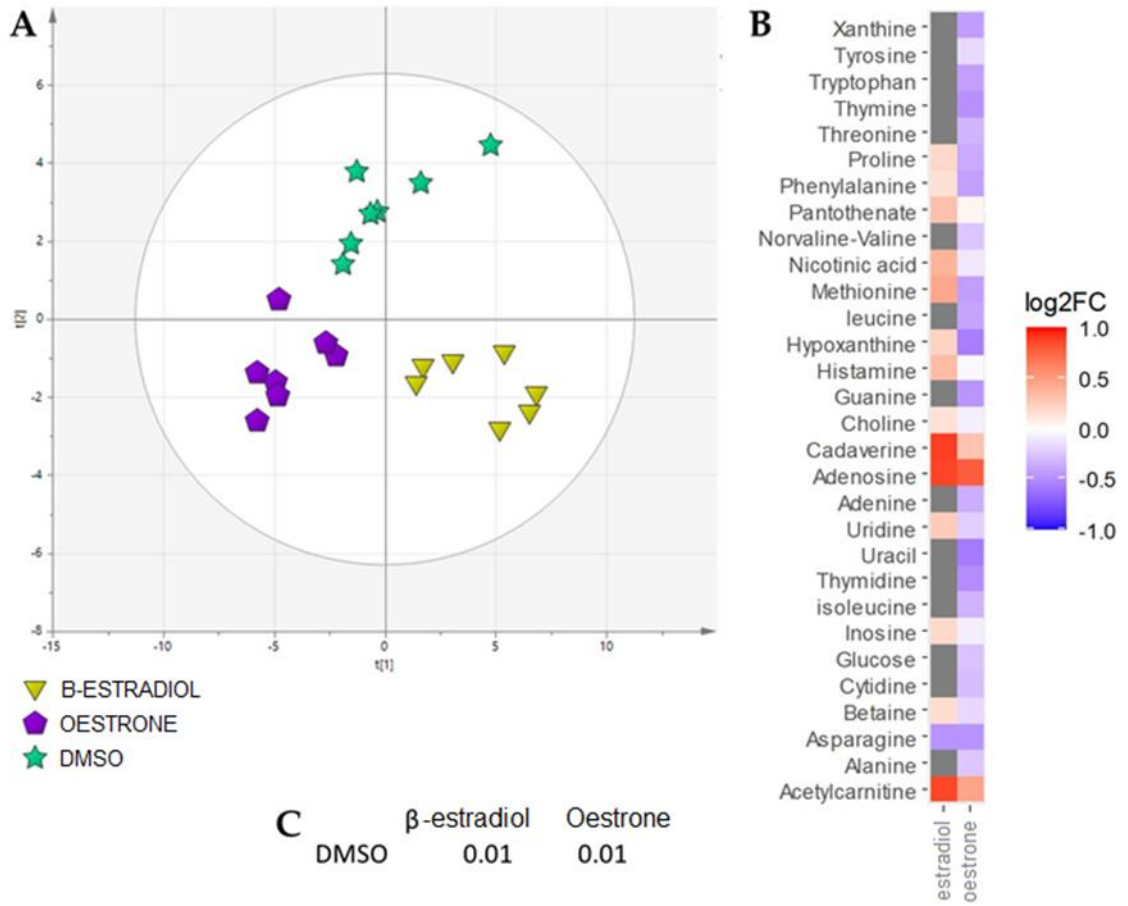

Figure 2.6. Multivariate statistical analysis of the metabolic impact of oestrogens on daphnids. (A) PLS-DA score plots based on data from targeted analysis LC-MS/MS of daphnids extracts exposed to oestrogens versus DMSO. (B) Heatmap of metabolites with significant changes in $\log_2$ fold change. (C) CV ANOVA p value of OPLS-DA models. OPLS-DA score plots based on data from targeted analysis LC-MS/MS extracts of daphnids exposed.

2.3.2 Chronic exposure to oestrogen compounds

To assess the chronic effect of oestrogens on *Daphnia magna*, neonates (<24 hours) were exposed for 21 days to oestrone, β-estradiol, ethinylestradiol and their combined mixture at a sublethal concentration of 1 mg/L. Following exposure, phenotypic endpoints including, size and fecundity and molecular endpoints such as protein content and enzyme activities were measured. Moreover, a targeted analysis of the core metabolites of daphnids was performed.

2.3.2.1 Reproduction

Fecundity of daphnids was assessed as per the OECD test guideline 211. This experiment evaluates the chronic effect a chemical exerts on the reproduction of

daphnids. In preparation for this phenotypic test, a preliminary chronic experiment with different food conditions was performed to optimize the experimental conditions. The objective of this test was to identify which concentration of algae and marinure (a seaweed extract of *Ascophyllum nodosum*, which provides additional nutrition for the daphnids) enabled daphnids to produce the threshold of >60 neonates per individual over a 21-day period, which is effectively the baseline for the validity of this phenotypic test. Conditions containing marinure resulted in a significantly higher reproductive output compared to conditions in the absence of marinure. Specifically, all conditions in the absence of the seaweed extract did not reach the aimed yield of 60 neonates per individual for 21 days. Reproduction efficiency was highest in the condition containing the highest concentrations of algae and marinure (Figure 2.7).

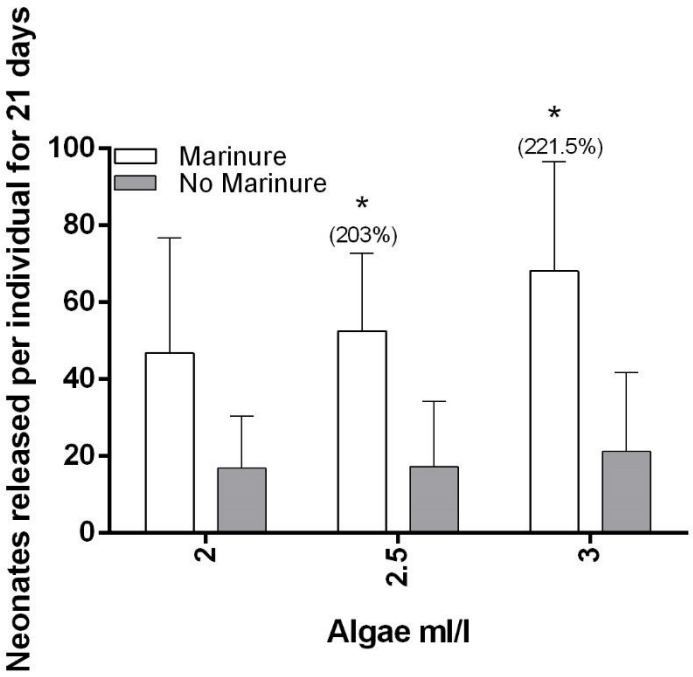

Figure 2.7. The impact of food supplements on fecundity of daphnids. Data represents average ± SD (N=8) replicates for each concentration. Statistically significant by Student's *t*-test compared to conditions without marinure ($p<0.05$). 2-way ANOVA comparison between algae and marinure inclusion.

Statistical analysis using a two-way ANOVA indicated that the addition of marinure to OECD media had a significant positive impact on the amount neonates produced throughout the 21-day period (*P* value = 0.0007). Furthermore, the student's T-test

revealed daphnids which were fed with 2.5 / 3 ml algae per l and 1.25 / 1.5 ml marinure per l produced 203% and 221.5% more neonates respectively to those fed only with algae. As a result, this prompted future chronic experiments to use the following concentrations of algae and include marinure; 3 ml algae per l and 1.5 ml marinure per l.

To assess the influence of oestrogen compounds on daphnid fecundity, a 21-day chronic experiment was performed. Daphnids were exposed to individual oestrogens and their triple mixture at a concentration of 1 mg/L. Results indicated that oestrogens did not pose a significant effect on daphnid fecundity (Figure 2.8). However, there was a noticeable trend that oestrone, ethinylestradiol and the mixture increased the number of neonates released by daphnids.

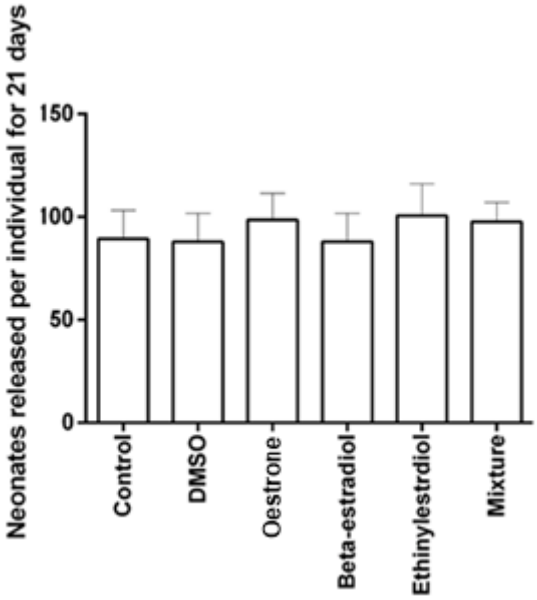

Figure 2.8. The effect of three oestrogens and their mixture on daphnid fecundity. Data represents average ± SD (N=8) replicates for each concentration. Statistically significant by Student's *t*-test compared to conditions without marinure ($p<0.05$).

Analysing the data per day showed that the brooding schedule of daphnids was similar among the control and test conditions (Figure 2.9). However, breeding began first in the control on day 9, while daphnids from the oestrogen treated groups and the DMSO began to breed shortly after, on day 10. Reproduction peaked at approximately day 14 in all test conditions.

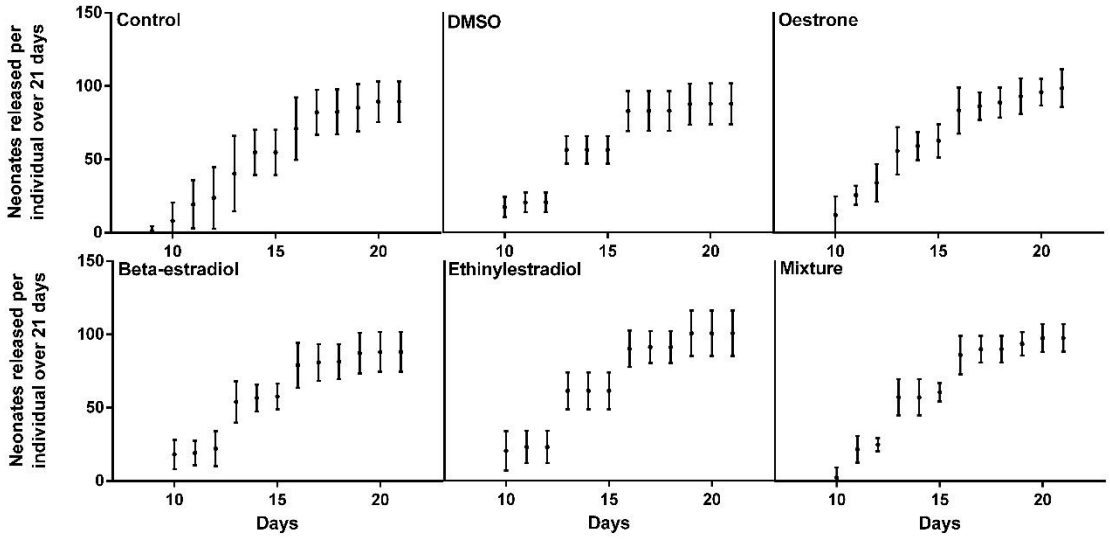

Figure 2.9. The number of neonates released per individual for several oestrogens and their mixture over a period of 21 days.

2.3.2.2 Biochemical markers of enzyme activities

Similar to acute experiments, enzyme markers of physiology and oxidative stress were used to assess the chronic impact of oestrogens on daphnids (Table 2.2). Interestingly, ethinylestradiol was the only exposure condition to impact both ALP and ACP activities, and was responsible for increases in both cases, of 13% and 19.4% respectively. The activity of β-galactosidase was notably altered by chronic exposure to oestrogens and their mixture. When compared to the control, DMSO increased the activity of β-galactosidase by 33.3%. Similarly, oestrone, ethinylestradiol and the mixture also increased BGAL activity by 25%, 32.9% and 32.1% respectively when compared to DMSO. Although β-estradiol did not cause a statistically significant change to BGAL activity, an obvious trend of β-estradiol to increase the activity of BGAL can be observed. Lipase activity was significantly altered by DMSO and two of the three individual oestrogens. The carrier solvent increased the activity by 171%, whereas the oestrogens; oestrone, ethinylestradiol and the mixture decreased the activity by 25.7% 50.4% and 21.2%. The oestrogen mixture was the only exposure group to significantly affect peptidase activity and was responsible for a 21.1% decrease. GST, which is an enzyme, related to xenobiotic metabolism was significantly altered by the oestrogen exposure groups, as well as DMSO (Figure 17). When compared to the OECD control, DMSO caused a significant increase (75.4%) of GST activity. Contrastingly, when compared to the DMSO control, the oestrogens,

β-estradiol and ethinylestradiol were responsible for decreases of -40.9% and -42.5% respectively, the most significant decrease was observed in the mixture condition (-51.6%).

Table 2.2. The chronic effect of oestrogens on multiple enzyme markers of *Daphnia magna* (age day 21).

Data represents average ± SD (N=4) replicates for each condition. Statistically significant by Student's t-test compared to control (*) or DMSO ($) (p<0.05). ALP, ACP, BGAL, LIP and PEP are expressed as milliunits/µg protein and GST as microunits/µg protein.

Enzyme	Control	DMSO	Oestrone	β-estradiol	Ethinylestradiol	Mixture
ALP	6.3±1.08	6.9±1.29	6.5±1.04	5.8±0.69	7.8±0.73**$** **(13%)**	7±0.56
ACP	2.4±0.44	3.1±0.33	3.3±0.34	3.4±0.22	3.7±0.07**$** **(+19.4%)**	3.6±0.47
BGAL	2.1±0.39	2.8±0.42***** **(33.3%)**	3.5±0.31**$** **(25%)**	3.2±0.31	3.7±0.52**$** **(32.1%)**	3.8±0.21**$** **(35.7%)**
LIP	26.1±9.38	70.7±8***** **(171%)**	52.5±2.04**$** **(-25.7%)**	66.2±1.56	35.1±7.14**$** **(-50.4%)**	55.7±7.83**$** **(-21.2%)**
PEP	1.7±0.13	1.9±0.13	1.6±0.2	2±0.14	1.7±0.13	1.5±0.21**$** **(-21.1%)**
GST	201.2±29.28	352.9±64.5***(75.4%)**	341±21.29	208.7±31.8**$** **(-40.9%)**	202.8±22.**8$** **(-42.5%)**	170.9±3**5$** **(-51.6%)**

2.3.2.3 Changes in protein and size growth rates

To determine the impact of oestrogens on growth, protein and size were measured, to calculate the rate of growth, Chopelet and Demott rates were used. The Chopelet growth rate was calculated for the incremental growth rate, i.e., day 1 to 7, day 7 to 14 and day 14 to 21, whereas, Demott rate was solely calculated growth rate at day 7, 14 and 21. These rates effectively provide information of how fast the animals grew over their 21 days exposure.

Chronic exposure to oestrogenic compounds (1 mg/L) and their mixture (3 mg/L) showed different patterns of impact on protein content depending on the compound (Figure 2.10). When compared to control (day 7) DMSO significantly increased protein content. When compared to their carrier solvent (DMSO) the oestrogens oestrone, ethinylestradiol and the mixture had significantly lower protein content, however they showed similar values compared to the control condition. In contrast, β-estradiol had similar protein content to that of DMSO and higher than the control condition. On day 14, DMSO had no statistical differences compared to the control, however β-estradiol, ethinylestradiol and the mixture were significantly lower in protein when compared to DMSO. Statistical differences of protein content between oestrogen compounds and

DMSO were not recorded on day 21, however DMSO caused considerably higher protein when compared to the control. By calculating protein growth rates using Chopelet, many statistically significant variances were recorded. Oestrone, ethinylestradiol and the mixture condition notably decreased growth rate between day 1 and 7 (compared to DMSO). Oestrone and β-estradiol were the only compounds to significantly impact growth rate between day 7 and 14, oestrone caused an increase in protein whereas β-estradiol had the opposite effect. The conditions, β-estradiol, ethinylestradiol and mixture caused a significant increase of protein between day 14 and 21. Similarly to Chopelet, there were numerous changes in Demott growth rate recorded. On day 7, oestrone, ethinylestradiol and the mixture decreased growth rate. Ethinylestradiol and mixture continued to have a reducing effect on protein on day 14 with the addition of β-estradiol. The mixture was the only condition to effect Demott growth rate on day 21, it demonstrated a positive effect on protein content.

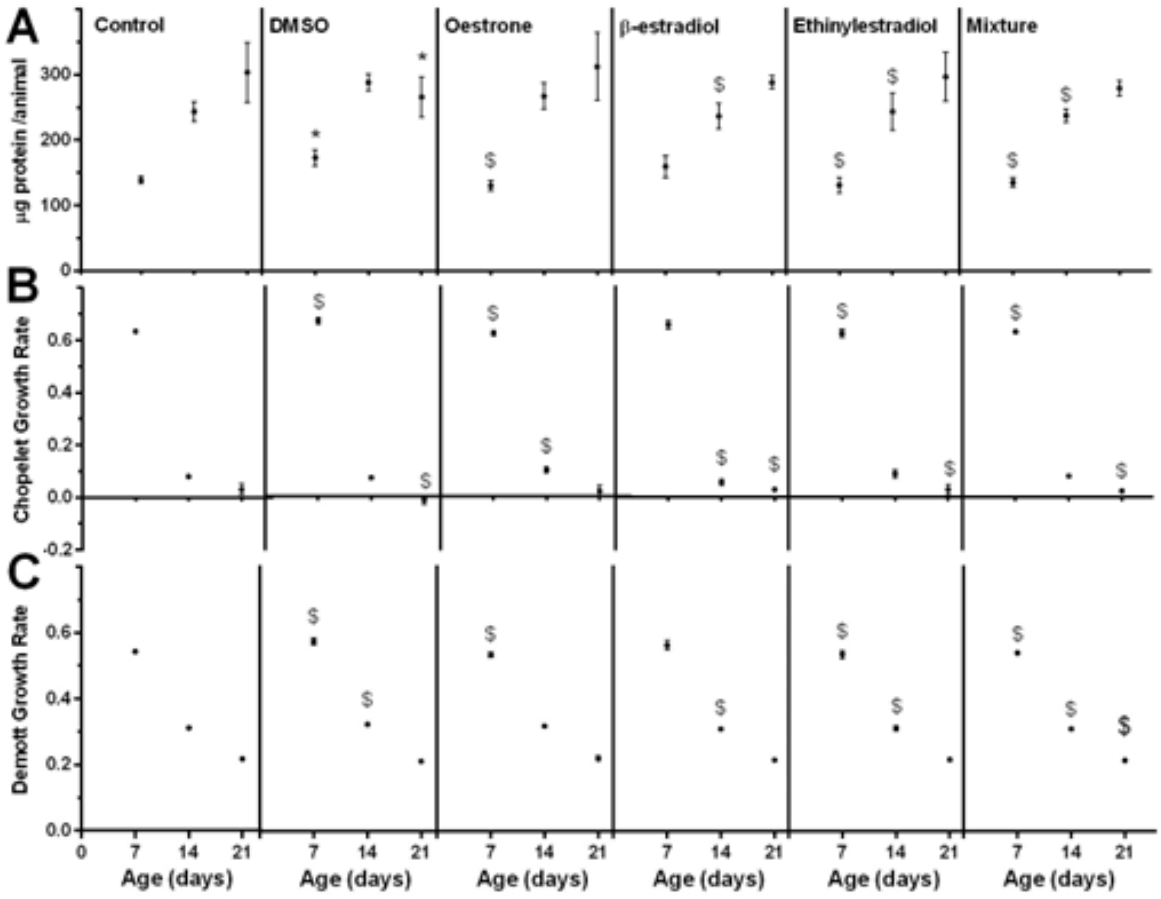

Figure 2.10. The chronic effect of three oestrogen compounds and their mixture on the endpoint protein in *Daphnia magna*. A depicts µg BSA / animal, B depicts Chopelet

growth rate and C depicts Demott growth rate. Statistically significant by Student's *t*-test compared to control (*) or DMSO ($) ($p<0.05$).

With regard to the size, measured as length per individual, significant differences were less common over the course of 21 days, however, the mixture condition did pose a negative effect on body length and caused a significant decrease (Figure 2.11). Between days 1 and 7 β-estradiol was the only chemical to impact Chopelet growth rate, it caused a significant reduction in growth rate. The only conditions to affect growth rate between day 14 and day 21 were oestrone and ethinylestradiol, which both illustrated a decreasing effect on growth rate. The mixture was the only condition to exert a significant impact on Demott growth rate, it caused a notable decrease in length at day 21.

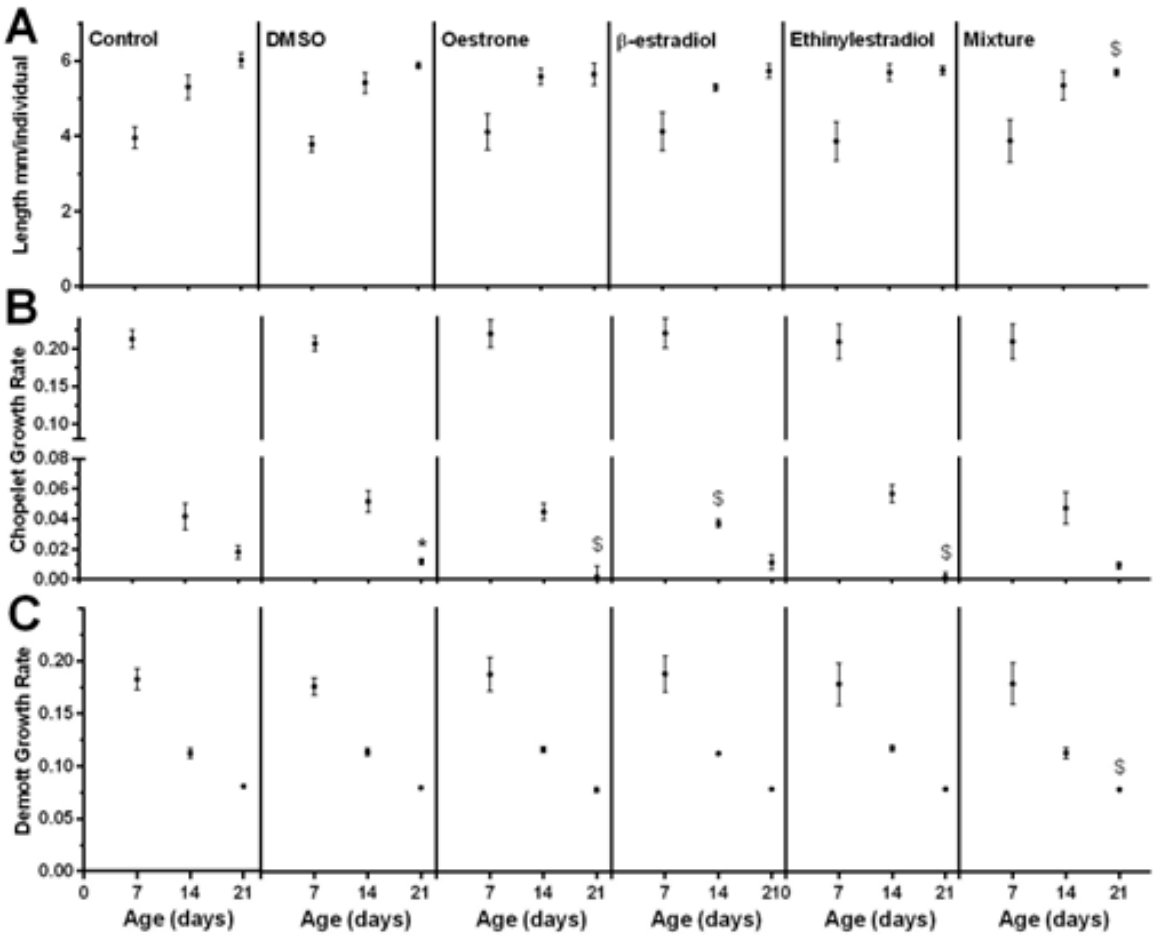

Figure 2.11. The chronic effect of three oestrogen compounds and their mixture on the endpoint size in *Daphnia magna*. A depicts length (mm) / individual, B depicts Chopelet growth rate and C depicts Demott growth rate. Statistically significant by Student's *t*-test compared to control (*) or DMSO ($) ($p<0.05$).

2.3.2.4 The effect of oestrogens and their mixture on metabolism of daphnids

A targeted metabolomics analysis of the hydrophilic content of daphnids, composed of amino acids, electron carrier molecules and intermediates of the central metabolic pathways was performed. Multivariate statistics via Pearson correlation hierarchical clustering (HCL) and Principal Component Analysis (PCA) revealed separation of the DMSO control from the oestrogen exposures, particularly the oestrogen mixture (Figure 2.12).

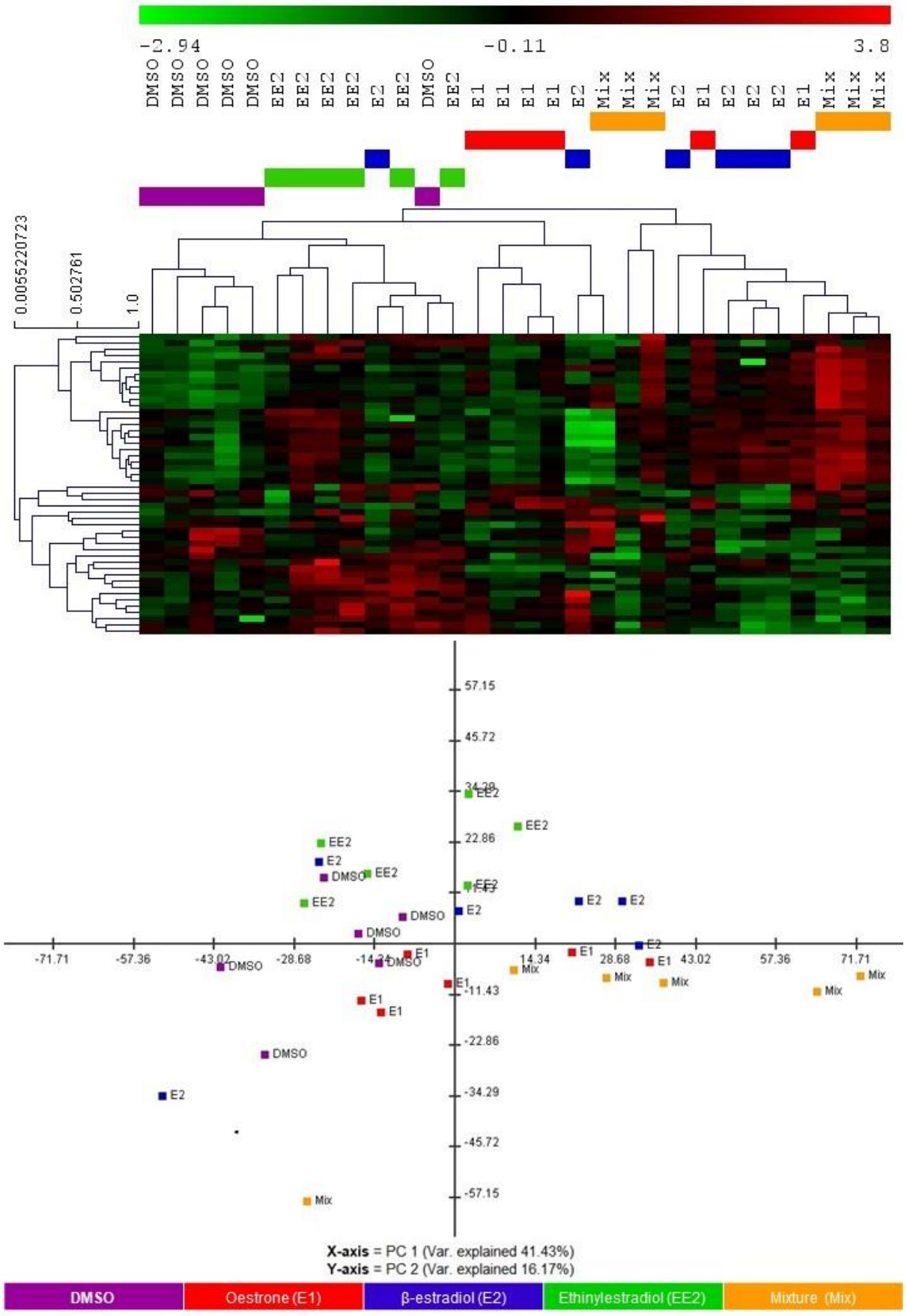

Figure 2.12. Multivariate statistical analyses; Pearson correlation (top) and PCA (bottom) show separation of DMSO from oestrogen exposures.

The fold changes in metabolites were calculated for oestrone, β-estradiol, ethinylestradiol and the mixture over the DMSO control (Figure 2.13). Twenty-eight molecules of energy metabolism were analysed, with the exception of D-sedoheptulose-7-phosphate, all metabolites were altered by exposure to at least one of the oestrogens tested. Thirty-five metabolites were up regulated by ≥1.5 and 18 down regulated by ≤0.5. Acetyl co-enzyme A was increased by each oestrogen exposure, the mixture was responsible for the greatest increase, suggesting an additive effect. Similarly, lactic acid was also upregulated by each oestrogen, particularly by the mixture, potentially demonstrating impairment of central metabolism. The oestrogens showed a trend to decrease the molecules GMP, CMP, UMP, and CDP. Overall, ethinylestradiol induced the most changes in metabolites (17), closely followed by the mixture (14), most of these differences were recorded as increases. In contrast, oestrone only altered 10 metabolites of this group, 6 of which were decreases, β-estradiol altered 12 molecules, most of which were upregulations. Interestingly, all fold changes of amino acids following oestrogen exposure were increases, and each amino acid was significantly altered by at least two oestrogen treatments, alanine, phenylalanine, serine, threonine, tyrosine and valine were increased by ≥1.5 by oestrone, β-estradiol, ethinylestradiol and the mixture. The mixture increased every amino acid with the exception of homoserine, and had the greatest impact on glycine. Ethinylestradiol increased all amino acids but four namely glutamic acid, isoleucine, proline and tryptophan. Interestingly, oestrone and β-estradiol demonstrated very similar effects on amino acids, and of the 5 amino acids oestrone did not alter, 4 were common with β-estradiol.

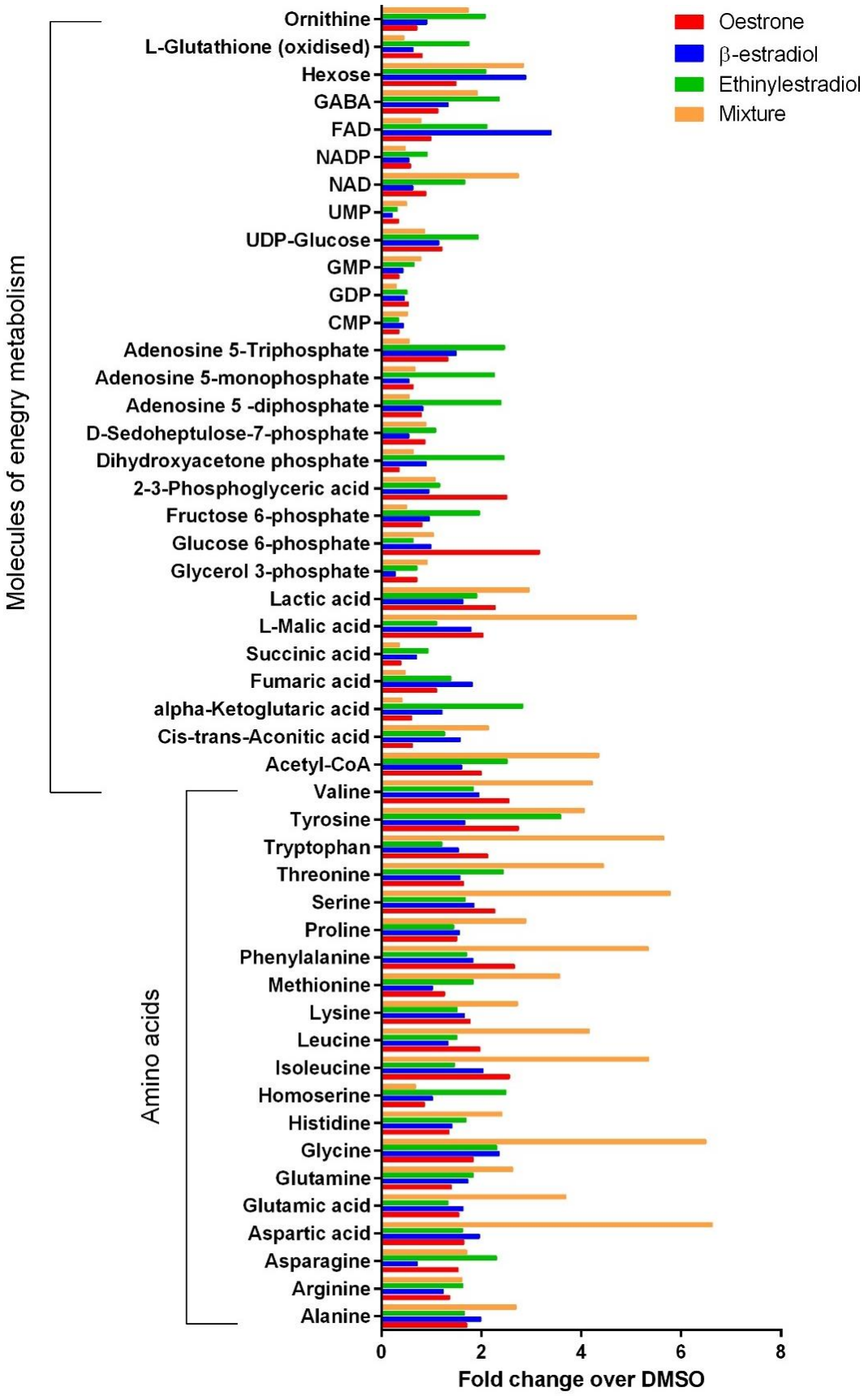

Figure 2.13. Fold change of metabolites for molecules of energy metabolism and amino acids. Data represents fold changes of oestrone, β-estradiol, ethinylestradiol and the mixture over DMSO.

2.4 Discussion

The emergence of endocrine disrupting compounds, oestrogens and their metabolites as contaminants of concern is of central importance in the scientific community of water monitoring, as their increased environmental concentrations threaten the physiology in fish (Pinto et al., 2014) and pose a potential threat to human health (Wocławek P et al., 2013). Specifically, for oestrogens, their impact on non-target biota species remains controversial as it is not fully understood how they impact the endogenous hormones of invertebrates. In this chapter the acute and chronic effects of oestrogens on daphnids was assessed through molecular, phenotypic and metabolic endpoints.

2.4.1 Oestrogen signalling pathways

Hormones, released by the endocrine system, function as chemical messengers that regulate communication between cells. Oestrogens are peripherally produced in the gonads (ovaries and testes) and locally synthesised by the nervous system (Dieni et al., 2020). In vertebrates and some invertebrates, oestrogen signalling is mediated by nuclear receptors, known as oestrogens receptors (ERs). An ER is composed of dimeric nuclear proteins, which binds to DNA to control gene expression. Oestrogens mediate cellular processes through two signalling pathways; (i) nuclear initiated steroid signalling and (ii) membrane-initiated signalling (Figure 2.14). Within the nuclear pathway, oestrogen molecules upon entering the cell, bind to the ERα or ERβ which then translocate to the nucleus where it binds to DNA at specific ER elements (ERE), in turn activating ERE dependant genes. In the membrane pathway, oestrogens can activate by binding to the ER at the plasma membrane (mER) or the novel G protein coupled E2 receptors (GPER). Following ER activation, various signalling pathways are triggered (i.e. $Ca^{+2,}$ cAMP, protein kinase cascades) which influence downstream transcription factors which are responsible for controlling gene expression.

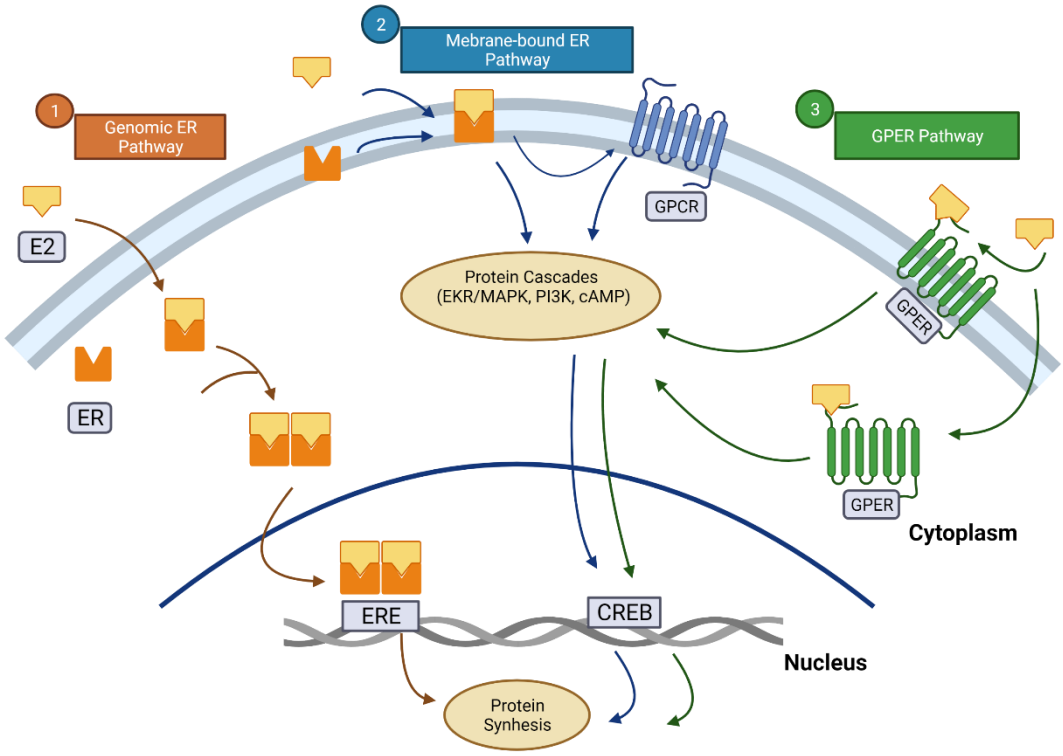

Figure 2.14. Oestrogen signalling pathways

2.4.2 Effect of oestrogens on freshwater species

Substances such as phthalates, insecticides, polychlorinated biphenyls and bisphenols are commonly referred to as endocrine disrupting compounds (EDCs). There is significant evidence indicating the capacity of these compounds to disrupt the communication between endogenous hormones and their receptors, resulting in the inaccurate interpretation of the message (Gonsioroski et al., 2020). The suspected mechanisms of action demonstrated by oestrogens are complex and not fully understood, however, there are four reported modes of action by which oestrogens effect normal endocrine functioning:

(i) oestrogens can mimic the action of endogenous hormones leading to undesirable responses such as a surplus of hormones being released and or hormones being released at the incorrect time (agonistic effect)

(ii) they can bind to proteins in the blood altering the concentration of hormones in circulation

(iii) they can bind to the receptor preventing natural occurring hormones to bind (antagonistic effect)

and (iv) they can affect the synthesis and concentration of endogenous hormones by disrupting metabolic processes (Nazari & Suja, 2016).

Naturally occurring oestrogens, excreted by humans and livestock are characteristically hydrophobic and generally have short half-lives in aquatic environments. However, synthetic forms of oestrogens such as EE2 is regarded especially persistent, and its stability can increase based on the oxidative state of the waterbody it infiltrates. It has been reported that anaerobic conditions can effect degradation rates and increase the half-life of oestrogens (Ying et al., 2003), and even with modern advances of filtration processes, removal of oestrogens from sewage influent is difficult (Huang et al., 2019; Tran et al., 2018). Invertebrates are considered especially vulnerable to oestrogen exposure (Wojnarowski et al., 2021), as its strongly accepted that hormones control physiological processes such as moulting, development and movement in invertebrates, and certain vertebrate analogues of these hormones can disrupt normal biological function by reducing fecundity or moulting frequency, however, this may not be the case for every oestrogen as studies using β-estradiol do not share the same findings (Kashian & Dodson, 2004). Although, in the acute component of our study, βestradiol was responsible for the most changes in enzyme activities, specifically it decreased ACP, PEP, LDH and increased BGAL activity. Overall, in the acute scenario, peptidase was the most impacted enzyme, and was decreased by each oestrogen. Acute exposures to low concentrations of oestrogens (0.1 to 1.0 ppm) has been documented to impact phenotypic endpoints, such as reduced heart rate in daphnids (Walker et al., 1998). In an actual environmental setting, this scenario could render daphnids more susceptible to predation, leading to the increased bioaccumulation of oestrogens in consumers of the food web (Almeida et al., 2020; Müller et al., 2020). In our investigation, oestrogens exhibited an influence on the physiology of daphnids, as evidenced by changes in their size and enzyme activities, but did not affect their reproductive output, as no significant differences between the number of neonates released by oestrogen exposed daphnids compared to the DMSO control was observed. This is supported by a study also employing *D. magna* in EE2 exposures between 62.5 – 1000 ng/L, and observed no negative impact on reproduction (Clubbs & Brooks, 2007). However, it has been documented that EE2 can impede on multiple endpoints such as survival and fertility in another invertebrate model *Drosophila melanogaster* (Bovier et al., 2018).

Moreover, Segner et al. reported the effect of EE2 on several model organisms namely; *Hydra vulgaris*, *Gammarus pulex*, *Chironarus riparius*, *Hyalella Azteca, and Lymnaea stagnalis,* and alluded to its effect on hatchability rate, body size, moulting, reproductive behaviour and brood size. Importantly, the study revealed at environmentally relevant concentrations, EE2 impacted the expression of ER2 and vitellogenin related genes in *Mytilus edulis*, suggesting its impact on reproduction (Segner et al., 2003). Regarding the chronic experiments of this study, ethinylestradiol and the mixture were responsible for the most significant alterations in both biochemical markers and metabolite levels. Ethinylestradiol and the mixture impacted 5 and 4 (out of 6) enzymes respectively. This same trend was observed in the metabolomics analysis, as EE2 and the mixture also induced the most changes in metabolites. GST was especially affected in the first-generation exposures by E2, EE2 and the mixture, whereas in the third-generation exposure the opposite was observed, and E1 was the only oestrogen to significantly affect GST. However, EE2 and the mixture remained responsible for the most changes in this scenario also. It has been documented that at low concentrations EE2 has effected GST in the copepods Calanoida (10, 100 and 1000 ng/L) and Cyclopoida (100 and 1000 ng/L), as well as Caspase 3, a marker for apoptosis at 1000 ng/L in both organisms (Souza et al., 2013). Literature is very scarce considering the findings of oestrone, and to our knowledge not much evidence exists on the direct mechanisms on daphnids. In one study it was shown that there was no effect on swimming behaviour in *D. pulex* following exposure to oestrone (Alla et al., 2021). Another study employing the copepod *Tisbe battagliai* indicated that at concentrations of 0.1-100 µg/L oestrone did not induce significant mortalities, reproductive dis- function or altered sex ratio (Hutchinson et al., 1999). Therefore, oestrone could be less hazardous to non-target species, although in our study the activity of beta-galactosidase was significantly impacted by oestrone as was Chopelet and Demott growth rate.

Beta estradiol or frequently referred to as E2 has been reported to cause various effects among aquatic organisms. It has been reported that E2 has no significant impact on sex determination and fecundity in daphnids when exposed to environmentally relevant concentrations (Kashian & Dodson, 2004). This is also supported by multi-generational studies performed by (Brennan et al., 2006). Although E2 has not been heavily reported to affect physiological endpoints such as

reproduction it has been described to exert effects at a deeper level. Alterations at the transcriptome level in daphnids have also been documented following E2 exposure. Specifically, following 3-12 hours of exposure to E2 at concentrations of 10 and 100 ng/L. 351-17’221 genes were upregulated and 505-10’282 genes were down regulated (Zheng et al., 2020). There is significantly more literature reporting the phenotypic effects of E2 on fish compared to daphnid models and there is notable evidence to suggest that E2 is harmful to fish even at concentrations as low as 1-2 ng/L. At such concentrations, male *Melanotaenia fluviatilis* (rainbow fish) demonstrated both male and female characteristics, possessing partially formed eggs and ova in the testes (Woods & Kumar, 2011). Moreover, at 8-9 ng/L, E2 caused reduced fertility and fecundity in male *Oryzias latipes* (Japanese medaka fish). Similarly, in another *Oryzias* species, E2 had a significant effect on fertilization and egg production (Imai et al., 2005), and reduced the number of eggs produced and successful spawns (Jukosky et al., 2008). Furthermore, negative effects of E2 have been recorded in other fish species e.g. the liver, kidney and gonad tissue of the sheepshead minnow were significantly affected as a result of E2 exposure, similarly to other reports daily reproduction was also reduced (Cripe et al., 2009). In our study, exposure to β-estradiol resulted in alterations of enzyme activities within the acute (ALP and GST) and chronic (GST and β-gal) scenarios of toxicity. Moreover, E2 affected protein content per animal and Chopelet and Demott growth rate.

There is strong evidence to suggest the feminization abilities of ethinylestradiol in aquatic wildlife, particularly in fish species (Daigle, 2010; Desbrow et al., 1999). However, invertebrates represent approximately 95% of all aquatic organisms, some of which play integral roles in ecosystems as keystone species and therefore it is important to understand the impact of oestrogens to invertebrates also. Exposure to low levels of EE2 for 21 days resulted in alterations of life history and biochemical parameters in daphnids; decreased growth rate, oxidative stress, lipid peroxidation and genotoxic damage were all detected (Rodrigues et al., 2021). During a multigenerational experiment, reduced body length of daphnids was recorded in the F0, F4 and F5 generations. Moreover, number of neonates released per individual was also significantly reduced in the F0 and F4 generation (Dietrich et al., 2010). In another multigenerational study, reduced fecundity was recorded in first generation daphnids after short term exposure (6-10 days) to EE2, whereas fecundity was similar to controls after prologued exposure (>10 days) (Clubbs & Brooks, 2007). Similar to

those effects demonstrated by fish exposed to E2, several genera of fish displayed phenotypic female characteristics. In the madeka species, *Oryzias latipes*, embryos derived from mothers injected with EE2 developed abnormal gonads, and XY males possessed ovaries which actively produced oocytes (Hano et al., 2005). Moreover, ethinylestradiol has been documented to exert oestrogenicity to invertebrates also, exposure to EE2 for 100 days at concentrations >1 mg/L resulted in significantly more females in the freshwater model *Gammarus pulex* (Watts et al., 2002). However, in our study the activities of ACP, and β-gal were significantly impacted after acute exposure to ethinylestradiol and β-gal, GST, protein content per animal and Chopelet and Demott after chronic exposure.

The metabolic profile of daphnids were significantly altered following acute and chronic exposure to oestrone, β-estradiol and ethinylestradiol. For acute exposures, daphnids exposed to β-estradiol resulted in increased levels of amino acids and amino acid derivatives (phenylalanine, proline and methionine) with an exception of asparagine, nucleosides (adenosine, inosine and uridine), vitamins (pantothenate and nicotinic acid) purine (hypoxanthine), 16 acetylcarnitine, cadaverine and histamine. Similarly, chronic exposure to E1 also resulted in the upregulation of phenylalanine and proline, and also no changes in asparagine. However, among the other shared amino acids of both analyses, chronic exposure also resulted in the increase of tyrosine, tryptophan, threonine, valine, leucine and isoleucine, which was not the case in the acute scenario. In contrast with β-estradiol, oestrone decreased the levels of most metabolites detected in daphnids including amino acids and amino acid derivatives, nucleosides and nucleobases, purines and others with the exception of acetylcarnitine, adenosine, cadaverine and pantothenate. Whereas, the opposite was observed in the chronic scenario, as E1 increased all shared amino acids. In agreement to our findings, previous studies have shown that acute exposure to ethinylestradiol (1 mg/L) resulted in the alterations of several amino acids in *Daphnia magna* (Kovacevic et al., 2019). In another study, isoleucine and leucine were significantly decreased by estrone, these branched chain amino acids are considered proteinogenic and to play key roles in protein turnover (Cappello et al., 2017). Similar to the other pharmaceuticals in this study, there is limited information available regarding the impact of oestrogens on the metabolome of daphnids. However, alterations in mRNA expression of vitellogenin and oestrogen receptor 2 has been documented in the blue mussel following ethinylestradiol exposure (Ciocan et al., 2010). These findings are supported by a

chronic study employing the rock oyster Saccostrea glomerata, increased vitellogenin levels were recorded in females and the display of intersex characteristics in males (Andrew et al., 2008). There is strong evidence suggesting oestrogenic mixtures behave in a concentration addition (CA) manner, the CA model alludes to synergistic effects of mixtures, even when the individual chemicals are at known ineffective concentrations (Brian et al., 2007; Payne et al., 2000; Silva et al., 2002). This coincides with the findings of this study as the mixture had a prominent impact on most endpoints assessed, particularly protein content, the metabolome of daphnids in the first generation, as well as the biochemical markers of third and fourth generation daphnids.

2.5 Conclusion

In this study, the oestrogen compounds oestrone, β-estradiol and ethinylestradiol induced significant changes in the activity of multiple enzyme markers of physiology and oxidative stress and these changes were accompanied by metabolic dysregulation. Within the acute scenario, β-estradiol was responsible for the most changes in enzyme markers, however within the metabolomics study oestrone was responsible for the most metabolic perturbations. In the context of the chronic exposures, ethinylestradiol and the mixture were responsible for the most changes at a metabolic level and were also coupled with extensive changes in enzyme activities.

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Chapter 3

The molecular impact of antibiotics on daphnids

Antibiotics have been widely detected in the environment including surface and ground waters, soil, and wastewater sludge. In recent years, the risk of enhanced presence of antibiotics in aquatic environments has gained attention, which is attributed to the incurred risk of the horizontal spread of antibiotic-resistant genes in the aquatic ecosystem. The phenomenon of antibiotic resistance was declared by the World Health Organisation as one of the most consequential challenges we face in society. As a result, antibiotics have a notable inclusion on the EU Surface Water list. Similar to other pharmaceuticals, antibiotics pose a threat to disrupt typical biocenosis and are reported to inhibit the biological functions of aquatic organisms. Modern water monitoring techniques produce greater insight into the potential toxicity of antibiotics by using effect-based methods and employing model species i.e. *Daphnia magna.* In this study, the ecotoxicology model organism *Daphnia magna* was exposed to the selected antibiotics as well as their combined mixture Combining molecular endpoints such as changes in enzyme activity and sensitive metabolomic approaches, the responses triggered by antibiotic exposures were assessed at a molecular level. The acute scenario revealed trimethoprim exposure to effect enzyme activities the most, while metabolism was impacted the greatest by amoxicillin and erythromycin. The transgenerational study, did not reveal an obvious trend of imprinted stress on latter generations. For example, the glycolysis pathway was more significantly impacted by first generation exposure, whereas, third generation exposure resulted in greater impairment of the TCA cycle.

3.1 Introduction

In this chapter, the ecotoxicological model *Daphnia magna* was exposed to a range of antibiotics with a different mode of action (Figure 3.1) and their mixtures. Following acute and chronic exposure to antibiotics, the responses of daphnids were assessed. The toxicity potential of antibiotic compounds was evaluated by of endpoints comprised of biochemical markers and a metabolomic analysis.

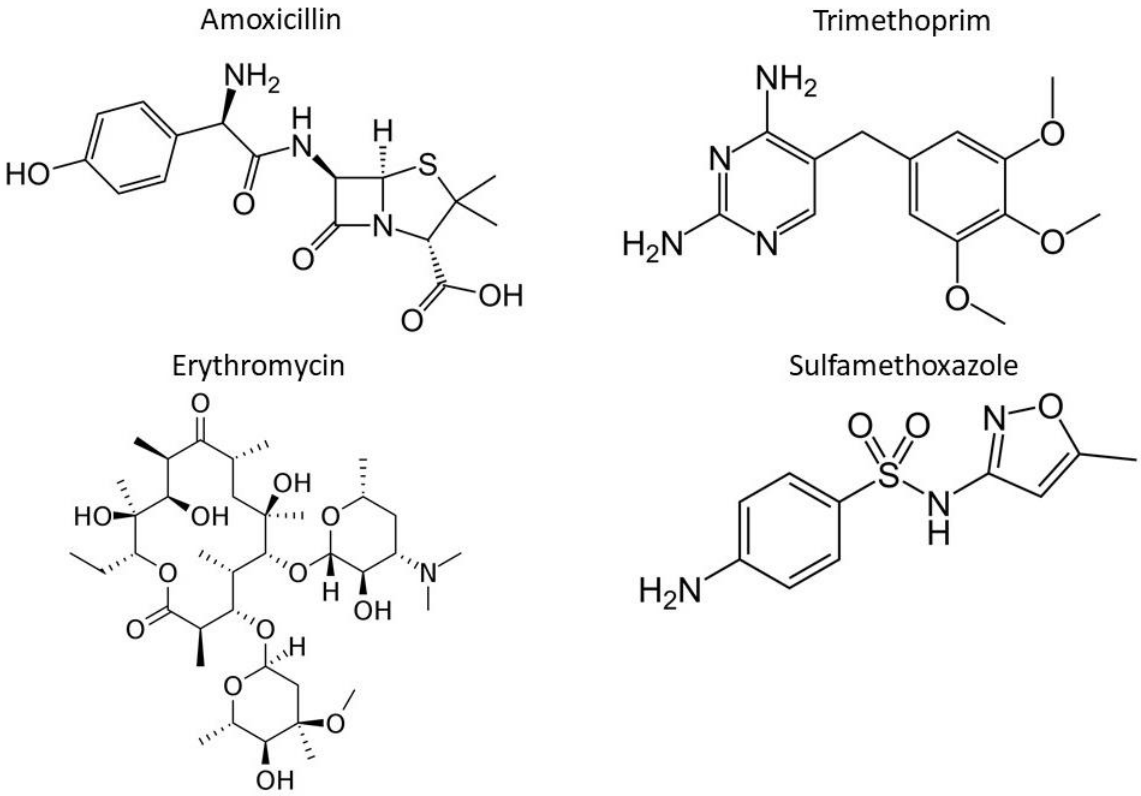


Figure 3.1. The antibiotics used in this chapter.

3.2 Materials and methods

3.2.1 Algae and daphnid culturing

Algae and daphnids were cultured following the same protocols described in the previous chapter (see section 2.2.1 and Appendix A).

3.2.2 Acute toxicity exposures

Acute toxicity exposures were executed following the same approach outlined in the previous chapter (see section 2.2.2).

3.2.3 Chronic toxicity exposures

Forty-five neonates (<24 hours) were cultured for 21 days in 1800 ml OECD media. Daphnids were fed daily with fresh algae (5.5ml) and a supplement of seaweed extract (2.7 ml) at the renewal of media every three days. Chemicals were also refreshed with media change for the duration of the experiment. Newly released neonates were removed daily by filtration. The biochemical endpoints alkaline phosphatase, acid phosphatase, β-galactosidase, and glutathione-s-transferase activities and protein were measured at day 21. Samples collected on day 21 were also snap-frozen for metabolomics and homogenized for biochemical assays (Figure 3.2).

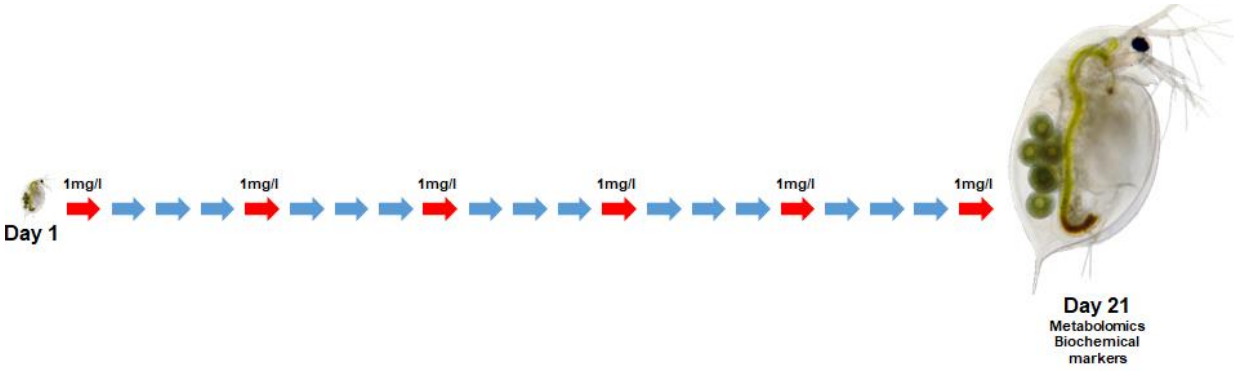


Figure 3.2. The basic design of chronic experiment (daily feeding and filtering are represented by the blue arrows, change of media and chemical renewal are represented by the red arrows).

3.2.4 Transgenerational exposures

During the initial chronic experiment neonates were collected from the mothers exposed for each condition and cultured using the same chronic exposure protocol for another generation (Figure 3.3). As previously outlined, once the second generation of animals was generating neonate (from 17 days old), their neonates were again collected and cultured in the same conditions for another generation. This third generation of daphnids was cultured until 21 days old and used in biochemical assays and frozen for metabolomics. In the same manner, once the animals from the third generation daphnids were producing neonates (older than 17 days old), neonates released were collected and cultured in clean media until day 4 to allow a recovery period. Specifically, 100 neonates (from each condition) were added per l OECD, and 3.75 ml seaweed extract per l was added on the day of the initial setup of these cultures. Daphnids were fed daily with 2.5 ml frozen algae per l and cultured until they

were collected at four days. In addition, the mothers of this third generation were also collected at 21 days, like the chronic (21 days) experiment for the first generation.

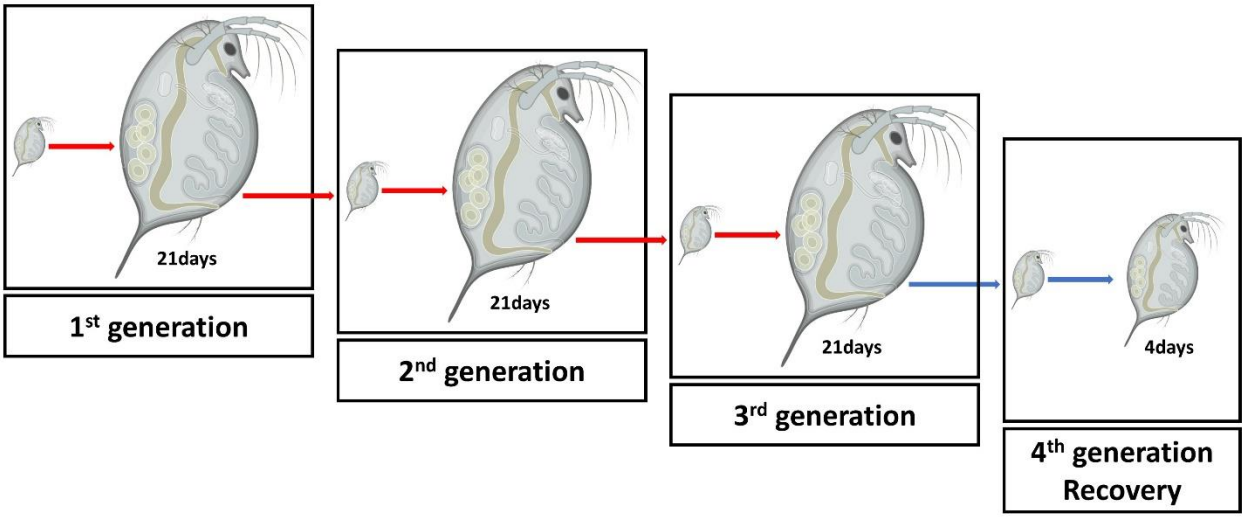


Figure 3.3. Experimental design of transgenerational and chronic exposures (daily feeding and filtering are represented by the blue arrows, change of media and chemical renewal are represented by the red arrows).

3.2.5 Sample homogenization and biochemical analyses

Samples were prepared using the same methods in the previous chapter (see section 2.2.6). Protein was quantified using an ultrasensitive Bradford method which is described in the materials and methods of the previous chapter (see section 2.2.7). The activity of enzymes (alkaline phosphatase, acid phosphatase, beta-galactosidase, lipase, peptidase, lactate dehydrogenase, glutathione reductase, glutathione -S-transferase) were measured as described in the previous chapter (see section 2.2.8).

3.2.6 Statistical analysis for biochemical markers

The acute toxicity and biochemical activity data were analysed using GraphPad as previously explained in Chapter 2 (see section 2.2.9).

3.2.7 Extraction of metabolites and metabolomic analyses

Metabolites were extracted from daphnid samples following the method outlined in the previous chapter (see section 2.2.10). For acute metabolomic analysis, the same approach with chapter 2 was followed (see section 2.2.11). For the chronic metabolomic analysis, a targeted LC-MS/MS analysis was performed with a QTRAP 6500+® system (Sciex, Framingham, MA, USA), coupled with an online HPLC system. Firstly, each sample was resuspended in 120 µl water and 10 µl were injected into an

Agilent 1290 II infinity UPLC system (Agilent Technologies Inc., Santa Clara, CA, USA). Chromatographic separation was achieved using an XSelect HSS T3 XP column (2.1 x 150 mm, 2.5 µm, 100 Å; Waters, Milford, MA, USA). Metabolites were eluted at a flow rate ranging from 0.4 ml/min to 0.15 ml/min with a non-linear 33 min gradient. Mobile phase A and B were 10 mM tributylamine, 10 mM acetic acid, 5% methanol and 2% 2-propanol (pH 7.1) and 100% 2-propanol, respectively. Autosampler was kept at 5°C and the column oven was set at 40 °C. Identification and relative quantification were based on specific MRM transi-tions measured in negative mode electrospray ionization. For the chronic metabolomic data, the values of peak area intensities were standardised by z-scoring and were then processed for multivariate statistical analysis using the open-access Multiexperiment View-er (MeV) software (Saeed et al., 2003). Principal Component Analysis (PCA) and Pearson and Euclidean hierarchical clustering were performed. To detect significant fold changes in metabolites, a Significance Analysis of Microarrays (SAM) between each exposed group and control was executed.

3.2.8 Reagents and chemicals

Amoxicillin and sulfamethoxazole were purchased from Sigma Aldrich. Trimethoprim was purchased from Acros Organics. Erythromycin was purchased from Thermo Scientific. See section 2.2.12 for all other chemical and reagent purchases.

3.3 Results

3.3.1 Acute exposure to antibiotics

Daphnids were individually exposed to the antibiotic's amoxicillin, erythromycin, trimethoprim, and sulfamethoxazole at 50 mg/L. Amoxicillin, erythromycin, and trimethoprim was dissolved in OECD media, and sulfamethoxazole was dissolved in DMSO. After 24 hours of exposure, several physiological and oxidative stress markers were measured (Table 3.1).

Phosphatase activity was significantly altered by antibiotic exposure, particularly acid phosphatase. DMSO, amoxicillin, and trimethoprim decreased ACP activity by 22.3%, 32.3%, and 22.2% respectively. Whereas erythromycin increased ACP activity by 17.5%. Antibiotic exposure had a lesser effect on ALP activity, DMSO and trimethoprim both decreased ALP activity by -24.1% and 25.4% respectively. However, there was only a slight trend of amoxicillin and erythromycin to increase ALP

activity. Increase in the activity of BGAL were recorded for trimethoprim (50.7%) and erythromycin (47.4%). Similarly, when compared to DMSO, sulfamethoxazole also increased BGAL actiivty by 10.4%. DMSO and trimethoprim decreased lipase activity by 12.7% and 16.9% respectively when compared to the control.

No significant changes in peptidase activity were recorded for the antibiotic compounds or the carrier solvent DMSO. However, LDH proved to be sensitive to antibiotic exposure as each antibiotic altered LDH activity. Significant decreases were detected for amoxicillin (31.4%), trimethoprim (14.3%), and erythromycin (25.7%) whereas an increase in activity was revealed for sulfamethoxazole 54.3%.

Sulfamethoxazole was the only antibiotic to significantly affect GR activity (77.8% increase). Like what was observed in ALP activity, DMSO and trimethoprim were the only conditions responsible for statistical differences in GST activity. Both DMSO and trimethoprim demonstrated a diminishing effect on GST, leading to a -38.2% and -69.6% respectively.

Table 3.1. The acute effect of antibiotics on multiple enzyme markers of *Daphnia magna* (age day 4).

Data represent average ± SD (N=4) replicates for each condition. Statistically significant by Student's t-test compared to control (*) or DMSO ($) ($p<0.05$). ALP, ACP, BGAL, LIP, PEP, and LDH are expressed as milliunits/animal and GST and GR as microunits/animal.

Enzyme	Control	DMSO	Amoxicillin	Trimethoprim	Erythromycin	Sulfamethoxazole
ALP	189.8±26.1	144±11.76* **(24.1%)**	206.9±20.34	141.5±5.66* **(-25.4%)**	218.3±13.64	133.5±9.18
ACP	85.2±8.3	66.2±5.46* **(-22.3%)**	57.7±2.48* **(-32.3%)**	66.3±1.01* **(-22.2%)**	100.1±7.84* **(17.5%)**	68.8±3.17
BGAL	81.8±3.82	85.2±3.79	85.3±7.15	123.3±6.62* **(50.7%)**	120.6±11.32* **(47.4%)**	94.1±5.99$ **(10.4%)**
LIPASE	1141.3±79	996.5±33.7* **(-12.7%)**	1264.2±136.13	948.7±102* **(-16.9%)**	1152.4±98.53	1096±103.34
PEP	3.7±0.38	3.4±0.57	4.1±0.54	3.8±0.28	3.2±0.48	3.1±0.31
LDH	3.5±0.09	3.5±0.2	2.4±0.35* **(-31.4%)**	3±0.23* **(-14.3%)**	4.4±0.25* **(25.7%)**	5.4±0.59$ **(54.3%)**
GR	222.2±50.92	200±57.74	211.1±19.25	258.3±41.94	222.2±38.49	355.6±19.25 **$(77.8%)**
GST	1025±95.7	633.3±72* **(-38.2%)**	937.7±107	311.1±96.2* **(-69.6%)**	1015.9±49.4	588.9±135

3.3.1.1 The acute effect of antibiotics on the metabolome of daphnids

A targeted HILIC LC-MS/MS analysis was performed, and the subsequent profiling data was assessed, data was based on the peak areas of the detected metabolites. Multivariate statistical analysis (OPLS-DA) showed distinct differences in the metabolic fingerprints of sulfamethoxazole compared to DMSO; erythromycin,

amoxicillin, and trimethoprim compared to the OECD control (Figure 3.4 A). The goodness of fit in the X and Y variables and the high predictability of the model (R2Y(cum) and Q2) were validated by model statistics. CV ANOVA resulted in a p-value <0.05, confirming the statistical significance detected in the model (Figure 3.4 D).

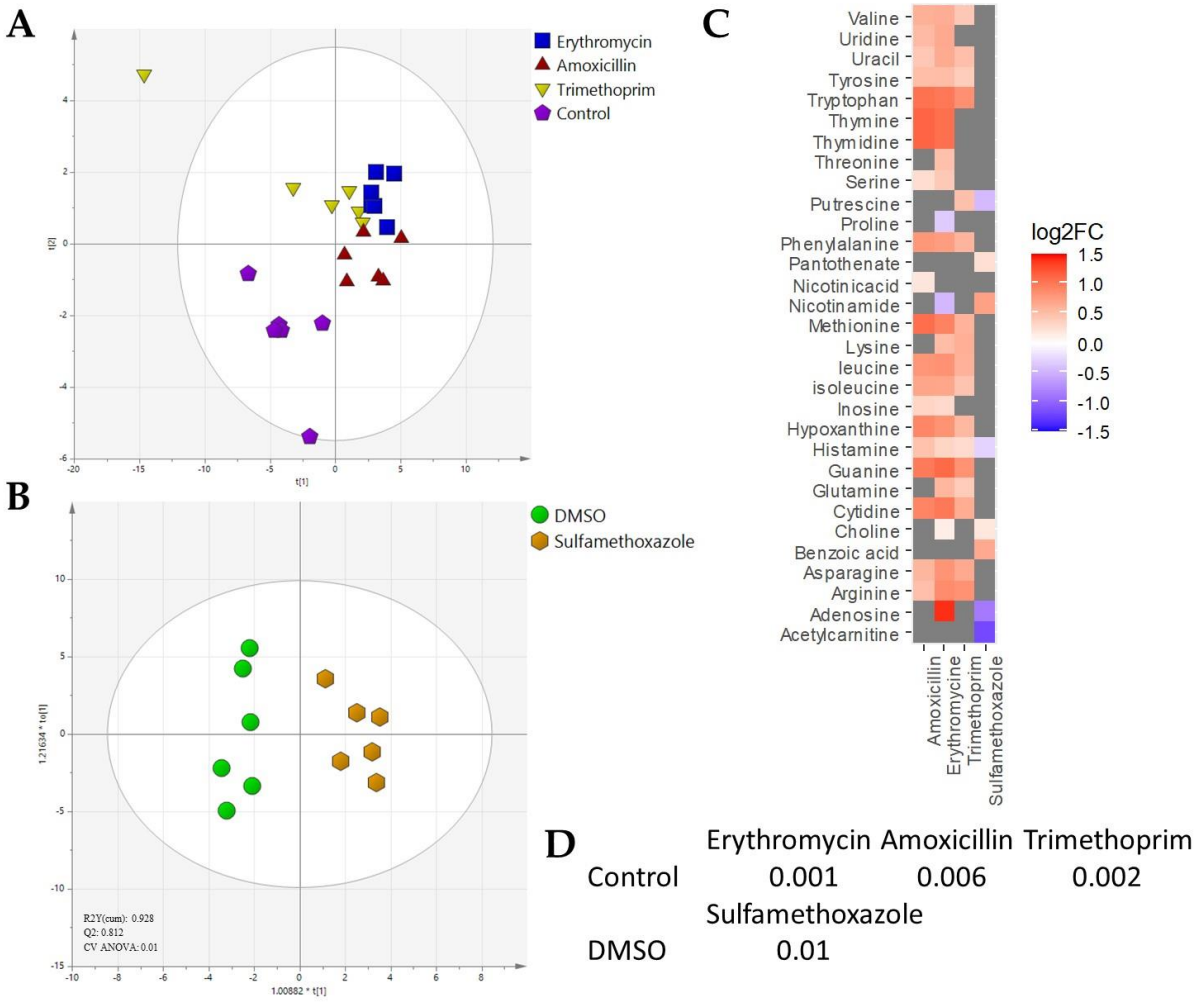


Figure 3.4. Multivariate statistical analysis of the metabolic impact of antibiotics on daphnids. (A) PLS-DA score plots based on data from targeted LC-MS/MS metabolic profiling of extracts of daphnids exposed to water soluble antibiotics (erythromycin, amoxicillin, trimethoprim) and (B) DMSO soluble antibiotic sulfamethoxazole. (C) Heatmap of Log2FC statistically significant compounds with p<0.05 and (D) CV ANOVA p value of OPLS-DA models. OPLS-DA score plots based on data from targeted analysis LC-MS/MS extracts of daphnids exposed.

Results revealed that 32 of all 40 metabolites detected were dysregulated as a result of different antibiotic exposure. Sulfamethoxazole was responsible for significant changes in 8 of the 40 metabolites; increased levels of acetylcarnitine, histamine, adenosine, and putrescine were detected while decreases in nicotinamide, benzoic

acid, choline, and pantothenate levels were recorded when in daphnids exposed to sulfamethoxazole compared to DMSO. Illustrated in supplementary Figure 3.2, are the box plots of the chromatographic peak areas of statistically significant metabolites of the metabolome for daphnids exposed to sulfamethoxazole. For the aqueous antibiotics, the metabolic fingerprints of amoxicillin, trimethoprim, and erythromycin were compared to the control using OPLS-DA models to identify significant alterations. Changes in 14 polar metabolites were frequently detected in daphnids previously exposed to water-soluble antibiotics. In particular, the amino acids arginine, asparagine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine nucleobases and nucleosides guanine, uracil, cytidine, hypoxanthine and histamine had elevated levels to varying degrees in exposed daphnids compared to the control group. Alterations in serine, inosine, thymidine, thymine, and uridine levels were also recorded for both amoxicillin and erythromycin-exposed daphnids compared to control, additionally; nicotinic acid was increased significantly in daphnids exposed to amoxicillin. The upregulation of adenosine, glutamine, and lysine levels in both amoxicillin and trimethoprim-exposed daphnids was observed. Exposure to only erythromycin caused changes in choline, threonine, proline, and nicotinamide, while putrescine levels were only impacted by exposure to trimethoprim Statistically significant metabolites with AUC, p and log 2-fold change values in antibiotic-treated daphnids about the control or DMSO group were summarized in supplementary table 3.1.

3.3.2 Chronic exposure to low concentrations of antibiotics

Animals exposed to selected antibiotics for 21 days showed diverse patterns of changes in phenotypical markers (Table 3.2). Activity of phosphatase was significantly affected by chronic antibiotic exposure, an increasing affect was recorded for acid phosphatase activity, whereas a negative effect can be observed for ALP activity. Trimethoprim was responsible for the most significant increase in ACP activity (53.3%), whereas DMSO caused the opposite effect and decreased ACP activity by 16.7%. In contrast to ACP activity, amoxicillin and mixture condition indicated a decrease in ALP activity (-25.5 % and 15.7% respectively). Three of the five antibiotic conditions had a notable impact on β – galactosidase activity as a result of chronic exposure. Trimethoprim, sulfamethoxazole, and the mixture exerted a diminishing effect on β – galactosidase activity (-12%, -16%, and -20% respectively). Lipase

activity was significantly increased by trimethoprim (20%), erythromycin (17.1%), and sulfamethoxazole (36.7%). GST activity was significantly altered by all antibiotics except for sulfamethoxazole. Trimethoprim, erythromycin and the mixture decreased GST activity by -24%, -11.9% and -17.2% respectively. Contrastingly, amoxicillin was responsible for a significant increase of 28.9%.

Table 3.2. The chronic effect of antibiotics on multiple enzyme markers of first-generation *Daphnia magna* (age day 21).
Data represent average ± SD (N=4) replicates for each condition. Statistically significant by Student's t-test compared to control (*) or DMSO ($) ($p<0.05$). ALP, ACP, BGAL, and lipase are expressed as milliunits/µg protein, and GST as microunits/µg protein.

Enzyme	Control	DMSO	Amoxicillin	Trimethoprim	Erythromycin	Sulfamethoxazole	Mixture
ALP	5.2±0.25	5.1±0.41	3.8±0.27**$** **(-25.5%)**	5.3±0.53	5±0.19	4.7±0.29	4.3±0.12**$** **(-15.7%)**
ACP	3.6±0.34	3±0.06***** **(-16.7%)**	3.6±0.39**$** **(20%)**	4.6±0.5**$** **(53.3%)**	4±0.26**$** **(33.3%)**	3.7±0.19**$** **(23.3%)**	3.9±0.41**$** **(30%)**
BGAL	2.5±0.05	2.5±0.11	2.3±0.18	2.2±0.08$ **(-12%)**	2.6±0.14	2.1±0.11**$** **(-16%)**	2±0.02**$** **(-20%)**
LIP	85.3±6.66	83.1±3.33	77.1±7.5	99.7±3.61**$** **(20%)**	97.3±1.61**$** **(17.1%)**	113.6±7.65**$** **(36.7%)**	88.9±7.62
GST	545.5±55.95	594.2±37	766.2±70.5**$** **(28.9%)**	451.6±30.5**$** **(-24%)**	523.7±42**$** **(-11.9%)**	536.1±39.5	492±48**$** **(-17.2%)**

3.3.3 Transgenerational exposures

The acute or chronic exposure of a chemical to a single generation often leads to an underestimation of a chemical's toxicity. Therefore, the chronic effects of several antibiotics were assessed over three generation lifecycles of daphnids to assess the biomolecular and physiological impact antibiotics pose to *Daphnia magna*. Three generations of daphnids were chronically exposed to amoxicillin, trimethoprim erythromycin, sulfamethoxazole, and a mixture containing all antibiotics at an environmentally relevant concentration (1mg/L). F_1 neonates were taken from the initial first-generation antibiotic chronic experiment and grown under the same conditions i.e., exposed to low-concentration antibiotics and fed daily according to the

protocol. The second generation were grown until approximately day 16 when neonates aged day 1 were taken from the exposures and used to set up an F_2 generation chronic exposure. These third generation daphnids were grown until day 21. Daphnids were snap-frozen and stored to be later extracted and analysed for metabolomics, and the remaining daphnids were used in biochemical assays. In addition to this, neonates produced by the third generation were collected and grown until day 4 in the absence of chemicals, and several biochemical markers were performed. Furthermore, fourth generation *Daphnia* cultured until day 4 in clean media were also used in a toxicity experiment to assess their sensitivity to an arbitrary mixture containing lithium and nicotine, and mortality was measured at 24, 48, and 72 hours.

3.3.3.1 The third generation of exposed animals to antibiotics

Neonates (<24 hours) were collected from the primary chronic experiment separated according to their condition and cultured for 21 days under the same concentration of antibiotic (1 mg /l). After 21 days of exposure, the enzyme activities of daphnids were recorded (Table 3.3). ACP activity was not impacted by any antibiotic, however, the carrier solvent DMSO caused a significant decrease (-17.2%). Contrastingly, ALP was significantly inhibited by several antibiotics in this experiment. Amoxicillin, trimethoprim, sulfamethoxazole, and the mixture all posed a decreasing effect on ALP activity by -24.8%, -15%, -16.8%, and -21.2% respectively. Erythromycin was the only antibiotic that did not cause significant alterations in ALP activity. The β-galactosidase activity was majorly impacted by all exposure conditions. The carrier solvent DMSO caused an increase in activity (10.1%) whereas the antibiotics posed the opposite effect. Amoxicillin, trimethoprim, erythromycin, sulfamethoxazole, and their mixture caused significant decreases of -30.3%. -22.4%, -19.7%, -31.6% and -34.2% respectively. GST activity was increased by 18.1% by individual antibiotic exposure to amoxicillin and erythromycin.

Table 3.3. The transgenerational effect of antibiotics on multiple enzyme markers of third-generation *Daphnia magna* (age day 21).

Data represent average ± SD (N=4) replicates for each condition. Statistically significant by Student's t-test compared to control (*) or DMSO ($) ($p<0.05$). ALP, ACP, BGAL, and GST are expressed as milliunits/µg protein.

Enzyme	Control	DMSO	Amoxicillin	Trimethoprim	Erythromycin	Sulfamethoxazole	Mixture
ALP	11.4±0.72	11.3±1.01	8.5±1.13**$** **(-24.8%)**	9.6±0.43**$** **(15%)**	10.8±0.75	9.4±0.54 **$** **(-16.8%)**	8.9±0.25**$** **(-21.2%)**

ACP	9.3±0.39	7.7±0.36***** **(-17.2%)**	7.7±0.75	8.4±0.56	8.4±0.56	7.1±0.75	7.6±0.9
BGAL	6.9±0.22	7.6±0.47***** **(10.1%)**	5.3±0.58**$** **(-30.3%)**	5.9±0.57**$** **(-22.4%)**	6.1±0.61**$** **(19.7%)**	5.2±0.92**$** **(-31.6%)**	5±0.55**$** **(-34.2%)**
GST	2.4±0.2	2.2±0.2	2.6±0.3**$** **(18.2%)**	2.1±0.11	2.6±0.2**$** **(18.2%)**	2.5±0.2	2.4±0.1

3.3.3.2 Fourth-generation offspring of animals exposed to antibiotics

To determine whether daphnid's biomolecular and physiological status could recover after transgenerational exposure to antibiotics, they were cultured in the absence of chemical to allow a potential recovery, before measuring biomolecular endpoints. On day 16 of the third-generation experiment, neonates were collected and cultured in clean media in the absence of chemicals. However, neonates remained separated according to their previous exposure conditions. Daphnids were fed daily and grown until day 4, they were then homogenized and centrifuged to collect the supernatant, which was used to measure several biochemical markers (ALP, ACP, β-GAL, and GST) (Table 3.4).

ACP activity was significantly impacted by its previous exposure to antibiotics. DMSO and antibiotic conditions posed opposite effects on ACP activity. DMSO exposure resulted in a significant decrease in activity (-29.4%), whereas the antibiotics caused significant increases in ACP activity. Amoxicillin, trimethoprim, erythromycin, sulfamethoxazole, and the mixture were responsible for significant increases of 23.8%, 26.2%, 25%, 35.7%, and 17.9% respectively. Significant alterations of ALP activity were recorded for DMSO and several antibiotic conditions. Similarly, to ACP, DMSO demonstrated a decreasing effect on ALP activity (-11.2%). Previous exposure to erythromycin, sulfamethoxazole, and the mixture resulted in an 18.7%, 24.2%, and 24.7% increase in ALP activity respectively.

The β-galactosidase activity was variously impacted by DMSO and several antibiotic conditions. DMSO, amoxicillin, trimethoprim and sulfamethoxazole exerted a diminishing effect on β-galactosidase activity, -12.5%, -12.4%, -10.5% and -13.4% respectively. Whereas the mixture hosed the opposite effect on β-galactosidase activity, causing a 6.8% increase. A slight trend of erythromycin to increase β-galactosidase activity was observed. Lipase activity was inherently impacted by previous exposure to antibiotics, although individual antibiotics caused various effects. Both amoxicillin and trimethoprim caused a decrease in lipase activity, by -11.1% and -22.2% respectively. Contrastingly, sulfamethoxazole and the mixture both generated significant increases in lipase activity by 25.9% and 40.7% respectively. GST activity was decreased by the carrier solvent DMSO (-9.8%), however exposure to amoxicillin and trimethoprim, caused increases of 53% and 44.6% respectively.

Table 3.4. The transgenerational effect of antibiotics on multiple enzyme markers of the fourth generation Daphnia magna (age day 4).

Data represent average ± SD (N=4) replicates for each condition. Statistically significant by Student's t-test compared to control (*) or DMSO ($) (p<0.05). ALP, ACP, BGAL, and lipase are expressed as milliunits/µg protein, and GST as microunits/µg protein.

Enzyme	Control	DMSO	Amoxicillin	Trimethoprim	Erythromycin	Sulfamethoxazole	Mixture
ALP	205±10.2	182±5.36***** **(11.2%)**	181±13.8	178±2.55	216±3.9**$** **(18.7%)**	226±9.9**$** **(24.2%)**	227±2.75**$** **(24.7%)**
ACP	119±6.45	84±2.64***** **(-29.4%)**	104±9.33**$** **(23.8%)**	106±3.56**$** **(26.2%)**	105±3.29**$** **(25%)**	114±6.5**$** **(35.7%)**	99±3.41**$** **(17.9%)**
BGAL	107±4.69	93.6±2.2***** **(-12.5%)**	82±5.24**$** **(-12.4%)**	83.8±5.56**$** **(-10.5%)**	93.9±8.96	81.1±9.95**$** **(-13.4%)**	100±3.21**$** **(6.8%)**
LIP	2.6±0.11	2.7±0.05	2.4±0.12**$** **(-11.1%)**	2.1±0.13**$** **(-22.2%)**	2.7±0.21	3.4±0.19**$** **(25.9%)**	3.8±0.27**$** **(40.7%)**
GST	9.2±0.4	8.3±0.5***** **(-9.8%)**	12.7±1.5**$** **(53%)**	10.2±2	12±1.81**$** **(44.6%)**	8.8±0.61	8.8±0.8

A targeted metabolomic analysis of the central metabolic pathways; glycolysis, TCA cycle and the pentose phosphate pathway was performed on first and third generation daphnids following chronic exposure. Hierarchical clustering was performed using Pearson correlation (Figure 3.5) and grouping was achieved by performing principal component analysis. Multivariate statistical analysis (PCA) showed the metabolic fingerprints of the DMSO control and each antibiotic treatment group (Figure 3.5). For the first generation, the PC1 did not reveal distinct separation between DMSO and exposure groups; trimethoprim and the mixture formed the most defined separation of all antibiotics. More defined separation among treatments can be observed in the third generation, DMSO, amoxicillin, and erythromycin formed the clearest groups.

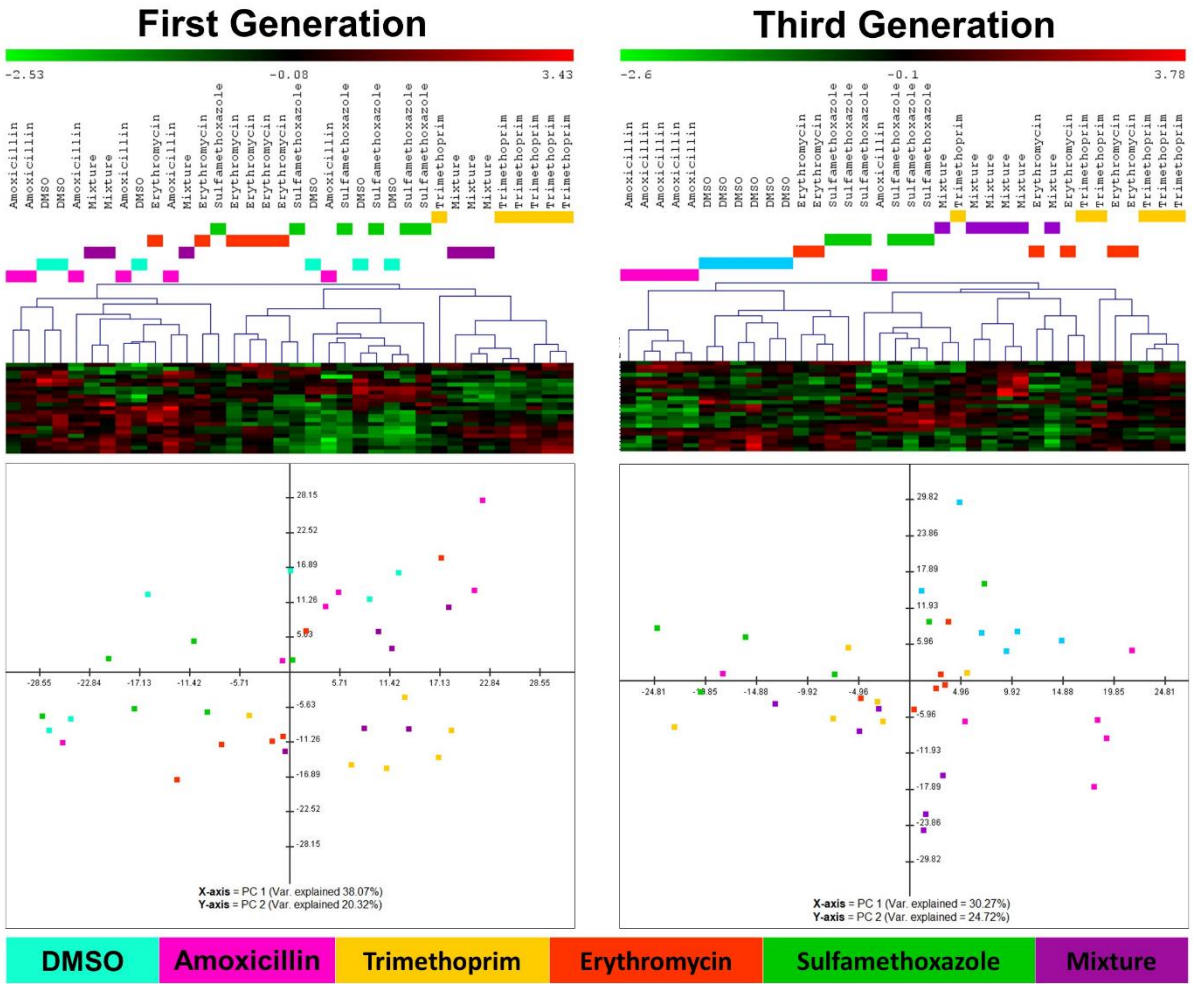


Figure 3.5. Metabolomic analysis for the first and third generation of exposures. Multivariate statistical analyses with hierarchical clustering and principal component analysis showed a distinct grouping among exposures.

A reconstruction of the central metabolic pathways, glycolysis, the TCA cycle and the pentose phosphate pathway was executed to illustrate the significant increases or decreases in metabolites detected for first and third generation daphnids (Figure 3.6). Changes were considered significant if the p-value was < 0.05 after ANOVA analysis of the log2 fold changes was performed and indicated in dark red (decrease) or dark green (increase); notable changes but not statistically significant are illustrated in light red (decrease) and light green (increase).

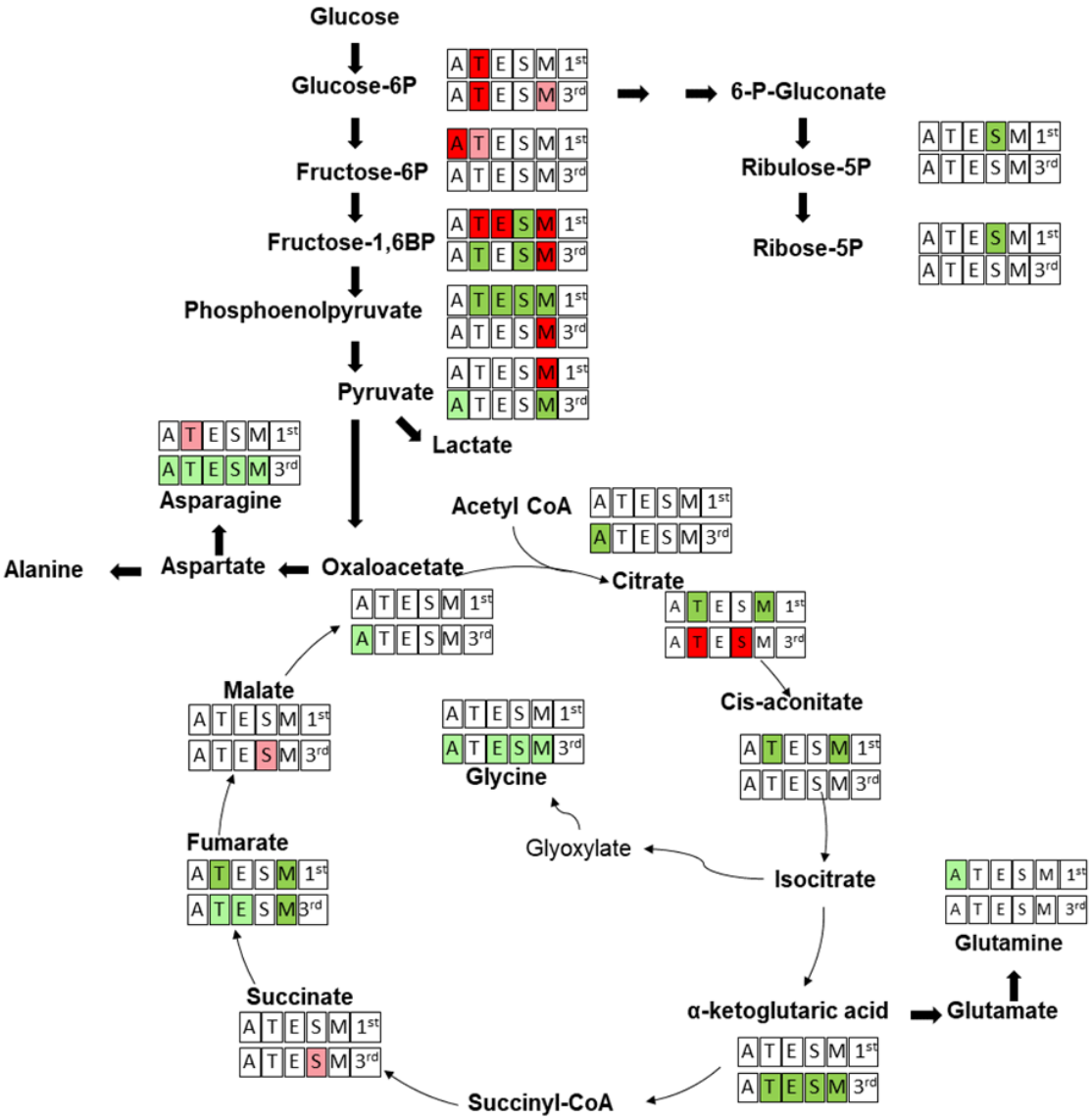


Figure 3.6. The reconstruction of the central metabolic pathways for chronic and transgenerational exposures, significant (P < 0.05) upregulations are indicated by dark red and significant downregulations are indicated by dark green. Log2fold changes which were detected but not considered significant are represented by light red (increases) and light green (decreases). The antibiotics are represented by their initial i.e. Amoxicillin = A, Trimethoprim = T, Erythromycin = E, Sulfamethoxazole = S.

Glucose, lactate, alanine, aspartate and glutamate were detected, but no significant differences or trends occurred when the exposed daphnids were compared to the controls. Within the glycolysis pathway, glucose levels were not changed by any antibiotic. Glucose-6P was increased by trimethoprim in both the first and third generations, and by the mixture in the third generation only. Amoxicillin and trimethoprim increased fructose-6P in the first generation. Fructose-1,6BP was the most affected metabolite within the glycolysis pathway; trimethoprim, erythromycin and the mixture increased fructose-1,6BP levels whereas sulfamethoxazole was responsible for decreasing levels in the first generation. Sulfamethoxazole and the mixture exerted the same effects in the third generation, whereas trimethoprim had the opposite impact resulting in a decrease. Significant decreases of phosphoenolpyruvate by trimethoprim, erythromycin, sulfamethoxazole and the mixture were recorded in the first generation; the mixture was the only condition responsible for significant change (increase) in the third generation. In the first generation, pyruvate was increased by the mixture and decreased by amoxicillin and the mixture in the third. Within the pentose phosphate pathway, sulfamethoxazole was the only compound to cause significant alterations and decreased ribulose-5P and ribose-5P in the first generation. Acetyl co-enzyme A was significantly decreased by only amoxicillin in the third generation. More changes are observed in citrate levels, decreases by trimethoprim and the mixture in the first generation were detected, and the opposite occurred in the third generation, whereby trimethoprim and sulfamethoxazole caused increases in citrate levels. A similar trend was observed in cis-Aconitate levels, which were decreased in the first generation by trimethoprim and the mixture. Alpha-Ketoglutaric acid levels were significantly increased by trimethoprim, erythromycin, sulfamethoxazole and the mixture in the third generation. No significant changes in glutamate levels were detected, although a slight decrease in glutamine by amoxicillin in the first generation was noted. Only slight increases in succinate and malate by sulfamethoxazole were recorded in the third generation, and no alterations were detected in the first. Significant decreases in fumarate levels in the first generation were documented by trimethoprim and the mixture. These decreases were also recorded for the third generation, with the addition of decreases by erythromycin. Oxaloacetate was slightly decreased in the third generation by amoxicillin alone. Finally, asparagine levels were significantly impacted by all antibiotics and the mixture in the third generation with apparent decreases being

observed, in the first-generation trimethoprim was responsible for slight increases in asparagine levels.

3.4 Discussion

As a result of their vast application in human and veterinary medicine, antibiotics are ubiquitously detected in the aquatic environment, where they remain biologically active. Not only do antimicrobial compounds exert toxic effects on non-target organisms, but they also contribute to the generation and spread of antibiotic-resistance genes (Nnadozie & Odume, 2019). Although the antibiotics of this study did not demonstrate toxicity in the immobilisation experiments, significant differences in enzyme activities and metabolism were recorded, and literature reports the ability of antibiotics to alter swimming behaviour (Pan et al., 2017), induce biochemical disruptions and genotoxicity (Nunes et al., 2018). Within the acute scenario of this study, the water-soluble antibiotics, trimethoprim, as well as erythromycin were responsible for the most significant alterations in enzyme markers, however sulfamethoxazole (DMSO soluble) also changed the activity of several enzymes. Overall, the water-soluble antibiotics were responsible for 32 changes in 40 of the metabolites detected, compared to sulfamethoxazole which only changed 8. Overall, the aqueous antibiotics had an up-regulatory effect on the metabolites significantly altered, in particular erythromycin was responsible for the most changes recorded. Specifically, the upregulation for adenosine, tryptophan, thymine, and thymidine was recorded, amoxicillin induced similar changes. Among the metabolism of daphnids acutely exposed to the water-soluble antibiotics, fourteen metabolites were commonly dysregulated. In particular, the amino acids arginine, asparagine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine and valine, nucleobases and nucleosides guanine, uracil, cytidine, hypoxanthine and finally histamine were all upregulated when compared to controls, however these changes in levels were in different extents. Literature reports the upregulation of alanine, glutamate, taurine, glycine and betaine in the blue mussel *Mytilus edulis*, after acute exposure to high concentrations of erythromycin (Liang et al., 2020). Arginine, isoleucine, leucine, phenylalanine, tryptophan, amino acids which play a role in aminoacyl-tRNA biosynthesis in daphnids. More specifically, tyrosine and phenylalanine are aromatic amino acids, that are precursors to biogenic amines (McCoole et al., 2012) and their

downstream products play pleiotropic (Christie, 2011; McCoole et al., 2012) roles in invertebrates. Methionine is responsible for controlling lifespan, egg quality and rate of fecundity in invertebrates (Grandison et al., 2009; Xu et al., 2021).

A clear trend can be seen in the chronic exposures, as amoxicillin and the mixture both majorly impacted daphnid enzyme activities and was responsible for the most dysregulations in daphnia metabolism. Interestingly, amoxicillin effected GST activity in the first, third and recovery exposures. The metabolic effect of amoxicillin and trimethoprim is particularly limited. However, penicillin has been documented to alter swimming behaviour and oxygen consumption in daphnids. More specifically, reduced swimming speed, track density, limb movement, and oxygen consumption over 24 hours was recoded (Bownik et al., 2019). Whereas, trimethoprim has been reported to affect reproduction and growth at high concentrations in daphnids. (De Liguoro et al., 2012). Moreover, in *Poecilia reticulata* (guppy), trimethoprim altered swimming activity and of the production of CYP1A protein which is an environmental marker of hypoxia of the gills, kidneys, and intestines.

It is accepted that antibiotics are often excreted by humans and animals incompletely metabolized, and therefore they eventuate in the freshwater environment biologically active and unchanged within excretory matter, studies report this is especially true for the macrolide antibiotic erythromycin (Szymańska et al., 2019). The antibiotic compounds aminosidine, bacitracin, erythromycin, and lincomycin were assessed for their toxicity to daphnids, and erythromycin demonstrated the second most toxicity (di Delupis et al., 1992). Moreover, it was reported that the other antibiotics altered phototaxis, specifically, lincomycin and bacitracin decreased phototactic behaviour, whereas aminosidine increased phototaxis and erythromycin did not induce significant changes to this endpoint. In this study erythromycin impacted enzyme activities in all toxicity scenarios including acute, chronic, and transgenerational exposures, however, it had a particular impact on first generation daphnids following chronic exposure. Erythromycin was also responsible for extensive metabolic dysregulation in the acute exposure scenario.

The effect of sulfamethoxazole on the phenotypic endpoints of daphnids such as ingestion, body length and fecundity has been reported (Lu et al., 2013). In a concentration-dependent manner (0.37-10 mg/L) sulfamethoxazole reduced the ingestion rate. Between concentrations of 0.12 and 10 mg/L, sulfamethoxazole increased days to first pregnancy, as well as days to first brood, decreased the number

of offspring per female in the first brood, decreased length (mm), and decreased the intrinsic rate of natural increase. Our findings showed that sulfamethoxazole exerted significant effects on the activities of several enzymes as a result of acute and chronic exposures to the compound. Similarly, to amoxicillin, fourth generation daphnids, whose mothers were previously exposed to sulfamethoxazole, demonstrated the highest level of sensitivity to the antibiotic in terms of biochemical endpoints. As mentioned previously, the DMSO soluble antibiotic, sulfamethoxazole only dysregulated 8 metabolites, specifically, acetylcarnitine, histamine, adenosine and putrescine levels were increased while nicotinamide, benzoic acid, choline and pantothenate were decreased. The purine nucleoside adenosine has a crucial role within the mitochondria of the cell and in phosphorylation processes. Although, in this study, adenosine levels were upregulated, literature reports the downregulation of adenosine following exposure to halogenated acetic acid's (Labine & Simpson, 2021). Generally, there is limited research regarding the effect of antibiotics on daphnids at the molecular level. However, in another ecotoxicological model *Mytilus galloprovincialis*, significant changes in the amino acids, aspartate, valine, phenylalanine and tryptophan and in the nucleotides guanosine and inosine were detected following sulfamethoxazole exposure. Alike our findings, these molecular changes were not coupled with biochemical disruption, specifically in markers of xenobiotic metabolism and oxidative stress (Serra-Compte et al., 2019). Histamine, the neurochemical, is considered to control phototactic behaviour in arthropods (McCoole et al., 2011) as well as histidine metabolism, in this study histidine was upregulated by sulfamethoxazole. However, the opposite was observed following exposure to bisphenol pollutants in *Daphnia magna*. Moreover, the downregulation of histamine, choline, cysteine, glutamic acid, histidine, isoleucine, leucine, malic acid, and phenylalanine (Oliveira Pereira et al., 2021) was recorded. On the contrary, in our study the upregulation of the metabolite choline, which is involved in glycine, serine and threonine metabolism was detected after sulfamethoxazole exposure.

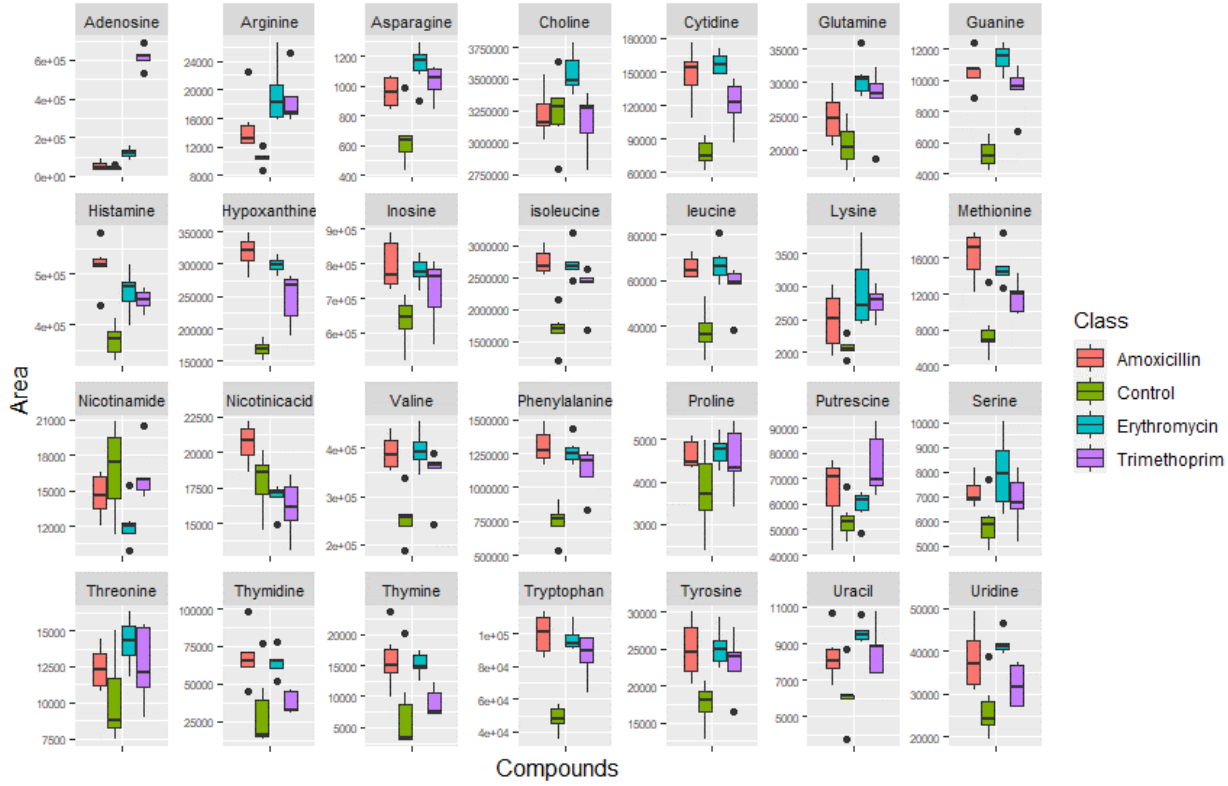
Antibiotics have been reported to alter the sex ratio of daphnids (Flaherty & Dodson, 2005). Acute exposure to a mixture containing erythromycin, trimethoprim, and triclosan (10 µg/L each) resulted in 20% fewer males than compared to controls. Acute and chronic exposure to a larger mixture containing 5 antibiotics (erythromycin, triclosan, trimethoprim, lincomycin, and sulfamethoxazole) had concentration-dependent effects on sex ratio but did not impact endpoints such as survival, growth,

and fecundity. At higher concentrations (100 µg/L each) an increase in males was recorded, whereas at lower concentrations (10 µg/L each) a decrease in males was noted. Our findings showed that the mixture did cause significant changes throughout all exposure scenarios, however it did not obviously induce an overall greater response in daphnids compared to the individual antibiotics. Although, the mixture, closely followed by amoxicillin, was responsible for the most metabolic dysregulations in the chronic analysis. Specifically for the biochemical markers, trimethoprim generated the greatest effect on enzyme activities, this could be a result of changes at the transcriptomic level, which may have resulted in altered expression of genes encoding for the specific enzymes, thus impacting their levels. On the other hand, it could solely be an effect on the metabolic level, as a result of altered enzyme activity or feedback inhibition.

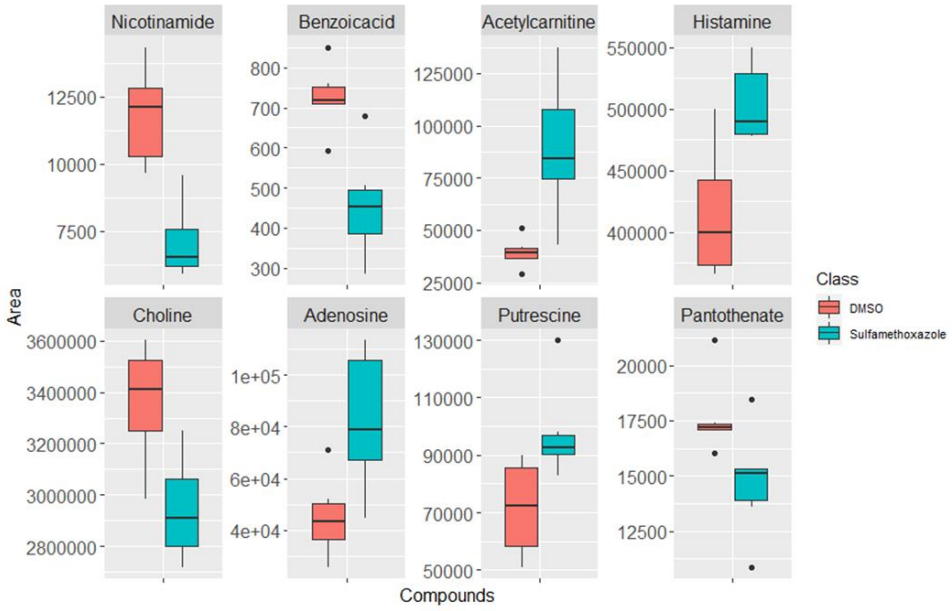
3.5 Conclusion

Major changes in the activity of several enzyme markers of physiology and oxidative stress, as well as metabolic perturbations were recorded for each antibiotic in both acute and chronic exposure experiments. Regarding acute exposures, trimethoprim demonstrated the most toxicity to daphnids, impacting 6 of 8 markers measured, whereas amoxicillin showed the least only impacting 2 of 8 markers. However, amoxicillin did have a greater effect on daphnid metabolism compared to trimethoprim. Daphnids did not demonstrate obvious sensitivity to transgenerational exposure with regard to their enzyme activities and metabolism, as significant changes in these endpoints were not greater or more frequent in the third-generation scenario.

3.6 Supplementary



Supplementary Figure 3.1. Box plot of chromatographic peak areas of statistically significant metabolites in daphnids exposed to amoxicillin, erythromycin trimethoprim and their aqueous OECD control.



Supplementary Figure 3.2. Box plot of chromatographic peak areas of statistically significant metabolites in daphnids exposed to sulfamethoxazole and the DMSO control.

Supplementary Table 3.1. Statistically significant metabolites with AUC, p and log 2-fold change values in antibiotic treated daphnids in relation to the control or DMSO group.

Name	AUC	T -test	Log2 FC	AUC	T -test	Log2 FC	AUC	T -test	Log2 FC	AUC	T -test	Log2 FC
	Amoxicillin Vs Control			Erythromycin Vs Control			Trimethoprim Vs Control			Sulfamethoxazole Vs DMSO		
Acetylcarnitine										0.97	4.64E-03	-1.18
Adenosine				1.00	5.45E-05	1.41	1.00	1.75E-09	3.78	0.88	2.14E-02	-0.86
Arginine	1.00	2.53E-02	0.50	1.00	4.95E-04	0.88	1.00	6.89E-04	0.84			
Asparagine	0.92	4.94E-03	0.57	0.97	3.95E-04	0.82	0.93	3.80E-03	0.66			
Benzoic acid										0.97	1.91E-03	0.66
Choline				0.89	4.56E-02	0.13				0.91	5.17E-03	0.19
Cytidine	1.00	6.93E-05	0.93	1.00	2.11E-07	1.02	0.93	2.57E-03	0.64			
Glutamine				1.00	1.95E-04	0.56	0.87	2.77E-02	0.40			
Guanine	1.00	4.81E-06	1.00	1.00	3.39E-07	1.11	1.00	4.81E-04	0.83			
Histamine	1.00	7.22E-05	0.48	0.97	1.17E-03	0.33	1.00	1.04E-03	0.28	0.88	1.56E-02	-0.28
Hypoxanthine	1.00	1.31E-07	0.92	1.00	4.16E-09	0.83	1.00	1.25E-03	0.55			
Inosine	1.00	2.67E-03	0.33	1.00	1.04E-03	0.30						
isoleucine	1.00	3.53E-05	0.70	1.00	8.07E-05	0.69	0.87	1.28E-02	0.46			
leucine	1.00	6.93E-05	0.81	1.00	1.65E-04	0.84	0.93	1.11E-02	0.60			
Lysine				0.85	1.98E-01	0.52	1.00	1.76E-02	0.62			
Methionine	0.97	3.45E-04	1.08	0.97	6.59E-04	0.95	0.87	3.14E-02	0.59			
Nicotinamide				0.86	2.23E-02	-0.46				1.00	5.03E-04	0.73
Nicotinic acid	0.89	2.39E-02	0.20									
Pantothenate										0.86	4.08E-02	0.26
Phenylalanine	1.00	1.69E-05	0.80	1.00	1.08E-05	0.76	0.97	2.51E-03	0.58			
Proline				0.81	4.13E-02	-0.33						
Putrescine							0.97	5.67E-03	0.49	0.91	7.87E-03	-0.44
Serine	0.86	2.73E-02	0.27	0.92	2.01E-02	0.42						
Threonine				0.89	1.47E-02	0.49						
Thymidine	0.83	1.70E-02	1.14	0.86	1.39E-02	1.07						
Thymine	0.83	2.85E-02	1.14	0.83	2.18E-02	1.07						
Tryptophan	1.00	5.39E-06	1.06	1.00	6.66E-07	1.02	1.00	3.15E-04	0.85			
Tyrosine	0.97	4.24E-03	0.51	1.00	6.54E-04	0.52	0.87	2.97E-02	0.39			
Uracil	0.89	2.71E-02	0.44	1.00	4.68E-04	0.65	0.93	2.03E-02	0.50			
Uridine	0.89	1.52E-02	0.54	1.00	4.39E-04	0.66						
Valine	1.00	2.52E-04	0.61	1.00	2.41E-04	0.63	0.87	2.13E-02	0.43			

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Chapter 4

The impact of environmentally relevant pharmaceuticals on daphnids

Abstract

Water monitoring at national and global levels has become increasingly important as research continues to reveal the hazards associated with specific chemical groups, including pharmaceuticals. Moreover, EU countries are required to inspect surface and ground waters in compliance with the Water Framework Directive. In this chapter, the pharmaceuticals investigated are referred to as “environmentally relevant”, attributed to their inclusion on past or present chemical Watch List or as a result of their ubiquitous prevalence within the aquatic environment. The acute and chronic effects of the five pharmaceuticals; diclofenac, metformin, gabapentin, gemfibrozil and carbamazepine, were investigated using daphnids as a model species in ecotoxicology. A suite of biochemical markers related to physiology and oxidative stress were measured in combination with the assessment of the central metabolic pathways of daphnids. The findings of this chapter revealed in both acute and chronic exposures, metformin and gabapentin to have the greatest impact on biochemical and molecular endpoints. In particular, following acute exposure these compounds altered 20 common metabolites, in particular thymine, thymidine, and acetylcarnitine. Chronic exposure to both metformin and gabapentin revealed increases in GST activity and other enzymes of metabolism, while also impairing central metabolism, in particular the TCA cycle and glycolysis.

4.1 Introduction

This chapter focused on the acute, chronic and transgenerational effects of a range of environmentally relevant pharmaceuticals on the ecotoxicological model *Daphnia magna*, namely diclofenac, metformin, gabapentin carbamazepine and gemfibrozil (Figure 4.1). The molecular and phenotypic response of daphnids were assessed as metrics for their effects and could be compared with the effects induced by daphnids exposed to environmental water samples.

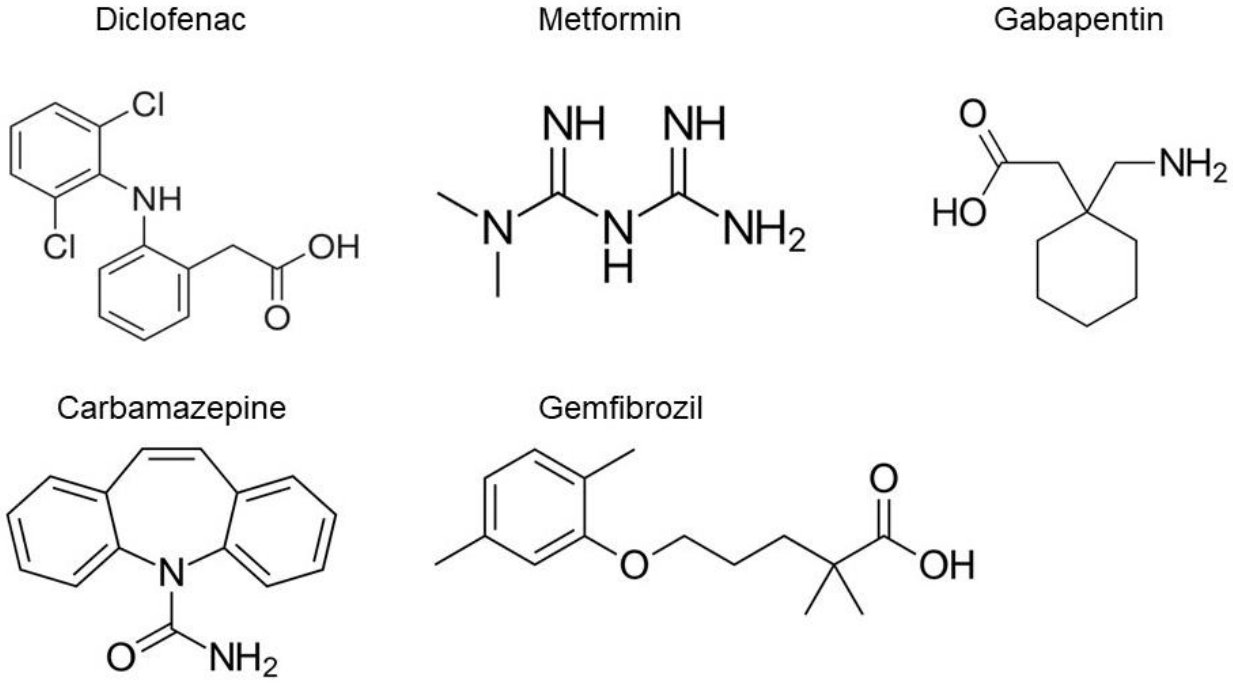


Figure 4.1. Chemical structures of pharmaceuticals used in this chapter.

4.2 Materials and methods

4.2.1 Algae and daphnids culturing

Algae and daphnids were cultured using the same protocols described in chapter 2 (see section 2.2.1 and Appendix A).

4.2.2 Acute and chronic toxicity exposures

Acute and chronic toxicity exposures were executed following the approach outlined in chapter 2 (see section 2.2.2 and 2.2.3).

4.2.3 Sample homogenization for biochemical analyses

Samples were prepared using the same methods in the previous chapter (see section 2.2.6). Protein was quantified using an ultrasensitive Bradford method as described in the materials and methods of chapter 2 (see section 2.2.7). The activity of enzymes (alkaline phosphatase, acid phosphatase, beta-galactosidase, lipase, peptidase, lactate dehydrogenase, glutathione reductase, glutathione-S-transferase) and the quantification of lipid hydroperoxides were measured as described in chapter 2 (see section 2.2.8).

4.2.4 Statistical analysis for biochemical markers

The data were analysed previously described in chapter 2 (see section 2.2.9).

4.2.5 Extraction of metabolites

Samples from acute and chronic exposures were prepared and analysed similar to previous chapters. Metabolites were extracted from daphnid samples following the method outlined in chapter 2 (see section 2.2.10).

4.2.6 Metabolomic analyses

The metabolomic analysis of samples from acute exposures was executed as described in chapter 2 for the corresponding samples (see section 2.2.11). For samples of chronic exposures, the protocol outlined for the targeted LC-MS/MS analysis in chapter 3 was followed (see section 3.2.7). Data acquisition and analysis were performed in Analyst® software (Version 1.7.1). All further analyses were done in in-house written R scripts.

4.2.7 **Reagents and chemicals**

Metformin, gabapentin, gemfibrozil and carbamazepine were purchased from Acros Organics. Diclofenac was purchased from Thermo Scientific. See section 2.2.12 for all other chemical and reagent purchases.

4.3 Results

4.3.1 The impact of environmentally relevant pharmaceutical compounds in acute exposures

A range of pharmaceuticals with diverse modes of action was investigated for their impact to several biochemical and molecular endpoints. Initially, to assess the toxicity potential of the selected pharmaceuticals, immobilisation tests were performed. Daphnids were exposed to each chemical at a non-environmentally relevant concentration of 50 mg/L for 24 hours to determine their toxicity potential. Chemicals which caused mortality were further investigated to generate full toxicity curves and EC values were calculated (Table 4.1). Toxicity curves for four days old daphnids over a range of concentrations for an exposure period of 24 hours showed different mortality levels for pharmaceuticals (Figure 4.2). The toxicity curves of each chemical were extremely steep, and the resulting EC values for diclofenac, gemfibrozil and metformin were all in a similar order of magnitude, specifically diclofenac and metformin. From this experiment, gemfibrozil was deemed to possess the highest toxicity potential as it caused 50% mortality at 27.86 mg/L, whereas EC_{50} for diclofenac and metformin was considerably higher (118.9 mg/L and 148 mg /l).

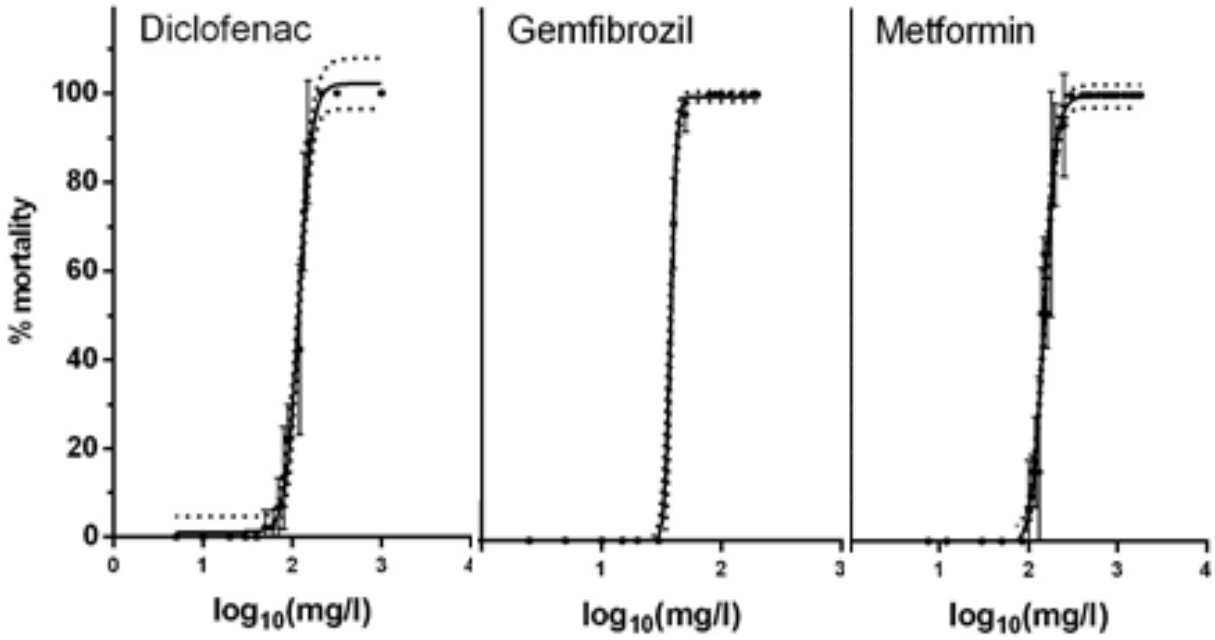


Figure 4.2. Toxicity curves for diclofenac, gemfibrozil, and metformin. Fifteen 4-day-old daphnids per replicate of each concentration were exposed to individual pharmaceuticals over a period of 24 hours. Data represents average±SD (N=3).

Table 4.1. EC values for mortality-causing pharmaceuticals investigated				
Pharmaceutical	**EC_1**	**EC_5**	**EC_{10}**	**EC_{50}**
Diclofenac	61.029	77.5	86.43	118.9
Gemfibrozil	19.12	21.89	23.27	27.86
Metformin	72.6	93.77	105.28	148

4.3.1.2 Phenotypic and biochemical markers of physiology

Daphnids were exposed to diclofenac, metformin and gemfibrozil at their EC_5 concentrations (Table 4.1) and gabapentin and carbamazepine at 50 mg/L. Following 24 hours of exposure, the protein content of daphnids was quantified, and enzyme activity was measured for multiple markers of physiology and oxidative stress (Table 4.2).

All conditions excluding DMSO, and carbamazepine induced statistical differences in acid phosphatase (ACP) activity. Metformin, diclofenac gabapentin and gemfibrozil caused significant decreases (-41.4%, -53.6%, -19.9% and -46.9%) in ACP activity. With the exception of DMSO and gabapentin, all exposure conditions initiated significant decreases in alkaline phosphatase (ALP) activity. Metformin, diclofenac, gemfibrozil and carbamazepine decreased ALP activity by -41.9%, -54.5%, -60.6% and -19.4% respectively.

The activity of β-galactosidase was altered significantly by three compounds, metformin, carbamazepine and gemfibrozil. Metformin caused the most notable decrease of -45.4%, whereas gemfibrozil decreased β-galactosidase by 27.8%. Carbamazepine was the only pharmaceutical to increase activity (26.1%).

Changes in lipase activity were recorded for all chemicals except for gemfibrozil. The chemicals which induced these changes all exerted a diminishing effect on lipase activity. Diclofenac was responsible for the most significant decrease (-24.1%). All pharmaceuticals except carbamazepine significantly altered peptidase activity, DMSO, metformin, diclofenac, gabapentin and gemfibrozil caused decreases of 23.6%, 82.1%, 49.1%, 47.2% and 37.7% respectively. DMSO, gabapentin and carbamazepine were the only compounds to affect LDH activity, DMSO and gabapentin caused increases of 48% and 112%, in contrast, carbamazepine caused a decrease of 21.6%.

All pharmaceuticals induced significant increases in GST activity. Gemfibrozil caused the greatest increase (1393.6%), whereas gabapentin caused the lowest increase

(9.1%). Metformin, diclofenac and carbamazepine produced similar increases in the level of GST activity of 90.9%, 148.1% and 175.6% respectively.

Table 4.2. The acute effect of environmentally relevant pharmaceuticals on multiple enzyme markers of *Daphnia magna* (age day 4).

Data represent average ± SD (N=4) replicates for each condition. Statistically significant by Student's t-test compared to control (*) or DMSO ($) (p<0.05). ALP, ACP, BGAL and GST are expressed as milliunits/µg protein, and LIP as units/µg protein.

Enzyme	Control	DMSO	Metformin	Diclofenac	Gabapentin	Gemfibrozil	Carbamazepine
ALP	57.1±0.8	71±4* **(24.3%)**	33.2±4.6* **(-41.9%)**	26±4.9* **(-54.5%)**	58.6±8.8	28.1±1.5$ **(-60.6%)**	57.2±6.4$ **(-19.4%)**
ACP	26.1±3.8	26.2±1	15.3±2.4* **(41.4%)**	12.1±3.5* **(-53.6%)**	20.9±1.8* **(-19.9%)**	13.9±2.1 **(-46.9%)**	26.9±3.7
BGAL	73.8±7	76.6±7.6	40.3±5.1* **(-45.4%)**	84.8±6.7	87.7±10.5	55.3±5.4$ **(-27.8%)**	96.6±4.6$ **(26.1%)**
LIP	2.9±0.1	2.5±0.1* **(-13.8%)**	2.4±0.1* **(-17.2%)**	2.2±0.4* **(-24.1%)**	2.4±0.1* **(-17.2%)**	2.4±0.3	2.2±0.1$ **(-12%)**
PEP	21.2±0.5	16.2±1.1* **(-23.6%)**	3.8±1.9* **(-82.1%)**	10.8±1* **(-49.1%)**	11.2±0.7* **(-47.2%)**	10.1±2.4$ **(-37.7%)**	18.3±1.4
LDH	2.5±0.4	3.7±0.4* **(48%)**	2.4±0.3	2.5±0.5	5.3±0.4* **(112%)**	3±0.5	2.9±0.2$ **(-21.6%)**
GST	7.7±0.5	7.8±0.2	14.7±1.4* **(91%)**	19.1±0.6* **(148.1%)**	8.4±0.3* **(9.1%)**	116.5± 1.2$ **(1393%)**	21.5±0.2$ **(175.6%)**

4.3.1.3 Metabolic perturbations induced by environmentally relevant pharmaceuticals

A targeted analysis of the hydrophilic content of daphnids was assessed in a metabolomic based analysis and revealed significant alterations in metabolite levels for metformin and gabapentin when compared to the control, and for carbamazepine and gemfibrozil in comparison with DMSO. The metabolomics data derived from the chromatic peak areas were extracted and further analysed. To validate the stability of the run, the QC samples were inspected using the PCA model. The QC samples were closely grouped together, therefore confirming the validity of the system during analysis. OPLS models revealed clear separation of the controls (OECD and DMSO) and the treatment groups (Figure 4.3) Model statistics, and the CV ANOVA p value indicated the high predictability of the significance of the models. The univariate statistical test, student's T-test was employed to identity the specific metabolites that were significantly altered. In the context of the OECD soluble compounds, metformin and gabapentin were responsible for the alteration of 26 and 23 polar compounds (20 common) respectively. With the exception of choline and cytosine for metformin and nicotinamide and thiamine for gabapentin, the compounds significantly changed were all upregulated (Supplementary Table 4.1). Metformin and gabapentin considerably

dysregulated the pathways associated with carbohydrate metabolism, amino acid metabolism and purine and pyrimidine metabolism. With regard to carbamazepine and gemfibrozil (DMSO soluble drugs), perturbations of 23 and 10 metabolites were recorded respectively. Decreases in essential / non-essential amino acids, purines, pyrimidines and majority of the nucleosides were detected after exposure to gemfibrozil, whereas increases in vitamins including riboflavin and nicotinic acid, uracil, betaine and acetylcarnitine were recorded. Carbamazepine had a lesser impact on the polar metabolic profile of daphnids when compared to the three other treatment groups. In total, 10 metabolites were detected as significantly different to DMSO treated daphnids, 5 of these metabolites were commonly changed by gemfibrozil, however, glucose was uniquely increased by carbamazepine. Moreover, carbamazepine was the only pharmaceutical to significantly alter benzoic acid levels, coupled with changes in uracil and cytidine, indicating the potential disruption of pyrimidine metabolism (Supplementary Table 4.1).

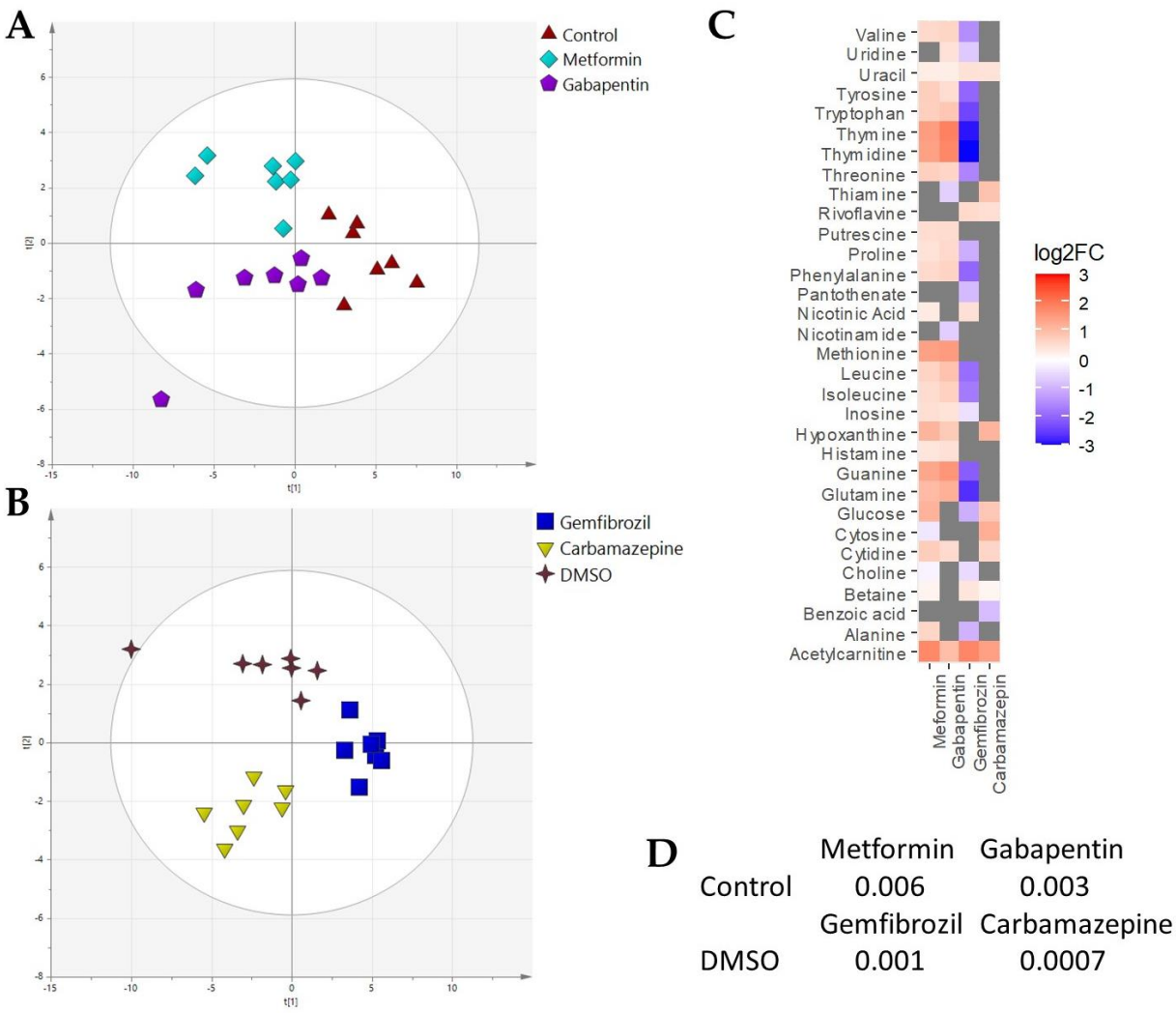


Figure 4.3. Multivariate statistical analysis of the metabolic impact of environmentally relevant pharmaceuticals on daphnids. PLS-DA score plots based on data from targeted LC-MS/MS metabolic profiling of extracts of daphnids exposed (A) to water soluble pharmaceuticals (metformin, gabapentin) and (B) DMSO soluble pharmaceuticals (gemfibrozil and carbamazepine). (C) heatmap of Log2FC statistically significant compounds with p<0.05 and (D) CV ANOVA p value of OPLS-DA models.

4.3.2 The chronic effects of environmentally relevant pharmaceutical compounds

Firstly, to identify working concentrations of the chronic experiments, acute toxicity exposures were executed using daphnids aged <24 hours old (Figure 4.4) to identify the toxicity potential of pharmaceuticals on the neonate. Toxicity curves were generated for the pharmaceuticals and EC values were calculated (Table 4.3). Gabapentin did not induce mortality even at extremely high concentrations (100 mg/L); hence a toxicity curve was not generated for it. Diclofenac proved to be the most toxic, as greater mortality occurred at lower concentrations compared to its counterparts.

However, overall, the pharmaceuticals had similar EC values when compared with each other.

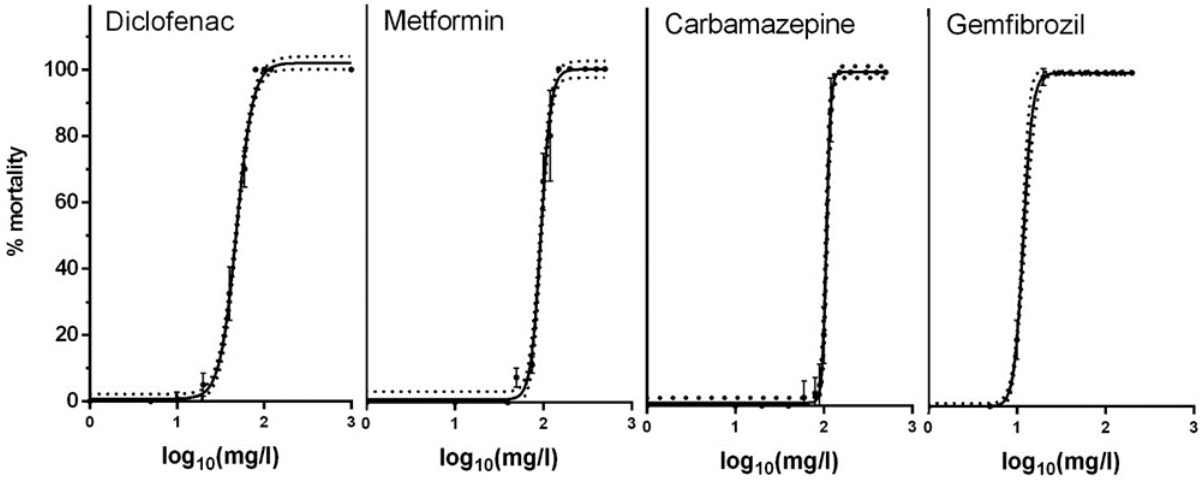


Figure 4.4. Toxicity curves of pharmaceuticals. Twenty neonates (<24 hours) were exposed for 24 hours to different pharmaceuticals in 50 ml OECD media. Data represent average±SD (N=4) for each concentration.

Table 4.3. Effect Concentration (EC) in mg/L for diclofenac, metformin, carbamazepine and gemfibrozil, calculated for day 1 daphnids exposed to pharmaceutical for 24 hours.

Pharmaceutical	**EC_1**	**EC_5**	**EC_{10}**	**EC_{50}**
Diclofenac	37.3	53.4	62.8	101.3
Metformin	54.8	67.8	74.6	99.04
Carbamazepine	83.5	91.5	95.3	107.5
Gemfibrozil	57.5	70.1	76.7	100

To assess the chronic effect of the pharmaceuticals, neonates <24 hours old were exposed the individual pharmaceuticals and their mixture for 21 days. The group of compounds were further split into two sub-groups based on their solubility in OECD media. Diclofenac, metformin and gabapentin are water-soluble chemicals and easily dissolved directly in OECD, whereas carbamazepine and gemfibrozil required DMSO as a carrier solvent. Daphnids were exposed to each chemical at 1 mg/L, and the mixture conditions comprised of each chemical (of their respective sub-group) at 1 mg/L. Thus, the OECD soluble mixture composed of diclofenac, metformin and gabapentin at 3 mg/L and the DMSO soluble mixture contained carbamazepine and

gemfibrozil at 2 mg/L. DMSO was compared against the control but acted as the control for the daphnids exposed to carbamazepine, gemfibrozil and their mixture. Following exposure to diclofenac, metformin, gabapentin and their mixture for 21 days, several enzyme markers were measured (Table 4.4).

Table 4.4. The chronic effect of diclofenac, metformin, and gabapentin and their mixture on the enzyme activity of daphnids. Data represents average±sd (n=4) replicates for each condition. Statistically significant differences in exposures compared to control (*) were identified by the student's t-test ($p<0.05$). ALP, ACP, BGAL, LIP and PEP are expressed as milliunits/µg protein, LDH and GST are expressed as microunits/µg protein.

Enzyme	Control	Diclofenac	Metformin	Gabapentin	Mixture
ALP	13.8±1.4	11.7±0.8* **(-15.2%)**	11.3±0.7* **(-18.1%)**	13.4±1.5	11.8±1.7
ACP	4.4±0.3	3.2±0.01* **(-27.3%)**	4.1±0.4	3.8±0.2* **(-13.6%)**	3.6±0.3* **(-18.2%)**
BGAL	3.2±0.4	2.7±0.3	2.5±0.3* **(-21.9%)**	2.8±0.1	3.1±0.3
LIP	12.2±0.4	12±1.4	14.1±2.4	14.8±0.4* **(21.3%)**	12±1.5
PEP	2.5±0.2	2.6±0.1	2.7±0.04* **(8%)**	2.8±0.2	2.4±0.2
LDH	39.4±8.4	31.2±6	50.1±4.2	46.2±6.7	71.9±11.2* **(82.5%)**
GST	213.9±21.3	223.3±71.6	265.6±11.6* **(24.2%)**	298.4±15.2* **(39.5%)**	303.7±22.2* **(42%)**

ACP and ALP activity were both affected by several of the compounds assessed. The only compound to exert a significant effect on both phosphatases was diclofenac, which significantly decreased ACP activity (-26.1%) but notably increased ALP activity (15.4%). Metformin was the only other compound to induce a significant increase (18.6%) in ALP activity. Gabapentin and the mixture condition initiated significant decreases in ACP activity (-14.2% and -17.4% respectively).

Metformin was the only pharmaceutical which exerted an effect on β-GAL activity, and caused a significant decrease of -21.9%. Similarly, metformin was also the only pharmaceutical to affect peptidase activity and was responsible for an 8% increase. All compounds except for diclofenac significantly altered GST activity. Metformin, gabapentin and the mixture condition caused a 24.2%, 39.5% and 42% increase in GST activity respectively.

After 21 days of exposure to carbamazepine, gemfibrozil and their mixture, several enzymes related to physiology and oxidative stress were measured (Table 4.5).

The carrier solvent DMSO was the only condition to cause a significant difference in both ACP and ALP activity, DMSO exposure resulted in an increase of 10.3% and 21.6% in both ACP and ALP. Carbamazepine and gemfibrozil did not significantly affect ALP activity but did decrease ACP activity by 15.6%and 9.4%.

Gemfibrozil was the only pharmaceutical to alter the activity of BGAL and was responsible for an increase of 38.9%. Similarly, to the phosphatases, lipase activity was only significantly changed by DMSO and not by the pharmaceuticals, DMSO caused a 20.6% increase in lipase activity when compared to the control. Peptidase activity was significantly impacted by every pharmaceutical condition and the carrier solvent. DMSO was responsible for an increase in peptidase activity (73.7%), whereas carbamazepine, gemfibrozil and the mixture caused decreases of 45.8%, 28.9% and 31.6% respectively.

GST activity was significantly altered by all conditions in the experiment. When compared to the OECD control, DMSO increased GST activity by 69.3%. Carbamazepine was responsible for the greatest decrease in GST activity (-43.9%), whereas gemfibrozil and the mixture caused similar decreases of -14.1% and -15.2% respectively.

Table 4.5. The chronic effect of a range of environmentally relevant pharmaceuticals on multiple enzyme markers of *Daphnia magna.*

Data represent average ± SD (N=4) replicates for each condition. Statistically significant by Student's t-test compared to control (*) or DMSO ($) ($p<0.05$). ALP, ACP, BGAL and LIP are expressed as milliunits/μg protein, PEP and GST are expressed as microunits/μg protein.

Enzyme	Control	DMSO	Carbamazepine	Gemfibrozil	Mixture
ALP	3.7±0.3	4.5±0.4***** **(21.6%)**	3.9±0.3	5±0.2	3.9±0.3
ACP	2.9±0.1	3.2±0.2***** **(10.3%)**	2.7±0.2**$** **(-15.6%)**	2.9±0.02**$** **(-9.4%)**	3±0.1
BGAL	1.6±0.2	1.8±0.2	1.9±0.2	2.5±0.2**$** **(38.9%)**	2±0.1
LIP	71.5±6.7	86.2±4.7***** **(20.6%)**	84.4±6.4	82.4±3.6	92.2±6.2
PEP	319±55.4	554±15.5***** **(73.7%)**	300±78.1**$** **(-45.8%)**	394±33.2**$** **(-28.9%)**	379±16.6**$** **(-31.6%)**
GST	221±51.3	374.2±24.4***** **(69.3%)**	210±29.8**$** **(-43.9%)**	321.6±2.2**$** **(-14.1%)**	317.3±10**$** **(-15.2%)**

The significant physiological responses of daphnids which were recorded were coupled with obvious metabolic perturbations in relevance to the central carbon metabolism with emphasis on glycolysis, pentose phosphate pathway and the Tricarboxylic Acid (TCA) cycle. A targeted analysis of key metabolites revealed clear metabolic differences based on the multivariate analysis of their fingerprints (Figure 4.5). Multivariate statistical analysis revealed distinct groups among the different exposures. For the dataset of aqueous dissolved pharmaceuticals, OECD controls separate on the principal component 1 (PC1) axis from metformin and even further from gabapentin. On the other hand, the separation of PC2 is observed for the mixture of all the aforementioned pharmaceuticals. Regarding the other dataset, for carbamazepine and gemfibrozil, there is a clear separation of the mixture on the PC1 axis from all groups, while DMSO seems to have an intermediate effect.

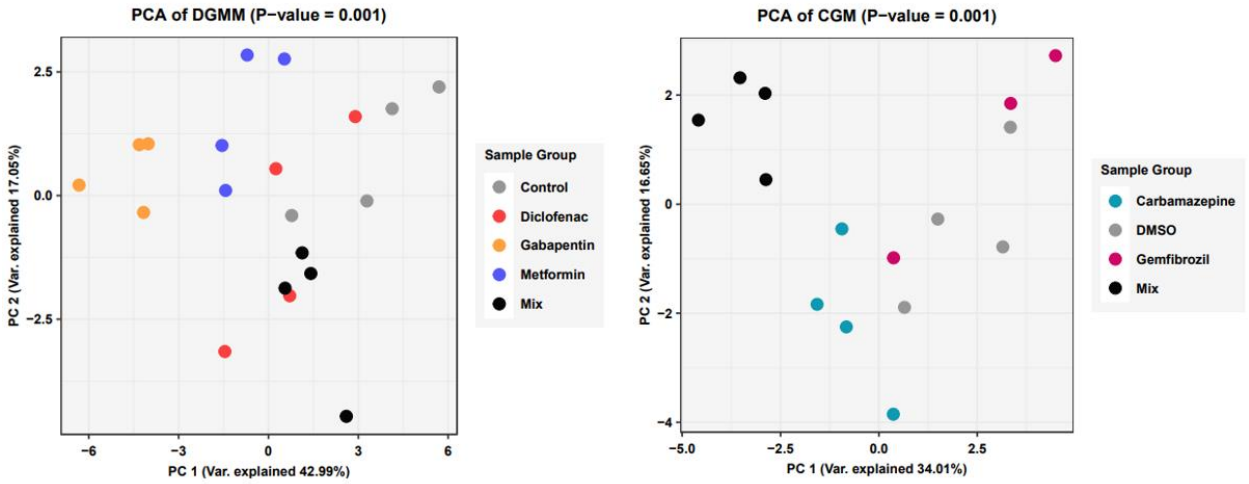
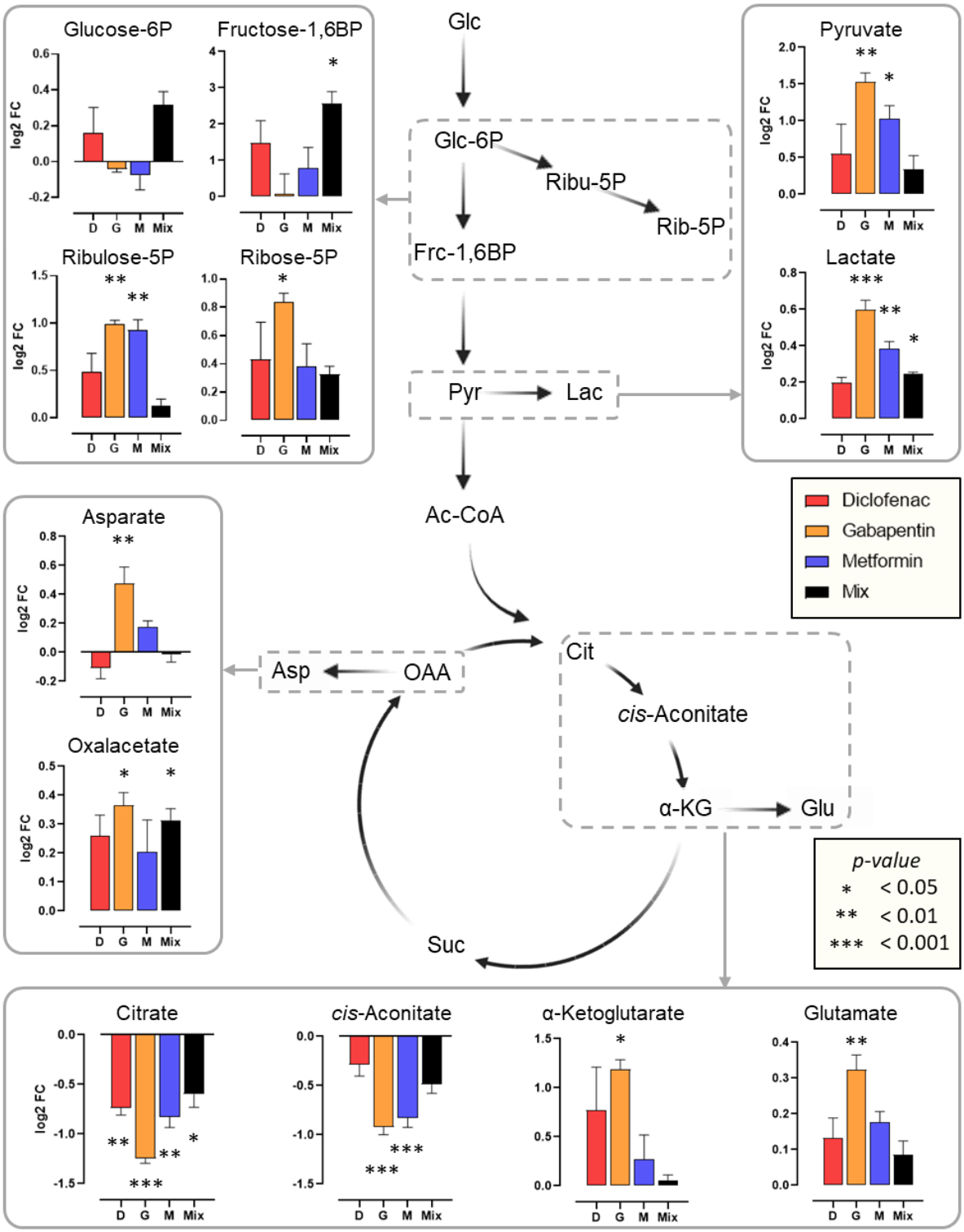


Figure 4.5. Principal component analysis for aqueous pharmaceuticals compared to the OECD control (left) and the non-aqueous pharmaceuticals compared to DMSO (right).

A reconstruction of the central metabolic pathways discussed revealed alterations in metabolites of the glycolysis, the pentose phosphate pathway and the TCA cycle (Figure 4.6). Exposure to aqueous pharmaceuticals resulted in decreased levels of citrate and cis-aconitate, which is accompanied by an increase in α-ketoglutarate, subsequently indicating a flux towards glutamate. In addition, there is an increase towards oxaloacetate, however, only gemfibrozil and metformin this was accompanied by a shift towards aspartate, therefore, exiting the TCA cycle. For glycolysis, glucose-6-phosphate was increased in diclofenac and the mixture but decreased in gabapentin and metformin, however, in all exposures a shift towards the pentose phosphate pathway was observed by the increase for ribulose-5-phosphate and ribose-5-phosphate. Chronic exposure to all DMSO-soluble pharmaceuticals increased citrate, malate and glutamate and a decrease in α-ketoglutarate. However, for other metabolites, there were more treatment-specific responses. Specifically, asparagine and glucose-6-phosphate were decreased in gabapentin but this was reversed for carbamazepine and the mixture.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Fructose-1,6BP
Ribulose-5P
Ribose-5P
log2 FC
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Rib-5P
Frc-1,6BP
Pyr
Lac
Pyruvate
Lactate
Ac-CoA
Asparate
Oxalacetate
Asp
OAA
Cit
cis-Aconitate
α-KG
Glu
Suc
Diclofenac
Gabapentin
Metformin
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*** < 0.001
Citrate
cis-Aconitate
α-Ketoglutarate
Glutamate

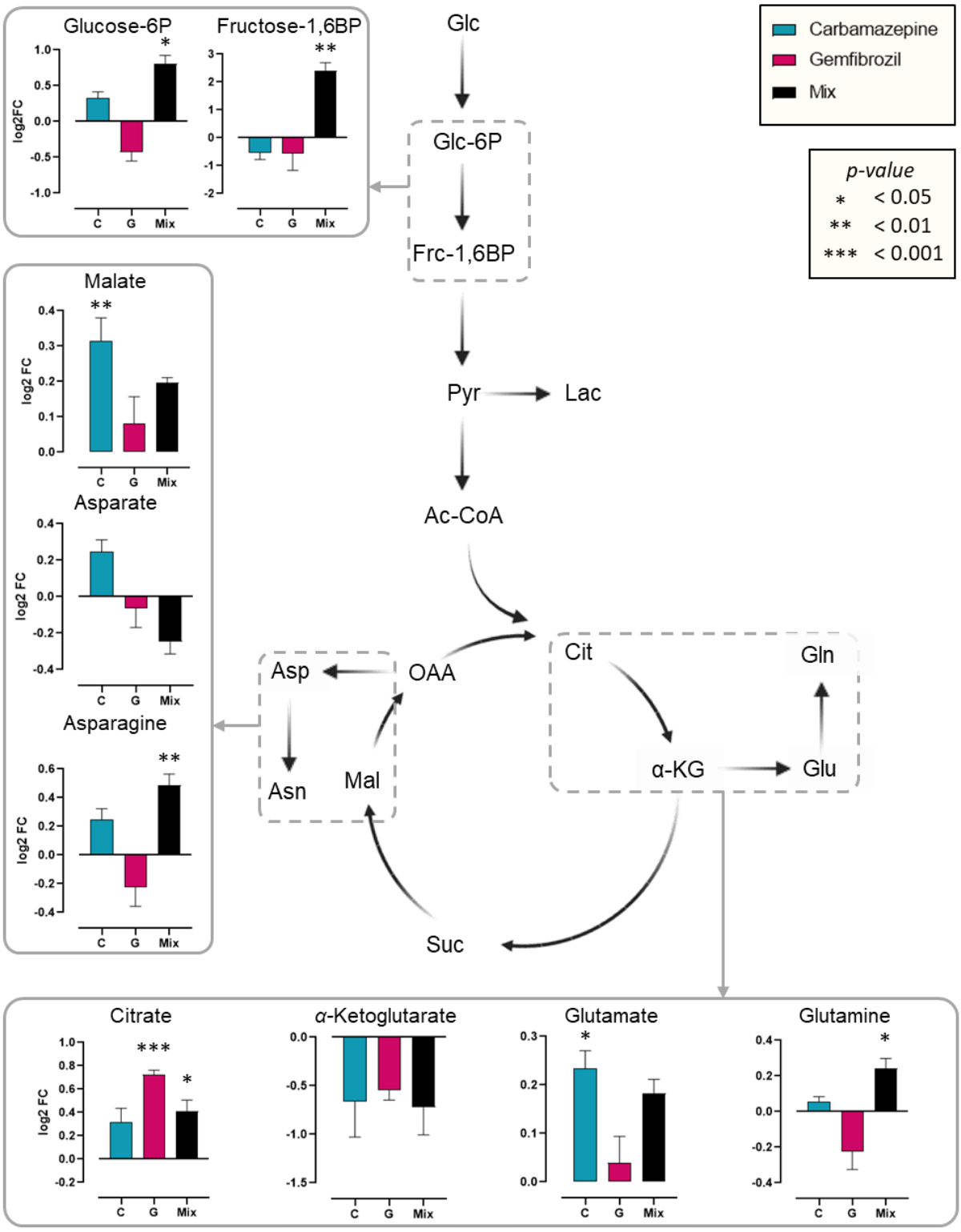

Figure 4.6. The impact of pharmaceuticals on the central metabolism of daphnids, specifically glycolysis, the pentose phosphate pathway and the tricarboxylic cycle . A. Statistical significance is displayed for diclofenac, gabapentin, metformin and their mixture compared with the OECD control. B. Carbamazepine, gemfibrozil and their mixture are compared with DMSO as their carrier solvent.

4.4 Discussion

In recent years, the assessment of pharmaceutical compounds in the aquatic ecosystem and their potential impact on non-target organisms has been of central focus in many research studies. Although pharmaceutical compounds play a pivotal role in mitigating both minor and serious health issues of humans and animals in society, their extensive use and complex modes of action threaten the normal functioning of aquatic ecosystems.

4.4.1 The toxic effect of diclofenac on aquatic biota

Diclofenac is a common NSAID that exists in many forms; oral tablets, suppositories and topical ointments, and have many applications in both human and veterinary medicine, for this reason, it is one of the most detected compounds in aquatic environmental matrices. As a result of its ubiquitous nature, diclofenac has been reported to disrupt the physiology of non-target organisms, particularly invertebrates (Bonnefille, Gomez, Courant, et al., 2018; Gonzalez-Rey & Bebianno, 2014; Leverett et al., 2021; Parolini, 2020). Diclofenac is classified as a selective inhibitor of the cyclooxygenase-2 (COX) enzyme, which catalyses the conversion of arachidonic acid to prostaglandins, which play a key role in the inflammatory response. As diclofenac is a selective inhibitor, it does not impede the activity of COX-1, which is essential for the maintaining of the stomach lining (Figure 4.7). Inhibition of the COX-2 enzyme results in a reduction of prostaglandins which promote pain, fever and inflammation.

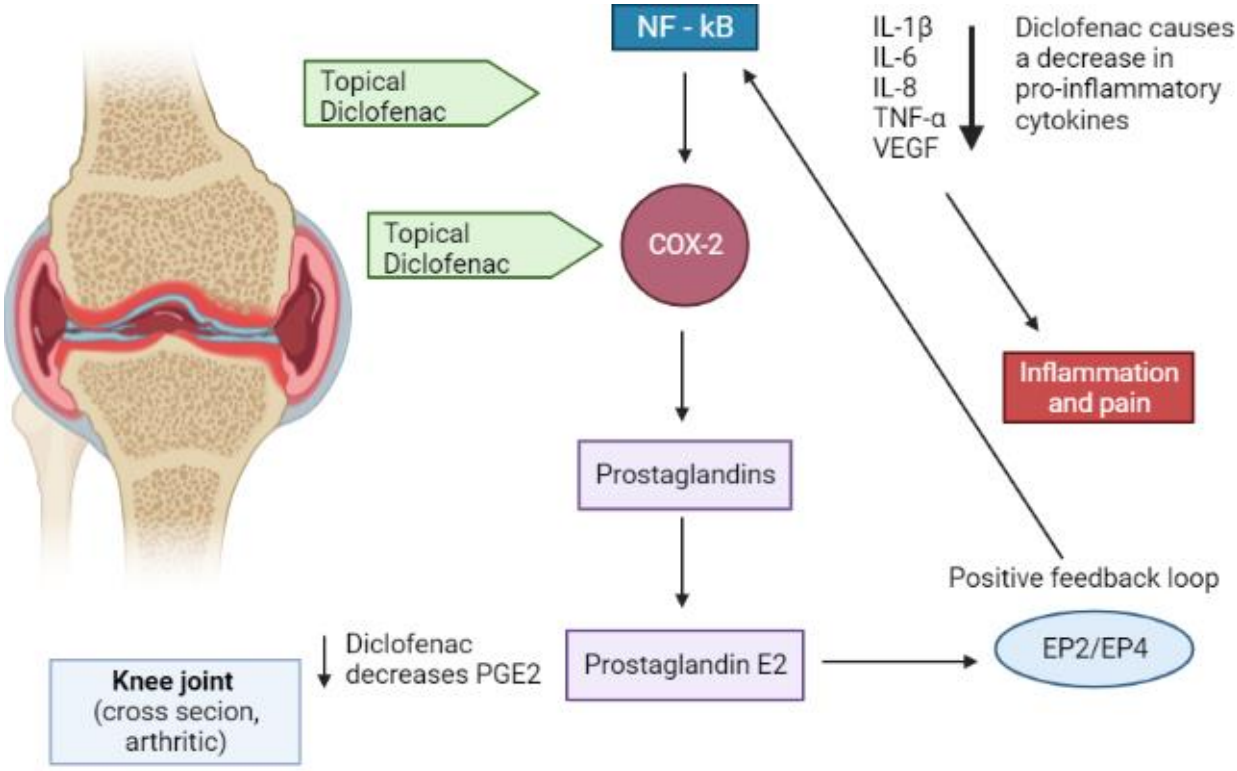

Figure 4.7. The mechanism of topical diclofenac via the modulation of COX-2 and the NFκB pathway, which results in decreased prostaglandin E2 plasma levels and pro-inflammatory cytokines (figure adapted from Bariguian Revel et al., 2020).

Acute exposure to diclofenac led to the inhibition and induction of expression for several selected genes related to metabolism, growth, development and reproduction ((Liu et al., 2017)/, P-gp, CYP36OA8, CYP31, GST, EcR and Vtg) in *Daphnia magna*. At environmentally relevant concentrations (50 µg/L), chronic exposure to diclofenac delayed the time to first egg production and time to first brood (Y. Liu et al., 2017b). In another study, at higher concentrations (>30 mg/L), diclofenac exposure resulted in an increase in the stress-induced protein Hsp70 in 12-19 day old daphnids (Haap et al., 2008). There are also reports of diclofenac altering enzyme activity, in a study daphnids were acutely exposed to diclofenac (0.08-18.4ng/L) and several enzymatic markers were measured (GST, CAT, ChE, Total GPx and selenium-dependent GPx) (L. L. D. Oliveira et al., 2015). Exposure to diclofenac resulted in decreased cholinesterase (ChE) activity and in selenium-dependent glutathione peroxidase (GPx) activity (L. L. D. Oliveira et al., 2015). Decreased filtration and ingestion rates coupled with enhanced oxidative stress via increased levels of ROS and increased AchE activity have also been documented (Nkoom, Lu, Liu, Dong, et al., 2019) Contrastingly, the opposite has also been reported, specifically increased glutathione

peroxidase and lipid peroxidation, and decreased superoxide dismutase (SOD) as well as DNA damage following 48- and 96-hours exposure (Gómez-Oliván et al., 2014). The findings of our study showed that diclofenac exerted alterations of biochemical markers after acute and chronic exposure. In chronic exposures, diclofenac significantly affected enzymes of metabolism, specifically ALP, ACP and PEP and decreased metabolites such as citrate and aconitate while increasing α-keto glutarate and glutamate, indicating a metabolic shift from the TCA cycle.

4.4.2 The impact of anti-convulsant drugs on freshwater organisms

Globally, carbamazepine is the most highly consumed anti-convulsant drug, due to its extensive use in the treatment of epilepsy and attributed to its low removal efficiency in WWTPs and persistence, it has been widely detected in the freshwater environment. For example, in a study conducted in China, carbamazepine had a 100% detection rate in 29 samples collected from Nansi Lake basin, confirming its ubiquitous nature (J. Wu et al., 2022). The underlying mechanism of action of carbamazepine is still not fully understood, however literature indicates its interaction with different channel types and synaptic transmission, specifically voltage-gated $Na^{2+,}$ Ca^{2+} and K^{+} channels (Figure 4.8) (Ambrósio et al., 2002; Worley & Baraban, 1987). Moreover, it is postulated that carbamazepine triggers the inhibition of glutamate release, adenosine receptors and the modulation of serotonin, dopamine and cyclic adenosine monophosphate (Biber et al., 2001; Okada et al., 1998).

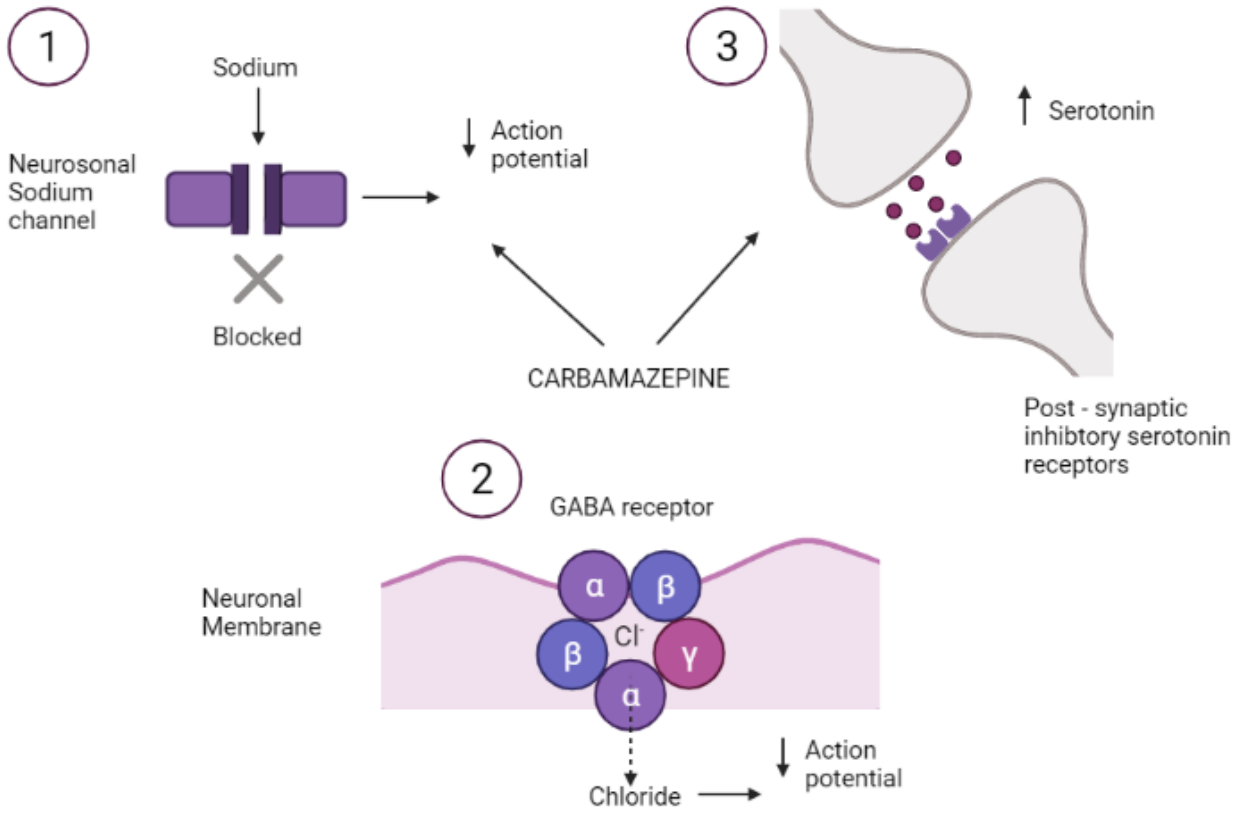

Figure 4.8. The mechanism of action of carbamazepine (Figure adapted from Harkin & Hopkinson, 2010). Carbamazepine exerts its mode of action by (1) blocking inactive sodium channels, inhibiting the generation of action potential, (2) acting as an agonist of the GABA receptor, allowing the influx of chloride into the cell and preventing the generation of action potential, (3) increasing the levels of the inhibitory neurotransmitter serotonin at neural synapses.

Carbamazepine has been reported to impact numerous life history parameters and physiological endpoints of fish and invertebrates (Contardo-Jara et al., 2011; Qiang et al., 2016). In a chronic study involving *Daphnia magna*, carbamazepine exposure (0.001 -0.5 mg/L) resulted in the decrease of multiple endpoints such as fecundity, fertility and growth rate (Tian et al., 2019). Similarly, in another chronic exposure to carbamazepine using maternal daphnids, the reproductive output and moulting frequency of daphnids was significantly decreased and induction of male offspring was recorded (Oropesa et al., 2016). In contrast, the opposite was documented in a study using *Daphnia pulex*, when exposed to carbamazepine (1µg/L), daphnids matured earlier compared to the controls, and at certain body lengths reproduction was increased. However, when exposed to higher concentrations (100 and 200 µg/L), the reproduction rate declined by 9 and 32% when compared to the controls and daphnids exposed to lower concentrations of carbamazepine (<10 µg/L) (De Lange et al., 2006). Moreover, there are reports of carbamazepine inhibiting feeding habits

(ingestion/filtration), negatively impacting phototactic behaviour, and affecting multiple enzymatic markers (acetylcholinesterase, superoxide dismutase, catalase, glutathione reductase) and induction of oxidative stress, leading to a compromised antioxidant defence system (Nkoom, Lu, Liu, Yang, et al., 2019b). Similar to diclofenac, carbamazepine exhibited toxicity in both acute and chronic exposure, GST was the most impacted enzyme marker in both scenarios. The dysregulation of 10 metabolites was recorded in the acute metabolomic analysis, of these 10, 5 were commonly altered by gemfibrozil also. Uniquely, carbamazepine increased glucose levels and significantly altered benzoic acid levels, while it also impacted uracil and cytidine, indicating a potential disruption of pyrimidine metabolism. Concerning chronic exposure to carbamazepine, minor and specific impacts of carbon metabolism were recorded at the metabolic level, in particular increases in malate and glutamate levels.

Gabapentin is another anticonvulsant drug used in the treatment of seizures and neuropathic pain of individuals with epilepsy. Exacerbated by increased consumption, poor metabolism in humans and low removal rates from WWTPs, gabapentin is now considered a contaminant of concern. However, information relating to gabapentin and its toxicity potential in an aquatic setting is scarce, and literature regarding its non-target effects on aquatic organisms is limited. Like carbamazepine the mode of action is not fully understood, although it is a structural analogue of the neurotransmitter γ-amino butyric acid (GABA), it does not directly interact with GABA receptors. The mode of action of gabapentin is similar to the action of carbamazepine, involving synaptic transmission. In the presynaptic nerve, gabapentin inhibits the voltage-gated Ca^{2+} channels, thus preventing the release of glutamate (Figure 4.9) (Sutton et al., 2002). Other reports suggest the inhibition of glutamatergic N-Methyl-D-aspartate (NMDA) receptors and increased release of GABA by gabapentin (Kang et al., 2014; Petroff et al., 2000).

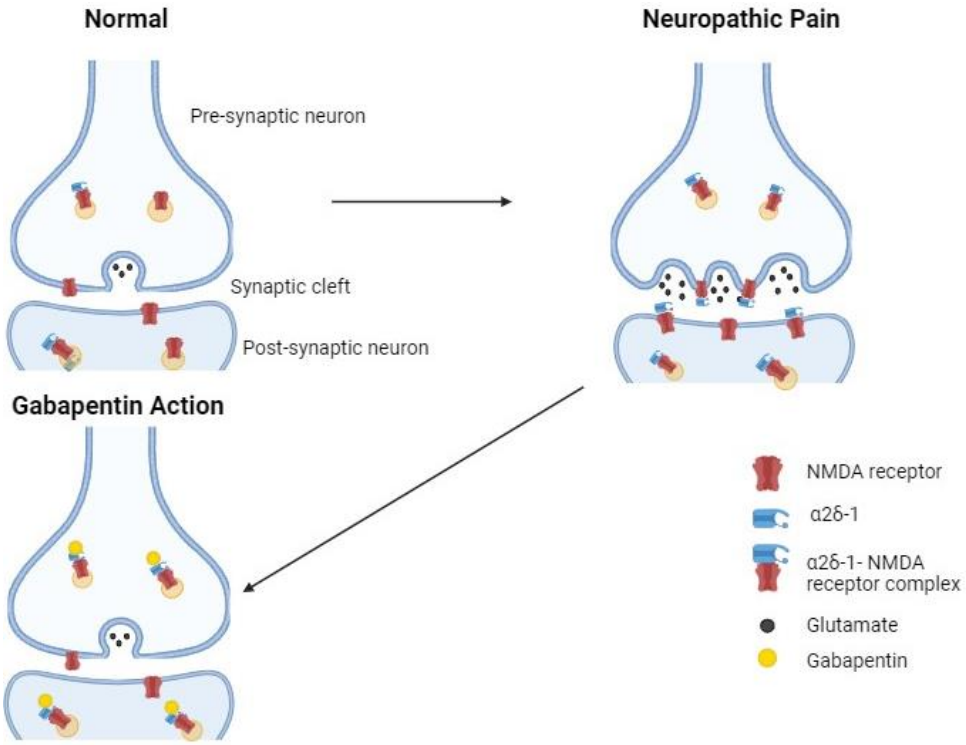

Figure 4.9. The mode of action of gabapentin (Figure adapted from J. Chen et al., 2018). Gabapentin binds to specific auxiliary subunits of voltage-gated calcium channels i.e α2δ subunit, this binding does not result in blockage of the channels but modulates their activity. In turn, this reduces the influx of calcium ions into the pre-synaptic terminal, leading to the decreased release of neurotransmitters i.e. glutamate from synaptic vesicles into the synaptic cleft for post-synaptic receptors to bind.

In a transcriptomics-based analysis investigating the toxicity potential of gabapentin on the embryos of zebrafish, gabapentin exhibited molecular and biochemical effects. Embryos were exposed to relevant concentrations (1 and 10 µg/L) of gabapentin, results revealed the differential expression of 136 and 750 genes. Gene ontology (GO) analysis and the Kyoto encyclopaedia of genes and genomes (KEGG) pathway analysis identified the involvement of these genes in the antioxidant, immune and nervous systems (He et al., 2019). Gabapentin has been documented to disrupt several phenotypic endpoints in zebrafish embryos such as swimming behaviour, body length and heart rate, as well as phenotypic changes, the generation of organ malformations was also recorded. Furthermore, biochemical endpoints were also impacted, the enzymes CAT, LDH and GST were significantly changed. In this study, gabapentin exerted significant effects on multiple biochemical markers in acute and chronic experiments, the endpoints which were most greatly impacted by short-term and long-term gabapentin exposure were PEP and GST respectively. Acute exposure

to gabapentin resulted in 23 significant metabolite alterations, which was the second highest number of changes recorded in this scenario, all significant changes were recorded as increases, with the exception of nicotinamide and thiamine. In the chronic metabolomic analysis, gabapentin was responsible for exerting the greatest impact on almost all metabolites except glucose-6-phosphate and fructose-1,6-bisphosphate in the glycolysis pathway, thus demonstrating the disruption of central carbon metabolism upon exposure.

4.4.3 The effects of the anti-diabetic drug metformin on aquatic invertebrates

Metformin or widely known as Glucophage is a medication used as the first line of treatment of type 2 diabetes, and also has applications in the treatment of polycystic ovary syndrome and individuals with insulin resistance (prediabetic). As a result of the global diabetes epidemic, coupled with metformin being the only anti-diabetic prescription available, it is frequently detected up to two orders of magnitude higher than other pharmaceutical compounds in the freshwater ecosystem (Briones et al., 2016). Metformin is a biguanide which combats insulin resistance through three main processes;

(i) Decreases blood glucose concentration by decreasing hepatic glucose production.
(ii) Reduces intestinal glucose absorption.
(iii) Improves insulin sensitivity by increasing preferable glucose uptake and utilization.

These processes are all mediated by metformin which activates activated protein kinase (AMP/AMPK) (Figure 4.10). AMPK is an enzyme produced in the liver which controls insulin signalling, energy balance, and the metabolism of glucose and fat.

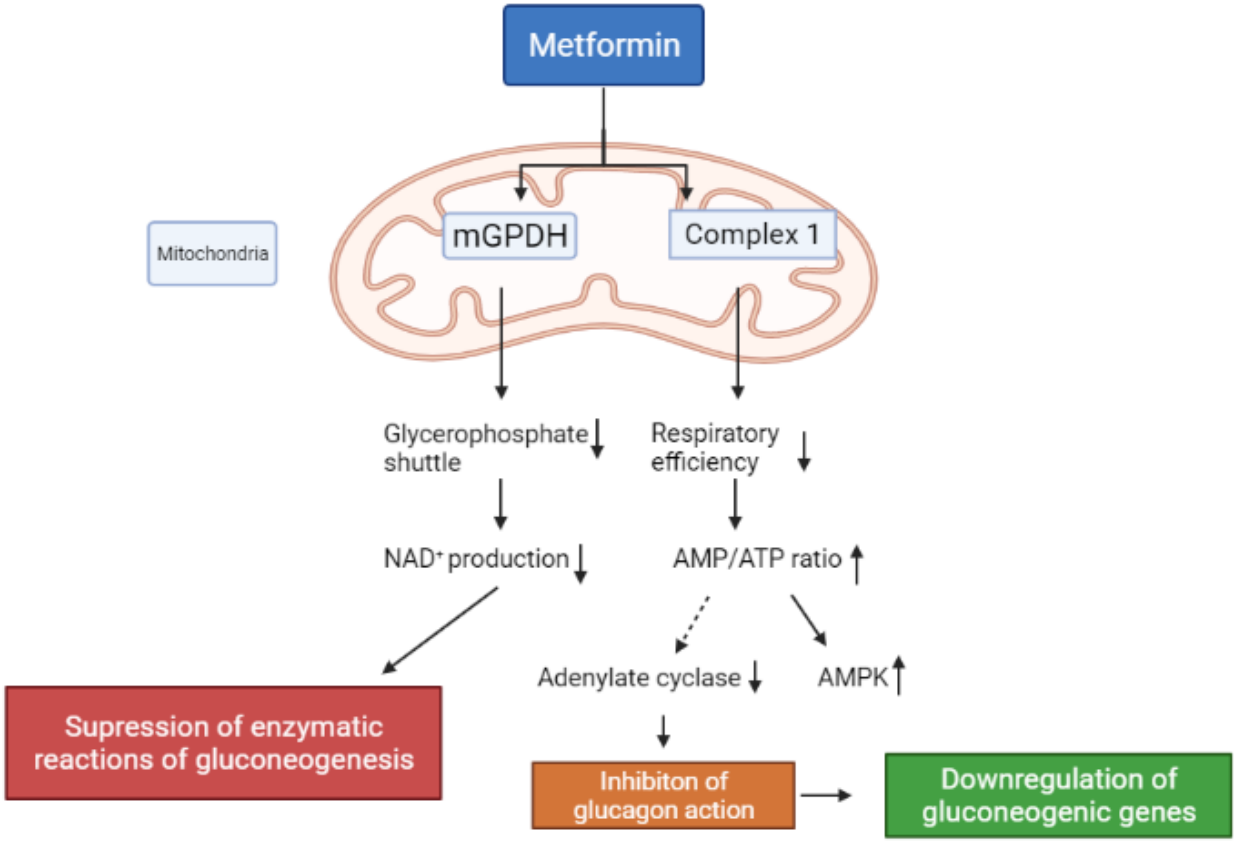

Figure 4.10. Mode of action of the biguanide metformin (Figure adapted from Minamii et al., 2018). Metformin reduces mitochondrial respiration through the inhibition of complex 1 of the electron transport chain, leading to an increase in the AMP:ATP ratio, and ultimately the activation of AMPK and the suppression of gluconeogenic genes. It is also thought that the increase of cellular AMP levels is likely to inhibit adenylate cyclase thus preventing glucagon action. Additionally, metformin inhibits the enzyme mGDPH, in turn supressing the production of NAD+ required for gluconeogenic reactions.

Information regarding the effect of metformin on non-target organisms, specifically *Daphnia*, is relatively low, however, limited studies are reporting its effects on various other invertebrates and fish (Jacob et al., 2019; Xie et al., 2021). Although structurally different to hormones, metformin has been named as a potential endocrine-disrupting compound (EDC). Male fathead minnows displayed several intersex traits such as oocytes developing in the testes and decreased reproduction between pairs with intersex males (Niemuth & Klaper, 2015). Similar findings were recorded in Japanese rice fish, metformin exposure resulted in intersex females and alterations of the biochemical system; including the production of ROS, decreased glutathione (males) and increased catalase (females) (Lee et al., 2019). Transcriptomic studies have revealed the ability of metformin to disrupt mRNA expression in the mussel *Mytilus*

edulis. Thermal stress coupled with metformin exposure led to an increase in vitellogenin mRNA expression thus inflicting severe pathologies on the gonads in turn leading to the destabilization of the lysosomal membrane of the haemocytes (Koagouw & Ciocan, 2018). At environmentally relevant concentrations, exposure to metformin caused an increase in heart rate, population decrease and reduced reproduction in daphnids (Das, 2016). In our study metformin, significantly affected multiple enzymes, in particular, it increased ALP and GST activity and decreased βGAL activity. Acute exposure to metformin resulted in the most significant changes (26) in metabolite levels recorded, particularly amino acids. The metabolic impact of metformin is extremely under reported, however fenoxycarb, another known endocrine disruptor has been reported to increase the amino acids, leucine, asparagine, methionine, and isoleucine in *Daphnia magna*, which was observed in this study. On the contrary, in the same study, putrescine was decreased but our findings showed the opposite (T. Y. Jeong & Simpson, 2020). Furthermore, the chronic metabolomic analysis revealed that metformin posed a significant impact on the glycolytic pathway in particular; as ribulose-5-phosphate, pyruvate and lactate were increased. A shift in energy use was revealed with obvious decreases in citrate and cis-aconitate, which was similarly seen in the case of diclofenac.

4.4.5 The impact of gemfibrozil on freshwater invertebrates

Gemfibrozil is a lipid-regulatory compound, used to treat dyslipidaemia in humans. As global obesity rates continue to rise, the consumption of gemfibrozil has increased and as a result it is now considered an emerging contaminant of concern that is ubiquitously detected in environmental matrices, particularly during warmer months (Couto et al., 2019; Fang et al., 2012). As a fibrate drug, gemfibrozil is a proliferator-activated receptor α (PPAR-alpha) agonist and controls blood lipid level, by binding to the receptor, increasing fatty acid oxidation and in turn reducing triglycerides (Figure 4.11).

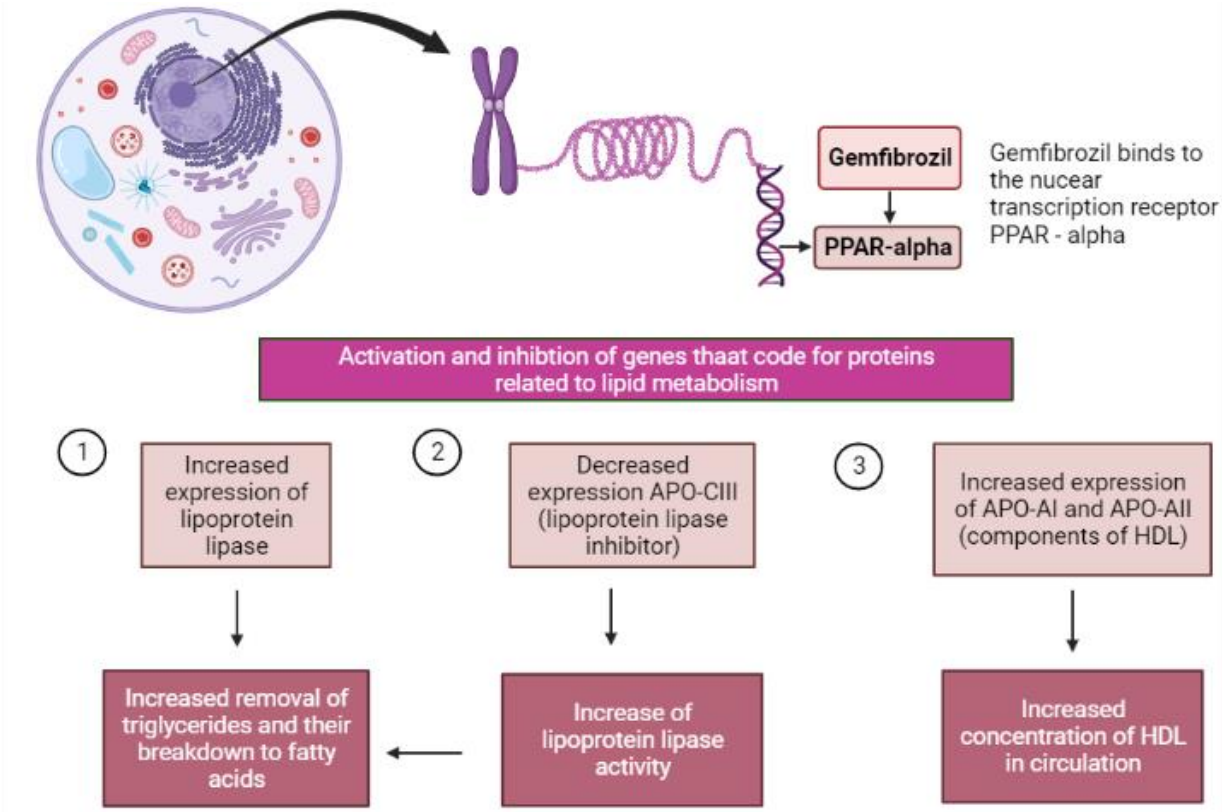

Figure 4.11. Mode of action of gemfibrozil (Figure adapted from Goldenberg et al., 2008). Gemfibrozil activates PPAR-alpha, a transcription factor of genes involved in lipid catabolism, which results in the increased expression of lipoprotein lipase, thus leading to increased removal of triglycerides. Moreover, lipoprotein lipase activity is enhanced by the decreased expression of APO-CIII, an inhibitory enzyme of lipoprotein lipase. Lastly, greater removal of cholesterol is achieved through increased HDLs in circulation, this is mediated via increased expression of the proteins APO-AI and APO-AII, which are components of HDLs.

There are limited studies regarding the impact of gemfibrozil on biochemical endpoints of *Daphnia*, however, there are reports of gemfibrozil disrupting biochemical activity in the zebra mussel *Dreissena polymorpha*. Exposure to this lipid regulator (in µg/L range) resulted in elevated levels of GST and metallothionein activities and increased lipid peroxidation, while DNA damage was recorded after 96 hours (Quinn et al., 2011). In this study, changes in enzyme activities were documented for both acute and chronic scenarios. Gemfibrozil showed trends to exert a stronger effect on enzymes of metabolism after acute exposure and stronger effects on enzymes of detoxification following chronic exposure. The lipid regulator gemfibrozil has been reported to disrupt normal biological functioning and life history traits of vertebrate and invertebrate organisms of the aquatic ecosystem (Barreto et al., 2018). Exposure to gemfibrozil at 50 ng/L resulted in increased mass, increased length and increased neonate

reproduction in daphnids, daphnids exposed to higher levels of the compound (500 ng/L) had increased cholesterol levels. In the same study, under conditions where food availability was low, daphnids previously exposed to gemfibrozil, grew just as well as the control group (Steinkey et al., 2018). The effect of gemfibrozil on feeding behaviour and reproduction at two temperatures (22°C and 28°C) was investigated (Salesa et al., 2017). Ingestion and filtration rates were not impacted by gemfibrozil at several environmentally realistic concentrations (0.1,0.5,1,5 and 7.5 mg/L) at either temperature. On the contrary, an increase in temperature resulted in decreased reproductive outputs including brood size and the total number of offspring per female. Furthermore, at the highest exposure concentration, cholesterol levels of daphnids decreased. Regarding the metabolomic analysis, acute exposure to gemfibrozil resulted in the alteration of 23 metabolites. Specifically, essential and non-essential amino acids, purines, pyrimides and most nucleosides were decreased, whereas vitamins like rivoflavine and nicotinic acid, uracil betaine and acetylcarnitine were upregulated. In this study, chronic exposure to gemfibrozil led to decreases in GST activity and several other key enzymes of metabolism, particularly significant increases in βGAL activity. Furthermore, at a metabolic level; prolonged exposure to gemfibrozil revealed increased citrate levels.

4.4.6 The combined mixture effect of pharmaceuticals on daphnids

In recent years, the said mixture or “cocktail” effect of pharmaceuticals, or chemicals in general has become of central focus in risk assessment and ecotoxicology studies. It is widely accepted that individual pharmaceuticals at their “No Effect” Concentration (NOEC).t concentration, can pose synergistic effects when they are combined. Although in the environment, pharmaceuticals are in the ng/L range, as they exist in complex mixtures, with different pharmaceuticals as well as other pollutants, together they can exert a combined effect to non-target organisms. Currently, chemical risk assessment follows a chemical-by-chemical approach, and does not assess mixture effects, therefore the magnitude of the threat chemical mixtures pose is not fully understood.

In this study, the mixture comprised of the aqueous soluble pharmaceuticals i.e. diclofenac, metformin and gabapentin did not obviously illicit a greater impact on the physiology or metabolome of daphnids. Although in certain instances, the mixture was

responsible for the greatest increase or decrease of an enzyme's activity, for example in the case of GST and LDH. This is supported by (Dietrich et al., 2010), who did not observe a more potent effect of a pharmaceutical mixture compared to individual compounds. However, the opposite impact has been reported also (Cleuvers, 2003, 2004), where an observable synergistic cocktail effect was documented.

The DMSO soluble, double mixture of carbamazepine and gemfibrozil did not have an obvious greater impact on the biochemical markers measured, and in most instances had a lesser impact compared to the individual treatment groups. In contrast, within the metabolomic analysis, the double mixture was responsible for the most significant changes in metabolite levels, including fructose-1,6BP, asparagine, citrate and glutamine. This is majorly different to what was observed in the aqueous mixture, which caused smaller effects, whereas the DMSO mixture had an apparent additive effect. Even so, a definitive effect on fructose-1 in both scenarios, indicates a junction between glycolysis and the pentose phosphate pathway and a disturbance in metabolic flow. Moreover, citrate was significantly altered in both datasets, although the mixtures had reversed effects, more profound effects were documented for the aqueous pharmaceutical mixture.

4.5 Conclusion

The selected environmentally relevant pharmaceuticals of this study induced significant alterations of enzyme activities related to physiology and oxidative stress following both short-term and long-term exposure. Within the acute experiments, metformin and gabapentin demonstrated the most toxicity, and were responsible for significant changes in 6 of the 8 markers measured. Regarding the acute metabolomics analysis, metformin induced the most perturbations, closely followed by gabapentin and carbamazepine. Similarly, metformin was also responsible for the most changes in enzyme activity within the chronic experiment. Overall, chronic exposure to the water-soluble pharmaceuticals had a greater impact to central metabolism compared to the DMSO soluble compounds, in particular gabapentin induced the most alterations in metabolites.

4.6 Supplementary Material

Supplementary Table 4.1. Statistically significant metabolites with p and log 2-fold change values in daphnids treated with drugs in relation to the control or DMSO group

Metabolite	T -test	Log2 FC	T -test	Log2 FC	T -test	Log2 FC	T -test	Log2 FC
	Metformin Vs Control		Gabapentin Vs Control		Gemfibrozil Vs DMSO		Carbamazepine Vs DMSO	
Acetylcarnitine	1.15E-03	1.81	5.53E-04	1.02	1.10E-03	1.83	2.58E-03	1.47
Alanine	1.64E-02	0.64			5.12E-02	-0.99		
Benzoic acid							1.98E-02	-0.85
Betaine	4.54E-02	0.17			3.84E-03	0.40	4.40E-02	0.16
Choline	4.28E-02	-0.15			3.63E-02	-0.47		
Cytidine	1.06E-03	0.78	1.81E-02	0.56			1.62E-03	0.64
Cytosine	5.61E-02	-0.31					2.89E-06	1.24
Glucose	1.89E-04	1.18			1.50E-02	-1.04	6.22E-03	0.86
Glutamine	3.59E-03	1.06	5.65E-04	1.22	2.00E-02	-2.67		
Guanine	3.41E-04	1.37	1.79E-03	1.62	1.26E-03	-2.10		
Histamine	3.30E-02	0.39	2.72E-03	0.48				
Hypoxanthine	1.00E-04	1.16	1.22E-03	0.82			2.75E-03	1.16
Inosine	1.12E-06	0.51	3.29E-04	0.45	3.42E-02	-0.38		
Isoleucine	1.02E-02	0.55	3.98E-03	0.72	1.83E-02	-1.73		
Leucine	4.54E-03	0.68	2.95E-03	0.95	2.82E-02	-1.90		
Methionine	1.32E-03	1.45	2.19E-02	1.54				
Nicotinamide			2.97E-02	-0.62				
Nicotinic Acid	1.39E-02	0.31			3.66E-03	0.48		
Pantothenate					4.68E-04	-0.88		
Phenylalanine	8.30E-03	0.58	1.50E-02	0.67	1.46E-02	-1.98		
Proline	5.07E-02	0.44	4.66E-02	0.60	1.78E-02	-1.06		
Putrescine	2.74E-02	0.53	1.72E-02	0.55				
Rivoflavine					3.22E-04	0.58	2.35E-04	0.50
Thiamine			1.94E-02	-0.60			1.09E-03	0.95
Threonine	4.93E-02	0.73	3.89E-02	0.64	4.05E-02	-1.58		
Thymidine	6.76E-03	1.45	6.77E-03	1.82	4.06E-03	-2.99		
Thymine	5.39E-03	1.51	5.58E-03	1.91	3.80E-03	-2.90		
Tryptophan	2.09E-03	0.75	9.73E-03	0.88	1.46E-02	-2.39		
Tyrosine	1.54E-03	0.76	7.92E-03	0.54	3.11E-03	-1.99		
Uracil	2.77E-02	0.29	3.02E-02	0.26	4.40E-04	0.46	1.58E-02	0.45
Uridine			1.22E-02	0.46	1.21E-02	-0.68		
Valine	5.75E-03	0.60	5.26E-03	0.66	2.24E-02	-1.48		

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Chapter 5

Mixture toxicology:

A realistic approach for multi-omics analyses

Abstract

Pharmaceutical compounds are continuously released via many routes into the freshwater environment, where they exist in complex mixtures and pose their mechanism of action to non-target organisms. In this study, daphnids were first acutely and then chronically exposed to a pharmaceutical mixture comprised of diclofenac, metformin, gabapentin, amoxicillin, trimethoprim and erythromycin. Acute exposures showed a small number of physiological changes, while daphnids exposed transgenerationally to the mixture using a culture media were significantly affected. To apply these methods to real-life scenarios, daphnids were then cultured in four different river samples and their corresponding spiked river sample. A suite of biochemical enzyme markers was measured for first and third generation daphnids grown in culture media, and for first generation daphnids of the environmental sample exposures. Additionally, a metabolomics analysis was performed for the daphnids exposed in culture media, which revealed many metabolic perturbations. A dose-dependent response to the mixture was seen in the acute exposures, in particular feeding was decreased significantly by the highest mixture concentration. A slight dose-dependent response can be observed in the chronic exposures, which became more obvious in the third-generation daphnids. Interestingly, within the metabolomics analysis, the lower mixture concentration induced the most metabolic impairment, additionally a generational impact was observed. Exposure to the unspiked river samples, resulted in altered enzyme activity when compared to the media control (OECD), in particular ALP and LIP, this suggests the possible contamination of the rivers from anthropogenic activity.

5.1 Introduction

In recent decades, demand and consumption of pharmaceuticals have continuously increased; with over 3000 pharmaceutical compounds in existence coupled with greater access to over-the-counter medication, pharmaceuticals are now ubiquitous in the aquatic environment. As a result of the existence of many pharmaceutical classes with inherently different mechanisms of action and a vast number of applications in human and veterinary medicine; pharmaceuticals do not occur as isolated, pure substances but exist in complex mixtures in surface waters. Upon excretion or eventuation in the environment, pharmaceuticals are either subjected to physical or chemical transformation processes or taken by an organism and biotransformed. For this reason, even individual pharmaceuticals have to be regarded as a multi-component chemical mixture (parent compound, degradation and metabolites). Generally, the ecotoxicity of a mixture of pollutants is almost always higher than the effects of its components, even in cases when individual compounds have not displayed toxicity. As a mixture, pharmaceuticals present a synergistic threat to plants and wildlife.

As a result of their ambiguous nature attributed to spatial and temporal variability; the antagonism and synergism of chemical mixtures are difficult to predict and assess. In the context of the aquatic environment, there are two main approaches which are employed to determine the toxicity potential of chemical mixtures. Concentration addition is a frequently employed method for the assessment of chemical combinations, based on the theory of Loewe & Muischnes, that components of a specific mixture have a common site of action. Independent action or also commonly referred to as response addition and effect multiplication is an approach often used in the context of risk assessment. This method is based on the assumption that the compounds of a given mixture act independently in a statistical sense. Compounds within a mixture which possess different molecular receptor sites may act on different physiological systems.

A series of acute, chronic and transgenerational experiments were executed using a mixture of six pharmaceutical compounds, comprised of the aqueous pharmaceuticals of the previous chapters, namely; diclofenac, metformin, gabapentin, amoxicillin, trimethoprim and erythromycin. The selection was based on their solubility within the lab media (OECD), as to avoid the requirement of a carrier solvent such as DMSO and a subsequent secondary control. Moreover, these compounds represent a broad

range of pharmaceuticals which are ubiquitously detected in the aquatic environment, and have been previously reported in literature to cause sub-lethal effects in non-target organisms, emphasising the importance of their assessment. The six pharmaceuticals were mixed at environmentally relevant concentrations of 0.1 and 1 mg/L within chronic experiments, and at an additional higher concentration of 10 mg/L for acute experiments. Daphnids were exposed in acute, chronic and transgenerational scenarios in the daphnid culture media (OECD) and, subsequently, the biomolecular and phenotypic responses of daphnids to these exposures were measured. Following these experiments which used OECD culture medium, the experimental concept was applied to environmental samples, to assess the toxicity potential of four rivers located in Co. Dublin.

5.2 Materials and methods

5.2.1 Algae and daphnid culturing

Algae and daphnids were cultured using the same protocols described in chapter 2 (see section 2.2.1 and Appendix A).

5.2.2 Acute toxicity exposure

Acute toxicity exposures were executed following the approach outlined in chapter 2 (see section 2.2.2), 24-hour toxicity curves were generated for neonates and 24- and 48-hour curves were executed for daphnids aged day 4 (Figure 5.1). For the toxicity curve using day 4 animals, 200 daphnids < 24 hours were cultured in 4l OECD and 15 ml seaweed extract and fed 10 ml frozen algae until day 4 and were then used in toxicity experiments.

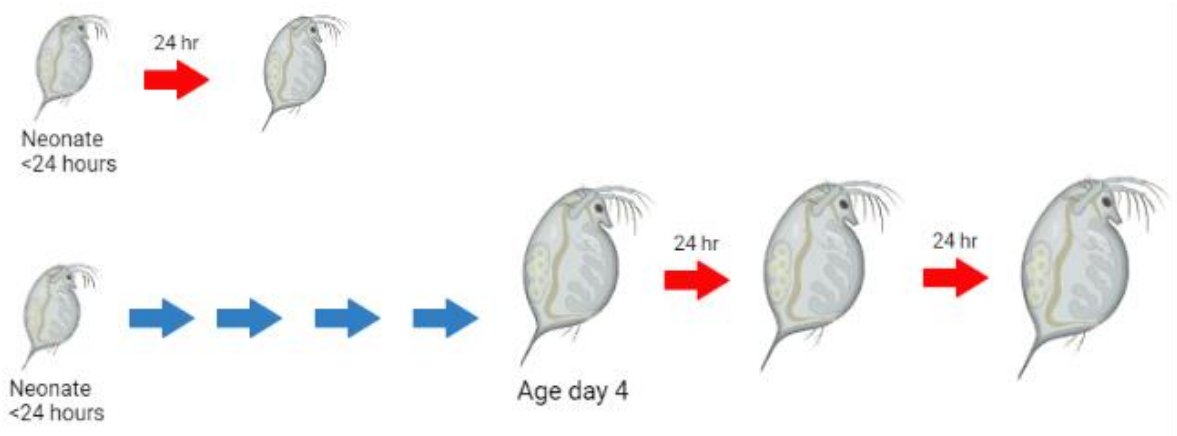

Figure 5.1. Acute exposures in neonates (<24 hours) and four days old daphnids. Blue arrows indicate culturing in the absence of the pharmaceutical mixture and red arrows in the presence of the pharmaceuticals.

5.2.3 Acute Feeding

Feeding is a phenotypic endpoint which can be measured to assess the physiology of an organism, it is convenient in *Daphnia* studies as they are non-selective filter feeders of a range of particles (1-50 µM) (Eltemsah & Bøhn, 2019). In this study, a feeding assay was performed based on the method recently described by (Giannouli et al., 2023;), this approach quantifies ingestion by measuring the fluorescence of microparticles (average size of 2 µM) present in the gut of daphnids. but also, they are fluorophores that can be visualized through fluorescence microscopy. Initially neonates less than 24 hours old were cultured until day 4 and then exposed to their relative mixture concentration following the protocols previously described above. After 24 hours of exposure, daphnids were collected and separated according to their exposure condition, using extra daphnids from these exposures, several animals were pooled together, but were not fed microparticles, this acted as a reagent blank to account for potential background fluorescence of daphnids. To a falcon tube, 9 ml OECD was added, followed by 15 daphnids and finally 9 ml of the microparticle stock (latex beads, carboxylate-modified polystyrene, fluorescent red – 1000 µl/l), daphnids were allowed to feed for 20 minutes before being transferred to clean OECD media to cease feeding. Internal fluorescence was calculated by homogenizing the 14 animals in 0.465 ml water and measuring fluorescence at an excitation of 560 nm and emission of 590 nm. Fluorescence was calculated by converting the fluorescent content of the samples to microparticle µg/L using the corresponding standard curve (2.6 -26 mg/L microplastic).

5.2.4 Chronic and transgenerational toxicity exposures

For biochemical experiments, 15 daphnids were cultures in 600 ml OECD and fed 1.8 ml fresh algae daily, on the day of setting up and biweekly thereafter, media was changed, and chemical renewed with the inclusion of 0.9 l seaweed extract for additional nutrition. For daphnids required for metabolomic analysis, 75 daphnids were cultured in 3 l of OECD, fed 9.1 ml fresh algae daily and at the time of set up / media change, 4.55 ml of seaweed extract was given. These cultures followed the same

biweekly media change protocol i.e., every Monday and Friday. Furthermore, neonates were taken from the previous generation only on or after day 13 (Figure 5.2).

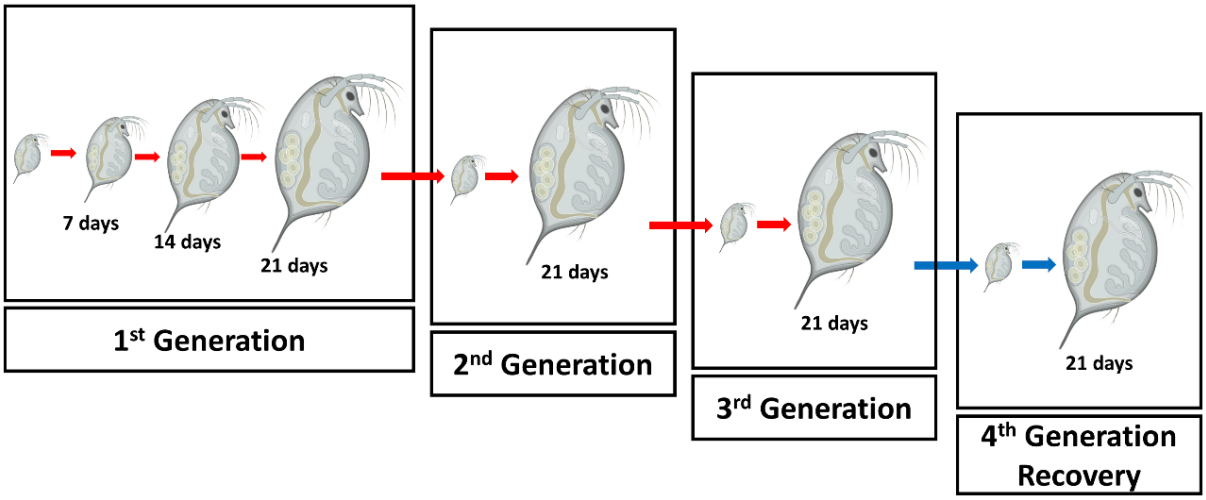

Figure 5.2. Chronic and transgenerational exposure design. For each generation, a 21 days exposure was followed. Following the third generation of exposure, animals were cultured for another 21 days in the absence of chemicals as a recovery period.

5.2.5 Environmental samples

Water samples from four rivers were collected on the 9th of October 2022 (Figure 5.3). Two rivers, the Broadmeadow (BM) (53.4715817, -6.2214660) and Kettle's (K) (53.4371616, -6.2131980) are located in north county Dublin, surrounded by mainly agricultural land. The other two rivers are located in urban areas, the Grand Canal (GC) (53.3300154, -6.2669666) and Tolka (T) (53.3737491, -6.2716325). Water samples were collected and stored in plastic sampling bags or plastic bottles.

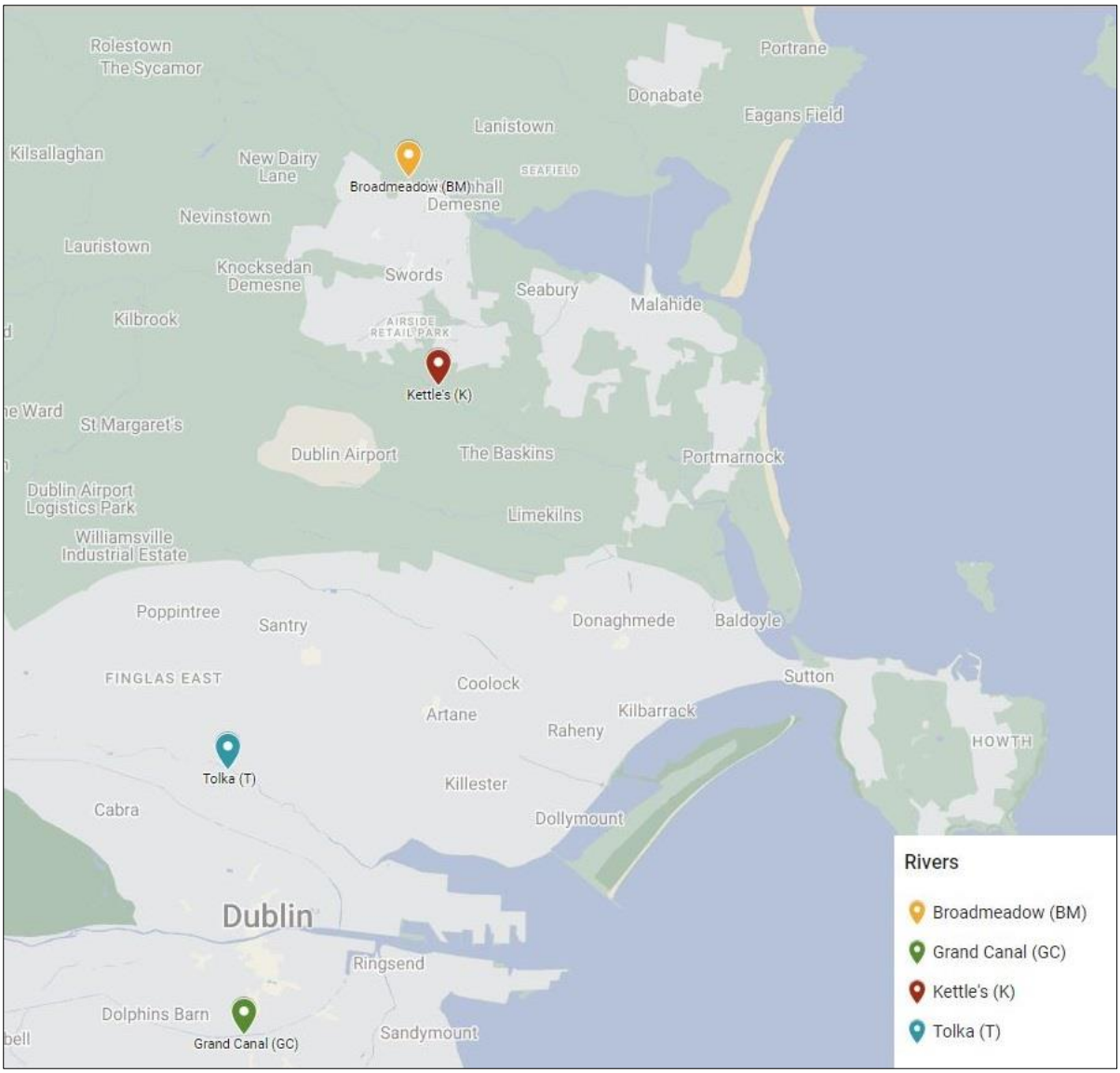

Figure 5.3. Sampling locations for rivers; Broadmeadow (BM), Kettle's (K), Grand Canal (GC), and Tolka (T).

5.2.6 Environmental sample transgenerational exposure

For the first-generation exposure,75 neonates were cultured in 3 liters of OECD and the four river samples (unspiked). Following the same procedure, neonates were also cultured in OECD/river with the addition of the pharmaceutical mixture with a final concentration of 0.1 mg/L (spiked) (Figure 5.4). The animals were grown until day 21, on day 10 of the exposure, the media was changed and the chemical mixture was refreshed. On day 21, daphnids were snap-frozen for metabolomic analysis and the remaining were homogenized and used in biochemical experiments.

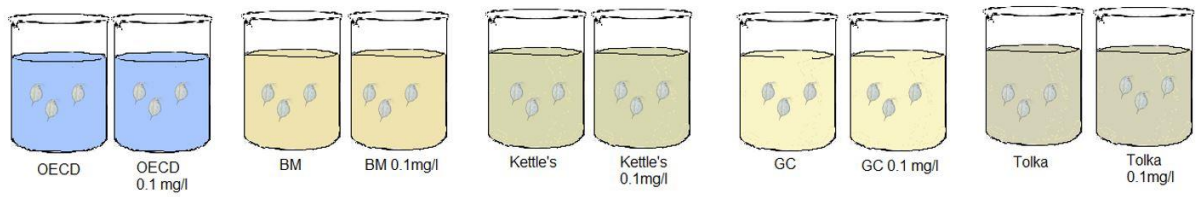

Figure 5.4. Exposure design for laboratory media (OECD) and environmental samples for the rivers Broadmeadow (BM), Kettle's (K), Grand Canal (GC), Tolka (T).

5.2.7 Sample homogenization and biochemical analyses

Samples were prepared using the same methods in the previous chapter (see section 2.2.6). Protein was quantified using an ultrasensitive Bradford method which is described in the materials and methods of the previous chapter (see section 2.2.7). The activity of enzymes (alkaline phosphatase, acid phosphatase, beta-galactosidase, peptidase, lactate dehydrogenase, glutathione reductase, and glutathione-S-transferase) was measured as described in the previous chapter (see section 2.2.8).

5.2.8 Extraction of metabolites and metabolomic analyses

Animals were snap-frozen in liquid nitrogen and stored at -80°C until their extraction and analysis. Samples were analysed in the Metabolomics Core Facility of University College Dublin. Briefly, frozen samples were thawed and reconstituted in 100 µL of Ethanol:PBS (85:15), samples were homogenised and centrifuged at 24000 x g for 5 minutes at 4°C, supernatant was collected and stores at -80°C until data acquisition. Following manufacturer's instructions, the *AbsoluteIDQ*® p180 assay (Biocrates Life Sciences, Innsbruck Austria) was applied. Metabolomics data was acquired by LCMS/MS using a SCIEX QTRP 6500plus mass spectrometer coupled with a SCIEX ExionLC™ Series UHPLC capability, fitted with a column provided by the p180 assay. The mobile phase consisted of water and acetonitrile with 0.2% formic acid (A and B). All metabolites were quantified by a multiple reaction monitoring method.

5.2.9 Statistical analysis

The data were analysed and plotted using the Excel and GraphPad Prism software packages. Student's *t*-test analyses were performed for comparisons, and the EC values were calculated using the GraphPad Prism plotting functions. Metabolomics data was processed in Excel, and multivariate statistical analyses were performed in

MeV software. Heat maps for the metabolomic data were created using the Heat Map function in GraphPad Prism.

5.2.10 Reagents and chemicals

Latex beads, carboxylate-modified polystyrene, fluorescent red microparticles were purchased from Sigma Aldrich. See sections 2.2.12, 3.2.8, and 4.2.7 for all other chemical and reagent purchases.

5.3 Results

In the aquatic environment, pollution is not likely the result of a single chemical, but rather many chemicals at a low concentration. To reflect this daphnids were exposed to a pharmaceutical mixture of diclofenac, metformin, gabapentin, amoxicillin, trimethoprim, and erythromycin at several concentrations. The 0.1, 1 and 10 mg/L mixture comprised of each pharmaceutical at 0.1, 1 and 10 mg/L respectively.

5.3.1 The acute impact of a 6 pharmaceutical mixture on the biochemical activity of daphnids

To assess the toxicity potential of the mixture, neonates (<24 hours) were exposed to the combination of pharmaceuticals for 24 hours (Figure 5.5). Alternatively, neonates were separately cultured until four days old and then exposed for 24 and 48 hours to the pharmaceutical mixture (Figure 5.6). Toxicity curves were generated and the Effective Concentration (EC) values were calculated for both toxicity scenarios to assist in the selection of the working concentrations for both acute and chronic experiments (Table 5.1). As expected, neonates were more sensitive to the pharmaceutical mixture, compared to the day 4 animal. Additionally, the 48-hour exposure resulted in lower EC values, compared to the 24-hour exposure, as anticipated.

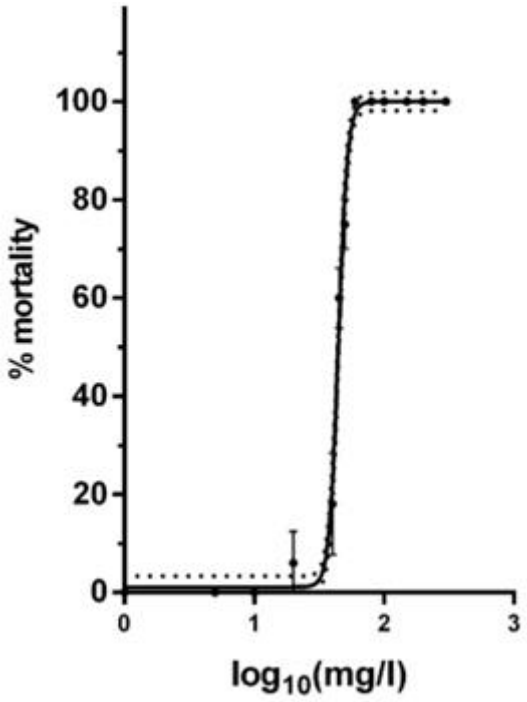

Figure 5.5. Toxicity curve for neonates (<24 hr), exposed to pharmaceutical mixture for 24 hours. Data represents average±SD (N=4) for each concentration.

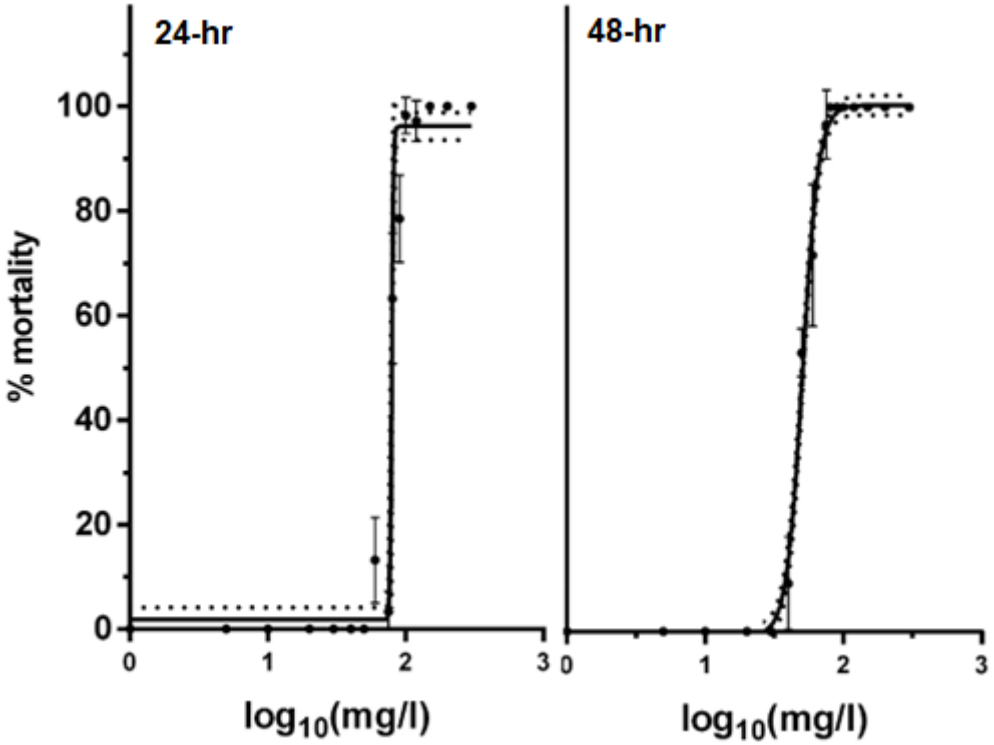

Figure 5.6. Toxicity curve for daphnids aged day 4 exposed to pharmaceutical mixture, for 24 hours (left) and 48 hours (right). Data represents average±SD (N=4) for each concentration.

Table 5.1. EC values (mg/L) calculated for neonates (<24 hours) and four days old acutely exposed to a pharmaceutical mixture. Data represents average±SD (N=4) for each concentration.

Daphnid age (days)	Exposure duration (hours)	EC_1	EC_5	EC_{10}	EC_{50}
<1	24	30.6	35	37.2	44.5
4	24	74.3	76.1	76.9	79.3
4	48	27.5	34.3	37.9	50.7

Daphnids aged less than twenty-four hours were grown until day four, they were then exposed to several concentrations of the pharmaceutical mixture (0.1, 1 and 10 mg/L) for twenty-four hours and forty-eight hours. Following the acute exposure to the mixture, multiple biochemical endpoints were measured to assess the impact of the mixture on the physiology of daphnids (Table 5.2).

Table 5.2. The acute effect of the pharmaceutical mixture on enzyme markers of four days old daphnids following 24- and 48-hours exposures.
Data represent average ± SD (N=4) replicates for each condition. Statistically significant by Student's t-test compared to control (*) ($p<0.05$). ALP, ACP, BGAL, LIP PEP and GST are expressed as milliunits/animal.

Time (hours)	Enzyme	Control	0.1 mg/L	1 mg/L	10 mg/L
24	ALP	88.5±6.6	86.3±9.2	77.1±9.6	81.3±5.4
	ACP	53.2±1.9	52.7±3.2	48.7±6.3	51.8±3.8
	BGAL	66.7±3.8	62.7±3.2	72.5±3.4	83.1±1.9* **(24.6%)**
	LIP	152.5±10.7	124.3±17.3* **(-18.3%)**	140.8±13.7	123.5±6.7* **(-19%)**
	PEP	4±0.7	4.1±0.4	3.7±0.4	3.7±0.4
	GST	11.8±1	10.8±0.6	12.1±0.5	11.9±0.8
48	ALP	70.1±9	80±4.8	71±12.2	77.1±8.9
	ACP	38.7±3.6	38.1±3.7	38.3±2.3	42.1±4.5
	BGAL	26.3±1.9	26.8±1.6	30.8±0.6* **(17.1%)**	39.2±2.8* **(49%)**
	LIP	71.8±1.6	80.1±9.4	75.6±4.6	69.1±6.7
	PEP	6±0.8	5.5±0.4	4.9±0.6	5±0.4
	GST	7±0.8	6.3±0.8	6±0.4* **(-14.3%)**	5.6±0.8* **(-20%)**

Phosphatase activity (ALP/ACP) was not altered after 24- or 48-hour exposure to any concentration of the pharmaceutical mixture. Exposure to the mixture at 0.1 mg/L resulted in significant changes in lipase activity only (-18.3%). No statistical differences were recorded after 24 hours of exposure to 1 mg/L, although several changes were observed after 48 hours. BGAL activity was increased by 17.1%; whereas GST activity was decreased by -14.3%. Most significant changes occurred after 10 mg/L exposure, in particular after 48 hours. After 24 hours, BGAL activity was increased by 24.6% and LIP was decreased by 19%. Further exposure to the 10 mg/L mixture (48 hours) caused a 49% increase and a 20% decrease in BGAL and GST activity, respectively.

5.3.2 Acute Feeding

Following acute exposure to the pharmaceutical mixture for 24 hours, a feeding assay was executed and internal fluorescence was measured (Figure 5.7. A).. To illustrate the ingestion of microparticles by daphnids, optical stereoscopy was performed, and representative images were taken for each condition (Figure 5.7. B), to validate there was no autofluorescence from daphnids, animals which were not fed microplastic were also imaged.

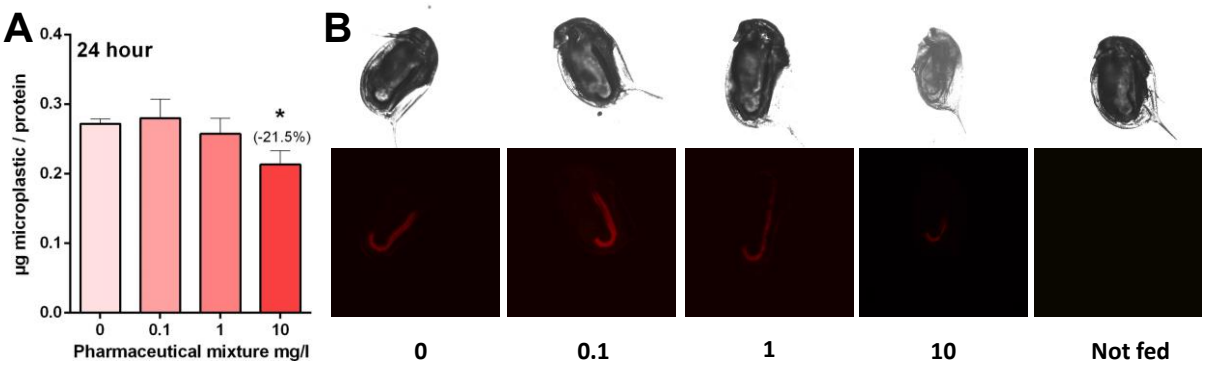

Figure 5. 7. The impact of a pharmaceutical mixture on feeding ability of *Daphnia* aged day 5. Total ingested microplastic was quantified based on their fluorescence in the incubation media and expressed as µg microplastic / protein (A). The ingested microparticles were visualized by optical stereoscopy (B), brightfield images (top) and fluorescence microscopy (bottom). Data represent average ± standard deviation (N = 4 replicates). * Statistically significant by Student's *t*-test denotes significant difference in comparison to the OECD control.

There is a clear trend of a dose dependent effect of the mixture on feeding of daphnids, the 0.1 mg/L treatment had similar ingestion to the control, in fact it fed slightly more. However, as the concentration increased to 1 and 10 mg/L, feeding decreased. A

significant decrease of -21.5% was recorded for the highest concentration of the mixture (10 mg/L).

5.3.3 Chronic and transgenerational effect of a pharmaceutical mixture on daphnids

The synergistic and transgenerational effect of a pharmaceutical mixture was assessed in three generations of *Daphnia magna*; first and third-generation daphnids were exposed chronically to the mixture at 0.1 and 1 mg/L, and on day 21 the activities of several enzymes were measured, on the case of first-generation daphnids only, biochemical markers were also measured on day 7 and 14 (Table 5.3). In addition to this, daphnids were also frozen on day 21 for metabolomic analysis. To determine if *Daphnia* could recover from the generational exposure of their mothers to the pharmaceutical mixture, fourth-generation *Daphnia* were grown for 21 days in the absence of chemicals and the same enzyme activities were measured.

Table 5.3. The chronic effect of the pharmaceutical mixture on enzyme markers of daphnids on the first, third and fourth generation exposure. Data represent average ± SD (N=4) replicates for each condition. Statistically significant by Student's t-test compared to control (*) (p<0.05). ALP, ACP, BGAL, LIP and PEP are expressed as milliunits/µg protein, GST is expressed as microunits/µg protein.

Generation/days of exposure	Enzyme	Control	0.1 mg/L	1 mg/L
1st generation 7 days	ALP	4.5±0.3	4.9±0.2	5±0.1***(11.1%)**
	ACP	6.3±0.2	5.2±0.3***(-17.5%)**	4.6±0.1*(27%)
	BGAL	5.7±0.4	6±0.3	6.7±0.4***(17.5%)**
	LIP	75.3±4.4	89.9±3.1***(19.4%)**	84.1±6
	PEP	4.1±0.1	4.2±0.4	4.4±0.1***(7.3%)**
	GST	242±16.1	284.9±14.4***(15.1%)**	293.3±22.3***(21.2%)**
1st generation 14 days	ALP	7.2±1	8.6±0.6	7.2±0.8
	ACP	4.8±0.1	5.2±0.2***(8.3%)**	5.6±0.5***(16.7%)**
	BGAL	4.3±0.4	4.4±0.2	3.6±0.1***(-16.3%)**
	LIP	68.2±6.2	60.1±1.9***(-11.9%)**	49.4±5.1***(-27.6%)**
	PEP	3±0.3	4.1±0.2***(36.7%)**	3.7±0.1***(23.3%)**
	GST	582.1±35.5	565.3±54.7	617.6±54.04
1st generation 21 days	ALP	9.2±1.04	12.1±0.5***(32%)**	9.5±0.6
	ACP	7.6±0.4	8.2±0.7	7±0.5
	BGAL	7.5±0.5	9.5±0.2***(26.7%)**	8.1±0.5
	LIP	179.7±22.8	174.2±16.8	145.7±9.9***(-18.9%)**
	PEP	7.3±0.4	10.1±0.8***(38.4%)**	7.3±0.1
	GST	398.2±58.9	640.2±50.3***(60.8%)**	648.2±3***(62.8%)**
3rd generation 21 days	ALP	4.7±0.3	4.7±0.5	4.1±0.3***(-12.8%)**
	ACP	5.8±0.6	4.1±0.4***(-29.3%)**	4.3±0.3***(-25.9%)**
	BGAL	4.4±0.4	6.1±0.5***(38.6%)**	5.8±0.5***(31.8%)**
	LIP	121.2±10.8	135.7±6.7	124.1±6.3
	PEP	2.3±0.2	2.5±0.1***(8.7%)**	3±0.3***(30.4%)**
	GST	1175.9±74.6	1258.5±45.04	1302±34.9***(10.8%)**
4th generation 21 days	ALP	5.4±0.3	4.4±0.8***(-18.5%)**	4.5±0.4***(-16.6%)**
	ACP	4.6±0.4	4±0.2***(-13%)**	3.3±0.1***(-28.3%)**
	BGAL	7.8±0.4	6.3±0.5***(-19.2%)**	5.8±0.5***(-25.6%)**
	LIP	153.6±1.8	135.2±9.1***(-12%)**	147.3±8.1
	PEP	2.8±0.2	2.4±0.14***(-14.3%)**	2.7±0.2
	GST	1115.7±87	1091.8±96	1064.9±101.1

First generation

The activity of ALP was significantly altered in first generation daphnids, in particular by exposure to the 1 mg/L mixture. After 7 and 14 days of exposure, ALP activity remained unchanged within the 0.1 mg/L exposure scenario. Although, after 21 days, a significant increase of 32% was observed. Contrastingly, the 1 mg/L mixture elicited an increasing effect on ALP after 7 and 14 days, of 11.1% and 16.7% respectively. At

21 days, daphnids exposed to the higher mixture concentration showed similar ALP activities as the control. ACP activity was affected differently compared to ALP, significant changes in activity were recorded for both concentrations after 7 and 14 days of exposure. At a concentration of 0.1 mg/L, ACP activities decreased by 17.5%, whereas at 14 days, an increase of 8.3% was revealed. The higher concentration of 1 mg/L induced significant increases at both periods; after one week of exposure, ACP activity was increased by 27%, and by 16.7% in the case of the two-week exposure.
Significant changes in BGAL activity were detected at each time point, similarly to ALP activity BGAL was only affected after 21 days by the lower mixture concentration, and after 7 and 14 days by the higher concentration. Exposure to 0.1 mg/L for 21 days resulted in a 26.7% increase in BGAL activity. After 7 days, daphnids exposed to the mixture at 1 mg/L, had increased BGAL activity (17.5%), however after 14 days this activity was decreased by 16.3%. Lipase activities were significantly different to controls after 7- and 14-days exposure to 0.1 mg/L. A significant increase of 19.4% was recorded after 7 days, whereas a 11.9% decrease was observed after 14 days. No apparent differences in LIP activity were detected after 21 days exposure to the 0.1 mg/L mixture. Exposure to the 1 mg/L mixture led to significant differences in LIP activity after 14 and 21 days, decreases of 27.6% and 18.9% were recorded respectively. Peptidase activity was impaired at each exposure scenario, in particular by the lower concentration of 0.1 mg/L. No changes in activity were recorded after 7 days, although significant increases of 36.7% and 38.4% were recorded after 14 and 21 days respectively. Exposure to the 1 mg/L concentration, resulted in a minor increase of 7.3% after 7 days and 23.3% after 14 days. Daphnids exposed to the higher concentration of the mixture demonstrated the same PEP activity as the controls. The two mixture concentrations demonstrated similar impacts on GST activity. After 7 days of exposure, the mixture of 0.1 and 1 mg/L increased GST activity by 15.1% and 21.2% respectively. Following 14 days of exposure, no significant changes in GST activities were recorded for either concentration. Although after 21 days of exposure, significant increases of 60.8% and 62.8% were recorded for 0.1 and 1 mg/L exposure scenarios.

Third generation

When compared to first-generation daphnids (21 days), third-generation daphnids revealed more significant alterations of enzyme activities; in some cases, opposite

effects or lesser / higher percentage changes were demonstrated. For example, ALP activity was impacted only by the 1 mg/L mixture (-18.9%), whereas in the first generation 1 mg/L did not induce any significant changes. In a similar trend, the lower concentration did not significantly impact ALP activity in the third generation, although it was responsible for a 32 % increase in the first generation. Significant alterations of ACP activity were recorded for both mixture concentrations in the third generation, 0.1 and 1 mg/L elicited similar decreasing effects of -29.3% and -225.9% respectively. This is in contrast to the findings within the first generation, where no significant changes were observed. BGAL activity was more affected in the third generation, bigger increases (38.6%) in activity were recorded in daphnids exposed to the 0.1 mg/L mixture. Furthermore, the 1 mg/L mixture increased activity by 31.8%, whereas no changes were observed in this scenario within the first generation. No significant changes of lipase activity were detected in the third-generation exposure; however, the 1 mg/L mixture was responsible for decreases in the first generation. In relation to peptidase activity, third generation daphnids exposed to 0.1 and 1 mg/L, demonstrated increased PEP activity of 8.7% and 30.4% respectively; significant differences in PEP activity were only detected at 0.1 mg/L in the first generation. GST activity was less impacted in the third generation when compared to the first generation, no changes were recorded for the 0.1 mg/L exposed, which is in contrast to the first generation. A significant increase of 10.8% in GST activity was observed in the third generation daphnids exposed to the 1 mg/L mixture, however more significant fold changes were recorded in the first generation.

Fourth generation

To assess the ability of daphnids to recover from transgenerational exposure to the pharmaceutical mixture, fourth-generation daphnids were collected from the mothers of each condition and cultured in clean media in the absence of chemicals. After 21 days of culture, the aforementioned array of biochemical assays was performed, to measure the enzyme activities of the fourth-generation daphnids. Significant alterations were detected for both concentrations in 3/5 enzymes of metabolism; in the remaining enzymes of metabolism, at least one concentration of the mixture posed a significant impact on enzyme activity. The enzyme GST which is responsible for the detoxification of xenobiotics was not significantly impacted by either pharmaceutical mixture concentration. Decreases in phosphatase activity were detected, 0.1 mg/L

caused a decrease of 18.5% and 13% for ALP and ACP activity; 1 mg/L decreased their activities by 16.6% and 28.3% respectively. Similarly, BGAL activities decreased by 19.2% and 25.6%. Lipase activity was disrupted only by the 0.1 mg/L conditions, a 12% decrease was observed, and no significant changes were recorded in the previous generation. Similarly, peptidase activity was impacted only by the lower concentration of 0.1 mg/L, resulting in a 14.3% decrease.

5.3.4 The transgenerational effect of a pharmaceutical mixture on Daphnia metabolism

Chronic exposure to the pharmaceutical mixture revealed many metabolic perturbations in daphnids, and multivariate statistics revealed differences following generational exposure, as increased separation among treatments can be observe in the third-generation daphnids (Figure 5.8). Overall, there was a trend for biogenic amines and amino acids to be increased, these upregulations were more frequently recorded in the third-generation exposures and greater increases were measured and can be seen with increasing red intensity (Figure 5.9). In particular, the amines; kynurenine and symmetric dimethylarginine (SDMA), methionine sulfoxide (Met-so), and asymmetric dimethylarginine (ADMA) were upregulated the most significantly. Whereas taurine was the most downregulated biogenic amine, as was carnosine and putrescine to a lesser degree. Within the first generation, an upregulation of 19/21amino acids in daphnids exposed to the 0.1mg/L was observed, and exposure to the higher concentration of 1 mg/L resulted in only changes in 2 out of 21 amino acids measured. The opposite was observed in the third-generation scenario, with less changes occurring in the 0.1 mg/L exposed (12/21) compared to the 1 mg/L mixture. All amino acids with the exception of tyrosine were increased following exposure to the 1 mg/L exposure, tyrosine had a tendency to decrease in both concentrations. Citrulline, was the only amino acid to be significantly decreased, and this was only recorded for first generation daphnids following exposure to 1 mg/L.

Contrastingly, the sphingolipids demonstrated a trend to decrease, in particular Hydroxysphingomyelin C16:1 (SM (OH) C16:1) and Hydroxysphingomyelin C22:2 (SM (OH) C22:2) were the most down regulated in the first generation, whereas in the third generation, Hydroxysphingomyelin C22:1 (SM (OH) C22:1) and Sphingomyelin C24:0 (SM C24:0) were upregulated, although the other sphingolipids tended to decrease.

The significant fold changes ≤≥ 20% in lysophosphatidcholines were all recorded as decreases, while decreases in 7/12 lysophosphatidcholines were observed in the 1 mg/L exposure of the first generation, further generational exposure to the 0.1 mg/L mixture resulted in the down regulation of 9/12 lysophosphatidcholines, including a 320-fold decrease in lysoPhosphatidylcholine acyl C20:4 (lysoPC a C20:4). LysoPC a C18:0 and lysoPC a C20:4 were the only lysophosphatidcholines to be upregulated, this occurred in the 1 mg/L exposure in third generation daphnids.

Upregulations and downregulations of acylcarnitines were recorded for both concentrations over both generations, however more changes were observed in the third-generation scenario. In particular Acetylcarnitine (C2) and Octadecanoylcarnitine (C18) were the most altered of all acylcarnitines. C2 was decreased by both mixture concentrations in both generations. The lower concertation was responsible for the greatest fold changes in both generations, 29-fold in the first generation and by 103-fold in the third generation, whereas the higher concentration caused a decrease of 27-fold in the first -generation and 78 in the third. C18 was increased 73-fold by both mixture concentrations in the first generation, but decreased 94-fold by the 0.1mg/L mixture in the third generation. Overall, there was a generational effect observed as greater fold changes were recorded in the third generation.

Phosphatidylcholines were mostly down regulated as a result of exposure to the pharmaceutical mixture (Figure 5.10), all significant differences were recorded as decreases in the first generation with the exception of one metabolite (PC ae C44:5) which was upregulated by both concentrations. In particular, downregulations occurred following exposure to the lower concentration. Most of the fold changes observed were also decreases in the third generation, however some upregulations also occurred. Similar to what was demonstrated in the first generation, most of the significant fold changes were recorded in the lower mixture concentration.

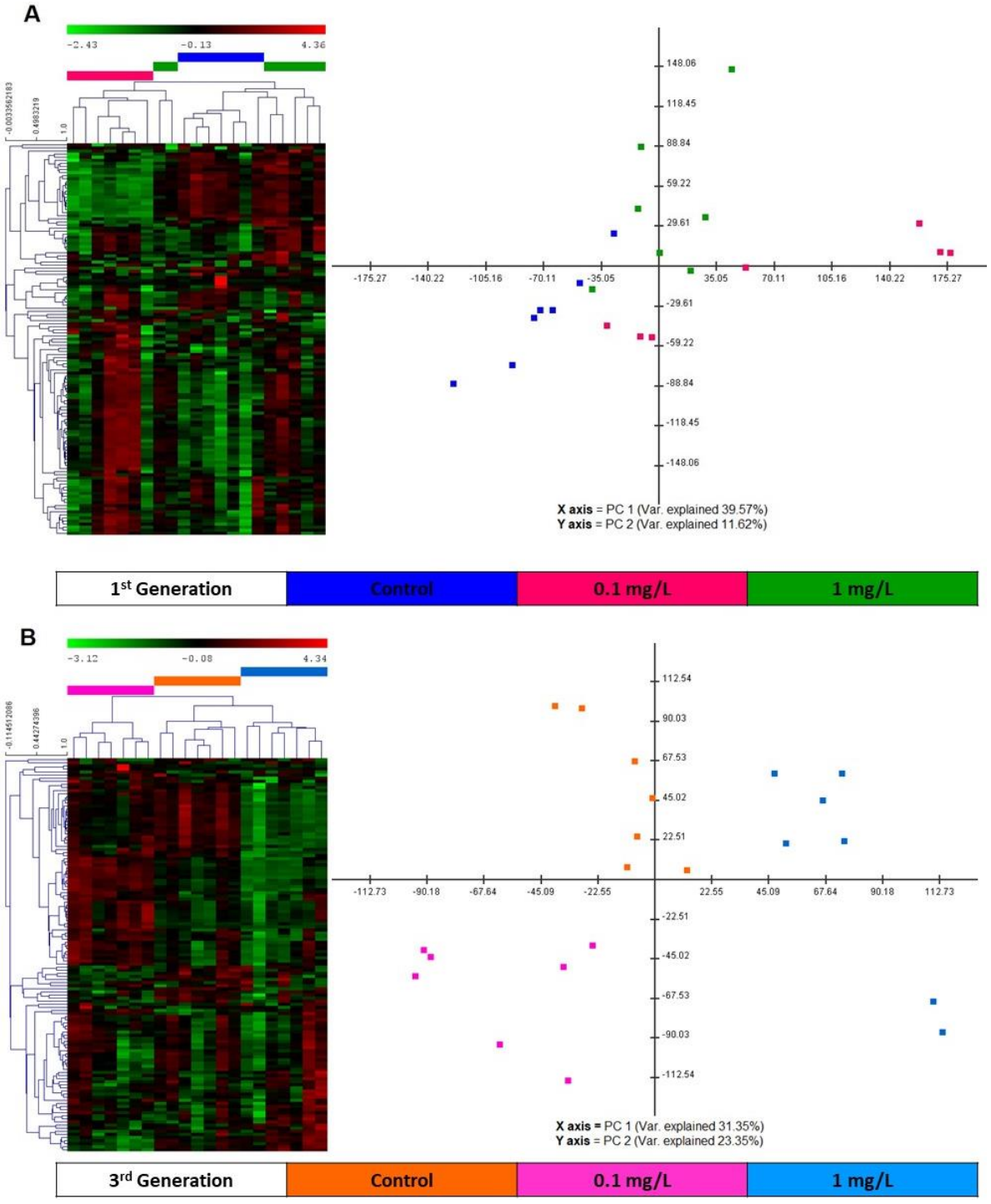

Figure 5.8. Metabolomic analysis for first generation (A) and third generation (B) daphnids exposed to the mixture at 0.1 and 1 mg/L. Multivariate statistical analyses with Pearson correlation hierarchical clustering and principal component analysis revealed moderate separation between treatments.

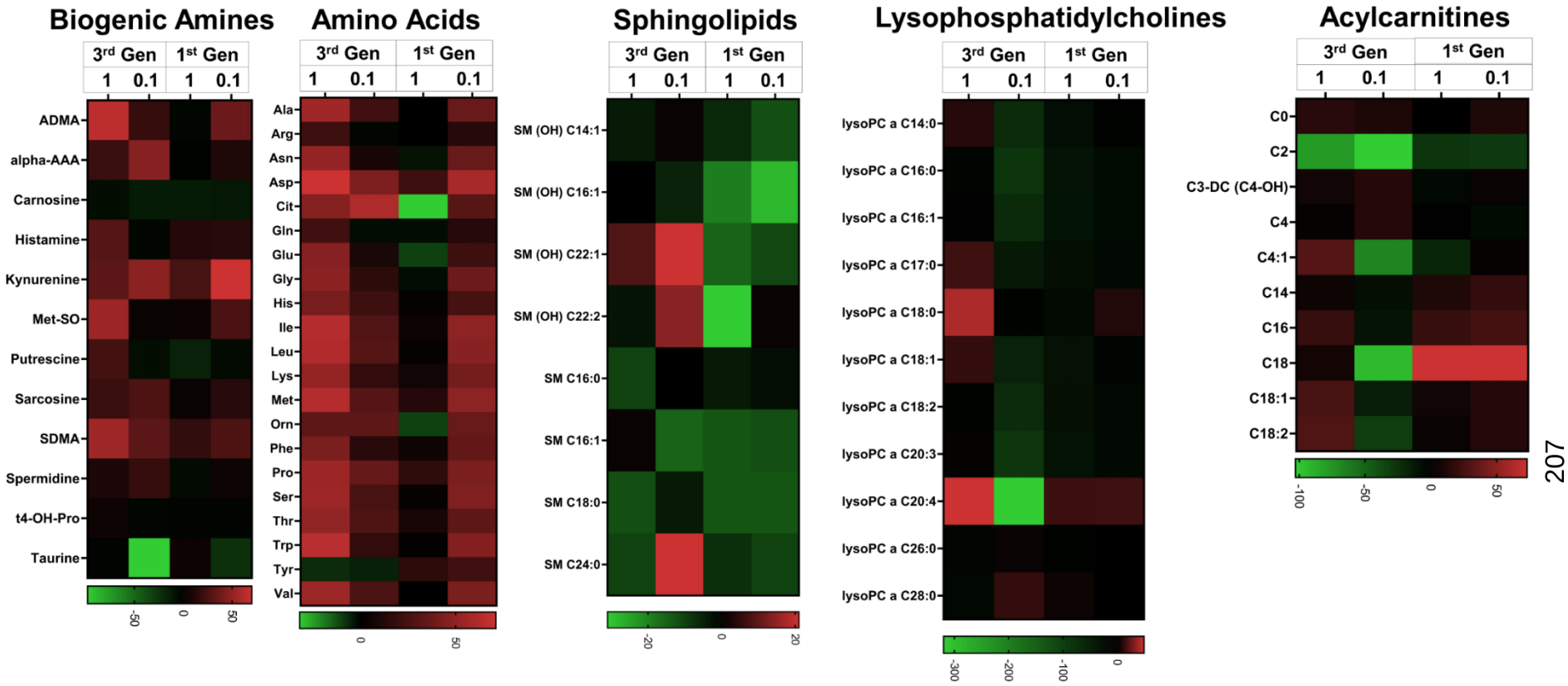

Figure 5.9. The transgenerational effect of a pharmaceutical mixture at 0.1 mg/L and 1 mg/L on the metabolism of daphnids. A targeted analysis of polar metaboites ranging from biogenic amines, amino acids, sphingolipids, lysophosphaidylcholines and acylcarnitines was performed. Heat map represents fold changes of < or > 20% in the exposed (0.1 mg/L and 1 mg/L) daphnids compared to the OECD control for the first generation (two right columns) and the third generation (two left columns), red indicates fold increase, and green indicates fold decreases.

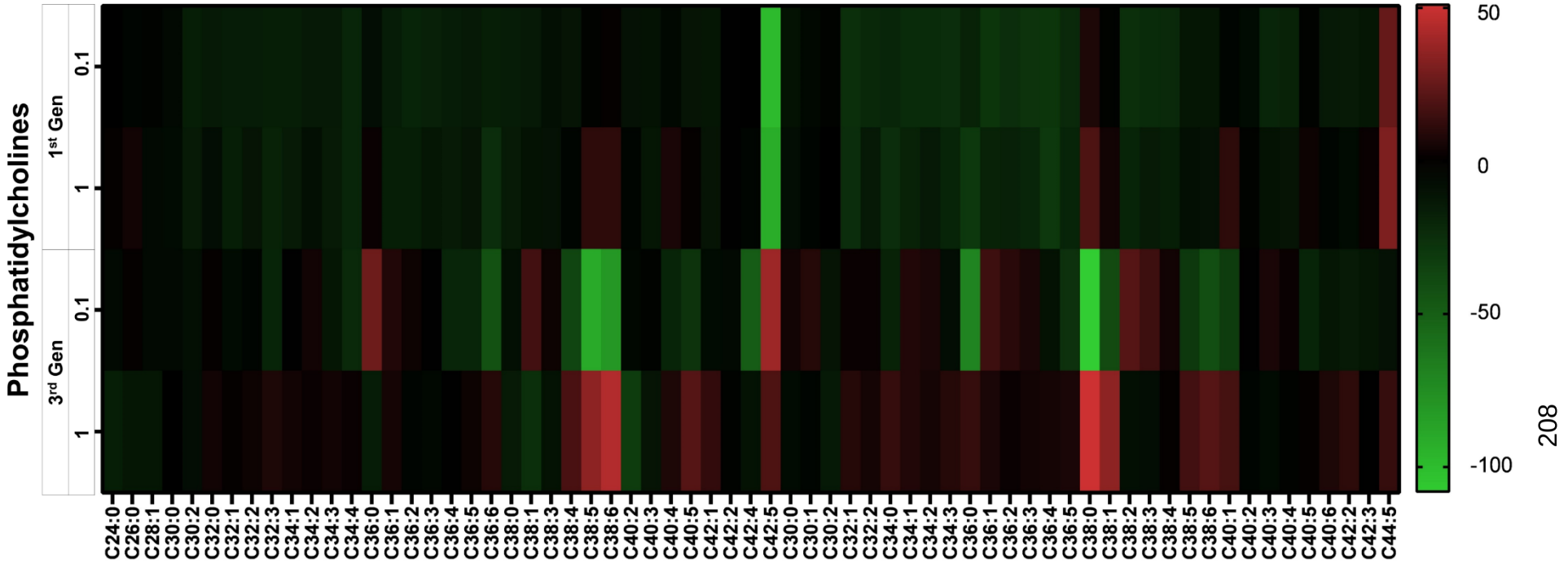

Figure 5.10. The transgenerational effect of a pharmaceutical mixture at 0.1 mg/L and 1 mg/L on the metabolism of daphnids. A targeted analysis of phosphatidycholines. Heat map represents fold changes of < or > 20% in the exposed (0.1 mg/L and 1 mg/L) daphnids compared to the OECD control for the first generation (two right columns) and the third generation (two left columns), red indicates fold increase, and green indicates fold decreases.

5.3.3 Exposure of daphnids to pharmaceuticals in the spiked environmental samples

To simulate a more realistic scenario and to apply our *in vitro* research to environmental samples, a chronic exposure comprising of OECD media and 4 rivers was performed. In total there were 10 exposure conditions, consisting of an unspiked and spiked (0.1 mg/L mixture) version of each media/river. Comparisons between the unspiked rivers were drawn as was comparisons between the unspiked and spiked river samples. After 21 days, the enzyme activities of daphnids were measured (Table 5.4). Most significant changes recorded were between the unspiked rivers and the unspiked OECD media. ALP and followed by LIP were the most sensitive to significant changes following chronic exposure. Decreases in ALP activity was detected for each river exposure, with the greatest decrease observed in the Broadmeadow (BM) exposure (-39%). Similarly, Kettle's (K), Grand Canal (GC), and Tolka (T) all decreased ALP activities by -35.6%, -24.1% and -34.9% respectively. Moreover, a decrease of 49.2% was recorded for OECD 0.1 mg/L when compared to the unspiked OECD condition. Contrastingly, ACP activities were less impacted compared to ALP, significant changes in ACP activity were only recorded for the Grand Canal (GC) river, which was responsible for a 49.9% increase when compared to OECD, and no differences between unspiked and spiked media/rivers were detected. This is consistent with what was observed in BGAL activity, a significant decrease of -67.7% was only recorded between the spiked and unspiked OECD condition. In relation to LIP activity, mainly decreases were recorded with the exception of the spiked Tolka (T) river which increased LIP by 35.1% when compared to its unspiked counterpart. When compared to the OECD control, the Broadmeadow (BM) and Kettle's (K) river similarly decreased LIP by -30.6% and -31% respectively. Furthermore, the spiked OECD daphnids demonstrated decreased LIP activity when compared to the OECD control (-42.5%). PEP activity was impacted in the same manner as BGAL, a decrease in activity was observed only in the spiked OECD exposure, when compared to the OECD control (-52.1%). GST The Kettle's unspiked river exposure was the only condition to significantly impact the activity of GST and was responsible for a -21.4% decrease when compare with the OECD control.

Table 5.4. The impact of the pharmaceutical mixture in OECD media and river matrix. Data represent average ± SD (N=4) replicates for each condition. Statistically significant by 2way ANOVA, by Sidak's multiple comparisons test ($p<0.05$). ALP, ACP, BGAL, LIP and PEP are expressed as milliunits/µg protein, GST is expressed as microunits/µg protein. Statistically different significance between river control and OECD control is represented by (*) and significant difference between spiked media and it's unspiked control by (**$**).

Enzyme	OECD Control / 0.1 mg/L	Broadmeadow Control / 0.1 mg/L	Kettle's Control / 0.1 mg/L	Grand Canal Control / 0.1 mg/L	Tolka Control / 0.1 mg/L
ALP	4.3±0.2 / **2.2±0.6 $(-49.2%)**	**2.6±0.1** / 2.9±0.2 ***(-39%)**	**2.8±0.3** / 2.8±0.1 ***(-35.6%)**	**3.3±0.1** / 3.2±0.1 ***(-24.1%)**	**2.8±0.3** / 3.2±0.1 ***(-34.9%)**
ACP	2.7±0.2 /2.6±0.4	3.3±0.4 / 3.4±0.1	2.3±0.2 / 2.4±0.4	**4.1±0.4** / 4.3±0.3 ***(49.9%)**	3.4±0.3 / 3.6±0.6
βGAL	1.7±0.1 / **0.5±0.2 $(-67.7%)**	1.9±0.2 / 1.7±0.1	1.7±0.1 / 1.7±0.1	1.6±0.1 / 1.7±0.1	1.7±0.2 / 1.8±0.1
LIP	67.9±3.4 / **39±4.3 $(-42.5%)**	**47.1±2.1** / 46±4.8 ***(-30.6%)**	**46.8±6.4** / 41.5±2.9 ***(-31%)**	62.9±4.9 / 58.7±5.1	57.3±7.5 / **77.4±7 $(35.1%)**
PEP	8±0.6 / **3.8±0.4 $(-52.1%)**	8.1±0.5 / 8.6±0.5	7.6±0.7 / 8.6±0.2	8.4±0.2 / 8.3±0.8	8.3±1 / 8.3±0.2
GST	468±25.7 /536.5±37.3	396.2±31.6 / 400.4±17.9	**367.7±31.2** /359.2±23.9 ***(-21.4%)**	452.3±52.2 / 451±41.8	462.4±42.8 /528.7±41.5

5.4 Discussion

Pharmaceuticals in the environment

As a result of their increased rates of production and diversification, pharmaceuticals now present a greater threat to the planet compared to other known agents of global change such as rising atmospheric carbon dioxide levels, biodiversity loss, habitat destruction and nutrient pollution (Bernhardt et al., 2017; OECD, 2019). Pharmaceuticals were designed to exert their mechanism of action in the biological system at low concentrations, and to ensure they reach target receptors and molecules, they were also created to be stable in nature. Subsequently, pharmaceutical release rates often exceed degradation rates in the environment and for this reason pharmaceuticals are ubiquitously detected in the aquatic system, where they can interact with non-target organisms (Bungau et al., 2018; OECD, 2019; Rogowska & Zimmermann, 2022). Pharmaceutical compounds have been detected up to the µg/L range in the receiving waters of WWTPs and hospital effluent (Orias Frederick, 2014), however in surface waters they are genuinely detected in the ng/L range. A critical issue in the estimation of pharmaceutical concentrations in the environment is that most studies employ different analytical instrumentation with non-standardised methods. Moreover, geographically many countries have been ignored, therefore most data are largely based on North America and Western Europe and as a result the full extent of global pharmaceutical pollution is unknown. A recent study, which measured pharmaceutical levels in 258 rivers over 137 countries revealed that

most significant pollution occurred in low-middle income areas, as a result of poor WWTP infrastructure, specifically in sub-Saharan Africa, South America and South Asia. Carbamazepine, metformin and caffeine were the most frequently detected pharmaceuticals, and occurred in over half of the sampling locations. Moreover, in 25.7% of the sampling sites, at least one pharmaceutical was at a concentration considered unsafe for aquatic organisms (Wilkinson et al., 2022).

Reported mixture effects in aquatic organisms

Perhaps one of the most important aspects of ecotoxicology is that chemicals in the environment co-exist in complex mixtures with other contaminants, transformation products, and by-products of WWTPs (Kahatagahawatte & Hara-Yamamura, 2020). Subsequently, these compounds can interact and cause synergistic, antagonistic or additive effects to non-target organisms (Liess et al., 2020). Although, the scientific community recognizes mixture effects, risk assessment and chemical safety is still often approached in a single chemical by chemical manner. Limited research exists of pharmaceutical mixture effects on aquatic organisms, moreover, these studies are primarily lab based using simple or binary mixtures, which generally do not fully reflect an environmental scenario.

The synergistic effect of NSAIDs on *Daphnia* immobilization has been reported (Cleuvers, 2003, 2004). For example, when combined at half the concentration of diclofenac and ibuprofen which elicited ~2% and ~12% immobilization respectively, synergism was demonstrated and 95% immobilization resulted, this binary mixture exceeded the predicted effects calculated using the Concentration Addition (CA) model. The binary mixture exhibited a synergistic effect at every EC value, whereas a quarterly mixture of diclofenac, ibuprofen, naproxen, and acetylsalicylic acid only exhibited synergism at EC_{50} and EC_{80}. The same mixtures exerted synergism at every EC value in algae growth inhibition tests and followed the predictive CA model. This illustrates the variance with such models, proving the difficulty of these mathematical models to estimate mixture effects, this also supported by another study that concluded CA or Independent Action (IA) models were not suitable in the predictability of the pharmaceutical mixtures investigated (Białk-Bielińska et al., 2022).

In a similar study, growth inhibition of the algae *Pseudokirchneriella subcapitata* was investigated following exposure to mixtures of antibiotics (González-Pleiter et al., 2013). Binary mixtures of erythromycin/levofloxacin, norfloxacin/erythromycin,

erythromycin/tetracycline, levofloxacin/norfloxacin, levofloxacin/tetracycline, and norfloxacin/tetracycline exerted a synergistic effect on growth inhibition. However, some of the same binary mixtures exerted an antagonistic effect on a stimulatory target organism *Cyanobacterium anabaena.* Norfloxacin, erythromycin, levofloxacin, norfloxacin demonstrated antagonism, whereas the other binary mixtures exhibited synergism. The chronic mixture effect of erythromycin, triclosan, trimethoprim, lincomycin, and sulfamethoxazole, resulted in decreased sex ratio in daphnids, this did not correlate with the results of individual exposures, proving the low predictability of mixtures (Flaherty & Dodson, 2005).

The mixture effect of several synthetic steroidal hormones, namely ethinylestradiol, trenbolone, beclomethasone dipropionate, desogestrel, and levonorgestrel was assessed on egg production in the fathead minnow (Thrupp et al., 2018). The lowest concentration (351.4 ng/L) of the mixture reduced egg production by 50%, whereas the median concentration (1159.4 ng/L) completely inhibited egg production.

In a multi-generational study, which assessed the individual and combined effect of the pharmaceuticals diclofenac, carbamazepine, ethinylestradiol, and metoprolol at environmentally relevant concentrations, on phenotypic endpoints body length and brood size of daphnids, revealed no significant differences from single or mixture exposures, emphasizing the unpredictable effects of mixtures (Dietrich et al., 2010).

Metabolites in relation to physiology of daphnids

In arthropods, biogenic amines are known to control energy metabolism (Gruntenko et al., 2005). Predominantly, they act as neurotransmitters, neuromodulators, and neurohormones, to regulate processes such as growth, development, behavior and reproduction, by binding to G coupled protein receptors, they are important in toxicological response (N. Liu et al., 2021; Zou & Fingerman, 1997). For example, the biogenic amine, histamine is reported to play a crucial signalling role in the visual system, thus is responsible for the modulation of phototaxis in response to UV exposure (McCoole et al., 2011). Similarly, to chordates, dopamine is an important neurotransmitter, which plays a crucial role in several metabolic pathways. The endocrine system of non-insect arthropods is still poorly understood, however in insects dopamine acts as a neurohormone and controls the synthesis and degradation of the juvenile hormone (JH). Although it is not well researched, it is postulated that the juvenile hormone has a critical role in daphnids also, as when exposed to JH or

its analogues, reduced offspring and altered sex-ratio occurred (Bodar et al., 1990; Miyakawa et al., 2018; Wang et al., 2011). In this study, the mixture induced the up-regulation of biogenic amines, particularly kynurenine. A seemingly generational impact can be observed, as more significant alterations can be seen for the third - generation daphnids. Moreover, a slight dose-dependent trend can also be observed as the higher concentration was responsible for the most changes. A similar trend was observed for the amino acids, with the majority of obvious changes recorded being increases, however a generational effect was not noted, as many alterations in amino acids were recorded for the first-generation following exposure to 0.1 mg/L. Interestingly, exposure to 1 mg/L led to only notable change of one metabolite i.e. citrulline, this is in contrast to the third-generation where a dose dependant trend can be seen, exposure to 1mg/L in the third generation led to the alteration of 20/21 amino acids measured.

Phospholipids are the main component of cell membranes, their structure consists of a lipid bilayer, which serves as a barrier of protection to the cell from environmental factors (Dai et al., 2021). There are two main groups of phospholipids, glycerophospholipids e.g. phosphatidylcholines and sphingolipids e.g. sphingomyelin (F. Yang & Chen, 2022), when phosphatidylcholine is cleaved by phospholipase, lysophosphatidylcholine is formed. In humans lysophosphatidylcholines are a marker for several diseases such as cancer and diabetes, in the liver it upregulates genes of cholesterol biosynthesis and down-regulates hepatic fatty oxidation (Law et al., 2019). It is also reported that lysophosphatidylcholines have significant roles in metabolic pathways of oxidative stress and inflammatory response (Carneiro et al., 2013). Sphingomyelin is the most abundant form of sphingolipid and is thought to play a key role in daphnid development and maturation. Moreover, toxicant exposure is reported to affect lipid metabolism and allocation as lipid receptors also act as xeno-sensor nuclear receptors. It has been reported that xenobiotic exposure negatively impacted development and reproduction (Sengupta et al., 2017),as well as enhance fecundity in daphnids (Fuertes et al., 2020). In this study, the lysophosphatidylcholines were mostly down-regulated, particularly following exposure to the 1 mg/L mixture in the first generation, and the 0.1 mg/L in the third generation. Overall, sphingolipids were not majorly affected by mixture exposure when compared to the other categories of metabolites. However, of the notable changes detected, most occurred in the first generation. Acylcarnitines play a significant role in cellular metabolism, specifically

fatty-acid β-oxidation, and are essential in the balance of intracellular lipid and sugar metabolism (Li et al., 2019). The findings of this study revealed the dysregulation of acylcarnitines in the first and third generation, particularly following 0.1 mg/L exposure, acetylcarnitine was down-resulted by both concentrations over both generations, in both instances the lower concentration induced a greater decrease.

Environmental samples

The inclusion of water samples in lab-based exposure experiments, is imperative to simulate realistic conditions as well as to demonstrate the validity of the experimental methodology. There is limited literature available directly related to daphnids exposed to surface water with emphasis on pharmaceuticals. However, a multigenerational study, which employed daphnids exposed to stream water contaminated with organic matter, revealed the adaption capability of daphnids (Chatterjee et al., 2019). The physiological endpoints, hemoglobin level, DNA methylation (epigenetic biomarker) and the proteome profile of daphnids was assessed over three generations. More instances of increased hemoglobin, DNA hypermethylation and differentially expressed proteins (DEPs) were recorded in the first generation, and decreased with generation, suggesting phenotypic plasticity and adaption of daphnids. Swimming behaviour was also assessed in the original generation, and proved to be a sensitive marker for toxicity as daphnids exposed to the stream water showed significantly decreased swimming speed, acceleration and locomotion rate as well as increased stopping instances and stop durations. This is supported by the work of (Egan et al., 2023), which observed negatively impacted swimming behaviour and altered phototaxis of daphnids exposed to river water contaminated by agricultural run-off. Another study which employed daphnids in chronic exposures to river water collected on three different dates revealed differences in survival (Sakai, 2001). No mortality was observed in 2 of 3 dates, but 100% mortality was recorded for water taken on the third date, the pesticides, fenitrothion, and thiobencarb were detected in this particular water sample. However *in vitro* exposures to thiobencarb, revealed no differences in the number of live neonates produced when compared to the controls, this study highlights the variance in results, the importance of sampling across several time points as well as the potential impact of mixture effects unknown chemicals and transformation products present in the water samples. Exposure to the environmental water-samples of this study resulted in many changes of enzyme activities of

daphnids, more so than their spiked counterparts, suggesting their likely contamination, these results indicate how holistic approaches could be adapted and employed to identify pollution hot-spots in the environment

5.5 Conclusion

Acute exposure to the pharmaceutical mixture of this study did not reveal many significant changes in biochemical markers, although when chronically and transgenerationally exposed to the same concentrations (0.1 and 1 mg/L), extensive alterations of enzyme activities and of metabolism were recorded. This highlights the importance of chronic exposures when assessing mixture toxicology. Moreover, in many instances throughout this study, the lower concentration posed the greatest impact to the molecular endpoints assessed, and or the effects only occurred following transgenerational exposure, thus emphasising the requirement to investigate mixtures at low concentrations and in multi-generational studies. This chapter was a pilot study of mixture toxicology, which validated the use of our methodology to investigate mixture effects in lab media and environmental sample exposures. This work could be strengthened by the addition of a water analysis to identify the potential contaminants present in the collected river samples, as well as an investigation into how the six selected pharmaceuticals of this mixture interact with each other in our OECD lab media.

5.6 References

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5.7 Supplementary material

Abbreviation	Analyte
Biogenic Amines	
Ac-Orn	Acetylornithine
ADMA	Asymmetric dimethylarginine
alpha-AAA	α-Aminoadipic acid
c4-OH-Pro	cis-4-Hydroxyproline
Carnosine	Carnosine
Creatinine	Creatinine
DOPA	Dihydroxyphenylalanine
Dopamine	Dopamine
Histamine	Histamine
Kynurenine	Kynurenine
Met-SO	Methionine sulfoxide
Nitro-Tyr	Nitrotyrosine
PEA	Phenylethylamin
Putrescine	Putrescine
Sarcosine	Sarcosine
SDMA	Symmetric dimethylarginine
Serotonin	Serotonin
Spermidine	Spermidine
Spermine	Spermine
t4-OH-Pro	trans-4-Hydroxyproline
Taurine	Taurine
Amino Acids	
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
Cit	Citrulline
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Orn	Ornithine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
Sphingolipids	
SM (OH) C14:1	Hydroxysphingomyelin C14:1
SM (OH) C16:1	Hydroxysphingomyelin C16:1

SM (OH) C22:1	Hydroxysphingomyelin C22:1
SM (OH) C22:2	Hydroxysphingomyelin C22:2
SM (OH) C24:1	Hydroxysphingomyelin C24:1
SM C16:0	Sphingomyelin C16:0
SM C16:1	Sphingomyelin C16:1
SM C18:0	Sphingomyelin C18:0
SM C18:1	Sphingomyelin C18:1
SM C20:2	Sphingomyelin C20:2
SM C22:3	Sphingomyelin C22:3
SM C24:0	Sphingomyelin C24:0
SM C24:1	Sphingomyelin C24:1
SM C26:0	Sphingomyelin C26:0
SM C26:1	Sphingomyelin C26:1
Lysophosphatidylcholines	
lysoPC a C14:0	lysoPhosphatidylcholine acyl C14:0
lysoPC a C16:0	lysoPhosphatidylcholine acyl C16:0
lysoPC a C16:1	lysoPhosphatidylcholine acyl C16:1
lysoPC a C17:0	lysoPhosphatidylcholine acyl C17:0
lysoPC a C18:0	lysoPhosphatidylcholine acyl C18:0
lysoPC a C18:1	lysoPhosphatidylcholine acyl C18:1
lysoPC a C18:2	lysoPhosphatidylcholine acyl C18:2
lysoPC a C20:3	lysoPhosphatidylcholine acyl C20:3
lysoPC a C20:4	lysoPhosphatidylcholine acyl C20:4
lysoPC a C24:0	lysoPhosphatidylcholine acyl C24:0
lysoPC a C26:0	lysoPhosphatidylcholine acyl C26:0
lysoPC a C26:1	lysoPhosphatidylcholine acyl C26:1
lysoPC a C28:0	lysoPhosphatidylcholine acyl C28:0
lysoPC a C28:1	lysoPhosphatidylcholine acyl C28:1
Acylcarnitines	
C0	Carnitine
C2	Acetylcarnitine
C3	Propionylcarnitine
C3-DC (C4-OH)	Hydroxybutyrylcarnitine
C3-OH	Hydroxypropionylcarnitine
C3:1	Propenoylcarnitine
C4	Butyrylcarnitine
C4:1	Butenylcarnitine
C5	Valerylcarnitine
C5-DC (C6-OH)	Glutarylcarnitine
C5-M-DC	Methylglutarylcarnitine
C5-OH (C3-DC-M)	Hydroxyvalerylcarnitine
C5:1	Tiglylcarnitine
C5:1-DC	Glutaconylcarnitine
C6 (C4:1-DC)	Hexanoylcarnitine
C6:1	Hexenoylcarnitine
C7-DC	Pimeloylcarnitine
C8	Octanoylcarnitine
C9	Nonaylcarnitine

C10	Decanoylcarnitine
C10:1	Decenoylcarnitine
C10:2	Decadienoylcarnitine
C12	Dodecanoylcarnitine
C12-DC	Dodecanedioylcarnitine
C12:1	Dodecenoylcarnitine
C14	Tetradecanoylcarnitine
C14:1	Tetradecenoylcarnitine
C14:1-OH	Hydroxytetradecenoylcarnitine
C14:2	Tetradecadienoylcarnitine
C14:2-OH	Hydroxytetradecadienoylcarnitine
C16	Hexadecanoylcarnitine
C16-OH	Hydroxyhexadecanoylcarnitine
C16:1	Hexadecenoylcarnitine
C16:1-OH	Hydroxyhexadecenoylcarnitine
C16:2	Hexadecadienoylcarnitine
C16:2-OH	Hydroxyhexadecadienoylcarnitine
C18	Octadecanoylcarnitine
C18:1	Octadecenoylcarnitine
C18:1-OH	Hydroxyoctadecenoylcarnitine
C18:2	Octadecadienylcarnitine
Phosphatidylcholines	
PC aa C24:0	Phosphatidylcholine diacyl C24:0
PC aa C26:0	Phosphatidylcholine diacyl C26:0
PC aa C28:1	Phosphatidylcholine diacyl C28:1
PC aa C30:0	Phosphatidylcholine diacyl C30:0
PC aa C30:2	Phosphatidylcholine diacyl C30:2
PC aa C32:0	Phosphatidylcholine diacyl C32:0
PC aa C32:1	Phosphatidylcholine diacyl C32:1
PC aa C32:2	Phosphatidylcholine diacyl C32:2
PC aa C32:3	Phosphatidylcholine diacyl C32:3
PC aa C34:1	Phosphatidylcholine diacyl C34:1
PC aa C34:2	Phosphatidylcholine diacyl C34:2
PC aa C34:3	Phosphatidylcholine diacyl C34:3
PC aa C34:4	Phosphatidylcholine diacyl C34:4
PC aa C36:0	Phosphatidylcholine diacyl C36:0
PC aa C36:1	Phosphatidylcholine diacyl C36:1
PC aa C36:2	Phosphatidylcholine diacyl C36:2
PC aa C36:3	Phosphatidylcholine diacyl C36:3
PC aa C36:4	Phosphatidylcholine diacyl C36:4
PC aa C36:5	Phosphatidylcholine diacyl C36:5
PC aa C36:6	Phosphatidylcholine diacyl C36:6
PC aa C38:0	Phosphatidylcholine diacyl C38:0
PC aa C38:1	Phosphatidylcholine diacyl C38:1
PC aa C38:3	Phosphatidylcholine diacyl C38:3
PC aa C38:4	Phosphatidylcholine diacyl C38:4
PC aa C38:5	Phosphatidylcholine diacyl C38:5
PC aa C38:6	Phosphatidylcholine diacyl C38:6

PC aa C40:1	Phosphatidylcholine diacyl C40:1
PC aa C40:2	Phosphatidylcholine diacyl C40:2
PC aa C40:3	Phosphatidylcholine diacyl C40:3
PC aa C40:4	Phosphatidylcholine diacyl C40:4
PC aa C40:5	Phosphatidylcholine diacyl C40:5
PC aa C40:6	Phosphatidylcholine diacyl C40:6
PC aa C42:0	Phosphatidylcholine diacyl C42:0
PC aa C42:1	Phosphatidylcholine diacyl C42:1
PC aa C42:2	Phosphatidylcholine diacyl C42:2
PC aa C42:4	Phosphatidylcholine diacyl C42:4
PC aa C42:5	Phosphatidylcholine diacyl C42:5
PC aa C42:6	Phosphatidylcholine diacyl C42:6
PC ae C30:0	Phosphatidylcholine acyl-alkyl C30:0
PC ae C30:1	Phosphatidylcholine acyl-alkyl C30:1
PC ae C30:2	Phosphatidylcholine acyl-alkyl C30:2
PC ae C32:1	Phosphatidylcholine acyl-alkyl C32:1
PC ae C32:2	Phosphatidylcholine acyl-alkyl C32:2
PC ae C34:0	Phosphatidylcholine acyl-alkyl C34:0
PC ae C34:1	Phosphatidylcholine acyl-alkyl C34:1
PC ae C34:2	Phosphatidylcholine acyl-alkyl C34:2
PC ae C34:3	Phosphatidylcholine acyl-alkyl C34:3
PC ae C36:0	Phosphatidylcholine acyl-alkyl C36:0
PC ae C36:1	Phosphatidylcholine acyl-alkyl C36:1
PC ae C36:2	Phosphatidylcholine acyl-alkyl C36:2
PC ae C36:3	Phosphatidylcholine acyl-alkyl C36:3
PC ae C36:4	Phosphatidylcholine acyl-alkyl C36:4
PC ae C36:5	Phosphatidylcholine acyl-alkyl C36:5
PC ae C38:0	Phosphatidylcholine acyl-alkyl C38:0
PC ae C38:1	Phosphatidylcholine acyl-alkyl C38:1
PC ae C38:2	Phosphatidylcholine acyl-alkyl C38:2
PC ae C38:3	Phosphatidylcholine acyl-alkyl C38:3
PC ae C38:4	Phosphatidylcholine acyl-alkyl C38:4
PC ae C38:5	Phosphatidylcholine acyl-alkyl C38:5
PC ae C38:6	Phosphatidylcholine acyl-alkyl C38:6
PC ae C40:1	Phosphatidylcholine acyl-alkyl C40:1
PC ae C40:2	Phosphatidylcholine acyl-alkyl C40:2
PC ae C40:3	Phosphatidylcholine acyl-alkyl C40:3
PC ae C40:4	Phosphatidylcholine acyl-alkyl C40:4
PC ae C40:5	Phosphatidylcholine acyl-alkyl C40:5
PC ae C40:6	Phosphatidylcholine acyl-alkyl C40:6
PC ae C42:0	Phosphatidylcholine acyl-alkyl C42:0
PC ae C42:1	Phosphatidylcholine acyl-alkyl C42:1
PC ae C42:2	Phosphatidylcholine acyl-alkyl C42:2
PC ae C42:3	Phosphatidylcholine acyl-alkyl C42:3
PC ae C42:4	Phosphatidylcholine acyl-alkyl C42:4
PC ae C42:5	Phosphatidylcholine acyl-alkyl C42:5
PC ae C44:3	Phosphatidylcholine acyl-alkyl C44:3
PC ae C44:4	Phosphatidylcholine acyl-alkyl C44:4

PC ae C44:5	Phosphatidylcholine acyl-alkyl C44:5
PC ae C44:6	Phosphatidylcholine acyl-alkyl C44:6

Appendix A

A. 1 Daphnia magna culturing protocols

A.1.1 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.

***A.1.2 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.

A.1.3 Proposed plan for culturing daphnids. The following culturing scheme is followed only to generate clonal populations of mothers who will breed animals for experiments.

Daphnid age (days)	Procedure		OECD	Yeast	Fresh Algae	Seaweed Extract
1	**Setup new culture**		4 l	2 ml	4 ml	12 ml
2				2 ml	4 ml	
3				2 ml	4 ml	
4				2 ml	6 ml	
5				2 ml	6 ml	
6	Change media		4 l	2 ml	6 ml	12 ml
7	Remove neonates every day and throw them			2 ml	6 ml	
8				2 ml	8 ml	
9				2 ml	8 ml	
10				2 ml	8 ml	
11				2 ml	8 ml	
12				2 ml	8 ml	
13	Change media		4 l	2 ml	8 ml	16 ml
14	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	

A.2 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.

A.2.1 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 sterilized by autoclaving for 15 minutes at 121˚C and stored in the cold room.

A.3 Metabolomic extraction protocol

Animals which were previously snap-frozen using liquid nitrogen were homogenised in Eppendorf's with an 80:20 HPLC methanol and water mixture, using a pestle. Following this, Eppendorf's were centrifuged at 10'000 rpm for 5 minutes, to remove unhomogenized tissue. The resulting clear supernatant was collected and a fixed volume was evaporated using a speedvac. Quality Control (QC) samples were created by pooling a fixed volume from each sample, and reagent blanks (RBs) of 80:20 HPLC methanol and water were also created. In the same manner as the other samples, a fixed volume of QCs an RBs were transferred to Eppendorf's, and evaporated using the speedvac. All samples were stored at -80°c, until time of analysis.

Appendix B

B.1 Papers as first author

O'Rourke, K.; Engelmann, B.; Altenburger, R.; Rolle-Kampczyk, U.; Grintzalis, K. Molecular Responses of Daphnids to Chronic Exposures to Pharmaceuticals. *Int. J. Mol. Sci.* 2023, *24*, 4100. https://doi.org/10.3390/ijms24044100

O'Rourke, K.; Virgiliou, C.; Theodoridis, G.; Gika, H.; Grintzalis, K. The impact of pharmaceutical pollutants on daphnids – A metabolomic approach. *Environmental Toxicology and Pharmacology*, 100, 104157. https://doi.org/10.1016/j.etap.2023.104157

B.2 Oral Presentations

O'Rourke Katie and Grintzalis Konstantinos, (2020). Investigation of the potential effect of oestrogenic substances on *Daphnia magna*. BRS Research Day 2021.

O'Rourke, Katie and Gkrintzalis, Konstantinos, (2021). The acute, chronic and transgenerational effects of antibiotics on *Daphnia magna.* OpenTox 2021.

O'Rourke Katie, O'Rourke Katie, Engelmann Beatrice, Altenburger Rolf, Rolle-Kampczyk Ulrike E., Grintzalis Konstantinos (2022). Responses of daphnids upon chronic exposures to pharmaceuticals. Animal Biology Early Career Researcher Symposium, Helsinki, Finland, 2022.

B.3 Posters

O'Rourke, Katie and Gkrintzalis, Konstantinos, (2019). The impact of medicinal compounds on the physiology of *Daphnia magna.* SETAC Europe 30th Annual Meeting – 2020 (Dublin).

O'Rourke, Katie and Gkrintzalis, Konstantinos, (2021). The acute and chronic impact of a range of environmentally relevant compounds on Daphnia magna enzyme activity. EnvChem 2021.

O'Rourke, Katie and Gkrintzalis, Konstantinos, (2021). Acute and chronic exposure to oestrogenic compounds results in biochemical alterations in *Daphnia magna*. SEB 2021 Annual Conference.

O'Rourke Katie, Engelmann Beatrice, Altenburger Rolf, Rolle-Kampczyk Ulrike E., Grintzalis Konstantinos (2022). Chronic exposure to pharmaceuticals results in metabolic and physiological responses in *Daphnia magna.* Metabolomics Conference, Valencia, Spain 2022.

O'Rourke Katie, Engelmann Beatrice, Altenburger Rolf, Rolle-Kampczyk Ulrike E., Grintzalis Konstantinos (2022). The metabolic and physiological responses of daphnids upon chronic exposures to pharmaceuticals. The Biochemistry Global Summit, Lisbon, Portugal, 2022.