

**The response of *Daphnia pulex* to multiple environmental stressors**

Shlair AbdulRazzaq Sadeq

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

The University of Sheffield

Faculty of Science

Department of Animal and Plant Sciences

October 2018

*My husband,*

*My family,*

&

*Home*

*Thank you for being my inspiration and drive to succeed*

**Acknowledgments**

Throughout my research journey, many people have had significant roles in my career. I am grateful to all of them.

First, I would like to express my special thanks to Andrew Beckerman for his invaluable guidance, endless support and lovely times during the lab meetings. Thanks Andrew for introducing me to the *Daphnia*, which made my time in the lab very special. Thanks also to Dr. Dörthe Becker for assisting me in most parts of my project. Big thanks to Dr. John Moody and Andrew Atfield from Plymouth University for helping me with the enzyme assays. I would also like to thank Sophie and Will for being keen on answering my questions.

To all the lab members, PhD and generations of master students thank you for your support. Thanks Erika for reviewing my papers. Richard, thank you for helping me with the microbiome experiment.

To all my friends here and home who have put a smile on my face thank you. I have not have the space to thank you individually, but you have been truly supportive of everything I have done.

Thanks to all the stuff in the department: technicians and administration office’s members. I would especially like to thank Allison and Lynsey, they have always been helpful and excellent.

Finally, I am grateful to the Ministry of Higher Education and Scientific Research/Iraq for funding this project and the Iraqi attaché in London for the facilitating student’s requests.

I would have not got to this point without your help. Thank you so much, everyone.

**Author’s Declaration**

The research presented in this thesis is my own and the thesis has not been submitted for any other award at this or any institution.

Chapter 2

This chapter has been published as:

**Sadeq, S.A. & Beckerman, A.P. 2019. The chronic effects of copper and cadmium on life history traits across Cladocera species: A meta-analysis. Archives of Environmental Contamination and Toxicology.** <https://doi.org/10.1007/s00244-018-0555-5>**.**

The manuscript is reproduced fully in the thesis with minor formatting alterations. SAS and APB conceived the study. SAS collected the data. SAS and APB performed the statistical analyses. SAS wrote the manuscript with contributions from APB.

Chapter 3

This chapter has been accepted to the Journal of Enviromental Science and pollution Research as:

**Sadeq, S.A. & Beckerman, A.P. 2019. Evaluating additive vs. interactive effects of copper and cadmium on *Daphnia pulex* life history.**

The manuscript has been accepted with revision at Enviromental Science and pollution Research. It is reproduced fully in the thesis with minor formatting alterations. SAS and APB conceived the study. SAS performed the experiments and collected the data. SAS and APB performed the statistical analyses. SAS wrote the manuscript with contributions from APB.

Chapter 4

This chapter is currently in preparation for submission to the Journal of Functional Ecology as:

**Sadeq, S.A. & Beckerman, A.P. 2019. Biomarker responses in *Daphnia pulex* exposed to copper and cadmium: Digestive and antioxidant enzymes**

It is reproduced fully in the thesis with minor formatting alterations. SAS and APB conceived the study. SAS performed the experiments and collected the data. SAS and APB performed the statistical analyses. SAS wrote the manuscript with contributions from APB.

Chapter 5

This chapter has been submitted to the Journal of Proceedings of the Royal Society B as:

**Sadeq, S.A. & Beckerman, A.P. 2019. The microbiome mediates an interaction between natural and anthropogenic stress on life history, morphology and condition.**

It is reproduced fully in the thesis with minor formatting alterations. This work also involved Richard Lloyd Mills, an APS MRes student. SAS, RLM and APB conceived the study. SAS and RLM performed the experiments and collected the data. SAS, RLM and APB performed the statistical analyses. SAS wrote the manuscript with contributions from RLM and APB.

**Table of contents**

Abstract…………………………………………………………………………………..viii

Chapter 1 ……………………………………………………………………………….....1

1.1 Introduction: Stressors and mechanisms…………………………………..........1

1.1.1 The Ecological Scales of Multiple Stressors……………………………….....3

1.1.2 Metals and Predation as a Case Study of Multiple Stressors…………………………8

1.2 Heavy metals: Background, classification and effects………………..……….10

1.3 Feeding Behaviour in *Daphnia*………………………………………………...13

1.4 Biomarker responses…………………………………………………………..16

1.5 Predation, metals and the microbiome ………………………………………..17

1.6 Model system……………………………………………………………….....18

Chapter 2…………………………………………………………………………………22

2.1 Introduction……………………………………………………………………22

2.2 Materials and methods………………………………………………………...27

2.3. Results………………………………………………………...……………….29

2.3.1 Copper: Aqueous and dietary delivery……………………………………....29

2.3.2 Cadmium: Aqueous and dietary delivery…………………………....………34

2.3.3 Copper and cadmium: Experimental factors………………………………....37

2.4. Discussion……………….…………………………………………………….42

Chapter 3…………………………………………………………………………………46

3.1 Introduction…………………………………………………………………….46

3.2 Material and methods…………………………………………………………..49

3.3 Results: Life history traits…………………………………..………………….52

3.4 Discussion…………………………………………………………………….58

Chapter 4………………………………………………………………………………..62

4.1 Introduction…………………………………………………………………..62

4.2 Material and methods………………………………………………………...65

4.3 Results: Enzyme assays…………………………………...…………………69

4.5 Discussion……………………………………………………………………77

Chapter 5……………………………………………………………………………….82

5.1 Introduction…………………………………………………………………..82

5.2 Material and methods………………………………………………………...85

5.3 Results: Enzyme assays…………………………………...………………….89

5.5 Discussion……………………………………………………………………95

Chapter 6 General discussion……...………………………………………………….100

References…………………………………………………………………………….109

Appendix…………………………………………………………………………...….151

**Thesis abstract**

Natural and anthropogenic sources produce a wide range of stressors, which influence the water quality and food web’s components including *Daphnia* populations. Current environmental risk assessment remains dominated by evaluation of single substances and single species. However, typically, aquatic organisms are exposed to various chemicals and stressors, which together cause additive, synergistic or antagonistic effects. Therefore, understanding population and community response to various stressors requires understanding how the stressors interact and how different genotypes/species respond. Both are necessary to evaluate the risks associated with pollutants.

Because *Daphnia* spp. are keystone species regulating algae biomass, supporting invertebrate and vertebrate consumers and influencing the provision of freshwater, impacts on them may influence the structure of entire communities. Within populations, stressors and other conditions, such as water chemistry, may directly influence phenotype and digestive physiology of daphnids. In this thesis, I examined how copper (Cu), cadmium (Cd) and predation risk influence *Daphnia pulex* life history, foraging activity and digestive, and oxidative stress responses.

I first examined, via meta-analysis of 32 published papers, the sub-lethal effects of Cu and Cd  
concentrations on Cladoceraspp. life history (reproduction, maturation age, and somatic growth rate). I found that Cladocera spp. showed different variations in their responses to both metals, and *D. magna* was found to be less sensitive to sub-lethal changes in reproduction. The effects were largely consistent for aqueous vs. dietary food. Further, water hardness and exposure duration (only Cu) had no detectable effect for *D. magna* reproductive response.

Literature-based toxicity data on Cladocera is focused on examination of single toxicants and single genotypes/species. Further, in ecotoxicology, a major concern is that genotypes of a certain species can respond differently to stressors in both laboratory and field tests. Therefore, I examined, using response surface design, whether the effects of Cu and Cd are additive or interactive on foraging and life history traits using three genotypes of *Daphnia pulex*. I further replicated our experiment under standard and high food levels. I found that that the studied traits were affected in non-additive ways by the following factors: mixtures of stressors, genotypes and resource levels.

Next, I examined the response of different biomarkers (digestive such as Amylase, Trypsin and Esterase and antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and glutathione S-transferases) under lethal exposures of Cu and Cd (alone and in a mixture). I found, as with the life history responses, numerous instances of synergistic interactions of Cu and Cd on both digestive and antioxidant enzyme activity.

Finally, I examined the interaction between the anthropogenic stress (Cu) and natural stress (predation risk) by examining morphology, life history and body condition. I also manipulated the microbiome of *D. pulex* to test whether the response to each and to multiple stressors was influenced by the gut bacterial community. I found multiple instances of non-additive, interactive effects of Cu and predation risk across life history traits. I also found that the community structure of the natural gut microbiota responds to any stress in non-additive way. However, manipulation of the gut microbiota at young ages generated unique communities later in life and many instances where life history responses were reversed from untreated conditions.

This thesis contributes to further understanding of the risk associated with multiple stressors, in particular highlights the metals interaction together and with other stressors in the context of risk assessment and water quality guidelines.

**Chapter 1**

**1.1. Introduction: Stressors and mechanisms**

In ecology, a stress refers to any external conditions that can cause a detrimental effect to living organisms such as climate change, loss of food availability and pollution (Bijlsma and Loeschcke 2005, Steinberg et al. 2010, Altshuler et al. 2011, Killen et al. 2013). In the natural ecosystems, organisms are exposed simultaneously to a wide range of stressors. Anthropogenic sources of stressors are becoming a serious concern due to their potential impacts directly on ecosystem services such as freshwater (via releasing different classes of substances to the natural water), but also on living organisms’ physiology and fitness (growth and reproduction), which pose a further risk to ecosystem functions that underpin these services and ultimately human wellbeing (Altshuler et al. 2011, Bergman Filho et al. 2011, Hani et al. 2019). Against this context, multiple stressor research has grown in the field of ecotoxicology, and stressors are typically classified as either chemical or non-chemical. The latter are further characterized into extrinsic abiotic factors such as low oxygen, temperature, pH, UV-radiation and salinity, and extrinsic biotic factors such as predation, parasites and bacteria (Fig. 1.1) (Coors and Meester, 2008, Hani et al., 2019).

When there are multiple stressors, their impacts may be additive or interactive and interactions may be synergistic or antagonistic (Folt et al. 1999,Piggott et al. 2015). Understanding of whether and how stressors interact, in the context of effects on organisms and responses by these organisms, is increasingly important to ecotoxicologists (Altshuler et al. 2011, Shaw et al. 2017). Responses (e.g. phenotypic and genomic responses) to multiple stressors are now studied in ecology and ecotoxicology to identify the effects of anthropogenic activities at different ecological scales such as physiology, life history and communities (e.g. distributions) and to ecosystem services such as water quality (Feder and Hofmann 1999, Meaney and Szyf 2005, Eads et al. 2008, Crespi et al. 2013, Todgham and Stillman 2013). The evaluation of their effects continues to focus on molecular and physiological biomarkers which may provide rapid insights into the impacts and also the mechanisms giving rise to them (Demirak et al. 2006, Ferreira et al. 2008, Killen et al. 2013).

**Environmental stressors**

**A biotic factors**

**Biotic factors**

**Predation Bacterioplankton Organic matter Heavy metals Temperature Nutrients**

**Freshwater Ecosystem (Stressors’ effects)**

**Daphnia Populations**

**Figure 1.1.** A diagram showing the environmental stressors and their effects on ecosystem: Food web and water quality.

*1.1.1 The Ecological Scales of Multiple Stressors*

In order to reveal additive versus interactive effects of multiple stressors and identify the mechanisms giving rise to these patterns, research must be done at multiple ecological scales. In the following sections I review conceptual ideas about life history, behaviour, physiology, and genetics that guide our thinking and research about multiple stressor effects. In this section I provide some examples too, largely from the *Daphnia* literature – *Daphnia magna* is the focal organism in this thesis and is a model organism for ecological and ecotoxicological research on anthropogenic and natural forms of stress.

1.1.1.1 *A framework for the process of multi-stressor effects and responses*

An important challenge for developing a predictive understanding of how organisms respond to multiple stressors is to describe the mechanisms by which a first stressor modulates physiological responses to a second stressor. At the organismal level, interactions can arise when a first stressor elevates tolerance to a second stressor (possible antagonism) or causes the organisms to be more susceptible to the second stressor (possible synergy; Todgham and Stillman 2013). At the cellular level, multiple molecular pathways, each stimulated by a separate stressor, may converge on one physiological function, or each stressor may modulate the same pathway, producing distinct physiological outcomes (Todgham and Stillman 2013). The magnitude and duration of stress will also impact on these physiological processes. Ultimately, organisms may divert variable amounts of available energy away from growth and reproduction to cellular defense and maintenance of homeostasis (Barata et al. 2005, Jemec et al. 2007, Todgham and Stillman 2013). In the context of a multiple stressors and a changing climate, it is vital to assess these processes and assess the bio-energetic costs of and downstream consequences for fitness.

1.1.1.2 *Life History and Behaviour*

A cornerstone of research on multiple stressors is linked to evaluating behavioural and life history responses to multiple stressors (Folt et al. 1999, Piggott et al. 2015). Fundamentally, work at this scale involves factorial experiments that are designed to reveal whether the effects of multiple stressors on life history trait and behavioural responses are additive or interactive via statistical inference. Though there has been considerable exploration into the toxicity of stressors on aquatic organisms, particularly Cladocera, the majority of work has focused on single stressors and under standardised conditions that are beneficial to a testing environment but not always reflective of natural communities (Brix et al. 2001, Barata et al. 1998, Griffitt et al. 2008, Sadeq and Beckerman 2019).

For example, the effects of thermal stress, cyanotoxin exposure, and low food on life history traits (somatic growth rate, reproduction) of *Daphnia* have been widely studied(Folt et al. 1999, Coors and De Meester 2008, Steinberg et al. 2010). The study of [De Coninck](https://www.sciencedirect.com/science/article/pii/S0166445X1300180X#!) et al. (2013) examined the interactive effect between metal (Cd) and a natural stressor (Microcystis) on D. magna reproduction in 20 different clones. It has been found that both stressors did not inhibit the reproductive performance of Daphnia. Also, *no* inter-clonal variation was found of the interactive effect between stressors. Furthermore, temperature has been shown to have great effects on the life history of *Daphnia*, inducing earlier emergence (Carvalho and Kirika 2003) and shorter lifespan (Bottrell 1975). In addition, as temperature increases, the filtering rate, metabolic rate, and demand for food also increase (Burns 1969). Even little increase in temperature during a short seasonal period can adversely affect *Daphnia* populations, and induce significant changes in entire food webs (Wagner and Benndorf 2007). Moreover, temperature changes can cause rapid microevolution of *Daphnia* and alter the community structure of freshwater habitats (Van Doorslaer et al. 2010).

In ecotoxicological research, there is a strong theoretical template for defining multiple stressor effects based on the way that doses/concentrations of stressors combine – the concentration addition (CA) model – or because of the way that responses to the stressors combine – the independent action (IA) model. The CA model was historically formulated for exploring how chemicals with similar modes of action combine and assumes that compounds with similar modes of action will behave as if they are simply higher doses of a single compound. Critically, the dose of each compound is expected to behave additively on the response variables. In contrast, the IA model was developed based on an assumption that the effects of the compounds, not the compounds themselves, behave additively and typically applies to chemicals with vastly different modes of action, for example anthropogenic stress versus natural stress.

Against this template, there is growing literature investigating the effects of metal mixtures/stressors and *Daphnia* spp. Metals remain a major pollutant and toxicant, even though levels have reduced below toxic and are now often classed as sub-lethal (Shuhaimi-Othman et al. 2010, Fernández-Gonzáles et al. 2011). This research focuses particularly on foraging rates because they are linked to growth and reproduction. For example, in recent research, mixtures of nickel (Ni), zinc (Zn), copper (Cu0, and cadmium (Cd) produced a more than additive effect on food consumption rate of *D. magna* at low concentration of metals(Lari et al. 2017). Inter-clonal variation in *D. magna* in the response of ingestion rate to Cd and temperature (Muyssen and Janssen 2010) reveals a mixture of additive and interactive effects depending on genotypes. These examples and other work suggests a key role of foraging and digestion in metal effects.

In contrast, many studies have found only antagonistic effects. For example, recent research by Pérez and Hoang (2018) on mixture toxicity to *D. magna* showed that the sub-lethal concentration of Cd and Ni offset each other in their effects on life history traits. Other studies on metal mixture toxicity have also reported similar less than additive effects to *D. magna* (Komjarova and Blust 2008, Meyer et al. 2015, Traudt et al. 2017, Pérez and Hoang 2017, 2018). Further, Mahar and Watzin (2005) examined the impacts of Cu, Zn, and insecticide mixtures on the survival and reproduction in *Ceriodaphnia dubia*. It has been found that metals had a great impact on toxicity of diazinon. Also, less‐than‐additive interactions were observed in most mixture treatments on reproduction.

1.1.1.3 *Physiology*

There are many ways in which the physiological response to multiple stressors are studied. The research approach is often linked to the specificity and target of particular stressors (Feder and Hofmann 1999, Knops et al. 2001, Eads et al. 2008), but most often research focuses on respiration and metabolism. For example, Barber et al. (1990) studied the respiration rates in different clones of *D. magna* under cadmium and 3,4-dichloroaniline exposures. The rate of oxygen consumption was reduced in both clones at concentrations 5 and 20 µg/l. Furthermore, [Knops](https://www.sciencedirect.com/science/article/pii/S0166445X00001703#!) et al. (2001) investigated the alterations in the physiological energetics, growth and reproduction of *D. magna* in exposure to toxicants stress: Cu, Cd and cetyltrimethylammonium bromide (CTAB)*.* It has been observed that toxicant exposures impaired growth rate and affected reproduction while no change on metabolic costs of exposed animals.

Aspects of stress physiology are also used as biomarkers/indicators of individual performance and fitness. Biomarkers include reactive oxygen species profiles (Doyotte et al. 1997, Barata et al. 2005, Jemec et al. 2007) and heat shock proteins (Kagawa and Mugiya 2002, Sørensen et al. 2003, Pijanowska and Kloc 2004). The study of Atienzar et al. (2001) used the molecular and population levels to assess toxicants. The work examined the random amplified polymorphic DNA profiles and some ecological fitness parameters (age-specific survival, age-specific fecundity, net reproductive rate, and intrinsic rate of population increase) in *D. magna* under Cu exposures. It has been suggested that most of the fitness parameters and genomic template stability were affected at high concentrations of the metal.

Finally, more specific work is guided by the targets of stressors. For example, stressors that target feeding and assimilation have been examined in the context of digestive enzymes (De Coen and Janssen 1997, Chen et al. 2002). De Coen and Janssen (1997) found that the sub-lethal concentrations of CdCl2 and HgCl2 inhibited the activity of cellulase, amylase, galactosidase, trypsin and esterase after 48h exposure of both toxicants. Stressors such as pesticides or endocrine disruptors, which target neural or reproductive machinery, elicit research focused on physiological responses associated with neuro-endocrine hormones [(Olmstead](https://setac.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Olmstead%2C+Allen+W) and [LeBlanc](https://setac.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=LeBlanc%2C+Gerald+A) 2000, [Snyder](https://www.sciencedirect.com/science/article/pii/S0166445X01001734" \l "!) [and Mulder](https://www.sciencedirect.com/science/article/pii/S0166445X01001734#!) 2001, Tatarazako and Oda 2007) or with reproductive hormones ([Olmstead](https://setac.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Olmstead%2C+Allen+W) and [LeBlanc](https://setac.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=LeBlanc%2C+Gerald+A) 2000, [Brennan](https://www.sciencedirect.com/science/article/pii/S0045653505014098#!) et al. 2006). For example, the study of [Schlotz](https://www.sciencedirect.com/science/article/pii/S1095643312001286#!) et al. (2012) found that the rich food (dietary polyunsaturated fatty acids) had a beneficial effect on reproduction and population growth rates of D. magna accompanying by an increased vitellogenin (DmagVtg1) gene expression.

1.1.1.4 *Genetics*

The development of molecular genetic technologies has allowed even deeper investigation into the genes or gene networks that might underpin or even regulate the physiological responses to multiple stressors. One of the most insightful has centred around metal detoxification processes and specifically in *Daphnia* spp. with its sequenced and annotated genome of *D. pulex* (Shaw et al. 2007, Colbourne et al. 2011). At the cellular level, the key detoxification process of metals implies a metalloprotein, the metallothioneins (MTs), which are non-enzymatic proteins with a low molecular weight, high cysteine content, no aromatic amino acids and heat stability. MTs are involved in several cellular processes: metal transport and storage, de-activation of zinc regulated proteins and scavenging of free radicals. MTs detoxify or control the internal availability of trace metals in many aquatic invertebrates (Roesijadi 1992, Wallace et al. 2003). The thiol groups (–SH) of cysteine residues enable MTs to bind particular heavy metals. Because of its structure, it is considered to be important in the homeostatic control of essential metals (e.g., Cu, Zn) and the detoxification of nonessential metals like Cd and Hg (Tsui and Wang 2007).

Genetic insights are not limited to metals with similar insights arising from work on the stressors (individuals and combination) (Neumann and Galvez 2002, Poynton et al., 2007, Soetaert et al. 2007, Asselman et al. 2012). *Daphnia* is an important model system for crustaceans and freshwater organisms due to the availability of its whole genome sequence (Colbourne et al. 2011). Several ecotoxicogenomic studies employed *Daphnia* to characterise profiles of gene expression for various metal contaminants such as copper, cadmium, zinc, and nickel (Poynton et al. 2007, Vandegehuchte et al. 2010, Poynton et al. 2011).

1.1.2 *Anthropogenic and Natural Stress:* *Metals and Predation as a Case Study of Multiple Stressors*

Natural ecosystems are permanently under threat of exposure to multiple stressors, such as metal contaminants, acidification and eutrophication (Moore et al. 1997, Carpenter et al.1998, Demirak et al. 2006, Ferreira et al. 2008, Shaw et al. 2017). However, these largely anthropogenic sources of stress on populations and communities are operating against a backdrop of natural/ecological stress linked to predation, disease and food supply variation. My thesis focuses on sub-lethal exposure to metals and to the risk of predation in freshwater aquatic communities, particularly on *Daphnia spp.*

and on three environmental stressors: Copper (Cu), cadmium (Cd) and predation risk, all of which are known to induce, at sub-lethal ‘concentrations’, changes in physiology, foraging and life history. I specifically examine their effects on the water flea *D. pulex,* focusing on mixtures of the metals. I combined feeding assays and life table analyses to explore additive vs. interactive (synergy or antagonism) effects of the metals on traits with experimental assessment of digestive and antioxidant enzymes associated with exposure to different metals (individually and mixtures) to better understand mechanisms. Finally, I explored the interaction between Cu and predation, specifically focusing on the role of digestive physiology and the microbiome by experimentally removing the bacterial microbiome from the daphnids. Here I review the motivation for this focus.

1.1.2.1 *Metal contamination and sub-lethal exposure in freshwater*

The contamination of aquatic ecosystems by metals has received a great deal of attention (Shalaby 2000, Zhou et al. 2008). Current levels of contamination in many parts of the world are now considered sub-lethal, meaning exposure to and acquisition of contaminants, rather than lethality, are vital to understand their effects. At low and threshold concentrations, evidence, much of it from research on *Daphnia spp.* suggest that metals exert a series of toxicological impacts on living organisms and water quality. These impacts include alterations in reproduction (Wong 1993, Poynton et al. 2007, Vandegehuchte et al. 2010, Poynton et al. 2011), somatic growth rate (Koivisto et al. 1992, De Schamphelaere and Jaanssen 2004 a, De Schamphelaere et al. 2007, Cooper et al. 2009), respiration and metabolism (Winner et al. 1977, Bohrer and Lampert 1988), and the relationship between predators and prey (Pyle and Mirza 2007, Boyd 2010, DeMille et al. 2016). Metals also induce genotoxic effects including molecules damage as well as increased mutation rate which may be damaging to the integrity of the genome (Conners and Black 2004, Rogalski 2017). Chronic experimental exposure to sub-lethal concentrations is typically used to examine responses that may scale up to ecologically relevant information at the population level (Sarma and Nandini 2006, Zhao and Wang 2011, Nys et al. 2015).

1.1.2.2 *Predation risk in freshwater*

In freshwater ecosystems, organisms are exposed to a wide range of biotic and abiotic stressors including predation (Hunter and Pyle 2004, Long et al. 2004, Coors and De Meester 2008). Predation is a major biological stress to *Daphnia* populations. Several decades of research have revealed a wide array of responses to predation risk in habitat use ([Dodson](https://setac.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Dodson%2C+Stanley+I) et al. 1995, De Meester and Cousyn 1997), foraging (Noonburg and Nisbet 2005, Balseiro et al. 2007), life history (Rose et al. 2001, Schulz and Dabrowski 2001, Campero et al. 2007, Pestana et al. 2009, Beckerman et al. 2010, Trekels et al. 2013) and morphology (Tollrian 1995, Hammill et al. 2008). The phenotypic plasticity, and associated genetic adaptation, underpin the structure, distribution and abundance of *Daphnia* communities (Reger et al. 2018). Moreover, predation can cause changes in the composition and diversity of natural communities in addition to influences on ecosystem through recycling nutrients (Attayde and Hansson 1999, Berga et al. 2015). More details are found section 1.5 and 1.6.

In the following sections, I review heavy metal research and the mechanisms by which such metals, specifically Cu and Cd, might impact on organismperformance, focusing on *Daphnia* spp. I also review various other environmental variables that might interact with the effects of Cu and Cd effects on *Daphnia spp*. Finally, I formally introduce the model system used in my experiments and the thesis aims and objectives, with an overview of a meta-analysis, experiments focused on Cu and Cd interactions and mechanism and experiments focused on digestive physiology and Cu–predation interaction mediated by the microbiome.

**1.2. Heavy metals: Background, classification and effects.**

Due to their persistence, non-biodegradability and potential risks to living organisms and ecosystem quality, metals are considered the most important category of environmental contaminants (Shalaby 2000, Zhou et al. 2008, Boyd 2010). The presence of heavy metals in water bodies comes from anthropogenic and natural discharges such as agricultural and industrial activities, atmospheric deposition, domestic effluents and coal combustion (Demirak et al. 2006, Ferreira et al. 2008, Shaw et al. 2017). In natural waters, metals show different physico-chemical forms either as free ions or complexes with organic and inorganic substances (Barata et al. 1998). However, in mixtures, metals can interact with each other causing synergistic, additive or antagonistic influences (Kim et al. 2006, Syberg et al. 2008).

Metals are categorized into two groups: essential metals, such as Cu, Zn, chromium (Cr) and manganese (Mn), are important for sustaining the development and growth of living organisms (Bossuyt and Janssen 2004), as well as synthesis of DNA and RNA molecules (Giusto and Ferrari 2014, Ackerman et al. 2017). However, these metals become toxic at high concentrations (Wang et al. 2004 b, Duarte et al. 2017). Whereas, non-essential elements, such as Cd, lead (Pb) and mercury (Hg**)**, induce toxicity at low concentrations (Duarte et al. 2017).

Food and water represent the major routes for metals uptake in animals (Barata et al. 2002 a, Géret et al. 2002, Rainbow, 2002). However, the way of taking up metals depends on feeding behaviour, body size, exposure duration and life cycle (Gerhardt 1993). Animals may take metals via the ingestion of food contaminated with toxic chemicals (Bryan and Langston 1992, Weltens et al. 2000, Rainbow 2002). In many invertebrates (e.g. daphnids), the effects of contaminated particles on foraging have been documented by the reduction in the food intake that causes serious sub-lethal impacts on populations (Allen et al. 1995, Weltens et al. 2000). Furthermore, in aquatic invertebrates, metals can also enter the body through the cuticle (Bodar et al. 1988a, Krantzberg and Stokes 1988, Robinson et al. 2003). Ions of Fe (iron), Mn and Pb are sorbed on the body surface and bind to the cuticle, whereas, Cd, Zn and Cu are accumulated in the cytosol within cells (Cain et al. 1992).

Aquatic organisms may thus accumulate metals in their tissues and organs (Rainbow 2002, Heugens et al. 2003). Metal ions can penetrate into cells in different ways i) simple diffusion, ii) interaction with proteins and/or ion channels in the cell membrane (Veltman et al. 2008). Cd ions may penetrate cell membranes by cell diffusion through calcium channels, while Cu and Zn via active transfer of transporters (Grosell and Wood a 2002).

At the optimal environmental concentration of essential elements, organisms may regulate the internal concentrations of certain elements in three ways: i) active regulation, ii) storage, iii) via both mechanisms (Brix and Deforest 2000). Active regulation happens via excreting metals at rates higher than intake rate (Brix and Deforest 2000). Storage instead keeps these metals in a detoxified form (Brown 1982) or bound to metallothioneins (Roesijadi 1992). There is much evidence that metals such as Cu and Zn are actively regulated in algae, fish and decapod crustaceans (Amiard et al. 1987, Rainbow and White 1989, Kraak et al. 1993). Whereas, these metals are stored in barnacles, bivalve molluscs, and aquatic insects in detoxified forms like MT-thiol (Amiard et al. 1987, Krishnakumar et al. 1990).Moreover, some metals have the ability to bind to molecules like proteins (Nieboer and Richardson 1980, Rainbow 1997).

Among these metals, Cu and Cd are very common stressors in aquatic environments and have received much attention associated with risk assessment regulations (Shuhaimi-Othman et al. 2010, Fernández-Gonzáles et al. 2011, Al-Reasi et al. 2012, Pérez and Hoang 2018). Both metals interfere with digestion, limiting food intake and consequently assimilation in *Daphnia* (De Coen and Janssen 1997, Lv et al. 2017), as well as interfering with *Daphnia*’s metabolism (De Coen and Janssen 1998). Each metal tends to have different modes of action (Shanker 2008). However, toxic substances may have multiple modes of action (Barata et al. 2002 b, Barata et al. 2004). Gerhardt (1993) indicated that the toxic action of metals arises from binding -SH (thiol group) of proteins (like Metallothionein) to metals in a number of biochemical reactions. This results in malfunctions of cell metabolism, permeability of cell membranes, inhibition oxidative phosphorylation, enhanced lipid peroxidation, and damage in Ca2+ homeostasis.

1.2.1. Copper

Copper is an abundant element in freshwater bodies and an essential component for all living organisms. In natural water, Cu can exist in complexes with organic and inorganic substances (Zhang et al. 2014). It can classified into five forms: Cu2+, CuCl+, CuCl2, CuCl3- and CuCl4-2 depending on chloride concentrations and salinity (De Vreese et al. 2012, Zhao et al. 2013, Zhang et al. 2014). In freshwater, the sub-lethal concentration of Cu for *Daphnia* populations is 30 μg/l (Al-Reasi et al. 2012).

Copper redox(Redox is an electron transfer reaction) nature makes it essential to biological processes as well as inducing toxicity at high concentrations (Grosell and Wood 2002 a, Bossuyt and Janssen 2004, Xie et al. 2006). Cu plays an important role in some physiological processes like antioxidant defence, neurotransmitter function, cellular iron metabolism, respiration and electron transport (Atienzar et al. 2001, Bossuyt and Janssen 2004, Daniel et al. 2004, de Oliveira-Filho et al. 2004, Letelier et al. 2005). It can also produce reactive oxygen species (ROS), which result in oxidative damage in biological targets (Letelier et al. 2005). Through Fenton reactions, Cu also binds to DNA generating double-strand breaks, putative intra-strand cross-links and 8-hydroxydeoxyguanosine (LIoyd and Philips1999). Further, Cu disturbs sodium uptake and increases sodium loss through cell diffusion (Grosell et al. 2002 a). Sodium uptake is important for freshwater organisms due to its role in enhancing the natural membrane potential (Grosell et al.2002 b).

1.2.2. Cadmium

Cd is a highly toxic metal, classified as a type I carcinogen by the USA National Toxicology Program International Agency for Research on Cancer(IARC) and the. In surface water, Cd sub-lethal concentrations for *Daphnia* populations range from 0.01 to 0.31 μg**/**L (Tan and Wang 2011, De Coninck et al. 2014). In the natural environments, Cd can exist in different forms, however, cadmium chloride (CdCl2) is the most commonly found (Canton and Slooff 1982).

Cd induces toxicity via inhibition of DNA resulting in apoptosis (Nzengue et al. 2011). Furthermore, oxidative stress is identified as the direct effect of Cd exposure where the metal has no redox activity. This may enhance ROS production by suppressing free-radical scavengers such as glutathione and by inhibiting detoxifying enzymes such as glutathione peroxidase (GPX), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT), or through other indirect mechanisms. Available data confirm that the formation of free radicals such as superoxide ions, hydrogen peroxide, and hydroxyl radicals involves depletion of GSH and changes in the activity of antioxidant enzymes (Matovic et al. 2011).

**1.3. Feeding Behaviour in *Daphnia***

Foraging is the primary route of exposure to sub-lethal concentrations of Cu and Cd (De Coen and Janssen 1997,1998, Wilding and Maltby 2006). Food is central to growth and reproduction, and thus any change in acquisition or assimilation of resources associated with metals/stressors will likely impact on individual’s growth and reproduction and then the whole population (Boggs 1992, Wilding and Maltby 2006). Life history theory indicates that resource shortage during development may cause serious consequences for individuals such as delayed development, reduced growth rate and small body size at maturity (Fig. 1.2.) (Martin-Creuzburg et al. 2006, Gorbi et al. 2011). For example, malfunction of foraging and digestion has been widely investigated (Wilding and Maltby 2006, Macedo-Sousa et al. 2007, Pestana et al. 2007, Rocha et al. 2016). This include impairment in response to toxicants that directly affect organism’s survival, growth and reproduction (life history traits) (Wilding and Maltby 2006).

**Foraging**

**Energy allocation**

**Under stressed conditions**

**Under normal conditions**

The amount of energy allocated in each trait

**Growth (-)**

**Reproduction**

**(-)**

**Metabolism**

**(+)**

**Reproduction**

**Growth**

**Metabolism**

**Figure 1.2.** The energy expenditure to each trait in stressed and un-stressed daphnids.

The relationship between physiological and organismal levels (growth and reproduction) in response to sub-lethal metals are crucial in the eco-toxicological studies in order to understand how stressed populations may respond as well as giving insight on the mode of action of toxicants (De Coen and Janssen 1998). Further, considerable research has documented that metals (prolonged exposures), in particular Cu and Cd, impact feeding activity in water fleas and consequently enzymatic responses (digestive enzymes) (Barata and Baird 2000, McWilliam and Baird 2002, Dedourge-Geffard et al. 2009). Increased energy expenditure under metal exposures would decrease the energy available for reproduction and growth and consequently lead to reduced population growth and survival (Calow and Sibly, 1990). Therefore, feeding assays are considered an important indicator to detect metal insult and offer rapid insight in to fitness and ecosystem functioning.

The effect of food availability and metals, in which daphnids allocate resources metabolism, growth and reproduction has been documented (Ferrando and Andreu 1993, Barry et al. 1995, McWilliam and Baird 2002) where it has been found that filtration and ingestion rates declined in *Daphnia* spp. under metal exposures. These responses may differ among Cladocera spp. depending on resources input and the metabolic status, which reflect the cost of feeding and consequently the costs to life history traits (Bohrer and Lampert 1988, Boggs 1992). Furthermore, the interaction among stressors/toxicants can be also framed by food availability. For example, [Heugens](https://setac.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Heugens%2C+Evelyn+H+W)et al. (2006) found that negative effects of cadmium on the growth rate of *D. magna* were enhanced at elevated temperatures, whereas high levels of food protected the exposed daphnids from adverse effects of cadmium.

The effects of foraging disruption can extend from the individual to the ecosystem. Water fleas are keystone species in freshwater communities because they have a high grazing rate (Lynch and Shapiro 1981, Lampert et al. 1986). Any stress or toxic substances that impairs ingestion rates of *Daphnia* individuals and populations can increase in the phytoplankton biomass (Werner et al. 1983, Day et al. 1987, Bradley et al. 1991, Jak 1997, Barata et al. 2002 b).

Metals are known to be transferred across the food web’s components causing a series of toxicological effects (Chen et al. 2000). One of their effects is bottom–up, which refers to the effects of nutrient and food on other trophic levels (Fleeger et al. 2003).It has been demonstrated that the phytoplanktonic *Chlorella* tends to accumulate high concentrations of metals (Hart and Scaife 1977).

**1.4. Biomarker responses (linking to feeding and metabolism)**

Rapid assessment of sub-lethal effects of metals is increasingly important. While molecular tools are slowly emerging, enzymatic biomarkers have a less well known but longstanding reputation. Their value is that they are associated potentially with feeding and stress. Here, we focus on two enzymatic responses: digestive and antioxidant enzymes detecting-via sub-lethal exposures-the impact of Cu and Cd concentrations. Theses enzymes are involved in different physiological pathways in test organisms potentially capturing different metal modes of action.

Food ingestion process releases energy that required for maintenance, growth, and reproduction of organisms (De Coen and Janssen 1997, Dedourge-Geffard et al. 2009). This process take place in the alimentary tract of organisms and involves a range of enzymes allocated in breaking down food particles (Lv et al. 2017). Toxicants impact on digestive enzymes, such as amylase, trypsin and chymotrypsin, could affect the digestive process and consequently impact the energy allocated to metabolism (De Coen and Janssen 1997, Demott and Gulati 1999, Dedourge-Geffard et al. 2009). Thus, detection of digestive enzyme activities could be potentially an early warning of stress insult especially these enzymes are directly linked to organism’s metabolism (De Coen and Janssen 1997, Lv et al. 2017). The ecotoxicological influences on digestive enzymes activity produced by metal exposures have been little documented across a number of aquatic species (De Coen and Janssen 1997, Chen et al. 2002, Dedourge-Geffard et al. 2009, Rocha et al. 2016).

Oxidative stress also closely linked to the disruption of physiological processes. Metals induce oxidative stress by generation of free radicals called reactive oxygen species (ROS) (Barata et al. 2005, Jemec et al. 2007). Increasing ROS levels cause damage to cellular macromolecules and lipid peroxidation and a change in antioxidant enzymes, which consider the defence line against stressors effects (Barata et al. 2005, Letelier et al. 2005). The effect of multiple stressors on digestive and antioxidant enzymes in *D. pulex* has not been investigated. These enzymes are directly linked to feeding ecology as well as being considered to be an early indicator of metals stress conditions.

**1.5. Predation, metals and the microbiome**

As noted above, anthropogenic sources of stress on populations and communities are operating against the backdrop of natural/ecological stress linked to predation, disease and food supply variation. I have reviewed the role of food above, and here review the potential role of predation. Because of the central role of digestive physiology in mediating metal effects, I also suggest that the gut microbiota may be important in research on metals and predation because of the central role of energy acquisition in performance, which is impacted by metals and predation.

*1.5.1. Predation*

Like the response to Cu and Cd, predation risk and starvation risk are intimately connected. Behavioural responses to predation include changes in foraging activity either by refuge use or habitat shifts, which impacts food acquisition and predation risk **(**Lima and Dill 1990, Abrams and Rowe 1996). Theory further suggests that predation risk affects life history by altering the allocation of resources to growth versus development (Ball and Baker 1995, Beckerman et al. 2007). Under predation and food stress, increasing energy allocation to development may decrease maturation time as well as body size (Noonburg and Nisbet 2005, Bekerman et al. 2007). Finally, size selective predation theory indicates that growth and development rate can be decoupled. If predation is not size selective, theory predicts that the response to predation will be delayed reproduction at a small size which matches the effects of low food. However, predation that selects small individuals can lead to delayed reproduction at a larger size and predation that selects for large individuals can lead to early reproduction at a small size. These two responses are potentially antagonistic to the effects of low food, generated naturally or by metals (Beckerman et al. 2007).

*1.5.2. Microbiome*

Against this idea that digestion and the gut are a focal point for both metals and predation, we might consider the microbiome as important to combined stress. Intestinal microorganisms play an important role in host’s nutrition via transformation and degradation of complex biopolymers (Freese and Schink 2011). It has been demonstrated that the bacteria community in three species of Cladocera guts is dominated by the genus *Limnohabitans* of the class Protobacteria (Hahn et al. 2010, Freese and Schink 2011), which remain relatively stable having similar community structures of bacteria. The microbiome is linked to life history and population growth. Removal or disruption of bacterial symbionts in *D. pulex*, lead to altered population dynamics (Peerakietkhajorn et al. 2015), behavioural changes (Gorokhova et al. 2015) as well as lower fecundity and slower growth rates (Sison-Mangus et al. 2015).

Thus, under stress such as predation risk or metals, which influences organisms via foraging behaviour and digestive physiology, the bacterial community comprising the gut microbiome can change. Against this background, we can hypothesize that gut microbiota mediates the life history responses in *D. pulex* in presence of multiple stressors, particularly those like Cu, Cd and predation that act on foraging and digestion.

**1.6. Model system**

The species *D. pulex* was selected as it is considered a model system in ecology, ecotoxicology and genotoxicology due to their sensitivity to toxic chemicals. They are common species of the water flea in freshwater environments (Colbourne et al. 2011). They also represent a crucial link in the aquatic food web, typically the principal grazers of algae, protozoa, bacteria, and the primary forage of fish. *Daphnia* is an ideal system for studying multiple stressors because of its short generation time, well-studied ecology and evolutionary history, wide geographical distribution across many limnetic systems, high mutation and recombination rates, high sensitivity to changes in environmental conditions, unique cyclical parthenogenetic life history, and recent availability of many genomic tools (Sarma and Nandini 2006, Colbourne et al. 2011).

*Daphnia* typically reproduces by cyclical parthenogenesis; this strategy entails both clonal reproduction during optimal environmental conditions and sexual reproduction otherwise. The sexual phase is often triggered by environmental stresses such as crowding, cooling, or change in photoperiod, as well as by predation (Paland et al. 2005). This unique reproductive system of *Daphnia* allows us to maintain both lines of genetically identical individuals in the laboratory, as well as lines of genetically variant clones.



**Figure 1.3.** Adult of *D. pulex* (Female)

**1.7. Thesis aims**

Cladocera spp. encounter a wide range of environmental stressors. Evaluation of these stressors and their effects on natural populations remains a key challenge in environmental risk assessment. There remains, however, a lack of research on multiple stressor interaction.

This thesis reports on several explorations into interactions among two metals: Cu and Cd, and a metal (Cu) and predation, specifically focusing on their effects on foraging and life history. In the context of the metal–metal interactions (e.g. two anthropogenic stressors), I explored additive vs. interactive effects and enzyme kinetics in the gut linked to metabolism and detoxification. In the context of metal–predation interactions (e.g. anthropogenic and natural stressors), I explore the role of the microbiome in regulating those interactions.

It is important to understand metal-associated risk with focus on life history traits, digestive and antioxidant enzymes and microbiome. To do this, I:

1. Evaluated life history responses (reproduction, maturation time, size at maturity and growth rate) to Cu and to Cd in multiple species via a meta-analysis. This work identified significant, quantifiable variability in the response to Cu and Cd among various species of Cladocera and highlight that *D. magna*, the most studied species, may not be the most typical species.
2. Evaluated experimentally the following questions linked to combined metal stress: a) does the effect of Cu on trait vary by Cd?; b) is the effect of Cu or Cd non-linear in isolation?; c) does the effect of Cu or Cd vary by food level?; d) does the effect of Cu or Cd vary by clone identity?; and e) does the effect of food level vary by clone? Our results indicated pervasive interactions between the metals, numerous instances where the effects of each metal were non-linear, numerous instances where the effect of a metal varied by *D. pulex* genotypesand numerous instances where the effect of a metal varied by food levels. Overall, our results indicate that these five traits are affected in non-additive ways by three factors that are often discussed and rarely estimated together: mixtures of stressors, genotypes and resource levels. This study highlights that metals can and do interact with each other and food levels in the context of risk assessment and water quality guidelines, but that the effects appear to be tractable/ understandable in this experimental system.
3. Evaluated experimentally the biochemical responses to Cu and Cd in *D. pulex*, with specific attention to digestive and antioxidant enzymes activity. We evaluated the activity of three digestive and antioxidant enzymes in response to short exposures of Cu and Cd (alone and in a mixture). We found that enzyme responses (digestive and antioxidant) were signifcantly affected to both metals (Cu:Cd interaction) and that the effects of the metal vary with the duration of the experiment for the most tested enzymes. This study highlights the importance of digestive physiology and antioxidant responses in metal’s toxicity and mechanisms.
4. Evaluated experimentally the role of the microbiome in *D. pulex* with specific attention to the interaction between predation risk and Cu. We tested whether the gut microbiome mediates the responses of life history to multiple stressors (predation and metal). We also tested whether the microbiome (community structure) was affected by the two stressors and antibiotics. The study highlights the role of bacteria-host interactions in response to biotic and abiotic stressors and how this ecologically linked to *Daphnia*’s population and community shifts.

**Chapter 2**

**The Chronic effects of copper and cadmium on life history traits  
across Cladocera species: A meta‑analysis**

**2.1. Introduction**

Anthropogenic and natural processes introduce a great deal of pollutants and stress into natural ecosystems (Mountouris et al. 2002, Demirak et al. 2006, Dedourge-Geffard et al. 2009, Martins et al. 2017). Over the past few decades, trace levels of metals have received much attention (Jing et al. 2006), because they generate negative biological effects on aquatic organisms, such as algae grazers and subsequently alter water quality. Such “sub-lethal” effects include changes in reproduction (Bodar et al. 1988b, Wang et al. 2009, Kim et al. 2017), somatic growth rate (Chandini 1989, Koivisto et al. 1992, De Schamphelaere and Janssen 2004a), feeding rate (Ferrando and Andreu 1993, McWilliam and Baird 2002, De Schamphelaere et al. 2007), and respiration and metabolism (Dave 1984, Bodar et al. 1988a, Khangarot and Rathore 2003).

Copper and cadmium are widely found as pollutants in natural water systems and are well-known toxicants for aquatic invertebrates, particularly Cladocera. These chemicals continue to occupy a large portion of the research agenda on sub-lethal effects of metal pollution (Bellavere and Gorbi 1981, Shuhaimi-Othman et al. 2010, Fernández-Gonzáles et al. 2011). However, each element has a different mode of action (Shanker 2008). Cu is an essential element to living organisms where it is primarily involved as a co-factor in enzymatic reactions of biological processes (Bossuyt and Janssen 2004, de Oliveira-Filho et al. 2004). It is considered to be a potent toxicant only at high concentrations and works largely by disrupting digestive physiology, which is linked to energy intake and hence resources acquired for growth and reproductive activities (Barata and Baird 2000, Bui et al. 2016). Its toxicity at high concentrations derives from its redox potential where it can induce reactive oxygen species formation (ROS) that lead to oxidative stress (Stoddard and Harper 2007, Giusto and Ferrari 2014). These conditions can cause damage to biological structures, such as DNA, lipoproteins, and organelles (Letelier et al. 2005, Giusto and Ferrari 2014). Furthermore, it has been demonstrated that Cu inhibits the activity of enzymes involved in cell metabolism, such as Na+/K+-ATPase and Mg+2-ATPase, which are responsible for the exchange of ions across the cell membrane (Pagliarani et al. 1996, Handy et al. 2002, Katranitsas et al. 2003).

On the other hand, Cd is a nonessential element and highly toxic even at very low concentrations (Gama-Flores et al. 2006, Wang et al. 2009). The International Agency for Research on Cancer of USA (1997) classified cadmium as a Type 1 carcinogen. Exposure causes a series of changes in cellular homeostasis, such as DNA damage, acidification of the cytoplasm, and oxidative stress linked to ROS formation via elevation of lipid peroxidation in tissues (Stohs and Bagchi 1995, Soetaert et al. 2007). In addition to its role in reduction of the activity of antioxidant enzymes, such as glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD) (Waisberg et al. 2003, Wang et al. 2004 a, Sandrini et al. 2008). Cd also strongly impacts feeding activity which hence impairs reproduction and growth (Baird et al. 1990, Barata and Baird 2000). In freshwater systems, the concentrations associated with sub-lethal effects in Cu range from 0.2 to 30 μg/L (Al-Reasi et al. 2012), whereas it is lower than 0.1 μg/L for Cd (Tan and Wang 2011).

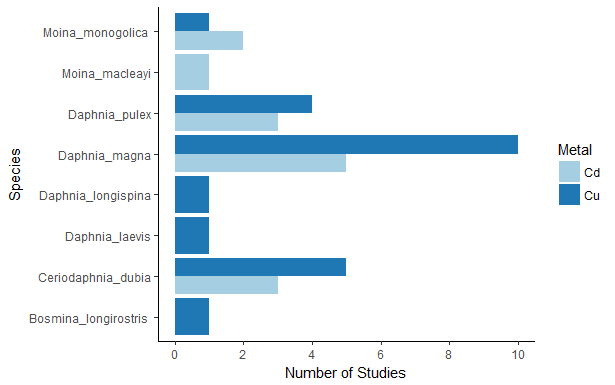
In natural environments, contaminants often are diluted and occur at low concentrations. Therefore, aquatic organisms are considered to be under risk of chronic exposure to low concentrations over long periods. To evaluate the risks associated with pollutants, chronic exposure to sub-lethal concentrations of a stressor is typically used to examine life history responses of different species. Furthermore, short-term toxicity tests represent an early warning indicator of toxicant impacts on aquatic organisms (Stephan et al. 1985). These ecotoxicological assays are a typical method for evaluating how an organism or a population respond to contaminants exposure (Barata and Baird, 2000). The data from such assays are used to define standards for ecosystem services, such as water quality monitoring, and to define impacts on natural populations, their habitats, and ecosystems (Lopes et al. 2004, Cooper et al. 2009).

Recent work suggests that the responses to sub-lethal concentrations of metals may be influenced by species identity (e.g., interspecific variation) and genotype (e.g., intraspecific variation) (Barata et al. 2000, Hoang and Klaine 2007). Furthermore, metal complexity, metal toxicity, and aqueous versus dietary exposure influencing bioavailability to an organism may be important (Gusso-Choueri et al. 2012). This may be further influenced by redox potential, pH, hardness, and the amount of existing metal present in water (Long et al. 2004, Bae and Freeman 2007, Hoang and Klaine 2007).

It has been demonstrated that bioavailability and toxicity of metals to aquatic species may be affected by a number of environmental factors, such as water hardness, alkalinity, pH, and dissolved organic carbon (DOC) (Santore et al. 2001). The biotic ligand model (BLM) is a promising model to assess how the water chemistry influence the toxicity of Cu to *D. magna*. This model is based on the complexation of free ions and competition with other cations (from the natural and artificial media) (Bossuyt et al. 2004, De Schamphelaere and Janssen 2004 b, c, De Schamphelaere et al. 2004, Clifford and McGeer 2010). Data collected from the literature agree on the decrease of Cu toxicity as a result of hardness, pH, and dissolved organic carbon (Di Toro et al. 2001, Santore et al. 2001, De Schamphelaere et al. 2004). Furthermore, BLM has recently been developed for other metals across species of invertebrates, including *Daphnia* (Keithly et al. 2004, De Schamphelaere et al. 2006, Kozlova et al. 2009, Clifford and McGeer 2010, Esbaugh et al. 2012).

Toxicity tests on a species level (single and multiple) remain the core biological level of assessment, and it is assumed that the link between ecosystems function and taxonomic variation in the response to toxicants can offer insight into how population and ecosystems may respond (Cairns, 1983, Rahbek 2005). This is crucial in the context of ecotoxicology where very few species or even taxa are used to make inference about the environmental impacts of contaminants and other stressors. Whilst species differences may seem to be “common sense,” identifying such differences should provide important insight into how the evaluation of toxicant impacts is conducted. Although environmental risk assessment routinely focuses on responses of one or several species to chemicals, little is known regarding species specificity in life history responses under short- or long-term exposure.

The cladoceran species are regarded as the ideal model for ecotoxicological tests of monitoring natural ecosystems. They have a global distribution, play a central role in the aquatic food chain, are sensitive to a vast range of pollutants, are easy to handle, have a short lifespan, and demonstrate dramatic phenotypic plasticity (Lampert 2006, Stollewerk 2010). Considerable laboratory research has documented effects of sub-lethal concentrations of Cu and Cd on life history traits of different species of Cladocera (Baird et al. 1990, Khangarot and Rathore 2003, Sofyan et al. 2007, Dao et al. 2017) (Fig. 2.1). However, to date, we are unaware of any systematic review of this literature that would allow estimation of whether the effect of increasing Cu or Cd concentration on life history varies by species, how experimental conditions affect these responses, and which species demonstrate strong or weak responses (e.g., appear resistant or sensitive).

**Figure 2.1.** Number of studies (1970-2017) for each species of Cladocera in response to sub-chronic exposure of Cu and Cd.

**2.1.1. Expectations from theory**

Existing research centers on several aspects of *Daphnia* spp. behaviour and life history: feeding rates, the number of neonates per female per day, age at maturity, and somatic growth rate. Life history theory provides a template against which the effects of Cu and Cd can be evaluated. Because they interfere with digestion, acting to limit resource intake and assimilation (De Coen and Janssen, 1997, Lv et al. 2017), and ultimately interfere with metabolism (De Coen and Janssen 1998), life history theory predicts that as metal concentrations rise, foraging, and assimilation of energy may decline leading to reduced reproduction, delayed maturity, and slower somatic growth rates.

However, data reviewed on a case by case basis provide equivocal alignment with such expectations. While many published data showed that Cu and Cd have negative impacts on the production of neonates in many species (Knops et al. 2001, Luciana et al. 2014), feeding rate (McWilliam and Baird 2002), and somatic growth rate (Agra et al. 2011), several studies also demonstrated that each metal can cause an increase in neonate production (Dave 1984, Bodar et al. 1988b, Roux et al. 1993, Agra et al. 2011).

Thus, while theory predicts marked changes in life history, there are equivocal empirical patterns among studies and species requiring meta-analytic tools to make cross study inference. The objective of our systematic review is to analyse the effects of Cu and Cd across studies, capturing variation in both species identity and specific lab conditions that might generate variation in the responses to metals.

First, we asked whether increasing Cu or Cd concentrations lead to decreased reproduction, delayed maturity and slower somatic growth rates, and whether this varies byspecies. Second, where possible, we tested whether aqueous versus dietary Cu and Cd induced different responses among *Daphnia* species. Finally, where possible, we considered whether lab conditions, including water hardness and exposure period, interact with Cu and Cd toxicity on life history parameters. Overall, species identity matters substantially; the most common species studied (*D. magna*) appeared to be more resistant (i.e. less sensitive) to metals than other species.

**2.2. Materials and methods**

**2.2.1. Literature search**

We gathered evidence from the literature on the effect of sub-lethal Cu and Cd on the number of neonates per female per day, age at maturity, and somatic growth rate in different species of Cladocera. We searched in seven databases: Web of Science, Scopus, JSTOR, BIOSIS, Science Direct, Google Scholar and StarPlus (University of Sheffield library collection) for relevant publications, using the following key word combinations: (effect OR impact\* OR influence\*) AND (metals OR copper\* OR cadmium\*) AND (life history OR reproduction\* OR age at maturity\* OR growth rate\*) AND (*Daphnia* OR Cladocera\*). We collected studies between 1970 and 2017.

More than 200 references were obtained that measured relevant endpoints under chronic exposures of Cu and Cd. However, to be included, studies must report accessible information on the mean, standard deviation, or standard error of effects and sample sizes (Vilá et al. 2011). Publications were excluded when any of this information was missing or not estimable from the information provided. Only 32 of > 200 papers reported all data required for each trait/metal.

**2.2.2. Data extraction and effect size calculation**

For each study, quantitative data was extracted on metal type (Cu, Cd), species identity, traits (reproduction, maturation age, and somatic growth rate), aqueous or dietary delivery of the metal, exposure duration, water hardness, and the sample size, standard deviations or standard errors, and the mean of both the control and the experimental groups. These data were obtained directly from tables or digitized from graphs or bars using Graph-Click (Arizona Software, version 3.0.3). Where necessary, we calculated the standard deviation by multiplying the standard error by the square root of the sample size.

We performed a quantitative meta-analysis focusing on whether the effect of the metals varied by species. We also explored the effects of delivery method, exposure duration and water hardness. These analyses were conducted using Osenberg et al. (1997) method allowing comparisons among studies. For each trait and trial, we calculated Cohen’s *d* according to the following equation:

(1)

Where *X* c is the mean of the control group, *X*e is the mean of the experimental group and *s* is the pooled standard deviation of the control and experimental groups, and *J* is the corrector for bias.

(2)

(3)

*S*e is the standard deviation of the experimental group, *S*c is the standard deviation of the control group, *N*e is the number of cases of the experimental group, and *N*c is the number of cases of the control group.

The primary goal of our study was to identify the species specificity of the effects of nominal concentrations of Cu and Cd on reproduction, maturation age, and somatic growth rate. Where possible, we evaluated whether an aqueous versus dietary source of food influenced this pattern and whether water hardness and exposure period influenced this pattern. To assess the overall significance of an interaction between concentration and species OR concentration and condition, we fit random effects models using maximum likelihood via the Metafor package in R (Viechtbauer 2010, R Core team 2013) version 1.1.453. We performed a likelihood ratio test (LRT) between a model with and without the interaction between metals and species to test formally whether the effect of Cu and Cd on traits varied by species/ experimental conditions. Regression coefficients were then evaluated on the model using restricted maximum likelihood. Furthermore, *D. magna* was the most common species reported in the literature, and we present specific inference about the interactions (or lack of) via comparison to the *D.* *magna* response (reference species).

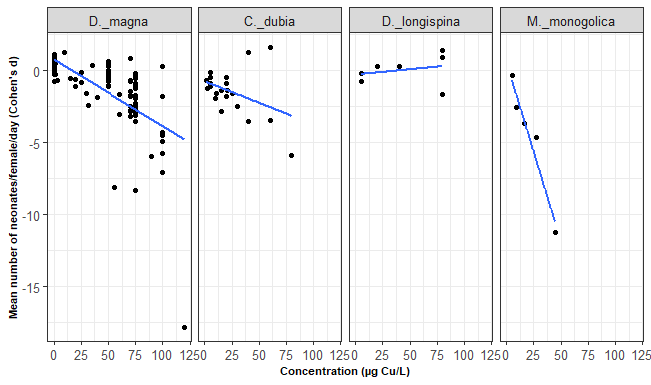
**2.3. Results**

We first report whether the effect of aqueous Cu and Cd concentration on traits depend on species. This includes comparing aqueous versus dietary delivery. We then report on the experimental factors of water hardness and exposure duration.

2.3.1. Copper‑reproduction: Aqueous and dietary delivery

Of the 12 independent studies reporting on aqueous Cu concentration and reproduction, 95% reported a nominal concentration between 0 and 120 µg/L. A total of 161 trials provide data on effect of Cu concentration on reproduction among four species of Cladocera: *Daphnia magna*, *D. longispina, Moina monogolica*, and *Ceriodaphnia dubia*.

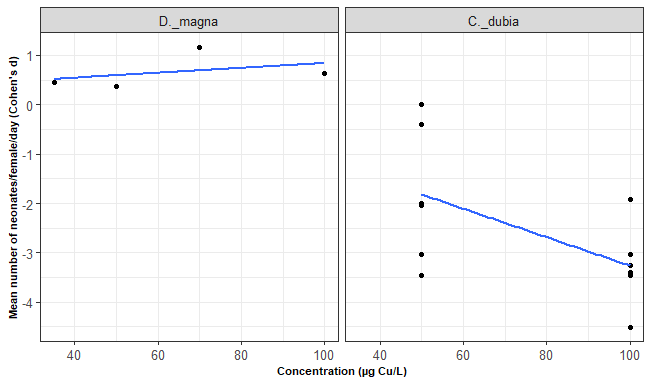
Overall, increasing Cu concentration led to a decrease in the mean number of neonates produced per female per day and the effects of copper on reproduction varied by species (Fig.  2.2; LRT = 19.76, *p* = 0.0002). As Cu concentration increased, *D. magna* reproduction declined (*D. magna* slope = − 0.04, *z* = − 8.8, *p* = 0.0001, intercept= 0.64, *z* = 2.6, *p* = 0.009). The effect of Cu on *C*. *dubia* was indistinguishable from *D. magna* (gradient change from *D. magna* = 0.015, *z* = 0.95, *p* = 0.34). By comparison, Cu had a weaker effect on *D. longispina* (gradient change from *D. magna* = 0.05, *z* = 2.8, *p* = 0.005), reflecting a slightly positive effect of Cu for this species. Cu had an even stronger negative effect on *M. monogolica* (gradient change from *D. magna* = − 0.19, *z* = −3.3, *p* = 0.001).



**Figure 2.2.** Mean number of neonates produced per female per day for four species of Cladocera exposed to < = 120 µg/L (nominal aqueous concentrations of Cu). The black dots represent *d* values, n=161. All species are compared to the reference species (first panel). Only *D. magna* has a range of Cu concentrations up to <=120 µg/L.

In the subset of data that documents the effect of dietary Cu (≤ 120 µg/L,), 19 trials present data across three studies of two species of Cladocera: *D. magna* and *C. dubia*. We found that the effect of Cu concentration on reproduction does not vary by species (Fig.  2.3a; LRT = 2.66, *p* = 0.102). Cu increased *D. magna* reproduction (*D. magna* intercept = 1.96, *z* = 2.21, *p* = 0.027), but the concentration had no effect (*D. magna* slope = − 0.02, *z* = − 1.82, *p* = 0.067). The effect of Cu on *C. dubia* was to reduce reproduction on average (difference in intercept to *D. magna* = 2.86, *z* = 4.54, *p* = 0.0001). For dietary Cu, concentrations ≥120 µg/L had a strong negative effect, with data only available for *D. magna* reproduction, strongly driven by a single trial (from one study) at 500 µg/L Cu (Fig.  2.3b).

**a)**



**b)**

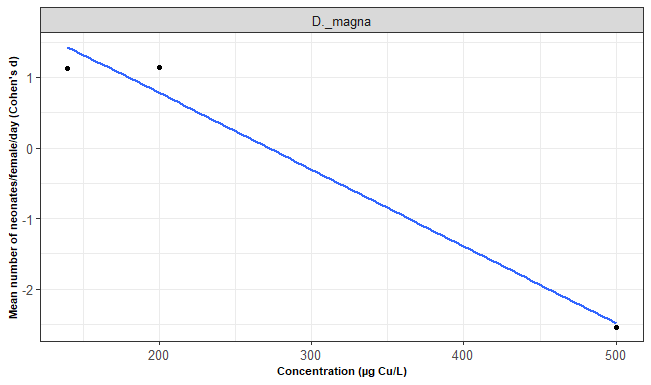


Figure 2.3. Mean number of neonates produced per female per day for (a) two species of Cladocera exposed to < = 120 µg/L dietary Cu (nominal), the black dots represent *d* values, n=16 (b) *D. magna* exposed to > 120 µg/L dietary Cu (nominal), n= 3. All species are compared to the reference species (*D. magna*).

2.3.2. Copper‑age at maturity

Our meta-analysis for age at maturity included 41 trials of four independent studies. All data are referenced to reported nominal Cu concentrations and involve four species: *D.* *magna, D. pulex, Bosmina longirostris,* and *D. longispina*. Increasing copper concentration delayed maturation and this effect varied by species (Fig.  2.4; LRT = 9.56, *p* = 0.022). For *D. magna,* increasing Cu concentration delayed the maturation age (*D. magna* slope = 0.08, *z* = 3.10, *p* = 0.001, intercept = − 0.66, *z* = − 1.22, *p* = 0.022). The effects of Cu concentration on maturation age for both *D. pulex* (gradient change from *D. magna* = − 0.04, *z* = − 0.77, *p* = 0.436) and *B. longirostris* (gradient change from *D. magna* = 0.005, *z* = 0.06, *p* = 0.951) were not different from *D. magna*. By contrast, increasing Cu concentration delayed less severely the maturation age of *D. longispina* (gradient change from *D. magna* = − 0.07, *z* = − 2.77, *p* = 0.005).

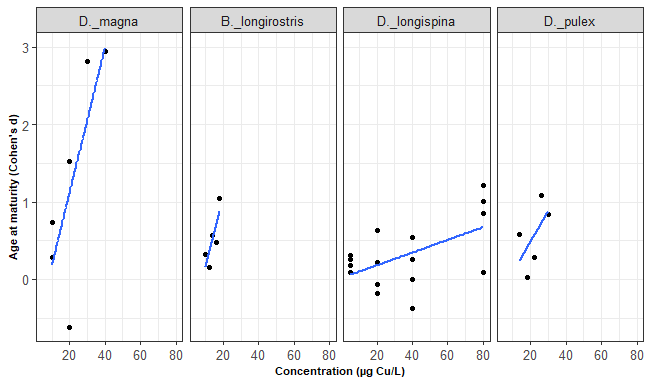
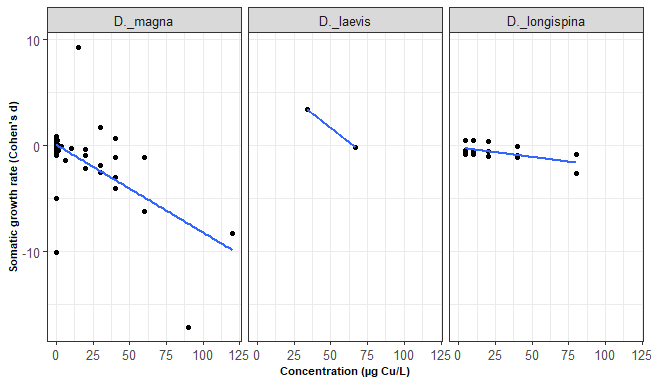
****

Figure 2.4. The maturation age response of four species of Cladocera exposed to nominal sub-lethal concentrations of Cu. The black dots represent *d* values, n=41. All species are compared to the reference species (*D. magna*).

2.3.3. Copper‑somatic growth rate

Our meta-analysis of somatic growth rate involved 7 studies providing 69 trials among 3 species: *D. magna, D. longispina,* and *D. laevis*. The effect of Cu concentration on somatic growth rate depends on species (Fig.  2.5; LRT = 8.26, *p* = 0.016). With increasing Cu concentrations, the somatic growth rate decreased for *D. magna* (*D.* *magna* slope = − 0.07, *z* = − 6.7, *p* = 0.0001, intercept= 0.17, *z* = 0.61, *p* = 0.54). The effect of Cu concentration on the somatic growth rate of *D. laevis* was not different from *D.* *magna* (gradient change from *D. magna* = − 0.03, *z* = − 0.47, *p* = 0.64). The effect of Cu concentration also reduced the *D.* *longispina* somatic growth rate but significantly less than for *D. magna* (gradient change from *D. magna* = 0.053, *z*= 2.72, *p* = 0.006).



(-)

Figure 2.5. The somatic growth rate response of *Daphnia* spp. to sub-lethal concentrations of Cu (nominal). The black dots represent *d* values, n=69. All species are compared to the reference species (*D. magna*).

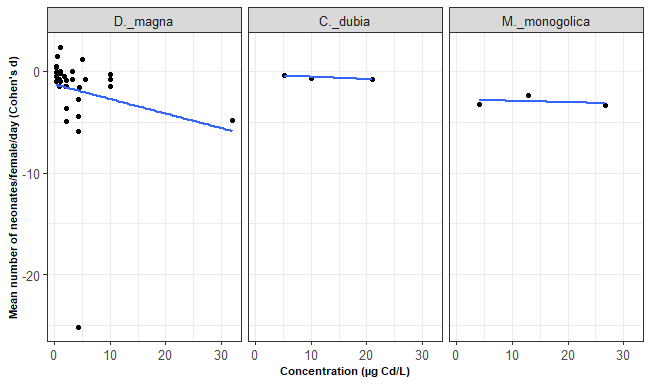
2.3.4. Cadmium‑reproduction: Aqueous and dietary delivery

Seventy-two trials from ten studies were available to explore the effects of Cd on reproduction in three species: *D. magna*, *M. monogolica,* and *C. dubia.* Ninety-five percent of the studies reported nominal concentration between 0 and 40 µg/L Cd.

For aqueous Cd delivery, we analyzed 40 trials from 6 studies. Increasing Cd concentration reduced reproduction, but this did not vary by species (Fig.  2.6a; LRT = 2.39, *p* = 0.30). As Cd concentration increased, *D. magna* reproduction decreased (*D. magna* slope = − 0.097, *z* = − 2.84, *p* = 0.004, intercept = − 0.50, *z* = − 2.02, *p* = 0.043). The average effects of Cd on reproduction on *C. dubia* (difference in intercept to *D. magna* = 1.04, *z* = 1.43, *p* = 0.150) and on *M. monogolica* (difference in intercept to *D. magna* = − 1.07, *z* = − 1.16, *p* = 0.242) were also not distinguishable from *D.* *magna*.

For the dietary Cd data, we analyzed 32 trials from four studies of three species: *D. magna, C. dubia,* and *M. monogolica*. The effect of dietary Cd concentration on reproduction depended on species (Fig.  2.6b; LRT = 13.52, *p* = 0.0012). Increase of dietary Cd concentrations led to no detectable reduction in *D. magna* reproduction (*D. magna* slope = − 0.012, *z* = − 0.18, *p* = 0.855, intercept = − 0.76, *z* = − 0.44, *p* = 0.66). Cd concentration also did not reduce *C. dubia* reproduction compared with *D. magna* (gradient change from *D. magna* = − 0.13, *z* = − 1.78, *p* = 0.074; note influential data point at 80 µg/L Cd). The effect of Cd concentration on *M. monogolica* also was not different from *D. magna* (gradient change from *D. magna* = 0.05, *z* = 0.72, *p* = 0.47). However, the effect of Cd concentration on *C. dubia* was very different to *M. monogolica* (gradient change *C. dubia* to *M. monogolica* = 0.18, *z* = 3.57, *p* = 0.003). This difference drives the interaction between Cd concentration and species.

**a)**



**b)**

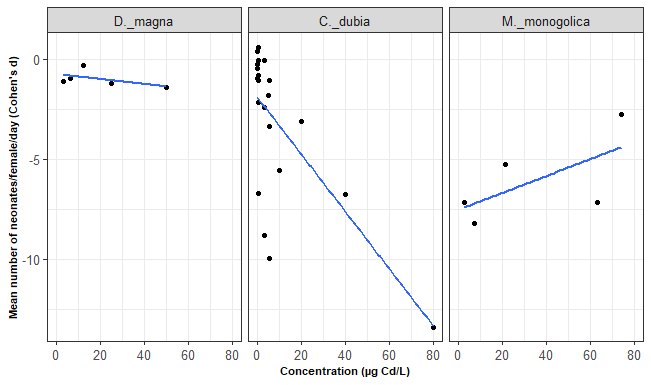


Figure 2.6. Mean number of neonates produced per female per day of three species of Cladocera exposed to (a) < = 40 µg/L nominal aqueous concentrations of Cd, n=40 (b) > 40 µg/L nominal dietary concentrations of Cd, n=32. The black dots represent *d* values. All species are compared to the reference species (*D. magna*).

2.3.5. Cadmium‑age at maturity

Data on Cd and maturation age came from two studies representing 41 cases in two species: *D. magna* and *M. macleayi.* The effect of Cd concentration on age at maturity did not depend on species (Fig.  2.7; LRT = 0.17, *p* = 0.68). For *D. magna,* Cd delayed on average the maturity age (*D. magna* intercept = − 5.69, *z* = − 11.82, *p* = 0.0001), but the concentrations had no effect (*D.* *magna* slope = 0.013, *z* = 0.08, *p* = 0.93). However, for *M.* *macleayi*, Cd did not delay the maturation age (difference in intercept to *D. magna* = 5.75, *z* = 9.91, *p* = 0.0001; *M.* *macleayi* has an intercept of 0).

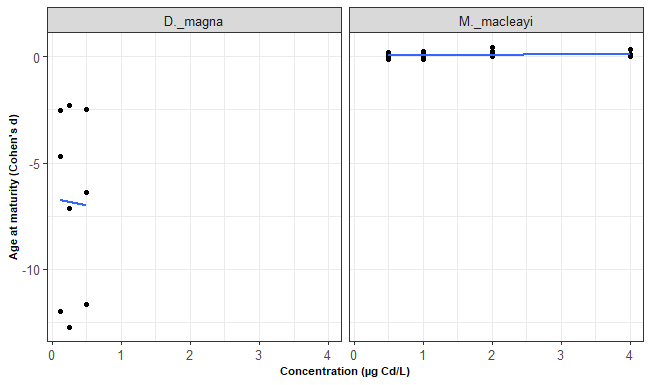


Figure 2.7. Sub-lethal effect of nominal Cd on maturation age of species of Cladocera. The black dots represent *d* values, n=41. Species are compared to the reference species (*D. magna*).

2.3.6. Cadmium‑growth rate

The data on the effects of Cd on somatic growth rate came from four studies representing 43 trials of three species: *D. magna*, *D. pulex,* and *M. macleayi.* The effect of Cd concentration on somatic growth rate varied by species (Fig.  2.8; LRT = 10.77, *p* = 0.0046). There was no effect of Cd concentrations on *D. magna* somatic growth (*D. magna* slope = − 0.03, *z* = − 0.38, *p* = 0.71, intercept = − 0.37, *z* = − 0.83, *p* = 0.41). The effect of Cd on growth in *M.* *maclaeyi* was indistinguishable from *D. magna* (gradient change from *D. magna* = − 0.07, *z* = − 0.82, *p* = 0.408). The negative effect of Cd concentration on *D. pulex* was substantially stronger than on *D. magna* (gradient change from *D. magna* = − 0.88, *z* = − 3.26, *p* = 0.001).

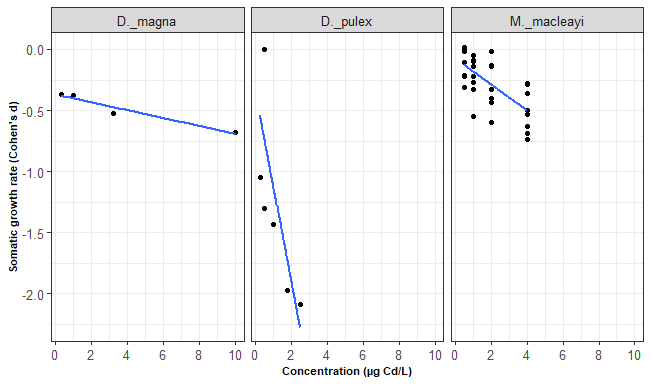
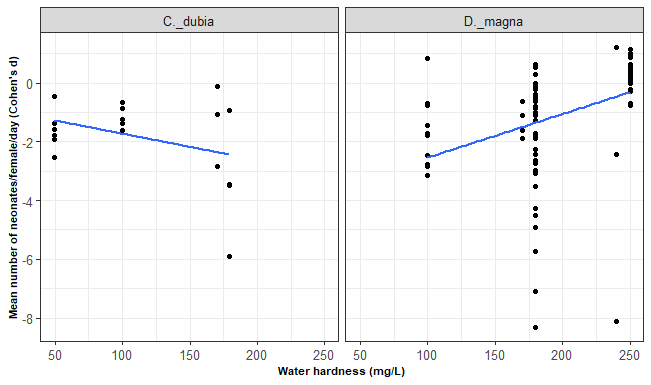


Figure 2.8. The mean somatic growth rate for *D. magna, D. pulex* and *M. macleayi* in response to nominal Cd. The black dots represent *d* values, n=43. All species are compared to the reference species (*D. magna*).

2.3.7. Copper and cadmium‑water hardness

According to the literature, water hardness levels (WH) (i.e., soft, moderate and hard) range between 40 and 300 mg/L as calcium carbonate (CaCO3). We asked for these species and trials whether the interaction between Cu or Cd concentration and species varied by WH. We constructed four models to evaluate this hypothesis. Model 1 was our full model with main effects of WH, species and metal, 2-way interactions, and the 3-way interaction. Model 2 removed the 3-way interaction. Model 3 removed both the 3-way interaction and the 2-way interactions with WH. Model 4 was the core model from all analyses above that include only the metal, species, and metal\*species interaction (i.e., no effect of WH). Comparing M1 to M2 via a LRT tested the 3-way interaction. If this was not significant, we compared M2 to M3 via a LRT to test for interactions between WH and Cu or WH and species. In the absence of these interactions, comparing M3 to M4 tested for a simple additive effect of WH. We found no evidence to support the effect of WH on the interaction between Cu and species (7 studies representing 130 trials and 2 species; *D. magna* and *C. dubia*; M3 vs. M4: LRT = 2.44 and *p* = 0.12; Fig.  2.9a). I found no evidence to support the effect of WH on the interaction between Cd and species (3 studies and 30 trials, *D. magna* only; M3 vs. M4: LRT = 2.73, *p* = 0.097; Fig. 2.9b).

**a)**



**b)**

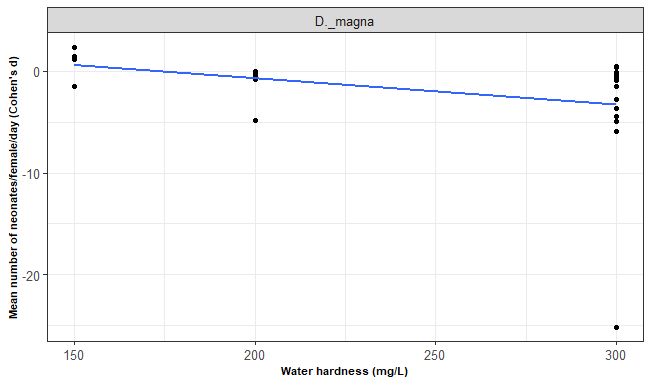
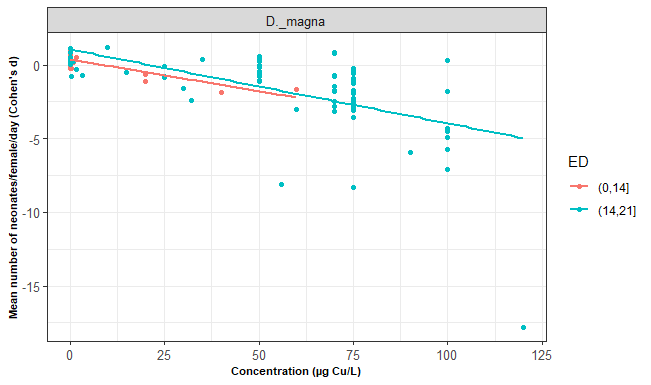


Figure 2.9. Water hardness effect on the toxicity of a) Cu, n= 130 b) Cd, n= 30 for Cladocera species reproduction. The black dots represent *d* values where species are compared to the reference species (*D. magna*).

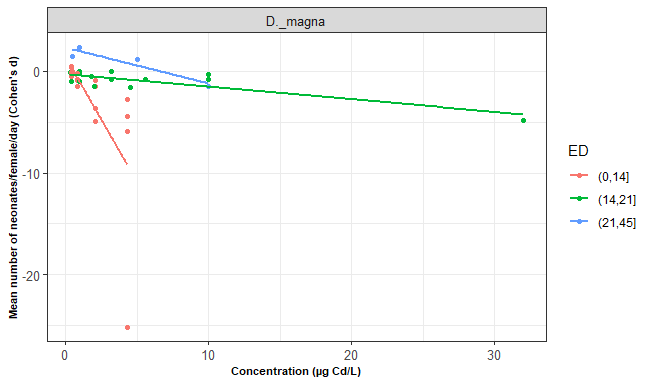
2.3.8. Copper and cadmium‑exposure duration

We analyzed 7 studies and a total of 113 trials that quantified the impact of exposure duration and Cu on reproduction. Data were only available on *D. magna* exposed to sub-lethal concentrations of Cu over two exposure periods: 0–14 and 14–21 (our reference duration) day trials. We found that the effect of Cu concentration on *D. magna* reproduction did not differ by test duration (Fig.  2.10 a; LRT = 0.001, *p* = 0.97). The 21-day exposure had no effect on the toxicity of Cu (slope = − 0.04, *z* = − 7.99, *p* = 0.11, intercept= 0.51, *z* = 1.1, *p* = 0.27). The effect of Cu on reproduction over 14 days was not different from the reference duration (difference in intercept to the reference = 0.36, *z* = 0.99, *p* = 0.32). In contrast, 4 studies represented 34 trials that quantified the impact of exposure duration and Cd concentration on reproduction. Three test periods were examined for *D. magna:* 0–14, 14–21 (our reference duration), and 21–45 days exposure. The effect of Cd concentration on *D. magna* reproduction varied by test duration (Fig.  2.10 b; LRT = 29.17, *p* = 0.0001). Increasing Cd concentration decreased reproduction over 21 days of exposure (slope = − 1.08, *z*= − 5.38, *p* = 0.0001). The 45-day exposure was indistinguishable from the reference (gradient change to the reference = 0.73, *z* = 3.41, *p* = 0.0007). However, 14-day exposure was not affected the toxicity of Cd (gradient change to the reference = 0.28, *z* = 0.77, *p* = 0.44).

**a)**



**b)**



**Figure 2.10.** Effect of exposure duration (ED) on the toxicity of a) Cu, n= 113 b) Cd, n=34 for *D. magna* reproduction. The black dots represent *d* values where exposure duration compared to the reference duration: 14-21.

**2.4. Discussion**

Cladocerans, and particularly *Daphnia* species, are classic test organisms for evaluating the sub-lethal effects of metals, which globally represent a serious problem to the aquatic food chain and water quality (Sarma and Nandini 2006, Altshuler et al. 2011, Martins et al. 2017). The sub-lethal levels of metals have received a great deal of attention, because they cause a range of ecotoxicological effects on those species. However, the importance of species specificity in understanding responses to contaminants remains challenging. Therefore, the main objective of this analysis was to assess quantitatively whether the effects of Cu and Cd on life history traits varies among species of Cladocera. Because *D. magna* is the most common test species, we were able to evaluate by direct comparison whether other species are more or less sensitive to Cu and Cd (e.g., whether *D. magna* can be considered representative). Furthermore, the selected parameters for this analysis-reproduction and somatic growth rate-are considered to be sensitive to sub-lethal exposure of contaminant.

Many laboratory reports have showed that the effect of contaminants or other stressors vary across species of aquatic organisms (Brix et al. 2001, Griffitt et al. 2008). However, this analysis is the first to have assessed the ecological effects of Cu and Cd across different species of Cladocera, the dominant test species in environmental monitoring and ecotoxicology. Recent meta-analysis studies have assessed the effect of contaminants on different biological levels, including species. For example, O’Brien and Keough (2014) suggest that we may expect substantial variation in life history response responses to a range of contaminants, driven by identity of contaminant, the identity of organism (taxa), and the scale of biological organization at which assessment is made (individuals, population, and community). A meta-analysis using 216 studies on the effect of toxic pollutants on marine communities also detected a strong reduction in species richness (Johnston and Roberts 2009). Furthermore, Jan Hendriks et al. (2005) and Blanar et al. (2009) detected via meta-analysis that the response to aquatic pollutants vary by population and community levels (crustaceans and parasites).

**4.1. The influence of metals on life history traits**

Our systematic review supports theory on the sub-lethal effect of Cu and Cd across traits/species. The analysis demonstrated that overall Cu and Cd reduce reproduction, increase the age at maturity, and reduce growth, as expected by theory linked to their mode of action. However, there was substantial variation among species and *D. magna* was not uniformly the most responsive species.

We found that the effect of Cu on reproduction (only aqueous delivery), maturation age, and somatic growth rate depended on species identity. Likewise, the effects of Cd on reproduction (only dietary delivery) and somatic growth rate also depend on species identity. In many cases, the dominant test species *D. magna* often was quite resistant to nominal metal concentrations up to 120 µg/L Cu and 40 µg/L Cd. Furthermore, *D. longispina* reproductive response to aqueous Cu and *D. magna* response to dietary Cu were positive. Our data indicate that reproduction is likely the most sensitive endpoint showing strong responses to both metals among species. Life history endpoints may respond differently to the same stressor or a combination of stressors. For example, Bednarska et al. (2009) found that the reproduction of adult ground beetles was most sensitive to nickel concentrations at low and high temperatures (10 and 25 °C), but at survival was less affected by the combined effect of Ni and chlorpyriphos at both temperatures. Furthermore, Laskowski et al. (2010) conducted a meta-analysis on the interactions between toxic chemicals and natural environmental factors across a range of vertebrates and invertebrates species (including metals and *D. magna*), finding that the effects of toxicants on organisms may differ depending on external factors. Whilst, Vijver et al. (2011) suggested via meta-analysis that the effect types (additive, antagonistic, and synergistic) were significantly different across toxicological endpoints and combinations of Cu, Cd, and Zn.

Our quantitative review highlights that species vary in their sensitivity to metals. This can arise for several reasons. Modes of action vary, the effects of pollutants can be regulated through different physiological pathways that may be species-specific, and we also expect variation due to choice of genotype used in the studies (Baird et al. 1990, Brix et al. 2001, Barata et al. 2004, Bossuyt and Janssen 2005). While it was impossible in our analysis of published data to deduce whether the same or different genotypes were being used, within species genetic variation is also likely important to consider at population and community levels. Overall, the limited number of studies reporting appropriate information for meta-analyses (sample size, means and standard deviation/error) (Furukawa et al. 2006), combined with missing detail on genotype identity, means that there is ample opportunity to pursue more rigorous assessment of these sources of variation and their impact on specificity of responses.

**4.2. The influence of the experimental factors** **on individuals’ responses to metals**

Given natural variation in pH, water hardness, and other environmental factors in ponds and lakes, and the theoretical expectation that these factors may influence bioavailability, we also explored whether variation in experimental conditions influenced experimental effects of Cu and Cd concentrations. According to data available in this analysis, for both metals, variation among trials did not appear to arise from variation in water hardness. This correspond with the study of De Schamphelaere and Janssen (2004 b) who found that water hardness had no effect on the chronic toxicity of Cu in *D. magna.* Keithly et al. (2004) showed that chronic toxicity of Ni was less dependent on hardness in *C. dubia* than acute toxicity. Our results showed that test duration had no pronounced effects on the chronic toxicity of Cu, suggesting perhaps rapid acclimation. However, under Cd exposure, *D.* *magna* reproduction was affected by test duration.

Our data on water hardness contrasts with what may be suggested by simply looking at results (vote counting) from the published literature. Many consider water hardness to be a crucial factor affecting metals toxicity; many reports showed that the high levels of hardness may decrease the toxicity of Cu and Cd (Heijerick et al. 2003, Wang et al. 2016). However, other works indicated that heavy metals are more toxic in soft water (Ebrahimpour et al. 2010, Taylor et al. 2000). Furthermore, the effect of water chemistry on metals toxicity may be varied across the acute and chronic exposures (De Schamphelaere and Jansen 2004c). For example, Belanger and Cherry (1990) found that pH had negligible influences on the toxicity of Cu to reproduction of *C. dubia*. However, increasing pH caused a decrease in the acute toxicity (48-h mortality). Moreover, the effect of water chemistry variables on metals toxicity may show different responses under the same experimental conditions. Kozlova et al. (2009) found that sodium, potassium, and chloride ions did not affect the toxicity Ni on *D.* *pulex,* whereas pH effect on Ni toxicity varied in presence of HCO3. Similarly, the study of De Schamphelaere and Janssen (2004c) showed that dissolved organic carbon (DOC) and pH had a significant impact on chronic toxicity of Cu to *D. magna*, but water hardness did not.

Meta-analytic methods provide substantially more reliable insights into the effects across studies. While our results show limited effects, it is important to recognize that our data are a subset of all studies that report sufficient information to include in meta-analyses. A more thorough and standardized reporting of results linked to water hardness and test duration is thus warranted. Our analysis data is not always in line with literature, but the BLM and better availability of data for meta-analyses in the future will help reconcile these issues.

This analysis is the first meta-analytic consideration of the ecological effects of Cu and Cd concentration across different species of Cladocera. The data highlight several species’ specific responses to the sub-lethal concentrations of both metals and several traits that, on average, appear tolerant to metals in some species. The substantial omission of numerous studies due to incomplete reporting of means, standard deviations/errors, and sample size is sobering given the importance of drawing generalized conclusions from test species. Detailed meta-analyses (and associated effective reporting of data) on water quality parameters, such as hardness, pH, and dissolved organic components (DOC), are needed to elucidate the role of water chemistry on the toxicity of metals across different biological organizations.

**Chapter 3**

**Evaluating additive vs. interactive effects of copper and cadmium on *Daphnia pulex* life history**

**3.1. Introduction**

Metal toxicity is a worldwide concern arising from natural and anthropogenic discharges such as domestic effluents, agricultural and industrial activities (Ferreira et al. 2008, Shaw et al. 2017). At low and threshold concentrations, metals are known to induce a range of effects on living organisms and their communities, ranging from reductions in reproduction and growth to associated changes in food web interactions. Their persistence, non-biodegradability, and these associated effects on life history and species interactions ultimately impact on ecosystem services and human health (Monserrat et al. 2007, Nzengue et al. 2011, Piscia et al. 2015).

How stressors such as metals interact with each other and the environment remains a central question in ecology and ecotoxicology because most investigations have historically been limited to single stressors. However, numerous approaches now exist to assess whether the effects of multiple stressors are additive, synergistic, or antagonistic including methods linked to rejecting the null additive models of independent action or concentration addition through to response surface experiments designed to detect in species traits whether and how interactions among stressors manifest (Barata et al. 2006, Ferreira et al. 2008, Laskowski et al. 2010, Pavlaki et al. 2011).

Here we performed a response surface experiment and evaluated the effects of Cu and Cd on feeding rates and four life history traits. In aquatic communities, Cu and Cd are two major stressors from among inorganic pollutants and have received much attention associated with risk assessment regulations (Bellavere and Gorbi 1981, Shuhaimi-Othman et al. 2010, Fernández-Gonzáles et al. 2011). Each metal has a different cellular and ecological mode of action (e.g. impact on life history) because of the differences in each metal’s absorbability, solubility, chemical reactivity, transport and formation of complexes within the body (Shanker 2008, Stohs and Bagchi 1995).

At the molecular level, metal–metal interaction is a chemical reaction which can change the oxidation state of the metals, cleave organic radicals as well as change the state of organometallic compounds (Magos and Webb 1978). In organism’s bodies, metal–metal interactions (among essential and non-essential metals) may occur due to the similarity in physical and chemical properties among elements via the mechanisms of ionic and molecular mimicry (Brzóska and Moniuszko-Jakoniuk 2001, Bridges and Zalups 2005). These interactions may cause substantial alterations in the apparent properties of components as well as producing complexes that induce negative effects in organisms (Altenburger et al. 2013). For example, Cd effects arise from interactions with micro- and macro-elements (essential analogues) such as Ca, Zn, Cu and Se through ionic mimicry (Feng et al. 2018). Via ionic mimicry, species of a certain metal are able to either mimic essential element or the cationic form of the element (Zalups and Ahmad 2003). Cd can act as an ionic mimic via substitution for other metal ions (mainly Zn2+, Cu2+ and Ca2+) in metalloenzymes (Brzóska and Moniuszko-Jakoniuk 2001). In contrast, molecular mimicry affects the binding of nucleophilic groups of certain biomolecules to metal ions (Zalups 2000). For example, Cd tends to bind to structures containing –SH groups, such as enzymes, proteins and nucleic acids (Stohs and Bagchi 1995).

Despite these emerging insights that offer cellular level hypotheses about how interactions (synergy or antagonism) between metals might arise, we still lack robust data on the outcomes of these cellular level effects, including how Cu and Cd combine to affect foraging and life history. Here we focus on identifying whether the effects of Cu and Cd are additive or interactive on foraging and life history traits using three genotypes of *Daphnia pulex* exposed to Cu and Cd via a response surface exposure experiment. We further replicated our experiment under standard and high food levels.

*Daphnia* spp. are a model system for detecting the effects of metal pollution in aquatic environments. They have a significant role in the aquatic food chain linking between algae, higher invertebrates, and fish. Given their susceptibility to various contaminants, short generation times, easy culturing, and high fecundity rates, they are widely used in experiments (Sarma and Nandini 2006, Colbourne et al. 2011). Furthermore, water flea individuals are clonal, reproducing asexually and populations are typically comprised of many genetically distinct clones that can respond differently to environmental pollutants. This makes it possible to explore easily how interactions among stressors may vary amonggenotypesof *Daphnia* spp. Several studies have identified the substantial variability of genotypes in response to metals (Baird et al. 1990, 1991, Soares et al. 1992, Barata et al. 1998). Understanding whether there is genotypic variation in response to stressors is crucial to the management of natural water bodies which will typically be populated by multiple genotypes and to eco-toxicological test outcomes which are often based on single genotypes.

Our work here builds on a rich body of literature reporting on the toxicity of each of Cu or Cd (Agra et al. 2011, Piscia et al. 2015, Gama-Flores et al. 2006, Guan and Wang 2006, Sadeq and Beckerman 2019). While there is no consistent guideline for the assessment of mixture toxicity, one current approach is to explore how the individual components combine in mixture using the concentration addition (CA) versus independent action (IA) models (Cedergreen et al. 2008). The CA model was historically formulated for exploring how chemicals with similar modes of action combine and assumes that compounds with similar modes of action will behave as if they are simply higher doses of a single compound. Critically, the dose of each compounds is expected to behave additively on the response variables. In contrast, the IA model was developed against an assumption that the effects of the compounds, not the compounds themselves, behave additively and typically applies to chemicals with vastly different modes of action, for example anthropogenic stress versus natural stress (Rodea-Palomares et al. 2015**)**.

Here, based on detail presented above, we assume that Cu and Cd operate with different modes of action and that effects may combine additively or with interaction (e.g. the IA assumptions). To assess this, we employ a response surface experiment and analysis, manipulating exposure to single and combined concentrations of each metal. We tested whether mixture concentrations of Cu and Cd affect ingestion rate, size at maturity, age at maturity, somatic growth rates, and reproduction in an additive vs. synergistic manner as revealed by patterns in a response surface, where the life history traits are measured along single and combined concentration gradients of Cu and Cd.

A long history of statistical inference from response surface theory forms the basis by which we draw conclusions: additive effects are revealed by planar surfaces, while numerous forms of interactions are revealed by non-linearities in the surfaces. Our use of the response surface approach follows concepts in pharmacology called ‘isoboles’ (Rodea-Palomares et al. 2015) which do not necessarily depend on mechanistic assumptions (IA vs. CA) and are independent of the shapes of the dose-response curves so that they might apply for both CA and IA models of additivity (Berenbaum 1981, Rodea-Palomares et al. 2015). Our approach focuses attention on life history endpoints. When combined with genetic variation and multiple levels of food, we gain further insight into how natural variation in aquatic communities impacts on our assessment of multiple stressors.

**3.2. Material and methods**

*3.2.1 Daphnia culturing*

Three genotypes of *D. pulex,* LD33, D86A and D84A, were collected from field populations in the UK and have been in culture for 10 years in the Department of Animal and Plant Sciences, University of Sheffield. These three genotypes have different intrinsic body sizes, life history traits and sensitivties to predation risk (Reger 2013, 2018).

Stock cultures were acclimated in ASTM (American Society for Testing and Materials) hard water under controlled conditions of temperature 20±2 ºC, photoperiod 16:8h light:dark and light intensity 19 µmol m -2s-1. Prior to experiments, animals were acclimated to test media over three weeks as recommended in the OECD (Organisation for Economic Co-operation and Development) guidelines ([Aljaibachi](https://peerj.com/articles/4601/author-1) and [Callaghan](https://peerj.com/articles/4601/author-2), 2018[​](mailto:a.callaghan@reading.ac.uk)). The cultures were maintained in 2L tanks with approximately 25 individuals of each *Daphnia* genotype and fed every day with the green algae *Chlorella vulgaris* fo*. viridis* (strain number: CAAP 211/12) (2 x 105 cells/ml). The algae cultures were grown in Ebert medium (Ebert group, Zoologisches Institut Evolutionsbiologie, Switzerland) and kept on a table shaker in controlled room at 20±2 ºC under an 16h light:8h dark photoperiod with 20 µmol m -2s-1.

*3.2.2 Metals preparation*

To prepare metals stock solutions, analytical grade dihydrous copper and cadmium chloride (Fisher Scientific, UK) were dissolved in distilled water and these stocks used to prepare solutions for the experimental treatments. For chronic toxicity exposures (single and mixture), each treatment concentration (single or combined metals) was prepared daily in 1L volume ASTM hard water and subsequently delivered into experimental jars. Nominal Cu concentrations were 0, 5 and 10 µg/L and nominal Cd concentrations were 0, 0.5 and 1 µg/l. These sub-lethal concentrations were chosen based on an extensive literature review (Sadeq and Beckerman, 2019) investigating sub-lethal effects of Cu and Cd on Cladoceran species. All pairwise combinations of Cu and Cd were assayed, generating a complete response-surface grid of nine metal treatments.

Actual metal concentrations, estimated from solutions containing algae food resources, were calculated using ICP-MS (Inductively coupled plasma mass spectrometry). As in our experimental design, we estimated realised concentration in medial preparations that were provided daily to the replicate organisms. Realised concentrations did not deviate from nominal concentrations (Cu: R2 = 0.99, F = 1913, p < 0.002; Cd: R2 = 0.98, F = 996, p < 0.002).

*3.2.3 Experimental Design*

Our exposure tests were performed in accordance with the protocol OECD *Daphnia* *sp*., No. 212 (OECD, 2002). Our experimental design follows a completely factorial response surface defined by three genotypes, two food conditions (standard food level = 2 x 105 cells/ml and high food level = 5 x 105 cells/ml), and nine metal treatments defined by the three concentrations each of Cu and Cd and their combinations. This standard food level has been used for more than a decade with these genotypes (Beckerman et al 2010, Dennis et al 2011, Lind and Jeyasingh 2015, Reger et al. 2018) and produce life history trait values in line with other research labs and the published literature.

We replicated each metal treatment x food level x genotype combination five times for a total of 270 individuals, each observed over 21 days in 100 ml jars (9 metal treatments x 2 food levels x 3 genotypes x 5 replicates). Animals were fed and media replaced daily.

*3.2.3.1. Life History Traits*

We measured five traits in the daphnids over the 21 day experimental period: ingestion rate, size at maturity, age at maturity, somatic growth rate, and reproduction as the sum of three clutches. Life history traits in all treatments were assessed by daily observation during the transfer to new media in new jars. Photographs taken under a microscope Leica MZ6 modular stereomicroscope (GmbH, Wetzler, Germany) with Canon EOS 350D DSLR camera. Size at maturity was measured from the top of the head to the base of the carapace spine using ImageJ (Hooper et al. 2006, Beckerman et al. 2010). Age and size at maturity were estimated on the day neonates first appear in the brood pouch. Reproductive output was recorded by counting neonates produced over three clutches. Somatic growth rate was calculated as ln (size at maturity/initial size) / (age at maturity (days)).

Ingestion rate was measured on animals at their day of maturity in all treatments. As media was replaced daily with known food concentrations, this is a straightforward assay recording algae concentration on transfer to the new media and 24 hours later, before transfer to new media again. It was calculated as the number of algae cells digested by daphnids over 24 h (cells/day) (final measurement (after 24 h) - initial measurement (0 time)) using spectrophotometry at wavelength 440 nm (Ferrando and Andreu 1993, Beckerman et al. 2007).

*3.3 Statistical Analysis*

For each trait, we implemented the following analysis pipeline that maps onto our experimental design. We fit a response surface model that combined first order main effects of Cu and Cd, second order polynomials of Cu and Cd, and the interaction between Cu and Cd. This is the classic response surface model used to reveal additive and several forms of interactive (synergistic /antagonistic) effects on the life history endpoints (Khuri and Cornell, 1996). We combined this standard response surface model with the main effects of, and interaction between, algae food levels and *Daphnia* genotype, algae levels and each of the metals and *Daphnia* genotype and each of the metals. Our statistical model is thus defined as

Trait ~ (Cu + Cu2 + Cd + Cd2 + Cu \* Cd) +

Food Level + Genotype +

(Cu \* Food Level) + (Cd \* Food Level) +

(Cu \* Genotype) + (Cd \* Genotype) +

(Food level \* Genotype)

The terms in the first line define the classic response surface model that allows for planar or non-linear shapes to be detected over the nine treatment combinations for each metal. The second line identifies the additional main effects of food level and genotype and the third line the interactions between these terms and between them and the metals. Other terms represent effects estimated with respect to genotype and food levels.

This model allows us to answer the questions a) does the effect of Cu on trait vary by Cd?; b) is the effect of Cu or Cd non-linear?; c) does the effect of Cu or Cd vary by food level?; d) does the effect of Cu or Cd vary by genotype?; and e) does the effect of food level vary by genotype?

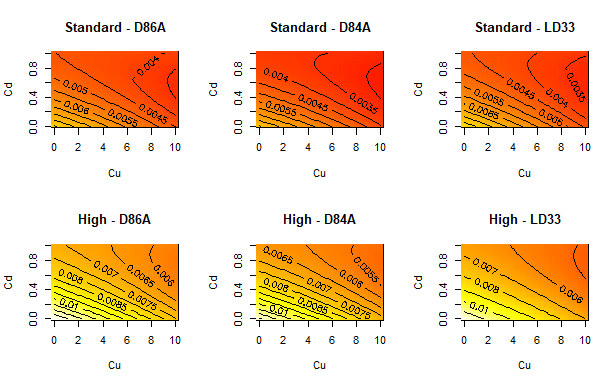
All data were analysed using the statistical software programme R (R Core team, 2013) version 3.4.2. We fit the model using the lm () function in R followed by Type II sums of squares implemented by the Anova() function in the *car* package for R for significance testing.

**3.4. Results**

In each of the following sections, we report for each trait on interactions between Cu and Cd, algae levels and genotype, algae levels and the metals and genotype and the metals for each measured trait. The results for each trait are visualised by a set of six contour plots graphically depicting the shape of the estimated response surface among genotypes and between food levels. Note that in all presentations of the effect of interactions, the “:” is used to represent the interaction.

*3.4.1 Ingestion Rate*

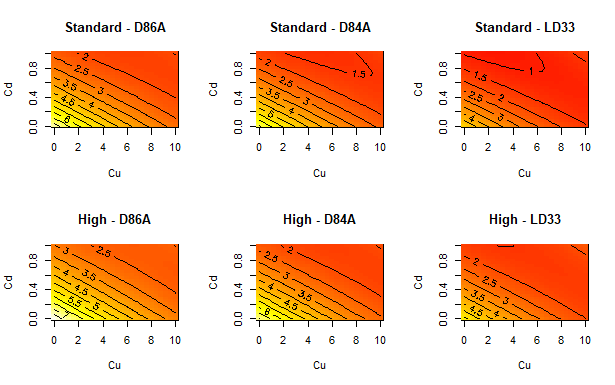
The effects of Cu and Cd on ingestion rates are defined by several interactions (Fig. 3.1). The two metals interact to shape ingestion rate (cell/day); the effect Cu on ingestion varied by Cd in all genotypes (Cu:Cd interaction; F = 54, df = 1, p<0.01). Furthermore, the effect of Cd, but not Cu was nonlinear, as indicated by the significant Cd2 term (F = 10.15, P<0.01; t-Cu^2 = -0.1 1, df = 2, p = 0.9, t-Cd^2 = 4.2, df = 1, p<0.01). The effect of Cu and Cd also varied by food levels (F-Cu:Food Level = 5.08, df = 1, p = 0.025, F-Cd:Food Level = 11.9, df = 1, p <0.01). Ingestion declined more with increasing Cu and Cd at high food. In contrast to the above interactions, the effect of Cu and Cd on ingestion did not vary by genotype (F-Cu:Genotype = 0.7, df= 1, p=0.49; F-Cd:Genotype = 0.23, df= 1, p = 0.79). However, genotypes differ in their average rate of ingestion (F = 3.76, df= 1, p = 0.025).



**Fig. 3.1.** Contour plots for the effect of Cu/Cd mixture on the ingestion rate (cell/day) of different genotypes of *D. pulex*. Each panel is a genotype-food combination (panel title) and the x- and y-axes are the concentrations of the metals (μg/L). The plots display the contours and colours associated with fitted (predicted) values from the response surface model with red colours = higher values of ingestion (trait). Details of significant terms that underpin the shapes that can be seen are described in the text.

*3.4.2 Reproduction*

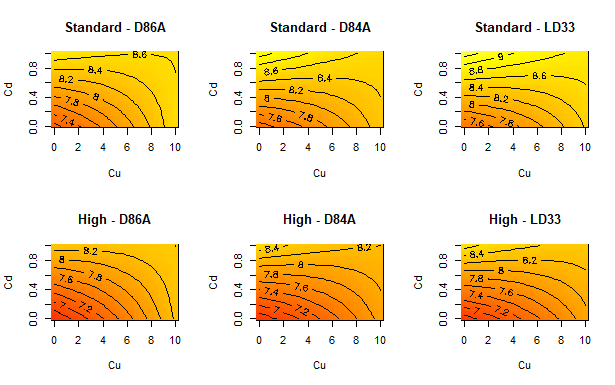
The effects of Cu and Cd on reproduction are defined by several interactions (Fig 3.2). The effect of Cu on reproduction of *D. pulex* varied by Cd; the two metals interact to shape reproduction (Cu:Cd interaction; F = 194, df = 2, p<0.01). Further, the effects of Cu and Cd were nonlinear defined by significant squared terms for each (F = 18.8, P<0.01; t-Cu^2 = 3.1, df = 2, p = 0.01, t-Cd^2 = 3.7, df= 1, p<0.01). The effect of Cu and Cd on reproduction varied by genotype (F-Cu:Genotype = 5.7, p=0.004; F-Cd:Genotype = 4.6, df= 1, p = 0.01). However, The effect of each metal on reproduction did not vary by food levels (F-Cu:Food Level = 0.65, df = 1, p = 0.42, F-Cd:Food Level = 0.03, df = 1, p <0.84).



**Fig. 3.2.** Contour plot for the effect of Cu/Cd mixture on reproduction (mean number of neonates per female) across three genotypes of *D. pulex.* Each panel is a genotype-food combination (panel title) and the x- and y-axes are the concentrations of the metals (μg/L). The plots display the contours and colours associated with fitted (predicted) values from the response surface model with red colours = higher values of ingestion (trait). Details of significant terms that underpin the shapes that can be seen are described in the text.

*3.4.3. Maturation Time*

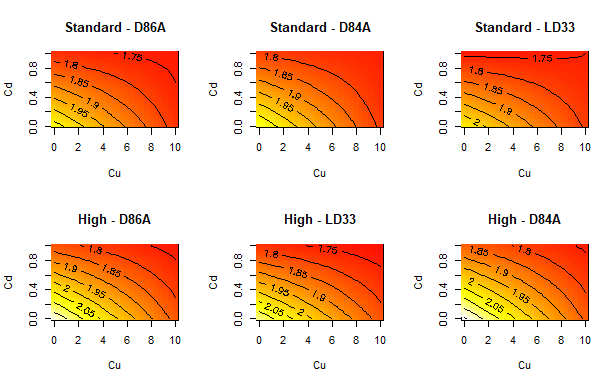
The effects of Cu and Cd on maturation time are defined by several interactions (Fig 3.3.). The effect of Cu on maturation time varied by Cd (Cu:Cd interaction; F = 84, df = 1, p<0.01). On their own, neither Cu nor Cd showed nonlinear relationship (F = 0.28,, P<0.76; t-Cu^2 = 0.66, df = 2, p = 0.51, t-Cd^2 = 0.07, df =1, p<0.94). Cu and Cd effects did not vary by food levels (F-Cu:Food Level = 1.81, df = 1, p = 0.27, F - Cd:Food Level = 0.21, df = 1 , p <0.64) or by genotypes (F-Cu:Genotype = 1.84, df= 1, p=0.16; F-Cd:Genotype = 0.54, df= 1, p = 0.58). Genotypes did not differ in their maturation time with food levels (F = 0.4, df = 1, p = 0.67).



**Fig. 3.3.** Contour plot for the effect of Cu/Cd mixture on the maturation age (days) of different genotypes of *D. pulex.* Each panel is a genotype-food combination (panel title) and the x- and y-axes are the concentrations of the metals (μg/L). The plots display the contours and colours associated with fitted (predicted) values from the response surface model with red colours = higher values of ingestion (trait). Details of significant terms that underpin the shapes that can be seen are described in the text.

*3.4.5. Size at Maturity*

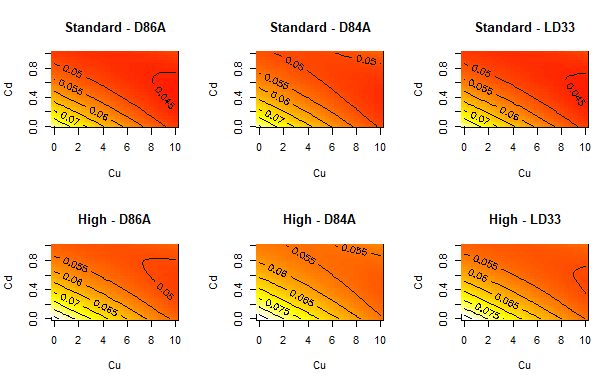
The effects of Cu and Cd on size at maturity are defined by several interactions (Fig 3.4). The effect of Cu on size at maturity varied by Cd; size reductions caused by Cd were less pronounced at high levels of Cu (Cu:Cd interaction; F = 145.1, df = 1, p<0.01). On their own, Cu and Cd did not show nonlinear relationship (F = 0.35, P<0.70; t-Cu^2 = 0.4, df = 2, p = 0.68, t-Cd^2 = 0.52, df =1, p<0.6). The effect of Cu and Cd on size at maturity varied by food level (F-Cu:Food Level = 8.47, df = 1, p = 0.004, F-Cd:Food Level = 8.68, df = 1, p <0.003). The effect on size at maturity of each metal did not vary by genotype (F-Cu:Genotype = 2.64, df =1, p=0.07; F-Cd:Genotype = 0.01, df =1, p = 0.98). Average size at maturity did not vary by genotype (F = 0.54, df =1, p = 0.59).



**Fig. 3.4.** Contour plot for the effect of Cu/Cd mixture on body size at maturity (mm) across three genotypes of *D. pulex.* Each panel is a genotype-food combination (panel title) and the x- and y-axes are the concentrations of the metals (μg/L). The plots display the contours and colours associated with fitted (predicted) values from the response surface model with red colours = higher values of ingestion (trait). Details of significant terms that underpin the shapes that can be seen are described in the text.

*3.4.6. Somatic Growth Rate*

The effects of Cu and Cd on somatic growth rate are defined by several interactions (Fig 3.5.). The effect of Cu on somatic growth rate varied by Cd across genotypes (Cu:Cd interaction; F = 214.85, df = 1, p<0.01). Growth rates were significantly lower with increasing Cu and Cd at standard food level. We also found evidence of quadratic interaction between Cu and Cd (Cu:Cd; F = 17.52, df = 2, p<0.01). On their own, Cd only had a nonlinear effect (t-Cu^2 = 1.92, df =1, p = 0.56, t-Cd^2 = 4.41, df =1, p<0.01). The effect of Cu and Cd did not vary by food levels (F-Cu:Food Level = 0.15, df = 1, p = 0.7, F-Cd:Food Level = 0.82, df = 1, p <0.37). Furthermore, the effect of Cu and Cd on somatic growth rate did not vary by genotype (F-Cu:Genotype = 0.08, df = 1, p=0.92; F-Cd:Genotype = 0.71, df= 1, p = 0.53). Genotypes did not differ in their average growth rate (F = 0.09, df = 1, p = 0.91).



**Fig. 3.5.** Contour plot for the effect of Cu/Cd mixture on somatic growth rate g (d-1) of different genotypes of *D. pulex.* Each panel is a genotype-food combination (panel title) and the x- and y-axes are the concentrations of the metals (μg/L). The plots display the contours and colours associated with fitted (predicted) values from the response surface model with red colours = higher values of ingestion (trait). Details of significant terms that underpin the shapes that can be seen are described in the text.

**3.5. Discussion**

Evaluation of the effect of metal mixtures at sub-lethal levels on life history traits of organisms in aquatic communities is of particular importance to the assessment of ecosystem services (e.g freshwater, recreation), to chemical risk assessment and subsequently to water quality criteria. In the last few decades, metal pollution has drawn much attention due to their persistence and deleterious effects on aquatic food chains. Though there has been considerable exploration into the toxicity of Cu and Cd on aquatic organisms, particularly Cladocera, the majority of work has focused on single stressors and single genotypes under standardised conditions that are beneficial to a testing environment but not always reflective of natural communities (Brix et al. 2001, Barata et al. 2002, Griffitt et al. 2008, Sadeq and Beckerman 2019). Assessing the interaction among stressors (metals), the role of genetic variation, and the impact of variation in food levels remains a key agenda in ecological and ecotoxicological risk assessment.

We assessed the presence of additive vs. interactive effects of the metals Cu and Cd on life history of three *D. pulex* genotypes under two food conditions. We employed a response surface experimental design to capture patterns of additive or interactive effects in ingestion rates and life history traits of three *D. pulex* genotypes via a chronic exposure experiment over 21 days. We focused on five classic ecological endpoints linked to daphnid biology and impact in aquatic communities: ingestion rate, reproduction, age at maturity, size at maturity and somatic growth rate.

Despite the presence of several interactions (among stressors, genotypes and food levels), our data indicate overall (Fig 1-5) that the presence of two metals reduced ingestion rates, impaired reproduction, extended the time to maturation, reduced size at maturity and lowered somatic growth rates. The nonlinearities we detect do not, we suggest, paint too complex a picture for understanding the combined effects Cu and Cd. Fig 1–5 do not show peaks or troughs of responses at sub-lethal levels of Cu and Cd. There are no saddle points that arise across the concentrations. In general, combined effects of Cu and Cd equate with increases or decreases in the traits we have measured and thus indicate in summary what logic and conventional thinking suggest: a combination of Cu and Cd are substantially worse for *Daphnia* performance.

While, our findings showed a variation in the interaction between metals and food levels and metals and genotypes across traits, our data highlight the importance of context in assessment of life history endpoints in response to different stressors. With respect to Cu, which is known to interfere with digestion, we expected and found that the effect of Cu on ingestion rates varied by food. We also found this pattern of effect for Cd. Metals disrupt digestive physiology, which is linked to energy intake and hence resources acquired for growth and reproductive activities (Barata and Baird 2000, Bui et al. 2016). The relationships between energy intake and the life history traits (more food should increase size at maturity, reduce age at maturity, increase reproduction and increase somatic growth rate) suggests that the interaction between metals and food we see for these traits may arise in part via these relationships between life history and food.

Our work contributes to a growing literature investigating the effects of metal mixtures and *Daphnia* spp. For example, in recent research, binary mixtures of nickel (Ni), zinc (Zn), Cu, and Cd produced a more than additive effect on food consumption rate of *D. magna* at low concentration of metals(Lari et al. 2017). Inter-clonal variation in *D. magna* was found in the response of ingestion rate to Cd and temperature (Muyssen and Janssen 2010). This body of data suggests a key role of foraging and digestion in metal effects. Compounding these effects, under metal exposure, organisms may also spend more energy to increase their tolerance (Calow 1991). Supporting our data on reproduction, growth and maturity, data also suggest changes in allocation to growth and reproduction that effect life history responses depending on duration and exposure concentration (Muyssen and Janssen 2001, Bossuyt and Janssen 2004, Canli 2005, Durou et al. 2005).

Detecting consistency in the type of response (additive, synergistic, antagonistic) is proving challenging. A recent meta-analysis evaluating Cu, Cd and Zn toxicity showed variable interactions for many endpoints measured under the same experimental conditions. The effects were strongly associated with the identity of the endpoint on which the metal combinations were tested (Vijver et al. 2011). Similarly, different patterns of responses have been observed in *D. magna* across traits studiedand different metal mixtures (Loureiro et al. 2010, Pavlaki et al. 2011). Data also indicate that interactions between metals may vary from synergistic to antagonistic depending on the metals, properties of contaminants and test species (Shuhaimi-Othman and Pascoe 2007, Meyer et al. 2015, Kim et al. 2017).

While numerous studies found a combination of additive or synergistic effects, many studies have found only antagonistic effects. For example, recent research by Pérez and Hoang (2018) on mixture toxicity to *D. magna* showed that the sub-lethal concentration of Cd and Ni caused antagonistic effects across traits studied. However, other studies on metal mixture toxicity have reported less than additive effects to *D. magna* (Komjarova and Blust 2008, Meyer et al. 2015, Traudt et al. 2017, Pérez and Hoang 2017, 2018). Mahar and Watzin (2005) examined the impacts of Cu, Zn, and insecticide mixtures on the survival and reproduction in *Ceriodaphnia dubia*. It has determined less than additive effect for the binary mixture of Cu and Zn on survival, but more than additive effect on reproduction. Further, an antagonistic effect was observed on reproductive activity of *C. dubia* under exposure to high concentrations of benzalkonium chloride with binary mixtures of anticancer drugs, but additive effects in all mixtures at low concentrations (Russo et al. 2018).

Our research, and that of others noted above, highlight that if we are to move towards generalising and predicting the effect of multiple stressors, experiments need to account not only for the diversity of traits that can be evaluated, but also for context defined by the metal identity, concentrations, food levels and genotype.

Interestingly, apart from reproduction, our data show that the effects of metals rarely varied among our three genotypes. While three genotypes is certainly too few to generalise about the nature of genetic variation, it is data that suggests we should assess whether uniformity in responses to metals is more common than say to food or predation. Baird et al. (1990) found small differences among *D. magna* clones in response to chronic Cd and 3,4-dichloroanilin. These responses may be related to specific mechanisms of heavy metals (e.g. metallothionein vs. detoxification). In contrast, Barata et al. (2001) suggested that differences in *D. magna* clones’ response to ethyl parathion was due to genetic differences in tolerance.

**Chapter 4**

**Biomarker responses in *Daphnia pulex* exposed to copper and cadmium: Digestive and antioxidant enzymes**

**4.1. Introduction**

Exposure of living organisms to metals may induce toxicological effects via several biological processes tied to physiology and biochemistry (Soetaert et al. 2007, Wu and Wang 2010). Biomarker responses such as digestive and antioxidant enzymes activity are increasingly considered a promising indicator of toxicant effects on organism’s metabolism (De Coen and Janssen 1997, McWilliam and Baird 2002, Sancho et al. 2009). Biomarker responses are valuable because they are a potentially rapid assessment of biological response to sub-lethal levels of contaminants – they don’t require traditional, time consuming life-table experiments (e.g. OECD 21 day trials) (De Coen and Janssen 1998).

Furthermore, they are considered to be more aligned with the mode of action of contaminants (Golovanova 2008) (Fig. 4.1). The biochemical endpoints of biomarkers are considered an early warning of stress caused by metal exposures (De Coen and Janssen 1998) and of the mechanisms by which interactions between metals might arise to generate additive or interactive effects (antagonistic or synergistic). They thus provide a mechanistic perspective on how contaminants affect individuals and populations and contribute to our understanding of how contaminants drive changes in ecosystem services.

Cladocerans such as *Daphnia* spp. remain one of the core test species in ecotoxicology of freshwater systems. Water fleas play a central link in the food webs as an energy transfer between producers and secondary consumers. Furthermore, they are widely utilized as an indicator species to assess the response of natural populations to stressors (Sarma and Nandini 2006, Colbourne et al. 2011). The current understanding of metals toxicity in Cladocera mainly focuses on life history responses as a way to diagnose the potential effects of toxicants (Urabe and Sterner 2001, Sarma and Nandini 2006, Zhao and Wang 2011, Kovács et al. 2012, Nys et al. 2015, Sadeq and Beckerman, 2018). Here we advance knowledge on sub-lethal effects of copper Cu and Cd stress by focusing on digestive enzyme and antioxidant responses to them in *D. pulex*. These metals remain widely distributed in the aquatic environment and induce toxic influences to living organisms (Bellavere and Gorbi 1981, Shuhaimi-Othman et al. 2010, Fernández-Gonzáles et al. 2011).

**Heavy metals exposure**

**Oxidative stress (Production of ROS)**

**Enzymatic modification DNA damage Production of free radicals**

**Cell damage**

**Metallothionein** (e.g. Apoptosis) **Decrease of Antioxidants**

(SOD, GPX and GST)

Metals bind to MT

MT redox cycle

**-SH** (thiol group) **Scavenging of free radicals**

Oxidation of thiolate clustyers

Release of metal ions

MT-disulphide MT-thiol

MT-disulphide degradation

**Fig. 4.1.** The proposed mechanisms of heavy metals.

Furthermore, there has been an increased interest in the interaction between sub-lethal levels of metal stressors and associated biomarker responses (Company et al. 2004, Nicholson and Lam 2005, Rajalakshmi and Mohandas 2005, Jing et al. 2006). In particular, the additive or interactive effects (synergistic, antagonistic) of multiple metals are likely linked to toxicity mechanism of metals within cells and tissues which can damage or inhibit enzyme activity via interaction of metal ions with active sites of those enzymes resulting in alterations in their configurations and functions (Chen et al. 2002, Khoury et al. 2009).

Evaluation of digestive physiology in response to environmental stressors is tightly connected to feeding ecology, energy availability for metabolism and ultimately growth and reproduction (Dedourge-Geffard et al. 2009). The alimentary canal is the direct target of metal uptake in daphnids and rich in digestive enzymes that drive digestion processes (De Coen and Janssen 1997, Chen et al. 2002). Changes in digestive enzyme activity in early developmental stages may drive changes in digestive capabilities and influence developmental and life history transitions (Hammer et al. 2000).

Hasler (1935) was the first to describe the digestive enzymes in *Daphnia* spp. through monitoring proteinase, amylase and lipase activities. Subsequently, more digestive enzymes have been described (De Coen and Janssen 1997, 1998, Diamantino et al. 2001, Schwarzenberger et al. 2012, Koussoroplis et al. 2017). It is now known that digestive enzymes such as amylase, trypsin and esterase are responsible for breaking down of large food molecules into nutrients (De Coen and Janssen 1998, Dedourge-Geffard et al. 2009). This process releases energy for maintenance, reproduction and growth of organisms (McWilliam and Baird 2002, Dedourge-Geffard et al. 2009, Khoury et al. 2009). Investigating daphnid digestive physiology under metal stress is likely to be informative to our understanding of toxicity and daphnid roles in maintaining water quality.

In addition to digestive enzymes, antioxidant enzymes are considered to be a defence mechanism against increasing reactive oxygen species (ROS) levels and maintainers of cell homeostasis (Doyotte et al. 1997, Barata et al. 2005). Many studies documented that the high levels of ROS can cause a reduction in antioxidant enzyme activities and increasing levels of oxidative damage such as lipid peroxidation and reduced animal life span (Barata et al. 2005, Sohal et al. 2000, Khangarot and Rathore 2003, Jemec et al. 2007). Failure of antioxidant defence under metal exposure condition may activate oxidative stress which targets important macromolecules such as DNA, lipid, and proteins (Giusto and Ferrari 2014).

For example, superoxide dismutase (SOD) transforms superoxide anion (O2-) into hydrogen peroxide (H2O2), and GPX has complementary roles in detoxification of H2O2 (Barata et al. 2005). Furthermore, GSH is a major antioxidant play an important role in protection of organism’s cells through binding to radicals and metals (Klaassen et al. 1985, Mah and Jalilehvand 2012). Thus, the antioxidant defence systems are representative biomarkers for the estimation of the effects of stressors on freshwater invertebrates (Doyotte et al. 1997).

We evaluated the activity of three digestive enzymes involved in digesting the major food particles in *D. pulex*: trypsin (proteins), amylase (carbohydrates) and esterase (lipids). We also evaluated three antioxidant enzymes activities that play important roles in protection of organisms from oxidative stress damage: superoxide dismutase, glutathione peroxidase and glutathione S-transferases. We evaluated these as potential biomarkers to detect toxicants-related digestive and oxidative stress under short term exposure of Cu and Cd alone and in a mixture.

**4.2. Material and methods**

4.2.1. *Daphnia* culturing

The clone *D. pulex* LD33 was collected from field populations in the UK and cultured in the Department of Animal and Plant Sciences, University of Sheffield. Stock cultures were acclimated in ASTM hard water (Physicochemical parameters as follow: pH of 7.6, Ca+2 concentration of 21 mg L-1) under controlled conditions of temperature 20±2 ºC, photoperiod 16h:8h light/dark and light intensity 16 µmol m -2s-1. Prior to experiments, animals were acclimated to test media over three weeks as recommended in the OECD guideline for *Daphnia* *sp*., No. 212 (OECD 2002). The cultures were maintained in 2L tanks with 25 individuals and fed every day with the green algae *C. vulgaris* fo*. viridis* (strain number: CAAP 211/12) at a concentration of 2 x 10^5 cells ml-1. The culture medium was changed once a week. The algae cultures were grown in Ebert medium (Ebert group, Zoologisches Institut Evolutionsbiologie, Switzerland) and kept on a table shaker in controlled room at 20±2 ºC under an 8 h dark16 h light photoperiod with 18 µmol m -2s-1.

4.2.2. Preparation of chemicals

All chemicals used for enzymatic assays were analytical grade and obtained from Sigma Aldrich, UK. To prepare metals stock solutions, analytical grade dihydrous copper and cadmium chloride (Fisher Scientific, UK) were dissolved in distilled water. For acute toxicity exposures (single and mixture), for each treatment, experimental concentrations of each treatment (single or combined metals) were added into 1L ASTM hard water and decanted into experimental vessels containing the animals.

4.2.3. Experimental setup

Acute exposure for enzyme assay tests were performed in accordance with the standard OECD protocol *Daphnia* *sp*., No. 202 (OECD 2004). After acclimations to lab conditions, *D. pulex* neonates (1st and 2nd instars) were placed in 1 L glass jar containing hard ASTM with a standard food level. For each enzyme and metal, five sets of treatments (5 replicates each of 20-30 neonates) were exposed to dissolved Cu and Cd and a combination of both metals. The concentrations of Cu used in the exposure experiment were 0, 10, 20, 30 and 40 µgL-1, and for Cd were 5, 10, 5, 15 and 20 µg L-1. For mixture exposures, our experimental design comprised a) a gradient of increasing copper concentrations (control +4 concentrations); b) a gradient of increasing cadmium risk (control +4 concentrations); and c) combinations of each for a total of 9 treatments. We evaluated the digestive and antioxidant enzymes: Trypsin, amylase, esterase, SOD, GPX and GST over 24 and 48h experimental period on this experimental design.

For both groups of enzymes, after the exposure, all live daphnids (per replicate) were collected, weighed after removing the water residue, pooled in a 1.5 mL microcentrifuge tube and frozen in liquid nitrogen (-80 ºC) and then kept in the freezer (-20 ºC). All enzymatic assays were performed in five technical replicates using plate reader (VersaMaxTM Microplate Reader) at 25±0.5 ºC for 10 min where the absorbency of treatment was determined every 30s. For each replicate, 10 µL of crude extract has been added into 10 ml buffer in addition to substrate. Thoses three elements represent the master mix that applies to all assays.

4.2.4. Enzyme assays

4.2.4.1. Digestive enzymes

4.2.4.1.1. Trypsin assay

Water flea samples (exposed neonates and control) were homogenized in a micro-centrifuge tube in buffer of 0.1mol/l sodium phosphate and pH 6.5 using pellet pestles (cordless motor). The enzyme activity was measured with the substrate N-benzoyl-DL-arginine-para-nitroanilide using the method described by Dölling et al. (2016). The final concentration of substrate is 1.88 mmol/l. all stock solutions were prepared in DMSO at 0.5% (the final concentration). The absorbance was monitored at 390 nm.

4.2.4.1.2. Amylase assay

Crude extracts of exposed samples were prepared in 10 mM Tris-HCl buffer, pH 7.5, with 0.15 M NaCl (Castro et al. 2012). The activity was measured using 2% (w/v) starch solution as substrate. The reaction solution consisted of 375 μ L of starch solution, 375 μ L of 10 mM phosphate buffer (pH 7.5) and 20 μ L of crude extract. Samples were incubated for 10 min at 37°C. Then, 100 μ L of this mixture was added to 1 mL of a 3.5-dinitrosalicylic acid (DNSA) solution using heating block bath in order to stop the reaction. The absorbance was recorded at 570 nm.

4.2.4.1.3. Esterase assay

*Daphnia* samples were homogenized with sodium phosphate buffer (0.1 M, pH 7.4) using pellet pestles. Esterase assays were performed using the substrate of p-nitrophenyl acetate (PNPA) and sodium phosphate buffer (0.1 M, pH 7.4). Porcine esterase (catalyze the hydrolysis of pentaacetyl catechin) was used at a final concentration of 0.1 mg/ml. Activity was monitored at wavelength 405 nm (Homola and Chang 1997).

4.2.4.2. Antioxidant enzymes

Daphnids samples were homogenized in a micro-centrifuge tube in 4 volume ratio of extraction buffer (100 mM phosphate buffer, pH 7.4, containing 100 mM KCl and 1 mM EDTA) on ice using pellet pestles. Then, the homogenates were centrifuged at 1000g for 10 min using Eppendorf 5417R Centrifuge at 4 ºC. The supernatant was separated from the pellet and used immediately for analysis. For each replicate, 10 µL of crude extract has been added into 10 ml buffere in addition to substrate. These three elements represent the master mix that applies to all assays.

4.2.4.2.1. Superoxide dismutase assay

The SOD activity was measured using the method of Barata et al. (2005) method by measuring the reduction of cytochrome c where 1 U of SOD is defined as the amount of sample causing 50% inhibition of cytochrome c reduction. SOD competes with cytochrome c for O2 generated by hypoxanthine and xanthine oxidase action. The reduction of cytochrome c by O2 (absorbance) was measured at 550 nm. Samples were assayed in a solution of 50 mM phosphate buffer, pH 7.8, 0.1 mM EDTA, 50 AM hypoxanthine, 5.6 mU xanthine oxidase and 10 AM cytochrome c. The results of this assay are expressed in units of SOD activity per milligram of protein (U mg -1).

4.2.4.2.2. Glutathione peroxidase assay

The GPX activity was measured using the method described by Barata et al. (2005). The mix  
solution contained 100 mM phosphate buffer (pH 7.5), 2 U glutathione reductase, 2 mM GSH, 0.12 mM NADPH. The activity was measured by following the reduction in NADPH concentration at wavelength of 340 nm (extinction coefficient 0.00622 µM-1 cm-1).

4.2.4.2.3. Glutathione S-transferases assay

GST activity was measured by the method of Barata et al. (2005). The mix contained 100 mM phosphate buffer (pH 7.5), 1 mM GSH and 1 mM CDNB. The formation of S-2,4-dinitrophenyl glutathione conjugate was recorded by monitoring the change in absorbance at 340 nm.

4.2.5. Data analysis

All data were analysed using the statistical software programme R (3.4.2) (R Core team 2013). For each enzyme, we fit a response surface model that combined first order main effects of Cu and Cd, second order polynomials of Cu and Cd, the interaction between Cu and Cd. This is the classic response surface model that can reveal additive and several forms of interactive effects on enzyme activity. We combined this standard response surface model with the main effects exposure period (24/48 hour) and the interaction between exposure period and metal. For each enzyme, we present a response surface contour plot showing the effects of single and combined metals on enzyme activity in each of the i) 24 and ii) 48-hour exposures. We report on the significance of the main effects and interactions using type II sums of squares tests from the Anova() function in the car library.

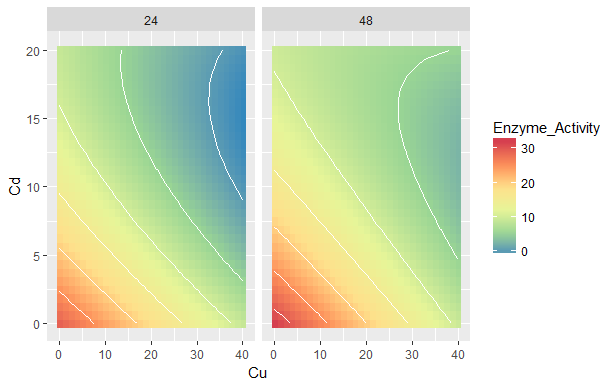
**4.3. Results**

We report the toxicity effects of single and combination of Cu and Cd on two different classes of biomarkers (digestive and antioxidants enzymes) in *D. pulex* over two different experimental durations (24 and 48h). Overall, metals decreased enzyme activity. We report however, on nearly ubiquitous interactions where the negative effects of Cu vary by Cd concentration (statistical Cd:Cu interaction), where the negative effects of Cu and Cd are non-linear (Cu and Cd effects are fit best by polynomials) and where the negative effects vary by experiments duration (metal:duration interaction). We detail these results for each assay below and present a response-surface plot to help visualise the patterns captured by the statistics.

4.3.1. Digestive enzymes

4.3.1.1. Trypsin

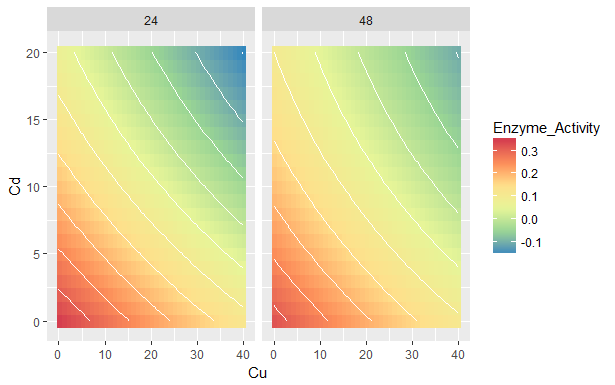
The effects of Cu and Cd on trypsin activity are defined by several interactions (Fig. 4.2). The two metals interact to shape the response of trypsin activity. The data indicate that the effect of Cu on trypsin activity varied by the concentration of Cd (Cu:Cd interaction; F = 50, df = 1, p<0.0002) where the negative effect of Cu on trypsin activity was reduced by increasing concentrations of Cd. The independent effects of Cu and Cd were non-linear and caused a decrease quadratically in trypsin activity with increasing concentration of the metal (more pronounced in Cd than Cu) defined by significant squared terms for each metal (F-Cu^2 = 5, df = 1, p = 0.03, F-Cd^2 = 236, df = 1, p<0.0002). Furthermore, we found that the effect of Cu and Cd on enzyme activity varied by exposure time (F-Cu:Exposure time = 8.2, df = 1, p=0.005; F-Cd:Exposure time = 6, df = 1, p = 0.02) where the negative effect of Cu and Cd on trypsin decreased as experiment duration increased.



**Figure 4.2.** Contour plot for the effect of Cu and Cd on trypsin activity of *D. pulex.* Unit of activity: Micromol/min/g protein. Each line represents a response value.

4.3.1.2. Amylase

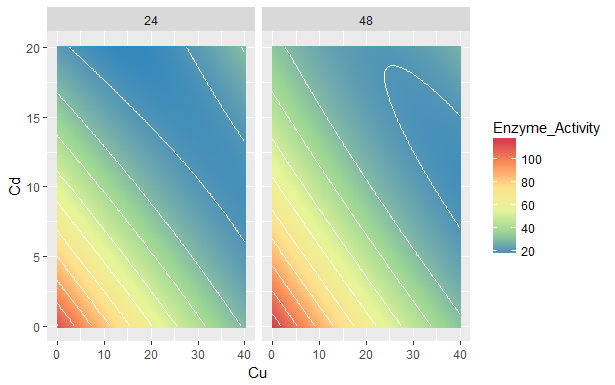
The effects of Cu and Cd on amylase activity are defined by several interactions (Fig. 4.3). The data indicate that the effect of Cu on amylase activity did not vary by Cd (Cu:Cd interaction; F = 0.005, df = 1, p=0.95). The effect of Cd, but not of Cu was nonlinear defined by a significant squared term for Cd but not Cu (F-Cu^2 = 0.7, df = 1, p = 0.42, F-Cd^2 = 7.4, df = 1, p<0.007). The data indicate that the effect of Cd, but not Cu, on amylase activity varied by exposure time (F-Cu:Exposure time = 0.6, df = 1, p=0.5; F-Cd:Exposure time = 8, df = 1, p = 0.006). The negative effect of Cd on amylase decreased as experiment duration increased.



**Figure 4.3.** Contour plot for the effect of Cu and Cd mixture on amylase activity of *D. pulex.* Unit of activity: U/mg protein. Each line represents a response value.

4.3.1.3. Esterase

The effects of Cu and Cd on esterase activity are defined by several interactions (Fig. 4.4). The two metals interact to shape the response of esterase activity. The data indicate that the results showed that the effect of Cu on esterase activity varied by Cd concentrations (Cu:Cd interaction; F = 23, df = 1, p<0.0005) where the negative effect of Cu on esterase activity was reduced by increasing concentrations of Cd. The independent effects of Cu and Cd on esterase activity were non-linear and declined quadratically under metal exposures defined by significant squared terms for each metal (F-Cu^2 = 14, df = 1, p = 0.0003, F-Cd^2 = 20, df = 1, p<0.0001). The data also indicate that the effect of Cu and Cd on enzyme activity did not vary by exposure time (F-Cu:Exposure time = 0.3, df = 1, p=0.61; F-Cd:Exposure time = 0.98, df = 1, p = 0.32). However, esterase activity decreased with increased experimental duration overall (F = 14, df = 1, p = 0.0003).

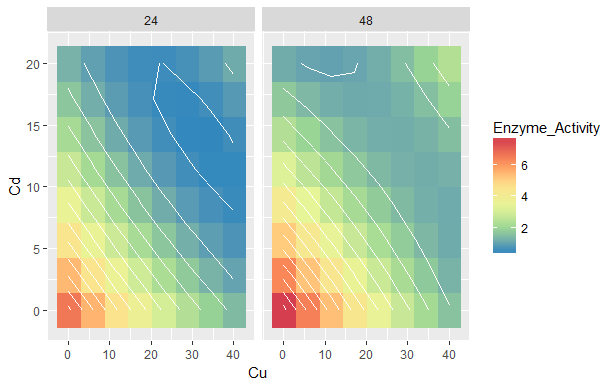


**Figure 4.4.** Contour plot for the effect of Cu and Cd mixture on esterase activity of *D. pulex.* Unit of activity: U/ml. Each line represents a response value.

4.3.2. Antioxidant enzymes

4.3.2.1. SOD

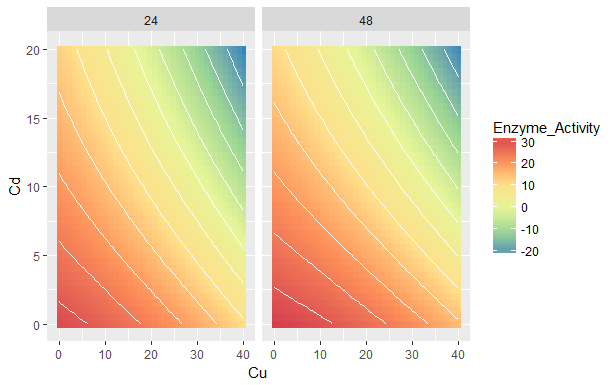
The effects of Cu and Cd on SOD activity are defined by several interactions (Fig. 4.5). The two metals interact to shape the response of SOD activity. The data indicate that the effect of Cu on SOD activity varied by the concentration of Cd (Cu:Cd interaction; F = 43, df = 1, p<0.0002) where the negative effect of Cu on SOD activity was reduced by increasing concentrations of Cd. On their own, the effects of Cu and Cd had non-linear relationship and SOD activity decreased quadratically with increasing concentration of the metals defined by significant squared terms for each metal (F-Cu^2 = 24, df = 1, p = 0.0003, F-Cd^2 = 35, df = 1, p<0.0005). The data also show that the effect of Cu and Cd on enzyme activity varied by exposure time (F-Cu:Exposure time = 6, df = 1, p=0.02; F-Cd:Exposure time = 11, df = 1, p = 0.001). The negative effect of Cu and Cd on SOD decreased as experiment duration increased.



**Figure 4.5.** Contour plot for the effect of Cu and Cd on SOD activity of *D. pulex.* Unit of activity: U/min/mg protein. Each line represents a response value.

4.3.2.2. GPX

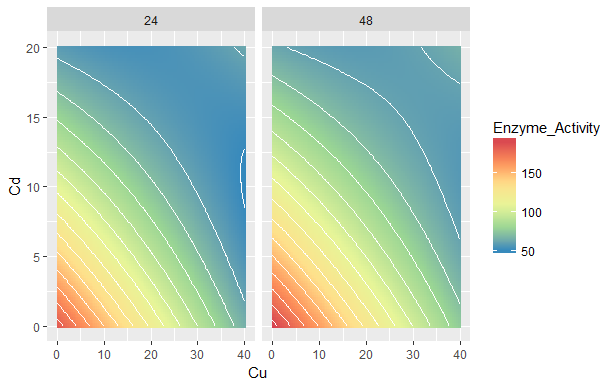
The effects of Cu and Cd on GPX activity are defined by several interactions (Fig. 4.6). The two metals interact to shape the response of GPX activity. The data indicate that the effect of Cu on GPX activity varied by the concentration of Cd (Cu:Cd interaction; F = 8, df = 1, p<0.006) where the negative effect of Cu on GPX activity was reduced by increasing concentrations of Cd. On their own, the effect of Cu, but not Cd, caused GPX to decline non-linearly as indicated by significant squared terms for Cu (F-Cu^2 = 8.2, df = 1, p = 0.005, F-Cd^2 = 3, df = 1, p<0.08). Furthermore, the data show that the effect of Cu and Cd on enzyme activity did not vary by exposure time (F-Cu:Exposure time = 3, df = 1, p=0.09; F-Cd:Exposure time = 3, df = 1, p = 0.11). However, GPX activity declined negatively by the experimental duration overall (F = 11, df = 1, p = 0.001).

**

**Figure 4.6.** Contour plot for the effect of Cu and Cd on GPX activity of *D. pulex.* Unit of activity: Nmol/min/ml. Each line represents a response value.

4.3.2.3. GST

The effects of Cu and Cd on GST activity are defined by several interactions (Fig. 4.7). The two metals interact to shape the response of GST activity. The data show that the effect of Cu on GST activity varied by the concentration of Cd (Cu:Cd interaction; F = 44, df = 1, p<0.0002) the negative effect of Cu on GST activity was reduced by increasing concentrations of Cd. The data show that the effects of Cu and Cd on GST was negative and non-linear with increasing concentration of the metals (more pronounced in Cd) defined by significant squared terms for each metal (F-Cu^2 = 6, df = 1, p = 0.02, F-Cd^2 = 26, df = 1, p<0.0001). Furthermore, we found that the effect of Cu and Cd on enzyme activity did not vary by exposure time (F-Cu:Exposure time = 0.01, df = 1, p=0.9; F-Cd:Exposure time = 0.7, df = 1, p = 0.4). However, there was a negative effect on GST activity by the experimental duration overall (F = 24, df = 1, p = 0.0003).

**

**Figure 4.7.** Contour plot for the effect of Cu and Cd on GST activity of *D. pulex.* Unit of activity: Nmol/min/mg protein. Each line represents a response value.

**4.4. Discussion**

In recent years, biomarker assays have been applied in environmental risk assessments in order to assess the effect of multiple stressors, including understanding the mechanisms of metals toxicity (De Coen and Janssen 1997, Chandran et al. 2005, Sancho et al. 2009). Metals can generate substantial changes in living organisms by binding to enzymes and reducing their function (Skaggs and Henry 2002). Sub-lethal responses like those of enzyme activity to stressors are efficient and quick assays to characterize changes in stressed organisms. These physiological and biochemical responses are the first target of toxicants within organisms (De Coen and Janssen 1997) and a critical step towards linking contaminant mode of action to population and community responses (Barata et al. 2002 b).

Biomarker assays have emerged as a popular idea for assessing environmental health because they can use organisms directly from the environment and don’t require medium to long-term life-table experiments in the lab. Biomarkers continue to offer immense potential to assess the interaction among environmental stressors such as metals (Barata et al. 2005). We focused on biomarker responses of *D. pulex* in response to Cu, Cd and their mixture. These metals remain important chronic stressors in aquatic and terrestrial ecosystems at sub-lethal levels. We carried out multi-biomarker approaches to obtain an insight into the digestive physiological and biochemical responses to the metals focusing on lipid, carbohydrate and protein digestive enzymes and on antioxidant enzymes. In the present study, three digestive and three antioxidant (stress) responses enzymes have been tested in response to lethal concentrations of Cu and Cd over two exposure periods: 24 and 48h.

4.4.1. The influence of Cu and Cd on the activity of digestive enzymes

Overall, the results indicate that the activity of enzymes declined nonlinearly with increasing concentrations of Cu and Cd (Fig 2-4). However, our data reveal that enzyme responses to one metal are influenced by the presence of another metal (e.g. Cu:Cd interaction) and that the effects of the metal vary with the duration of the experiment, a proxy for exposure to the contaminants. For trypsin and esterase, the effects of a metal on enzyme activity varied by the concentration of the other metal (e.g. an interaction between metals), but it did not for amylase. We also found that the effects of the metals vary by experiment duration trypsin and amylase (Cd only), but it did not for esterase.

This variation may be related to the differences in metabolic rates associated with food supply and/or malfunction in food assimilation in presence of metal ions and then digestion process efficiency (Bohrer and Lampert 1988, Golovanova 2008). DeLong et al. (2014) suggested that the metabolic rate may be affected by the interaction between organisms and their environments. Furthermore, the study of Biesiot and Capuzzo (1990) indicated that digestive enzyme activity appears to be associated with development of gut where they show less activity in the early larval stages compared to the successive stages.

Our work contributes to the growing understanding of how enzyme inhibition is linked to responses to metal concentration. Reductions in digestive enzyme activity underpin reduced acquisition of resource which results in very well-established life history responses: delayed maturation, smaller size at maturity and reduced fecundity. There are few eco-toxicological studies reporting the activity of *Daphnia* spp. digestive enzymes in response to single metals in particular Cu and Cd, but relatively few combining the two metals. Dedourge-Geffard et al. (2009) suggested some hypotheses that explain how digestive enzyme are affected by toxic chemicals: (i) contaminants may influence directly on synthesis of digestive enzymes; (ii) contaminants can impact organism’s behaviour via decreasing the foraging activity (iii) the quality and quantity of food.

De Coen and Janssen (1997) demonstrated a reduction in digestive enzymes activity (amylase, β-galactosidase, trypsin and esterase) in *D. magna* under acute exposures to CdCl2 over 48 h. However, prolonged exposure (96 h) led to an increase in the activity of all digestive enzymes. Furthermore, the study of De Coen and Janssen (1998) assessed the activity of digestive enzymes under short exposure to CdCl2 at different exposure times (30-360 min). Esterase activity significantly decreased to 62% after 90 min exposure to metal concentrations, whilst the activity of cellulase and amylase were not affected by any of the concentrations tested and across exposure periods. Also, the activity of lactate dehydrogenase was decreased in *Daphnia magna* after acute exposure to zinc chloride (Diamantino et al. 2001).

In metal mixture exposures, Binelli et al. (2006) found an interaction between the terbutilazine and organophosphate insecticide chlorpyrifos, which led to decrease the activity of Acetylcholinesterase (AChE) in the mussel *Dreissena polymorpha*. The study of Canesi et al. (2008) showed that acute exposure of EDC mixtures (natural estrogens and estrogenic chemicals) significantly affected lipid metabolism in the digestive gland of *M. galloprovincialis.* Furthermore, like in our work,the effect of exposure duration influenced the effects with a significant increase in neutral lipids accumulation via lipid peroxidation observed under mixture exposure at 72h compared to 24 h. the study of Chen et al. (2002) indicated the impact of Cu protease activity across invertebrate species depend on species considered as well as duration of exposure.

4.4.2. The influence of Cu and Cd on the activity of antioxidant enzymes

Our data suggest a consistent decrease in SOD, GPX and GST activities across Cu and Cd concentrations (Fig 5-7) suggesting that stress response/homeostasis mechanisms are being adversely affected by metals. We found that the effect of Cu on enzyme activity vary by the Cd, this applies to all antioxidant enzymes. We also found that the effects of metals vary by exposure periods for SOD only, but it did not for GPX and GST. The reduction in the activities of antioxidant enzyme may be due to increasing oxyradicals levels during metals exposure. Further, at the cellular level, inhibition of enzymes by metals may disrupt the functions of endoplasmic reticulum and mitochondrial respiration (Orbea et al. 2000, Chandran et al. 2005). March (1988) suggested that metals may disrupt the function of cells by interference with ion regulation, distressing of calcium (Ca) uptake and metal accumulation in lysosomes.

Studies published demonstrated that metals can produce oxidative stress, but the response varies depending on the duration of exposure, metals concentration, species, and species-specific resistance to metals (Hoffman et al. 2000, Mateo and Hoffman 2001, Ji et al. 2006, Berglund et al. 2007, Martinez-Haro et al. 2011, Espín et al. 2014 a,b). Further, García-Fernández et al. (2002) suggested that an antioxidant defence mechanism is activated at low metal doses, and this system may be collapsed when metal exposure is high. The study of Chandran et al. (2005) indicated that Cd exhibits different mechanisms of toxicity across different species and experimental conditions. Additionally, toxic responses of chemicals may depend on their ratios, their physio-chemical properties and age of the exposed animals (Alberto et al. 2011, Tang et al. 2011). Also, the interactive relationship among chemicals (less or greater than additive) occurs depending on availability and uptake rates as well as physiologic processes of organisms (Tang et al. 2011).

Further, GSH is known to form stable GSH–Cu(I) complexes, which could explain the complete protection afforded by GSH against the effects of Cu. It has been demonstrated that Cu exposure induces oxidative stress in the tissue, as indicated by the increased accumulation of lipid peroxidation products (Bagchi et al. 1997, Correia et al. 2003).

Our findings are in line with previous research, for example, Jing et al. (2006) who demonstrated that the activity of Se-GPx for the species *Pinctada fucata* was sensitive to acute exposures of Cu. In bivalve molluscs *Tapes philippinarum* and *Macoma balthica* (L.), Cu and Cd ions change significantly the activities of some glutathione-dependent and antioxidant enzymes (Regoly et al. 1998). Further, the enzyme GPX was found to be sensitive to Pb and Cd exposures in previous studies (Congiu et al. 2000, Mateo et al. 2003). Liu et al.(2014) revealed that Pb could cause oxidative stress by generating ROS and inhibiting antioxidant enzyme activities in crabs. Moreover, Kim et al. (2017) found that antioxidant enzymes (CAT and SOD) were sensitive to 24h-Hg and 48h-Pb exposures in *D. magna*. Our results are also in line with previous data where it has been demonstrated that neonates and juveniles are the most sensitive life stages in acute toxicity bioassays than older organisms (Hoang and Klaine 2007, Muyssen and Janssen 2007).

A number of studies have reported the response of antioxidant enzymes to mixtures of metals. For example, the mixture of Cu and Cr caused a more pronounced effect on GST in *Carcinus maenas* than individual metals (Elumalai et al. 2002). The study of Tang et al. (2011) found Cu and Cd had greater toxicity effect than Penta-BDE on the activity of antioxidant enzymes in *D. magna* over 48h. Also, the responses of SOD and CAT activities were less than additive in binary metal treatments, while GST exhibited an additive response in the same treatments. Further, differences in antioxidant responses has found across *M. galloprovincialis* organs exposed to mixtures of benzo (a) pyrene and Cu. Also, interaction and no interaction effects were observed in the digestive gland across all the binary mixtures of both chemicals (Maria and Bebianno 2011).

This is the first evaluation of the effect of two stressors, Cu and Cd, on digestive and antioxidant enzymes in *D. pulex*. Our data show reduction in digestive and antioxidant enzymes via exposed to Cu and Cd. Reductions in digestive enzyme activity may lead to altered growth and reproduction in this critical species to freshwater ecosystem function. Reduced oxidative stress activity by these metals may increase *D. pulex* sensitivity to other stressors as well.

Because the response of biomarkers to one metal varied by the other metal and by exposure time, our data suggest that more studies need to work with mixtures so that we me assess the generality of multiple stressor response patterns. These assays remain a good standard for rapid toxicity, but their value will only increase with an understanding of this generality and further links to population growth and whether they increase sensitivity to other stressors. Enzymatic responses are a reliable, sensitive and informative tool in detecting initial damages caused by multiple stressors (Cu and Cd), provide insight into mechanisms and thus important to toxicant risk assessment and water quality management.

**Chapter 5**

**The microbiome mediates an interaction between natural and anthropogenic stress on life history, morphology and condition**

**5.1. Introduction**

In freshwater ecosystems, organisms are exposed to a wide range of biotic and abiotic stressors including predation, bacterioplankton, metals, nutrition, pH and temperature ([Hecky](https://aslopubs.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Hecky%2C+R+E) and [Kilham](https://aslopubs.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Kilham%2C+P) 1988, Hunter and Pyle 2004, Long et al. 2004, Coors and De Meester 2008, Hahn et al. 2010, Dölling et al. 2016, Martins et al. 2017). Two major research agendas, centred on these multiple forms of stress, are whether life history, morphological and behaviour responses are shaped by interactions (additive vs. non-additive effects) between stressors and whether the response to these stressors is mediated by the microbiome of the target species.

Multi-stressor research has grown enormously in the past decades as our understanding of modes of action of stressors have allowed hypotheses and experiments to address whether stressors combine additively or interactively (Folt et al. 1999, Loureiro et al. 2010, [Altshuler](javascript:;) et al. 2011, Jansen et al. 2011). Among stressors in freshwater communities, heavy metals such as Cu and Cd are worthy of such attention because they are major contaminants that cause substantial changes to the fitness of aquatic organisms and ecosystem processes (Shuhaimi-Othman et al. 2010, Martins et al. 2017).

Ecotoxicological research on heavy metals routinely uses *Daphnia* spp. as a model test organism (McWilliam and Baird 2002, Khangarot and Rathore 2003, Sarma and Nandini 2006, Loureiro et al. 2010, Tang et al. 2011, Sadeq and Beckerman, 2019). Theory and data from the literature indicate that metal concentrations influence foraging and assimilation of nutritional resources which can lead to a reduction in reproduction, delayed maturity, and slower somatic growth rate. Feeding rate and hence digestive physiology are the main targets of metals and this influences the energy budget and resource allocation to growth and reproduction (Barata and Baird 2000, Bui et al. 2016). Furthermore, research on metal mixture toxicity suggest a combination of interactive and additive effects on feeding, digestive activities and other life history traits across a range of invertebrates including *Daphnia* (Barata and Baird 2000, Cooper et al. 2009, Sadeq and Beckerman,2019).

Predation is a major biological stress to *Daphnia* populations. Several decades of research have revealed a wide array of responses to predation risk in habitat use ([Dodson](https://setac.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Dodson%2C+Stanley+I) et al. 1995, De Meester and Cousyn 1997), foraging (Noonburg and Nisbet, 2005, Balseiro et al. 2007), life history (Schulz and Dabrowski 2001, Rose et al. 2001, Campero et al. 2007, Pestana et al. 2009, 2010, Beckerman et al. 2010) and morphology (Tollrian 1995, Hammill and Beckerman, 2010). In parallel to recent work on metals, much of predation risk research is framed around how risk of predation alters foraging, habitat use and life history and on the allocation of energy to growth versus reproduction. (Werner et al. 1983, Stoks and McPeek 2003, Benard 2004, Noonburg and Nisbet 2005, Pauwels et al. 2005, Beckerman et al. 2007)

While there is extensive research on anthropogenic stressors like metals, and on natural stressors like predation, research on their interactions remain rare. This is despite the clear potential for interaction via resource allocation: the central role of food availability, quality and assimilation in life history and allocation to predator defences suggests that any abiotic (e.g. metal) stress that interferes with digestive physiology may provide a pathway for interaction with predation risk.

*5.1.1. The relationship between stress responses and microbiota*

A focal point for this potential interaction between stressors is thus the gut. This raises the hypothesis that the microbiome may mediate interactions between stressors like metals and predation risk. Microbiome research is growing rapidly with evidence that gut microbiota may be affected by host diet (Bolnick et al. 2014, Gorokhova et al. 2015), nutrients in the gut (Laparra and Sanz 2010) and indirectly by host physiology (Hooper et al. 2012, Nicholson et al. 2012), foraging success and body condition (Bolnick and Lau 2008). In both plants and animals, the microbial community in a gut or on an organism’s surface may play a role in mediating the response to various stressors like disease, resource stress, or predation.

The *Daphnia* gut and body surface is colonized by a wide range of symbiotic bacteria. They obtain these microbes via the transmission from host parents to offspring (Salem et al. 2015) or acquisition from sediments of ponds and lakes that enter organism’s gut via filter feeding (Grossart et al. 2009,Mushegian et al. 2018). Under normal conditions and during filtration activity, *Daphnia* feed mainly on algae and bacteria from the surrounding water (Porter et al. 1983, Gillis et al. 2005). However, in the case of lack of food, they move towards the bottom of the water column ingesting sediments, which may also contain contaminants such as metals in addition to bacteria (Gillis et al. 2005). As a result, *Daphnia* offer a niche for selective microbes that provide benefits and services to their hosts and where the gut and exoskeleton of *Daphnia* provide suitable surfaces for bacterial attachment (Shapira 2016). Such symbioses are involved in a pathway, the “microbial loop”, where bacteria may transfer energy to zooplankton and higher levels via dissolving organic matter released from phytoplankton (Degans et al. 2002, Tang et al. 2009).

In this context, gut microbiota offer a variety of functions and physiological processes to their hosts associated with metabolism, development, fecundity immunity, and behaviour (Dattagupta et al. 2009, Nicholson et al. 2012, Sommer et al. 2013, Gorokhova et al. 2015, McKenney and Pamer 2015, Sampson and Mazmanian 2015, Sison-Mangus et al. 2015). Although gut bacteria may compete with the host’s resources, they also make resources available for the host (Tremaroli and Backhed 2012, Kamada et al. 2013) by supplying nutrients and/or producing certain enzymes such as lipids, amino acids and vitamins (Dillon and Dillon 2004, Peter and Sommaruga 2008). Considering the ubiquitous abundance of bacteria in the natural water, quantitative studies demonstrated that the bacterial abundance per unit of zooplankton body volume ranges from 107- 1011 cells/ ml (Tang 2005, Tang et al., 2010).

Bacterial communities may thus influence the potential interaction between metals and predation (Gorokhova et al. 2015). Here we combine these two research agendas, manipulating exposure of *D. pulex* to predation risk and the heavy metal Cu to assess their interaction and additionally manipulating exposure to antibiotics to alter the diversity of their gut microbiota. Our research focuses on the general question of whether the response to multiple stressors is mediated by the microbiome and specifically whether the effect of predation risk varied by the presence of a heavy metal, and whether this interaction or any of the effects of predation risk/metal were mediated by the microbiome. Our experiments are motivated by the potentially shared importance of digestive physiology on the response to predation risk and metals. In this context, we report on changes in microbiota diversity under control and stress treatments, with and without antibiotics. These data underpin our analyses of whether the effect of predation on traits varies by copper exposure and the revelation that many of the responses to multiple stressors are reversed when the microbiome is heavily manipulated.

**5.2. Materia and methods**

*5.2.1 Daphnia culturing*

The clone *D. pulex* (LD33) was collected from field populations in the UK and cultured in the Department of Animal and Plant Sciences, University of Sheffield. Stock cultures were acclimated in ASTM hard water under controlled conditions at a temperature of 20±2 ºC, photoperiod 16h light: 8h dark and light intensity 26 µmol m -2s-1. Prior to experiments, animals were acclimated to test media over 3 weeks as recommended in the OECD guideline. The cultures were maintained in 2L tanks with approximately 25 individuals of the *Daphnia* clone and fed every day with the green algae *C. vulgaris* fo*. viridis* (strain number: CAAP 211/12). The algal cultures were grown in Ebert medium (Ebert group, Zoologisches Institut Evolutionsbiologie, Switzerland) and kept on a table shaker in controlled room at 20±2 ºC under an 8 h dark16 h light photoperiod with 22 µmol m -2s-1.

*5.2.2. Experimental media*

Daphnids were exposed in a factorial experiment to copper, predator cues (*Chaoborus flavicans* extract) (kairomone) and antibiotics. The control medium and other treatment groups consisted of autoclaved ASTM water, 300µl marinure, and the green algae *C. vulgaris* at standard food level (2 x 10^5 cells/ml).

In the Cu treatments, a concentration of 5µg/l aqueous copper (II) chloride dihydrate (Fisher Scientific UK C/7920/48) was pipetted into the media (see chapters 2 and 3). For predation exposure, concentrated chemical cues were extracted using the procedure of Tollrian et al. 1995, Beckerman et al. 2010, Lind et al. 2015) and added to treatments medium at a concentration of 1µl / ml. Nominal and realised concentrations of Cu were strongly correlated (ICP-MS; r2 = 0.99, F = 1.91, p < 0.002)

The antibiotic treatments were comprised of ampicillin (Sigma-Aldrich Company Ltd A9393-25G) and kanamycin sulphate (Sigma Aldrich Company Ltd 60615-5G) delivered at 9.5µg and 4µg per 150ml.

*5.2.3. Experiment set up*

Neonates from a single *Daphnia* clone (LD33) <24 h old were assigned to two experiments: the life table experiment and the microbiome experiment. The two experiments were required because analysis of the microbiome requires destructive sampling before the life table assays are completed. Both groups were exposed to the same conditions and following treatments: Control, Cu, Predation, Cu-Predation, Antibiotics (AB), AB-Cu, AB-Predation and AB-Cu-Predation. The life table experiment followed 15 replicates in each treatment. The microbiome experiment followed an additional 30 replicates in each treatment.

*5.2.3.1. Life History Experiment (Chronic exposure)*

Experiments were initiated by transferring <24 h old neonates individually into six-well plates (10ml) until first clutch. The daphnids were fed daily, their media changed and photographed every day using a Canon camera (EOS 350D DSLR) placed onto a Leica MZ6 modular stereomicroscope (Leica Microsystems GmbH, Wetzler, Germany).

Using these photos and observations, we captured six life history variables in the life history experiment: clutch size, size at maturity, age at maturity, induction of neck teeth, somatic growth rate and lipid status. Age and size at maturity were estimated on adults on the day neonates first appear in their brood pouch. Size was estimated with image analysis (the head to the base of the carapace spine) using ImageJ (Hooper et al. 2006). Reproductive output (clutch size) was recorded by counting the number of eggs in the first clutch (Gliwicz and Boavida 1996). Somatic growth rate was calculated as ln (size at maturity/initial size) / (age at maturity (days). Lipids were counted daily and calculated as follow (sum of lipid droplets/exposure period) ([Gilbert](https://onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Gilbert%2C+John+J) 2004). The induction score was calculated based on a composite of pedestal size and spike number (Naraki et al. 2013).

*5.2.3.2. Microbiome Methods (Bacterial communities’ identification)*

Guts of 30 individuals were picked up from each treatment and transferred into petri dish to characterize the gut microbiota. Antibiotics were applied to embryos/neonates and guts were collected from adults (at first clutch). Thus, the guts were expected to have a microbiome acquired from living and feeding naturally after antibiotic exposure but under the control or experimental treatments. The guts were dissected under a stereo-microscope with sterilised needles (Fig. 5.1) and transferred into phosphate buffered saline buffer (PBS) and then to micro-centrifuge tubes (1.5ml). The guts from each treatment were pooled to ensure sufficient material for sequencing and as such represent an average microbiome community among 30 individuals in each treatment. Samples were frozen in liquid nitrogen and then stored in the freezer at -20 ºC for bacteria abundance and diversity analysis.

B

A

**Figure 5.1.** Dissected guts of *D. pulex* A) Un-stressed daphnids B) Stressed daphnids.

Samples of *D. pulex* guts were analysed using 16S rDNA sequencing (DNA sequencing of bacteria; RTL Genomics, Texas, USA). This technique is a well-established method for identifying taxonomy and phylogeny of bacteria (Woo et al. 2008). The analysis yielded numerous OTU (operational taxonomic unit) data (Woo et al. 2008). OTU were taxonomically classified by identifying sequences to the highest similarity among bacterial taxa (Pruesse et al. 2012).

*5.3. Statistical analysis*

Phenotype data were analysed using R software (R Core team 2013, version 3.5.1). We first analysed our data as a MANOVA testing whether the effect of predation risk on all phenotypic traits varied by Cu and then whether this interaction (or not) varied by microbiome treatment.MANOVA was then followed by univariate ANOVA for each individual trait. We used Type II sums of squares implemented in the Anova() function of the car package (Fox and Weisberg, 2011; version 3.0-2) for R to assess significance in the MANOVA and ANOVA models. We also report effect sizes (partial eta-squared) from the heplots package (Friendly 2007, version 1.3-5).

These microbiome data were further analysed using the phyloseq package for R (McMurdie and Holmes 2013). We specifically assessed the changes in bacterial diversity and community composition among treatments using hierarchical clustering and non-metric multidimensional scaling (NMDS).

**5.4. Results**

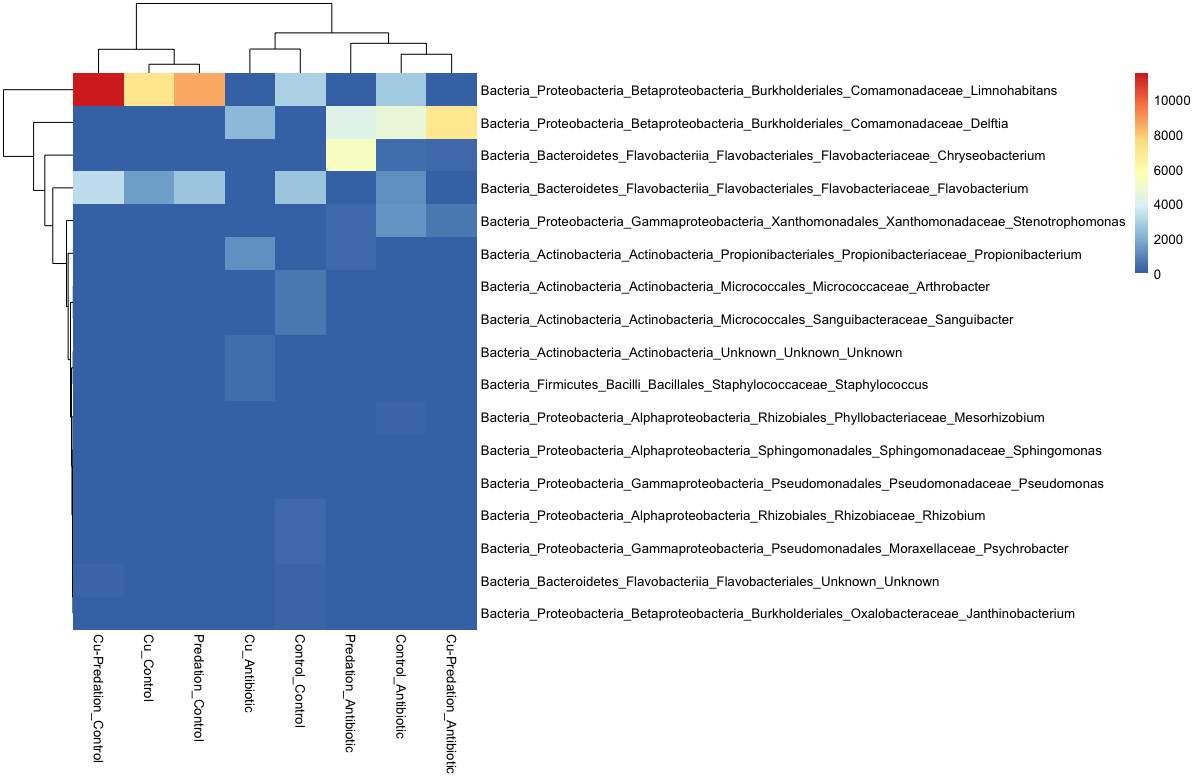
We first report on whether and how the microbiome was affected by the two stressors under control and antibiotic conditions. We then report on the effects of stress and antibiotic on life history, morphology and condition (lipids). Specifically we report on a set of interactive and additive effects of Cu and predation that are often reversed by manipulation of the microbiome.

*5.4.1. Microbiome outcomes*

*5.4.1.1. The diversity and composition of the bacterial communities in the digestive tract in response to stressors*

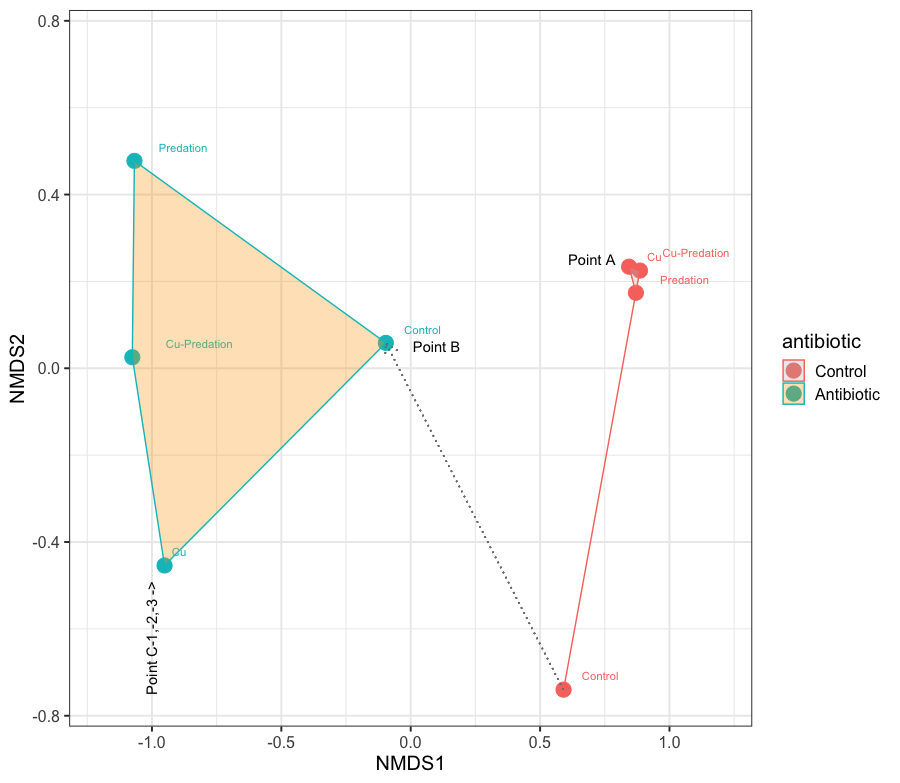
We detected ~10,000 unique OTUS among our samples. Specifically, we detected OTUs in Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes phyla, Betaproteobacteria, Flavobacteriia, Gammaproteobacteria, Actinobacteria, Bacilli and Alphaproteobacteria classes, Burkholderiales, Flavobacteriales, Xanthomonadales, Propionibacteriales, Micrococcales, Bacillales, Rhizobiales, Sphingomonadales and Pseudomonadales orders, Comamonadaceae, Flavobacteriaceae, Xanthomonadaceae, Propionibacteriaceae, Micrococcaceae, Sanguibacteraceae, Staphylococcaceae, Phyllobacteriaceae, Sphingomonadaceae, Pseudomonadaceae, Rhizobiaceae, Moraxellaceae and Oxalobacteraceae families and *Limnohabitans, Delftia, Chryseobacterium, Flavobacterium, Stenotrophomonas, Propionibacterium, Arthrobacter, Sanguibacter,**Staphylococcus, Mesorhizobium,**Sphingomonas, Pseudomonas, Rhizobium, Psychrobacter and Janthinobacterium* genera.

Hierarchical clustering of bacterial abundances (Fig. 5.2) revealed a diverse bacterial community in the gut of *D. pulex* that was influenced by the stressors/treatments. Across treatments, the dominant classes were Betaproteobacteria, Gammaproteobacteria, Alpaproteobacteria and Flavobacteria. The data suggest that antibiotics and our stressors alter the relative abundance of four key genera: *Limnohabitans, Delftia, Chryseobacterium* and *Flavobacterium. Limnohabitans* and *Flavobacterium* dominated the no antibiotic conditions, but these were replaced by *Delftia, Chryseobacterium* and *Stenotrophomonas* under antibiotic treatments (Fig 5.2).



**Figure 5.2.** Heatmap based on hierarchical clusturing of the bacterial community composition in different taxa (row labels; phylum, class, order, family and genus) associated with the *Daphnia pulex* gut in six treatments (column names; chronic exposure to different stress (ors). OTUs are classified using 16S rDNA gene sequences (~10000 reads/OTU).

We gained further insight into the community changes linked to predation, copper and antibiotic treatments via nonmetric-multidimensional scaling analysis (Fig. 5.3). First, under conditions with no antibiotics, copper, predation and their combination each shifts the microbiome to the same community structure (Fig 5.3, point A). Second, the addition of antibiotics shifts the control microbiome (Fig 5.3, point B). Finally, under antibiotic treatment, each stressor also shifts the community (Fig 5.3, points C 1,2,3). In contrast to their effects under no-antibiotic conditions, each stressor is here associated with a unique community under antibiotic treatments. These data show that our antibiotic treatments had substantial effects on the gut and that the response of the microbiome to stressors also varies by antibiotic treatments.



**Figure 5.3.** The bacterial diversity in *D. pulex* gut in response to two stressors, Cu and predation and to antibiotic treatment. The data are analysed via NMDS using bray-curtis dissimilarity. The red dots represent the control treatments (without antibiotics), while the blue dots are the treatments under antibiotics. Point A design representes the community under copper, predation or combined stress, but in the absence of antibiotics. Point B represents the shift in the control community in the absence of antibiotics to their presence (grey dotted arrow). Points C-1, -2, and -3 indciate the community under copper, predation or mixed stressors, but after the antibiotic treatments.

*5.4.2. Life history*

We analysed the trait response data using a combination of MANOVA to evaluate the overall response of the phenotype comprised of life history, morphology and condition traits, and univariate ANOVAs. For all analyses, we report first whether the three-way interaction among stressors (predation, Cu and antibiotics) was significant. If this was not significant, we then report on significant two-way interactions.

*5.4.2.1. Whole phenotype (MANOVA)*

The effect of predation risk on the phenotype comprised of the six traits we measured varied by the presence of copper, and this interaction varied by the antibiotic treatment (3-way interaction; Pillai’s trace = 0.156, approximate F = 2.84, p = 0.013). Against the background that the microbiome community shifted dramatically with treatments, this suggests a strong association between how organisms respond to multiple stressors and their gut bacteria community.

*5.4.2.2. Size at maturity*

There was no evidence of a three-way interaction (Cu: Predation:Antibiotics interaction; F = 1.4, df = 1, p = 0.25; Fig. 5.4a). We found that the effect of Predation on size at maturity varied by antibiotics (Predation:Antibiotics interaction; F = 14.3, df = 1, p<0.0002) where Predation reduced body size for the bacteria-free daphnids, but increased body size under antibiotics. Furthermore, Cu had significant effect on body size which varied by antibiotic treatments (Cu:Antibiotics interaction; F = 56, df = 1, p<0.0003). Cu reduced body size in control treatment, but increased the size under antibiotic treatments.

*5.4.2.3. Age at maturity*

We found no evidence of a three-way interaction (Cu:Predation:Antibiotics interaction; F = 2.7, df = 1, p = 0.1; Fig. 5.4b). Cu had significant effect on maturity age and this varied by antibiotics (Cu:Antibiotics interaction; F = 49, df = 1, p<0.0003) where maturation happened later as Cu increased under control, but earlier under antibiotic conditions. The effect of Cu on age varied by predation (Cu:Predation interaction; F = 40, df = 1, p<0.0008) where maturation happened later as Cu increased under control (no predation and antiobiotics), but earlier under predation treatments.

*5.4.2.4. Growth*

The results indicated that the impact of predation on somatic growth rate did vary by Cu and this interaction varied by antibiotic exposures (Cu:Predation:Antibiotics interaction; F = 8.5, df = 1, p<0.004; Fig. 5.4c). Cu reduced somatic growth rate under control, but increased growth under antibiotics. The reduction without antibiotics was much weaker under predator conditions and the increase under antibiotics was much stronger under predator conditions.

*5.4.2.5. Clutch size*

There was no evidence of a three-way interaction (Cu: Predation:Antibiotics interaction; F = 2.4, df = 1, p = 0.12; Fig. 5.4d). However, we found that the effect Cu on clutch size varied by antibiotics (Cu:Antibiotics interaction; F = 12.2, df = 1, p<0.0007) where Cu reduced number of eggs in the absence of antibiotics and increased them in their presence.

*5.4.2.6. Induced defence*

We found that the effect of predation on neck-teeth production varied by Cu and this interaction varied by antibiotics (Cu: Predation:Antibiotics interaction; F = 7.4, df = 1, p<0.008; Fig. 5.4e). Cu reduced neck-teeth production under control, but increased spike production under antibiotics.

*5.4.2.7. Lipid status*

There was no evidence for a three-way interaction (Cu: Predation:Antibiotics interaction; F = 0.6, df = 1, p = 0.5; Fig. 5.4f). We found that the effect of predation on lipid droplets varied by antibiotic exposures (Predation:Antibiotics interaction; F = 9.2, df = 1, p<0.003). Predation risk reduced the number of lipid droplets under control conditions, but had no effect under antibiotics where condition was on average lower than control-control conditions.



**Figure 5.4.** Interaction plot of the effect of multi-stressors: Copper, predation and antibiotics on *D. pulex* life history traits as following (in order) size at maturity, age at maturity, somatic growth rate, clutch size, induction and lipid. Mean SE, n=15.

**5.5. Discussion**

Bacterial communities are ecologically fundamental components in the aquatic food web that play a significant role in biochemical cycles, transfer of nutrients to higher trophic levels and mediation of a range of functions and physiological processes in their hosts when comprising their microbiota (Falkowski et al. 2008, Gorokhova et al. 2015). However, the relationship between microbiota and their potential to mediate how multiple environmental stressors affect organisms remains understudied. Here we focused on exploring how manipulations of the gut microbiome of *Daphnia*, a keystone species in aquatic communities, influences the response of life history, morphology and body condition to combined anthropogenic (metal) and natural (predation) stress. Our work thus centres on both the interactive effects of multiple simultaneous stressors (predation and metals) and on the potential role that the microbiome plays in mediating the effects of the multiple stressors. Evaluation of the interaction among natural and anthropogenic stressors, and the implications of this for species and ecosystem functioning, is a core challenge for environmental risk assessment.

Our experiments were motivated by the idea that the responses of *D.pulex* to predation and Cu are both mediated in part by digestive physiology. Under this assumption, then predicted interactions between these stressors and predicted that alterations to the microbiome would impact on these interactions. Our data reveal a significant effect of antibiotics on the relative abundance of bacterial taxa in the *D. pulex* microbiome. In parallel, our data reveal that these changes in the microbiota are associated with numerous examples where the interactive effects of predators and Cu are reversed. The gut microbiome is clearly linked to the maintenance of adaptive responses to stress.

In the following sections we first review the effects of Cu on predator induced changes under no-antibiotic conditions (no manipulation of the microbiome). Then we discuss in more detail how these patterns changed and often reversed under manipulation of the gut microbiome. Finally, we discuss potential functional links between the substantial change in the abundance of the four genera of bacteria, feeding/digestive physiology and our life history, morphology and condition traits.

*5.5.1. Copper–predation interactions (No antibiotics)*

The left-hand panels of Fig. 4 provide substantial insight into whether and how the natural predation risk stress and the anthropogenic Cu metal stress interact among six traits. In order to make these patterns clearer and facilitate comparison with other similar research, we re-analysed these data on their own.

Overall, the effect of predation on the phenotype under control conditions varied by Cu (MANOVA, Pillai’s Trace = 0.56, approximate F = 7.9, p <0.001). More specifically (all described patterns p<0.05), Cu reduced reproductive output (clutch size), but there was no effect of predation. Predation risk reduced body condition, but there was no effect of Cu. Predation risk and Cu both reduced size at maturity, but their effects were additive. The positive effects of predation on age at maturity, somatic growth rate and induction of morphological defence all varied by Cu. Cu increased age at maturity in the absence of predation, but because age was later under predation risk without copper, there was no Cu effect under predation risk. Cu reduced somatic growth from a much higher point in the absence of predation risk. Finally, Cu reduced the morphological defence induced by predation risk.

These results demonstrate that the effects of predation risk on several life history traits varies by the presence of copper. The additive and interactive patterns in life history traits are predictable from life history theory associated with resource limitation and predation risk by small size-selective predators experienced by *D. pulex*. Under resource limitation, life history theory predicts later maturity, decreased somatic growth rates, smaller size at maturity and smaller clutches. In contrast predation risk research and theory predicts that under threats from small size-selective predators, individuals mature later, but at the same or larger sizes, experience faster juvenile somatic growth and have the same or larger clutches (Beckerman et al 2010).

Some of our results, in the absence of antibiotics, are also in line with the findings of several other groups. For example, Pyle and colleagues (2004, 2009) have investigated the interaction between predation risk and Cu in several *Daphnia* species and for several traits. In their 2004 work, they found that concentrations of Cu and Ni had inhibitory impacts on neck tooth induction in *D. pulex* (more clearly in Cu). In their 2009 work, they showed that Cu and predation had morphological changes in *D. pulex* neonates leading to fewer and shorter neckteeth compared to kairomone treatments alone. DeMille et al. (2016) further revealed that clones of *D. pulicaria* collected from lakes representing a gradient of Cu concentrations exhibited different responses to *Chaoborus* kairomones in the presence of Cu. This work suggests that acclimation or adaptation to local metal concentrations is linked to changing capacity to respond to predation risk. Whilst, working with sodium chloride (NaCl) instead of metals, Robison et al. (2018) found that the metabolic rate and toxicity of NaCl to *D. pulex* were decreased in the presence of predation cues.

Much of this work remains at the scale of responses, and does not evaluate how metals (or salts) might interfere with predator induced morphological and life history. While, we, Pyle and colleagues (2004, 2007, 2016) suggest that because Cu interferes with digestive physiology it may impact on induced defences by altering the pathways for allocating energy to defences, we extended this conjecture to invoke the microbiome. We now review in more detail the results from our manipulation of the microbiome.

*5.5.2. Microbiome mediation*

Our experiments generated four distinct patterns in the bacterial microbiome community (Fig 5.3). First, in the absence of any antibiotic treatment, Cu, predation risk or their combination shifted the community to the same location (Point A). Second, our antibiotic treatment shifted the community of control organisms to a new community (Point B). Third, Cu and predation risk each generated unique communities from this antibiotic altered community (Point C1,2). Finally, the Cu-predation risk community under the antibiotic treatment was intermediate to the Cu and predation risk communities (Point C3).

Figure 5.2 and 5.3 combine to highlight the change in relative abundance of bacteria taxa among the treatments. Our data show that *Limnohabitans* and *Flavobacterium* dominated the control conditions, but these were replaced by *Delftia, Chryseobacterium* and *Stenotrophomonas* under antibiotic treatments. Given our experimental design, the new gut communities under antibiotic treatment are acquired throughout development via feeding from the existing bacterial community in the water column. The most striking pattern in the data are that under no antibiotic conditions, where parents and offspring experience the same bacterial community, either, and both, forms of stress generate a change in community, but the same change. This suggests, that against the background, natural microbial community, these natural and anthropogenic stressors act generically to alter gut biota in a similar way. We are, however, unable to assess whether these changes are caused by stress induced alteration of the gut flora, stress induced limited uptake of the bacteria from the community or stress induced change in the external bacterial community. The latter is unlikely, however, as under antibiotic treatment, the subsequent adults contain a vastly different community depending on the stressors. This suggests that stressors either alter the gut community or the uptake, but only when the community starting point is first altered by antibiotics in the first place.

A striking result of our antibiotic treatments and associated change in gut bacterial community are the several instances where the pattern under no antibiotics is reversed under antibiotic treatments. For example, not only did antibiotics increase age at maturity but the effect of copper on age shifted from positive to flat under no-predator conditions and from flat to negative under predation conditions (Fig. 5.4). Antibiotic treatment also shifted the negative effect of copper on somatic growth and reproduction to a positive one. Finally, the negative effect of copper on induced morphological defences under predation risk with un-manipulated microbiota was reversed when the gut flora had been manipulated.

In the absence of information on the chemical, functional and nutritional properties of all bacteria genera that comprise the change in microbiota community structure, we are unable to ascribe causal inference to these patterns. However, it is clear that the community composition of *Daphnia* guts is deeply connected to their life history, to morphological defences and to body condition.

Our results complement evidence focused on life history and the role of the microbiome in the absence of other stressors. This work suggests a functional relationship between *Limnohabitans sp* (Betaproteobacteria), who’s relative relative abundance was dramatically altered in our experiments and multiple traits. Qi et al. (2009) found that re-infection of aposymbiotic *D. pulex* to *Limnohabitans sp*., the dominant bacterial species in *D. pulex,* led to elevated reproduction. Peerakietkhajorn et al. (2015) found that *Limnohabitans* sp. play an essential role in conferring fecundity by showing that bacteria-free *Daphnia* recovers fecundity when inoculated with *Limnohabitans* sp. However, host-microbiota interactions within *D. magna* are known to be highly specific, e.g., only certain strains of the genus *Limnohabitans* are able to recover the fitness of germ-free individuals after re-inoculation.

More generally, several studies show that microbial communities can sometimes, but not always increase fecundity, growth and population size of aposymbiotic *Daphnia magna* (Sison-Mangus et al. 2015, Peerakietkhajorn et al. 2015, Callens et al. 2016). In relation to our energetics driven hypothesis, short-term exposure to the antibiotic trimethoprim can negatively affect host growth by decreasing the digestion and incorporation of food (Gorokhova et al. 2015). Taipale et al. (2012) found a positive correlation between the proportions of the bacterial fatty acids in the *Daphnia* lipids (fed on bacteria–phytoplankton).

Overall, these data support our hypothesis and data which indicate a substantial influence of the microbiota in *D. pulex* on their response to multiple simultaneous stressors. This study is the first to combine a study of interactions between natural and anthropogenic stress with manipulation of the gut microbiota. Our data highlight stressor interactions, stress mediated changes in microbiota and microbiota linked changes and even reversals of stressor interactions.

**Chapter 6**

**General discussion**

**6.1. Synopsis of the thesis and summary of the chapters**

Organisms face an array of anthropogenic and natural stress. Understanding how multiple forms of stress impact on organism physiology and life history is important for preserving biodiversity and maintaining ecosystem function. In this thesis, I focused on interactions between multiple anthropogenic stressors and between anthropogenic and natural stressors on a keystone grazer in freshwater communities, *D. pulex*. My investigations ranged from examining effects and interactions between the heavy metals copper (Cu) and cadmium (Cd), which remain pollutants of many freshwater communities, interactions between Cu and predation risk. I used a range of methods, including traditional life table analyses, assessment of traits linked to survival and reproduction, to assays of digestive physiology, and ultimately manipulations of gut microbiomes. My data revealed interactions between Cu and Cd, and Cu and predation at multiple scales of biology with responses in life history, behaviour, body condition and digestive physiology.

In **Chapter 1** (Literature review), I introduced metal pollution as an ongoing issue in freshwater ecosystems, specifically focusing on two common stressors: Cu and Cd. I review the literature on the mode of action of these metals with specific attention reference to digestive physiology and to life history. I introduce the issues with anthropogenic and natural stress and ideas about additive vs. non-additive (interactions; antagonistic and synergistic) effects. I also introduce *Daphnia* spp. as central to research on freshwater stressors and specifically *D. pulex* as my study organism.

In **Chapter 2,** I performed the first quantitative, meta-analytic review of Cu and Cd effects on *Daphnia* spp. This work focused on the mode of action of these metals with specific reference to digestive physiology and life history. I highlighted the importance of species-specificity in response to metals and other experimental conditions (e.g. water hardness). I assessed the life history trait responses in different species of Cladocera including their reproduction, age at maturity and somatic growth rate across sub-lethal concentrations of Cu and Cd. These data revealed highly variable, species specific susceptibility and response to metals across traits and experimental conditions, in particular in reproduction. The work sheds light on the risks of focusing on one species (e.g. *D. magna* is the most commonly used test organism) and on the number of studies failing to provide sufficient detail (sample sizes, standard deviations) to be included in systematic, quantitative reviews.

In **Chapter 3**, I focused on metals occurring as mixtures and more specifically on whether metals interact to shape the life history and behaviour responses in *D. pulex*. I employed a response-surface experimental design to assess three levels of Cu and Cd and their combinations on three genotypes of *D. pulex* in a classic 21 day life table experiment, and under two food levels. I assessed whether Cu and Cd interacted, and whether Cu or Cd interacted with food levels to shape patterns of ingestion rate, reproduction, maturation time, size at maturity and somatic growth rate. The data revealed numerous instances of non-additive metal-metal interactions (the effects of one vary by the other).

To further investigate the mode of action associated with these sub-lethal effects detailed in Chapter 3, in **Chapter 4**, I explored the effects of Cu, Cd and their combination on three digestive and three antioxidant enzymes. These enzymatic assays represent a potential biomarker/early warning of metal stress that do not rely on 21-day life table experiments. We found, as with the life history, numerous instances of synergistic interactions of Cu and Cd on digestive and antioxidant enzymes.

For my last piece of research, **Chapter 5**, I examined the interaction between the anthropogenic stress of Cu and the natural stress of predation risk. Several studies had hinted at an interaction between metals and predation cues, and I expanded on this work by examining morphology, life history and body condition. I also manipulated the microbiome of the *D. pulex* to test the hypothesis that the response to each and to multiple stressors was influenced by the gut bacterial community. The data revealed multiple instances of non-additive, interactive effects of Cu and predation risk. The data also revealed that Cu, predation or their combination shift the microbiota community in the same way. However, early life manipulation of the gut microbiota by antibiotics resulted in stress specific communities re-colonising the gut. Furthermore, after antibiotic treatment and acquisition of these unique communities, several effects of Cu and predation on life history, morphology and body condition were reversed. This work adds to the growing knowledge of how important gut bacteria are to the life history of organisms facing multiple stressors.

**6.2. Meta-analysis on the chronic toxicity of Cu and Cd**

In the eco-toxicological literature, via field and laboratory experiments, life history-associated stress has been widely used to assess the effects of contaminants on biota including *Daphnia* (Johnston and Roberts 2009, O'Brien and Keough 2014). Traits that are commonly assessed include reproduction, time to first reproduction, size at maturity, somatic growth rate and population growth rate (Chandini 1989, Koivisto et al. 1992, Khangarot and Rathore 2003, De Schamphelaere and Janssen 2004, De Schamphelaere et al. 2007, Wang et al. 2009, Kim et al. 2017). Data from experiments and life history theory suggested that increased metal concentrations, foraging, and assimilation of energy may decrease, leading to reduced reproduction, delayed maturity, and slower somatic growth rate.

In this chapter, I used meta-analytic methods to quantitatively review responses to Cu and Cd in Cladocera species. I examined more than 200 papers, reporting on variation in species identity (resistant or sensitive) and specific lab conditions that may generate variation in the responses to metals. Effect sizes are the core to a meta-analysis (Osenberg et al. 1997, Blanar et al. 2009). Due to lack of information on mean, standard deviation, or standard error and sample sizes, among these 200+ papers, only 32 experimental studies could be included.

Despite this, I found that the Cladocera species chronically exposed to Cu and Cd, varied in their susceptibility to metals exhibiting different responses, in particular in reproduction. This was expected because organisms may have heterogeneous tolerances (sensitivity to toxicity) to metals such as Cu (Millward and Grant 2000), and Cd (Knapen et al. 2004). Furthermore, it has been suggested that the responses to sub-lethal concentrations of metals may be affected by species identity (e.g., interspecific variation) and genotype (e.g., intraspecific variation) (Barata et al. 2000, Hoang and Klaine 2007). Despite these suggestions, there had never been a systematic review of the effects of Cu and Cd among multiple species. Two critical messages that emerge from this work are that *D. magna*, the most commonly used test species, is not the most responsive to metal pollution and that reporting of sample size and variation from these studies has been historically too poor to allow systematic, quantitative assessment of generality of Cu and Cd effects.

**6.3. Evaluating additive vs. interactive effects of Cu and Cd**

The effect of multiple, simultaneous stressors remains an important research topic because the interaction among stressors and the relationship with environmental factors may generate vastly different outcomes to single stressor responses. This is best contextualised by recognising that stressors have a mode of action and as such, they might add up, multiply (synergy) or antagonise each other (De Zwart and Posthuma 2005). These interactions remain a core topic in ecotoxicology especially as most investigations and hypotheses have been restricted to single toxin evaluation. Despite the impact and continuing importance of heavy metal pollution in freshwater community, the number of studies investigating mixtures is limited. In this chapter,

I demonstrated that five traits are affected in non-additive ways by mixtures of stressors, genotypes and resource levels. The combined stressors are likely to yield ecological surprises in real ecosystems. A review on multiple environmental stressors, Crain et al. (2008) suggested that interaction types (synergistic, antagonistic or additive) vary with the specific stressor combination, response level (population vs. community), and the trophic level (herbivores vs. predators).

The current knowledge for the assessment of mixture toxicity based on two traditional models: the concentration addition (CA) versus independent action (IA) models (Barata et al. 2006). However, due to differences in Cu and Cd mechanisms, we employed a response surface experiment and analysis, manipulating exposure to single and combined gradients of each metal. Despite the numerous forms of interactions are revealed by non-linearities in the surfaces. Our use of the response surface approach does not depend on mechanistic assumptions (IA vs. CA), but is independent of the shapes of the dose-response curves so that they might apply for both CA and IA models of additivity.

**6.4. Biomarker responses in *D. pulex* exposed to Cu and Cd**

The sub-lethal toxicity of metals is hypothesized to operate via impacts on physiological processes. It has been suggested that the Cu and Cd modes of action operate via digestive physiology and feeding behaviour in Cladocera which arise from direct impact of metals on metabolism. This would influence energy production and consequently resource allocation for growth and reproduction (Barata and Baird 2000, Bui et al. 2016). The sub-lethal exposure of metals induces oxidative stress by generation of reactive oxygen species (Barata et al. 2005, Sohal et al. 2000, Jemec et al. 2007). It has been suggested that the production of ROS, localized particularly around mitochondria (the major site of oxygen consumption and ROS production), resulting in lipid peroxidation, protein oxidation, and nucleic acid damage.

In this chapter, I estimated experimentally the responses of digestive and antioxidant enzymes to sub-lethal exposures of Cu and Cd. The outcomes indicated that the activity of digestive and antioxidant enzymes declined nonlinearly with increasing concentrations of Cu and Cd. However, our findings showed variation in enzyme responses to metals and specifically interactions between metals, and metals and exposure periods. For trypsin and esterase, the effects of Cu on enzyme activity varied by Cd concentrations, but it did not for amylase. We also found that the effects of the metals vary by experiment duration trypsin and amylase (Cd only), but it did not for esterase. We also found that the effect of Cu on all antioxidant enzymes activity varied by Cd. We also found that for only SOD, the effects of metals varied by exposure periods.

The variation in digestive enzyme responses is linked to the differences in metabolic rates associated with food supply and to malfunctions in food assimilation in presence of metal ions and associated digestion process efficiency (Bohrer and Lampert 1988, Golovanova 2008). Such responses of digestive enzymes linked to metal exposure have been documented in a number of invertebrate species including *Daphnia* (Biesiot and Capuzzo 1990, Yan et al. 1996, De coen and Janssen 1997,1998).

As a results of metal exposure, antioxidant defences in *Daphnia* were also affected due to oxidative damage causing from high level of ROS (Barata et al. 2005). One of the detoxidative mechanisms in exposed organisms is that maintaining the balance between production and removal of ROS. *Daphnia* spp. possesses a number of antioxidant enzymes, CAT, SOD and GPX, which are considered the first line of defence against metal stress (Barata et al. 2005). Moreover, alteration in physiology may lead to changes in antioxidant status. This has been tested in the enzymes CAT, SOD and GPX of *D. magna.* (Barata et al. 2005). Under metal stress, oxyradicals as a result of lipid peroxidation can impair cellular function and cause alterations in physicochemical properties of cell membranes, which hence disrupt vital functions (Rikans and Hornbrook 1997).

The study of Barata et al. (2005) found that SOD activity was significantly induced by Cd and marginally increased by Cu. Across exposure levels of Cu, SOD activity did not show a clear pattern. Jemec et al. (2007) reported the inability of endosulfan to increase CAT activities and its ability to induce GPX in the fish *Channa punctatus*. The study of Halliwell and Gutteridge (1999) indicated that enhanced levels of total-GPX activity found in *Daphnia* juveniles exposed to high endosulfan levels may be related to the enzymatic detoxication of hydroperoxides produced by increased levels of hydroxyl radicals. It has been demonstrated that the enzymes SOD and GPX play an important antioxidant role against ROS in many invertebrate species (Halliwell and Gutteridge 1999, Doyotte et al. 1997). These enzymes are crucial due to their role in metabolising O2 by SOD, and organic hydroperoxides by GPX.

Furthermore, it has been suggested that the low levels of GST under Cu and Cd exposures induced compared to endosulfan and menadione may be due to an excess of ROS and hence of lipid peroxidation levels (Barata et al. 2005). The potential for Cu to induce oxidative tissue damage relative to Cd may be related to the redox cycling properties of Cu (Stohs and Bagghi 1995). Livingstone et al. (1992) results suggested that toxicants may induce different antioxidant/ prooxidant responses depending on their ability to produce ROS and of antioxidant enzymes to detoxify them. Since environmental stressors generate toxic impacts related to oxidative stress, this may be important for further biomarker development.

**6.5. The microbiome of *D. pulex* in response to stressors**

Intestinal microorganisms play an important role in shaping host’s responses to stressors by contributing in the digestion and nutrition by degradation and transformation of complex biopolymers. They may produce methane and nitrous oxide as side products (Dillon and Dillon 2004), but may also improve the host’s resistance to pathogens and infections (Bolnick and Lau 2008). However, the research on the composition and stability of microbiome communities in cladocerans and their responses in presence of stressors are still limited.

I investigated the interaction between multiple stressors (Cu and predation risk cues) on *Daphnia* morphology, life history and body condition and whether the gut microbiota might mediate these interactions. I manipulated the gut microbiota at birth using antibiotics and then explored *D. pulex* traits and how the composition of the re-colonised microbiome was affected by these stressors.

The results clearly showed that stressors had strong, interactive effects on the responses of *D. pulex* traits that antibiotic treatments early in life dramatically influence the bacterial diversity and communities in the gut, and that such changes in the gut are associated with reversal of many patterns found under natural gut communities.

Our data are in line with the study ofGorokhova et al. (2015) where antibiotic treatments caused a strong decrease in feeding activity, growth, digestion efficiency and carbon uptake in *D. magna*.Antibiotic treatment also has an effect on growth rates (Callens et al. 2018). Further, Berga et al. (2015) suggested that reduction in the bacterial diversity in *Daphnia magna* may be caused by the presence of competitors, such as heterogeneous nanoflagellates.

One of the most interesting results of my experiment was that in the absence of antibiotics, any stress exposure (Cu, predation, Cu+predation) cause the community to shift to the same new community (Fig 5.3, Chapter 5). However, not only did antibiotic treatment early in life shift the community of control organisms, the community acquired by the *Daphnia* was unique to the type of stress after exposure – Cu, predation and the mixture all have different communities after initial treatment. These unique communities are associated with often dramatic reversals of pattern in life history, morphology and body condition responses to Cu and predation.

Previous work shows that the microbiota assembly mechanisms are strongly affected when germ-free *Daphnia* are inoculated with microbiota (Callens et al. 2018). The increased abundance of specific taxa could furthermore benefit the host if these are better suited for providing a specific service. Callens et al. (2018) also demonstrated that Oxytetracycline exposure had a strong effect on the abundance of *Neisseriaceae* sp. growth. The occurrence of *Neisseriaceae* sp. was either strongly reduced or completely removed in inocula exposed to higher concentrations of oxytetracycline. This reduction resulted in either the establishment of a more diverse community on *Daphnia* or allowed other taxa to dominate the microbiota in the absence of *Neisseriaceae* sp. It has also been observed large differences in microbiota community composition between inocula not exposed to oxytetracycline and the *Daphnia* colonized by these inocula.

In our study, *Limnohabitans* sp. was found to be the dominant species across treatments under control conditions. Our double-antibiotic treatment clearly altered community assembly through development where *Delftia, Chryseobacterium* and *Stenotrophomonas* substantially increased relative to *Limnohabitans* (see Figure 5.2 from Chapter 5). While, antibiotics and stressors alter the relative abundance by prevailing the following genera: *Limnohabitans, Delftia, Chryseobacterium* and *Flavobacterium.*

**6.6. Conclusions and recommendations**

The research I have made highlights the importance of species and genotype responses in the assessing of natural population responses to stressors (anthropogenic and natural). Our data are the first attempt on species specificity evaluation to chronic effects of Cu and Cd across Cladocera species and the first application of the taxonomical and functions of *Daphnia* gut microbiome. Via life history and biomarker assays, we demonstrated a great variation in species and genotypes responses at multiple biological scales for multiple forms of stress*.* Future research must focus on interactions and sources of variation, including the concentration (acute vs. chronic), organisation level (individual, community, population) and type (essential vs. non-essential) of metals.

Enzyme assays bring much insight into the mechanism of stressors and how interactive effects may arise. Assessments on other metals and/or environmental stressors using ecologically-relevant organisms are recommended to further understanding of the behaviour and mechanistic toxicity of contaminants.

Experiments on gut microbiota are valuable to obtain information on their role in mediating interactions among stressors. The microbiota can be a crucial factor in determining *Daphnia* responses to alterations in environmental circumstances. Hence, further research is needed on the response of host–microbiota interactions to other environmental stressors in order to accurately quantify the functional role of microbiota for *Daphnia.*

**References**

Abrams, P. A. and Rowe, L. (1996). The effects of predation on the age and size of maturity of prey. *Evolution* 50, pp. 1052– 1061.

Ackerman, C.M., Lee S. and Chang C.J. (2017). Analytical methods for imaging metals in biology: From transition metal metabolism to transition metal signalling. *Analytical Chemistry*, 89, pp. 22­-41.

Agra, A.R, Soares, A.M.V.M. and Barata, C. (2011). Life-history consequences of adaptation to pollution. *Daphnia longispina* clones historically exposed to copper. *Ecotoxicology,* 20, pp. 552-562.

Alberto, A.C., Rocío, O.B. and Fernando, M.J. (2011). Age effect on the antioxidant activity of Daphnia magna (Anomopoda: Daphniidae): does younger mean more sensitivity? *Journal of Environmental Biology*, 32(4), pp. 481–487.

Aljaibachi, R. and Callaghan, A. (2018). Impact of polystyrene microplastics on *Daphnia magna* mortality and reproduction in relation to food availability. *PeerJ 6*: e4601.

Allen, Y., Calow P., and Baird, D.J. (1995). A mechanistic model of contaminant-induced feeding Inhibition in *Daphnia magna*. *Environmental Toxicology and Chemistry,* 14, pp. 1625-1630.

Al-Reasi, H.A, Smith D.S, and Wood C.M. (2012). Evaluating the ameliorative effect of natural dissolved organic matter (DOM) quality on copper toxicity to *Daphnia magna*: Improving the BLM. *Ecotoxicology,* 21, pp. 524-537.

Altenburger, R., Backhaus, T., Boedeker, W., Faust M., and Scholze, M. (2013). Simplifying complexity: Mixture toxicity assessment in the last 20 years. *Environmental Toxicology and Chemistry,* 32, pp. 1685-1687.

Altshuler, I., Demiri, B., Xu, S., Constantin, A., Yan, N.D. and Cristescu, M.E. (2011). An integrated multi-disciplinary approach for studying multiple stressors in freshwater ecosystems: *Daphnia* as a model organism. *Integrative and Comparative Biology*, 51, pp. 623-633.

Amiard, J.C., Amiard-Triquet, C., Berthet, B., and Mktayer, C. (1987). Comparative study of the patterns of bioaccumulation of essential (Cu, Zn) and non- essential (Cd, Pb) trace metals in various estuarine and coastal organisms. *Journal of Experiental Marine Biology and Ecology*, 106, pp. 73-89.

Asselman, J., Glaholt, S.P., Smith, Z., Smagghe, G., Janssen, C.R., Colbourne, J.K., Shaw, J.R. and De Schamphelaere, K.A.C. (2012). Functional characterization of four metallothionein genes in Daphnia pulex exposed to environmental stressors. *Aquatic Toxicology*, 110–111, pp. 54–65.

[Atienzar](javascript:;), F.A., Cheung, V.V., Jha, A.N. and Depledge, M.H. (2001). Fitness parameters and DNA effects are sensitive indicators of copper-induced toxicity in *Daphnia magna*. *Toxicological Sciences,* 59, pp. 241-250.

Attayde, J.L. and Hansson, L.A. (1999). Effects of nutrient recycling by zooplankton and fish on phytoplankton communities. *Oecologia*, 121, pp. 47-54.

Bae, J.-S., and Freeman, H.S. (2007) Aquatic toxicity evaluation of copper complexed direct dyes to the *Daphnia magna*. *Dyes Pigment*, 73, pp. 126–132.

Bagchi, D., Bagchi, M., Balmoori, J., Ye, X. and Stohs, S.J. (1997). Comparative induction of oxidative stress in cultured J774A.1 macrophage cells by chromium picolinate and chromium nicotinate. *Research Communication in Moleclar Patholology and Pharmacology,* 97, pp. 335-346.

Baird, D.J, Barber, I. and Calow, P. (1990). Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. I. Chronic life history effects. *Functional Ecology*, 4, pp. 399-407.

Baird, D.J., Barber, I., Bradley, M., Soares, A.M.V.M. and Calow, P. (1991). A comparative study of genotype sensitivity to acute toxic stress using clones of *Daphnia magna* Straus. *Ecotoxicology and Environmental Safety*, 21, pp. 257–265.

Ball, S.L. and Baker, R.L. (1995). The nonlethal effects of predators and the influence of food availability on life-history of adult *Chironomus tentans* (Diptera, Chironomidae). *Freshwater Biology*, 34, pp. 1–12.

Balseiro, E., Modenutti, B., Queimaliños, C. and Reissig, M. (2007). Daphnia distribution in Andean Patagonian lakes: Effect of low food quality and fish predation. *Aquatic Ecology*, 41, pp. 599-609.

Barata, C. and Baird, D.J. (2000). Determining the ecotoxicological mode of action of chemicals from measurements made on individuals: Results from instar-based tests with *Daphnia magna* Straus. *Aquatic Toxicology*, 48, pp. 195–209.

Barata, C., Baird, D.J. and Markich, S.J. (1998). Influence of genetic and environmental factors on the tolerance of *Daphnia magna* Straus to essential and non-essential metals. *Aquatic Toxicology,* 42, pp. 115–137.

Barata, C., Baird, D.J and Markich, S.J. (1999). Comparing metal toxicity among *Daphnia magna* clones: An approach using concentration–time response surfaces. *Archives of Environmental Contamination and Toxicology*, 37, pp. 326–331.

Barata C., Baird, D.J., Miňarro, A. and Soares, A.M.V.M. (2000). Do genotype responses always converge from lethal to nonlethal toxicant exposure levels? Hypothesis tested using clones of *Daphnia magna* Straus. *Environmental Toxicology and Chemistry,* 19, pp. 2314–2322.

Barata, C., Baird, D.J., Soares, A.M.V.M. and Guilhermino, L. (2001). Biochemical factors contributing to response variation among resistant and sensitive clones of *Daphnia magna* straus exposed to ethyl parathion. *Ecotoxicology and Environmental Safety*, 49, pp. 155–163.

Barata, C., Markich, S.J., Baird, D.J. and Soares, A.M.V.M. (2002 a). The relative importance of water and food as cadmium sources to *Daphnia magna* Straus. *Aquatic Toxicology*, 61, pp. 143-154.

Barata, C., Baird, D.J., Medina, M., Albalat, A. and Soares, A.M.V.M. (2002 b). Determining the ecotoxicological mode of action of toxic chemicals in meiobenthic marine organisms: stage-specific short tests with *Tisbe battagliai*. *Marine Ecology Progress Series*, 230, pp. 183-194.

Barata, C., Porte, C., and Baird, D.J. (2004). Experimental designs to assess endocrine disrupting effects in invertebrates: A review. *Ecotoxicology*, 13, pp. 511-517.

Barata, C., Varob, I., Navarrob, J.C., Arunc, S. and Porte, C. (2005). Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. *Comparative Biochemistry and Physiology*, C (140), pp. 175-186.

Barata, C., Baird, D.J., Nogueira, A.J.A, Soares, A.M.V.M and Riva, M.C. (2006). Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. *Aquatic Toxicology*, 78, pp. 1–14.

Barber, I., D. J. Baird, and Calow, P. (1990). Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. II. Physiological effects. *Functional Ecology,* 4, pp. 409-414.

Barry, M.J., D.C. Logan, J.T. Ahokas, and Holdway, D.A. (1995). Effect of algal food concentration on toxicity of two agricultural pesticides to *Daphnia carinata*. *Ecotoxicology and Environmental Safety*, 32, pp. 273–279.

Beckerman, A.P., Wieski, K. and Baird, D.J. (2007). Behavioural versus physiological mediation of life history under predation risk. *Oecologia*, 152, pp. 335–343.

Beckerman, A.P., Rodgers, G.M., and Dennis, S.R. (2010). The reaction norm of size and age at maturity under multiple predator risk. *Journal of Animal Ecology,* 79, pp. 1069-1076.

Bednarska, A.J., Portka, I., Kramarz, P.E. and Laskowski, R. (2009). Combined effect of environmental pollutants (nickel, chlorpyrifos) and temperature on the ground beetle, *Pterostichus oblongopunctatus* (Coleoptera: Carabidae). *Environmental Toxicology Chemistry*, 28, pp. 864–872.

Belanger, S.E., Cherry, D.S. (1990). Interacting effects of pH acclimation, pH, and heavy metals on acute and chronic toxicity to *Ceriodaphnia dubia* (Cladocera). *Journal of Crustacean Biology*, 10, pp. 225–235.

Bellavere C, Gorbi J (1981) A comparative analysis of acute toxicity of chromium, copper and cadmium to *Daphnia magna*, *Biomphalaria glabrata* and *Brachydanio rerio*. *Environmental Technology Letters*, 2, pp. 119–128.

Benard, M.F. (2004). Predator‐induced phenotypic plasticity in organisms with complex life histories. *Annual Review of Ecology Evolution and Systematics* 35, pp. 651–673.

Berenbaum, M.C. (1981). Criteria for analyzing interactions between biologically active agents. *Advance in Cancer Research.* 35, pp. 269–335.

Berga, M., [Östman](https://onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=%C3%96stman%2C+%C3%96rjan), Ö., [Lindström](https://onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Lindstr%C3%B6m%2C+Eva+S), E.S. and [Langenheder](https://onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Langenheder%2C+Silke), S. (2015). Combined eﬀects of zooplankton grazing and dispersal on the diversity and assembly mechanisms of bacterial metacommunities. *Environmental Microbiology*, 17, pp. 2275-2287.

Berglund, A.M.M., Sturve, J., Forlin, L. and Nyholm, N.E.I. (2007). Oxidative stress in pied ﬂycatcher (*Ficedula hypoleuca*) nestlings from metal contaminated environments in northern Sweden. *Environmental Research*, 105, pp. 330–339.

Bergman Filho, T., Soares. A. and Loureiro, S. (2011). Energy budget in Daphnia magna exposed to natural stressors. *Environmental Science and Pollution Research*, 18, pp. 655-662.

[Biesiot](https://www.sciencedirect.com/science/article/pii/002209819090190N#!), P.M. and [Capuzzo](https://www.sciencedirect.com/science/article/pii/002209819090190N#!), J.M. (1990). Changes in digestive enzyme activities during early development of the American lobster Homarus americanus Milne Edwards. [*Journal of Experimental Marine Biology and Ecol*](https://www.sciencedirect.com/science/journal/00220981)*ogy*, [136, pp, 2](https://www.sciencedirect.com/science/journal/00220981/136/2)107-122.

Bijlsma, R. and Loeschcke, V. (2005). Environmental stress, adaptation and evolution: An overview. *Journal of Evolutionary Biology*, 18, pp.744-749.

Binelli, A., Ricciardi, F., Riva, C. and Provini, A. (2006). New evidence s for old biomarkers: Effects of several xenobiotics on EROD and AChE activities in Zebra mussel (*Dreissena polymorpha). Chemosphere,* 62, pp. 510–519.

Blanar, C.A., Munkittrick, K.R., Houlahan, J., MacLatchy, D.L. and Marcogliese, D.J. (2009). Pollution and parasitism in aquatic animals: A meta-analysis of effect size. *Aquatic Toxicology*, 93, pp.18–28.

Bodar, C.W., van der Sluis, I., Voogt, P.A. and Zandee, D.I. (1988 a). Effect of cadmium on consumption assimilation and biochemical parameters of *Daphnia magna*: Possible and implications. *Comparative Biochemistry and Physiology,* 90C, (2), pp. 341-346.

Bodar, C.W.M., Van Leeuwen, C.J., Voogt, P.A. and Zandee, D.I. (1988 b). Effect of cadmium on the reproduction strategy of *Daphnia* *magna.* *Aquatic Toxicology*, 12, pp. 301–310.

Boggs, C. L. (1992). Resource allocation: Exploring connections between foraging and life history*. Functional Ecology*, 6, (5), pp. 508–518.

Bohrer, R.N. and Lampert W. (1988). Simultaneous measurement of the effect of food concentration on assimilation and respiration in *Daphnia magna* Straus. *Functional Ecology,* 2, (4), pp. 463-471.

Bolnick, D. and Lau, O. (2008). Predictable patterns of disruptive selection in stickleback in postglacial lakes. *The American Naturalist*, 172, pp. 1–11.

Bolnick, D., Snowberg, L., Hirsch, P., Lauber, C., Org, E., Parks, B., Lusis, A., Knight, R., Caporaso, J. and Svanbäck, R. (2014). Individual diet has sex-dependent effects on vertebrate gut microbiota. *Nature Communications*, 5:4500.

Bossuyt, T.A.B. and Janssen, C. R. (2004). Influence of multigeneration acclimation to copper on tolerance, energy reserves and homeostasis of *Daphnia magna* Straus. *Environmental Toxicology* *and Chemistry*, 23, pp. 2029-2037

Bossuyt, B.T.A. and Janssen, C.R. (2005). Copper regulation and homeostasis of *Daphnia magna* and *Pseudokirchneriella subcapitata*: Influence of acclimation. *Environmental Pollution*, 136, pp. 135–144.

Bossuyt, B.T.A., De Schamphelaere, K.A.C. and Janssen, C.R. (2004). Using the biotic ligand model for predicting the acute sensitivity of cladoceran dominated communities to copper in natural surface waters. *Environmental Science and Technology,* 38, pp. 5030–5037.

Bottrell, H.H. (1975). Generation time, length of life, instar duration and frequency of moulting, and their relationship to temperature in eight species of cladocera from the River Thames, reading, [*Oecologia*](https://link.springer.com/journal/442)*,* 19, pp. 129-140.

Boyd, R.S. (2010). Heavy metal pollutants and chemical ecology: Exploring new frontiers. *Journal of Chemical Ecology*, 36, pp. 46-58.

Bradley, M.C., Baird, D.J., and Calow, P. (1991). Mechanisms of energy allocation to reproduction in the cladoceran *Daphnia magna* Straus. *Biological Journal of the Linnean Society,* 44, pp. 325–333.

Brennan, S.J., Brougham, C.A., Roche, J.J. and Fogarty, A.M. (2006). Multi-generation effects of four selected environmental oestrogens on Daphnia magna. *Chemosphere*, 64, pp. 49–55.

Bridges, C.C. and Zalups, R.K. (2005). Molecular and ionic mimicry and the transport of toxic metals. *Toxicology and Applied Pharmacology*, 204, pp. 274–308.

Brix, K.V., and Deforest, D.K. (2000). Critical review of the use of the bioconcentration factors for hazard classification of metals and metal compounds. Washington, USA: Parametrix Inc. Report No. 555-3690-001.

Brix, K.V., Deforest, D.K. and Adams, W.J. (2001). Assessing acute and chronic copper risks to freshwater aquatic life using species sensitivity distribution for different taxonomic groups. *Environmental Toxicology and Chemistry*, 20(8), pp. 1846-1856.

Brown, B.E. (1982). The form and function of metal-containing “granules” in invertebrate’s tissues. *Biological Reviwers,* 57, pp. 621-667.

Bryan, G.W. and Langston, W.J. (1992). Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: A review. *Environmental Pollution*, 76, pp. 89-131.

Brzóska, M.M. and Moniuszko-Jakoniuk, J. (2001). Interactions between cadmium and zinc in the organism. *Food and Chemical Toxicology*, 39, pp. 967–980.

Bui, T.-K.L., Do-Hong, L.C., Dao, T.-S. and Hoang, T.C. (2016). Copper toxicity and the influence of water quality of Dongnai River and Mekong River waters on copper bioavailability and toxicity to three tropical species. *Chemosphere*, 144, pp. 872–878.

Burns, C.W. (1969). Relation between filtering rate, temperature, and body size in four species of *Daphnia*. *Limnology and Oceanography*, 14, pp. 693-700.

Cain, D.J., Luoma, S.N., Carter, J.L. and Fend S.V. (1992). Aquatic insects as bioindicators of trace element contamination in Cobble-Bottom Rivers and streams. *Canadian Journal of Fisheries and Aquatic Sciences*, 49(10), pp. 2141-2154.

Cairns, J.Jr. (1983). Are single species toxicity tests alone adequate for estimating environmental hazard? *Hydrobiologia*, 100, pp. 47–57.

Callens, M., Watanabe, H., Kato, Y., Miura, J. and Decaestecker, E. (2008). Microbiota inoculum composition affects holobiont assembly and host growth in *Daphnia*. *Microbiome*. 6(1), 56.

Callens, M., Macke, E., Muylaert, K., Bossier, P., Lievens, B., Waud, M. and Decaestecker, E. (2016). Food availability affects the strength of mutualistic host–microbiota interactions in *Daphnia magna*. *The ISME Journal*, 10(4), pp. 911-920.

Calow, P. (1991) Physiological costs of combating chemical toxicants: *Ecological implications*. *Comparative Biochemistry and Pysiology*, 100C, pp. 3-6.

Calow, P. and Sibly, R.M. (1990). A physiological basis of population processes: Ecotoxicological implications. *Functional Ecology,* 4, pp. 283-288.

Campero, M., Slos, S., Ollevier, F. and Stoks, R. (2007). Sublethal pesticide concentrations and predation jointly shape life history: Behavioral and physiological mechanisms. *Ecological Applications*, 17, pp. 2111-2122.

Canesi, L., Borghi, C., Ciacci, C., Fabbri, R., Lorusso, L.C., Vergani, L., Marcomini, A. and Poiana, G. (2008). Short-term effects of environmentally relevant concentrations of EDC mixtures on *Mytilus galloprovincialis* digestive gland. *Aquatic Toxicology,* 87, pp. 272–279.

Canli, M. (2005). Dietary and water-borne Zn exposures affect energy reserves and subsequent Zn tolerance of *Daphnia magna*. *Comparative Biochemistry and Physiology* *C: Toxicology and Pharmacology*, 141, pp. 110–116.

Canton, J.H. and Slooff, W. (1982). Toxicity and accumulation studies of cadmium (Cd+2) with freshwater organisms of different trophic levels. *Ecotoxicological Enironmental and Safety*, 6, pp. 113–128.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N. and Smith, V.H. (1998). Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological Applications*, 8, (3), pp. 559-568.

Carvalho, L. and Kirika, A. (2003). Changes in shallow lake functioning: Response to climate change and nutrient reduction. *Hydrobiologia,* 506, pp. 789-796.

Castro, P.F., Freitas, Jr. A.C.V., Santana, W.M., Costa, H.M.S., Carvalho, L.B. Jr. (2012). Comparative study of amylases from the midgut gland of three species of penaeid shrimp. *Journal of Crustacean Biology,* 32, pp. 607–613.

Cedergreen, N., Christensen, A.M., Kamper, A.; Kudsk, P., Mathiassen, S., Streigig, J.C. and Sørensen, H. (2008). A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. *Environmental Toxicology and Chemistry*, 27(7), pp. 1621–1632.

Chandini, T. (1989). Survival, growth and reproduction of *Daphnia* *carinta* (Crustacea: Cladocera) exposed to chronic cadmium stress at different food (*Chlorella*) level. *Environmental Pollution*, 60, pp. 29–45.

Chandran, R., Sivakumar, A.A., Mohandass, S. and Aruchami, M. (2005). Effect of cadmium and zinc on antioxidan t enzyme activity in the gastropod, *Achatina fulica. Comparative Biochemistry Physiology*, Part C 140, pp. 422-426.

Chen, Z., Mayer, L.M., Weston, D.P., Bock, M.J. and Jumars, P.A. (2002). Inhibition of digestive enzyme activities by copper in the guts of various marine benthic invertebrates. *Environmental Toxicology and Chemistry,* 21, pp. 1243–1248.

Chen, C.Y., Stemberger, R.S., Klaue, B., Blum, G.D. Pickhardt, P.C. and Folt, C.L. (2000). Accumulation of heavy metals in food web components across a gradient of lakes. *Limnology Oceanography* 45, (7), pp. 1525–1536.

Clifford, M. and McGeer, J.C. (2010). Development of a biotic ligand model to predict the acute toxicity of cadmium to *Daphnia pulex*. *Aquatic Toxicology*, 98, pp. 1–7.

Colbourne, J.K., Pfrender, M.E., Gilbert, D., Thomas, W. K., Tucker, A., Oakley, T.H., Tokishita, S., Aerts, A., Arnold, G.J., Basu, M.K., Bauer, D.J., Cáceres, C.E., Carmel, L., Casola, C., Choi, J.-H., Detter, J.C., Dong, Q., Dusheyko, S., Eads, B.D., Fröhlich, T., Geiler-Samerotte, K.A., Gerlach, D., Hatcher, P., Jogdeo, S., Krijgsveld, J., Kriventseva, E.V., Kültz, D., Laforsch, C., Lindquist, E., Lopez, J., Manak, J.R., Muller, J., Pangilinan, J., Patwardhan, R.P., Pitluck, S., Pritham, E.J., Rechtsteiner, A., Rho, M., Rogozin, I.B., Sakarya, O., Salamov, A., Schaack, S., Shapiro, H., Shiga, Y., Skalitzky, C., Smith, Z., Souvorov, A., Sung, W., Tang, Z., Tsuchiya, D., Tu, H., Vos, H., Wang, M., Wolf, Y.I., Yamagata, H., Yamada,T., Ye, Y. Shaw, J.R., Andrews, J., Crease, T.J., Tang, H., Lucas, S.M., Robertson, H.M., Bork, P., Koonin, E.V., Zdobnov, E.M., Grigoriev, I.V., Lynch, M. and Boore, J.L. (2011). The ecoresponsive genome of *Daphnia pulex*. *Science*. 331, pp. 555–561.

Company, R., Serafim, A., Bebianno, M.J., Cosson, R., Shillito, B. and Fiala-Medioni, A. (2004). Effect of cadmium, copper and mercury on antioxidant enzyme activities and lipid peroxidation in the gills of the hydrothermal vent mussel *Bathymodiolus azoricus*. *Marine Environmental Research*, 58, pp. 377–381.

Congiu, L., Chicca, M., Pilastro, A., Turchetto, M. and Tallandini, L. (2000). Effects of chronic dietary cadmium on hepatic glutathione levels and glutathione peroxidase activity in starlings (*Sturnus vulgaris*). *Archives of Environmental Contamination and Toxicol*, 38, pp. 357–361.

Conners, D.E. and Black, M.C. (2004). Evaluation of lethality and genotoxicity in the freshwater mussel *Utterbackia imbecillis* (Bivalvia: Unionidae) exposed singly and in combination to chemicals used in lawn care. *Archives of Environmental Contamination and Toxicology,* 46(3), pp. 362–371.

Cooper, N.L., Bidwell, G.R. and Kumar, A. (2009). Toxicity of copper, lead, and zinc mixtures to *Ceriodaphnia dubia* and *Daphnia carinata.* *Ecotoxicology and Environmental Safety*, 72, pp. 1523–1528.

Correia, A.D., Costa, M.H., Luis, O.J. and Livingstone, D.R. (2003). Agerelated changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in whole body *Gammarus lacusta* (Crustacea: Amphipoda). *Journal of Experimental Marine Biology and Ecology*, 289, pp. 83–101.

Coors, A. and De Meester, L. (2008). Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in *Daphnia magna*. *Journal of Applied Ecology,* 45, pp. 1820–1828.

Crain, C.M. (2008). Interactive and cumulative effects of multiple human stressors in marine systems. *Ecological Letters*, 11, pp. 1304–1315.

Crespi, E.J., Williams, T.D., Jessop, T.S. and Delehanty, B. (2013). Life history and the ecology of stress: How do glucocorticoid hormones influence life-history variation in animals? *Functional Ecology*, 27, pp. 93–106.

Daniel, K., Harbach, R., Guida, W., and Dou, Q. (2004). Copper storage diseases: Menkes, Wilson's, and Cancer. *Frontiers in Bioscience.* 9, pp. 2652–2662.

Dao, T.-S., Le, V.-N., Bui, B.-T., Dinh, K.V., Wiegand, C., Nguyen, T.-S., Dao, C.-T., Nguyen, V.D., To, T.-H., Nguyen, L.-S.-P., Vo, T.-G. and Vo, T.-M.-C. (2017). Sensitivity of a tropical micro-crustacean (*Daphnia lumholtzi*) to trace metals tested in natural water of the Mekong River. *Science of the Total Environment*, 574, pp. 1360–1370.

Dattagupta, S., Schaperdoth, I., Montanari, A., Mariani, S., Kita, N., Valley, J.W. and Macalady, J.L. (2009). A novel symbiosis between chemoautotrophic bacteria and a freshwater cave amphipod. *ISME Journal*, 3, pp. 935-943.

Dave, G. (1984). Effects of copper on growth, reproduction, survival and haemoglobin in *Daphnia magna*. *Comparative Biothemistry and Physiology*, 78, pp. 439-443.

Day, K.E., Kaushik, N.K. and Solomon, K.R. (1987). Impact of fenvalerate on enclosed freshwater planktonic communities and on in situ rates of filtration of zooplankton. *Candian Journal of Fisheries and Aquatic Science,* 44, pp. 1714-1728.

De Coen, W.M. and. Janssen, C. R. (1997). The use of biomarkers in *Daphnia magna* toxicity testing II. Digestive enzyme activity in *Daphnia magna* exposed to exposed sublethal concentrations of cadmium, chromium and mercury. *Chemosphere,* 35, (5), pp. 1053–1067.

De Coen, W.M. and Janssen, C.R. (1998). The use of biomarkers in *Daphnia* *magna* toxicity testing. *Hydrobiologia,* 367, pp. 199-209.

De Coninck, D.I.M., Janssen, C.R. and De Schamphelaere, K.A.C. (2013). An investigation of the inter-clonal variation of the interactive effects of cadmium and Microcystis aeruginosa on the reproductive performance of *Daphnia magna*. *Aquatic Toxicology*, 140–141, pp. 425-431.

De Coninck, D.I.M., Asselman, J., Glaholt, S., Janssen, C.R., Colbourne, J.K., Shaw, J.R. and De Schamphelaere, K.A.C. (2014). Genome-wide transcription profiles reveal genotype-dependent responses of biological pathways and gene-families in *Daphnia* exposed to single and mixed stressors. *Environmental Science and Technology*, 48, pp. 3513–3522.

Dedourge-Geffard, O., Palais, F., Biagianti-Risbourg, S., Gefard, O. and Geffard A. (2009). Effects of metals on feeding rate and digestive enzymes in *Gammarus fossarum*: an in situ experiment. *Chemosphere,* 77, pp. 1569–1576.

Degans, H., Zöllner, E., Gucht, K., Meester, L., and Jürgens, K. (2002). Rapid *Daphnia*-mediated changes in microbial community structure: An experimental study. *FEMS* *Microbiology Ecology,* 42, pp. 137–149.

DeLong, J.P., Hanley, T.C. and Vasseur, D.A. (2014). Competition and the density dependence of metabolic rates. *Journal of Animal Ecology*, 83, pp.51–58.

De Meester, L. and Cousyn, C. (1997). The change in phototactic behaviour of a Daphnia magna clone in the presence of fish kairomones: The effect of exposure time. *Hydrobiologia*, 360, pp. 169-175.

DeMille, C., Arnott, S. and Pyle, G. (2016). Variation in copper effects on kairomone-mediated responses in *Daphnia pulicaria*. *Ecotoxicology and Environmental Safety*, 126, pp. 264­–272.

Demirak, A., Yilmaz, F., Tuna, A.L. and Ozdemir, N. (2006). Heavy metals in water, sediment and tissues of *Leuciscus cephalus* from a stream in southwestern Turkey. *Chemosphere*, 63, pp. 1451–1458.

Dennis, S.R. Carter, M.J. Hentley, W.T. and Beckerman A.P. (2011). Phenotypic convergence along a gradient of predation risk. *Procedings of the Royal Society B*, 278, pp. 1687–1696.

de Oliveira-Filho, E.C., Lopes, R.M. and Paumgartten, F.J.R. (2004). Comparative study on the susceptibility of freshwater species to copperbased pesticides. *Chemosphere*, 56, pp. 369–374.

De Schamphelaere, K.A.C. and Janssen, C.R. (2004 a). Effects of chronic dietary copper exposure on growth and reproduction of *Daphnia magna*. *Environmental Toxicology and Chemistry*, 23, pp. 2038–2047.

De Schamphelaere, K.A.C. and Janssen, C.R. (2004b). Development and field validation of a biotic ligand model predicting chronic copper toxicity to *Daphnia magna*. *Environmental Toxicology and Chemistry,* 23, pp. 1365–1375.

De Schamphelaere, K.A.C. and Janssen, C.R. (2004c). Effects of dissolved organic carbon concentration and source, pH and water hardness on chronic toxicity of copper to *Daphnia magna*. *Environmental Toxicology and Chemistry*, 23, pp. 1115–1122.

De Schamphelaere, K.A.C., Vasconcelos, F.M., Tack, F.M.G., Aallen, H.E. and Janssen, C.R. (2004) Effect of dissolved organic matter source on acute copper toxicity to *Daphnia magna*. *Environmental Toxicology and Chemistry,* 23, pp.1248–1255.

De Schamphelaere, K.A.C., Heijerick, D.G. and Janssen, C.R. (2006). Cross phylum comparison of a chronic biotic ligand model to predict chronic toxicity of copper to a freshwater rotifer, *Brachionus calyciforus* (Pallas). *Ecotoxicology and Environmental Safety*, 63, pp. 189–195.

De Schamphelaere, K.A.C., Forrez, I., Dierckens, K., Sorgeloos, P. and Janssen, C.R. (2007). Chronic toxicity of dietary copper to *Daphnia magna*. *Aquatic Toxicology*, 81, pp. 409-418.

De Vreese, P., Brooks, N., Van Hecke, K., Van Meervelt, L., Matthijs, E., Binnemans, K. and Van Deun, R. (2012). Speciation of copper (II) complexes in an ionic liquid based on choline chloride and in choline chloride/water mixtures. *Inorganic Chemistry*, 51(9), pp. 4972–4981.

De Zwart, D. and Posthuma, L. (2005). Complex mixture toxicity for single and multiple species: proposed methodologies. *Environmental Toxicology and Chemistry*, 24, pp. 2665-2676.

Diamantino, T.C., Almeida, E., Soares, A.M.V.M. and Guilhermino, L. (2001). Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* Straus. *Chemosphere*, 45, pp. 553-560.

Dillon, R.J., and Dillon, V.M. (2004). The gut bacteria of insects: nonpathogenic interactions. *Annual Review of Entomology,* 49, pp. 71–92.

Di Toro, D.M., Allen, H.E., Bergman, H., Meyer, J.S., Paquin, P.R. and Santore, C.S. (2001). Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environmental Toxicology and Chemistry*, 20, pp. 2383–2396.

Dodson, S.I. and Hanazato, T. (1995). Commentary on effects of anthropogenic and natural organic chemicals on development, swimming behavior, and reproduction of *Daphnia*, a key member of aquatic ecosystems. *Environmental Health Perspectives*. 103, pp. 7–11.

Dölling R., Becker, D., Hawat S., Koch M., Schwarzenberger A. and Zeis B. (2016). Adjustments of serine proteases of *Daphnia pulex* in response to temperature changes. *Comparative Biochemistry and Physiology, Part B* 194–195, pp. 1 –10.

Doyotte, A., Cossu, C., Jacquin, M.C., Babutb, M. and Vaseural, P. (1997). Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. *Aquatic Toxicology,* 39, pp. 93–110.

Duarte, D.A.L.F., de Souzaa, C.A., Pereirab, C.D.S. and Pinheiro, M.A.A. (2017). Metal toxicity assessment by sentinel species of mangroves: In situ case study integrating chemical and biomarkers analyses. *Ecotoxicology and Environmental Safety*, 145, pp. 367–376.

Durou, C., Mouneyrac, C. and Amiard-Triquet, C. (2005). Tolerance to metals and assessment of energy reserves in the polychaete Nereis diversicolor in clean and contaminated estuaries. *Environmental Toxicology*, 20, pp. 23–31.

Eads, B.D., Andrews, J. and Colbourne, J.K. (2008). Ecological genomics in Daphnia: Stress responses and environmental sex determination. *Heredity*, 100, pp. 184–190.

Ebert group, Zoologisches Institut Evolutionsbiologie, Switzerland. <http://evolution.unibas.ch/ebert/research/>

Ebrahimpour, M., Alipour, H. and Rakhshah, S. (2010). Influence of water hardness on acute toxicity of copper and zinc on fish. *Toxicology and Industrial Health*, 26(6), pp. 361–365.

Elumalai, M., Antunes C. and Guilhermino, L. (2002). Effects of single metals and their mixtures on selected enzymes of Carcinus Maenas. [*Water, Air, and Soil Pollution*](https://link.springer.com/journal/11270), 141, pp. 273–280.

Esbaugh, A.J., Brix, K.V., Mager, E.M., De Schamphelaere, K. and Grosell, M. (2012). Multi-linear regression analysis, preliminary biotic ligand modelling, and cross species comparison of the effects of water chemistry on chronic lead toxicity in invertebrates. *Comparative* *Biochemistry and Physiology,* Part C 155, pp. 423–431.

Espín, S., Martínez-López, E., Jiménez, P., María-Mojica, P. and García-Fernández, A.J. (2014 a). Effects of heavy metals on biomarkers for oxidative stress in *Griffon vulture* (Gyps fulvus). *Environmental Research*, 129, pp. 59-68.

Espín, S., Martínez-López, E., León-Ortega, M., Martínez, J.E., García-Fernández, A.J. (2014 b). Oxidative stress biomarkers in Eurasian *Eagle owls* (Bubo bubo) in three different scenarios of heavy metal exposure. *Environmental Research*, 131, pp. 134–144.

Falkowski, P.G., Fenchel, T., and Delong, E.F. (2008). The microbial engines that drive Earth’s biogeochemical cycles. *Science,* 320, pp. 1034–1039.

Feder, M.E. and Hofmann, G.E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology,* 61*,* pp. 243–282.

Feng, J., Gao, Y., Ji, Y. and Zhu, L. (2018). Quantifying the interactions among metal mixtures in toxicodynamic process with generalized linear model. *Journal of Hazardous Materials*, 345, pp. 97–106.

Fernández-Gonzáles, M.A., Gonzáles-Barrientos, J., Carter, M.J. and RamosJiliberto, R. (2011). Parent to-offspring transfer of sublethal effects of copper exposure: Metabolic rate and life-history traits of *Daphnia*. *Revista Chilena de Historia Natural*, 84, pp. 195–201.

Ferrando, M.D. and Andreu, E. (1993). Feeding behaviour as an index of copper stress in *Daphnia magna* and *Brachionus calyciforus*. *Comparative Biochemistry and Physiology,* 106C, pp. 327-331.

Ferreira, A.L.G., Loureiro, S. and Soares, A.M.V.M. (2008). Toxicity prediction of binary combinations of cadmium, carbendazim and low dissolved oxygen on *Daphnia magna*. *Aquatic Toxicol*ogy, 89, pp. 28–39.

Fleeger, J.W., Carman, K.R. and Nisbet, R.M. (2003). Indirect effects of contaminants in aquatic ecosystems. *Science of the Total Environent*, 317, pp. 207–233.

Folt, C.L., Chen, C.Y., Moore, M.V. and Burnaford J. (1999). Synergism and antagonism among multiple stressors. *Limnology and Oceanography*, 44, pp. 864–877.

Freese, H. and Schink, B. (2011). Composition and stability of the microbial community inside the digestive tract of the aquatic crustacean *Daphnia magna*. *Microbial Ecology*, 62(4), pp. 882-894.

Friendly, M. (2007). HE plots for multivariate linear models. *Journal of Computational and Graphical Statistics*. 16, pp. 421-444.

Furukawa, T.A., Barbui, C., Cipriani, A., Brambilla, P. and Watanabe, N. (2006) Imputing missing standard deviations in meta-analyses can provide accurate results. *Journal of Clinical Epidemiology*, 59, pp. 7–10.

Gama-Flores, J.L., Sarma, S.S.S. and Nandini, S. (2006). Effect of cadmium level and exposure time on the competition between zooplankton species *Moina macrocopa* (Cladocera) and *Brachionus calyciforus* (Rotifera). *Journal of Environmental Science and Health*, Part A 41, pp. 1057–1070.

García-Fernández, A.J., Bayoumi, A.E., Pérez-Pertejo, Y., Motas, M., Reguera, R.M., Ordóñez, C., Balaña-Fouce, R., and Ordóñez, D. (2002). Alterations of the glutathioneredox balance induced by metals in CHO-K1 cells. *Comparative Biochemistry and Physiology: Toxicology and Pharmacology*, CBP 132, pp. 365–373.

Géret, F., Jouan, A., Turpin, V., Bebianno, M.J. and Cosson, R.P. (2002). Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalve mollusks: The oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*). *[Aquatic Living Resources](https://www.alr-journal.org/)*[, 15, pp. 61–66.](https://www.alr-journal.org/)

Gerhardt, A. (1993). Review of impact of heavy metals on stream invertebrates with special emphasis on acid conditions. *Water, Air and Soil pollution*, 66, pp. 289–314.

Gilbert, J.J. (2004). Females from resting eggs and parthenogenetic eggs in the rotifer Brachionus calyciflorus: lipid droplets, starvation resistance and reproduction. *Freshwater Biology*, 49, pp. 1505–1515.

Gillis, P.L., Chow-Fraser, P., Ranville J.F., Ross, P.E. and Wood, C.M. (2005). *Daphnia* need to be gut-cleared too: the effect of exposure to and ingestion of metal-contaminated sediment on the gut-clearance patterns of *D. magna*. *Aquatic Toxicology,* 71, pp.143–154.

Giusto, A. and Ferrari, L. (2014). Biochemical responses of ecological importance in males of the austral South America amphipod *Hyalella* *curvispina* Shoemaker, 1942 exposed to waterborne cadmium and copper. *Ecotoxicology and Environmental Safety*, 100, pp. 193–200.

Gliwicz, Z.M. and Boavida, M.J. (1996). Clutch size and body size at first reproduction in *Daphnia* *pulicaria* at different levels of food and predation. *Journal of Plankton Research*, 18, pp. 863-880.

Golovanova, L. (2008). Effects of heavy metals on the physiological and biochemical status of fishes and aquatic invertebrates. *Inland Water Biology*, 1, pp. 93–101.

Gorbi, G., Moroni, F., Sei, S. and Rossi, V. (2011). Anticipatory maternal effects in two different clones of *Daphnia magna* in response to food shortage. *Journal of Limnology*, 70, pp. 222–230.

Gorokhova, E., Rivetti, C., Furuhagen, S., Edlund, A., Karin, E.K. and Breitholtz, M. (2015). Bacteria-mediated effects of antibiotics on Daphnia nutrition. *Environmental Science and Technology*, 49, pp. 5779–5787.

Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.-C. and Barber, D.S. (2008). Effect of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environmental Toxicology and Chemistry*, 27(9), pp. 1972-1978.

Grosell, M. and Wood, C.M. (2002 a). Copper uptake across rainbow trout gills: mechanisms of apical entry. *The Journal of Experimental Biology*, 205, pp. 1179-1188.

Grosell, M., Nielsen, C., and Bianchini, A. (2002 b). Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. *Comparative Biochemistry and Physiology.* Part C, 133, (1-2), pp. 287–303.

Grossart, H.P., Dziallas, C. and Tang, K.W. (2009). Bacterial diversity associated with freshwater zooplankton. Environmental Microbiogy Reports, 1, pp. 50–55.

Guan, R. and Wang, W.-X. (2006), Comparison between two clones of *Daphnia magna*: Effects of multigenerational cadmium exposure on toxicity, individual fitness, and biokinetics. *Aquatic Toxicology*, 76, pp. 217–229.

Gusso-Choueri, P.K., Choueri, R.B., Lombardi, A.T. and Melão, M.G.G. (2012). Effects of dietary copper on life-history traits of a tropical freshwater cladoceran. *Archives of Environmental Contamination and Toxicology*, 62, pp. 589–598.

Hahn, M., Kasalicky, V., Jezbera, J., Brandt, U., Jezberova, J. and Simek, K. (2010). *Limnohabitans* curvus gen. nov., sp. nov., a planktonic bacterium isolated from a freshwater lake. *International Journal of Systematic and Evolutionary Microbiology*, 60(6), pp. 1358–1365.

Halliwell, B. and Gutteridge, J.M.C. (1999). Free Radicals in Biology and Medicine. Oxford University Press, Oxford.

Hammer, H.S., Bishop, C.D. and Watts, S.A. (2000). Activities of three digestive enzymes during development in the crayﬁsh Procambarus clarkii (Decapoda). *Journal of Crustacean Biology*, 20, pp. 614–620.

Hammill, E. and Beckerman, A.P. (2010). Reciprocity in predator–prey interactions: exposure to defended prey and predation risk affects intermediate predator life history and morphology. *Oecologia*, 163, 193-202.

Hammill, E., Rogers, A. and Beckerman, A.P. (2008). Costs, benefits and the evolution of inducible defences: A case studywith *Daphnia pulex*. *Journal of Evolutionary Biology*, 21, pp. 705–715.

Handy, R.D., Eddy, F.B. and Baines, H. (2002). Sodium-dependent copper uptake across epithelia: a review of rationale with experimental evidence from gill and intestine. *Biochimica et Biophysica Acta*, 1566, pp. 104–115.

Hani, Y.M.I., Turies, C., Palluel, O., Delahaut, L., Bado-Nilles, A., Geffard, A., Dedourge-Geffard, O. and Porcher, J.-M. (2019). Effects of a chronic exposure to different water temperatures and/or to an environmental cadmium concentration on the reproduction of the threespine stickleback (*Gasterosteus aculeatus).* *Ecotoxicology and Environmental Safety*, 174, pp. 48-57.

Hart, B.A., and Scaife, B.D. (1977). Toxicity and bioaccumulation of cadmium in *Chlorelkt pyrenoidosa*. *Environmental Research,* 14, pp. 401–413.

Hasler, A.D. (1935). The physiology of digestion of plankton crustacean. I. Some digestive enzymes of *Daphnia*. *Biological Bulletin*. 68, pp. 207-2014.

[Hecky](https://aslopubs.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Hecky%2C+R+E), R.E. and [Kilham](https://aslopubs.onlinelibrary.wiley.com/action/doSearch?ContribAuthorStored=Kilham%2C+P), P. (1988). Nutrient limitation of phytoplankton in freshwater and marine environments: A review of recent evidence on the effects of enrichment. *Limnology and Oceanography*, 33, pp. 796–822.

Heijerick, D.G., Janssen, C.R. and De Coen, W.M. (2003). The combined effects of hardness, pH, and dissolved organic carbon on the chronic toxicity of Zn to *D. magna*: development of a surface response model. *Archives Environmental Contamination and Toxicology*, 44, pp. 210–217.

Heugens, E.H., Jager, T., Creyghton, R., Kraak, M.H., Jan Hendriks, A., van Straalen, N.M. and [Admiraal](https://pubs.acs.org/action/doSearch?field1=Contrib&text1=Wim++Admiraal), W. (2003). Temperature-dependent effects of cadmium on *Daphnia magna*: accumulation versus sensitivity. *Environmental Science and Technology,* 37, pp. 2145–2151.

Hoang, T.C., Klaine, S.J. (2007). Influence of organism age on metal toxicity to *Daphnia magna*. *Environmental Toxicology and Chemistry* 26, pp. 1198–1204.

Hoffman, D.J., Heinz, G.H., Sileo, L., Audet, D.J., Campbell, J.K. and LeCaptain, L.J. (2000). Developmental toxicity of lead-contaminated sediment to mallard ducklings. *Archives Environmental Contamination and Toxicology,* 39, pp. 221–232

Homola, E., and Chang, E.S. (1997). Distribution and regulation of esterases that hydrolyse methyl farnesoate in *Homarus americanus* and other crustaceans. *General and Comparative Endocrinolohy*, 106, pp. 62–72.

Hooper, L.V., Littman, D.R. and Macpherson, A.J. (2012). Interactions between the microbiota and the immune system*. Science*, 336, pp. 1268–1273.

Hooper, H.L., Connon, R., Callaghan, A., Maund, S.J., Liess, M., Duquesne, S., Hutchinson, T.H., Moggs J. and Sibly, R.M. (2006). The use of image analysis to estimate population growth rate in *Daphnia magna*. *Journal of Applied Ecology*, 43(4), pp. 828-834.

Hunter, K. and Pyle, G. (2004). Morphological responses of *Daphnia pulex* to *Chaoborus americanus* kairomone in the presence and absence of metals. *Environmental Toxicology and Chemistry*, 23, (5), pp. 1311–1316.

International Agency for Research on Cancer of USA (1997). Beryllium, cadmium, mercury and exposures in the glass manufacturing industry. Summary of data reported and evaluation. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.

Jak, R. (1997). Toxicant-induced changes in zooplankton communities and consequences for phytoplankton development. PhD thesis. TNO, Den Helder, The Netherlands.

Jan Hendriks, A., Maas-Diepeveen, J.L.M., Heugens, E.H.W. and van Straalen, N.M. (2005). Meta-analysis of intrinsic rates of increase and carrying capacity of populations affected by toxic and other stressors. *Environmental Toxicology and Chemistry*, 24, pp. 2267–2277.

Jansen, M., De Meester, L., Cielen, A., Buser, C.C. and Stoks, R. (2011). The interplay of past and current stress exposure on the water flea *Daphnia*. *Functional Ecology*, 25, pp. 974-982.

Jemec A., Drobne D., Tišler T., Trebše P., Roš, M. and Sepčić K. (2007). The applicability of acetylcholinesterase and glutathione S-transferase in *Daphnia magna* toxicity test. *Comparative Biochemistry and Physiology*, Part C*,* [*Toxicology and Pharmacology*](https://www.sciencedirect.com/science/journal/15320456)*,* 144, pp. 303–309.

Ji, X., Hu, W., Cheng, J., Yuan, T., Xu, F., Qu, L. and Wang, W. (2006). Oxidative stress on domestic ducks (Shaoxing duck) chronically exposed in a Mercury-Selenium coexisting mining area in China. *Ecotoxicology and Environmental Safety*, 64, pp. 171–177.

Jing, G., Li, Y., Xie, L. and Zhang, R. (2006). Metal accumulation and enzyme activities in gills and digestive gland of pearl oyster (*Pinctada fucata*) exposed to copper. *Comparative Biochemistry and Physiology*, 144, pp. 1841–1890.

Johnston, E.L. and Roberts, D.A. (2009). Contaminants reduce the richness and evenness of marine communities: a review and meta-analysis. *Environmental Pollution*, 157, pp. 1745–1752.

Kagawa, N. and Mugiya, Y. (2002). Brain HSP70 mRNA expression is linked with plasma cortisol levels in goldfish (*Carassius auratus*) exposed to a potential predator. *Zoological Science*, 19, pp. 735- 740.

Kamada, N., Chen, G. Y., Inohara, N. and Nunez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. *Nature Immunology,* 14, pp. 685−690.

Katranitsas, A, Castritsi-Catharios, J. and Persoone, G. (2003). The effects of a copper-based antifouling paint on mortality and enzymatic activity of a non-target marine organism. *Marine Pollution Bulletin*, 46, pp. 1491–1494.

Keithly, J., Brooker, J.A., Deforest, D.K., Wu, B.K. and Brix, K.V. (2004). Acute and chronic toxicity of nickel to a cladoceran (*Ceriodaphnia* *dubia*) and an amphipod (*Hyalella azteca*). *Environmental Toxicology and Chemistry*, 23, pp. 691–696.

Khangarot, B.S. and Rathore, R.S. (2003). Effects of copper on respiration, reproduction, and some biochemical parameters of water flea *Daphnia magna* Straus. *Bullutein of Environmental Contamination sand Toxicology*, 70, pp. 112–117.

Khoury, J.N., Powers, E., Patnaik, P. and Wallace, W.G. (2009). Relating disparity in competitive foraging behavior between two populations of fiddler crabs to the subcellular partitioning of metals. *Archives Environmental Contamination and Toxicol*ogy, 56, pp. 489-499.

Khuri, A.I. and Cornell, J.A. (1996). Responses Surfaces: Design and Analyses. 2nd edition. Marcel Dekker, Monticello, NY.

Killen, S.S., Marras, S., Metcalfe, N.B., McKenzie, D.J. and Domenici, P. (2013). Environmental stressors alter relationships between physiology and behaviour. *Trends in Ecology and Evolution*, 28, pp. 651–658.

Kim, K.T., Lee, Y.G. and Kim, S.D. (2006). Combined toxicity of copper and phenol derivatives to *Daphnia magna*: Effect of complexation reaction. *Environment International,* 32, pp. 487-492.

Kim, H., Yim, B., Bae, C., Lee and Y.-M. (2017). Acute toxicity and antioxidant responses in the water flea *Daphnia magna* to xenobiotics (cadmium, lead, mercury, bisphenol A, and 4 nonylphenol). *Toxicology and Environmental Health Science*, 9, pp. 41–49.

Klaassen, C.D., Bracken, W. M., Dudley, R. E., Goering, P.L., Hazelton, G.A., and Hjelle, J.J. (1985). Role of sulfhyrdyls in the hepatoxicity of organic and metallic compounds. *Fundamental and Applied Toxicology*, 5, pp. 806-815.

Knapen, D., Bervoets, L., Verheyen, E. and Blust, R. (2004). Resistance to water pollution in natural gudgeon (*Gobio gobio* L.) populations may be due to genetic adaptation. *Aquatic Toxicology*, 67, pp. 155–165.

Knops, M., Altenburger, R. and Segner, H. (2001). Alterations of physiological energetics, growth and reproduction of *Daphnia magna* under toxicant stress. *Aquatic Toxicology,* 53, pp. 79–90.

Koivisto, S., Ketolab, M. and Walls, M. (1992). Comparison of five cladoceran species in short- and long-term copper exposure. *Hydrobiologia.* 248, pp. 125–136.

Komjarova, I. and Blust, R. (2008). Multi-metal interactions between Cd, Cu, Ni, Pb, and Zn in water flea *Daphnia magna*, a stable isotope experiment. *Aquatic Toxicology*, 90, pp. 138-144.

Koussoroplis, A.M., Schwarzenberger, A. and Wacker, A. (2017). Diet quality determines lipase gene expression and lipase/esterase activity in *Daphnia pulex*. *Biology Open*, 6, pp. 210-216.

Kovács, A.; Abdel-Hameid, N.-A., Ács A.; Ferincz, Á. and Kováts, N. (2012). A novel protocol for assessing aquatic pollution, based on the feeding inhibition of *Daphnia magna*. Knowl. Manag. *Aquatic Ecology*, 404, pp. 1-19.

Kozlova, T., Wood, C.M. and McGeer, J.C. (2009). The effect of water chemistry on the acute toxicity of nickel to the cladoceran *Daphnia* *pulex* and the development of a biotic ligand model. *Aquatic Toxicology*, 91, pp. 221–228.

Kraak, M.H.S., Toussaint, M., Bleeker, E.A.J. and Lavy, D. (1993). Metal regulation in two species of freshwater bivalves. In Dallinger R, Rainbow PS, eds, *Ecotoxicology of Metals in Invertebrates.* Lewis, Boca Raton, FL, USA, pp. 175–186.

Krantzberg, G. and Stokes, P.M. (1988). The importance of surface adsorption and pH in metal accumulation by chironomids. *Environmental Toxicology and Chemistry*, 7, pp. 653–670.

Krishnakumar, P. K., Asokan, P. K. and Pillai, V. K. (1990). Physi- ological and cellular responses to copper and mercury in the green mussel *Perna viridis* (Linnaeus). *Aquatic Toxicology*, 18, 163–174.

Lampert, W. (2006). *Daphnia*: Model herbivore, predator and prey. *Polish Journal of Ecology*. 54, (4), pp. 607–620.

Lampert, W., Fleckner, W., Rai, H. and Taylor, B.E. (1986). Phytoplankton control by grazing zooplankton: A study on the clear water spring phase. *Limnology and Oceanography.* 31, pp. 478–490.

Laparra, J.M. and Sanz, Y. (2010). Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacological Research*, 61, pp. 219.225.

Lari E., Gauthier, P., Mohaddes, E. and Pyle, G.G. (2017). Interactive toxicity of Ni, Zn, Cu, and Cd on *Daphnia magna* at lethal and sub-lethal concentrations. *Journal of Hazardous Materials*, 334, pp. 21-28.

Laskowski, R., Bednarska, A.J., Kramarz, P.E., Loureiro, S., Scheil, V., Kudłek, J. and Holmstrup, M. (2010). Interactions between toxic chemicals and natural environmental factors: A meta-analysis and case studies. *Science of the Total Environment.* 408, pp. 3763–3774.

Letelier, M.E., Lepe, A.M., Faundez, M., Salazar, J., Marına, R., Aracena, P. and Speisky, H. (2005). Possible mechanisms underlying copper induced damage in biological membranes leading to cellular toxicity. *Chemico-Biological Interactions*, 151, pp. 71–82.

Lima, S.L. and Dill, L.M. (1990). Behavioral decisions made under the risk of predation\_\_a review and prospectus. *Candian Journal of Zoology*, 68, pp. 619–640.

Lind P.R. and Jeyasingh, P.D. (2015). Genotypic differences in phosphorus use physiology in producers (*Chlamydomonas reinhardtii*) and consumers (*Daphnia pulex*) interact to alter primary and secondary production. *Evolutionary Ecology*, 29, pp. 551–563.

Liu, J., Wu, H., Feng, J., Li, Z. and Lin, G. (2014). Heavy metal contamination and ecological risk assessments in the sediments and zoobenthos of selected mangrove ecosystems, South China. *Catena,* 119, pp. 136–142.

Livingstone, D.R., Lips, F., Garcia Martinez, P. and Pipe, R.K. (1992). Antioxidant enzymes in digestive gland of the common mussel *Mytilus edulis* L. *Marine Biology*, 112, pp. 265–276.

Long, K.E., Van Genderen, E.J. and Klaine, S.J. (2004). The effects of low hardness and pH on copper toxicity to *Daphnia magna*. *Environmental Toxicology and Chemistry,* 23, pp. 72–75.

Lopes I, Baird D.J. and Ribeiro R. (2004). Genetic determination of tolerance to lethal and sublethal copper concentrations in field populations of *Daphnia longispina*. *Archives Environmental Contamination and Toxicology*, 46, pp. 43–51.

Loureiro S., Svendsen, C., Ferreira A.L.G., Pinheiro, C., Ribeiro F. and Soaresy A.M.V.M. (2010). Toxicity of three binary mixtures to *Daphnia magna*: Comparing chemical modes of action and deviations from conceptual models. *Environmental Toxicology and Chemistry*, 29(8), pp. 1716–1726.

Luciana, R, Ulises, R, Susana, G, Horacio, T. and Ana María, G. (2014). Effect of metals on *Daphnia magna* and cladocerans representatives of the Argentinean fluvial littoral. *Journal of environmental Biology*, 35, pp. 689–697.

Lv, X., Huang, B., Zhu, X., Jiang, Y., Chen, B., Tao, Y., Zhou, J. and Cai, Z. (2017). Mechanisms underlying the acute toxicity of fullerene to *Daphnia* *magna*: Energy acquisition restriction and oxidative stress. *Water Research*, 123, pp. 696–703.

Lynch, M. and Shapiro, J. (1981). Predation, enrichment, and phytoplankton community structure. *Limnology and Oceanography.* 26, pp. 86–102.

Macedo-Sousa, J.A., Pestana, J.L., Gerhardt, A., Nogueira, A.J., Soares, A.M. (2007). Behavioural and feeding responses of *Echinogammarus meridionalis* (Crustacea, Amphipoda) to acid mine drainage. *Chemosphere,* 67, pp. 1663–1670.

Magos, L. and Webb, M. (1978). Theoretical and practical considerations on the problem of metal-metal interaction. *Environmental Health Perspectives,* 25, pp. 151–154.

[Mah](https://pubs.acs.org/author/Mah%2C+Vicky), V. and [Jalilehvand](https://pubs.acs.org/author/Jalilehvand%2C+Farideh), F. (2012). Lead (II) Complex Formation with Glutathione. *Inorgic Chemistry*, 51, pp. 6285-6298.

Mahar, A.M. and Watzin, M.C. (2005). Effects of metal and organophospate mixtures on *Ceriodaphnia dubia* survival and reproduction. *Environmental Toxicology and Chemistry*, 24, pp. 1579-1586.

March, B.G.E. (1988). Acute toxicity of binary mixtures of five cations (Cu2+, Cd2+, Zn 2+, Mg2+, and K+) to the freshwater amphipod *gammarus lacustris* (Sars): Alternative descriptive models. *Canadian Journal of Fisheries of Aquatic Science,* 45(4), pp. 625-633.

Maria, V.L. and Bebianno, M.J. (2011). Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. *Comparative Biochemistry and Physiology, Part C*, 154, pp. 56-63.

Martin-Creuzburg, D., Bec, A. and von Elert, E. (2006). Supplementation with sterols improves food quality of a ciliate for *Daphnia magna*. *Protist* 157, pp. 477–486.

Mateo, R. and Hoffman, D.J. (2001). Differences in oxidative stress between young Canada geese and mallards exposed to lead-contaminated sediment. *Journal and Toxicological and Environmental Health* A, 64, pp. 531–545.

Mateo, R.W., Beyer, W.N., Spann, J.W., Hoffman, D.J. and Ramis, A. (2003). Relationship between oxidative stress, pathology, and behavioral signs of lead poisoning in mallards. *Journal of Toxicology and Environmental Health* Part A 66, pp. 1371–1389.

Martinez-Haro, M., Green, A.J., and Mateo, R. (2011). Effects of lead exposure on oxidative stress biomarkers and plasma biochemistry in waterbirds in the field. *Environmental Research*, 111, pp. 530–538.

Martins, C., Jesus, F.T. and Nogueira, A.J.A. (2017). The effects of copper and zinc on survival, growth and reproduction of the cladoceran *Daphnia longispina*: introducing new data in an “old” issue. *Ecotoxicology*, 26, pp. 1156–1169.

Matovic, V., Buha, A., Bulat, Z. and Đukiccosic, D. (2011). Cadmium toxicity revisited: Focus on oxidative stress induction and interactions with zins and magnesium. *Arhiv za Higijenu Rada i Toksikologiju*, 62, pp. 65–76.

McKenney, P.T. and Pamer, E.G. (2015). From hype to hope: the gut microbiota in enteric infectious disease. *Cell* 163, pp. 1326–1332.

McMurdie, P.J. and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoSONE* 8: e61217.

McWilliam, R.A. and Baird, D.J. (2002). Postexposure feeding depression: A new toxicity endpoint for use in laboratory studies with *Daphnia magna. Environmental Toxicology and Chemistry,* 21, (6), pp. 1198–1205.

Meaney, M.J. and Szyf, M. (2005). Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome. *Dialogues Clin Neurosci*, 7(2), pp. 103–123.

Meyer, J.S., Ranville, J.F., Pontasch, M., Gorsuch, J.W. and Adams, W.J. (2015). Acute toxicity of binary and tertiary mixtures of Cd, Cu and Zn to *Daphnia magna*. *Environmental Toxicology and Chemistry*, 34 (4), pp. 799-808.

Millward, R.N. and Grant A. (2000). Pollution-induced tolerance to copper of nematode commu- nities in the severely contaminated Restronguet Creek and adjacent estuaries, Cornwall, United Kingdom. *Environmental Toxicology and Chemistry*, 19, pp.454-461.

Mirza, R.S. and Pyle, G.G. (2009). Waterborne metals impair inducible defences in *Daphnia pulex*: morphology, life-history traits and encounters with predators. *Freshwater Biology.* 54, pp. 1016–1027.

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M.G., Pinho, G.L.L., Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J. and Bianchini, A. (2007). Pollution biomarkers in estuarine animals: Critical review and new perspectives [*Comparative Biochemistry and Physiology. Part C: Toxicology and Pharmacology*](https://www.sciencedirect.com/science/journal/15320456)*,* [146, pp. 2](https://www.sciencedirect.com/science/journal/15320456/146/1)221-234.

Moore, M. V., Pace, M. L., Mather, J. R., Murdoch, P. S., Howarth, R. W., Folt, C. L., Chen, C. Y., Hemond, H. F., Flebbe, P. A. and Driscoll, C. T. (1997). Potential effects of climate change on freshwater ecosystems of the new England/Mid-Atlantic region. *Hydrobiological Processes*.11, pp. 925–947.

Mountouris, A., Voutsas, E. and Tassios, D. (2002). Bioconcentration of heavy metals in aquatic environments: the importance of bioavailability. *Marine Pollution Bulletin*, 44, pp. 1136–1141.

Mushegian, A.A., Arbore, R., Walser, J.-C. and Ebert, D. (2018). Environmental sources of bacteria and genetic variation in behavior influence host-associated microbiota. *Ecotoxicology and Environmental Safety*, 68, pp. 416-422.

Muyssen, B.T.A. and Janssen, C.R. (2001). Multigeneration zinc acclimation and tolerance in Daphnia magna: Implications for water-quality guidelines and ecological risk assessment. *Environmental Toxicology and Chemistry,* 20, pp. 2053-2060.

Muyssen, B.T.A. and Janssen, C.R. (2007). Age and exposure duration as a factor influencing Cu and Zn toxicity toward *Daphnia magna*. *Ecotoxicology and Environmental Safety*, 68, pp. 436-442.

[Muyssen](https://www.sciencedirect.com/science/article/pii/S0045653501000479#!), B.T.A. and [Janssen](https://www.sciencedirect.com/science/article/pii/S0045653501000479#!), C.R. (2010). Combined cadmium and temperature acclimation in *Daphnia magna*: Physiological and sub-cellular effects. *Ecotoxicology and Environmental Safety,* 73, pp. 735-742.

Naraki, Y., Hiruta, C. and Tochinai, S. (2013). Identification of the precise kairomone-sensitive period and histological characterization of neck tooth formation in predator-induced polyphenism in Daphnia pulex. *Zoological Science,* 30, pp. 619-625.

Neumann, N.F. and Galvez, F. (2002). DNA microarrays and toxicogenomics: Applications for ecotoxicology? ***Biotechnology Advances*,** 20, pp. 391-419.

Nicholson, S. and Lam, P.K.S. (2005). Pollution monitoring in Southeast Asia using biomarker in the mytilid mussel *Perna viridis* (Mytilidae: Bivalvia). *Environment International*, 31, pp. 121–132.

Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W. and Pettersson, S. (2012) Host-gut microbiota metabolic interactions. *Science* 336, pp. 1262–1267.

Nieboer, E., and Richardson, D.H.S. (1980). The replacement of the non-descript term ‘heavy metals’ by a biologically and chemically significant classification of metal ions. *Environmental Pollution Series B,* 1, pp. 3–26.

Noonburg, E.G. and Nisbet, R.M. (2005). Behavioural and physiological responses to food availability and predation risk. *Evolutionary Ecology Research*, 7, pp. 89–104.

Nys, C., Asselman, J., Hochmuth, J.D., Janssen, C.R., Blust, R., Smolders, E. and De Schamphelaere, K.A.C. (2015). Mixture toxicity of nickel and zinc to *Daphnia magna* is noninteractive at low effect sizes but becomes synergistic at high effect sizes. *Environmental Toxicology and Chemistry,* 34, pp. 1091–1102.

Nzengue, Y., Candéias, S. M., Sauvaigo, S., Douki, T., Favier, A., Rachidi, W. and Guiraudc, P. (2011). The toxicity redox mechanisms of cadmium alone or together with copper and zinc homeostasis alteration: Its redox biomarkers. *Journal of Trace Elements in Medicine and Biology*, 25, pp. 171–180.

O’Brien, A.L. and Keough, M.J. (2014). Ecological responses to contamination: A meta-analysis of experimental marine studies. *Environmental Pollution*, 195, pp. 185–191.

OECD 202 (2004). Guideline for testing of chemicals. *Daphnia* sp-Acute immobilisation test.

OECD 2012. OECD guideline for testing of Chemicals—*Daphnia magna* Reproduction test. Experimental guideline No.211, OECD.

Olmstead, A.W. and LeBlanc, G.A. (2000). Effects of endocrine-active chemicals on the development of sex characteristics of Daphnia magna. *Environmental Toxicology and Chemistry*, 19, pp. 2107–2113.

Orbea, A., Fahimi, H.D. and Cajaraville, M.P. (2000). Immunolocalization of four antioxidant enzymes in digestive glands of mollusks and crustaceans and fish liver. Histochem. *Cell Biology*, 114, pp. 393–404.

Osenberg, C.W., Sarnelle, O. and Cooper, S.D. (1997). Effect size in ecological experiments: the application of biological models in meta-analysis. *The American Naturalist*, 150, pp. 798–812.

Pagliarani, A., Ventrella, V., Trombetti, F., Pirini, M., Trigari, G. and Borgatti, A.R. (1996). Mussel microsomal Na+, Mg2+-ATPase sensitivity to waterborne mercury, zinc and ammonia. *Comparative Biochemistry and Physiology,* C 113, pp. 185–191.

Paland, S., Colbourne, J.K. and Lynch, M. (2005). Evolutionary history of contagious asexuality in *Daphnia pulex*, *Evolution,* 59, pp. 800-813.

Pauwels, K., R. Stoks, and de Meester. L. (2005). Coping with predator stress: interclonal differences in induction of heat-shock proteins in the water flea *Daphnia magna*. *Journal of Evolutionary Biology,* 18, pp. 867-872.

Pavlaki, M.D., Pereira, R., Loureiro, S. and Soares A.M.V.M. (2011). Effects of binary mixtures on the life traits of *Daphnia magna*. *Ecotoxicology and Environmental Safety,* 74, pp. 99-110.

Peerakietkhajorn, S., Tsukada, K., Kato, Y., Matsuura, T. and Watanabe, H. (2015). Symbiotic bacteria contribute to increasing the population size of a freshwater crustacean. *Daphnia magna. Environmental Microbiology Reports,* 7, pp. 364–372.

Pérez, E. and Hoang, T.C. (2017). Chronic toxicity of binary-metal mixtures of cadmium and zinc to *Daphnia magna.* *Environmental Toxicology and Chemistry*, 36, pp. 2739-2749.

Pérez, E. and Hoang T.C. (2018). Responses of *Daphnia magna* to chronic exposure of cadmium and nickel mixtures. *Chemosphere*, 208, pp. 991-1001.

Pestana, J.L.T., Re, A., Nogueira, A.J.A. and Soares, A.M.V.M. (2007). Effects of cadmium and zinc on the feeding behaviour of two freshwater crustaceans: *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda). *Chemosphere*, 68, pp. 1556–1562.

Pestana, J.L.T., Loureiro, S., Baird, D.J. and Soares, A. (2009). Fear and loathing in the benthos: Responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. *Aquatic Toxicology*, 93, pp. 138–149.

Pestana, J.L.T., Loureiro, S., Baird, D.J. and Soares, A.M.V.M. (2010). Pesticide exposure and inducible antipredator responses in the zooplankton grazer, *Daphnia magna* Straus. *Chemosphere*, 78, pp. 241–248.

Peter, H. and Sommaruga, R. (2008). An evaluation of methods to study the gut bacterial community composition of freshwater zooplankton. *Journal of Plankton Research*, 30, pp. 997-1006.

Piggott, J.J., Townsend, C.R., and Matthaei, C.D. (2015). Reconceptualizing synergism and antagonism among multiple stressors. *Ecology and Evolution,* 5, pp. 1538–1547.

Pijanowska, J. and Kloc, M. (2004). *Daphnia* response to predation threat involves heat‐shock proteins and the actin and tubulin cytoskeleton. *Genesis*, 38, pp. 81– 86.

Piscia, R., Colombini, M., Ponti, P., Bettinetti, R., Monticelli, D., Rossi, V. and Manca, M. (2015). Lifetime response of contemporary versus resurrected *Daphnia galeata* Sars (Crustacea, Cladocera) to Cu (II) chronic exposure. *Bulletin of Environmental Contamination and Toxicology*, 94, pp. 46–51.

Porter, K.G., Feig, Y.S. and Vetter, E.F. (1983). Morphology, flow regimes, and filtering rates of *Daphnia, Ceriodaphni and Bosminafed* natural bacteria. *Oecologia,* 58, pp. 156-163.

Poynton, H.C., Varshavsky, J.R., Chang, B., Cavigiolio, G., Chan, S., Holman, P.S., Loguinov, A.V., Bauer, D.J., Komachi, K. and Theil, E.C. (2007). *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. *Environmental Science and Technology,* 41, pp. 1044–1050.

Poynton, H.C., Lazorchak, J.M., Impellitteri, C.A., Smith, M.E., Rogers, K., Patra, M., Hammer, K.A.; Allen, H. J. and Vulpe, C.D. (2011). Differential gene expression in *Daphnia magna* suggests distinct modes of action and bioavailability for ZnO nanoparticles and Zn ions. *Environmental Science and Technology,* 45, pp. 762–768.

Pruesse, E., Peplies, J. and Glockner, F.O. (2012). SINA: accurate high throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics*, 28, pp. 1823–1829.

Pyle, G.G. and Mirza, R.S. (2007). Copper-Impaired Chemosensory Function and Behavior in Aquatic Animals. *Human and Ecological Risk Assessment*, 13, pp. 492–505.

Qi, W., Nong, G., Preston, J., Ben-Ami, F. and Ebert, D. (2009). Comparative metagenomics of *Daphnia* symbionts. *BMC Genomics*, 10(1), 172.

R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org>.

Rahbek, C. (2005). The role of spatial scale and the perception of largescale species-richness patterns. *Ecological Letters*, 8, pp. 224–239.

Rainbow, P.S. (1997). Trace metal accumulation in marine invertebrates: marine biology or marine chemistry? *Journal of the Marine Biological Association* *UK,* 77, pp. 195–210.

Rainbow, P.S. (2002). Trace metal concentrations in aquatic invertebrates: why and so what? *Environmental Pollution,* 120, pp. 497–507.

Rainbow, P. S. and White, S.L. (1989). Comparative strategies of heavy metal accumulation by crustaceans: zinc, copper and cadmium in a decapod, an amphipod and a barnacle. *Hydrobiologia*, 174, pp. 245–262.

Rajalakshmi, S. and Mohandas, A. (2005). Copper-induced changes in tissue enzyme activity in a freshwater mussel. *Ecotoxicological and Environmental safety*, 62, pp. 140–143.

Reger, J. (2013). The quantitative genetic basis of inducible defences and life-history plasticity in *Daphnia pulex*. PhD thesis. Sheffield University.

Reger, J., Lind, M.I., Robinson, M.R. and Beckerman, A.P. (2018). [Predation drives local adaptation of phenotypic plasticity](https://doi.org/10.1038%2Fs41559-017-0373-6). *Nature Ecology and Evolution*, 2, pp. 100-107.

Regoly, F., Hummel, H. and Amiard-Triquet, C. (1998). Trace Metals and Variations of Antioxidant Enzymes in Arctic Bivalve Populations, *Archives Environmental Contamnation and* *Toxicology*, 35, 4, pp. 249–256.

Rikans, L.E. and Hornbrook, K.R. (1997). Lipid peroxidation, antioxidant protection and aging. *Biochimica et Biophysica Acta.* 1362, pp. 116–127.

Robinson, K. A., Baird, D. J. and Wrona, F. J. (2003). Surface metal adsorption on zooplankton carapaces: implications for exposure and effects in consumer organisms. *Environmental Pollution,* 122, pp. 159–167.

Robison, A.L., Chapman, T. and Bidwell, J.R. (2018) Predation cues influence metabolic rate and sensitivity to other chemical stressors in fathead minnows (*Pimephales promelas*) and *Daphnia pulex.* *Ecotoxicology*, 27, pp. 55–68.

Rocha, G.S., Tonietto, A.E., Lombardi, A.T. and Melão, M.G.G. (2016). Eﬀect of copper contaminated food on the life cycle and secondary production of *Daphnia laevis*. *Ecotoxicology and Environmental safety*, 133, pp. 235–242.

Rodea-Palomares, I., González-Pleiter, M., Martín-Betancor, K., Rosal, R. and Fernández-Piñas, F. (2015). Additivity and interactions in ecotoxicity of pollutant mixtures: Some patterns, conclusions, and open questions. *Toxics*, 3, pp. 342-369.

Roesijadi, G. (1992). Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology,* 22, pp. 81–113.

Rogalski, M.A. (2017). Maladaptation to acute metal exposure in resurrected *Daphnia ambigua* clones after decades of increasing contamination. *The American Naturalist*, 189(4), 443–452.

Rose, R.M., Warne, M.S.J. and Lim, R.P. (2001). The presence of chemicals exuded by fish affects the life-history response of *Ceriodaphnia dubia* to chemicals with different mechanisms of action. *Environmental Toxicology and Chemistry*, 20, pp. 2892–2898.

Roux, D.J., Kempster, P. L., Turter, E. and Van der Merwe, L. (1993). Effect of cadmium and copper on survival and reproduction of *Daphnia pulex*. *Water* *SA*, 19, (4), pp. 269–274.

Russo, C., Kundi, M., Lavorgna, M., Parrella, A. and Isidori M. (2018). Benzalkonium chloride and anticancer drugs in binary mixtures: Reproductive toxicity and genotoxicity in the freshwater crustacean *Ceriodaphnia dubia*. *Archives Environmental Contamnation and Toxicology,* 74, pp. 546–556.

Sadeq, S.S. and Beckerman, A.P. (2019). The chronic effects of copper and cadmium on life history traits across Cladocera species: A meta-analysis. *Archives of Environmental Contamination and Toxicology*, 76, pp. 1–16.

Salem, H., [Florez](https://royalsocietypublishing.org/doi/full/10.1098/rspb.2014.2957), L., [Gerardo](https://royalsocietypublishing.org/doi/full/10.1098/rspb.2014.2957), N.and [Kaltenpoth](https://royalsocietypublishing.org/doi/full/10.1098/rspb.2014.2957), M. (2015). An out-of-body experience: The extracellular dimension for the transmission of mutualistic bacteria in insects. *Proceedings of the Royal Society* *B*, 282(1804), pp.1–10.

Sampson, T.R. and Mazmanian, S.K. (2015). Control of brain development, function, and behavior by the microbiome. *Cell Host and Microbe* 17, pp. 565–576.

Sancho, E., Villarroel, M.J., Andreu, E. and Ferrando, M.D. (2009). Disturbances in energy metabolism of *Daphnia magna* after exposure to tebuconazole. *Chemosphere* 74, pp. 1171-1178.

Sandrini, J.Z. (2008). Antioxidant responses in the Nereid *Laeonereis* *acuta* (Annelida, Polychaeta) after cadmium exposure. *Ecotoxicological and Environmental Safety*, 70, pp. 115–120.

Santore, R.C., Di Toro, D.M., Paquin, P.R., Allen, H.E. and Meyer, J.S. (2001). Biotic ligand model of the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and *daphnia*. *Environmental and Toxicological Chemistry*, 20(10), pp. 2397–2402.

Sarma, S.S.S. and Nandini, S. (2006). Review of recent ecotoxicological studies on cladocerans. *Journal of Environmental Science and Health, Part B,* 41, pp. 1417–1430.

Schlotz, N., Sørensen, J.G. and Martin-Creuzburg, D. (2012). The potential of dietary polyunsaturated fatty acids to modulate eicosanoid synthesis and reproduction in *Daphnia magna*: A gene expression approach. [*Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*](https://www.sciencedirect.com/science/journal/10956433)*,* 162(4), pp. 449-454.

Schulz, R. and Dabrowski, J.M. (2001). Combined effects of predatory fish and sublethal pesticide contamination on the behavior and mortality of mayfly nymphs. *Environmental Toxicology and Chemistry*, 20, pp. 2537–2543.

Schwarzenberger, A, Kuster, C.J. and Von Elert, E. (2012). Molecular mechanisms of tolerance to cyanobacterial protease inhibitors revealed by clonal differences in *Daphnia magna*. *Molecular* *Ecology*, 12, pp. 4898-4911.

Shalaby, A.M. (2000). Sublethal effects of heavy metals copper, cadmium and zinc alone or in combinations on enzymes activities of common carp *Cyprinus carpio* L. *Egypt Journal of Aquatic Biology and Fisheries*, 4, (2), pp. 229–246.

Shanker, A.K. (2008). Mode of action and toxicity of trace elements. In: Prasad MNV (ed.) Trace elements as contaminants and nutrients: consequences in ecosystems and human health. Wiley, Hoboken, pp. 525–555.

Shapira M. (2016). Gut microbiotas and host evolution: scaling up symbiosis. *Trends in Ecology and Evolution.* 31, pp. 539-549.

Shaw, J.L.A., Judy, J.D., Kumar, A., Bertsch, P., Wang, M.-B. and Kirby, J.K. (2017). Incorporating transgenerational epigenetic inheritance into ecological risk assessment frameworks. Environmental Science and Technology*,* 51 (17), pp. 9433–9445.

Shuhaimi-Othman, M. and Pascoe, D. (2007). Bioconcentration and depuration of copper, cadmium and zinc mixtures by the freshwater amphipod *Hyalella azteca*. *Ecotoxicological and Environmental Safety.* 66, pp. 29-35.

Shuhaimi-Othman, M., Nadzifah, Y. and Ahmad, A.K. (2010). Toxicity of copper and cadmium to freshwater fishes. *World Academy of Science, Engineering and Technology*, 6(5), pp. 319–321.

Sison-Mangus, M., Mushegian, A.A. and Ebert, D. (2015). Water fleas require microbiota for survival, growth and reproduction. *The ISME Journal*, 9(1), pp. 59–67.

Skaggs, H.S. and Henry, R.P. (2002). Inhibition of carbonic anhydrase in the gills of two euryhaline crabs, *Callinectes sapidus* and *Carcinus maenas*, by heavy metals. *Comparative Biochemistry and Physiology* C 133, pp. 605-612.

Soares, A.M.V.M., Baird, D.J. and Calow, P. (1992). Interclonal variation in the performance of *Daphnia magna* Straus in chronic bioassays. *Environmental Toxicology and chemistry*, 11, pp. 1477-1483.

Soetaert, A., Vandenbrouck, T., van der Ven, K., Maras, M., van Remortel, P., Blust, R. and De Coen W.M. (2007). Molecular responses during cadmium-induced stress in *Daphnia magna*: integration of differential gene expression with higher-level effects. *Aquatic Toxicol*ogy, 83, pp. 212–222.

Sohal, R.S., Mockett, R.J. and Orr, W.C. (2000). Current issues concerning the role of oxidative stress in aging: a perspective. *Results and Problems in Cell Differentiation*, 29, pp. 45–66.

Sofyan, A., Rosita, G., Price, D.J. and Birge, W.J. (2007). Cadmium uptake by *Ceriodaphnia dubia* from different exposures: Relevance to body burden and toxicity. *Environmental Toxicological and Chemistry*, 26, pp. 470–477.

Sommer, F. and Backhed, F. (2013). The gut microbiota: Masters of host development and physiology. *Nataural and Revlution Microbiology*, 11, pp. 227–238.

Sørensen, J.G., Kristensen, T.N. and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecology Letters.* 6, pp. 1025– 1037.

Steinberg, C.E.W., Ouerghemmi, N., Hermann, S., Bouchnak, R., Timofeyev, M.A. and Menzel, R. (2010). Stress by poor food quality and exposure to humic substances: *Daphnia magna* responds with oxidative stress, lifespan extension, but reduced offspring numbers. *Hydrobiologia*, 652, pp. 223-236.

Stephan, C.E., Mount, D.L., Hansen, D.J., Gentile, J.H., Chapman, G.A. and Brungs, W.A. (1985). Guidelines for deriving numerical national water quality criteria for the protection of aquatic organisms and their uses, Report # PB85-227049. Environmental Protection Agency, Washington.

Stoddard, J.L. and Harper, R. (2007). Effects of multi-generational exposure of *Daphnia magna* to copper. Ph.D. thesis. Huxley College of the Environment, Western Washington University.

Stohs, S.J. and Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology and Medicine,* 18, pp. 321–336.

Stoks, R., and McPeek, M.A. (2003). Antipredator behaviour and physiology determine *Lestes*  
species turnover along the pond-permanence gradient. *Ecology* 84: pp.3327-3338.

Stollewerk, A. (2010). The water flea *Daphnia*-a ‘new’ model system for ecology and evolution? *Journal of Biology,* 9, pp. 11-21.

Syberg, K., Elleby, A. Pedersen, H., Cedergreen, N. and Forbes, V.E. (2008). Mixture toxicity of three toxicants with similar and dissimilar modes of action to *Daphnia magna*. *Ecotoxicology and Environmental Safety*, 69, pp. 428–436.

Snyder, M.J. and Mulder, E.P. (2001). Environmental endocrine disruption in decapod crustacean larvae: Hormone titers, cytochrome P450, and stress protein responses to heptachlor exposure. *Aquatic Toxicology*, 55, 177-190.

Taipale, S., Brett, M., Pulkkinen, K. and Kainz, M. (2012). The influence of bacteria-dominated diets on *Daphnia magna* somatic growth, reproduction, and lipid composition. *FEMS Microbiology Ecology*, 82(1), pp. 50-62.

Tan, Q.-G. and Wang, W.-X. (2011). Acute toxicity of cadmium in *Daphnia* *magna* under different calcium and pH conditions: Importance of influx rate. *Environmental Science and Technology*, 45, pp. 1970–1976.

Tang, K.W. (2005). Copepods as microbial hotspots in the ocean: effects of host feeding activities on attached bacteria. *Aquatic Microbology and Ecology* 38, pp. 31–40.

Tang, B., Zhui, L. and Zhou, O. (2011). Joint effects of Penta-BDE and heavy metals on *Daphnia magna* survival, its antioxidant enzyme activities and lipid peroxidation. *Environmental Science and Enginginering* *China*, 5(1), pp. 99-110.

Tang, W.T., Turk, V. and Grossart, H.-P. (2010). Linkage between crustacean zooplankton and aquatic bacteria. *Aquatic Microbology and Ecology*, 61, pp. 261-277.

Tang, X., Gao, G., Qin, B., Zhu, L., Chao, J., Wang, J. and Yang, G. (2009). Characterization of Bacterial Communities Associated with Organic Aggregates in a Large, Shallow, Eutrophic Freshwater Lake (Lake Taihu, China). *Microbial Ecology*, 58(2), pp. 307-322.

Tatarazako, N. and Oda, S. (2007). The water flea Daphnia magna (Crustacea, Cladocera) as a test species for screening and evaluation of chemicals with endocrine disrupting effects on crustaceans. *Ecotoxicology,* 16, pp. 197-203.

Taylor, L.N., McGeer, J.C, Wood, C.M. and McDonald, D.G. (2000). Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: evaluation of chronic indicators. *Environmental and Toxicological Chemistry*, 19, pp. 2298–2308.

Todgham, A.E. and Stillman, J.H. (2013). Physiological responses to shifts in multiple environmental stressors: Relevance in a changing world, Integrative and Comparative Biology, 53(4), pp. 539–544.

Tollrian, R. (1990). Predator-induced helmet formation in *Daphnia cucullata* (Sars). *Archiv Hydrobiologia*, 119, pp. 191–196.

Tollrian, R. (1995). Predator-induced morphological defenses: Costs, life history shifts, and maternal effects in *Daphnia pulex*. *Ecology,* 76, pp. 1691–1705.

Traudt, E.M., Ranville, J.F. and Meyer, J.S. (2017). Effect of age on acute toxicity of Cd, Cu, Ni, and Zn in individual-metal exposures to Daphnia magna neonates. *Environmental Toxicology and Chemistry*, 36, pp. 113-119.

Trekels, H., Van de Meutter, F. and Stoks, R. (2013). Predator cues magnify effects of the pesticide endosulfanin water bugs in a multi-speciestestin outdoor containers. *Aquatic Toxicology*, 138−139, pp. 116−122.

Tremaroli, V. and Bäckhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature*, 489, pp. 242 −249.

Tsui, M. T. and Wang, W. X. (2007). Biokinetics and tolerance development of toxic metals in Daphnia magna. Environmental Toxicology and Chemistry, 26*,* pp. 1023–1032.

Urabe, J. and Sterner, R.W. (2001). Contrasting effects of different types of resource depletion on life-history traits in *Daphnia*. *Functional Ecol*ogy, 15, pp. 165-174.

USEPA (United States Environmental Protection Agency) (2000) (August) Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures U.S. Environmental Protection Agency, Washington, DC.

Vandegehuchte, M.B., Vandenbrouck, T., De Coninck, D., De Coen, W.M. and Janssen, C.R. (2010). Can metal stress induce transferable changes in gene transcription in *Daphnia magna*? *Aquatic Toxicology*, 97, pp. 188–195.

Van Doorslaer, W., Stoks, R., Swillen, I., Feuchtmayr, H., Atkinson, D., Moss, B. and De Meester, L. (2010). Experimental thermal microevolution in community-embedded *Daphnia* populations. *Climate Research*, 43, pp. 81-89.

Veltman, K., Huijbregts, M.A.J., Kolck, M.V., Wang, W.-X. and Jan Hendriks, A. (2008). Metal bioaccumulation in aquatic species: quantification of uptake and elimination rate constants using physicochemical properties of metals and physiological characteristics of species. *Environmental Science and Technology*, 42, pp. 852–858.

Viechtbauer, W. (2010). Conducting meta-analyses in R with the metaphor package. *Journal of Statistical Softwar*e, 36, pp. 1–48.

Vijver, M.G., Elliott, E.G., Peijnenburg, W.J.G.M. and Snoo, D. (2011). Response predictions for organisms water-exposed to metal mixtures: a meta-analysis. *Environmental and Toxicological Chemistry* 30, pp. 1482–1487.

Vilá, M., Espinar, J.L., Hejda, M., Hulme, P.E., Jarošík, V., Maron, J.L., Perg, J., Schafner, U., Sun, Y. and Pvšek, P. (2011). Ecological impacts of invasive alien plants: a meta-analysis of their effects on species, communities and ecosystems. *Ecological Letters*, 14, pp. 702–708.

Waisberg, M., Joseph, P., Hale, B. and Beyersmann, D. (2003). Molecular and cellular mechanisms of cadmium carcinogenesis. *Toxicology,* 192, pp. 95–117.

Wagner, A. and Benndorf, J. (2007). Climate-driven warming during spring destabilises a *Daphnia* population: A mechanistic food web approach. *Oecologia*, 151, pp. 351–364.

Wallace, W.G., Lee, B.-G. and Luoma, S.N. (2003). Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Marine Ecology Progress Series*, 249, pp. 183–197.

Wang, Z., Yan, C. and Zhang, X. (2009). Acute and chronic cadmium toxicity to a saltwater cladoceran *Moina monogolica* Daday and its relative importance. *Ecotoxicology* 18, pp. 47–54.

Wang, Z., Meador, J.P. and Leung, K.M.Y. (2016). Metal toxicity to freshwater organisms as a function of pH: A meta-analysis. *Chemosphere,* 144, pp. 1544–1552.

Wang, Y.D., Fang, J., Leonard, S.S. and Rao, K.M. (2004 a). Cadmium inhibits the electron transfer chain and induces reactive oxygen species. *Free Radical Biology and Medicine*, 36 (11), pp. 1434–1443.

Wang, Q.R., Kim, D., Dionysiou, D.D., Sorial, G.A. and Timberlake, D. (2004 b). Sources and remediation for mercury contamination in aquatic systems- a literature review. *Environmemtal Pollution*, 131, pp. 323–336.

Weltens, R., Goossens, R. and Puymbroeck, S.V. (2000). Ecotoxicology of contaminated suspened solids for fliter feeders (*Dahnia magna*). *Archives of Environmental Contamination and Toxicology,* 39, pp. 315–323.

Werner, E.E., Mittelbach, G.G., Hall, D.J. and Gilliam, J.F. (1983). Experimental tests of optimal habitat profitability. *Ecology*, 64, pp. 1525–1539.

Wilding, J. and Maltby, L. (2006). Relative toxicological importance of aqueous and dietary metal exposure to a freshwater crustacean: implications for risk assessment. *Environmental Toxicology and Chemistry*, 25, pp. 1795–1801.

Winner, R.W., Keeling, T., Yeager, R. and Farrell, M.P. (1977). Effect of food type on the acute and chronic toxicity of copper to *Daphnia magna. Freshwater Biology,* 7, pp. 343–349.

Wong, C.K. (1993). Effects of chromium, copper, nickel and zinc on longevity and reproduction of the cladoceran *Moina macrocopa*. *Bulletin of Environmental Contamination and Toxicology,* 50, pp. 663–639.

Woo, P.C.Y., Lau, S.K.P., Teng, J.L.L., Tse, H. and Yuen, K. (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infectection*, 14, pp. 908–934.

Wu, H. and Wang, W.-X. (2010). NMR-based metabolomic studies on the toxicological effects of cadmium and copper on green mussels *Perna viridis.* *Aquatic Toxicology,* 100, pp. 339–345.

Xie F., Koziar, S.A., Lampi, M.A., Dixon, D.G., Warren, N.P., Borgmann, U., Huang, X.D. and Greenberg, M. (2006). Assessment of the toxicity of mixtures of copper, 9, 10 phenanthrenequinone, and phenanthrene to *Daphnia magna*: evidence from a reactive oxygen mechanism. *Environmental Toxicology and Chemistry,* 25, (2), pp. 613–622.

Yan, T., Teo, L.H. and Sin, Y.M. (1996). Effects of metals on a-amylase activity in the digestive gland of the green mussel, Perna viridis L. *Bulletin of Environmental Contamnation and Toxicology*, 56, pp. 677–682.

Zalups, R.K. and Ahmad, S. (2003). Molecular handling of cadmium in transporting epithelia. *Toxicological and Applied Pharmacology,* 186, pp. 163–188.

Zhang, N., Zhou, Q., Yin, X. and Zeng, D. (2014). Trace amounts of aqueous copper (II) chloride complexes in hypersaline solutions: spectrophotometric and thermodynamic studies. *Journal of Solution Chemistry*, 43(2), pp. 326–339.

Zhao, C.-M. and Wang, W.-X. (2011). Comparison of acute and chronic toxicity of silver nanoparticles and silver nitrate to *Daphnia magna*. *Environmental Toxicology and Chemistry.* 30, (4), pp. 885–892.

Zhao, H., Chang, J., Boika, A. and Bard, A. (2013). Electrochemistry of high concentration copper chloride complexes. *Analytical Chemistry*, 85(16), pp.7696-7703.

Zhou, Q., Zhang, J., Fu, J., Shi, J. and Jiang, G. (2008). Biomonitoring: An appealing tool for assessment of metal pollution in the aquatic ecosystem. *Analytica Chimica Acta,* 606, (2), pp.135-150.

Zielinski, S. and Pörtner, H.O. (2000). Oxidative stress and antioxidative defense in cephalopods: a function of metabolic rate or age. *Comparative Biochemistry and Physiology*, B 125, pp. 147–160.

Zikic, R., Stajn, A., Saicic, Z., Spasic, M., Ziemnicki, K. and Petrovic, V. (1996). The activities of superoxide dismutase, catalase and ascorbic acid content in the liver of goldfish (Carassius auratus gibelio Bloch.) exposed to cadmium. *Physiological Research*, 45, pp. 479-481.

Zou E., and Bu, S. (1994). Acute toxicity of copper, cadmium, and zinc to the water flea, *Moina irrasa* (Cladocera). *Bulletin of Environmental Contamination and Toxicology*, 52, pp. 742–748.

**Appendix 1**

**Table 1**: Summary of a qualitative literature review (n= 446) examining the effect of Cu and Cd on life history traits across different species of Cladocera and showing the effect size’s elements including *d* values, *s* is the pooled standard deviation of the control and experimental groups, and *J* is the corrector for bias.

**Reference Metal Species Clone Trait *s* *J* *d***

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.05 0.96 - 0.21

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.08 0.95 0.63

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.12 0.95 0.92

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.05 0.96 0.87

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.08 0.95 1

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.89 0.96 0.46

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.76 0.96 0.34

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.11 0.96 0.55

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.05 0.96 0

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.06 0.96 0.31

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.04 0.96 -0.25

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.06 0.95 0.15

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.09 0.95 0.52

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.04 0.95 - 0.49

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.04 0.94 - 0.45

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.05 0.9 -0.34

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.75 0.9 -0.34

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.69 0.9 -0.34

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.54 0.9 -0.34

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.15 0.95 0.06

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.2 0.94 0.52

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.16 0.95 1.13

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.18 0.95 0.31

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.25 0.94 0.37

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.44 0.95 0.39

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.25 0.96 0.89

**Reference Metal Species Clone Trait *s* *J* *d***

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.19 0.95 0.25

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.18 0.95 0.41

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.16 0.95 0.23

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.14 0.95 - 0.76

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.24 0.93 0.29

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.17 0.92 -0.27

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.15 0.94 -0.71

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.18 0.9 -0.61

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.21 0.87 -0.51

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.67 0.99 -0.79

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.54 0.99 -0.43

Dave 1984 Aqueous Cu *D. magna* NA Number of neonates/female 0.48 0.96 -0.75

Moore and Winner 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.86 0.96 -0.44

Moore and Winner 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 1.02 0.96 1.27

Moore and Winner 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.85 0.96 1.64

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.86 0.96 -0.47

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.73 0.96 -1.93

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.91 0.96 - 1.36

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.75 0.96 -1.59

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.51 0.96 -2.52

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.53 0.96 -0.92

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.39 0.96 -3.5

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.41 0.96 -3.46

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.37 0.96 -5.89

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.75 0.96 -7.83

Belanger et al 1989 Aqueous Cu *C. dubia* NA Number of neonates/female 0.29 0.96 -7.83

Atienzar et al 2001 Aqueous Cu *D. magna* Clone 5 Number of neonates/female 0.21 0.99 -0.53

Atienzar et al 2001 Aqueous Cu *D. magna* Clone 5 Number of neonates/female 0.27 0.99 - 1.57

Atienzar et al 2001 Aqueous Cu *D. magna* Clone 5 Number of neonates/female 0.21 0.99 -3.04

Atienzar et al 2001 Aqueous Cu *D. magna* Clone 5 Number of neonates/female 0.4 0.99 -5.92

Atienzar et al 2001 Aqueous Cu *D. magna* Clone 5 Number of neonates/female 0.21 0.99 -17.81

**Reference Metal Species Clone Trait *s* *J* *d***

Khangarot and Rathore 2003 Aqueous Cu *D. magna* NA Number of neonates/female 0.16 0.9 1.23

Khangarot and Rathore 2003 Aqueous Cu *D. magna* NA Number of neonates/female 0.16 0.9 -8.1

Khangarot and Rathore 2003 Aqueous Cu *D. magna* NA Number of neonates/female 0.65 0.99 -1.2

Khangarot and Rathore 2003 Aqueous Cu *D. magna* NA Number of neonates/female 0.14 0.99 -11.2

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.67 0.96 -3.46

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.37 0.96 -5.89

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.29 0.96 -7.83

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.29 0.96 -7.83

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.21 0.99 -0.53

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.27 0.99 - 1.57

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.21 0.99 -3.0

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.4 0.99 -5.92

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.21 0.99 -7.81

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.16 0.9 1.23

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.04 0.94 - 0.45

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.32 0.9 -8.1

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.66 0.9 -18.2

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.14 0.9 -18.2

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.41 0.96 -3.46

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.37 0.96 -5.89

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.29 0.96 -7.83

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.75 0.96 -7.83

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.61 0.99 -0.53

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.37 0.99 - 1.57

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.21 0.99 -3.04

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.48 0.99 -5.92

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.21 0.99 -17.81

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.16 0.9 1.23

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.04 0.94 - 0.45

**Reference Metal Species Clone Trait *s* *J* *d***

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.67 0.9 -3.11

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.34 0.9 -0.2

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.14 0.9 -1.12

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.41 0.96 -3.46

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.37 0.96 -5.89

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.29 0.96 -7.83

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.29 0.96 -7.83

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.89 0.99 -0.53

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.27 0.99 - 1.57

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.28 0.99 -3.04

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.4 0.99 -5.92

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.21 0.99 -17.81

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.16 0.9 1.23

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.04 0.94 - 0.45

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.76 0.9 -8.1

Bossuyt and Janssen2003 Aqueous Cu *D. magna* Clone K6 Number of neonates/female 0.34 0.9 -0.2

DeSchamphelaere and Janssen2004 Aqueous Cu *D. magna* NA Number of neonates/female 0.54 0.9 -0.28

DeSchamphelaere and Janssen2004 Aqueous Cu *D. magna* NA Number of neonates/female 0.41 0.96 -3.46

DeSchamphelaere and Janssen2004 Aqueous Cu *D. magna* NA Number of neonates/female 0.37 0.96 -5.89

DeSchamphelaere and Janssen2004 Aqueous Cu *D. magna* NA Number of neonates/female 0.29 0.96 -7.83

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.29 0.96 -3.17

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.66 0.99 -0.53

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.27 0.99 - 1.57

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.21 0.99 -3.04

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.34 0.99 -5.92

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.71 0.99 -7.81

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.16 0.9 1.23

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.04 0.94 - 0.45

**Reference Metal Species Clone Trait *s* *J* *d***

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.89 0.9 -3.1

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.74 0.9 -18.2

Hoang et al., 2007 Aqueous Cu *D. magna* NA Number of neonates/female 0.88 0.9 -18.2

Wang et al., 2007 Aqueous Cu *M. monogolica* NA Number of neonates/female 0.41 0.96 -3.46

Wang et al., 2007 Aqueous Cu *M. monogolica* NA Number of neonates/female 0.37 0.96 -5.89

Wang et al., 2007 Aqueous Cu *M. monogolica* NA Number of neonates/female 0.01 0.96 -7.83

Wang et al., 2007 Aqueous Cu *M. monogolica* NA Number of neonates/female 0.39 0.96 -3.17

Wang et al., 2007 Aqueous Cu *M. monogolica* NA Number of neonates/female 0.21 0.99 -0.53

Cooper et al., 2009 Aqueous Cu *C. dubia* NA Number of neonates/female 0.89 0.99 - 1.57

Cooper et al., 2009 Aqueous Cu *C. dubia* NA Number of neonates/female 0.76 0.99 -3.04

Cooper et al., 2009 Aqueous Cu *C. dubia* NA Number of neonates/female 0.4 0.99 -5.92

Cooper et al., 2009 Aqueous Cu *C. dubia* NA Number of neonates/female 0.36 0.99 -7.81

Cooper et al., 2009 Aqueous Cu *C. dubia* NA Number of neonates/female 0.16 0.9 1.23

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.67 0.94 - 0.45

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.65 0.99 -3.11

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.43 0.9 -18.2

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.78 0.9 -18.2

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.62 0.96 -3.46

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.37 0.96 -5.89

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.67 0.96 -7.83

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.44 0.96 -3.17

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.76 0.99 -0.53

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.85 0.99 - 1.57

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.34 0.99 -3.04

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.11 0.99 -5.92

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.54 0.99 -7.81

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.87 0.9 1.23

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.04 0.94 - 0.45

**Reference Metal Species Clone Trait *s* *J* *d***

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.23 0.9 -5.1

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.54 0.9 -11.2

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.14 0.9 -1.6

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Number of neonates/female 0.41 0.96 -3.46

Luciana et al., 2014 Aqueous Cu *D. magna* NA Number of neonates/female 0.21 0.99 -0.53

Luciana et al., 2014 Aqueous Cu *D. magna* NA Number of neonates/female 0.29 0.96 -7.83

Luciana et al., 2014 Aqueous Cu *D. magna* NA Number of neonates/female 0.29 0.96 -3.17

Luciana et al., 2014 Aqueous Cu *D. magna* NA Number of neonates/female 0.21 0.99 -0.53

Luciana et al., 2014 Aqueous Cu *D. magna* NA Number of neonates/female 0.27 0.99 - 1.57

Luciana et al., 2014 Aqueous Cu *D. magna* NA Number of neonates/female 0.21 0.99 -3.04

Luciana et al., 2014 Aqueous Cu *D. magna* NA Number of neonates/female 0.4 0.99 -5.92

Luciana et al., 2014 Aqueous Cu *D. magna* NA Number of neonates/female 0.21 0.99 -7.81

Luciana et al., 2014 Aqueous Cu *D. magna* NA Number of neonates/female 0.16 0.9 1.23

Luciana et al., 2014 Aqueous Cu *C. dubia* NA Number of neonates/female 0.04 0.94 - 0.45

Luciana et al., 2014 Aqueous Cu *C. dubia* NA Number of neonates/female 0.76 0.9 -2.1

Luciana et al., 2014 Aqueous Cu *C. dubia* NA Number of neonates/female 0.04 0.94 - 0.45

Luciana et al., 2014 Aqueous Cu *C. dubia* NA Number of neonates/female 0.14 0.9 -18.2

Luciana et al., 2014 Aqueous Cu *C. dubia* NA Number of neonates/female 0.41 0.96 -3.46

Luciana et al., 2014 Aqueous Cu *C. dubia* NA Number of neonates/female 0.21 0.99 -0.53

Luciana et al., 2014 Aqueous Cu *C. dubia* NA Number of neonates/female 0.29 0.96 -7.83

Luciana et al., 2014 Aqueous Cu *C. dubia* NA Number of neonates/female 0.29 0.96 -3.17

Luciana et al., 2014 Aqueous Cu *C. dubia* NA Number of neonates/female 0.21 0.99 -0.53

DeSchamphelaere and Janssen 2004 Dietary Cu *D. magna* NA Number of neonates/female 0.06 0.96 1.02

DeSchamphelaere and Janssen 2004 Dietary Cu *D. magna* NA Number of neonates/female 0.21 0.99 -3.04

DeSchamphelaere and Janssen 2004 Dietary Cu *D. magna* NA Number of neonates/female 0.4 0.99 -5.92

DeSchamphelaere and Janssen 2004 Dietary Cu *D. magna* NA Number of neonates/female 0.21 0.99 -7.81

DeSchamphelaere and Janssen 2004 Dietary Cu *D. magna* NA Number of neonates/female 0.16 0.9 1.23

DeSchamphelaere and Janssen 2004 Dietary Cu *D. magna* NA Number of neonates/female 0.04 0.94 - 0.45

**Reference Metal Species Clone Trait *s* *J* *d***

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.11 0.9 -3.1

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.36 0.9 -0.2

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.14 0.9 -4.2

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.41 0.96 -3.46

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.21 0.99 -0.53

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.29 0.96 -7.83

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.29 0.96 -3.17

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.21 0.99 -0.53

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.27 0.99 - 1.57

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.21 0.99 -3.04

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.4 0.99 -5.92

Hauri and Horne 2004 Dietary Cu *C. dubia*  NA Number of neonates/female 0.21 0.99 -7.81

De Schamphelaere et al., 2007 Dietary Cu *D. magna* NA Number of neonates/female 0.16 0.9 1.23

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Age at maturity 1.19 0.96 0.74

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Age at maturity 1.67 0.96 1.52

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Age at maturity 2.24 0.96 2.82

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Age at maturity 2.55 0.96 2.95

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Age at maturity 1.14 0.97 0.28

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Age at maturity 1.11 0.97 -0.62

Koivisto and Ketola 1995 Aqueous Cu *D. pulex*  NA Age at maturity 0.68 0.95 0.58

Koivisto and Ketola 1995 Aqueous Cu *D. pulex*  NA Age at maturity 0.64 0.95 0.03

Koivisto and Ketola 1995 Aqueous Cu *D. pulex*  NA Age at maturity 0.74 0.95 0.28

Koivisto and Ketola 1995 Aqueous Cu *D. pulex*  NA Age at maturity 0.72 0.95 1.09

Koivisto and Ketola 1995 Aqueous Cu *D. pulex*  NA Age at maturity 0.7 0.95 0.84

Koivisto and Ketola 1995 Aqueous Cu *B. longirostris* NA Age at maturity 0.62 0.97 0.32

Koivisto and Ketola 1995 Aqueous Cu *B. longirostris* NA Age at maturity 0.68 0.97 0.16

Koivisto and Ketola 1995 Aqueous Cu *B. longirostris* NA Age at maturity 0.51 0.97 0.57

Koivisto and Ketola 1995 Aqueous Cu *B. longirostris* NA Age at maturity 1.66 0.97 0.48

**Reference Metal Species Clone Trait *s* *J* *d***

Koivisto and Ketola 1995 Aqueous Cu *B. longirostris* NA Age at maturity 0.76 0.97 1.05

Atienzar et al., 2001 Aqueous Cu *D. magna*  NA Age at maturity 0 0.99 0

Atienzar et al., 2001 Aqueous Cu *D. magna*  NA Age at maturity 0 0.99 0

Atienzar et al., 2001 Aqueous Cu *D. magna*  NA Age at maturity 0 0.99 0

Atienzar et al., 2001 Aqueous Cu *D. magna*  NA Age at maturity 0 0.99 0

Atienzar et al., 2001 Aqueous Cu *D. magna*  NA Age at maturity 0 0.99 0

Ponti et al., 2010 Aqueous Cu *D. galeata*  NA Age at maturity 2.21 0.95 -0.65

Ponti et al., 2010 Aqueous Cu *D. galeata*  NA Age at maturity 1.46 0.95 -0.79

Ponti et al., 2010 Aqueous Cu *D. galeata*  NA Age at maturity 1.74 0.98 0.09

Ponti et al., 2010 Aqueous Cu *D. galeata*  NA Age at maturity 1.38 0.98 -0.03

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.76 0.98 -0.06

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.48 0.98 0

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.82 0.98 0.09

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.94 0.98 0.74

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.11 0.98 0.26

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.05 0.98 0.29

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.64 0.98 0.26

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 3.85 0.98 0.85

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 2.05 0.98 0.18

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.42 0.98 -0.3

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.42 0.98 -0.18

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.49 0.98 -0.38

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.82 0.98 1.01

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.1 0.98 0.31

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 0.27 0.98 2.05

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.39 0.98 0.63

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.81 0.98 0.54

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 1.56 0.98 1.22

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 0.7 0.97 0.11

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 0.94 0.97 1.03

**Reference Metal Species Clone Trait *s* *J* *d***

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Age at maturity 0.23 0.99 -5.92

Winner 1981 Aqueous Cu *D. magna*  NA Somatic growth rate 0.05 0.9 0.11

Winner 1981 Aqueous Cu *D. magna*  NA Somatic growth rate 0.47 0.96 0.31

Winner 1981 Aqueous Cu *D. magna*  NA Somatic growth rate 0.41 0.96 -3.46

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -0.53

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Somatic growth rate 0.29 0.96 -7.83

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Somatic growth rate 0.29 0.96 -3.17

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -0.53

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Somatic growth rate 0.27 0.99 - 0.57

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -3.04

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Somatic growth rate 0.4 0.99 -5.92

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -7.81

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Somatic growth rate 0.16 0.96 1.23

Flickinger et al., 1982 Aqueous Cu *D. magna*  NA Somatic growth rate 0.01 0.99 0.04

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.34 0.99 0.5

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.13 0.9 -1.62

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.05 0.9 0.11

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.47 0.96 0.31

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.41 0.96 -3.46

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -0.53

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.29 0.96 -7.83

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.29 0.96 -3.17

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -0.53

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.27 0.99 - 0.57

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -3.04

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.4 0.99 -5.92

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -7.81

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.16 0.96 1.23

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.01 0.99 0.04

**Reference Metal Species Clone Trait *s* *J* *d***

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.07 0.96 0.32

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.13 0.9 0.91

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.45 0.96 0.31

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.41 0.96 -1.46

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -0.53

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.29 0.96 -7.83

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.29 0.96 -3.17

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -0.53

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.27 0.99 - 0.57

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -3.04

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.4 0.99 -5.92

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.21 0.99 -7.81

Dave 1984 Aqueous Cu *D. magna*  NA Somatic growth rate 0.16 0.96 1.23

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.31 0.99 -0.53

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.11 0.99 1.01

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.48 0.96 0.12

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.18 0.96 0.63

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.45 0.96 0.31

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.41 0.96 -1.46

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.21 0.99 -0.53

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.29 0.96 -7.83

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.29 0.96 -3.17

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.21 0.99 -0.53

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.27 0.99 - 0.57

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.21 0.99 -3.04

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.4 0.99 -5.92

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.21 0.99 -7.81

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.16 0.96 1.23

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.31 0.99 -0.53

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.11 0.99 1.01

**Reference Metal Species Clone Trait *s* *J* *d***

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.26 0.96 - 0.44

Agra et al., 2011 Aqueous Cu *D. longispina* R Clones Somatic growth rate 0.57 0.96 0.83

Rocha et al., 2016 Aqueous Cu *D. laevis*  NA Somatic growth rate 0.27 0.99 1.16

Rocha et al., 2016 Aqueous Cu *D. laevis*  NA Somatic growth rate 0.46 0.99 -1.37

Van Leeuwen et al., 1985 Aqueous Cd *D. magna*  NA Number of neonates/female 0.11 0.99 1.48

Van Leeuwen et al., 1985 Aqueous Cd *D. magna*  NA Number of neonates/female 0.07 0.96 -1.28

Van Leeuwen et al., 1985 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -0.53

Van Leeuwen et al., 1985 Aqueous Cd *D. magna*  NA Number of neonates/female 0.29 0.96 -7.83

Van Leeuwen et al., 1985 Aqueous Cd *D. magna*  NA Number of neonates/female 0.29 0.96 -3.17

Van Leeuwen et al., 1985 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -0.53

Van Leeuwen et al., 1985 Aqueous Cd *D. magna*  NA Number of neonates/female 0.27 0.99 - 0.57

Van Leeuwen et al., 1985 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -3.04

Van Leeuwen et al., 1985 Aqueous Cd *D. magna*  NA Number of neonates/female 0.4 0.99 -5.92

Van Leeuwen et al., 1985 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -7.81

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.27 0.99 -3.2

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.56 0.9 1.6

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.73 0.9 -18.2

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.41 0.96 -3.46

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -0.53

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.29 0.96 -7.83

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.29 0.96 -3.17

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -0.53

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.27 0.99 - 1.57

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -3.04

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.4 0.99 -5.92

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -7.81

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.16 0.9 1.23

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.04 0.94 - 0.45

**Reference Metal Species Clone Trait *s* *J* *d***

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.69 0.99 4.13

Knowles and Mckee 1987 Aqueous Cd *D. magna*  NA Number of neonates/female 0.75 0.9 1.7

Bodar et al., 1988 Aqueous Cd *D. magna*  NA Number of neonates/female 0.43 0.96 0.62

Bodar et al., 1988 Aqueous Cd *D. magna*  NA Number of neonates/female 0.41 0.96 -3.46

Bodar et al., 1988 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -0.53

Bodar et al., 1988 Aqueous Cd *D. magna*  NA Number of neonates/female 0.29 0.96 -7.83

Barata and Baird 2000 Aqueous Cd *D. magna*  NA Number of neonates/female 0.29 0.96 -3.17

Barata and Baird 2000 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -0.53

Barata and Baird 2000 Aqueous Cd *D. magna*  NA Number of neonates/female 0.27 0.99 - 1.57

Barata and Baird 2000 Aqueous Cd *D. magna*  NA Number of neonates/female 0.21 0.99 -3.04

Sofyan et al., 2007a Aqueous Cd *C. dubia*  NA Number of neonates/female 0.4 0.99 -5.92

Sofyan et al., 2007a Aqueous Cd *C. dubia* NA Number of neonates/female 0.21 0.99 -7.81

Sofyan et al., 2007a Aqueous Cd *C. dubia*  NA Number of neonates/female 0.16 0.9 1.23

Wang et al., 2009 Aqueous Cd *M. monogolica* NA Number of neonates/female 0.03 0.96 - 0.15

Wang et al., 2009 Aqueous Cd *M. monogolica* NA Number of neonates/female 0.01 0.99 1.03

Wang et al., 2009 Aqueous Cd *M. monogolica* NA Number of neonates/female 0.61 0.9 -1.56

Sofyan et al., 2007a Dietary Cd *C. dubia*  NA Number of neonates/female 0.39 0.96 0.12

Sofyan et al., 2007a Dietary Cd *C. dubia*  NA Number of neonates/female 0.43 0.96 0.62

Sofyan et al., 2007a Dietary Cd *C. dubia* NA Number of neonates/female 0.41 0.96 -3.46

Sofyan et al., 2007a Dietary Cd *C. dubia* NA Number of neonates/female 0.21 0.99 -0.53

Sofyan et al., 2007a Dietary Cd *C. dubia* NA Number of neonates/female 0.29 0.96 -7.83

Sofyan et al., 2007a Dietary Cd *C. dubia* NA Number of neonates/female 0.29 0.96 -3.17

Sofyan et al., 2007 b Dietary Cd *C. dubia* NA Number of neonates/female 0.21 0.99 -0.53

Sofyan et al., 2007 b Dietary Cd *C. dubia* NA Number of neonates/female 0.27 0.99 - 1.57

Sofyan et al., 2007 b Dietary Cd *C. dubia* NA Number of neonates/female 0.21 0.99 -3.04

Sofyan et al., 2007 b Dietary Cd *C. dubia* NA Number of neonates/female 0.4 0.99 -5.92

Sofyan et al., 2007 b Dietary Cd *C. dubia* NA Number of neonates/female 0.21 0.99 -7.81

Sofyan et al., 2007 b Dietary Cd *C. dubia* NA Number of neonates/female 0.16 0.9 1.23

Sofyan et al., 2006 Dietary Cd *C. dubia* NA Number of neonates/female 0.03 0.96 - 0.15

Sofyan et al., 2006 Dietary Cd *C. dubia* NA Number of neonates/female 0.11 0.9 1.03

**Reference Metal Species Clone Trait *s* *J* *d***

Sofyan et al., 2006 Dietary Cd *C. dubia* NA Number of neonates/female 0.47 0.99 -1.22

Sofyan et al., 2006 Dietary Cd *C. dubia* NA Number of neonates/female 0.21 0.99 -7.81

Sofyan et al., 2006 Dietary Cd *C. dubia* NA Number of neonates/female 0.16 0.9 1.23

Sofyan et al., 2006 Dietary Cd *C. dubia* NA Number of neonates/female 0.03 0.96 - 0.15

Geffard et al., 2008 Dietary Cd *D. magna* NA Number of neonates/female 0.61 0.99 0.46

Geffard et al., 2008 Dietary Cd *D. magna* NA Number of neonates/female 0.47 0.96 -0.18

Geffard et al., 2008 Dietary Cd *D. magna* NA Number of neonates/female 0.58 0.99 0.56

Wang et al., 2010 Dietary Cd *M. monogolica* NA Number of neonates/female 0.13 0.96 - 0.55

Wang et al., 2010 Dietary Cd *M. monogolica* NA Number of neonates/female 0.52 0.96 - 0.17

Wang et al., 2010 Dietary Cd *M. monogolica* NA Number of neonates/female 0.36 0.96 - 0.83

Barata et al., 2000 Aqueous Cd *D. magna*  NA Age at maturity 0.62 0.99 -0.5

Barata et al., 2000 Aqueous Cd *D. magna*  NA Age at maturity 0.47 0.96 -0.28

Barata et al., 2000 Aqueous Cd *D. magna*  NA Age at maturity 0.56 0.96 -1.65

Barata et al., 2000 Aqueous Cd *D. magna*  NA Age at maturity 0.67 0.96 -1.107

Barata et al., 2000 Aqueous Cd *D. magna*  NA Age at maturity 0.53 0.96 -0.18

Barata et al., 2000 Aqueous Cd *D. magna*  NA Age at maturity 0.13 0.96 - 0.55

Barata et al., 2000 Aqueous Cd *D. magna*  NA Age at maturity 0.52 0.96 - 0.17

Barata et al., 2000 Aqueous Cd *D. magna*  NA Age at maturity 0.36 0.96 - 0.83

Barata et al., 2000 Aqueous Cd *D. magna*  NA Age at maturity 0.62 0.99 -0.5

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.58 0.99 0.56

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.21 0.99 -3.04

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.27 0.99 - 1.57

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.41 0.96 -3.46

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.62 0.99 -0.5

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.21 0.99 -0.53

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.47 0.96 -0.18

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.34 0.99 -3.04

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.13 0.96 - 0.55

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.03 0.96 - 0.15

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.76 0.99 -0.38

**Reference Metal Species Clone Trait *s* *J* *d***

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.03 0.96 - 0.15

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.62 0.99 -0.5

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.21 0.99 -0.53

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.41 0.96 -3.46

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.21 0.99 -3.04

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.03 0.96 - 0.45

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.47 0.96 -0.18

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.03 0.96 - 0.15

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.58 0.99 0.56

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.03 0.96 - 0.15

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.13 0.96 - 0.55

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.27 0.99 - 1.57

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.34 0.99 -3.04

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.75 0.9 1.7

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.21 0.99 -7.81

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.21 0.99 -3.04

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.03 0.96 - 0.16

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.41 0.96 -3.46

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.62 0.99 -0.5

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.21 0.99 -0.53

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Age at maturity 0.27 0.99 - 1.57

Van Leeuwen et al., 1985 Aqueous Cd *D. magna* NA Somatic growth rate 0.13 0.96 - 0.55

Van Leeuwen et al., 1985 Aqueous Cd *D. magna* NA Somatic growth rate 0.47 0.96 -0.18

Van Leeuwen et al., 1985 Aqueous Cd *D. magna* NA Somatic growth rate 0.21 0.99 -3.04

Van Leeuwen et al., 1985 Aqueous Cd *D. magna* NA Somatic growth rate 0.03 0.96 - 0.15

Van Leeuwen et al., 1985 Aqueous Cd *D. magna* NA Somatic growth rate 0.03 0.96 - 0.15

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.21 0.99 -7.81

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.56 0.96 -1.65

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.75 0.9 1.7

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.34 0.99 -3.04

**Reference Metal Species Clone Trait *s* *J* *d***

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.13 0.96 - 0.55

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.62 0.99 -0.5

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.21 0.99 -3.04

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.03 0.96 - 0.15

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.75 0.9 1.7

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.67 0.96 - 0.34

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.21 0.99 -0.53

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.58 0.99 0.56

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.47 0.96 -0.18

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.34 0.99 -3.04

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.41 0.96 -3.46

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.27 0.99 - 1.57

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.03 0.96 - 0.15

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.87 0.99 -5.92

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.56 0.96 -1.65

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.41 0.96 -3.46

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.62 0.99 -0.5

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.34 0.99 -3.04

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.03 0.96 - 0.15

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.27 0.99 - 1.57

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.13 0.96 - 0.55

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.21 0.99 -0.53

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.47 0.96 -0.18

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.03 0.96 - 0.15

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.58 0.99 0.56

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.31 0.99 -5.92

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.56 0.96 -1.65

Barata et al., 2002 Aqueous Cd *M. macleayi* NA Somatic growth rate 0.21 0.99 -7.81

Shaw et al., 2007 Aqueous Cd *D. pulex* NA Somatic growth rate 0.03 0.96 - 0.15

Shaw et al., 2007 Aqueous Cd *D. pulex* NA Somatic growth rate 0.75 0.9 1.7

**Reference Metal Species Clone Trait *s* *J* *d***

Shaw et al., 2007 Aqueous Cd *D. pulex* NA Somatic growth rate 0.56 0.99 - 1.23

Shaw et al., 2007 Aqueous Cd *D. pulex* NA Somatic growth rate 0.41 0.96 -3.46

Shaw et al., 2007 Aqueous Cd *D. pulex* NA Somatic growth rate 0.13 0.96 - 0.55

Coninck et al., 2014 Aqueous Cd *D. pulex* NA Somatic growth rate 0.62 0.99 -0.5

Coninck et al., 2014 Aqueous Cd *D. pulex* NA Somatic growth rate 0.23 0.96 - 0.17

**Appendix 2**

**Nominal and actual concentrations of copper (Chapters 3-6)**

Throughout the work using copper, nominal concentrations of 5, 10 and 25 µg/l of copper (II) chloride dihydrate (Fisher Scientific UK C/7920/48) was delivered in aqueous solution. To prepare metals stock solutions, analytical grade dihydrous copper was dissolved in distilled water.

Chronic exposure tests were performed in accordance with the standard protocol OECD Daphnia *sp*., No. 212 (OECD, 2002). Neonates of *D. pulex* (<24h), each individual in 100 mL glass jar containing 50 mL of Hard ASTM, were exposed to the metal over 21 days.

We used ICP-MS (Inductively coupled plasma mass spectrometry) to assay control and standard solutions prepared in glass jars. Realised concentrations were consistently lower than nominal concentrations but related very well by a linear transformation. The best fitting regression line predicts realised concentration = 1.33 + 0.19 \* nominal concentration (Fig 1; r2 = 0.6, F = 46.5, p < 0.001, data presented as mean ± se, regression fit to raw data (n = 3 replicates)).

While concentrations are lower, the data do indicate a gradient of concentrations and the multiple dose response experiments reveal substantial differences in traits arising from these concentration differences.

It is well known that glass can have an affinity for metals in solution (Eichholz et al., 1965; Struempler 1973; Steinhauser and Bichler, 2008), and we believe that it is likely that Cu has adsorbed to the glass leading to lower realized concentrations than nominal. We also note that chronic exposure experiments replace the treatment solutions daily such that organisms are accumulating exposure over their lifetime.



**Fig. 1**. Actual vs. nominal concentrations of Cu

**Appendix 3**

**Table (2):** Mean acute metals toxicity (µg/l) (Effective concentration; 24 and 48-h EC50; *n* =50) in different clones of *Daphnia pulex.*

**Metal Clone 24-h EC50 28-h EC50**

Copper D86A 36.9 33.4

D84A 35.1 31.7

LD33 35.2 32.5

Cadmium D86A 193.4 111.32

D84A 191.6 113.84

LD33 197.4 109.23