“Valeu a pena? Tudo vale a pena

Se a alma não é pequena”

“Was it worth it? Everything is worthy

If the soul is not small.”

Fernando Pessoa

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The effect of temperature on metal toxicity and toxicokinetics in the aquatic model organism *Daphnia magna*

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Cecília

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List of abbreviations

$[Metal]_{daphnia}$	Internal metal concentrations in *Daphnia magna*
$[Ni]_{daphnia}$	Internal Ni concentrations in *Daphnia magna*
ABC	Approximate Bayesian Computation
AIC	Akaike information criterion
ATP	Adenosine triphosphate
BLM	Biotic ligand model
DEB-IBM	Dynamic Energy Budget theory and Individual-Based Modelling
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EC10	Effect concentration resulting in 10% effect
$EC10_{F0}$	EC10 for reproduction of Ni for F0
EC20	Effect concentration resulting in 20% effect
EC50	Effect concentration resulting in 50% effect
$EC50_{F0}$	EC50 for reproduction of Ni for F0
ECx	Effect concentrations
ERA	Ecological risk assessment
F0	First generation
F1	Second generation
F2	Third generation
F3	Fourth generation
FA	Fulvic acid
Fx	Generation
GEE	Generalized estimation equation
HR-ICP-MS	High Resolution - Inductively Coupled Plasma - Mass Spectrometry
ICP-OES	Inductively Coupled Plasma - Optical-Emission Spectrometry
k_e	Elimination rate constant
$k_{e,absence}$	The k_e in the absence of Ni
$k_{e,presence}$	The k_e in the presence of Ni
k_u	Uptake rate constant
$k_{u,48h}$	The k_u estimated after 48h of exposure to Ni

$k_{u,I}$	The k_u estimated with the data from part I of the experiment
$k_{u,init}$	The initial k_u estimated with the data points from 0 to 24 h of exposure to Ni
^{Nat}Ni	Ni with a natural isotope ratio
OC	Organic carbon
OECD	Organization for Economic Co-operation and Development
PMoAs	Physiological mode of actions
QQ	Quantile-quantile
R_0	Number of offspring per individual female
Rep3	Number of offspring per individual female produced until more than 50% of the organisms in control treatment released the third brood
Rep5	Number of offspring per individual female produced until more than 50% of the organisms in control treatment released the third brood
SD	Standard deviation
SE	Standard error
SSD	Species sensitivity distribution
SSE	Sum of squared errors
TCF	Temperature correction factor

Chapter 1

General introduction and conceptual framework

1. General introduction and conceptual framework

For human society it is crucial to preserve the good state of aquatic ecosystems. Aquatic ecosystems provide crucial resources as drinking water, water for agriculture uses, for industries and for recreation activities. However, aquatic ecosystems are suffering constant threats from human activities. Human activities are introducing in the aquatic environment stressors as metals, excess nutrients and pesticides.

Effects of natural and anthropogenic stressors to ecosystems are studied in ecotoxicology. Research in aquatic toxicology, a sub discipline in ecotoxicology, provides data for risk assessment of chemicals and water quality criteria. The goal of ecological risk assessment (ERA) is the protection of populations and ecosystems (EEA 2008). However, ERA is usually based on apical endpoints (e.g. generally survival and reproduction of single individuals) measured within a single generation at a single temperature. By doing so, the effects at the population-level are disregarded and the potential influence of temperature on chemical toxicity is not taken into account. To our knowledge no guideline for the testing of chemicals considers temperature as a factor. Yet, temperature is an abiotic factor of high importance that has a direct impact on water quality, species abundance and distribution (van Vliet *et al*. 2011).

This thesis is conducted in the field of aquatic toxicology. The focus of this thesis was to explore the effect of temperature on metal toxicity to *Daphnia magna*. Several approaches were followed from single generational (21-d life table experiments) and multigenerational tests assessing apical endpoints to a population experiment (assessing total abundance and population structure). The research of the present thesis also investigated the effects of temperature on metal toxicokinetic processes.

1.1 Temperature

Temperature fluctuations shape ecosystems over time. Temperature changes over geological time periods. It varies according with geographical region, along seasons and even can change according to the period of the day (Willmer *et al*.

2004). European surface water temperatures along the year vary for example from 3.8 to 22.7°C in Flanders in Belgium, from 7.9 to 27.0°C in Alentejo in Portugal and from 0.1 to 16.5°C in Västerbotten in Sweden (EEA 2014). Climate projections predict an increase in high temperature extremes (Dokken *et al*. 2014) and water temperatures in Europe are predicted to increase by 2°C due to climate change (van Vliet *et al*. 2013).

All chemical and biochemical reactions are temperature dependent (Willmer *et al*. 2004). Therefore, temperature is an abiotic factor of high importance for all organisms but especially for ectothermic organisms. Ectothermic organisms cannot regulate their own temperature. Therefore, the environmental temperature determines their body temperature and consequently their metabolic rate (Figure 1.1) (Heugens *et al*. 2001; Sokolova and Lannig 2008). In aquatic ecosystems, more than 95% of the species are estimated to be ectothermic (Willmer *et al*. 2004).

Species vary in their thermal tolerance range. For example, ectothermic organisms can be found between temperatures of -1.6 to 44.0°C (Willmer *et al*. 2004). Also, different populations and even different individuals from the same population can have different thermal tolerances (Carvalho 1987; Mitchell and Lampert 2000).

Across species, individuals can acclimate to environmental changes. However, organisms need time to acclimate to the environmental change in order to restore their homeostatic balance (Williams *et al*. 2012). The homeostatic balance can be defined as the tendency of organism to maintain some or all of the components of their internal environment close to the original level, irrespective of external conditions (Willmer et al. 2004). Acclimation processes increase the possibilities of the individual to survive and consequently also increase the chance for persistence of the whole population (Paul *et al*. 2004a; Paul *et al*. 2004b; Lagerspetz 2006; Ziarek *et al*. 2011).

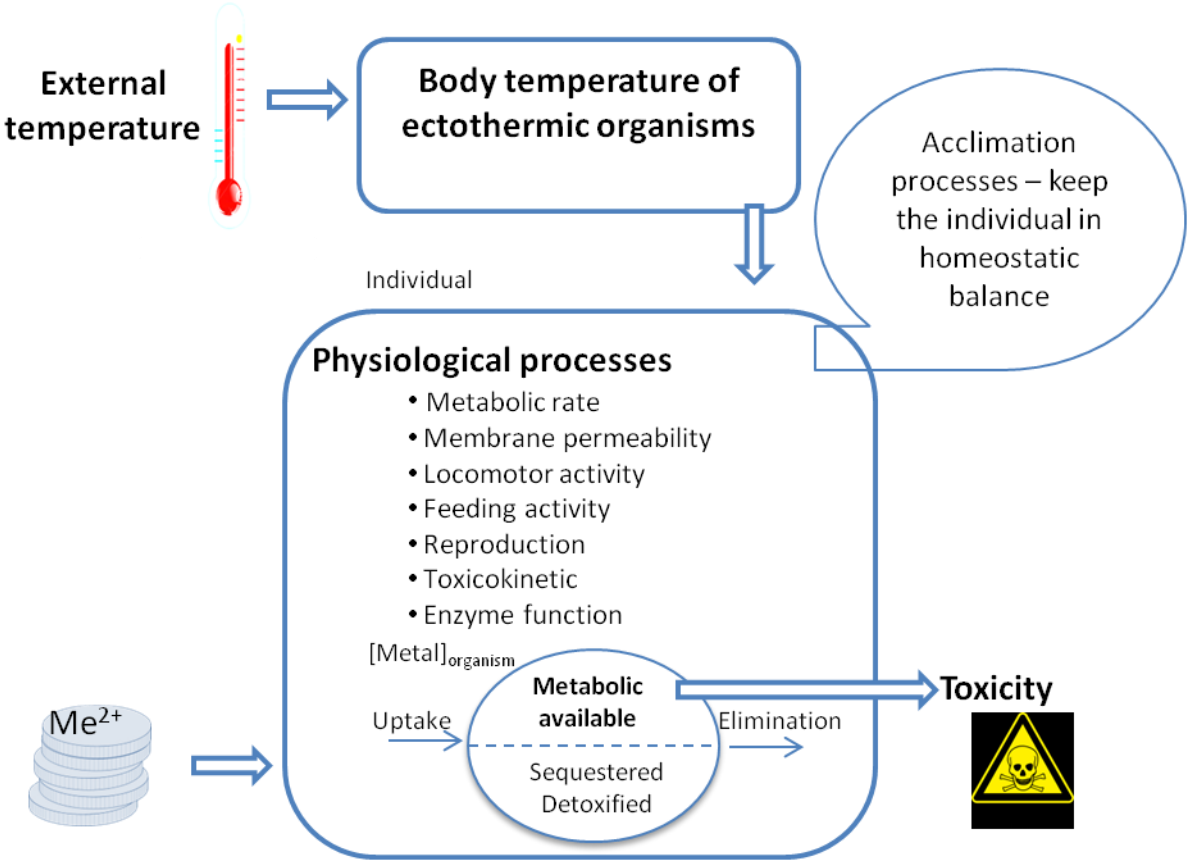

Figure 1.1. Conceptual diagram of possible interaction between temperature and metal toxicity.

1.1.1 Temperature and metal toxicity

Temperature influences the physiological processes of organisms. The increase of metabolic rate due to the increase of temperature can affect homeostatic processes of organisms, including energy demand and oxygen supply to tissues (Paul *et al*. 2004a; Paul *et al*. 2004b; Vergauwen *et al*. 2010). The effect of temperature on these physiological processes can indirectly determine the response of the organisms to other stressors e.g. the increase of temperature increases the energy demand of the organisms (Paul *et al*. 2004a) and therefore the available energy that could be used in detoxification processes. When one of these physiological processes is also the target of a chemical stressor then temperature and chemical toxicity interact (Heugens *et al*. 2001; Heugens *et al*. 2003; Mubiana and Blust 2007; Muyssen *et al*. 2010). Generally, the increase of temperature is associated with the increase of metal toxicity (Heugens *et al*. 2001; Sokolova and Lannig 2008). Sokolova and Lannig (2008) showed that the increase of temperature increased toxicant-induced mortality in 80% of the 115 studies available in aquatic

ectothermic organisms. However, the majority of the studies available focused on acute toxicity and acute exposure to temperature but the effect of temperature on metal toxicity depends on how much the organism is stressed by temperature (Willmer *et al*. 2004). Therefore, the effect of temperature on aquatic metal toxicity in situations where organisms are in homeostatic balance with the environment temperature is still unknown.

1.1.2 Temperature and metal toxicokinetics

For several biological processes the presence of a certain metal at a certain concentration is essential. However, if an excess of an essential metal or non-essential metal accumulates, their concentration and distribution needs to be regulated to prevent adverse effects (Luoma and Rainbow 2008). Toxicokinetic processes (i.e. uptake, elimination, detoxification and sequestration processes) regulate the internal metal concentrations in the organism and therefore determine metal toxicity (Figure 1.1) (Vijver *et al*. 2004; Rainbow 2007).

In the organism, the total internal metal concentration can be divided in two sub-compartments (Vijver *et al*. 2004; Luoma and Rainbow 2008). In the first sub-compartment the metal is metabolically available. The metabolic available metal can bind to target sites as proteins and DNA (deoxyribonucleic acid). In case of an essential metal, the metal has an essential role in the organism as for example activation of enzymes. However, if an excess of an essential metal or non-essential metal accumulates it may have a toxic effect to the organism (e.g. binding to target sites and preventing these from functioning in their normal metabolic role) and therefore it should be detoxified. In the second sub-compartment the detoxified metal is stored and it can or cannot be eliminated in the organism depending on the elimination strategy of the organism (Vijver *et al*. 2004; Luoma and Rainbow 2008).

Temperature can influence toxicokinetic processes by affecting chemical and biochemical reactions, membrane permeability, metabolic rate, reproduction, locomotors and feeding activity of an organism (Heugens *et al*. 2001; Willmer *et al*. 2004). The increase of temperature has been associated with the increase of uptake rates and with the increase of total internal metal concentration in the organism. Also, the increase of acute metal toxicity due to the increase of temperature has been

related with the increase of metal uptake rates (Heugens *et al*. 2001; Heugens *et al*. 2003; Sokolova and Lannig 2008). However, the information available about the influence of temperature on metal toxicokinetic processes when organisms are in homeostatic balance with the environmental temperature is scarce.

1.2 Multigenerational effects

Ecological risk assessment is often based on single generation ecotoxicological laboratory experiments. However, organisms in the field are often exposed to chemical stressors along multiple generations. Therefore, the potential toxic effects that may emerge across generations are assumed and this represents an uncertainty of the environmental quality standards.

Several studies showed that chemical toxicity can increase (Alonzo *et al*. 2008; Oliveira *et al*. 2009; Massarin *et al*. 2010; Bundschuh *et al*. 2012; Guo *et al*. 2012; Kim *et al*. 2012; Campos *et al*. 2016) decrease (Muyssen and Janssen 2004; Brausch and Salice 2011) or may not change along generations (Brennan *et al*. 2006). For example, the effect of 4-nonylphenol (detergent), piperonyl butoxide and tributyltin (insecticides) to *D. magna* reproduction and population growth rate increased in the second generation (Campos *et al*. 2016). Another study showed that the effect of a pesticide mixture (pyrethroid and diuron) to *D. magna* survival decreased in the second generation (survival increased from 81% to 100%) (Brausch and Salice 2011). Furthermore, multigenerational effects may depend on the chemical concentration tested (Muyssen and Janssen 2004; Amorim *et al*. 2017).

Multigenerational studies are complex and different trends can be observed according to the type of chemical stressor and the chemical concentration. Nevertheless, the importance of multigenerational studies cannot be disregarded.

1.3 Population effects

Ecological risk assessment extrapolates the effects of a chemical stressor from apical endpoints to the population-level. Chemical toxicity to a population can be influenced by factors such as population dynamics and timing of exposure (Gagneten and Vila 2001; Preuss *et al*. 2010; Gust *et al*. 2016). Previous studies showed the effect concentrations estimated based on apical endpoints may not reflect the effects

at population-level. For example, Gagneten and Vila (2001) showed a *Ceriodaphnia dubia* population exposed to a 50% effect concentration (EC50) for reproduction became extinct.

In a population, organisms compete for food and can be limited by other factors, such as crowding and light (Preuss *et al*. 2009; Martin *et al*. 2013a; Viaene 2016). One important ecological variable that determines population dynamics (i.e. density and structure) is temperature since it determines the metabolic rate of organisms (Pratt 1943; Jiang *et al*. 2014). Pratt (1943) showed that *D. magna* population density was 2-fold higher at 18 than at 25°C and population structure (i.e. age distribution along time) differed between temperatures.

Until now, to our knowledge, no study has considered whether the effect of temperature on chemical toxicity observed at apical endpoints corresponds to that at population-level.

1.4 Copper, zinc and nickel

Copper (Cu), zinc (Zn) and nickel (Ni) are trace elements occurring naturally in the Earth's crust. In aquatic systems, they can be found as a result of both natural and anthropogenic sources. Copper and Zn are essential elements. They have essential roles in organisms such as in the proper functioning of enzymes. Although Ni is an essential element in plants and microorganisms, Ni essentiality in animals has not yet been identified (e.g. no Ni-containing enzymes has been isolated from animal tissues) (Pyle and Couture 2011).

Anthropogenic emissions of Cu include mining activities (i.e. smelting and refining), agriculture with the use of fungicides, metal and electrical manufacturing (USEPA 2007). For Zn anthropogenic emissions include industrial activities (metal production, iron and steel mills), coal combustion and waste incineration (Luoma and Rainbow 2008). In the case of Ni, stainless steel production represents 65% of their use (Nickel Institute 2015). Anthropogenic emissions of Ni also results of the production of non-ferrous alloys, plating applications, coins, electronic and batteries (Nickel Institute 2015).

Van Regenmortel *et al.* (2017) showed that 0.3, 35 and 9% of the water samples sites in the Dommel river basin (The Netherlands) are potentially at risk due to Cu, Zn and Ni, respectively. The dissolved Cu, Zn and Ni concentrations in five European monitoring databases can be consulted in Table 1.1. Copper**,** Zn and Ni bioavailability and toxicity are determined by the physico-chemical characteristics of the water, such as the dissolved organic matter concentration, pH and water hardness (De Schamphelaere and Janssen 2004a; 2004b; De Schamphelaere et al. 2005; Deleebeeck et al. 2008; Deleebeeck et al. 2007).

Table 1.1. The dissolved nickel, zinc, and copper concentrations in different monitoring databases [adapted from Van Regenmortel et al. (2017)]. Data are median values, with 10th and 90th percentiles in parentheses. FOREGS: Forum of European Geological Surveys.

Metal (µg L^{-1})	Dommel	Belgium- Flanders	Rhine	Austria	FOREGS
Ni	8.3 (0.8–29.0)	2.5 (2.0–11.0)	1.1 (0.5–2.0)	0.5 (0.03–1.9)	1.9 (0.4–4.7)
Zn	28 (3.5–98.0)	15.0 (5.0–66.0)	2.8 (1.0–5.1)	1.9 (0.4–7.8)	2.7 (1.0–9.8)
Cu	2.1 (0.5–4.6)	1.0 (1.0–4.0)	1.6 (0.8–2.3)	0.5 (0.4–1.6)	0.9 (0.3–2.3)

Above certain concentrations even essential metals become toxic. Copper toxicity has been associated with the inhibition of acetylcholinesterase (i.e. neurotransmitters) (Untersteiner et al. 2003), oxidative stresss (Barata et al. 2005) and inhibition of Na^{2+} uptake (Chowdhury et al. 2016). Zinc toxicity has been associated with the disruption of Ca^{2+} homeostasis, with the decrease of feeding rates and energy reserves in *D. magna* (Muyssen et al. 2006). Nickel is an allergenic and genotoxic element (Cameron *et al.* 2011). The potential mechanisms of Ni toxicity in aquatic organisms are the disruption of Ca^{2+}, Mg^{2+} and Fe homeostasis and, the formation of reactive oxygen species (Brix et al. 2017). Nickel is listed as a priority substance by the Water Framework Directive (Directive 2008/105/EC).

1.5 Introduction to the model organism *Daphnia magna*

Daphnia magna (Strauss) is an ectothermic organism, a small planktonic crustaceous that belong to the class Branchiopoda and order Diplostraca (ITIS 2018) (Figure 1.2). It can be found in ponds and lakes at water temperatures between 5 and 26°C (Carvalho 1987; Lennon *et al*. 2001). The optimum temperature of *D. magna* is between 18 and 22°C (Goss and Bunting 1983). In the food web, in lakes and ponds, *D. magna* are keystone species. They are prey for planktivorous fish and they feed on algae.

Daphnia magna reproduces by cyclical parthenogenesis. During favourable periods *D. magna* reproduces by parthenogenesis (asexual reproduction). After every adult moult, a female produces a clutch of haploid eggs (i.e. genetically identical to their mother) that are released in the brood chamber and released in the next moult as neonates. Neonates (organisms with <24h) are similar to an adult daphnia but the reproductive system is not developed (Figure 1.2). The time to reach maturity (i.e. time to the first brood) and the time between broods decreases with the increase of temperature (Bae *et al*. 2016). Organisms change their reproduction strategy to sexual reproduction when they find stress conditions. Males are produced asexually and the females produce two haploids eggs that require fertilization by males. The sexual reproduction leads to the production of dormant eggs named ephippia. The ephippia pass through a diapause period and in the next favourable period the ephippia can hatch given origin to a genetically different individual.

Reproduction by cyclic parthenogenesis allows to culture different clone lines, which is important to study genetically different individuals (different clones) and how they react to different stressors. Previous studies have shown *D. magna* clones can have different thermal responses (Carvalho 1987; Van Doorslaer *et al*. 2009) and different tolerance to metal stressors (Barata *et al*. 1998; Messiaen *et al*. 2010).

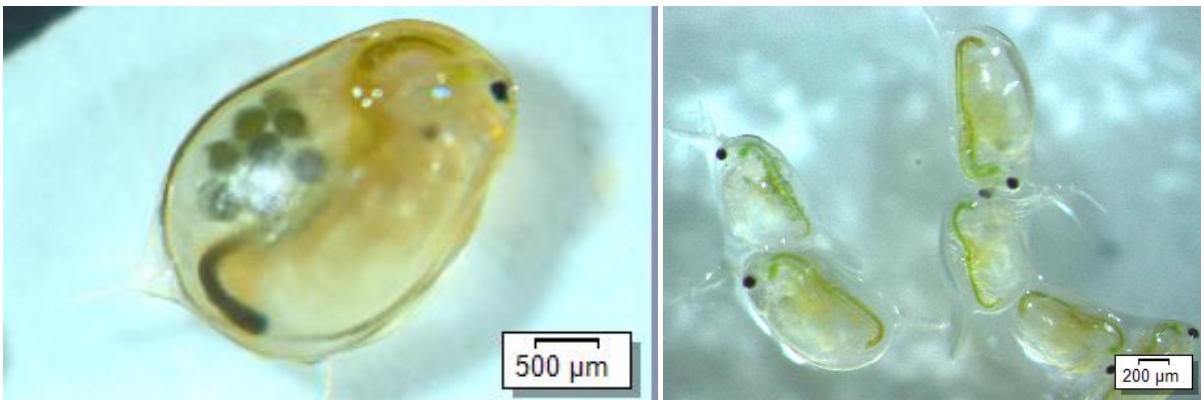

Figure 1.2. *Daphnia magna.* On the left an adult female carrying parthenogenic eggs and on the right neonates (<24h).

1.6 Conceptual framework of the study

Ecological risk assessment is often based on apical endpoints assessed in ecotoxicological laboratory experiment performed within a single generation at a standard temperature. However, organisms in the field are often exposed to chemical stressors along multiple generations and organisms are exposed to wide temperature ranges. Temperature is an important abiotic factor for ectothermic organisms determining the physiological state of the organism and therefore temperature can interact with chemical toxicity. Furthermore, in the field, in a population, organisms compete for food and are limited by other factors, such as crowding and light (Preuss *et al*. 2009; Martin *et al*. 2013a; Viaene 2016). Previous studies have shown food limitation and crowding can influence metal toxicity (Gagneten and Vila 2001; Smolders *et al*. 2005; Gust *et al*. 2016).

At the start of this study, the knowledge about the effect of temperature on metal toxicity was mainly focused on acute toxicity. The studies available indicate acute metal toxicity increases with the increase of temperature (Heugens *et al*. 2001; Sokolova and Lannig 2008). Furthermore, those studies did not acclimate the test organisms to the different temperature treatments, whereas an acclimation period is necessary to restore homeostasis (Williams *et al*. 2012).

The focus of this thesis was to investigate the effect of temperature on metal toxicity to pre-acclimated *D. magna* (i.e. in homeostatic balance with environment temperature). The outline of this thesis is described in Figure 1.3.

In **chapter 2**, the effect of temperature on chronic metal toxicity and whether this effect differed among four different *D. magna* clones were investigated. Life table experiments were performed with Cu, Zn and Ni at 15, 20 and 25°C.

The results of chapter 2 showed a significant effect of temperature on chronic toxicity of Cu, Zn and Ni to *D. magna.* The effect of temperature on chronic Ni toxicity was higher than on chronic Cu and Zn toxicity and it depended significantly on the clone. To help us to understand how temperature influences metal toxicity, in **chapter 3**, the effect of temperature on Ni uptake and elimination was investigated in four *D. magna* clones. Additionally, two assumptions that are often taken in toxicokinetic studies were explicitly tested. We investigated whether the *D. magna*

uptake rate remains constant over the exposure period and whether the elimination rate changes when transferred to a non-contaminated medium.

In **chapter 4**, the temperature dependence of multigenerational effects of Ni to *D. magna* (clone K6) was investigated. The effects of Ni on *D. magna* reproduction and offspring length were tested at 15, 20 and 25°C along four generations. In addition, to explore possible differences in the mechanisms of Ni toxicity at different temperature and different generations, internal Ni concentrations ($[Ni]_{daphnia}$) were measured in adult females. Also, to explore the mechanisms related to the effect of temperature on Ni toxicity to *D. magna* and how these mechanisms apply across a wide temperature range, the ion homeostasis of Cu, Zn, iron (Fe), magnesium (Mg), calcium (Ca), sodium (Na) and potassium (K) in the first generation was also measured.

To investigate whether the effect of temperature on chemical toxicity assessed in apical endpoints corresponds to effects at population-level, in **chapter 5**, a population experiment was conducted. A *D. magna* population was exposed to Ni at 15, 20 and 25°C during 9 weeks. Also, we investigated if a simplified dynamic energy budget individual based model (DEB-IBM) for *D. magna* (Martin *et al*. 2013a) that was calibrated using Ni toxicity data for individual daphnids at three temperatures could successfully predict effects occurring at the population-level.

In the final chapter, **chapter 6**, a general discussion about the information obtained in this thesis is performed. The main conclusions are summarized and suggestions for future research are formulated.

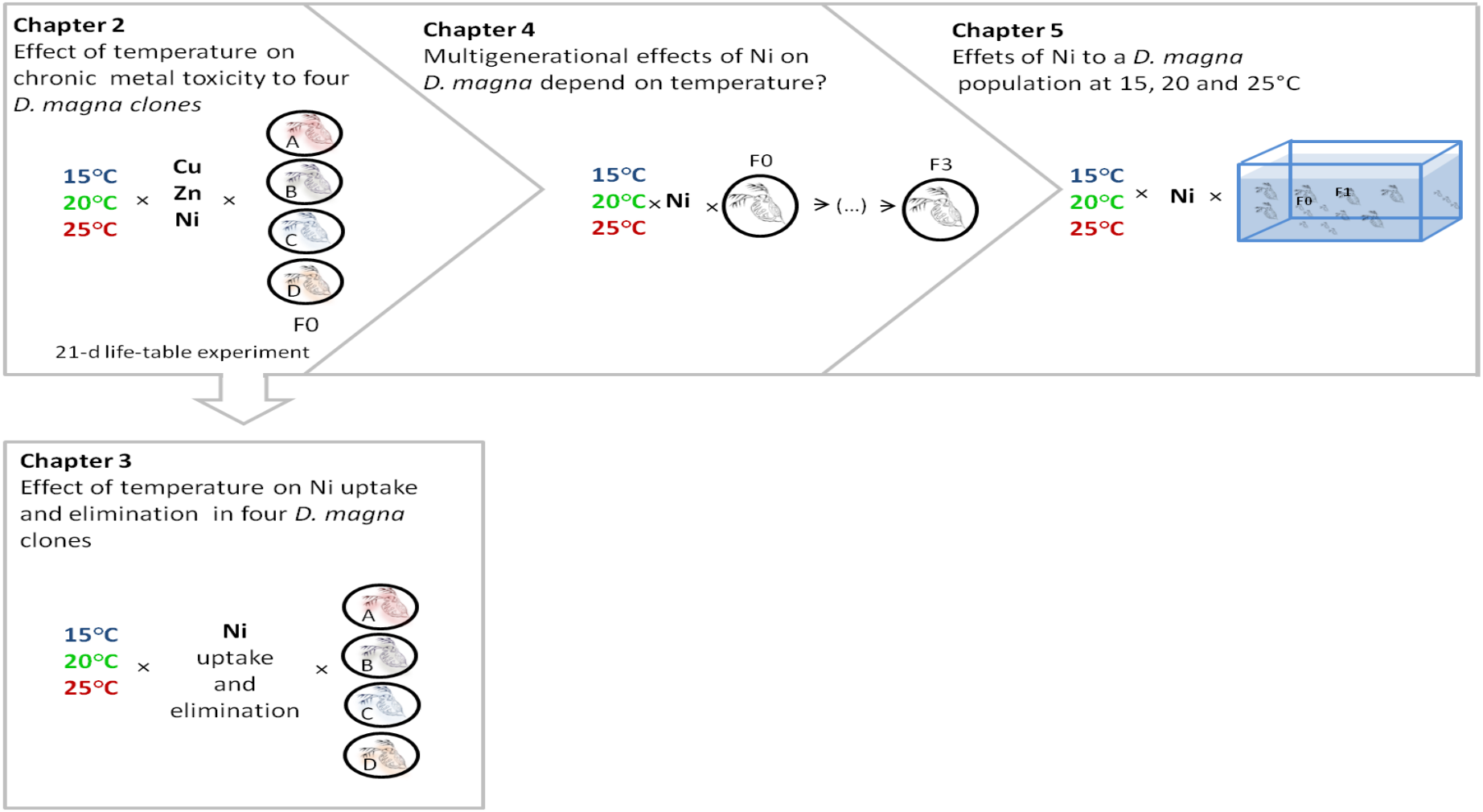

Figure 1.3. Outline of the PhD thesis.

Chapter 2

Effect of temperature on chronic toxicity of copper, zinc and nickel to *Daphnia magna*

Redrafted from:

Pereira CMS, Deruytter D, Blust R, De Schamphelaere KAC. 2017. Effect of temperature on chronic toxicity of copper, zinc, and nickel to *Daphnia magna*. Environmental Toxicology and Chemistry. 36(7):1909-1916.

2.1 Introduction

Temperature is an abiotic factor of high importance for ectothermic organisms due to its strong influence on their metabolic rates and on physiological processes. A well-known example of an ectothermic organism is *Daphnia magna*, one of the most studied species in ecotoxicology. Few studies have considered the effect of temperature on the sensitivity of *D. magna* to other stressors. Most ecotoxicology studies with *D. magna* are performed at 20°C, which is the standard temperature recommended by the OECD (Organisation for Economic Co-operation and Development) guideline for the *D. magna* reproduction test (OECD 2012). However, *D. magna* can be found in ponds and lakes at water temperatures between 5 and 26°C (Carvalho 1987; Lopez *et al*. 1991; EEA 2014). In European surface waters, mean temperatures are 15°C in late spring and 20°C in early summer. Due to climate change, water temperatures in Europe are predicted to increase by 2°C (van Vliet *et al*. 2013). It is assumed that higher temperatures would lead to higher metal toxicity because at higher temperatures metabolic rates are higher (Heugens *et al*. 2001; Paul *et al*. 2004a; Sokolova and Lannig 2008). In addition, with metabolic rates being raised by temperature increase, it is assumed that metal uptake rates would also increase (Heugens *et al*. 2003; Sokolova and Lannig 2008; Vandenbrouck *et al*. 2011). However, information concerning the effect of temperature on metal toxicity to *Daphnia* is until now limited to five acute studies and one chronic study (Persoone *et al*. 1989; Heugens *et al*. 2003; Boeckman and Bidwell 2006; Heugens *et al*. 2006; Ferreira *et al*. 2010; Vandenbrouck *et al*. 2011).

These studies indicate that metal toxicity increases with the increase of temperature. However, the influence of temperature on metal toxicity to *Daphnia* was in each of these studies only investigated with a single clone. In addition, none of these studies acclimated the test organisms to the different temperature treatments, whereas an acclimation period is necessary to restore homeostasis (Williams *et al*. 2012). In addition, inter-clonal variation of temperature effects on metal sensitivity has not been studied even though thermal tolerance differences and different sensitivities to metals have been reported among *D. magna* clones from a single population (Van Doorslaer *et al*. 2009; Messiaen *et al*. 2010).

Overall, there is a lack of information concerning the influence of temperature on chronic metal toxicity, which may be important for more realistic environmental risk assessments, especially in the light of seasonal effects and climate warming (Moe *et al*. 2013). We investigated the effect of temperature on chronic toxicity of Cu, Zn and Ni on four *D. magna* clones. The clones were randomly selected from a natural *D. magna* pond population from Belgium, and were acclimated for two generations to the temperature treatments prior to toxicity testing. Life table experiments were performed simultaneously for Cu, Zn and Ni at 15, 20 and 25°C, with 20°C acting as the standard test temperature and representing early summer, 15°C representing late spring, and 25°C representing summer in a warmer climate.

2.2 Material and methods

2.2.1 Organism maintenance cultures and test media

The four *D. magna* clones used in the present study were obtained from the KNO17 population that was established from ephippia collected from a temporal pond in Knokke, West Flanders, Belgium (details can be found in supportive information in Hochmuth *et al*. (2015)). These clones were maintained under laboratory conditions for two years at 20°C under a photoperiod regime of 16h: 8h (light: dark) and they were daily fed with *Pseudokirchneriella subcapitata*.

A modified M4 medium (Hochmuth *et al*. 2014) was used with following modifications: Na_2SiO_3, $NaNO_3$, KH_2PO_4, and K_2HPO_4 were excluded because these substances enhance algal growth and are not needed for daphnid growth and the Zn concentration was adjusted to 13 µg L^{-1}, which is in the optimal concentration range of this essential element for daphnids (Muyssen and Janssen 2004). This medium is further also called the control medium.

2.2.2 Experimental design

Prior to actual metal exposure, populations of each clone were acclimated for two generations to the test temperatures (Mitchell and Lampert 2000). Briefly, neonates

(<24h old, generation F0) were transferred from the maintenance culture at 20°C to climate rooms at 15°C or 25°C to establish populations in the same control medium but at the other two experimental temperatures. The juveniles (<24h old) resulting from their 3rd to the 5th brood were used to start generation F1. F2 neonates (<24h old) resulting from the 3rd to the 5th brood of F1 were exposed in the actual life table experiments.

The 21-d life table experiments were performed with *D. magna* at 15, 20 and 25°C for Cu, Zn and Ni. For each metal six concentrations were tested with four individual replicates per treatment for each clone. The test medium was spiked with Cu $CuCl_2 \cdot 2H_2O$, $ZnCl_2$ or $NiCl_2 \cdot 6H_2O$ three days before the start of the experiments and these test solutions were then kept at 20°C. One day before the start of the experiment or one day before the change of the medium the test solutions were placed in the different climate rooms at 15, 20 and 25°C. F2 Neonates (<24h old) from the third to the fifth brood were exposed in 50 ml of medium in polyethylene cups. Daphnids were fed daily with *Pseudokirchneriella subcapitata* with a food density of 2.5 mg C L^{-1} in the first week and 5 mg C L^{-1} in the second and third week. All experiments were performed under a controlled light cycle (16 h of light: 8 h of dark) (OECD 2012). The test medium was renewed three times a week. Survival and number of offspring were recorded daily. To avoid temporal variation of metal sensitivity, all life table experiments (i.e. all clones, all temperatures, and all metal concentrations) were performed simultaneously.

2.2.3 Chemical analysis

Dissolved metal concentrations, dissolved organic carbon (DOC), pH and O_2 were measured once a week, both in fresh and old medium. Reported concentrations refer to 0.45 µm membrane filtered concentrations (Acrodisc Filter, Supor Membrane, PALL, Newquay, Cornwall, UK). The samples for Cu, Zn and Ni analysis were taken from test solutions collected from all temperature treatments. Samples for metal analysis were acidified to a final concentration of 0.14 mol L^{-1} of HNO_3 (Normaton Ultrapure 69% HNO_3, Prolabo) prior to analysis.

Metal analyses were performed using graphite furnace atomic absorption spectrophotometry (GFAAS Furnace Autosampler, Thermo Fisher Scientific Inc.,

Waltham, MA, USA) for concentrations less than 50 µg L^{-1}(Cu) or 100 µg L^{-1} (Ni). The reference material was TM-24.3, lot 0510 [Environment Canada]. The limits of quantification were 2.5 µg L^{-1}(Cu) and 1.0 µg L^{-1}(Ni). The method detection limits were 0.8 µg L^{-1}(Cu) and 0.3 µg L^{-1}(Ni). For concentrations greater than 50 µg L^{-1}(Cu), 20 µg L^{-1} (Zn), or 100 µg L^{-1}(Ni) metal analysis was performed using flame atomic absorption spectrophotometry (SpectrAA100, Varian, Mulgrave, Australia). The reference material was TMDA-70, lot 0310 [Environment Canada]. The limits of quantification were 50 µg L^{-1}(Cu), 20 µg L^{-1} (Zn) and 60 µg L^{-1}(Ni) and the method detection limits were 16.6 µg L^{-1}(Cu), 6.6 µg L^{-1} (Zn), and 20 µg L^{-1}(Ni). Concentrations of major cations (Na, K, Ca, and Mg) were measured using iCAP 7000 Series Inductively Coupled Plasma Optical-Emission Spectrometry (ICP-OES) (Thermo Scientific; reference material TM 28-4). The concentrations of the major cations were measured at three different nominal test concentrations per metal. Samples were taken from test solutions that were pooled from all temperature treatments. This was performed once during the life table experiments.

The samples for DOC analysis from the Cu and Zn life table experiments were measured with the TOC5000A (Shimadzu, Duisburg, Germany; limit of quantification = 0.25 mg L^{-1}) and the samples from the Ni life table experiment were measured with the Total Organic Carbon L series CPH (TOC-L CPH) (Shimadzu, Duisburg, Germany; limit of quantification =1.5 mg DOC L^{-1}).

All data analyses were performed based on the mean dissolved concentrations of new and old medium. pH was measured with a pH meter 826 pH mobile (Metrohm, Swiss made). Dissolved oxygen was measured with an Oximeter WTW (probe WTW, cell Ox 325) and temperature was recorded daily.

2.2.4 Data analysis

We used the software WHAM (Windermere Humic Aqueuos Model) VII (Tipping *et al*. 2011) to calculate the speciation of Cu, Zn and Ni in the different temperature treatments. Metal complexation with dissolved organic matter (DOM) was taken into account by assuming DOM to contain 50% carbon (on weight basis), with 65% of binding sites being active with respect to cation binding. To this end, the dissolved

organic carbon measured (mg C L^{-1}) was multiplied by a factor of 1.3 to obtain the amount of fulvic acid (FA) (mg FA L^{-1}) to be used as the modelling input (Tipping *et al*. 2008). The stability constants used for inorganic metal-carbonate complexes were those proposed by the National Institute of Standards and Technology (Smith *et al*. 2004). In addition, we assumed that the activity of the metal cation Fe^{3+} was controlled by colloidal $Fe(OH)_3$ precipitates (Lofts and Tipping 2011).

Statistical analyses were performed using R software (version 2.3-96; R Core Team 2016). To determine the effect of temperature on chronic metal toxicity and whether this effect changes depending on which clone we tested, a general linear model was built to explore possible interactive effects between temperature, metal and clone. This general linear model predicted the metal effect on reproduction, expressed as the number of offspring per individual female (R_0), as a function of temperature and clone. Prior to analysis, the numbers of offspring per individual female were square root transformed to linearize the relationships.

The dose-response curves of the metal concentration (*Me*) on R_0 were modelled by a third order linear approximation:

Eq (2.1)

$$R_0 = \beta_0 + \beta_1 Me + \beta_2 Me^2 + \beta_3 Me^3$$

We tested whether the intercept (β_0) and regression coefficients i.e. gradient (β_1), curvature (β_2) and aberrancy (β_3) differed between temperatures (*T*), between clones and whether a clone × *T* interactive effect on each of these regression coefficients was observed. The most complex model, in which all regression coefficients are affected by clone, temperature and clone × *T* interaction, can thus be written as

Eq (2.2)

$$R_0 = \beta_0(T, clone) + \beta_1(T, clone)\, Me + \beta_2(T, clone)\, Me^2 + \beta_3(T, clone)\, Me^3$$

We fitted 46 different models to the data sets of each metal (Appendix A, Table A4). These 46 models resulted from the different combinations of possible interactions between temperature, metal and clones.

The interactive effects between temperature, clone (both factorial variables) and the metal concentration (continuous variable) were modelled to explore the possible interactive effects between temperature and metal on reproduction of *D. magna*, and to test whether these interactive effects differed among the four clones.

Optimal models were selected based on the Akaike information criterion (AIC). However, selecting for the lowest AIC can be arbitrary (Akaike 1974). Therefore, we also quantified the relative model support based on the Akaike weights (*w*) to compare how well the optimal model performed in comparison to the alternative models.

Eq (2.3)

$$w_i = \frac{e^{-\frac{\Delta_i}{2}}}{\sum_j^N e^{-\frac{\Delta_j}{2}}}$$

Where w_i is the Akaike weight for the i^{th} model and N is the number of models that are being compared. Δ_i is the difference in AIC between model i and the lowest AIC of all N models. The denominator is the sum of the relative likelihoods for all candidate models.

Normality was evaluated using a quantile-quantile (QQ) plot of the model residuals and the homogeneity of variances of residuals among all temperatures and clones was evaluated using a boxplot (Zuur *et al*. 2009).

The effect concentrations (ECx) and concentration response curves were calculated based on the average reproduction across all four clones (to mimic the effects on a population). As a result, the clones that had higher reproduction rates weighted more in the calculations of the effective concentrations and *vice versa*. Additionally, ECx were calculated for each individual clone at each temperature. Effect concentrations were determined using the *drc* package in R software (version 2.3-96; R

Core Team 2016) with the dose-response model that fitted best across the three temperature treatments (i.e., highest log likelihood). Three different dose-response models were fitted, i.e. the three-parameter log-logistic, the three-parameter Weibull and the four-parameter Brain and Cousens hormesis model. For the ECx calculated on the basis of the average reproduction of all four clones the three-parameter Weibull model was the best-fitting model for the Cu and Zn data sets and the three-parameter log-logistic model was the best-fitting model for the Ni data set. For the ECx calculated for each clone separately the three-parameter Weibull model was the best-fitting model for the Cu data sets (for each clone) and the three-parameter log-logistic model was the best-fitting model for the Zn data sets (for each clone). For the Ni data set, the best-fitting models across the three temperature treatments were the three-parameter log-logistic model for clones A, C and D and the Brain and Cousens hormesis 4-parameter model for clone B.

2.3 Results

Temperature remained stable during the life table experiments with Cu, Zn and Ni at 15°C (±0.8), 20°C (±0.5) and 25°C (±0.3) (mean ± standard deviation). The pH, the dissolved oxygen concentration, the dissolved organic carbon and the major ion concentrations also remained stable during the life table experiment, and so did the dissolved Cu, Zn and Ni concentrations (Appendix A, Table A1 and A2). The OECD validity criterion of reaching a mean of ≥ 60 living offspring per parent female surviving was not reached in the controls for some tested clones in some temperature treatments. However, this criterion was originally set in this guideline for 20°C and, in addition, our goal was to investigate a scenario that would be representative of the inter-clonal variation that may exist in a natural pond. Therefore, we have used four randomly selected clones from a single natural population and these four clones were not selected a priori for optimal performance under laboratory conditions (as is mostly the case with laboratory clones that are used for regulatory testing). Thus, due to inter-clonal variability the mean number of live offspring produced in control treatments differed among clones within each temperature treatment (Appendix A, Table A3).

To determine the effect of temperature on chronic metal toxicity and to test if this effect changes depending on the clone a general linear model was built (Appendix A, Table A5). The optimal third order linear models according to the Akaike weights for the Cu, Zn and Ni data sets indicate that temperature significantly affected metal toxicity to *D. magna* reproduction (Table 2.1, Figure 2.1; Appendix A, Table A6, A7, A8). The square root transformed data meet the assumption of normality based on the QQ plot of the model residuals. The residuals of the optimal models for the Cu, Zn and Ni data sets were homogeneously distributed around 0 when plotted against the variables temperature and clone indicating homogeneity of variance and the plot of predicted vs observed values indicates a good model fit (Appendix A, Figure A1, A2, A3) (Zuur *et al*. 2009)**.** In the case of the Cu data set, the significant interactions between temperature and Cu concentration, as Cu^2 and Cu^3 (p<0.001, p<0.01, respectively), explained 2.6 and 0.8% of the observed variation. The significant interactions between temperature and Zn, as Zn^2 (p<0.001), explained 2.5% of the observed variation. In the case of Ni, 1.6 and 1.3% of the observed variation was explained by the significant interactions between temperature and Ni, as Ni and Ni^2 (p<0.01, p<0.01, respectively).

The effect of temperature on chronic Cu and Zn toxicity was not significantly dependent on the clone (clone × T × Cu^2, p>0.05; clone × T × Cu^3, p>0.05; clone × T × Zn^2, p>0.05).

In contrast, the effect of temperature on chronic Ni toxicity significantly depended on the clone (clone × T × Ni, p<0.05; clone × T × Ni^2, p<0.01) and explained 1.55 and 1.95% of the observed variation, when Ni concentrations were expressed as Ni and Ni^2, respectively (Table 2.1).

The summary of the optimal models can be consulted in Appendix A (Table A6, A7 and A8).

When calculated on the basis of the average reproduction of the four clones, the 21-d EC50 for reproduction of Cu, Zn and Ni increased monotonously by 1.4, 1.2 and 2.0 fold with an increase of temperature from 15 to 25°C, respectively (Figure 2.2, Table 2.2). For the Cu and Ni data set, the same monotonous trend was observed for the 21-d

EC10 and EC20, i.e. the 21-d EC10 and EC20 for Cu increased 2.5 and 2.0-fold between 15 and 25°C, respectively, and the 21-d EC10 and EC20 for Ni increased 2.2-fold between 15 and 25°C. For the Zn dataset, a different (non-monotonous, inverse U-shaped) trend was observed for the 21-d EC10 and EC20, i.e. the 21-d EC10 and EC20 increased by 1.2-fold from 15 to 20°C and it decreased by 2.6 and 1.7-fold from 20 to 25°C, respectively (Figure 2.2 and Table 2.2). When toxicity was expressed on the basis of the free metal ion activity the 21-d EC50 between 15 and 25°C only varied 1.1-fold for Cu, whereas the same variation of 21-d EC50 with temperature was observed when expressed as dissolved metal concentration of free metal ion activity in the case of Zn and Ni (Table 2.2).

As mentioned above, the optimal third order linear model indicates that chronic metal toxicity was significantly affected by temperature and in case of Ni (but not in the case of Cu or Zn), this effect of temperature on chronic Ni toxicity significantly depended on the clone (Table 2.1). The concentration response curves and the ECx of Ni calculated for each individual clone support this statistically significant inter-clonal variation of the temperature effect (Figure 2.3; Appendix A, Table A9). For clone A, the estimated 21-d EC50 increased from 53.5 at 15°C to 70.6 µg Ni L^{-1} at 20°C, and to 67.0 µg Ni L^{-1} at 25°C. A significant hormesis effect was observed for clone B at 25°C, which was not observed at 20°C and 15°C. The 21-d EC50 slightly increased from 108.3 µg Ni L^{-1} at 15°C to 125.3 µg Ni L^{-1} at 20°C and to 134.3 µg Ni L^{-1} at 25°C. For clone C, the 21-d EC50 at 20°C was 81.5 µg Ni L^{-1}, it was lower at 15 and 25°C (55.3 and 61.5 µg Ni L^{-1}, respectively). The estimated values of the 21-d ECx of clone D at 15 and 20°C are questionable due to the very low reproduction in the control treatment.

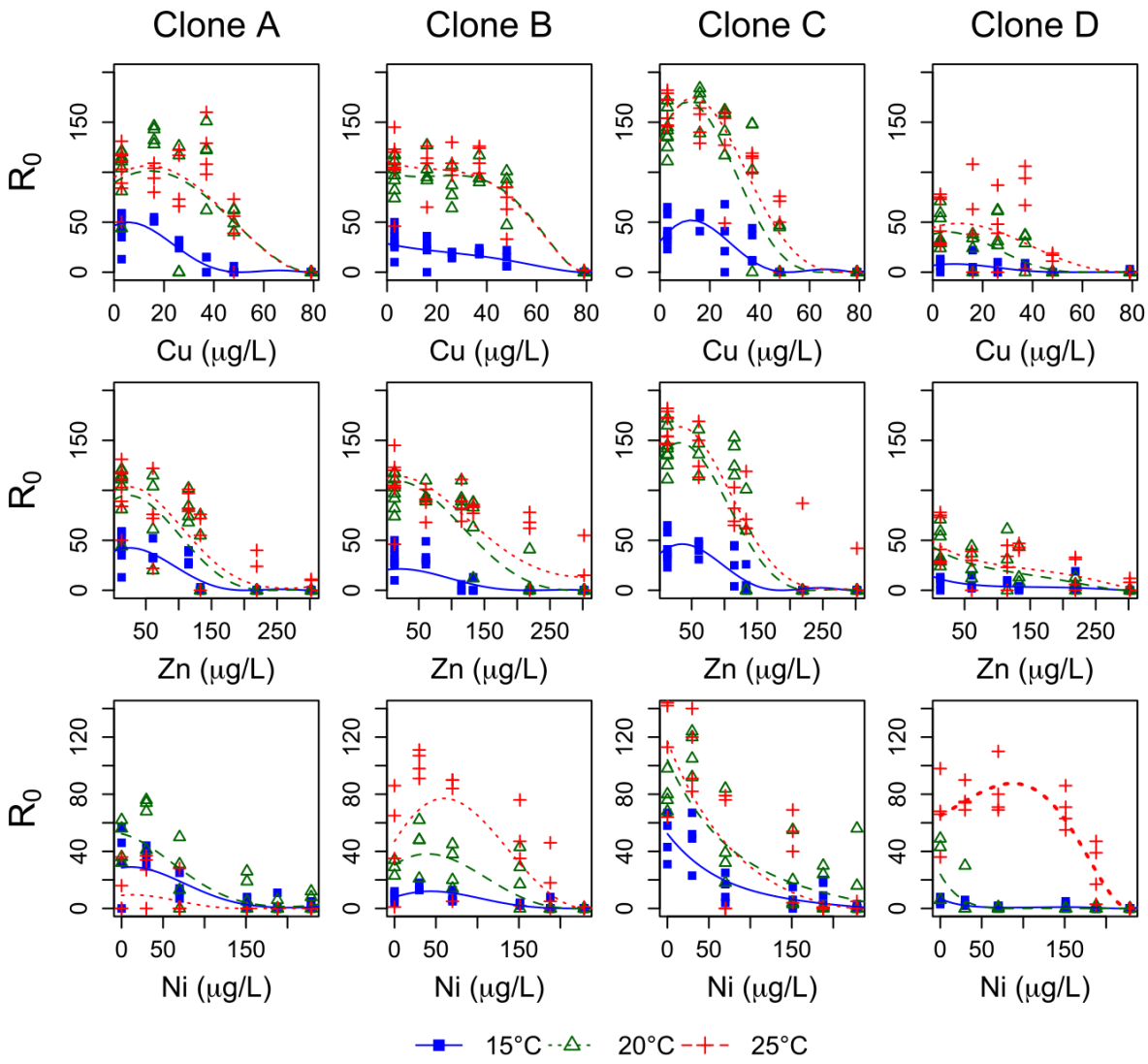

Figure 2.1. Observed and predicted number of offspring per individual female (R_0) as function of clones, temperature and metal concentration (Cu, Zn or Ni). Lines represent the predicted (fitted) concentration response curves and the symbols represent observed values (Ni, n=283; Cu, n=336, Zn, n=336).

Table 2.1. Summary of the analysis of variance of the optimal third order linear models (see supplemental data, Table A5) that predict $\sqrt{R_0}$ (number of offspring per individual female), as a function of temperature (T), clone, and metal concentration (Me) for the Cu, Zn and Ni data sets; with degrees of freedom (Df), explained variation (R^2) and significance (*p* value). n.a. – not applicable. The significant *p* values that are related to the two focal questions of the present study are marked with bold letters.

	Cu				Zn				Ni		
Predictor	Df	R^2 ×100%	*p* value		Df	R^2 ×100%	*p* value		Df	R^2 ×100%	*p* value
Clone	3	10.05	<0.001		3	5.54	<0.001		3	6.34	<0.001
T	2	18.76	<0.001		2	16.55	<0.001		2	6.60	<0.001
Me	1	39.82	<0.001		1	46.86	<0.001		1	36.67	<0.001
Me^2	1	2.70	<0.001		1	0.00	>0.05		1	0.09	>0.05
Me^3	1	0.51	<0.01		1	1.38	<0.001		1	0.02	>0.05
clone × T	6	0.79	>0.05		6	1.28	0.007		6	14.23	<0.001
Clone × Metal											
clone × *Me*	3	2.17	<0.001		3	3.66	<0.001		3	1.91	<0.001
clone × Me^2	3	0.90	<0.01		3	0.15	>0.05		3	1.68	<0.01
clone × Me^3	3	1.15	<0.01		3	1.23	<0.01		3	0.93	<0.05
Temperature × Metal											
T × *Me*	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.		2	1.58	**<0.01**
T × Me^2	2	2.63	**<0.001**		2	2.48	**<0.001**		2	1.33	**<0.01**
T × Me^3	2	0.84	**<0.01**		n.a.	n.a.	n.a.		n.a.	n.a.	n.a.
Temperature × Clone × Metal											
clone × *T* × *Me*	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.		6	1.55	**<0.05**
clone × *T* × Me^2	6	0.16	>0.05		6	0.32	>0.05		6	1.95	**<0.01**
clone × *T* × Me^3	6	0.71	>0.05		n.a.	n.a.	n.a.		n.a.	n.a.	n.a.
Residuals	287	18.81			291	20.55			243	25.12	

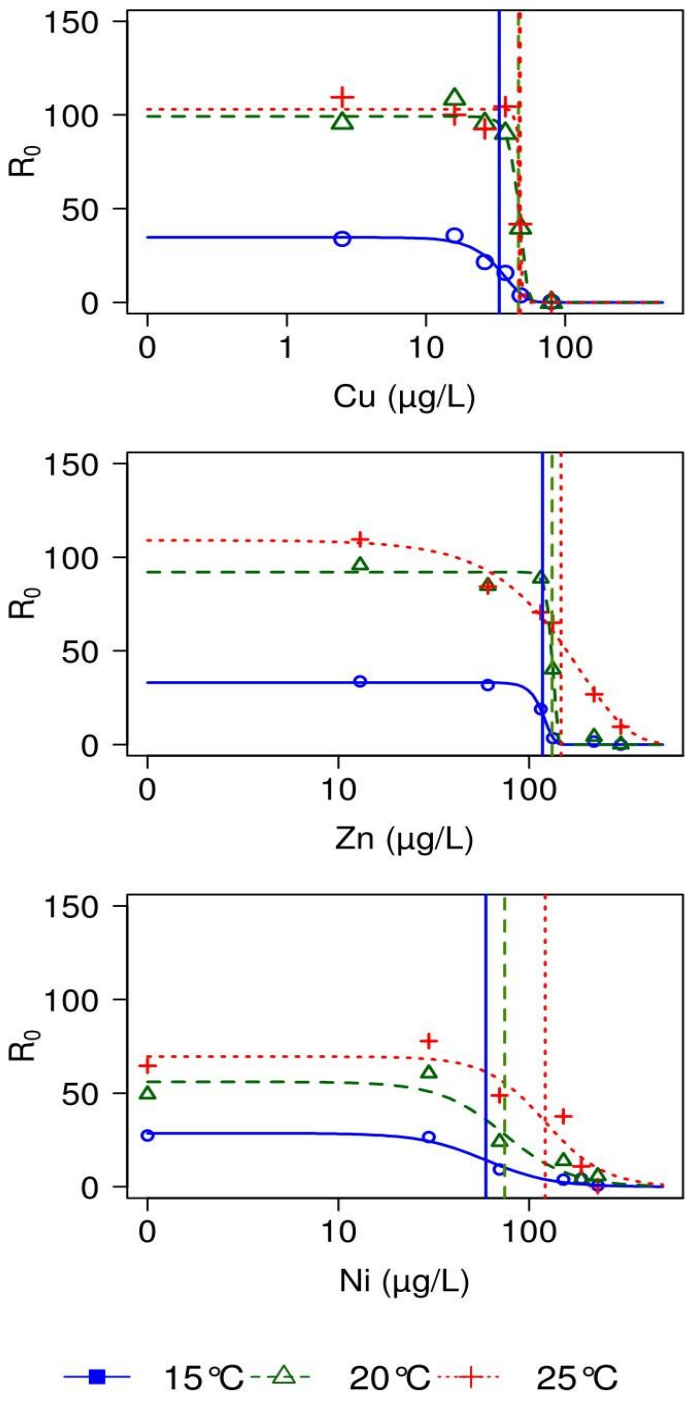

Figure 2.2. Twenty-one day concentration response curves of Cu, Zn and Ni at 15, 20 and 25°C calculated with the number of offspring per individual female (R_0), averaged over four *Daphnia magna* clones (to mimic the effects on a population). Marker points represent observations (average data) and lines are the fitted concentration response curves. Vertical lines indicate the EC50 values at 15, 20 and 25°C.

Table 2.2. The 21 day effective concentrations (ECx) (+/- standard error) of Cu, Zn and Ni at 15, 20 (the standard temperature) and 25°C calculated with the number of offspring per individual female (R_0), averaged over four *Daphnia magna* clones (to mimic the effects on a population).

		Dissolved metal (µg L^{-1})			Free metal ion activity (µg L^{-1})		
Metal	ECx	15°C	20°C	25°C	15°C	20°C	25°C
Cu	EC10	18.1 ± 9.9	37.4 ± 4.6	45.0 ± 21.3	3.0±1.6	5.4±0.7	5.7±2.7
	EC20	23.1 ± 9.1	40.7 ± 3.3	45.9 ± 14.2	3.8±1.5	5.9±0.5	5.8±1.8
	EC50	33.6 ± 6.7	46.2 ± 1.3	47.3 ± 2.8	5.5±1.1	6.7±0.2	6.0±0.4
Zn	EC10	96.3 ± 21.3	120.8 ±10.1	46.5 ± 14.9	63.4±14.1	78.5±6.6	29.9±9.6
	EC20	104.3 ± 16.0	125.1 ± 6.8	73.5 ± 16.6	68.6±10.5	81.7±4.4	47.3±10.7
	EC50	117.5 ± 8.4	131.9 ± 1.8	146.7 ± 14.9	77.1±5.5	85.6±1.2	94.1±9.5
Ni	EC10	24.9 ± 19.5	31.3 ± 9.3	55.6 ± 20.6	15.6±12.3	19.7±5.9	34.6±12.9
	EC20	34.3 ± 19.7	42.9 ± 9.9	74.1 ± 21.6	21.5±12.4	27.2±6.3	46.1±13.4
	EC50	59.2 ± 20.9	74.3 ± 13.0	121.2 ± 20.0	37.2±13.1	47.0±8.2	75.7±12.4

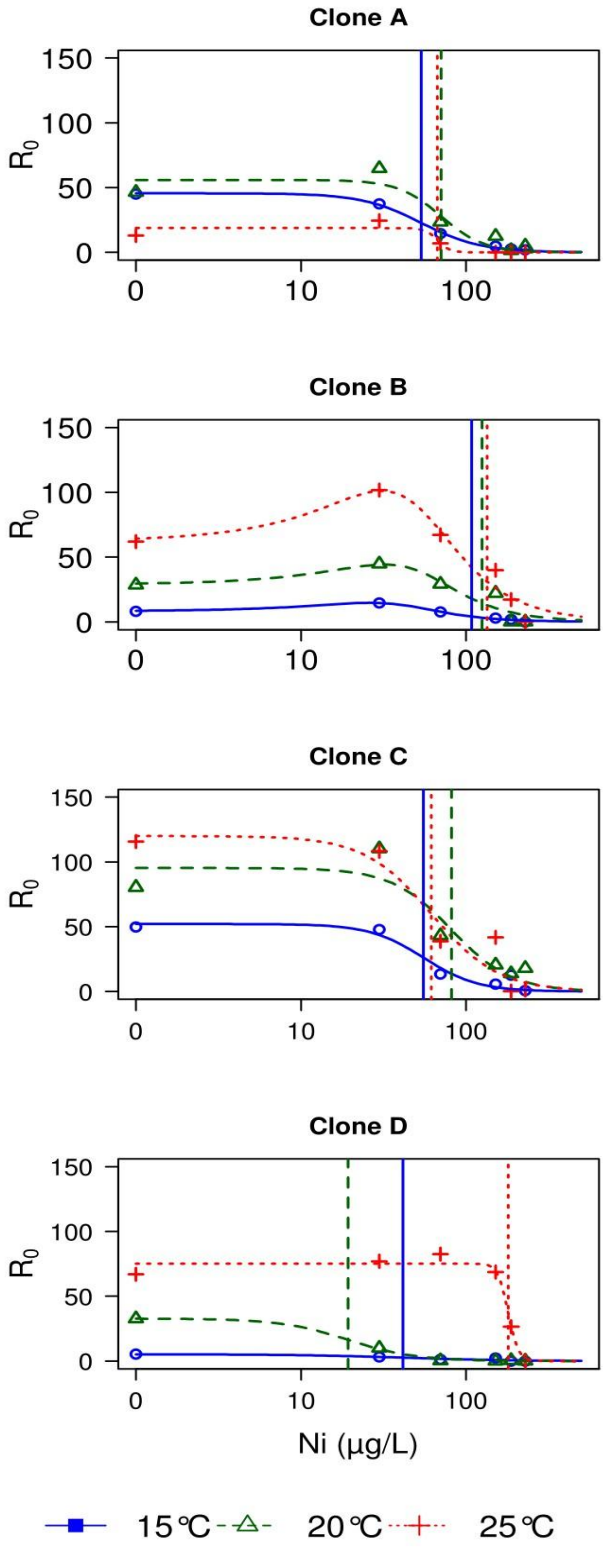

Figure 2.3. Twenty-one day concentration response curves of Ni at 15, 20 and 25°C for the four individual *Daphnia magna* clones calculated with the number of offspring per individual female (R_0). Marker points represent average data and lines the fitted concentration response curves. Vertical lines indicate the EC50 at 15, 20 and 25°C.

2.4. Discussion

We found that temperature had a significant effect on chronic toxicity of Cu, Zn and Ni to *D. magna* and that the effect of temperature on chronic Ni toxicity depended significantly on the clone. Our results indicate that chronic metal toxicity to *D. magna* was higher at 15°C than at 20°C, which is the temperature that is, used in standard chronic toxicity tests. Chronic Cu and Zn toxicity were not different between 25°C and 20°C, but chronic Ni toxicity was 1.6-fold lower at 25°C than at 20°C (i.e., the 21-d EC50 of Ni was 1.6 times higher at 25°C). Even considering that only a relatively small variation was observed in the 21-d EC50 among the three investigated temperatures (i.e., maximum 2-fold), the third order general linear model indicates a significant effect of temperature on metal toxicity for *D. magna* reproduction. Collectively, our results indicate a statistically significant trend of decreasing chronic toxicity of Cu, Zn and Ni in *D. magna* with increasing temperature. This trend is opposite to what is most commonly reported for the effect of temperature on acute metal toxicity. Thus, our study provides evidence that metal chronic toxicity does not generally increase with the increase of temperature when organisms are acclimated to the temperature treatments. Furthermore, the effect of temperature on chronic toxicity of Ni (but not of Cu and Zn) depended significantly on the clone.

The optimal third order linear models indicated that chronic metal toxicity to *D. magna* was significantly affected by temperature, but only for chronic Ni toxicity the effect of temperature depended on the clone. Conversely, the variation of the 21-d EC50 of Cu and Zn between *D. magna* clones was less than 1.5-fold at each temperature. The dissolved Cu 21-d EC50 at 25°C was 1.4-fold higher than at 15°C and was similar to 20°C. However, the variation of the 21-d EC50 between temperature treatments decreased to less than 1.1-fold when calculated as free Cu^{2+} ion activity. This suggests that the effect of temperature on the toxicity of Cu (when expressed on a dissolved metal concentration basis) is predominantly explained by the effect of temperature on Cu speciation and much less by an effect of temperature on the interaction of free Cu^{2+} ions with biological ligands at the water-organism interface (e.g. Cu uptake and sensitivity of the daphnids to Cu). WHAM 7 has temperature corrections

of stability constants for Cu, Zn and Ni complexes (Plyasunov and Grenthe 1994; Smith *et al*. 2004) and thus correct for the effect of temperature on metal speciation. Calculations in our test medium and at the investigated temperatures (15-25°C) showed that temperature did affect Cu and Zn speciation, but not Ni speciation. The effect of temperature on chronic metal toxicity diminished for Cu when toxicity was expressed as free Cu^{2+} ion activity (compared to when it was expressed as dissolved Cu), but remained similar for Zn and Ni (i.e., independent of being expressed as dissolved or free metal ion activity) (Table 2.2).

In the case of Ni, chronic toxicity was significantly affected by temperature and this temperature effect also varied among the four *D. magna* clones (Figure 2.1 and Table 2.1). The 21-d EC50, calculated with the total reproduction summed over all clones (to mimic the effects on a population), exhibited a two-fold variation between the temperature treatments (Figure 2.2 and Table 2.2). In comparison with the standard temperature (20°C), chronic Ni toxicity was 1.3 times higher at 15°C and it was 1.6 times less toxic at 25°C. The effect of temperature on chronic Ni toxicity significantly differed among clones. A previous study demonstrated an effect of pH on the chronic toxicity of Cu between two clones (Van Regenmortel *et al*. 2015). Therefore, the study of multiple clones is necessary to investigate the effect of environmental variables on chronic metal toxicity to *D. magna* within the same temperature treatment, the between-clone variation of the sensitivity to Ni was about 2-fold at 15, 20 and 25°C.

Our results suggest that chronic metal toxicity to *D. magna* at 25°C did not increase compared to 20°C, the standard test temperature. This is in contrast with many previous studies reporting that metal toxicity typically increases with the increase of temperature. Heugens *et al.* (2006) performed a chronic *D. magna* study that showed higher effects of cadmium (Cd) on the growth rate at 25°C in comparison to 20°C and 10°C. However, this was only investigated with a single clone, which was not acclimated to the temperature treatments. Based on the result obtained in our present study, we can easily see that our conclusions about the effect of temperature on chronic Ni toxicity to *D. magna* could have been different depending on which clone was tested (assuming that we would have conducted the study with a single, randomly chosen clone). For

example, if we would only have tested clone C we would have concluded that chronic Ni toxicity to *D. magna* is higher at 15 °C (1.5-fold) and 25°C (1.3 fold) than at 20°C (standard temperature). Yet, if we would only have tested clone B we would have concluded that the variation of chronic Ni toxicity between temperatures was small (1.2-fold) and that toxicity even slightly decreased with increased temperature. This observation demonstrates the necessity of being careful with our conclusions for ERA when extrapolating effects of temperature on chemical toxicity from single clone studies to the population-level (where multiple clones co-exist).

The lack of acclimation of the organisms to the temperature treatments may also contribute to the different outcomes. This is because an acclimation period is necessary to allow physiological adjustment to the new situation and reach homeostatic balance (Williams *et al*. 2012). Our results are in agreement with the study of Cedergreen *et al*. (2013) with a soil worm *Enchytraeus crypticus* acclimated to the temperature treatments, where it was reported that chronic metal toxicity was lower at higher temperatures (25°C). In acute *D. magna* studies, it was reported that metal toxicity increased with increased temperature, and that metal uptake rates increased with increased of temperature (Heugens *et al*. 2003; Vandenbrouck *et al*. 2011). However, no information is available about temperature effects on elimination rates and detoxification rates on *Daphnia*.

The processes of detoxification and elimination could also be raised by temperature but an acute test may be too short to affect toxicity in this way. Cedergreen *et al*. (2013) reported that at 25°C after two days of uptake the concentration of Cu inside the organism started to decrease, which gives an indication that elimination processes took place. This was not observed at 20 and 15°C. The balance of uptake, compartmentalization and elimination processes determines the internal metabolically available metal concentration, which determines the organism sensitivity (Rainbow 2007).

Future studies on metal uptake and elimination kinetics at different temperatures would be very usefully since they would allow better understanding of the mechanisms involved in metal toxicity from a toxicokinetic perspective.

For ERA purposes, some will argue that a variation within a factor two between 21-d EC50 values obtained at different temperatures is not extremely relevant or sufficiently pressing to incorporate temperature as a factor in metals risk assessment. However, various regulatory agencies (e.g. United States Environmental Protection Agency and legislative frameworks (European Water Framework Directive)) have previously integrated factors causing variations smaller than a factor two in risk assessment and water quality criteria setting through integration of such ("small") effects in the Biotic Ligand Model (BLM). The Biotic Ligand Model is a reference tool used to predict chronic metal toxicity based on the dissolved metal concentrations and the physico-chemistry in the water column (DEPA 2008; ECI 2008; Van Sprang *et al*. 2009). Specifically in the case of *D. magna*, the BLM to predict chronic Cu toxicity incorporated the effect of Na with only an overall effect of factor two on the 21-d EC50 over a wide range of Na concentration (De Schamphelaere and Janssen 2004b). In the case of the Zn BLM for chronic toxicity, an effect of about 2.3 and 1.6-fold on the 21-d EC50 was considered enough to include the effect of Na and Mg in the chronic Zn BLM, respectively (Heijerick *et al*. 2005). The effect of temperature on metal speciation is considered in the BLMs, yet evidence underlying temperature and speciation relations is relatively limited as thermodynamic stability constants are not generally measured across a range of temperature. The Biotic Ligand Model does not yet consider the effect of temperature on the interaction of the free metal ions with the biotic ligand of the organisms. Given that the present study demonstrated that temperature can significantly influence chronic metal toxicity to *D. magna* (with up to 2-fold variation among temperatures between 15 and 25°C), it would be worthwhile to consider explicit incorporation of temperature in BLMs beyond its effect on speciation alone.

Finally, it is also important to mention that the magnitude of metal toxicity considered for risk assessment (which varies across different jurisdictions) may influence the effect of temperature on chronic metal toxicity that is observed. In our

present study, a similar effect of temperature was observed for the 21-d EC50, EC20 and EC10 for Cu and Ni, but a quite different effect of temperature was observed for Zn, which suggests that slopes of concentration responses curves (of some metals) could be influenced by temperature. It would be worthwhile to further document temperature effects on chronic metal toxicity, including experiments with higher numbers of replicates and metal concentrations (especially at low effect sizes) to enable more precise low effect concentration estimates such as EC10 and EC20 and to enable further testing of the hypothesis that temperature can affect the slope of metal dose response curves.

2.5 Conclusion

The present study showed that temperature had a significant effect on chronic toxicity of Cu, Zn and Ni to *D. magna* and that the effect of temperature on chronic Ni toxicity depended significantly on the clone. Our results indicate that chronic metal toxicity to *D. magna* was generally higher at 15°C than at 20°C, which is the reference temperature that is used in standard chronic toxicity tests. At 15°C, the 21-d EC50 of Cu, Zn and Ni was 1.4, 1.1 and 1.3 times lower than at 20°C, respectively. Chronic Cu and Zn toxicity were not different between 25°C and 20°C, but chronic Ni toxicity was 1.6-fold lower at 25°C than at 20°C (i.e., 21-d EC50 of Ni at 25°C was 1.6 higher than at 20°C). The same trends were observed for Cu and Ni when the 21-d EC10 and EC20 were considered as the effect estimator, but not for Zn, which warns against extrapolating temperature effects on chemical toxicity across effect sizes. Collectively, our study provides evidence that metal chronic toxicity does not generally increase with the increase of temperature when organisms are acclimated to the temperature treatments. This trend is opposite to what is most commonly reported for the effect of temperature on acute metal toxicity.

The effect of temperature on the toxicity of Cu (when expressed on a dissolved metal concentration basis) is predominantly explained by the effect of temperature on Cu speciation and much less by an effect of temperature on the interaction of free Cu^{2+} ions with biological ligands at the water-organism interface (e.g. Cu uptake sites or toxic

action sites of Cu). However, the effect of temperature on chronic toxicity of Zn and Ni goes further than its effect on speciation alone and involves effects on the organism itself.

Finally, the effect of temperature on chronic toxicity of Ni (but not of Cu and Zn) depended significantly on the clone. This indicates that in the context of incorporating temperature influences in the environmental risk assessment of chemicals, it is necessary to be cautious when extrapolating data on the effects of temperature on chemical toxicity from single clone studies to the population-level (where multiple clones co-exist).

Chapter 3

Effect of temperature on nickel uptake and elimination in *Daphnia magna*

3.1 Introduction

For aquatic ectothermic organism the influence of temperature is critical as their body temperature is determined by the environmental temperature. The increase of temperature increases their respiration and metabolic rates which may contribute to the increase of metal uptake rates (Heugens *et al*. 2001; Paul *et al*. 2004; Sokolova and Lannig 2008; Bae *et al*. 2016). The increase of metal uptake rates has been associated with the increase of acute metal toxicity (Heugens *et al*. 2001; Heugens *et al*. 2003). This is in line with the critical body concentration hypothesis in which the internal concentration determines the toxicity to the organism (Vijver *et al*. 2004). However, the balance of uptake, compartmentalization, and elimination processes determines the internal metabolically available metal concentration and therefore the metal toxicity to the organism (Vijver *et al*. 2004; Rainbow 2007)**.**

A review by Sokolova *et al*. (2008) showed that in aquatic ectothermic organisms an increase of temperature increased metal uptake/accumulation, metal elimination and mortality in 85% (n=45), 26% (n=35) and 80% (n=115) of the studies available, respectively. Although, the majority of the studies reviewed in Sokolova *et al*. (2008) focused on acute toxicity and in the studies reviewed temperature acclimation was generally not included. For *Daphnia*, an ectothermic organism and a well-known ecotoxicological model, only one study is available concerning the effect of temperature on metal toxicokinetics (Heugens *et al*. 2003). Heugens *et al*. (2003) studied the influence of temperature (10, 20, 26°C) on Cd uptake kinetics in *Daphnia* (0 to 45 h of exposure) and showed the uptake rate constant (k_u) at 20°C was significantly higher than at 10°C and the k_u at 26°C was similar to 20°C. However, Heugens *et al*. (2003) did not pre-acclimate the test organisms to the different temperature treatments, whereas an acclimation period is necessary to physiologically adjust to the environmental temperature and reach homeostatic balance (Williams *et al*. 2012). In chapter 2, the effect of temperature on chronic metal toxicity to *Daphnia magna* (pre-acclimated to the temperature treatment) was studied. The results of chapter 2 showed that in comparison with 20°C, the standard temperature recommended by the OECD

guideline for the *D. magna* reproduction test (OECD 2012), chronic metal toxicity to *D. magna* increased at 15°C and decreased at 25°C, which is in contrast with previous acute studies (Persoone *et al*. 1989; Heugens *et al*. 2003; Boeckman and Bidwell 2006; Ferreira *et al*. 2010; Vandenbrouck *et al*. 2011). Furthermore, the results of chapter 2 showed that the effect of temperature on chronic Ni toxicity depended on the *D. magna* clone. The results of chapter 2 and the lack of information about the effect of temperature on toxicokinetic processes in pre-acclimated organisms leads us to our first and main objective (part I) which was to investigate the effect of temperature on Ni uptake and elimination in four *D. magna* clones (pre-acclimated, i.e. organisms in homeostatic balance with environment temperature).

In toxicokinetic studies, usually, a simple one-compartment kinetics model is used and experiments usually include two phases: an uptake phase in which organisms are exposed to metals via contaminated medium (elimination also can take place) (Heugens *et al*. 2003; Komjarova and Blust 2008, 2009b, 2009a; Lebrun *et al*. 2011) and an elimination phase for which organisms are transferred from the contaminated medium to a clean medium (Guan and Wang 2004; Lam and Wang 2006; Zhao *et al*. 2009; Lebrun *et al*. 2011; Adam *et al*. 2015). Two important assumptions are associated with this design. The first assumption is constant k_u over exposure duration. Most of the toxicokinetic studies assume that k_u does not change during the exposure period (Cedergreen *et al*. 2013; Adam *et al*. 2015). However, a previous study showed Ni assimilation rates can change along metal exposure (Laskowski *et al*. 2010). Laskowski *et al*. (2010) studied Ni toxicokinetics in *Pterostichus oblongopunctatus* (ground beetles) and in *Lumbricus terrestris* (earthworm) and showed that a three-phase model (one-compartment model), where after 1.5 d of Ni exposure k_u decreases or even becomes zero, better described metal toxicokinetics over time than the classic two-phase model with one k_u and one k_e. The second assumption is that the elimination rate constant (k_e) also remains the same in the presence and in the absence of the contaminant. This second assumption was tested in *Hydropsyche* larvae using a one-compartment model (Evans *et al*. 2002, 2006). Cadmium, Pb, Zn and Cu elimination in *Hydropsyche* larvae was similar in the presence vs. the absence of the contaminant. However, to our knowledge, this assumption was not validated in other species and also Ni was not

tested. The second part (II) of the objectives of the present study was to test these two assumptions in *D. magna*.

The use of stable isotopes has been shown as a useful technique to help to understand toxicokinetic processes (Evans *et al*. 2002; Pane *et al*. 2003; Evans *et al*. 2006; Balcaen *et al*. 2008; Komjarova and Blust 2009a). Therefore, in the experimental design of the present study, with the use of the stable isotope ^{62}Ni and Ni with a natural isotope ratio, the Ni isotope ratio of the exposure medium was switched in the course of the experiment. This experimental design allows answering the first part (I) and the second (part II) of our objectives:

I. To investigate the effect of temperature on Ni uptake and elimination in four *D. magna* clones;
II. a) to investigate whether k_u remains constant over the exposure period;
    b) to investigate whether k_e changes when the organisms are transferred to clean medium.

3.2 Material and methods

3.2.1 Organism cultures and test medium

The four *D. magna* clones used in the present study were obtained from the KNO17 population. This population was established from ephippia collected from a temporal pond in Knokke, West Flanders, Belgium (for details consult supportive information in Hochmuth *et al*. (2015). The four clones were maintained in laboratory condition during three years at 20°C under a controlled light cycle (16 h of light: 8 h of dark) (OECD 2012) and were fed daily with *Pseudokirchneriella subcapitata* with a food density of 2.5 mg C L^{-1}.

In order to reach physiological adjustment in each of the 3 temperature treatments, the daphnids were acclimated during 2 generations (Mitchell and Lampert 2000; Williams *et al*. 2012). More detailed information about the acclimation process can be found in chapter 2. Juveniles (5 d old) were used to guarantee enough biomass

for whole-body metal analysis and to avoid elimination trough the release of offspring. Neonates (<24h) collected from 3rd or 4th brood of the acclimated mothers were collected and cultured in aquaria for 5 days until (5-6 d old, juveniles) and then used to start the toxicokinetic experiment. A modified M4 medium was used (for details consult chapter 2).

3.2.2 Experimental design

We investigated Ni uptake and elimination in four *D. magna* clones at 15, 20 and 25°C. A scheme of the experimental design is given in Figure 3.1. Organisms were not fed during the experiment to avoid Ni uptake via food. The experimental set up was divided in two parts. In part I, organisms were exposed to ^{62}Ni during 72 h. Organisms were sampled at the time points 0, 8, 24, 48, and 72 h. In part II, after 48h of exposure to ^{62}Ni, organisms were transferred to test medium spiked with Ni with a natural isotope ratio ("presence of Ni") or to control medium ("absence of Ni"). Organisms were sampled at the time points 48, 56 and 72 h (i.e. 8 and 24 h after change of the isotope ratio).

Two Ni concentrations were tested per temperature, i.e. 56 and 73 µg Ni L^{-1} (measured dissolved concentration). These Ni concentrations correspond to the 21 d EC50 (estimated based on the average reproduction across all four clones i.e. to mimic the effect on a population) at 15 and 20°C, respectively (chapter 2). The treatment 73 µg Ni L^{-1} also corresponds to a 21-d EC20 at 25°C. A control treatment was performed and *Daphnia* samples were taken at 0 and 72 h. Three replicates with 5 organisms per replicate were taken for each clone at each temperature for each Ni concentration at each time point. Organisms were exposed in 200 ml of medium in polyethylene cups. Part I and part II of the experiment were performed in a single experiment.

The test medium was spiked with Ni three days before the start of the experiments and the test solutions were kept in the dark at 20°C. In part I, the stable isotope ^{62}Ni was used and obtained from Cortecnet (France) (Quality certificate: 28-01-62-4303) with the following composition 98.02, 1.02, 0.67, 0.25, 0.04 % of ^{62}Ni, ^{60}Ni, ^{58}Ni, ^{61}Ni and ^{64}Ni, respectively. In part II, a natural isotope ratio was used (i.e. 3.63,

26.22, 68.07, 1.13, 0.92 of ^{62}Ni, ^{60}Ni, ^{58}Ni, ^{61}Ni and ^{64}Ni, respectively (Gramlich *et al*. 1989), Ni (II) chloride hexahydrate from EMSURE analytical reagent was used.

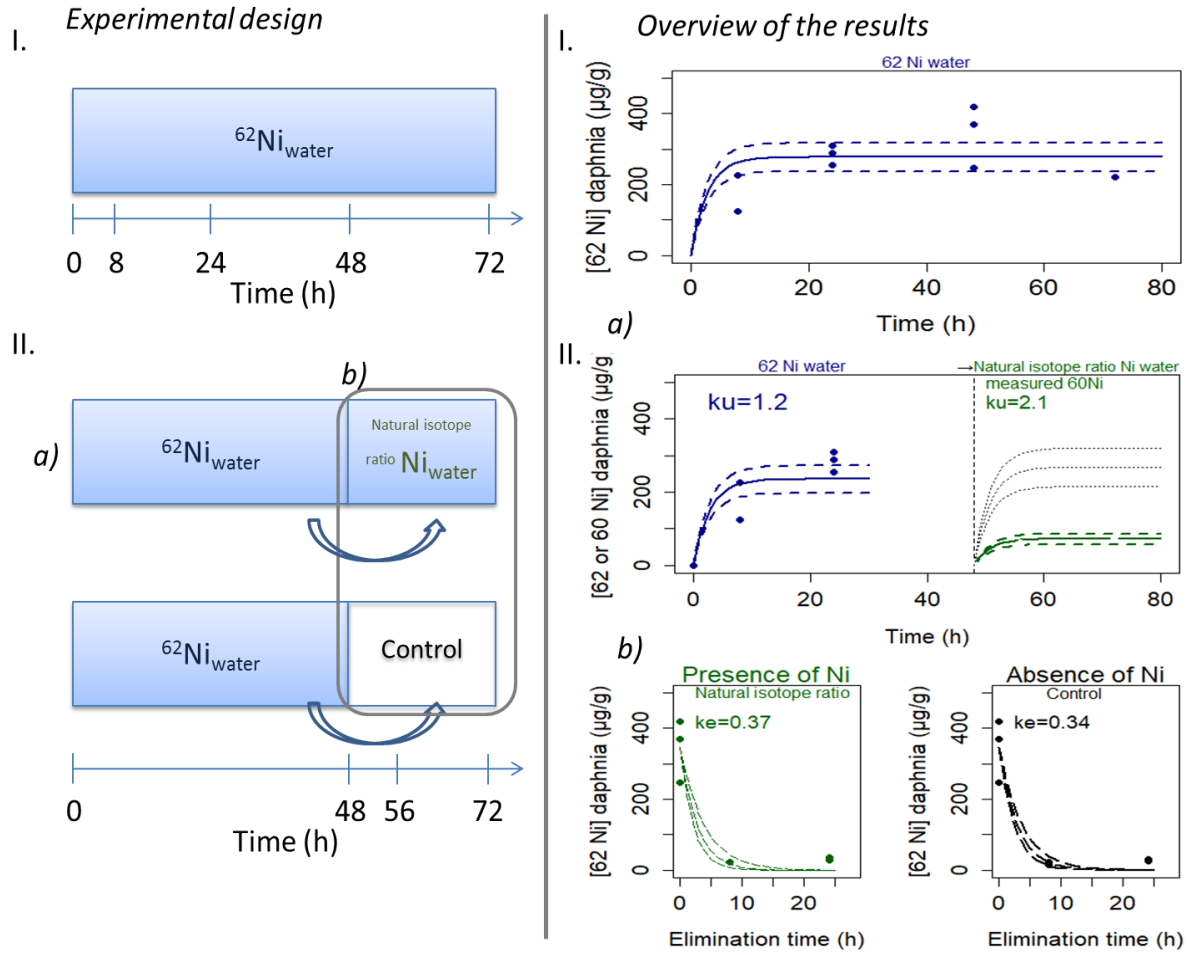

Figure 3.1. Overview of the experimental design used to investigate the influence of temperature in Ni uptake and elimination in *Daphnia magna* and of the results obtained. Four *D. magna* clones (5 d old) were exposed to Ni (56 and 73 µg L^{-1}) at 15, 20 and 25°C. The experiment design was divided in two parts: I) organisms were exposed to ^{62}Ni during 72h; II) after 48h of exposure to ^{62}Ni organisms were transferred to a test medium spiked $^{\text{Natural isotope ratio}}$Ni ("presence of Ni") switching the isotope ratio or to a control medium ("absence of Ni"). The sampling time is indicated. The results shown are of clone A at 20°C exposed to 56 µg Ni L^{-1}. Marker points represent observations and lines represent the estimated uptake or elimination curves and respective confidence limits. The letters specify the parts of the experiment design used and the type of results obtained to answer objective II a) and II b). Objective II a) was to investigate whether uptake rates remains constant over the exposure period. Objective II b) was to investigate whether the *D. magna* elimination rates change when the organisms are transferred for a non-contaminated medium. Dotted grey lines represent the predicted uptake curves after 48h of exposure with the water Ni concentration adjusted to the natural isotope ratio (^{60}Ni: 0.26%). Uptake rate constants (k_u) expressed as L g^{-1} h^{-1} and elimination rate constants (k_e) expressed as h^{-1}.

3.2.2.1 *Daphnia magna* sampling for nickel analysis

Organisms were transferred to a control medium for 10 minutes, after which they were transferred to a Na_2EDTA (5mM) (Sigma-Aldrich) solution were they remained during 1 minute (Adam *et al*. 2015). Organisms were then transferred to a sieve were they were quickly rinsed with deionized water. Organisms were transferred to a small piece of parafilm paper (PARAFILM M, Sigma-Aldrich) and all samples were placed in an oven at 60°C until constant dry weight (about 48h). Then organisms were weighed on a Sartorius Digital Micro balance (type 2405, Germany), and transferred to an Eppendorf tube (2 mL).

To each tube, containing dried daphnids, 100 µL HNO_3 (Normaton Ultrapure 69%, Prolabo) was added and the vial was left to stand overnight. Samples were microwave digested in 5 steps of 2 min at 90 and 5 steps of 2 min at 160 W, and five steps of 1 min at 360 W. A cooling interval of 2 min was included at the end of each step. Then 25 µL H_2O_2 was added to each sample (AnalaR NORMAPUR 30%) and 30 min later samples were microwave digested in 1 step of 2 min at 90, 1 step of 2 min at 160 W and 1 step of 1 min at 360 W. Samples were diluted with water (ultra-pure, Chem-lab NV) to 1 to 2% HNO_3. As quality control, two reference samples (mussel tissue 2976, NIST) and two procedural blanks were included in each 40-sample rack (Mubiana and Blust 2007). All samples with a total *Daphnia* body mass less than quantification limit (i.e.104 µg) were excluded from the analyses. Daphnid digests were measured with High Resolution Inductively Coupled Plasma - Mass Spectrometry (HR-ICP-MS) (Thermo Scientific Element 2 XR) to determine ^{60}Ni and ^{62}Ni concentrations at all time points. The recovery percentage of Ni in the reference samples was 70% of the certified values (10% coefficient of variation). The blanks were below quantification limit (0.001 µg $^{60}Ni\ L^{-1}$, 0.001 µg $^{62}Ni\ L^{-1}$). The quantification limits of *Daphnia* Ni body analysis were 4 µg $^{60}Ni\ g^{-1}$ and 19 µg $^{62}Ni\ g^{-1}$.

3.2.3 Water chemical analysis

The reference material used in all chemicals analysis and the quantification limits of water chemical analysis can be consulted in Appendix B (Table B1). Water

samples for metal and organic carbon analyses were taken at all time points, for all test solutions and for each temperature treatment. For the water analyses samples were taken for the total and dissolved concentrations. Dissolved concentrations refer to 0.45 µm membrane filtered levels (Acrodisc, PALL Life Sciences). Water samples were acidified to a final concentration of 0.14 mol L^{-1} of HNO_3 (Normaton Ultrapure 69% HNO_3, Prolabo) prior to analysis. Temperature was recorded daily. Dissolved oxygen concentration was measured at the beginning and at the end of the experiment. pH was measured at all time points in all test solutions at all temperature treatments. Concentrations of major cations (Na, K, Ca, and Mg) were measured using iCAP 7000 Series Inductively Coupled Plasma Optical-Emission Spectrometry (ICP-OES) (Thermo Scientific) in water samples collected at 72h.

Concentrations of ^{60}Ni and ^{62}Ni in water samples were measured with HR-ICP-MS (Thermo Scientific Element 2 XR). Samples for dissolved organic carbon analysis were measured with TOC-L CPH (Shimadzu). All data analyses were performed based on the mean dissolved concentrations. pH was measured with a pH meter 826 pH mobile (Metrohm). Dissolved oxygen was measured with an Oximeter WTW (probe WTW, cell Ox 325).

3.2.4 Data analysis

A one-compartment model was applied to calculate kinetic uptake and elimination parameters. The accumulation process can be described by the following differential equation:

Eq (3.1)

$$\frac{dC_{organism}}{dt} = k_u \cdot \mathrm{C_w} - k_e \cdot C_{organism}$$

which states that the change in concentration in an organism ($C_{organism}$) (µg g^{-1}) over time (t) (h) is a function of uptake minus elimination. C_w is the metal concentration in the exposure medium (µg L^{-1}), k_u is expressed by L g^{-1} h^{-1} and k_e is expressed by h^{-1} (Luoma and Rainbow 2008).

The k_e can be determined by the integrated version of equation 3.1

Eq (3.2)

$$C_{organism} = C_0 \cdot e^{(-k_e \cdot t)}$$

and the k_u can be determined by

Eq (3.3)

$$C_{organism} = C_0 + C_w \cdot \frac{k_u}{k_e} \cdot (1 - e^{(-k_e \cdot t)})$$

where C_0 is the metal concentration in the organism at t = 0.

As first steps of the analysis, the k_e in the presence ($k_{e,presence}$) and in the absence of Ni ($k_{e,absence}$) of the 4 clones at each temperature treatment were estimated using equation 3.2. To investigate whether the *D. magna* k_e change when the organisms are transferred to a clean medium, a paired t-test was performed to test significant differences between $k_{e,presence}$ vs. $k_{e,absence}$ (of the 4 clones at each temperature treatment). Significant differences were observed between $k_{e,\ presence}$ and $k_{e,\ absence}$ therefore the $k_{e,presence}$ values were used to estimate k_u according to equation 3.3.

As second step, the k_u of the 4 clones at each temperature treatment were estimated based on equation 3.3. The k_u were estimated with the data from part I of the experiment ($k_{u,I}$), the following function was used

Eq (3.4)

$$\left[^{62}\mathrm{Ni}\right]_{\mathrm{daphnia}} = \left[^{62}\mathrm{Ni}\right]_{\mathrm{daphnia,i}} + \left[^{62}\mathrm{Ni}\right]_{\mathrm{w}} \cdot \frac{k_{u,\mathrm{I}}}{k_{e,presence}} \cdot (1 - e^{(-k_{e,presence} \cdot t_{0\ to\ 72\ h})}$$

where $[^{62}Ni]_{daphnia}$ is the whole body ^{62}Ni concentration in the daphnia (µg g^{-1}), $[^{62}Ni]_{daphnia,i}$ is the ^{62}Ni concentration in the daphnia at t = 0 h, $[^{62}Ni]_w$ is the ^{62}Ni concentration in the exposure medium (µg L^{-1}). Also, the initial k_u were estimated with

the data points from 0 to 24 h ($k_{u,init)}$) from part I of the experiment, the following function was used

Eq (3.5)

$$\left[^{62}\mathrm{Ni}\right]_{\mathrm{daphnia}} = \left[^{62}\mathrm{Ni}\right]_{\mathrm{daphnia,i}} + \left[^{62}\mathrm{Ni}\right]_{\mathrm{w}} \cdot \frac{k_{u,init}}{k_{e,presence}} \cdot (1 - e^{(-k_{e,presence} \cdot t_{0\ to\ 24\ h})})$$

After 2 d of Ni exposure, the k_u were estimated with the data points from 48 to 72 h ($k_{u,48h}$), part II of the experiment, the following function was used

Eq (3.6)

$$\left[^{60}\mathrm{Ni}\right]_{\mathrm{daphnia}} = \left[^{60}\mathrm{Ni}\right]_{\mathrm{daphnia,i}} + \left[^{60}\mathrm{Ni}\right]_{\mathrm{w}} \cdot \frac{k_{u,48h}}{k_{e,presence}} \cdot (1 - e^{(-k_{e,presence} \cdot t_{48\ to\ 72h})})$$

where $[^{60}Ni]_{daphnia}$ is the whole body ^{60}Ni concentration in the daphnia (µg g^{-1}), $[^{60}Ni]_{daphnia,i}$ is the ^{60}Ni concentration in the daphnia at t = 48h, $[^{60}Ni]_w$ is the ^{60}Ni concentration in the exposure medium (µg L^{-1}).

The uptake and elimination rate constants of each clone at each temperature treatment were estimated using the *nlstool* package in R software (version 3.4.0; R Core Team 2017).

To explore the effect of temperature on Ni uptake in *D. magna* (objective I), paired t-tests were performed to: test significant differences of the $k_{u,I}$ values between 15, 20 and 25°C; and to compare the $k_{u,init}$ and $k_{u,48h}$ values between 15, 20 and 25°C.

To explore the effect of temperature on Ni elimination in *D. magna*, paired t-tests were performed to test significant differences of the $k_{e,presence}$ and the $k_{e,absence}$ values between 15, 20 and 25°C.

To investigate whether the k_u are constant over exposure duration, a paired t-test was performed to test significant differences between $k_{u,init}$ vs. $k_{u,48h}$ (of the 4 clones at each temperature treatment).

All statistical analyses were performed in R software (version 3.4.0; R Core Team 2017).

3.3 Results and discussion

3.3.1 Water chemistry

Temperatures remained stable along the experiment 14.9 (14.5-15.2°C), 19.3 (19.0-19.5°C) and 24.9°C (24.5-25.5°C) (average, and average minimum and average maximum temperature are given). The dissolved oxygen concentration (8.7±0.6 mg L^{-1}), the dissolved organic carbon (3.3±0.5 mg L^{-1}), the pH (7.6±0.1) and the major ion concentrations (17.7±0.3 mg Na L^{-1}, 8.8±0.2 mg Mg L^{-1}, 2.9±0.2 mg K L^{-1}, 52.1±1.2 mg Ca L^{-1}) also remained stable (average ± standard deviation) (Appendix B, Table B2).

The total and dissolved ^{60}Ni and ^{62}Ni concentrations remained stable during the toxicokinetic experiment (Appendix B, Table B3). The total Ni concentration presented <15% of variation in relation to the nominal concentration. In part I of the experiment, the measured dissolved Ni concentrations in water were 0.0±0.0, 56.8±3.4 and 73.7±13.4 µg L^{-1} (average ± standard deviation). In part II of the experiment (presence of Ni), the measured dissolved Ni concentrations in water were 0.0±0.0, 48.1±10.8, 69.5±19.2 µg L^{-1} (average ± standard deviation).

3.3.2 Uptake and elimination rate constants

An overview of the results obtained as well of the fits of the estimated uptake and elimination curves can be seen in Figure 3.1.

3.3.2.1 Effect of temperature on nickel uptake and elimination in *Daphnia magna*

The results of the present study indicated Ni uptake and Ni elimination in *D. magna* were affected by temperature. Furthermore, the same temperature patterns were observed on Ni uptake and on Ni elimination in *D. magna*. The k_u and k_e were lower at 25°C than at 20°C and they were similar between 20 and 15°C (Figure 3.2, 3.3 and 3.4). The Ni $k_{u,I}$ were significantly higher at 20°C than at 25°C by 2.6-fold (t-paired

test, n=4, $p<0.05$) but the $k_{u,I}$ at 15°C did not present significant differences in relation to 20°C and 25°C (t-paired tests: 15 vs. 20°C, n=3, $p>0.05$; 15 vs. 25°C, n=3, $p>0.05$) (Figure 3.2; Appendix B, Table B4, Figure B1 and B2). The same temperature trend was observed for $k_{u,init}$ and $k_{u,48h}$ values, i.e. the $k_{u,init}$ and $k_{u,\ 48h}$ values were significantly higher at 20°C than at 25°C by 1.5-fold (t-paired test, n=8, $p<0.05$) and the $k_{u,init}$ and $k_{u,48h}$ values at 15°C did not present significant differences in relation to 20°C and 25°C (t-paired tests: 15 vs. 20°C, n=6, $p>0.05$; 15 vs. 25°C, n=6, $p>0.05$) (Figure 3.3; Appendix B, Table B4, Figure B3 and B4). The $k_{e,\ absence}$ and $k_{e,\ presence}$ values were significantly lower at 25°C than at 15 and 20°C by 1.5-fold and 1.6-fold, respectively (t-paired test: 20 vs. 25°C, n=5, $p<0.05$; 15 vs. 25°C, n=5, $p<0.05$). The $k_{e,absence}$ and $k_{e,presence}$ values were similar between 15 and 20°C (t-paired test: 20 vs. 15°C, n=5, $p>0.05$) (Figure 3.4; Appendix B, Table B5).

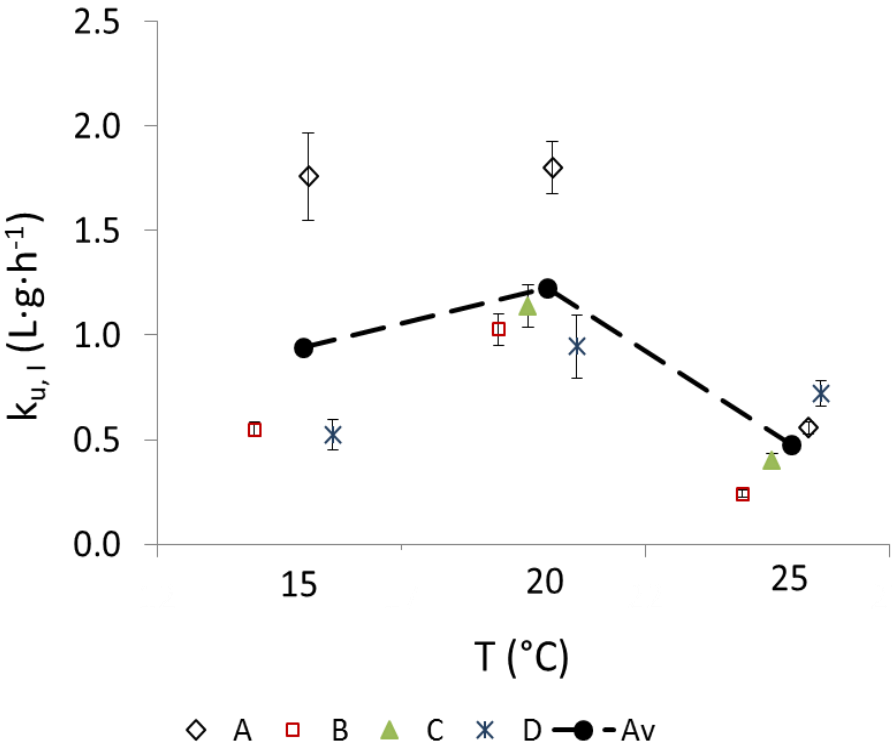

Figure 3.2. The effect of temperature on Ni uptake rate constant (k_u) (± standard error) in four *Daphnia magna* clones. The k_u were estimated with the data from part I of the experiment ($k_{u,\ I}$) where the organisms were exposed to 56 and 73 µg ^{62}Ni L^{-1} during 72h. Clones (A, B, C and D) are represented by different symbols. The averages of the $k_{u,\ I}$ of the four *D. magna* clones at 15, 20 and 25°C were plotted (Av).

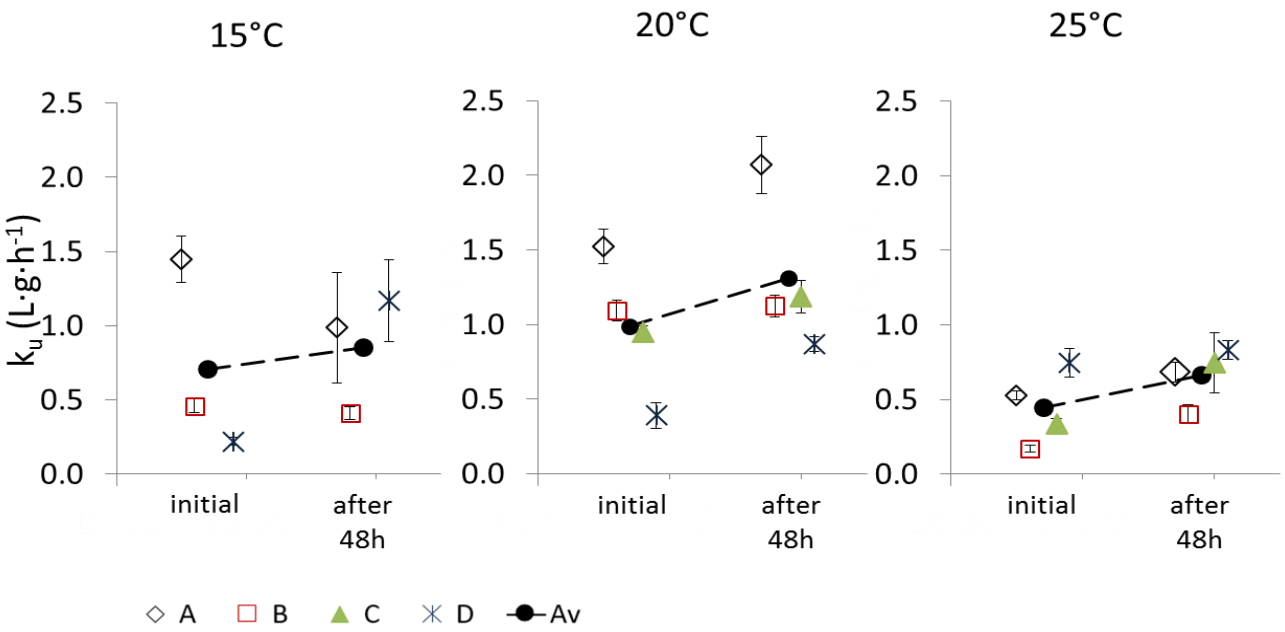

Figure 3.3. Uptake rate constants (k_u) (± standard error) estimated at the first day of Ni (56 and 73 µg ^{62}Ni L^{-1}) exposure (0-24h, initial) ($k_{u,init}$) and after 2d (48-72h, after 48h) ($k_{u,\ 48h}$) (48 and 70 µg ^{Nat}Ni L^{-1}) in four *Daphnia magna* clones at 15, 20 and 25°C. Clones are represented by different symbols. The averages of the k_u of the four *D. magna* clones at 15, 20 and 25°C were plotted (Av). Nat.: natural isotope ratio.

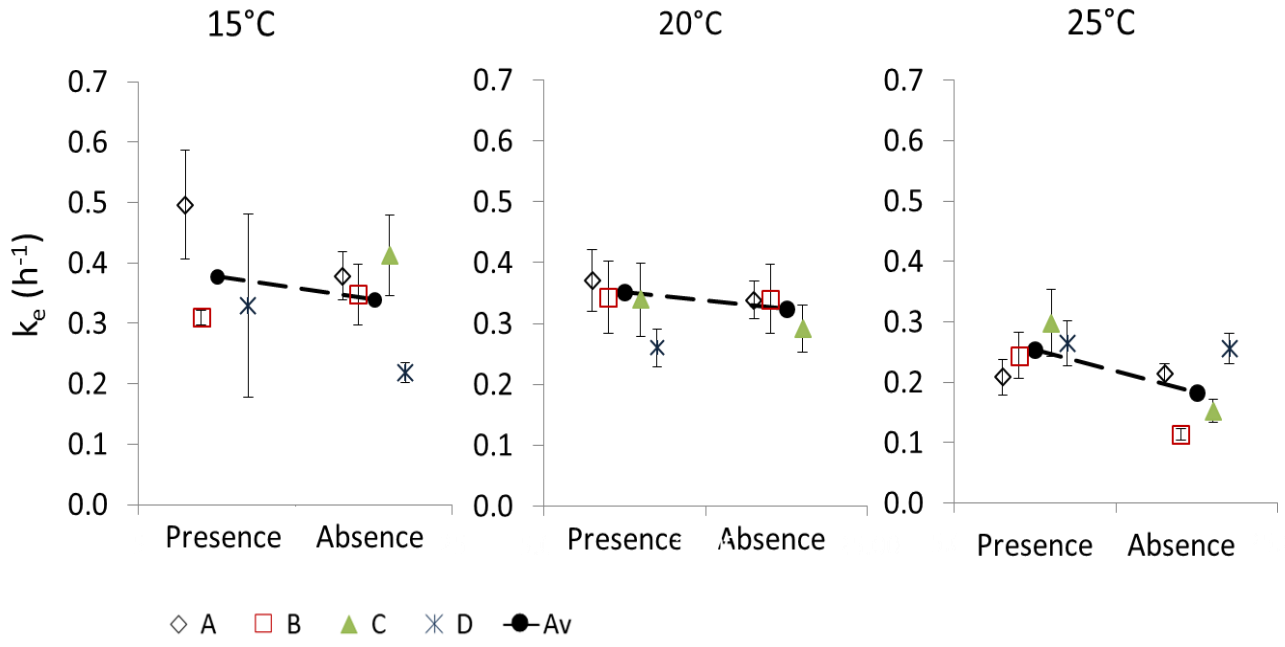

Figure 3.4. Elimination rate constants (k_e) of Ni estimated for the four *Daphnia magna* clones at 15, 20 and 25°C in the presence and absence of Ni. After 48h of exposure to 56 and 73 µg ^{62}Ni L^{-1}, *D. magna* were transferred to a medium spiked with Ni with a natural isotope ratio (presence) or to a control medium (absence). Clones are represented by different marker points. The averages of the k_e of the four *D. magna* clones at 15, 20 and 25°C were plotted (Av).

Until the present study, to our knowledge, only one study assessed the effect of temperature on metal toxicokinetics in *Daphnia* (Heugens *et al*. 2003). Heugens *et al*. (2003) indicated that an increase of temperature increased Cd k_u (i.e. the Cd k_u at 26°C ~ 20°C but the Cd k_u at 20°C> 10°C). Heugens *et al*. (2003) results apparently contrast with the present study. However, in Heugens *et al*. (2003) a single clone was tested, the test organisms were not pre-acclimated to the temperature treatments and another metal (Cd) was studied. In the present study, four clones pre-acclimated to the temperature treatments were used to guarantee that the effects of temperature on Ni toxicokinetics in *D. magna* were more broadly valid at population-level. The conclusions of the present study about the effect of temperature on Ni uptake in *D. magna* could have been different depending on which clone was tested (if a single clone was used). For example, with Clone A, the $k_{u,l}$ at 15°C and 20°C were similar and the $k_{u,l}$ at 25°C was 3-fold lower than at 15 and 20°C (the $k_{u,l}$ for clone A at 15, 20 and 25°C were 1.76±0.21, 1.80±0.13, 0.56±0.03 L g h^{-1}, respectively (±standard error)). Yet, if we only would have tested clone D we would have conclude the $k_{u,l}$ at 15°C was 1.8- fold lower than at 20°C and we would have concluded that the variation of the $k_{u,l}$ between 20 and 25°C was smaller (1.3-fold) (the $k_{u,l}$ for clone D at 15, 20 and 25°C were 0.52±0.07, 0.94±0.15 , 0.72±0.06 L g h^{-1} , respectively (±standard error)). The use of a single clone could have led to incorrect conclusions because the effects of temperature on Ni toxicokinetics in one clone may not represent the effects on the population. Furthermore, in ectothermic organisms an acclimation period is essential to physiologically adjust to the environmental temperature (Williams *et al*. 2012).

3.3.2.2 Effect of temperature on metal uptake and elimination in relation to chronic metal toxicity

Generally, the increase of metal uptake rate constants is associated with the increase of acute metal toxicity (Heugens *et al*. 2001; Heugens *et al*. 2003; Sokolova and Lannig 2008). However, little information is available concerning the effect of temperature on toxicokinetic processes in pre-acclimated organisms (i.e. organisms in homeostatic balance with environment temperature) and its association to chronic metal toxicity.

The effect of temperature on the ratio between k_u and k_e (k_u/k_e= Bioconcentration factor at equilibrium) determines the temperature effect on the internal metal concentration at equilibrium and whether the internal metal concentration can explain the effect of temperature on metal toxicity. The critical body concentration hypothesis states that the internal concentration of a chemical determines the toxicity to the organism (Vijver *et al*. 2004). However, the balance of uptake, compartmentalization, and elimination processes determines the internal metabolically available metal concentration and therefore the metal toxicity to the organism (Vijver *et al*. 2004; Rainbow 2007).

A previous in-house study, where the same *D. magna* clones and the same culture condition were tested as in the present study, indicated that chronic Ni toxicity to *D. magna* increased with the decrease of temperature (25°C<20°C<15°C) (chapter 2). The results of the present study indicated the ratio of $k_{u,I}$ and $k_{e,presence}$ was lower at 25°C (1.9 L g^{-1})) than at 20°C (3.7 L g^{-1}) (by 1.9-fold) hence the equilibrium internal Ni concentration observed after 48h of exposure ($[Ni]_{daphnia,48h}$) were lower at 25°C than at 20°C (Appendix B, Figure B1 and B2). The $[Ni]_{daphnia,48h}$ (present study) and the chronic Ni toxicity (chapter 2) were both lower at 25°C than at 20°C and these results are in line with the critical body concentration hypothesis (i.e. the internal concentration determine the toxicity to the organism) (Vijver *et al*. 2004). However, the ratio of $k_{u,I}$ and $k_{e,presence}$ was lower at 15°C (2.3 L g^{-1}) than at 20°C (3.7 L g^{-1}) (by 1.6-fold) hence the $[Ni]_{daphnia,48h}$ were lower at 15°C than at 20°C (and 15°C) (Appendix B, Figure B1 and B2). Although the $[Ni]_{daphnia,48h}$ were lower at 15°C than at 20°C, chronic Ni toxicity was higher at 15°C than at 20°C. Therefore, other toxicokinetic processes than Ni uptake and elimination are likely involved and contribute to explain the effect of temperature on chronic Ni toxicity to *D. magna* (Rainbow 2007; Luoma and Rainbow 2008; Rainbow and Luoma 2011). The results of chapter 4 showed that at lower temperatures a lower internal Ni concentrations in *D. magna* (clone K6) (adults) was needed to induce the same Ni toxicity than at higher temperatures (see chapter 4). Hence, in chapter 4, the internal Ni concentrations in *D. magna* also did not predict the effect of temperature on chronic Ni toxicity. Besides toxicokinetic processes, toxicodynamic processes, i.e. the metal interaction with the target sites, also determines metal toxicity.

3.3.2.3 Nickel kinetics in *Daphnia magna*

The results of the present study showed fast Ni kinetics in *D. magna*. At 20°C, the $k_{u\ (0\text{-}72h)}$ estimated varied from 0.94±0.15 to 1.80±0.13 L g^{-1} h^{-1} between clones and the k_e reached values from 0.26±0.03 to 0.37 ±0.05 h^{-1} (± standard error) (Figure 3.2 and 3.3; Appendix B, table B4 and B5).

Previously, Ni toxicokinetics have been studied in *D. magna* at 20°C (Komjarova and Blust 2009a). In Komjarova and Blust (2009a), *D. magna* was exposed simultaneously to low ecologically relevant concentration of Ni (18.6 µg L^{-1}), Cd (5.3 µg L^{-1}), Cu (9.8 µg L^{-1}), Pb (10.2 µg L^{-1}), and Zn (10.1 µg L^{-1}) at 20°C and, the Ni k_u was 0.04 L g^{-1} h^{-1} (875 L kg^{-1} d^{-1}). The Ni k_u values of the present study and of the Komjarova and Blust (2009a) present a difference of one order of magnitude but several differences are present between both studies. Firstly, organisms in different life stages were used, that is Komjarova and Blust (2009a) used adults (15-16 d old) and the present study used juveniles (5d old). Previous studies suggest Ni exposure can disrupt Mg homeostasis in *D. magna* and Mg is of crucial importance for the development and growth of organisms (Pane *et al*. 2003; Wolf and Cittadini 2003; Deleebeeck *et al*. 2008). Therefore, the higher k_u observed in the present study may be due to the juveniles need for more Mg. Secondly, in Komjarova and Blust (2009a), Ni uptake was study in the presence of other metals as Cu, Zn Pb and Cd which may influence each other's toxicokinetics. For example, previous studies have shown interactive effects between Ni, Cu and Zn on daphnid reproduction (Nys *et al*. 2015; Nys *et al*. 2017). Hence, the Ni k_u estimated in Komjarova and Blust (2009a) may not represent the Ni k_u in single exposure. Thirdly, different mediums with different water hardness were used. The water hardness of the mediuns used in the present study and in Komjarova and Blust (2009a) were 176 and 231 mg L^{-1} as $CaCO_3$, respectively. Deleebeeck *et al*. (2008), showed the increase of water hardness (Ca and Mg combined) protected *D. magna* against chronic Ni toxicity. Since, the cations Ca and Mg can compete with the Ni for binding at active sites (Deleebeeck *et al*. 2008) hence Ni uptake can be reduced. The competition between hardness cations (Ca and Mg) and Ni may explain the lower Ni k_u reported in Komjarova and Blust (2009a).

3.3.2.4 Is k_u constant with experimental time?

Nickel uptake rates increased over time in the four *D. magna* clones. The $k_{u,48h}$ values were significantly higher than the $k_{u,init}$ values (by 2.3, 1.5, 1.7-fold at 15, 20 and 25°C, respectively) (t-paired test, n=11, p<0.05) (Figure 3.4; Appendix B, Table B4).

A previous study showed Ni assimilation rates can change along metal exposure (Laskowski *et al*. 2010). Laskowski *et al*. (2010) studied Ni toxicokinetics in *P. oblongopunctatus* and in *L. terrestris* and showed that a three-phase model (one-compartment model), where after an initial period of exposure k_u decreases or even becomes zero, better described metal toxicokinetics over time than the classic two-phase model (i.e. one-compartment kinetics model) with one k_u and one k_e. Laskowski *et al*. (2010) suggested the physiological state of the organism along exposure can change with time in reaction to the contaminant exposure. Previous studies suggest Ni exposure can disrupt Mg homeostasis in *D. magna* (Pane *et al*. 2003; Deleebeeck *et al*. 2008). Nickel is very similar to Mg, it has the same size and water exchange constants as Mg (Wolf and Cittadini 2003). Magnesium is an essential ion important for several biological functions including structural stabilization of nucleic acids, cell membranes and to promote specific structural organization of enzymes and ribosomes (Wolf and Cittadini 2003). Therefore, in order to maintain Mg homeostasis, the mechanisms of Mg uptake may be triggered and hence Ni k_u may increase over time. Future toxicokinetic studies should consider that the k_u may not be constant over exposure period.

3.3.2.5 Is k_e affected by the presence and absence of the stressor?

Nickel k_e in *D. magna* decreased when the external Ni exposure stopped (Figure 3.3; Appendix B, Table B5). The $k_{e,absence}$ values were significantly lower than the $k_{e,presence}$ values by 1.2, 1.9 and 1.5-fold at 15, 20 and 25°C, respectively (t-paired test, n=10, p<0.05)(Figure 3.3; Appendix B, Table B5, Figure B5, B6, B7 and B8).

Previously, the studies of Evan *et al*. (2002, 2006) showed the elimination rates of Cd, Cu, Pb and Zn in *Hydropsyche* larvae were similar in the presence vs. the absence of the contaminant. However, different species from different classes were

tested: *Hydropsyche* larvae from the class Insecta and order Tricoptera (ITIS 2018) in Evan *et al*. (2002, 2006); *D. magna* from the class Branchiopoda and order Diplostraca in the present study (ITIS 2018). Differences among species on uptake and elimination processes can be due to physiological differences (e.g. locomotor and feeding activities) (Luoma and Rainbow 2008). Furthermore, different metals were tested in both studies.

The present study showed the differences between $k_{e,presence}$ and $k_{e,absence}$ although small were significant. Therefore in the design of future toxicokinetic studies should be considered that the k_e may differ in the presence and in the absence of the chemical stressor.

3.4 Conclusion

The results of the present study indicated Ni uptake and Ni elimination in *D. magna* were affected by temperature. The same temperature patterns were observed on Ni uptake and on Ni elimination in *D. magna* i.e. the k_u and k_e were lower at 25°C than at 20°C and they were similar at 20 and 15°C. Furthermore, our results showed the effects of temperature on Ni toxicokinetics in one clone may not represent the effects on the population, therefore the use of a single clone could guide us to incorrect conclusions.

In addition, the present study showed Ni uptake rates of *D. magna* increased over time and Ni k_e decreased when the external Ni exposure stopped. Therefore future toxicokinetic studies should considered the k_u may depend on the physiological state of the organism and the k_e may differ between the presence and in the absence of the chemical stressor.

Chapter 4

Multigenerational effects of nickel on *Daphnia magna* depend on temperature and the magnitude of the effect in the first generation

Redrafted from:

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4.1 Introduction

Ecological risk assessment is often based on single generation ecotoxicological laboratory experiments that are performed at a standard temperature. By doing so, the potential influence of temperature on chemical toxicity and effects from multigenerational exposures in the field are not taken into account. Temperature is an important abiotic factor for ectothermic organisms, including the ecotoxicological model organism *Daphnia magna*, which can be found in ponds and lakes at water temperatures between 5 and 26°C (Carvalho 1987; Lopez *et al*. 1991). The increase of surface water temperature increases the ventilation rates and the respiration rates and it reduces the generation time (i.e. time to first brood) of *D. magna* (Paul *et al*. 2004a; Walsh *et al*. 2014; Bae *et al*. 2016). A recent study of Bae *et al*. (2016) exposed *D. magna* to 20 and 25°C during three generations and it was found that the levels of reactive oxygen species increased at 25°C in comparison to 20°C. Moreover, the temperature dependency of the reactive oxygen species production increased along generations. This study also showed that the length of offspring (5 days old) was significantly reduced at 25°C. At 25°C, the shorter time to reach maturity and time to first brood could reduce offspring quality. Campos *et al*. (2016) found that negative effects on offspring quality (e.g. assessed based on offspring length) could enhance the adverse effects of chemicals in later generations. The results of chapter 2 indicated that the exposure temperature had a significant effect on chronic metal toxicity to *D. magna*. In chapter 2, the results showed that in comparison with 20°C, the standard temperature recommended by the OECD guideline for the *D. magna* reproduction test (OECD 2012), chronic Ni toxicity to *D. magna* increased at 15°C and decreased at 25°C.

Chemical toxicity can change along generations of *Daphnia* (Münzinger A. 1990; Pane *et al*. 2004; Guan and Wang 2006; Vandegehuchte *et al*. 2009; Massarin *et al*. 2010). However, the information about the multigenerational effects of metal exposure and of temperature on metal sensitivity is relatively limited. In two multigenerational studies performed at a single temperature (20°C) Ni toxicity to *D. magna* changed along generations and demonstrated differential effects on body length of offspring, mean life span, number of broods, brood size, and the intrinsic rate of population growth (Münzinger A. 1990; Pane *et al*. 2004). Two acute studies have reported higher internal metal concentrations at higher temperatures in

Daphnia, suggesting temperature effects on metabolic rates may influence metal uptake (Heugens *et al*. 2003; Sokolova and Lannig 2008; Vandenbrouck *et al*. 2011). However, the physiological state of the organism can influence metal uptake and also the detoxification, the sequestration and the elimination processes that determine metal toxicity (Vijver *et al*. 2004). It is therefore not known if differences in internal body concentrations of Ni could explain effects of temperature on chronic Ni toxicity.

Given the importance of temperature variation and multigenerational exposure in field conditions and the data gaps mentioned above, we investigated the temperature dependence on multigenerational effects of Ni. Therefore, the effects of Ni on *D. magna* reproduction and offspring length were tested at 15, 20 and 25°C along four generations. In addition, to explore possible differences in the mechanisms of Ni toxicity at different temperature and different generations, internal Ni concentrations ($[Ni]_{daphnia}$) were measured. Furthermore, little information is available concerning the mechanisms that are related to the effect of temperature on Ni toxicity to *Daphnia*. Brix *et al*. (2017) identified four potential mechanism of Ni toxicity in aquatic organisms, which are the disruption of Ca, Mg and Fe homeostasis and the generation of reactive oxygen species. Also, previous studies have shown interactive effects between Ni, Cu and Zn on daphnid reproduction (Nys *et al*. 2015; Nys *et al*. 2017), which might be related to these metals influencing each other's toxicokinetics. It is however, unknown if these potential Ni toxicity mechanisms apply across a wide temperature range. Therefore, ion homeostasis of Cu, Zn, Fe, Mg, Ca, Na and K (only in F0) were also measured.

4.2 Material and methods

4.2.1 Organism cultures and test media

The *D. magna* clone (K6) was cultured in aquaria in a modified M4 medium that was also used for exposure (see chapter 2). Prior to actual multigenerational Ni exposure, organisms were acclimated for two generations to the temperature treatments (Mitchell and Lampert 2000). Neonates (<24h) collected from 3rd to the 4th brood of the acclimated mothers were used to start the multigenerational Ni exposure. More detailed information about the acclimation process and test medium can be found in chapter 2.

4.2.2 Experimental design

A multigenerational toxicity test was performed with *D. magna* exposed to Ni at 15, 20 and 25°C. The test medium was spiked with Ni three days before the start of the experiment and these test solutions were then kept in the dark at 20°C. One day before the start of the experiment or one day before the change of the medium the test solutions were placed in the different climate rooms at 15, 20 and 25°C. Every 4 weeks new medium was prepared and spiked. A scheme of the experimental design can be found in Figure 4.1. Seven Ni concentrations (0, 12, 25, 50, 100, 200 and 300 µg L^{-1}) were tested per temperature along four generations. Organisms were continuously exposed along four generations to the same Ni concentration (further defined as: Ni treatment). The 7 Ni treatments and the 3 temperature treatments of the multigenerational experiment were investigated simultaneously. Fifteen individual replicates per Ni treatment were performed to guarantee a sufficient number of organisms to start the subsequent generation. Exposure of the first generation started with neonates (<24h) collected from 3rd to the 4th brood of pre-acclimated mothers in aquarium to the temperature treatments. Neonates (<24h) were exposed individually in 50 mL of medium in polyethylene cups. Once first generation females (F0) released their third brood of offspring, the second generation was initiated (F1). The same procedure was followed for the following generations. To start a next generation 10 neonates were collected from each of 5 randomly selected mothers and pooled together. When reproduction occurred in less than 5 mothers, neonates were collected from at least 3 mothers (10 neonates per mother). If less than 10 neonates were released per mother, 5 neonates were collected per mother from 5 to 10 mothers. Otherwise, the next generation of that Ni treatment was not started.

As *D. magna* is an ectothermic organism, time to reach maturity depends on temperature. Therefore, to ensure that *D. magna* reproduction could be assessed within each temperature treatment, the exposure of each generation continued until the daphnids had released the 5th brood in more than 50% of the replicates of the control treatment. In the present research a generation was defined as the time between the start of the Ni exposure of the neonates (<24h) and the release of the 5th brood by the adult females in control treatments. All experiments were performed under a controlled light cycle (16 h of light: 8 h of dark) and the test medium was

renewed three times a week as recommended by OECD guideline No. 211 (OECD 2012). Daphnids were fed daily with *Pseudokirchneriella subcapitata* with a food density of 2.5 mg C L^{-1} in the first week of each generation and 5 mg C L^{-1} on the following weeks.

Survival of each mother and number of offspring were recorded daily. To assess the quality of the offspring, the length of neonates (<24h) released at the end of each generation was measured in 3 replicates, 5 neonates per replicate. Different broods were used for different purposes, that is 3rd brood organisms were used to start the next generation and 5th brood organisms were used to measure length. This was logistically necessary because not enough organisms from the same brood were available for both purposes. To determine metal body concentrations in adult females, samples were taken at the end of each generation. The surviving adult females were randomly sampled and equally divided in 3 replicates to ensure sufficient body mass for the metal analysis. The internal Ni concentrations in *D. magna* ($[Ni]_{daphnia}$) were measured in all generations and the internal Cu, Zn, Fe, Mg, Ca, Na and K concentrations in *D. magna* were only measured in F0.

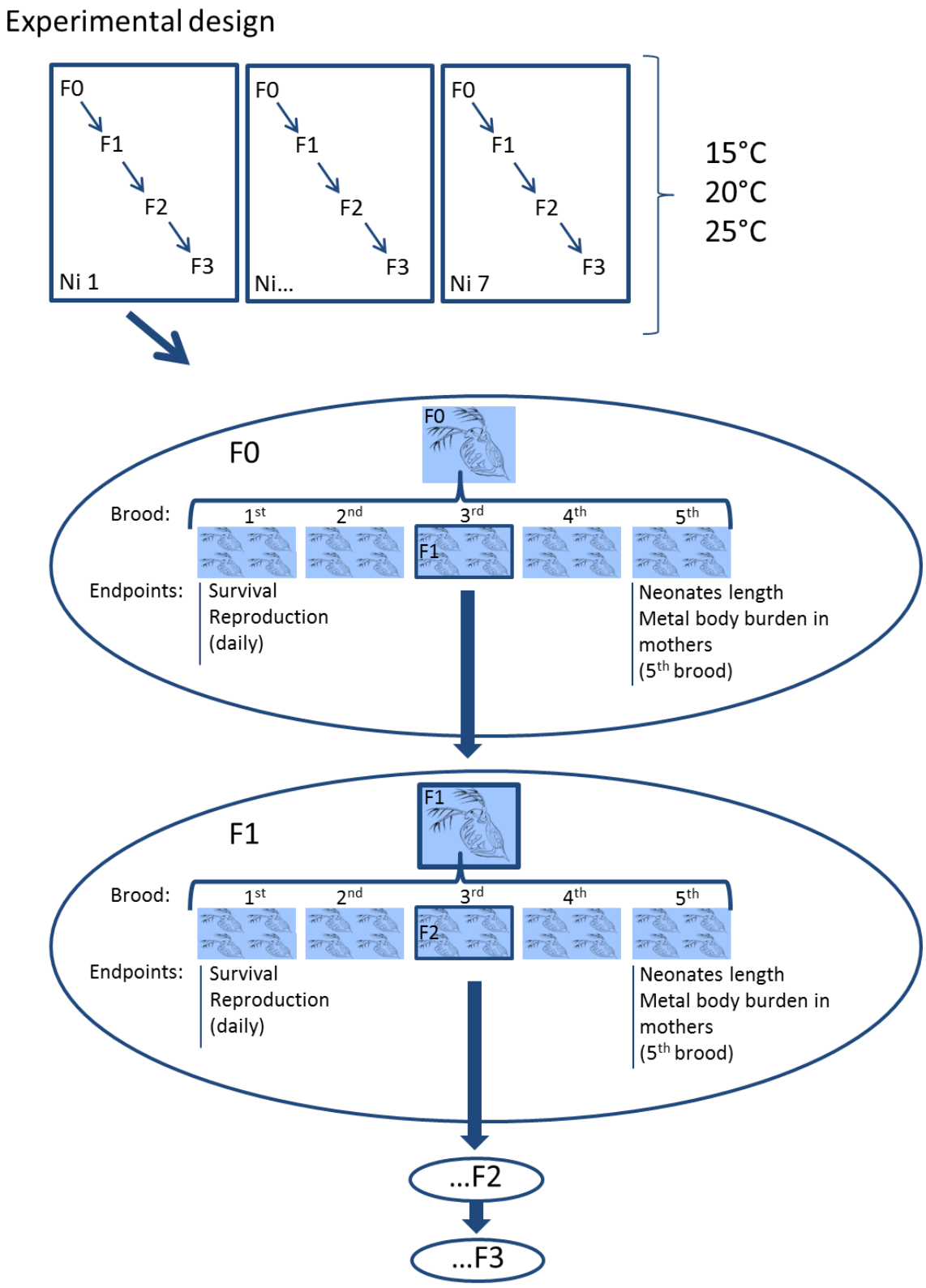

Figure 4.1. Experimental design used for the multigenerational exposure of *Daphnia magna* to Ni at 15, 20 and 25°C. The generations (Fx), the brood that was used to start the next generation (3rd brood) and the endpoints are indicated. Survival and reproduction were recorded daily. The length of neonates and metal body concentration in adult females were assessed at the end of each generation when 50 % of the organisms (adult females) in control treatments released the 5th brood.

4.2.3 *Daphnia magna* sampling for metal analysis

Daphnids were transferred to a control medium for 10 minutes, after which they were transferred to a Na_2EDTA (5mM) (Sigma-Aldrich) solution were they remained during 1 minute to remove Ni adsorbed to the carapace (Adam *et al*. 2015). The organisms were transferred to a sieve were they were quickly rinsed with deionized water. Daphnids were transferred to a parafilm paper and all samples were placed in an oven at 60°C for at least 48 h until a constant dry weight. The daphnids were weighed on a Sartorius Digital Micro balance (type 2405), and transferred to an Eppendorf tube (2 mL).

To each vial, containing dried daphnids, 200 µL HNO_3 (Normaton Ultrapure 69%, Prolabo) was added and the vial was left to stand overnight. The daphnids were microwave digested in five steps of 2 min at 90 and 160 W, and five steps of 1 min at 360 W. The different treatment steps were separated by a 2 min interval. Then 25 µL H_2O_2 was added to each sample (AnalaR NORMAPUR 30%) and 30 min later daphnids were microwave digested in 1 step of 2 min at 90, 160 W and 1 min at 360 W. The samples were diluted with water (ultra-pure, Chem-lab NV) to 1 to 2% HNO_3. As quality control, two reference samples (mussel tissue 2977, NIST) and two procedure blanks were included in each 40-sample rack (Mubiana and Blust 2007). Recoveries of all metals are reported in Appendix C (Table C1). The recovery of Mg in the reference samples was 8% of the certified values although very good recoveries (90%) had been found in earlier experiments, using the same procedure. Also, the $[Mg]_{daphnia}$ values found in the present study are in the same range as those reported in a previous study with *D. magna* (Pane *et al*. 2003). Therefore, despite the low recovery, we did report measured $[Mg]_{daphnia}$ values.

4.2.4 Chemical analysis

The reference material used in all chemical analyses and the quantification limits of water chemical analysis and *D. magna* metal body analysis can be consulted in Appendix C (Table C2). Water samples for metal analysis were taken from test solutions for each temperature and for each generation. During the four generations, samples of fresh and old medium of all treatments were collected weekly for analysis of total (only of fresh medium) and dissolved concentrations of Ni and also Cu, Zn,

Fe and major cations (Na, K, Ca and Mg). Dissolved concentrations refer to 0.45 µm membrane filtered concentrations (Acrodisc, PALL Life Sciences). Samples for metal analysis were acidified to a final concentration of 0.14 mol L^{-1} of HNO_3 prior to analysis. Water metal analysis was performed using iCAP 7000 Series ICP-OES (Thermo Scientific).

Weekly samples for total (only for fresh medium) and dissolved organic carbon analysis were taken for the 0, 12, 50, 200 µg Ni L^{-1} treatments in F0 and samples were taken only in the control treatment and in the highest Ni concentration tested in following generations. The samples for DOC analysis were measured with TOC-L CPH (Shimadzu).

All data analyses were performed based on the mean dissolved concentrations of new and old medium measured for F0. pH was measured with a pH glass electrode (P407; Consort) and dissolved oxygen was measured with an Oximeter WTW (probe WTW, cell Ox 325) were measured weakly. Temperature was recorded daily.

Daphnids digested samples were measured with ICP-OES to determine Cu, Zn, Fe, Na, K, Ca, Mg body concentrations and with graphite furnace atomic absorption spectrophotometry (GFAAS Furnace Autosampler, Thermo Fisher Scientific Inc., Waltham, MA, USA) to determine Ni body concentrations.

4.2.5 Data analysis

All statistical analysis were performed in R software (version 3.4.0; R Core Team 2017) according to the protocols of Zuur (2009).

4.2.5.1 *Daphnia magna* reproduction – Analysis of variance

To investigate whether the effect of temperature on Ni toxicity to *D. magna* reproduction changes across generations and to investigate whether the multigenerational effect on Ni toxicity to *D. magna* reproduction changes at different temperatures we tested for possible interactive effects between temperature, generation and Ni. The data that were generated were analysed by applying a general linear model. However, this analysis violates the independence assumption.

We observed that organisms (F_{x+1}) born from more healthy mothers (F_x) (expressed as a higher offspring production) also had more offspring regardless of temperature and Ni. We tested for possible correlations (Spearman correlation) and we observed significant positive correlations of reproduction between F0 vs. F1 (r^2 = 0.511, p<0.05), F1 vs. F2 (r^2 = 0.813, p<0.001) and F2 vs. F3 (r^2 = 0.593, p<0.05) (Appendix C, Figure C1).

As a tool to include a dependence structure in the analysis, we therefore applied to our data set a generalized estimation equation (GEE) with a three way-interaction between temperature, Ni and generation with an auto-regressive correlation structure between generation using the *geepack* package in R software (version 3.4.0; R Core Team 2017). The GEE predicted the Ni (continuous variable) effect on reproduction, expressed as the number of offspring per individual female produced until more than 50% of the organisms in control treatment released the fifth brood (*Rep5*), as a function of temperature (factorial variable) and generation (factorial variable).

Prior to analysis, *Rep5* was log10 transformed as $\log_{10}$ (*Rep5* +1). Normality was evaluated using a QQ plot of the model residuals and by plotting the residuals against the fitted values. The homogeneity of variances of residuals across all temperatures, generations, and Ni was evaluated using a boxplot (Zuur *et al*. 2009).

4.2.5.2 *Daphnia magna* reproduction - Effect and no-observed- effect concentrations

Pairwise Wilcoxon rank sum tests were used to test for statistical differences on the endpoints *Rep5* and offspring size (length) between control and Ni treatment at each generation and at each temperature. The Benjamini & Hochberg (1995) correction method was used to adjust the *p* values for multiple comparisons within each temperature at each generation. The determination of the ECx were performed with reproduction expressed as *Rep5* (% of control) using the *drc* package in R software (version 3.4.0; R Core Team 2017)). Effect concentrations were determined using the best fitted model to the data (Appendix C, Table C3). Pairwise Wilcoxon rank sum tests were performed using the *stats* package.

4.2.5.3 *Daphnia magna* internal nickel concentrations.

A GEE analysis was also applied to the endpoint $[Ni]_{daphnia}$ with a three way-interaction between temperature (factorial variable), Ni (continuous variable) and generation (factorial variable) with an auto-regressive correlation structure between generation using the *geepack* package in R software (version 3.4.0; R Core Team 2017) (Zuur *et al*. 2009). Prior to analysis, $[Ni]_{daphnia}$ and water Ni concentration were $\log_{10}$ (x+0.1) transformed. Normality and homogeneity of variances were evaluated as described in *D. magna* reproduction – Analysis of variance.

In addition, to investigate whether the effect of temperature on Ni toxicity to *D. magna* reproduction is explained by $[Ni]_{daphnia}$ we fitted the Michaelis-Menten equation to the data at each generation and each temperature. The Michaelis-Menten curve was fitted to the $[Ni]_{daphnia}$ using the *nlstool* package

Eq (4.1)

$$[\mathrm{Ni}]_{daphnia} = \beta_{max}\left(\frac{[Ni]_{water}}{[Ni]_{water} + Ks}\right)$$

where β_{max} is the maximum $[Ni]_{daphnia}$ (µg g^{-1}), $[Ni]_{water}$ is the water Ni concentration (µg L^{-1}) and Ks is the half saturation constant (µg L^{-1})(Buchwalter and Luoma 2005).

4.2.5.3 *Daphnia magna* internal cations concentrations.

A linear model was applied to each individual metal measured in *D. magna* and expressed as internal body concentrations ($[Metal]_{daphnia}$), i.e. Ca, K, Na, Mg, Cu, Zn and Fe with a two way-interaction between temperature (factorial variable) and Ni (continuous variable). Prior to the linear analysis, $[Metal]_{daphnia}$ and $[Ni]_{water}$ were log (x+0.1) transformed to normalize the data. To test for statistical differences on the $[Metal]_{daphnia}$ (i.e. Ca, K, Na, Mg, Cu, Zn and Fe) in F0 between temperatures a pairwise Wilcoxon rank sum tests were performed. The Benjamini & Hochberg (Benjamini and Hochberg 1995) correction method was used to adjust the p values for multiple comparisons.

4.3 Results

4.3.1 Test water characteristics

Temperature remained stable in the three treatments during the four generations (14.7 (±0.8), 19.9 (±0.5) and 24.8°C (±0.6) (mean ± standard deviation) (Appendix C, Table C4). The dissolved oxygen concentration (8.9±0.7 mg L^{-1}), the dissolved organic carbon (3.5±0.2 mg L^{-1}), the pH (8.0±0.1), and the major ion concentrations (21.2±1.3 mg Na L^{-1}, 9.2±0.7 mg Mg L^{-1}, 3.3±0.2 mg K L^{-1}, 62.1±3.7 mg Ca L^{-1}) (overall mean ± standard deviation) also remained stable during the multigenerational experiment (Appendix C, Table C4, C5 and C6).

4.3.2 *Daphnia magna* reproduction, offspring length

In the control treatments, at the end of each generation a mean of ≥ 60 living offspring per parent survival female was reached for all generations at 15 and 20°C but not at 25°C with a mean of ≥ 40 offspring per female (Appendix C, Table C8).

For F0 the length of the offspring was not reduced by Ni in any of the temperature treatments (p>0.05) with the exception of the 6 µg Ni L^{-1} treatment (nominal concentration: 12 µg Ni L^{-1}) at 25°C (p<0.05) (Appendix C, Table C9).

4.3.2.1 *Daphnia magna* reproduction – Analysis of variance

The GEE analysis revealed a significant interaction between temperature and Ni to *D. magna* reproduction, between generation and Ni, and a significant three-way interaction between generation, temperature and Ni (Table 4.1). The correlation coefficient between two sequential generations is 0.191± 0.056 (± standard error (SE)). The $\log_{10}$ (*Rep*5+1) transformed data met the assumption of normality based on the QQ plot of the model residuals and on the plot of the residuals vs. fitted values (Appendix C, Figure C2). The residuals of the optimal model were homogeneously distributed around 0 when plotted against the variables temperature, generation and Ni indicating homogeneity of variance. The plot of predicted vs. observed values indicates a good model fit (Appendix C, Figure C2) (Zuur *et al*. 2009).

Table 4.1. Summary of the analysis of variance performed with the generalized estimation equation that predicts the Ni (continuous variable) effect on reproduction as a function of temperature (factorial variable) and generations (factorial variable) (Ni × temperature × generation) with an auto-regressive correlation structure between generations. Reproduction expressed as log10-transformed number of offspring per individual female produced until the organisms in control treatment released the 5th brood plus 1 (log_{10} (*Rep5* +1)). Degrees of freedom (Df), Wald statistics (X^2), and significance (*p* value) are shown. The estimated correlation coefficient between Fx and Fx+1 was 0.191± 0.056 (± SE).

	Df	X^2	*p*
Ni	1	49.82	<0.001
Temperature	2	1.58	>0.05
Generation	3	11.09	<0.05
Ni × temperature	2	53.84	<0.001
Ni × generation	3	23.07	<0.001
Temperature × generation	6	22.77	<0.001
Ni × temperature × generation	6	79.10	<0.001

4.3.2.2 *Daphnia magna* reproduction - ECx and NOEC

To better understand the three-way interaction between generation, temperature and Ni toxicity to *D. magna* reproduction (p<0.001), we performed a more detailed analysis of our data, by calculating ECx values and by testing for statistical differences on *Rep5* between control and Ni treatment in each generation and at each temperature.

Corroborating the results of chapter 2, temperature had a significant effect on chronic Ni toxicity to *D. magna* in F0. The EC50 for reproduction of Ni for F0 ($EC50_{F0}$) increased 6.5-fold between 15 and 25°C (Figure 4.2 and Appendix C, Table C3). In some temperature treatments in later generations a concentration response curve could not be fitted because some of the Ni treatments that continue to the next generation did not present adverse effects. Therefore the effects of temperature on Ni toxicity in later generations could not be estimated on the basis of ECx values.

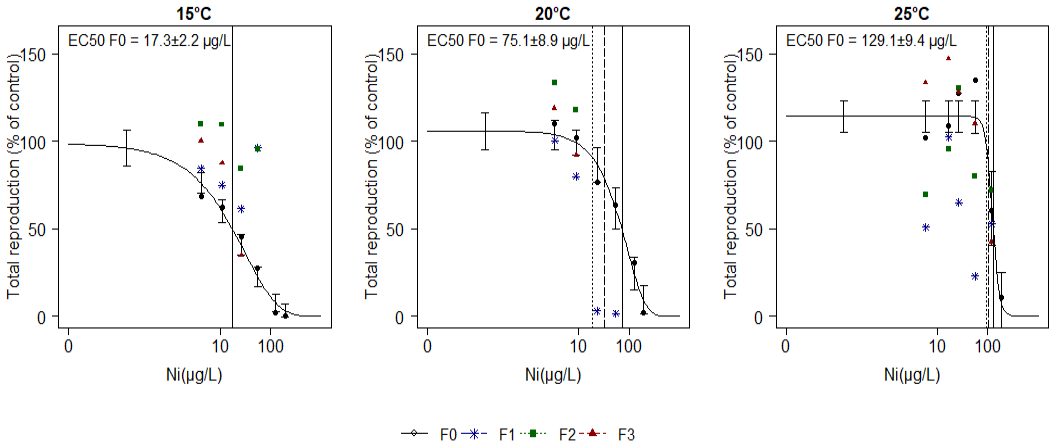

Figure 4.2. Reproduction of *Daphnia magna* exposed to Ni at 15, 20 and 25°C during four generations (F0, F1, F2, and F3). Reproduction is expressed as the number of offspring per individual female produced until the organisms in control treatment released the 5th brood as percentage of control treatment (total reproduction is expressed as % of control). Marker points represent observations (average data) and lines are the fitted concentration response curves for F0. Vertical lines indicate the effect concentrations (ECx) estimated for F0, the EC10 values are represented by dotted lines, the EC20 values by dashed line and EC50 values by full lines. At 15°C, the estimated Ni EC10 and EC20 were bellow the lowest concentration tested therefore that values were not reported.

Multigenerational Ni effects on *D. magna* reproduction showed different patterns at different temperatures (Figure 4.2; Table 4.1 and 4.2). At 20°C (standard temperature) when organisms were exposed to concentrations below the $EC10_{F0}$ (13.5 µg L^{-1} (±6.1 (SE))) chronic Ni toxicity did not increase along generations (Figure 4.2). However, chronic Ni toxicity significantly increased along generations when organisms were exposed to Ni concentrations higher than $EC10_{F0}$. Indeed, at 23 and 54 µg Ni L^{-1} reproduction was reduced by 23 to 36% in F0 but by 97 to 98% in F1 (Table 4.2). Therefore, it was also not possible to start exposures of F2 and F3 with these Ni treatments (due to a lack of neonates). At 15°C, chronic Ni toxicity was variable along generations, but was in all Ni treatments highest in F0, even though some increase of the measured total and dissolved Ni concentrations occurred along generations (Appendix C, Table C7). At 25°C, because we observed a relatively high within-Ni-treatment variation, an unexpected non-monotonic response in F1 and F2 and a strong effect in F1 at some concentrations below the $EC10_{F0}$, this temperature treatment was entirely repeated in an additional experiment. In both experiments, the same pattern was observed (e.g., non-monotonic response in F1 and F2 and a strong effect in F1 at some concentrations below the $EC10_{F0}$) but less variation within-Ni-treatment was observed in the 2nd experiment (Appendix C, Figure C4). Therefore, we present in the main manuscript the results of the 2nd experiment. At

25°C, when organisms were exposed to Ni concentrations below the $EC10_{F0}$ (88.1 µg L^{-1} (±13.3)), chronic Ni toxicity was not significantly affected in later generations at 17 and 27 µg L^{-1} (Table 4.2). However, at 6 and 56 µg L^{-1} chronic Ni toxicity increased from no effect in F0 to 49% and 77% of effect in F1, respectively. This increase of Ni toxicity became less pronounced in F2 and organisms completely recovered in F3 (Table 4.2). At 25°C when organisms were exposed to a Ni treatment higher than $EC10_{F0}$ (i.e. 120 µg L^{-1}) the Ni effect fluctuated along generations, but a consistent trend was not observed.

Table 4.2. The effect of Ni on *Daphnia magna* reproduction during four generations (F0, F1, F2, F3) at 15, 20 (standard temperature) and 25°C. Reproduction is expressed as the mean and standard deviation (SD) of the number of offspring per individual female produced until the organisms in control treatment released the 5th brood as percentage of control treatment. Nickel concentration (µg L^{-1}) expressed as nominal (nom) and dissolved (dis) and temperature as T (°C). The significant *p* values are marked with bold letters, the treatments below EC50 calculated for F0 at 15°C and EC10 values calculated for F0 at 20°C and 25°C are shaded. Within each generation and each temperature a pairwise Wilcoxon Rank Sum Tests were performed and the Benjamini & Hochberg (1995) correction method was used to adjust the *p* values for multiple comparisons.

T	Ni nom	ECx F0	F0					F1					F2					F3				
			Ni dis	Mean	SD	*p*	% effect	Ni dis	mean	SD	*p*	% effect	Ni dis	mean	SD	*p*	% effect	Ni dis	mean	SD	*p*	% effect
15	0	17.3	0	100.0	20.6			0	100.0	32.6			0	100.0	31.2			0	100.0	14.7		
	12		4	68.7	24.8	**<0.01**	**31**	9	84.5	35.1	>0.05	15	12	109.9	29.9	>0.05	-10	13	100.2	14.8	>0.05	0
	25		11	62.6	32.0	**<0.001**	**37**	19	75.0	35.1	>0.05	25	25	109.6	36.7	>0.05	-10	30	87.5	31.5	>0.05	13
	50		26	45.6	22.9	**<0.001**	**54**	42	61.4	27.0	**<0.01**	39	56	84.3	31.1	>0.05	16	54	34.8	14.9	**<0.001**	65
	100		55	27.3	12.6	**<0.001**	**73**	63	96.2	23.4	>0.05	4	86	95.6	31.7	>0.05	4					
	200		126	1.8	3.7	**<0.001**	**98**															
	300		194	0.4	1.3	**<0.001**	**100**															
20	0	18.8	0	100.0	41.2			0	100.0	32.7			0	100.0	34.4			0	100.0	27.8		
	12		3	109.9	35.9	>0.05	-10	4	100.5	27.4	>0.05	0	9	133.4	52.1	>0.05	-33	10	118.9	22.7	>0.05	-19
	25		9	101.8	28.6	>0.05	-2	10	79.9	39.2	>0.05	20	13	118.0	59.0	>0.05	-18	20	92.4	34.2	>0.05	8
	50		23	76.8	36.5	>0.05	23	25	3.2	9.7	**<0.001**	97										
	100		54	63.8	37.4	**<0.05**	36	49	1.5	6.7	**<0.001**	98										
	200		126	30.4	21.8	**<0.001**	70															
	300		193	1.8	3.6	**<0.001**	98															
25	0	93.3	0	100.0	40.6			1	100.0	44.1			0	100.0	21.1			0	100.0	46.1		
	12		6	101.9	48.7	>0.05	-2	10	50.9	49.2	**<0.05**	49	13	69.4	45.5	>0.05	31	12	133.5	38.5	>0.05	-34
	25		17	108.8	40.9	>0.05	-9	21	102.5	35.0	>0.05	-2	28	95.6	36.5	>0.05	4	26	147.2	60.1	>0.05	-47
	50		27	127.4	46.5	>0.05	-27	43	65.1	54.1	>0.05	35	61	130.4	40.2	>0.05	-30	57	127.8	56.2	>0.05	-28
	100		56	134.9	36.5	>0.05	-35	66	23.1	37.5	**<0.001**	77	73	80.2	34.7	>0.05	20	81	110.2	52.0	>0.05	-10
	200		120	60.2	36.6	>0.05	40	123	52.8	42.4	**<0.01**	47	121	71.9	30.1	**<0.05**	28	119	42.4	53.1	**<0.05**	58
	300		186	10.7	11.5	**<0.001**	89															

4.3.3 *Daphnia magna* internal nickel concentrations

The GEE analysis applied to the endpoint $[Ni]_{daphnia}$ indicates that Ni, temperature and generation are significant factors (Table 4.3). Significant interaction effects between Ni and generation were observed but not between Ni and temperature. A significant three-way interaction was observed between Ni, temperature and generation. The correlation coefficient between two sequential generations is 0.005±0.065 (± SE). The residuals of the model were homogeneously distributed around 0 when plotted against the variables temperature, generation and Ni indicating homogeneity of variance. The plot of predicted vs. observed values indicates a good model fit (Appendix C, Figure C3).

Table 4.3. Summary of the analysis of variance with the generalized estimation equation that predict the Ni (continuous variable) effect on internal Ni concentration ($[Ni]_{daphnia}$) as a function of temperature (factorial variable) and generation (factorial variable) (Ni × Temperature × Generation) with an auto-regressive correlation structure between generations. Internal Ni concentration expressed as log_{10}-transformed (log_{10} ($[Ni]_{daphnia}$ +0.1)). Degrees of freedom (Df), Wald statistics (X^2) and significance (*p* value) are shown. The estimated correlation coefficient between F_x and F_{x+1} was 0.005±0.065 (standard error).

Predictor	Df	X^2	*p*
Ni	1	401	<0.001
Temperature	2	22	<0.001
Generation	3	19	<0.001
Ni × Temperature	2	4	>0.05
Ni × Generation	3	14	<0.05
Temperature × Generation	6	111	<0.001
Ni × Temperature × Generation	6	185	<0.001

To test if the effect of temperature on Ni toxicity to *D. magna* reproduction is explained by $[Ni]_{daphnia}$ we fitted the Michaelis-Menten equation to the data at each generation and each temperature. In F0, we observed that at lower temperatures lower $[Ni]_{daphnia}$ was necessary to induce the same Ni toxicity (i.e. 50% reduction of reproduction) than at higher temperatures (Figure 4.3, Appendix C, Table C10). That is, when estimating the $[Ni]_{daphnia}$ based on the $[Ni]_{water} = EC50_{F0}$ and on the estimated values for ßmax and Ks (Appendix C, Table C11) the $[Ni]_{daphnia}$ was 2.5, 6.0 and 23.8 µg g^{-1} for 15, 20 and 25°C, respectively.

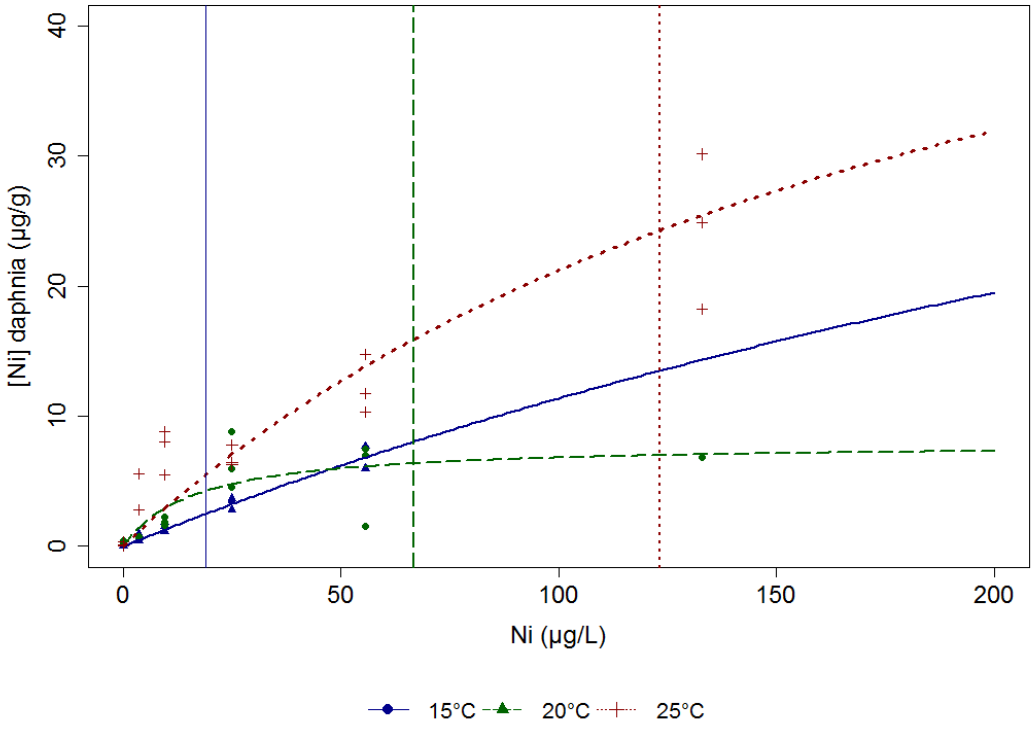

Figure 4.3. Plot of the internal Ni concentration in *Daphnia magna* exposed to Ni in the first generation of exposure (F0) at 15, 20 and 25°C. Internal Ni concentrations ([Ni]daphnia) expressed as µg per g dry weight and Ni water concentrations expressed as µg per L. Dots represent data points and lines are the fitted Michaelis-Menten curves. Vertical lines represent the effective Ni concentrations in the exposure medium that reduce *D. magna* reproduction by 50% at 15, 20 and 25°C.

The $[Ni]_{daphnia}$ presented a non-monotonous trend with generation-number that was different for every temperature, that is the $[Ni]_{daphnia}$ followed the order F1>F0≈F2>F3 at 15°C, F3≈F2>F0>F1 at 20°C and F0≈F3>F1>F2 at 25°C (Figure 4.4).

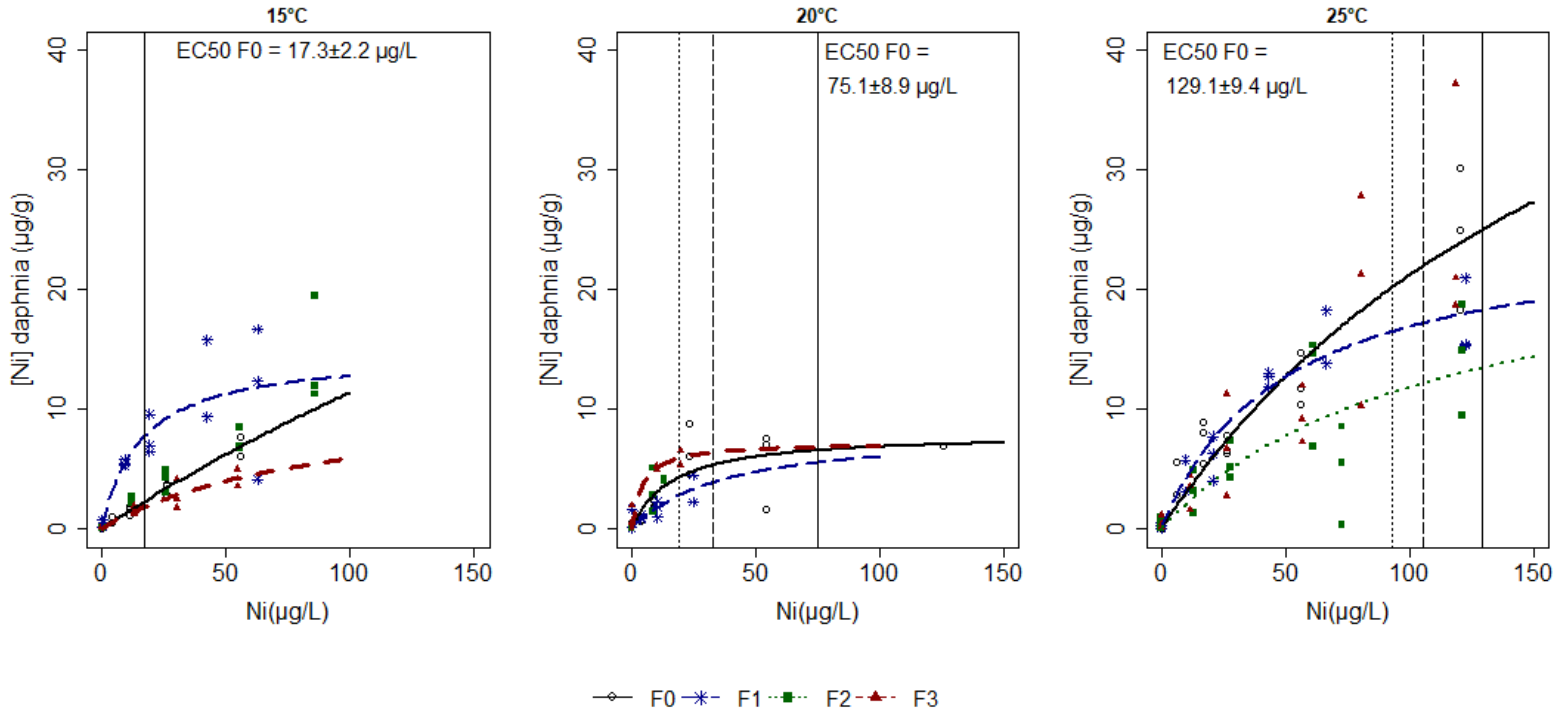

Figure 4.4. Plot of the internal Ni body concentration on *Daphnia magna* exposed to Ni during four generations (F0, F1, F2, and F3) at 15, 20 and 25°C. Internal Ni concentrations ([Ni]daphnia) expressed as µg per g dry weight, Ni water concentrations expressed as µg per L. Dots represent data points, and lines are the fitted Michaelis-Menten curves. Vertical lines indicate the effect concentrations (ECx) estimated for F0, the EC10 values are represented by dotted lines, the EC20 values by dashed line and EC50 values by straight lines.

4.3.4 *Daphnia magna* internal cations concentrations

In F0, Ni affected the $[Metal]_{daphnia}$ for Fe and Mg (p<0.05) (Table 4.4). Also, significant interactive effects were observed between Ni and temperature on the $[Metal]_{daphnia}$ for Ca, Na, Mg, Fe, Cu and Zn (p<0.05). Yet, no trends of $[Metal]_{daphnia}$ were observed as a function of Ni that were consistent across all temperatures (Figure 4.5 and 4.6). Temperature significantly affected the internal Ca, Mg, Fe, and K concentrations in daphnids but it did not affected internal Zn, Cu and Na concentration in daphnids (Table 4.4, Figure 4.5 and 4.6). The $[Ca]_{daphnia}$ significantly increased from 15°C (25.2±3.3 mg g^{-1}) to 20°C (30.7 ± 3.0 mg g^{-1}) and to 25°C (35.2 ± 4.1 mg g^{-1}). Also, the $[Mg]_{daphnia}$ significantly increased from 15°C (850.2±87.4 µg g^{-1}) to 20°C (936.3 ± 111.6 µg g^{-1}) and to 25°C (998.1 ± 103.5 µg g^{-1}). The $[Fe]_{daphnia}$ was significantly higher at 25°C (240.0±34.4 µg g^{-1}) compared to 20°C (178.4±27.9 µg g^{-1}) and 15°C (207.1±29.9 µg g^{-1}). The $[K]_{daphnia}$ was significantly lower at 25°C (5.5±0.5 mg g^{-1}) than at 20°C (6.0±0.4 mg g^{-1}) and 15°C (6.0±0.4 mg g^{-1}) and it was similar between 20°C and 15°C. All values were given as mean (± standard deviation).

Table 4.4. Summary of the linear model applied to each metal measured as internal body concentrations in *Daphnia magna*, i.e. Ca, K, Na, Cu, and Zn during the first generation of Ni exposure with a two way-interaction between temperature (factorial variable) and Ni (continuous variable).

Cations	Ni	Temperature	Ni × Temperature	r^2
Ca	>0.05	<0.001	<0.01	0.66
Mg	<.0.05	<0.01	<0.05	0.39
Fe	<0.05	<0.001	<0.05	0.54
K	>0.05	<0.01	>0.05	0.20
Cu	>0.05	>0.05	<0.01	0.23
Na	>0.05	>0.05	<0.05	0.19
Zn	>0.05	>0.05	<0.05	0.14

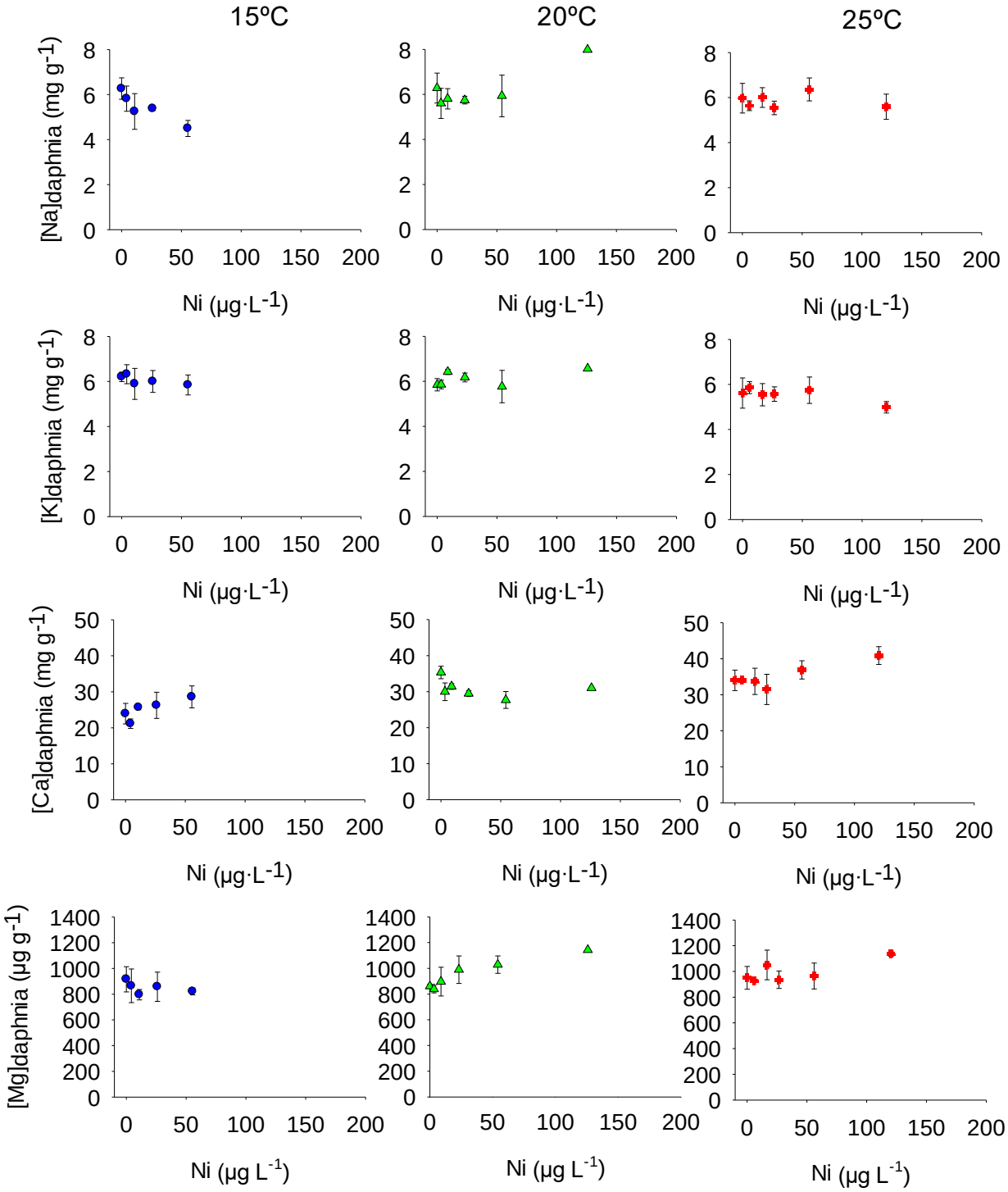

Figure 4.5. Plots of the internal Na, K, Ca and Mg concentrations in *Daphnia magna* (mean ± standard deviation) on the first generation (F0) of exposure to Ni at 15, 20 and 25°C.

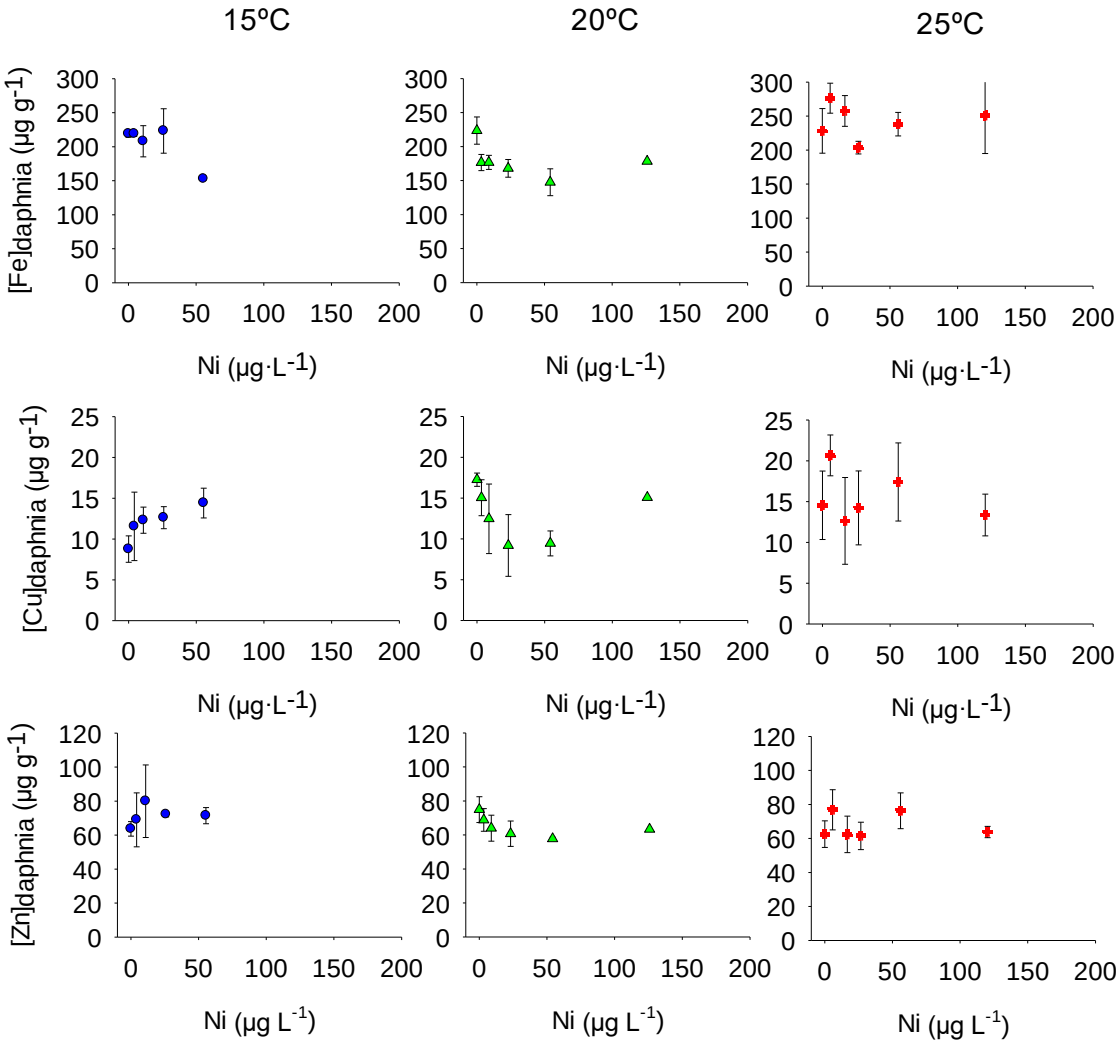

Figure 4.6. Plots of the internal Fe, Cu and Zn concentrations in *Daphnia magna* (mean ± standard deviation) during the first generation (F0) of exposure to Ni at 15, 20 and 25°C.

4.4 Discussion

Our multigenerational study with *D. magna* exposed to seven concentrations of Ni at 15, 20 and 25°C provided following main insights:

I. We found a three-way interaction between Ni, temperature and generation on *D. magna* reproduction (Table 4.1), and the multigenerational Ni effects on *D. magna* reproduction showed different patterns at different temperatures (Figure 4.2);
II. The $[Ni]_{daphnia}$ also showed a significant three-way interaction between Ni, temperature and generation (Table 4.3), but the $[Ni]_{daphnia}$ does not explain the different patterns of multigenerational Ni effects at different temperatures and neither the temperature effects on Ni toxicity in F0;
III. The internal ion concentrations in *D. magna* did not explain the effects of temperature on Ni toxicity.

Below, we will discuss each of these findings in more detail, including their implications for risk assessment.

4.4.1 Different multigenerational nickel effects on *Daphnia magna* reproduction

The multigenerational Ni effects on *D. magna* reproduction showed different patterns at different temperatures (Table 4.1, Figure 4.2). At 20°C, the magnitude of the effect in F0 determined if Ni effects weakened (at low effect levels: $<EC10_{F0}$) or strengthened (at high effect levels: $>EC10_{F0}$) in the following generations. At 15°C, chronic Ni toxicity at low and at high effect levels did not increase along generations. In addition, at low effect levels ($<EC50_{F0}$), a recovery was observed along generations (Table 4.2). At 25°C, at low effect level concentrations ($<EC10_{F0}$), an increase on Ni toxicity was observed in some Ni treatments in F1 and F2. This increase of Ni toxicity did, however, not persist and even a full recovery was observed in F3 (Figure 4.2).

It is important to acknowledge that the three studies about the multigenerational effects of Ni to *D. magna* available, that are the present study, Pane *et al*. (2004) and Munzinger (1990), have similarities but also differences. These differences can be due to different endpoints but also to the fact that different *D. magna* clones were used.

Indeed, it is well-known that different clones can have different tolerance to metals (Barata *et al*. 1998).

The study of Pane *et al*. (2004), performed at 20°C, indicated an increase of Ni sensitivity in F1. In their study, F1 organisms continuously exposed to 42 µg Ni L^{-1} (reported as no-observable-effect concentration for survival, reproduction and growth in F0) weighed significantly less than controls. This is in contrast with our results at 20°C in which Ni toxicity did not increase at low effect level concentrations.

Munzinger (1990) stated that *D. magna* acclimated to Ni during seven generations of exposure to 160 µg Ni L^{-1} (~EC50 for brood size) (i.e. lower Ni toxicity along generations), because the brood size increased along generations and with that the intrinsic rate of population increased. However, the mean life span and body length were significantly reduced along generations indicating a stronger Ni effect that is similar to our results at high effect level concentrations at 20°C.

4.4.2 $[Ni]_{daphnia}$ did not explain temperature and generation effects

The change of $[Ni]_{daphnia}$ along generations presented different patterns at different temperatures (Table 4.3 and Figure 4.4). These results suggest that the rates of metal uptake, detoxification and/or elimination change with both temperature and generation. The $[Ni]_{daphnia}$ did not explain the temperature effects on Ni toxicity (Figures 4.3 and 4.4; Appendix C, Table C10). In F0, at lower temperature lower $[Ni]_{daphnia}$ was necessary to induce the same Ni toxicity than at higher temperature (Figure 4.3). This could be explained by the fact that metal toxicity is determined by the metabolically available concentration and not by the total accumulation in the organism (Rainbow Philip S. 2002; Vijver *et al*. 2004). From the few studies available about the effect of temperature on metal toxicity, only two also report internal metal concentrations. These two acute studies, one with Cd and the other with Ni, indicated that metal toxicity to *D. magna* is enhanced by the increase of temperature and that there was a trend of increasing internal metal concentrations in *D. magna* with increasing temperature (Heugens *et al*. 2003; Vandenbrouck *et al*. 2011). This is in agreement with the hypothesis that, with metabolic rates being raised by temperature increase, metal uptake rates would also increase (Sokolova and Lannig 2008). The results of these

studies are in contrast with the present study, which also showed an increase in the $[Ni]_{daphnia}$ with increasing temperature, but which was not accompanied by increased chronic Ni toxicity with increased temperature. The contrast to the acute studies may be explained by the lack of an acclimation period in the acute studies necessary to reach a homeostatic balance to the new temperature environment (Williams *et al*. 2012) or to reach full sequestration capacity.

The exact mechanisms of uptake, elimination, sequestration and detoxification of Ni in *Daphnia* remain unclear (Pyle and Couture 2011; Brix *et al*. 2017). For instance, the metallothionein proteins are known to be involved in the regulation of some intracellular metal concentration such as Cu and Zn (Fan *et al*. 2009; Asselman *et al*. 2013) but unlikely in the case of Ni (Denkhaus and Salnikow 2002; Pyle and Couture 2011; Asselman *et al*. 2012). Some studies indicate that the levels of this protein can increase with the increase of temperature (Serafim *et al*. 2002; Baykan *et al*. 2007). This could be a possible explanation for the fact that lower toxicity at higher temperatures is observed. Yet, a study with *Daphnia pulex* showed no significant influence of Ni on metallothionein gene expression indicating that metallothionein is likely not involved in Ni detoxification processes in *Daphnia* (Asselman *et al*. 2012). Other proteins such as serum albumin, L-histidine and 2-macroglobulin in blood serum are known to bind to Ni (Denkhaus and Salnikow 2002). The study of Hall (1982) revealed that Ni was deposited in newly formed *D. magna* carapace but it was not deposited in the newly released eggs. It also revealed that 25 to 33% of Ni in *D. magna* is lost at moulting. It is therefore possible that at higher temperatures, more Ni is eliminated by the carapace. Future toxicokinetic and toxicodynamic studies performed at different temperatures would be useful to understand the exact mechanisms involved in the effect of temperature on Ni toxicity to *Daphnia*.

4.4.3 Effects on cations homeostasis *in Daphnia magna*

The $[Metal]_{daphnia}$ for Cu, Zn, Fe, Mg, Ca, Na, K and Mg did not explain the effect of temperature on chronic Ni toxicity. For none of the $[Metal]_{daphnia}$ was a consistent trend observed in relation to Ni across all temperatures, although some statistically significant interactions between temperature and Ni were found (Table 4.4, Figure 4.5

and 4.6). The study of Pane *et al*. (2003) showed that at 20°C Ni did not disrupt Ca and Na homeostasis in *D. magna* (which is confirmed by our results) but Mg was significantly reduced by Ni. In contrast, the results of present study showed that the $[Mg]_{daphnia}$ did not decrease with the increase of Ni. The contrasting results among both studies could be due to the experimental conditions, as in Pane *et al*. (2003) adults were exposed to Ni during 14 d and in the present study neonates (<24h) were exposed to Ni until the control treatment released the 5^{th} brood (~ 40, 22 and 17 d at 15, 20 and 25°C respectively). Our results, however, do show that temperature by itself has an important effect on metal ion concentrations in *D. magna*. Temperature significantly influenced the internal levels of Ca, Mg, Fe and K in F0 (Table 4.4). The $[Ca]_{daphnia}$ and the $[Mg]_{daphnia}$ were significantly higher at the highest temperature 25°C which is also the temperature at which lower Ni toxicity was observed. Calcium is important for several biological processes such as gene expression, cell proliferation, muscle coordination, etc. (Bootman 2012). Also, Mg is important for several biological functions including structural stabilization of nucleic acid, cell membranes and to promote specific structural organization of enzymes and ribosomes (Wolf and Cittadini 2003). Internal Fe concentrations are intrinsically associated to haemoglobin concentrations in *Daphnia* (Smaridge 1956; Paul *et al*. 2004b). Therefore, in case of Ni impairment of one of these biological processes, the highest internal concentration of Ca, Mg and Fe in *D. magna* observed at 25°C could have been an advantage and could be involved in the lower Ni toxicity at this temperature.

4.4.4 Risk assessment implications

Our study showed that at low effect level concentrations (estimated in the first generation) which are most relevant for ERA, Ni toxicity at 15 and 20°C did not increase along four generations, and the increase of Ni toxicity at 25°C observed in F1 and F2 in some Ni treatments did not persist into F3, in which effects were not stronger than in F0 (Figure 4.2). These results suggest that the low effect levels of Ni (<EC10) observed in the first generation are also protecting *D. magna* populations against multi-generational exposure. Future multigenerational studies with other substances would be useful to understand weather EC10 values, which are often considered as basis for chronic ERA,

are generally protective in a mutigenerational context. This is important, since risk assessment still largely ignores the possibility of multigenerational effects.

Ecological risk assessment also largely ignores the effect of temperature on metal toxicity. In the present study, for F0 the EC50 increased 6.5-fold with the increase of temperature from 15 to 25°C. This finding is in line with and even stronger than the results in chapter 2 who reported a 2-fold increase of the EC10 and of the EC50 with the increase of temperature from 15 to 25°C. Together, the present chapter and chapter 2 provide strong evidence that temperature should be integrated as a factor in metal risk assessment.

4.5 Conclusion

In our study the multigenerational Ni effects on *D. magna* reproduction showed very different patterns at different temperatures. Also, patterns of multigenerational effects on $[Ni]_{daphnia}$ were also very different at different temperatures. However, Ni accumulation did not explain the influence of temperature on chronic Ni toxicity to *D. magna* reproduction: at lower temperature a lower $[Ni]_{daphnia}$ was necessary in the first generation to induce the same Ni toxicity than at higher temperatures. In F0, the $[Metal]_{daphnia}$ of Ca, K, Na, Mg, Fe, Cu and Zn did not explain the effect of temperature on chronic Ni toxicity.

At relevant concentrations for ERA, that is at low effect level concentrations (EC10 or lower), chronic Ni toxicity at 15 and 20°C did not increase along four generations, and the increase of Ni toxicity at 25°C observed in F1 and F2 in some Ni treatments did not persist into F3. At higher effect level concentrations, the multigenerational Ni effects depended on the tested temperature and effects were in some cases stronger than in the first generation.

Finally, the present chapter and chapter 2 showed that temperature affected chronic Ni toxicity to *D. magna*, that is the EC50 increased 2 to 6.5-fold with an increase of temperature from 15 to 25°C. Together both studies provide strong evidence that temperature should be integrated as a factor in metal risk assessment.

Chapter 5

The unexpected absence of nickel effects on a daphnia population at three temperatures is correctly predicted by a Dynamic Energy Budget Individual-Based Model

* Karel Vlaeminck contributed to this chapter by calibrating the DEB-IBM and performing the model predictions.

5.1 Introduction

In aquatic ecosystems, where 95% of the species are ectothermic, temperature plays a prominent role (Willmer *et al*. 2004). The external temperature determines the body temperature of ectothermic organisms, which in turn determines all physiological rates (Paul *et al*. 2004a; Walsh *et al*. 2014). Population dynamics are also determined by temperature (Pratt 1943; Jiang *et al*. 2014). For instance, Pratt *et al*. (1943) showed that maximum *Daphnia magna* population abundance was higher at 18°C than at 25°C and that population structure differed among temperatures.

An increase of temperature is commonly associated with an increase of metal toxicity (Heugens *et al*. 2001; Sokolova and Lannig 2008). However, the majority of the available studies have focused on acute toxicity. *For D. magna*, studies on the effect of temperature on chemical toxicity have, to our knowledge, only assessed apical endpoints (e.g. survival and reproduction of single individuals). An important goal of Ecological risk assessment (ERA) is to protect populations against chemical exposure but ERA is still most commonly based on apical endpoints (Preuss *et al*. 2009; Galic *et al*. 2010; Jager *et al*. 2014). However, previous studies have shown that effect concentrations estimated based on apical endpoints may not reflect the effects at population-level (Gagneten and Vila 2001; Viaene *et al*. 2015).

Chapter 2 and chapter 4 showed that temperature affects chronic Ni toxicity to *D. magna* on apical endpoints. The EC50 increased 2.0 to 6.5-fold with an increase of temperature from 15 to 25°C, indicating lower toxicity at higher temperatures (chapter 2 and chapter 4). Furthermore, in chapter 4, the multigenerational Ni effects on *D. magna* reproduction showed different patterns at different temperatures. In addition, in chapter 4, the EC10 estimated in the first generation were considered to be protective for *D. magna* in a long-term context. However, the EC10, most commonly used for chronic ERA, were based on apical endpoints thus disregarding the population-level effects. Therefore, in the present study, we investigated whether the effect of temperature on chronic Ni toxicity to *D. magna* assessed on apical endpoints could be extrapolated to population-level. A population experiment was performed with *D. magna* exposed to

three Ni concentrations, i.e. 0 µg L^{-1} (control), and 12 and 100 µg L^{-1}, previously reported to significantly reduce the apical endpoint reproduction (chapter 4).

In a population, organisms compete for food and can be limited by other factors (i.e. intraspecific interaction), such as crowding and light (Preuss *et al*. 2009; Martin *et al*. 2013a; Viaene 2016). Daphnids can detect the presence of other individuals through the presence of metabolites or pheromones released into the environment (Gust *et al*. 2016), which can influence their growth and reproduction (Nishikawa and Ban 1998). Therefore, the fitness of an organisms and their response to chemical stressors not only depend on the temperature effect, but may also depend on population density (Jiang *et al*. 2014; Gust *et al*. 2016).

Differences between the Ni effects on apical reproduction and the Ni effect at population-level were expected but it was also expected that at 100 µg Ni L^{-1}, a concentration that reduced apical reproduction by 70, 50 and 20% at 15, 20 and 25°C, respectively (chapter 4), effects at population-level would be observed. However, the results showed an absence of any consistent Ni effects on total *D. magna* abundance and population structure at 15, 20 and 25ºC (see results and discussion section). In an attempt to try to explain this unexpected result, the effect of temperature on Ni toxicity to a *D. magna* population was also studied with a population model: Dynamic Energy Budget individual-based model (DEB-IBM) (Martin *et al*. 2013a). The DEB-IBM is a generic individual-based implementation of the Dynamic Energy Budget theory (Martin *et al*. 2012). The IBM describes population dynamics through individual behaviour, while DEB describes physiological process on an individual-level. In IBM, each individual organism is generated (at birth) and simulated individually (Galic *et al*. 2010). Hence, the population properties emerge from the individuals (Galic *et al*. 2010). In DEB, environmental conditions, including the concentrations of chemical stressors, are translated to metabolic functions (i.e. energy assimilation, maintenance, growth, and reproduction) (Kooijman S.A.L.M. 2009). Hence, DEB-IBM links individual metabolism to population dynamics. The simplified DEB-IBM for *D. magna* can extrapolate the effect of toxicants measured at apical endpoints to the effects on population dynamics (Martin *et al*. 2013b). We wanted to test whether a DEB-IBM, calibrated on the apical toxicity

data at different temperatures (chapter 4), would be able to predict the unexpected absence of effects at population-level.

5.2 Material and methods

5.2.1 Daphnia magna population experiment

5.2.1.1 Organism cultures and test media

The *D. magna* clone K6 was cultured in aquaria in a modified M4 medium (chapter 2). This medium was also used for exposure. Prior to actual Ni exposure, organisms were acclimated for two generations to each temperature treatment (Mitchell and Lampert 2000). More detailed information about the acclimation process and test medium can be found in (chapter 2).

5.2.1.2 Experimental design

A *D. magna* population was exposed to three Ni concentrations at 15, 20 and 25°C. The three Ni concentrations tested were 0 µg L^{-1} (control), 12 µg L^{-1} (nominal concentration), corresponding to ~ 30% of effect on *D. magna* reproduction at 15°C and 0 % of effect at 20 and 25°C, and 100 µg L^{-1} (nominal concentration) corresponding to 50% effect at 20°C, 20% at 25°C and 70% of effect at 15°C (chapter 4).

The population experiment started with 6 egg-carrying females that had already released their 3rd or 4th brood and 10 neonates (<24h) per replicate. Four replicates were performed for each Ni treatment at each temperature treatment. Organisms were exposed in 2 L of medium in aquaria that were continuously aerated. Daphnids were fed daily with *P. subcapitata* with a food density of 2.5 mg C L^{-1} in the first week and 5 mg C L^{-1} in the following weeks. To consider the different generation time (i.e. time to first brood) observed at the different temperature treatments (Paul *et al*. 2004a; Walsh *et al*. 2014; Bae *et al*. 2016) the experiment was conducted during 9 weeks (63-d) instead of 6 weeks as in Martin *et al*. (2013b). Half of the medium was renewed three times a week (every Monday, Wednesday and Friday). The total abundance and the population structure (i.e. number of neonates, juveniles and adults) were recorded twice a week,

every Monday and Thursday. When the number of organisms in the aquaria was above 300, it was necessary to take subsamples to facilitate counting (Wetzel and Likens 2000). Two subsamples of each aquarium were taken after gently spreading the organisms in order to have a homogeneous distribution of the organisms in the aquarium. The volume of the subsamples was measured and was between 125 and 200 mL. Three population size classes of *D. magna* were quantified by sieving the medium through sieves with different mesh sizes, i.e. adults (> 800 µm), juveniles (between 800 and 500 µm) and neonates (< 500 µm) (Preuss *et al*. 2009). The mean of both subsamples was used to estimate the number of organisms in 2L aquaria. The coefficient of variation was calculated for all size classes and if the coefficient of variation was larger than 20% a 3rd or even a 4th subsample was taken. Every two weeks (14 days) 50% of each of the 3 size classes were removed at random (i.e. culling) to avoid population collapses due to overcrowding (Van Doorslaer *et al*. 2009). The exposure was performed under a controlled light cycle (16 h of light: 8 h of dark) (OECD 2012).

5.2.1.3 Chemical analysis

Dissolved metal concentrations, dissolved organic carbon (DOC), pH and O_2 were measured once a week, both in fresh and old medium. Water samples of fresh and old medium of all treatments were collected weekly for analysis of total (only of fresh medium) and dissolved concentrations of Ni, major cations (Na, K, Ca and Mg) and DOC. Dissolved concentrations refer to 0.45 µm membrane filtered concentrations (Acrodisc Filter, Supor Membrane, PALL, Newquay, Cornwall, UK). Samples for metal analysis were acidified to a final concentration of 0.14 mol L^{-1} of HNO_3 prior to analysis. Metal analyses were performed using iCAP 7000 Series ICP-OES (Thermo Scientific). The samples for DOC analysis were measured with TOC-L CPH (Shimadzu, Duisburg, Germany). All data analyses were performed based on the mean dissolved concentrations of new and old medium.

The reference material used in all chemical analyses and the quantification limits can be consulted in Appendix D (Table D1). Temperature remained stable in the three

treatments during the 9 weeks of the population experiment (15.2°C (±0.6), 19.7°C (±1.3) and 25.0°C (±1.0); mean ± standard deviation). The dissolved organic carbon (3.5±0.4 mg L^{-1}) and the pH (7.9±0.1) also remained stable during the experiment (Appendix D, Table D2). At 20ºC, the measured concentration in the 12 µg Ni L^{-1} treatment was below the target concentration at four sampling time points, but the concentration in the 100 µg Ni L^{-1} treatment remained stable during the whole exposure (which was 9 weeks) (Appendix D, Table D2). At 15 and 25ºC, the 12 and 100 µg Ni L^{-1} treatments remained stable during the whole exposure (Appendix D, Table D2).

5.2.1.4 Data analysis – Population experiment

For each endpoint at each counting day and at each temperature a Dunn's test was applied to test for statistical differences between control and Ni treatments. The Benjamini & Hochberg (Benjamini and Hochberg 1995) correction method was used to adjust the *p* values for multiple comparisons within each temperature. At 15°C, reliable data was only available until 21 days of exposure as a result of an experimental error (tap water was mistakenly used as dilution water for media preparation instead of deionised water). The statistical analyses were performed using the *dunn.test* package in R software (version 3.4.0; R Core Team 2017). Only consistent effects of Ni were considered reliable and take further for data interpretation. A consistent effect of Ni was defined as an effect in the same direction that was found for at least 2 consecutive sampling dates (Van de Perre *et al*. 2016).

5.2.2 DEB-IBM

An IBM based on the DEB theory for *D. magna* was used to extrapolate effects of Ni to the population-level at 15, 20 and 25°C. An existing simplified DEB-IBM for *D. magna* (Martin *et al*. 2013a) was calibrated using apical Ni toxicity data (endpoints: reproduction and survival) at 15, 20 and 25°C (chapter 4). The calibrated model was used to predict (blindly) Ni effects at the population-level, i.e. it was independently validated against the dataset from the population experiment described above. Similar methods have been applied in the past to extrapolate effects of chemicals from the individual to the population-level (Martin *et al*. 2013a; Hochmuth 2016; Viaene 2016).

5.2.2.1 Calibration of the simplified DEB-IBM

A generic DEB-IBM implementation in NetLogo by Martin *et al*. (2013a) was used to predict Ni effects for *D. magna* populations. To simulate the conditions of the population experiment, the original model needed to be adjusted for different temperatures (1), different food conditions (2) and the effects of Ni (3).

In the first adjustment the temperature effects were implemented through the Arrhenius equation. The Arrhenius relationship (Equation 5.1) is used to implement temperature-dependent metabolic rates in the simplified DEB-IBM (Kooijman 2009; Hochmuth 2016). The Arrhenius temperature parameter T_A is species-specific and has been determined before for *D. magna* (Kooijman 1988).

Eq (5.1)

$$k(T) = k_1 * \exp\left(\frac{T_A}{T_1} - \frac{T_A}{T}\right)$$

where k is the metabolic rate at temperature T *(K)*, k_1 is the metabolic rate at the reference temperature T_1 (293 K) and T_A is the Arrhenius temperature (6400 K for *D. magna*) (Kooijman 2009).

The second adjustment considers the implementation of correction factors to adjust food-related parameters, in order to account for the differences in algal food and species-specific physiological characteristics (e.g. interclonal differences in *D. magna* in terms food size preference, food handling time, digestion time, etc.). In the original model, food-related parameters were based on the algae *Desmodesmus subspicatus* as food source (Martin *et al*. 2013b; Preuss *et al*. 2009). However, in the present study the *P. subcapitata* was used as food. To account for such differences, the maximum surface-specific assimilation rate (p_{Am}) (J $d^{-1}cm^{-2}$) and the food half-saturation constant (K) (number of cells L^{-1}) were corrected (Kooijman *et al*. 2008) (Equation 5.2 and 5.3)

Eq (5.2)

$$K_{food} = K * corr_K$$

Eq (5.3)

$$p_{Am,food} = p_{Am} * corr_{pAm}$$

By multiplying the p_{Am}, we thus implemented a correction factor to take into account differences in energy assimilation efficiency due to clone-specific physiological characteristics and due to food type compared to the original model. Additionally, knowing the food type is different and feeding characteristics may differ between clones, the *K* was corrected as well. Correction factors were determined for each temperature treatment separately since assimilation rates depend on the temperature (Yurista 1999). Optimization of the food correction factors was based solely on the control data of the standard reproduction experiment described in chapter 4. The calibrated correction factors for the p_{Am} ($corr_{pAm}$) were 0.98, 0.94 and 0.85 respectively for 15, 20 and 25°C. For *K*, the correction factors ($corr_K$) were 155, 71 and 34 for 15, 20 and 25°C respectively.

The third adjustment was the estimation of the effect parameters for the different physiological mode of actions (PMoAs) to account for Ni toxicity (Martin *et al*. 2013a). In the DEB theory, stressors affect the metabolic rates, and thus the energy flow, by changing one or more parameters. Depending on the PMoAs, the life history of *D. magna* will be altered. To describe the relationship between the environmental stressor concentration (in this case Ni) and the level of stress on *D. magna* two dose-response models were considered, the hockey-stick and the log-logistic relationship. We finally selected the hockey-stick relationship as this described the effects of Ni on size and reproduction better (Appendix D, Figure D1 and D2).

The PMoAs considered were inhibition of assimilation, general maintenance (with an increase on general maintenance costs i.e. both structural maintenance and

maturity maintenance), structural maintenance (increase of structural maintenance costs), maturity maintenance (increase of maturity maintenance costs), growth costs (an increase in costs for growth) and on reproduction costs (Martin *et al*. 2013b; Hochmuth 2016). The models with the different PMoAs (as indicated by Martin *et al*. 2013a and Martin *et al*. 2013b) were calibrated with the apical Ni toxicity data of *D. magna* from chapter 4. The best fitting model, i.e. the one that showed the lowest Euclidean distance (ρ), was further used in the DEB-IBM calibrated model.

For calibrating the food correction factors and the effect parameters for Ni toxicity, an Approximate Bayesian Computation (ABC) was used (van der Vaart *et al*. 2016). The model was run for 10 000 iterations, with randomized values of the parameters to be optimized in each iteration. The optimized parameter values were calculated from the 100 best fits based on the ρ between the model output (predictions) and apical Ni toxicity data of *D. magna* (endpoints: size and reproduction) from chapter 4 (observations) (Equation 5.4; van der Vaart *et al*. 2016).

The Euclidean distance is determined as

Eq (5.4)

$$\rho(m_i, D) = \sqrt{\sum_j \left(\frac{m_{i,j-D_j}}{sd(m_j)}\right)^2}$$

where $m_{i,j}$ is the model output of endpoint j in iteration run i, D_j is the observed data of endpoint j, and $sd(m_j)$ the standard deviation of endpoint j of all simulation runs.

5.2.2.2 Validation of the calibrated DEB-IBM

With the calibrated model (i.e. with the temperature adjustment, optimized food correction factors, and calibrated effect parameters for Ni toxicity), we mimicked the population experiment described above to know how well the model predicted the observation on population dynamics. The same conditions as the experiment were applied in the model simulation (i.e. daily food addition, medium replacement three

times per week, and culling every 14 days). The measured dissolved Ni concentrations were imposed to the simulations as for the experiment. The PMoA that best predicted the data of the apical experiment (based on the ρ) was used for these simulations. The predictions of the calibrated DEB-IBM were validated against the (independent) dataset from the population experiment.

5.2.2.3 Extrapolation of the effect concentrations for a *Daphnia magna* population

The calibrated model was further used to extrapolate the Ni effect concentrations for a *D. magna* population at 15, 20 and 25°C. We again imposed the same conditions as in the experiment, but now varying the Ni concentrations from 0 to 300 µg L^{-1}. Population-level effect concentrations were determined for equilibrium population abundance (i.e. the mean population density between 8 and 9 weeks of simulation). With the *drc* package in R, population-level ECx values were calculated based on regression of a log-logistic dose-response curve (*llogistic* – 3 parameters log-logistic curve) [Ritz *et al*. 2016; (version 3.4.0; R Core Team 2017)].

5.3 Results and discussion

5.3.1 Population experiment

The present study showed no consistent Ni effects on total *D. magna* population abundance at 15, 20 and 25°C (Figure 5.1), although the Ni concentrations tested (0, 12, 100 µg L^{-1}) were previously reported to significantly reduce the apical endpoint reproduction in a standard reproduction test (chapter 4). The tested concentration 12 µg Ni L^{-1} corresponds to ~ 30% of effect on *D. magna* reproduction at 15°C and to 0% of effect at 20 and 25°C (chapter 4). And, 100 µg Ni L^{-1} corresponds to 70, 50 and 20% of effect at 15, 20 and 25°C, respectively (chapter 4). Furthermore, population structure was also not affected by Ni that is no consistent effect of Ni on the number of neonates, juveniles and adults were observed at any temperature (Figure 5.2).

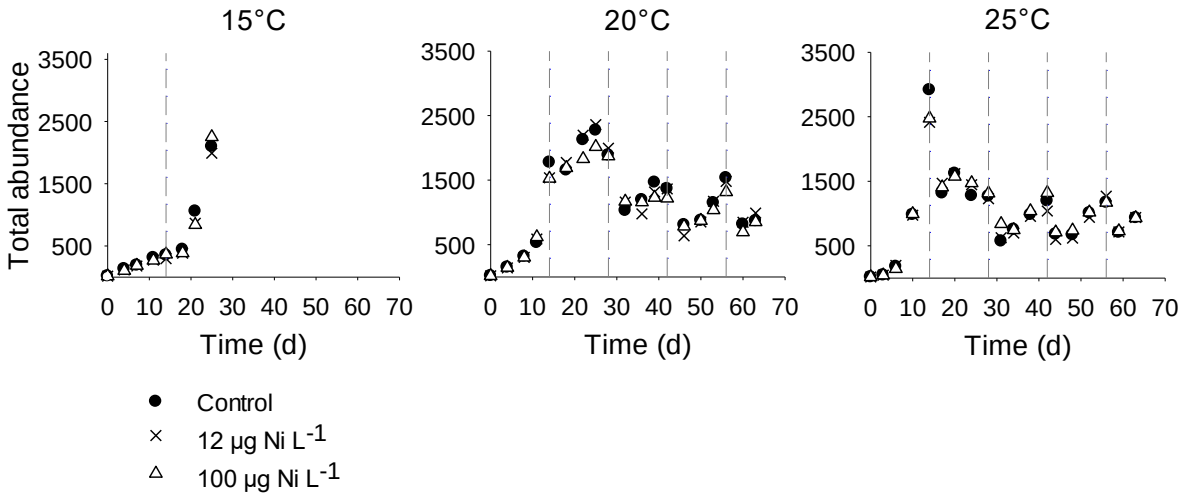

Figure 5.1. Average total abundance of *Daphnia magna* population exposed during 9 weeks (63 d) to Ni (0, 12 and 100 µg Ni L^{-1}) at 15, 20 and 25°C. Every 14 days half of the population was reduced by random culling. Vertical lines represent the days (every 14 days) that 50% of each of the 3 size classes (i.e. neonates, juveniles and adults) were removed at random to avoid population collapses due to overcrowding. At those time points, the total abundance of *D. magna* population reported refers to the abundance just prior to the culling.

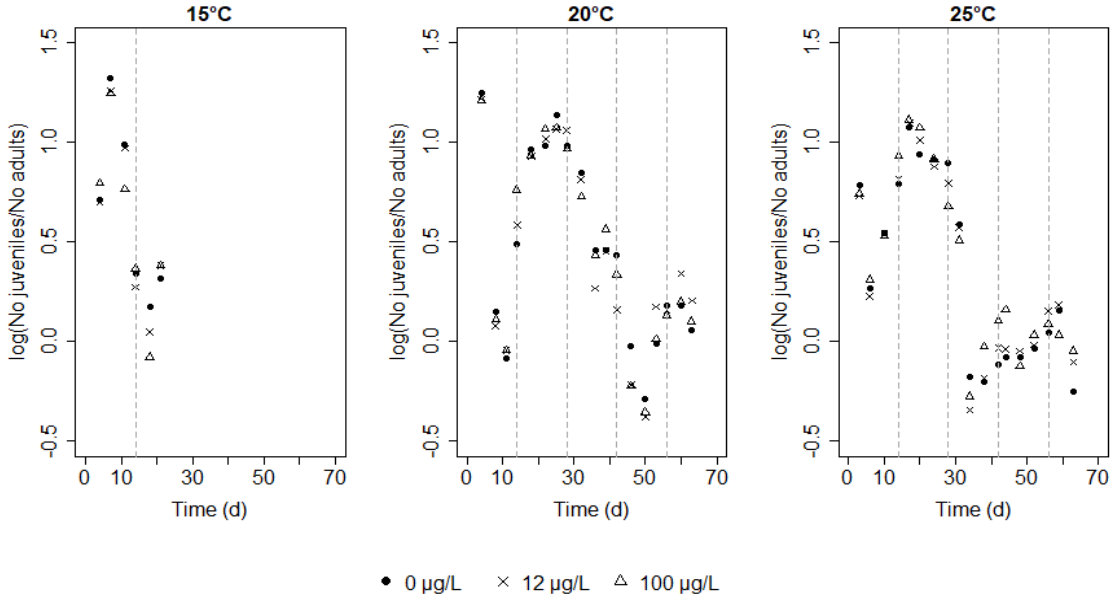

Figure 5.2. Ratio of the number (No) of juveniles and the number of adults of *Daphnia magna* population exposed during 9 weeks (63 d) to Ni (0, 12 and 100 µg Ni L^{-1}) at 15, 20 and 25°C. Vertical lines represent the days (every 14 days) that 50% of each of the 3 size classes (i.e. neonates, juveniles and adults) were removed at random to avoid population collapses due to overcrowding.

A previous study has also shown that effect concentrations estimated based on apical endpoints may not reflect the effects at population-level (Gagneten and Vila 2001). Gagneten and Vila 2001 showed that a *Ceriodaphnia dubia* population became extinct when exposed to 20 µg Cu L^{-1}, a concentration that reduced the apical endpoint reproduction by 50% (Gagneten and Vila 2001). To our knowledge, until now the effect of Ni to a *D. magna* population was only studied by Münzinger (1994) and only at 20°C in an 8 week study. In that study, the total abundance of the population held in flow through cultures was significantly reduced by 120, 160 and 200 µg Ni L^{-1} compared with the control treatment. The study did, however, not report effect concentrations on apical endpoints. Therefore, it is difficult to compare to the present study. The present study and the studies of Viaene *et al*. (2015) and Gagneten and Vila (2001) demonstrate that very different outcomes (quantitatively) can be obtained for population-level endpoints than for apical endpoints. Thus conventional ecotoxicological results (i.e. based on apical endpoints) cannot be straight forward extrapolated to population-level effects.

Along the 9 weeks of the experiment the major cations concentrations remained stable at 15°C and 25°C but not at 20°C (Appendix D, Table D3). One could argue that the different major ion concentrations at 20°C could compromise the comparison of the effect of Ni to the *D. magna* population with that at other temperatures. However, this is unlikely because the biotic ligand model for chronic Ni toxicity to *D. magna* (Deleebeeck *et al*. 2008) that we applied to media composition only estimated a 1.2-fold difference between the estimated EC10 values between the media at 15, 20 and 25°C.

5.3.2 DEB-IBM predictions

In the population experiment of the present study the effect of Ni on a *D. magna* population were absent at 15, 20 and 25°C. Therefore, to further investigate the effect of temperature on Ni toxicity to a *D. magna* population a simplified DEB-IBM calibrated (Martin *et al*. 2013a) using apical Ni toxicity data at 15, 20 and 25°C was used (chapter 4).

5.3.2.1 Physiological mode of action

To predict the population response to Ni we firstly had to identify the model parameters (i.e. the PMoAs) which are affected by Ni. The PMoA with an effect on growth costs (an increase in costs for growth) was able to predict the Ni effect on the apical endpoints size (length) and reproduction observed in chapter 4. Furthermore, it presented the lowest ρ and the best fits to the observations (Figure 5.3 and 5.4; Appendix D, Table D4). The PMoAs with an effect on general maintenance costs (i.e. both structural maintenance and maturity maintenance), on structural maintenance costs and on assimilation (decrease in feeding ability) also predicted the Ni effect on the apical endpoints size and reproduction relatively well but however the ρ were considerably higher (Figure 5.3 and 5.4; Appendix D, Table D4). The PMoAs with an effect on reproduction costs and maturity maintenance costs did not predict an effect on growth. Overall, these results indicate that the PMoA with an effect on growth costs best predicted the Ni effects on size and reproduction. A previous study showed that the effects of Cu and Zn on *Daphnia longispina* growth was associated with a decrease of reproduction (Martins *et al*. 2017) as smaller daphnids produce smaller broods and consequently the cumulative fertility (total number of neonates produced) is reduced (Campos *et al*. 2016).

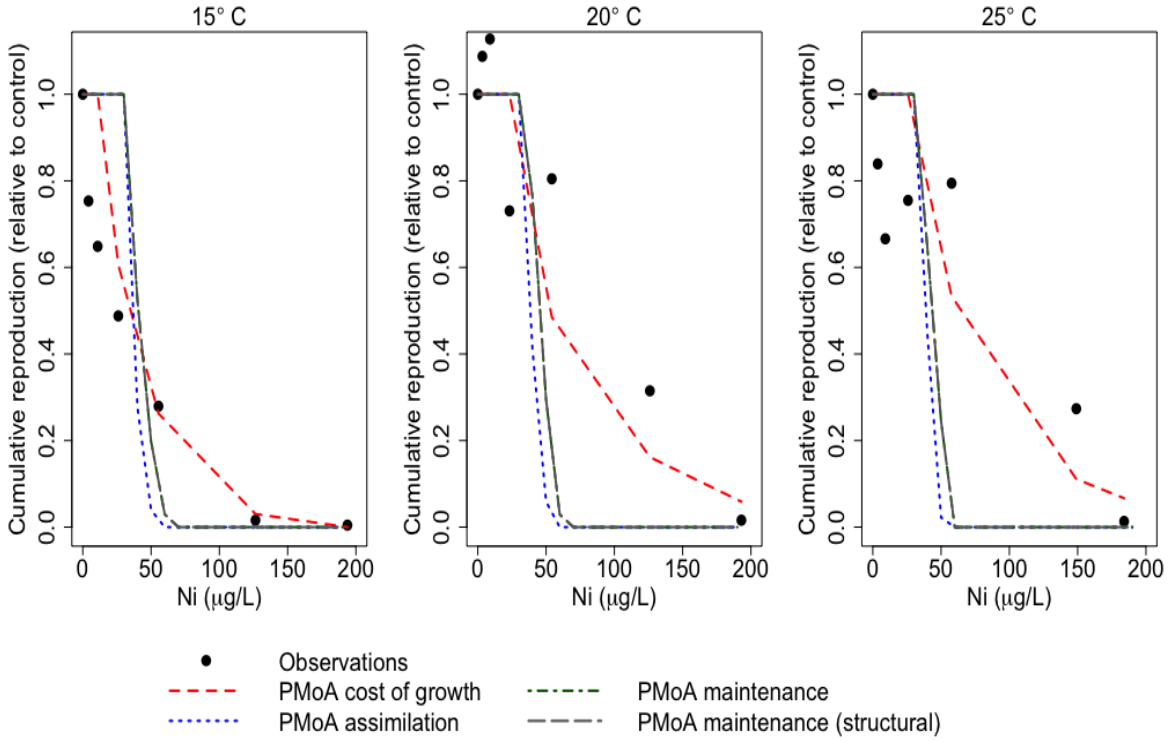

Figure 5.3. Calibration of the DEB-IBM for *Daphnia magna* with different physiological modes of action, i.e. an effect on growth costs (an increase in costs for growth), on general maintenance costs (both structural maintenance and maturity maintenance), on structural maintenance costs and on assimilation (decrease in feeding ability). Predictions are plotted versus the observations from the apical Ni toxicity dataset from chapter 4. The hockey-stick relationship between the Ni concentration and the level stress on the PMoAs was used. Each graph shows *D. magna* cumulative reproduction as a function of the Ni concentration at 15, 20 and 25°C. Cumulative reproduction is expressed as the number of offspring per individual female produced until the organisms in the control treatment released the fifth brood as percentage of control treatment (cumulative reproduction (relative to control). Lines represent predictions and dots observations.

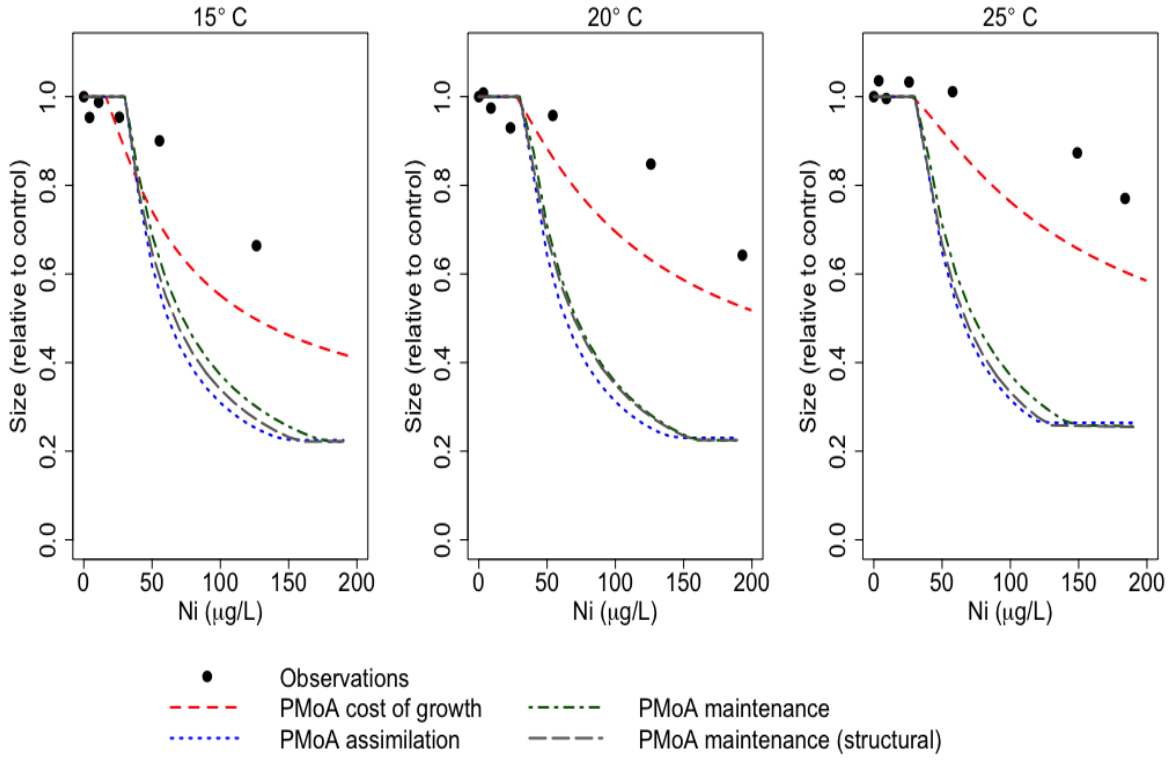

Figure 5.4. Calibration of the DEB-IBM for *Daphnia magna* with different physiological modes of action, i.e. an effect on growth costs (an increase in costs for growth), on general maintenance costs (both structural maintenance and maturity maintenance), on structural maintenance costs and on assimilation (decrease in feeding ability). Predictions are plotted versus the observations from the apical Ni toxicity dataset from chapter 4. The hockey-stick relationship between the Ni concentration and the level stress on the PMoAs was used. Each graph shows *D. magna* relative to control as a function of the Ni concentration at 15, 20 and 25°C. Lines represent predictions and dots observations.

The relation between the best-fit PMoA in this study (increased costs of growth) and the known mechanisms of Ni toxicity is not straightforward. Firstly, the mechanisms of Ni toxicity are not fully understood. Brix *et al*. (2017) identified as potential mechanisms of Ni toxicity in aquatic organisms the disruption of Ca, Mg and Fe homeostasis. Also, previous studies showed Ni exposure decreased respiration rates, glycogen and adenosine triphosphate (ATP) concentrations in *Daphnia* (Pane *et al*. 2004; Pane *et al*. 2003). Pane *et al*. (2004) showed that the exposure of *D. magna* to Ni decreased Mg concentrations and reduced the ATP levels. That study suggested those two endpoints could be related since the Mg^{2+} is a cofactor for ATP (Stryer 1995). The ATP, the main source of energy in cells, must be bound to an Mg^{2+} ion in order to be biologically active (Stryer 1995). It would be worthwhile to investigate if the effect of Ni on Mg and ATP levels that are necessary for the growth of organisms (to build new units of structure (e.g. a new cell)) could manifest into an increased cost for growth

Of course, the mechanisms of Ni toxicity reported above could equally well manifest into other PMoAs, since, ATP is necessary for all biological functions and therefore can have an effect on all PMoAs. Magnesium is essential for several biological functions including structural stabilization of nucleic acid (Wolf and Cittadini 2003), which could relate to the PMoAs of general maintenance costs and on structural maintenance costs. Also, Mg is essential for cell membranes and to promote specific structural organization of enzymes (Wolf and Cittadini 2003) which may translate into an effect on the PMoA assimilation. Ashauer and Janger (2018) already pointed to the importance to link the PMoA as defined in the DEB to physiological level endpoints (what happens at the cellular level) to enable the extrapolation from *in vitro and in vivo* toxicity studies to *in silico* studies (Ashauer and Jager 2018).

5.3.2.2 Predictions vs. observations

Overall, the DEB-IBM calibrated to our control condition and based on the apical Ni toxicity data (chapter 4) predicted the absence of Ni effect to a *D. magna* population at 12 and 100 µg Ni L^{-1} at 15, 20 and 25°C in accordance with the observations from the population experiment (Figure 5.5, 5.6 and 5.7). The total abundance of the population

over time, and also the size structure prediction were in accordance with the observations (Figure 5.5, 5.6 and 5.7).

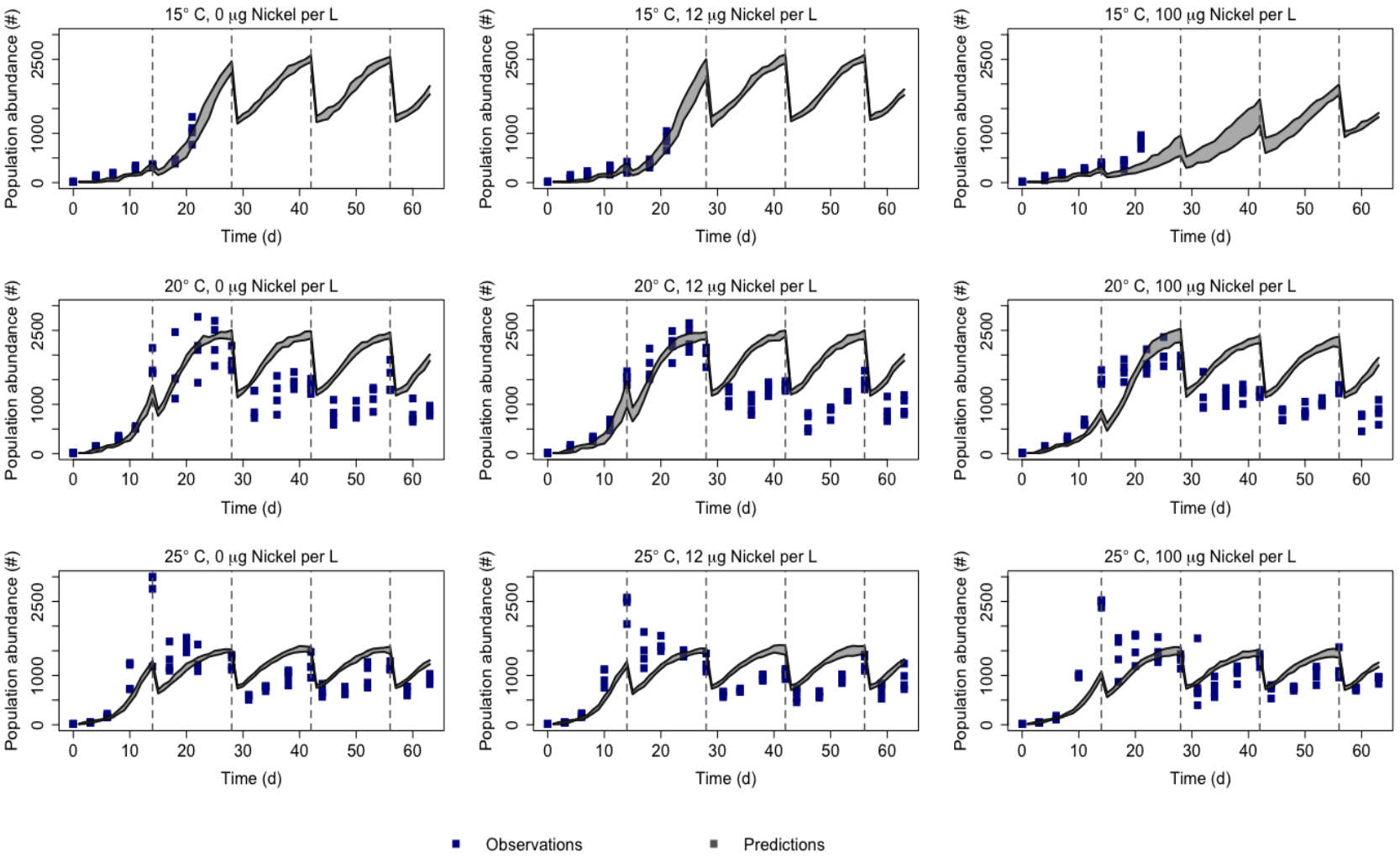

Figure 5.5. Observed total population abundance and predicted population dynamics for *Daphnia magna* under 0, 12 and 100 µg Ni L^{-1} during 9 weeks at 15 (data available only until 21 d), 20 and 25°C. Dots represent data points of the total population abundance and line the predictions of the model using the physiological mode with an effect on growth costs (an increase in costs for growth). Vertical lines represent the days (every 14 days) that 50% of each of the 3 size classes (i.e. neonates, juveniles and adults) were removed at random to avoid population collapses due to overcrowding.

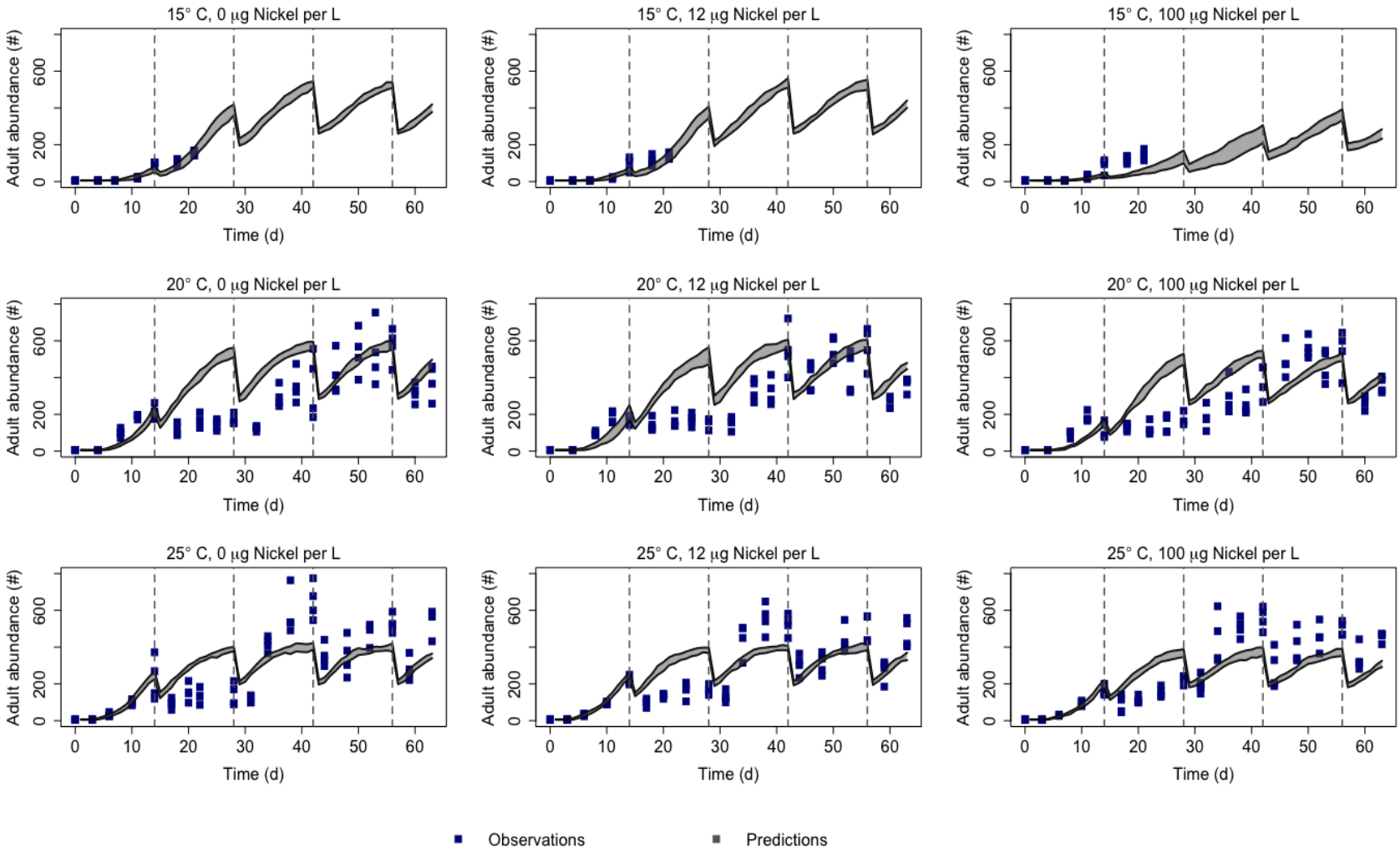

Figure 5.6. Observed adult abundance and predicted population dynamics for *Daphnia magna* under 0, 12 and 100 µg Ni L^{-1} during 9 weeks at 15 (data available only until 21 d), 20 and 25°C. Dots represent data points of the adult abundance and line the predictions of the model using the physiological mode of action mode with an effect on growth costs (an increase in costs for growth). Vertical lines represent the days (every 14 days) that 50% of each of the 3 size classes (i.e. neonates, juveniles and adults) were removed at random to avoid population collapses due to overcrowding.

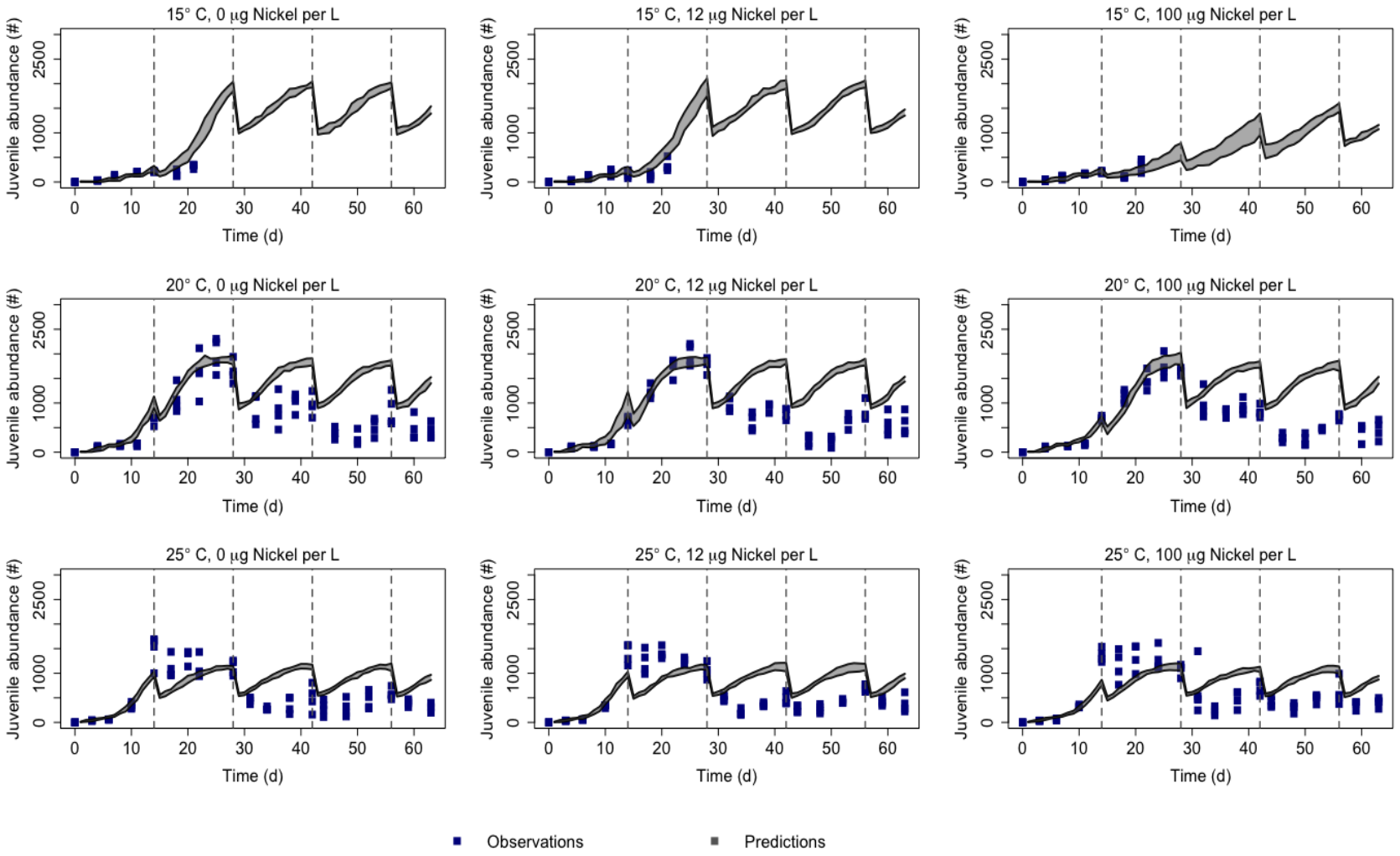

Figure 5.7. Observed juvenile abundance and predicted population dynamics for *Daphnia magna* under 0, 12 and 100 µg Ni L^{-1} during 9 weeks at 15 (data available only until 21 d), 20 and 25°C. Dots represent data points of the juveniles (and neonates) abundance and line the predictions of the model using the physiological mode of action mode with an effect on growth costs (an increase in costs for growth). Vertical lines represent the days (every 14 days) that 50% of each of the 3 size classes (i.e. neonates, juveniles and adults) were removed at random to avoid population collapses due to overcrowding.

5.3.2.3 Extrapolation of the nickel effect concentrations for a *Daphnia magna* population at 15, 20 and 25°C

The extrapolated EC50 for a *D. magna* population of Ni (i.e. using the average of the total population abundance from week 8 to week 9) was lower at 15°C<25°C<20°C and it was 2.3-fold higher at 20°C than at 15°C and 1.2-fold higher at 20°C than at 25°C (Figure 5.8, Table 5.1). The extrapolated EC50 values predict that the effect of temperature on Ni toxicity to a *D. magna* population was smaller than the effect of temperature on Ni toxicity to *D. magna* (apical) reproduction. Our previous experiment showed chronic Ni toxicity to *D. magna* increased with the increase of temperature with a 6.5-fold difference between the estimated EC50 values for *D. magna* apical reproduction (i.e. 17.3±2.2, 75.1±8.9, 129.1±9.4 µg L^{-1} at 15, 20 and 25°C, respectively) (chapter 4). The effect of temperature on Ni toxicity on *D. magna* population was thus smaller than on *D. magna* apical reproduction.

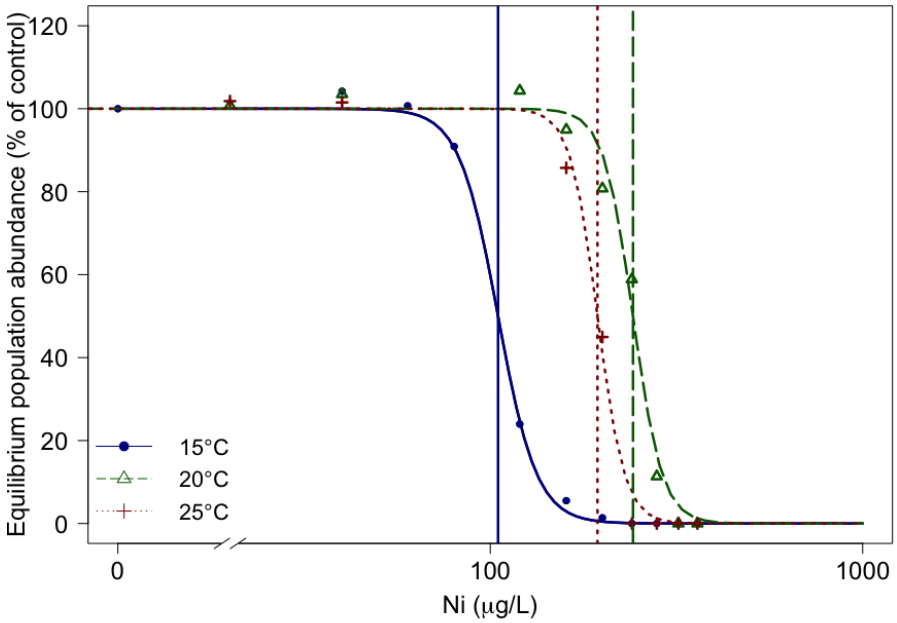

Figure 5.8. The extrapolated concentration response curves of Ni to a *Daphnia magna* population abundance (i.e. using the average of the total population abundance from week 8 to week 9) at 15, 20 and 25°C. Marker points represent average of the predictions and lines are the fitted concentration response curves. The concentration response curve were fitted through the results of the simulations with the *drc* package (three parameter log-logistic curve) in R. Vertical lines indicate the extrapolated EC50 values at 15, 20 and 25°C.

Table 5.1. The extrapolated effect concentrations (ECx) Ni to a *Daphnia magna* population (i.e. using the average of the total population abundance from week 8 to week 9) (+/- standard error) (µg L^{-1}) at 15, 20 and 25°C.

ECx	15°C	20°C	25°C
EC10	74.5 ± 0.9	191.7 ± 1.7	159.8 ± 1.0
EC20	83.9 ± 0.8	206.4 ± 1.3	171.4 ± 0.8
EC50	103.2 ± 0.6	234.3 ± 0.8	193.2 ± 0.5

The present study together with Viaene *et al*. (2015) and Gagneten and Vila (2001) showed that ERA should not extrapolate directly from apical to population-level endpoints. Population experiments are time consuming and expensive but the use of population models as DEB-IBM can help the scientific community in optimizing the population experiments. In the present study, the calibrated DEB-IBM predicted the unexpected absence of Ni effect to a *D. magna* population at the tested concentrations. Furthermore, we were able to use the calibrated DEB-IBM to extrapolate the ECx for a *D. magna* population of Ni. This type of information would have been very useful to optimize the experiment design e.g. choosing test concentrations. It has been shown that the DEB-IBM can be calibrated to different test species and different environmental conditions to extrapolate stressor effects to the population-level (Martin *et al*. 2013b; Jager *et al*. 2014; Hochmuth 2016; Viaene 2016; Ashauer and Jager 2018). For future population studies the prior use of the DEB-IBM to estimate population-level effects of chemical stressors would be helpful to improve the design of the experiment and therefore to optimize the time and the financial resources.

5.4 Conclusion

The present study showed the absence of consistent Ni effects on a *D. magna* population at 15, 20 and 25°C at Ni concentrations previously reported to significantly reduce the apical endpoint reproduction. This result supports the idea that ERA should not directly extrapolate from apical endpoints to population-level endpoints.

Interestingly, the simplified DEB-IBM calibrated using apical Ni toxicity data at 15, 20 and 25°C (chapter 4) predicted this unexpected absence of Ni effect to a *D. magna*

population. Furthermore, the extrapolated EC50 values predict that the effect of temperature on Ni toxicity to *D. magna* population density was considerably smaller than the effect of temperature on Ni toxicity to *D. magna* apical reproduction. DEB-IBM can help in to optimize the experimental design of population studies or even to replace population experiments by in *silico* simulations.

Chapter 6

General discussion and conclusions

6. General discussion and conclusions

Temperature has a major role in aquatic ecosystems where more than 95% of the species are estimated to be ectothermic (Willmer *et al*. 2004). The homeostatic balance of ectothermic organisms can be influenced by temperature fluctuations which therefore can interact with the effect of chemicals in the organisms (Heugens *et al*. 2001; Muyssen and Janssen 2001; Heugens *et al*. 2003). However, ERA within the chemical management context is still mainly based on standard tests performed at a standard temperature. Consequently, the potential influence of temperature on chemical toxicity is not taken into account. Furthermore, the standard tests rely on apical endpoints (e.g. survival and reproduction of single individuals) measured within a single generation. Hence population-level effects and the effects from multigenerational exposures in the field are also disregarded in ERA.

The focus of this thesis was to investigate the effect of temperature on chronic metal toxicity to acclimated *Daphnia magna* (i.e., in homeostatic balance with their environmental temperature). To this end single generation and multigenerational studies were performed assessing apical endpoints, the effect of temperature on Ni toxicokinetics was investigated and a population study was performed.

Temperature had a significant effect on chronic metal toxicity to *D. magna*. In chapter 2, where apical endpoints of four *D. magna* clones were assessed during one generation, chronic toxicity of Cu, Zn and Ni decreased with the increase of temperature. This trend is opposite to what is most commonly reported for the effect of temperature on acute metal toxicity (to non-acclimated organisms). Before the start of this doctoral study, the majority of the published studies had mainly focused on temperature effects on acute metal toxicity in non-acclimated organisms and those studies indicated that metal toxicity usually increased with the increase of temperature (Heugens *et al.* 2001; Sokolova and Lannig 2008). This was also the case for most published studies with *Daphnia* but all of those studies had only investigated a single clone and had not applied a prior acclimation to the temperature treatments (Persoone *et al.* 1989; Heugens *et al.* 2003; Boeckman and Bidwell 2006; Heugens *et al.* 2006; Ferreira *et al.* 2010; Vandenbrouck *et al.* 2011). There are a few more recent studies in

which an acclimation period to the temperature treatments was considered in the experimental design. Overall the results of those studies indicate that metal toxicity is not necessarily higher at higher temperatures (Cedergreen *et al.* 2013; Vergauwen *et al.* 2013). The study of Cedergreen *et al.* (2013) with *E. crypticus* (a terrestrial model oligochaete) acclimated to the temperature treatments (i.e. 11, 13, 15, 18, 21 and 25°C) during 6 months reported that chronic Cu and Cd toxicity was lower at higher temperatures. Bae *et al.* (2016) studied the effects of multigenerational exposure to elevated temperature on Cu acute toxicity in *D. magna* and showed that acute Cu toxicity assessed on the 2nd or 3rd brood of F0 (≈10 d of acclimation), F1 and F2 was lower at 25 than at 20°C. The study of Vergauwen *et al.* (2013) with adult zebrafish (*Danio rerio*) acclimated during 1 month to 18, 26, 30 and 34°C showed that acute Cd toxicity did not increase with increasing temperature.

In this thesis, the results showed inter-clonal variation of temperature effects on chronic Ni (but not of Cu and Zn) toxicity to *D. magna* (chapter 2) and on Ni toxicokinetics (Ni uptake and Ni elimination) (**chapter 3**). Previous studies reported inter-clonal variations of thermal tolerance (Van Doorslaer *et al*. 2009) and of metal sensitivities (Barata *et al*. 1998; Messiaen *et al*. 2010) among *D. magna* clones from a single population. The results of this thesis reinforce the work of those studies and together they indicate that the use of a single clone is not representative of the effect on a population (Barata *et al*. 1998; Van Doorslaer *et al*. 2009; Messiaen *et al*. 2010; Van Regenmortel *et al*. 2015). Therefore, it is necessary to be cautious when extrapolating data from single clone studies to population-level effects.

An acclimation period is necessary to restore homeostasis following a change in an organism's environment (Williams *et al*. 2012). For example, Paul *et al*. (2004a) showed that at 30°C, the lactate production rate was higher in organisms that were acclimated at 10°C than in organisms acclimated to 20 or 30°C. These results indicated that the organisms acclimated at 10°C had to produce energy trough anaerobic respiration because there was not enough oxygen to supply the rising oxygen demand in the warm temperature. Furthermore, Paul *et al.* (2004a) also showed that organisms that were acclimated at higher temperatures (20 and 30°C) had a higher concentration and oxygen affinity of haemoglobin. The subunit composition of haemoglobin and

consequently its function (i.e. oxygen affinity) varied depending on acclimation temperature (Paul *et al*. 2004a). Acclimation processes increase the possibilities of the individual to survive and therefore those processes are present across species (Paul *et al*. 2004a; Paul *et al*. 2004b; Lagerspetz 2006; Ziarek *et al*. 2011).

In acute studies with non-acclimated organisms, the increase of temperature was also associated with the increase of metabolic rates and hence of metal uptake rates (or accumulation) (Heugens *et al*. 2001; Paul *et al*. 2004a; Sokolova and Lannig 2008; Bae *et al*. 2016). In chapter 3, the effect of temperature on Ni uptake and elimination was investigated in four *D. magna* clones. The results of chapter 3 showed temperature affected Ni uptake and Ni elimination in *D. magna*. The same temperature trends were observed on Ni uptake and Ni elimination in *D. magna* i.e. the k_u and k_e were lower at 25°C than at 20°C and they were similar between 20 and 15°C. These results indicate that the general idea that metal uptake increases with the increase of temperature may not be applicable when organisms are in homeostatic balance with the environment temperature.

Also in chapter 3, with the use of an experimental design where the Ni isotope ratio of the exposure medium was changed from ^{62}Ni to ^{Nat}Ni, two important assumptions associated with the classical one-compartment kinetics experiment design were investigated: constant k_u over exposure duration and; k_e remain the same in the presence and in the absence of the contaminant. The results showed that Ni uptake rates of *D. magna* increased over time and Ni elimination decreased when the external Ni exposure stopped. These results indicate that in future toxicokinetic studies we should be cautious and consider that k_u may depend on the physiological state of the organism that may change along the exposure and the k_e may differ between the presence and the absence of the chemical stressor.

The effect of temperature on the $[Ni]_{daphnia}$ which is determined by the effect of temperature on the ratio between k_u and k_e (chapter 3) did not explain the effect of temperature on chronic Ni toxicity to *D. magna* (chapter 2). The same $[Ni]_{daphnia}$ was more toxic at 15°C than at 20 and 25°C. Previous studies also showed that the same metal body burden can have different toxicity to the organisms acclimated at different temperatures (Cedergreen *et al.* 2013; Vergauwen *et al.* 2013). Vergauwen *et al.* (2013)

and Cedergreen *et al.* (2013) showed that in *Danio renio* (zebrafish) and in *Enchytraeus crypticus* (acclimated to the temperature treatments), respectively, Cd accumulation was generally higher at higher temperatures but toxicity was not correspondingly higher at higher temperatures. Hence, it is possible that the detoxification mechanisms (e.g. immobilization of the metal, damage repair) are more efficient at higher than at lower temperatures. The higher metal toxicity observed at lower temperatures could be explained by the effect of temperature on enzyme activity or structure (Willmer *et al.* 2004). A rise in temperature will increase the activity of most enzymes (Willmer *et al.* 2004). For example, oxidative stress enzymes are involved in minimizing the effects of reactive oxygen species, which was reported to be induced by Ni exposure (Huang *et al.* 1994), Therefore, the lower activity of that enzymes at lower temperatures could explain the higher toxicity observed. It would be worthwhile to further investigate the mechanisms involved in the effects of temperature on chronic metal toxicity. The link between the effect of temperature on metal uptake and metal elimination processes and chronic metal toxicity is not straightforward. This is because the balance of uptake, compartmentalization, and elimination processes determines the internal metabolically available metal concentration and therefore the metal toxicity to the organism (Vijver *et al*. 2004; Rainbow 2007). In addition, toxicodynamic processes (i.e. metal interaction with target sites) can also determine the metal toxicity to the organism (Vijver *et al*. 2004; Rainbow 2007).

Organisms in the field are often exposed to chemical stressors along multiple generations. However this is still largely ignored in risk assessment. In **chapter 4**, the effect of Ni on *D. magna* (clone K6) (apical endpoints) was investigated at 15, 20 and 25°C along four generations. The results showed that multigenerational Ni effects on *D. magna* reproduction depended on the magnitude of the effect in F0 and showed very different patterns at different temperatures. At low effect level concentrations (EC10 or lower) determined in F0, chronic Ni toxicity at 15 and 20°C did not increase along four generations and at 25°C the increase of Ni toxicity observed in some Ni treatments in F1 and F2 did not persist and even a full recovery was observed in F3. At higher effect level concentrations, the multigenerational Ni effects depended on the tested temperature and effects were in some cases stronger than in the first generation.

Chapter 2 and 4 have provided strong evidence that temperature affected chronic metal toxicity *to D. magna*. However, only apical endpoints were assessed in those chapters. In a population, organisms compete for food and can be limited by other factors, such as crowding and light (Preuss *et al*. 2009; Martin *et al*. 2013a; Viaene 2016). Therefore, in **chapter 5**, the effect of temperature on Ni toxicity to a *D. magna* population was investigated.

The results of chapter 5 showed an absence of consistent Ni effect on a *D. magna* population at 15, 20 and 25°C although the Ni concentrations tested were previously (chapter 2 and 4) reported to significantly reduce the apical endpoint reproduction. To further investigate the effect of temperature on Ni toxicity to a *D. magna* population a simplified DEB-IBM (Martin *et al*. 2013a) was used. The DEB-IBM links individual metabolism to population dynamics (Kooijman 2009). The simplified DEB-IBM was calibrated using only the independent apical Ni toxicity data at 15, 20 and 25°C from chapter 4 (only F0). The calibrated DEB-IBM correctly predicted the unexpected absence of Ni effect (at the Ni concentrations tested) to a *D. magna* population at 15, 20 and 25°C, in accordance with the observations from the population experiment. The extrapolated EC50 values predict that the effect of temperature on Ni toxicity to a *D. magna* population is smaller (the extrapolated EC50 was 1.9-fold higher at 25°C than at 15°C) than on Ni toxicity to *D. magna* apical reproduction (the EC50 was 6.5-fold higher at 25°C than at 15°C). The results of chapter 5 together with two previous studies Viaene *et al*. (2015) and Gagneten and Vila (2001) showed that the direct extrapolation from apical endpoints to population-level effects can mislead us. Ecological risk assessment is based on apical endpoints extrapolating to population-level effects. Therefore there is an uncertainty in the environmental quality standards. One of the reasons for this extrapolation is the low number of population studies available compared with apical studies. Population experiments are time consuming and expensive. An approach that could help in the optimization of population experiment designs is the use of population models such as DEB-IBM. Recent studies have shown that DEB-IBM can be successfully used to extrapolate the effect of toxicants on apical endpoints to the effects on population dynamics (Martin *et al*. 2013b; Jager *et al*. 2014; Hochmuth 2016; Viaene 2016). The use of this type of tools can help

the scientific community to generate more data which would help to determine more accurate environmental quality standards.

The policies of risk assessment have to be continuously updated with the incorporation of new scientific knowledge to allow a better management of our resources (Luoma and Rainbow 2008). There are several examples of this in European chemical legislation. For example now environmental quality standards of metals are determined according to the environmental conditions as water hardness, pH and DOC (DEPA 2008; ECI 2008). For this the BLM, a reference tool used to predict chronic metal toxicity based on the dissolved metal concentrations and the physico-chemistry in the water column, is used according to legislative frameworks (European water Framework Directive) (DEPA 2008; ECI 2008; Van Sprang *et al*. 2009). Even though the influence of environmental conditions on metal toxicity to the organisms is addressed in ERA the effect of temperature is still disregarded. This thesis showed that temperature had a significant effect on chronic metal toxicity to *D. magna* this indicates an uncertainty in the current environmental quality standards. For most of European regions (except for regions with a Mediterranean climate) water temperatures are usually under 20°C (EEA 2014) and the results showed metal toxicity was generally highest at the lowest temperature investigated (15°C). Therefore, the current environmental quality standards established could be considered under-protective, but on the other hand population-level effects occur at higher concentrations than the effect concentrations estimated for apical reproduction.

Future work is needed to integrate temperature as a factor in metal risk assessment. In the ideal situation, temperature should be integrated in a mechanistic manner in metal bioavailability models such as the BLM. However, at this moment, there is not sufficient data for that option. Therefore, we propose an initial simpler alternative based on the type of data collected in this thesis. The integration of temperature in metal risk assessment - if temperature would have a significant effect on chronic metal toxicity across species - could occur through the introduction of a temperature correction factor (TCF). The TCF would be estimated by dividing an ECx at a certain temperature (ECx (T°C)) by an ECx at 20°C (which we define here as the reference temperature), the following equation was used

Eq (6.1)

$$TCF\ (Tx°C) = \frac{EC10\ (Tx°C)}{EC10(Reference\ temperature)}$$

We propose as future work to investigate the effect of temperature on chronic metal toxicity in at least one species from each of three trophic levels representing the primary producers (algae), the primary consumers (invertebrates e.g. *Daphnia*) and the predators (fish). In the cases that metal risk assessment is based on species sensitivity distribution (SSD) curves, the SSD curves could be normalized to each temperature by multiplying reported chronic EC10's of any species with the TCF derived for the corresponding trophic level. As an illustration of this proposal, we performed a preliminary application of this concept to the Ni risk assessment in the European Union. The Ni SSD curve was determined based on ECx values for a number of species from different taxonomic groups, but ECx values in this dataset have been obtained at various test temperatures (DEPA,2008; Nys *et al.* 2016). Hence, as a first step we grouped the ECx values for the different species into three temperature classes, according to their test temperature, i.e. 12.5°C-17.5°C, 17.5-22.5°C and 22.5-27.5°C. The SSD curve estimated for Ni was normalized to the midpoint of each of these classes, i.e. 15°C, 20°c and 25°C (see further). The second step was to estimate a TCF to be used for the normalisations. The TCF was estimated for *D. magna* (data of the present thesis) and, in absence of available chronic data for the other tropic groups, this TCF was assumed to be the same for all trophic levels. Hence, the reported results are for illustrative purposes only. For *D. magna* the estimated TCF(15°C) was 0.8 and the TCF(25°C) was 1.8 using the EC10 values for Ni reported in chapter 2. Each EC10 (or NOEC) was normalized to the midpoint of the three temperature classes, by multiplying by the respective TCF. A normalized SSD curve was then estimated for each temperature range (Figure 6.1 and 6.2) In this example, the normalised SSD curves merely reflect the lower chronic toxicity (higher EC10 values) of Ni at higher temperature for *Daphnia magna*, on which the TCF's were based in this example. If further work is performed on at least algae and fish and their corresponding TCF's are implemented, a more realistic picture of the SSD curve shifts could be obtained and the

different temperature classes could then represent different climatic regions (Deliège and Nicolay 2016) of Europe or different seasons within a climatic region. The proposed procedure could then be used as an initial starting point to integrate temperature as a factor in metal risk assessment, i.e. by using the normalized SSD curves as a basis for temperature-dependent PNEC or environmental quality standards values.

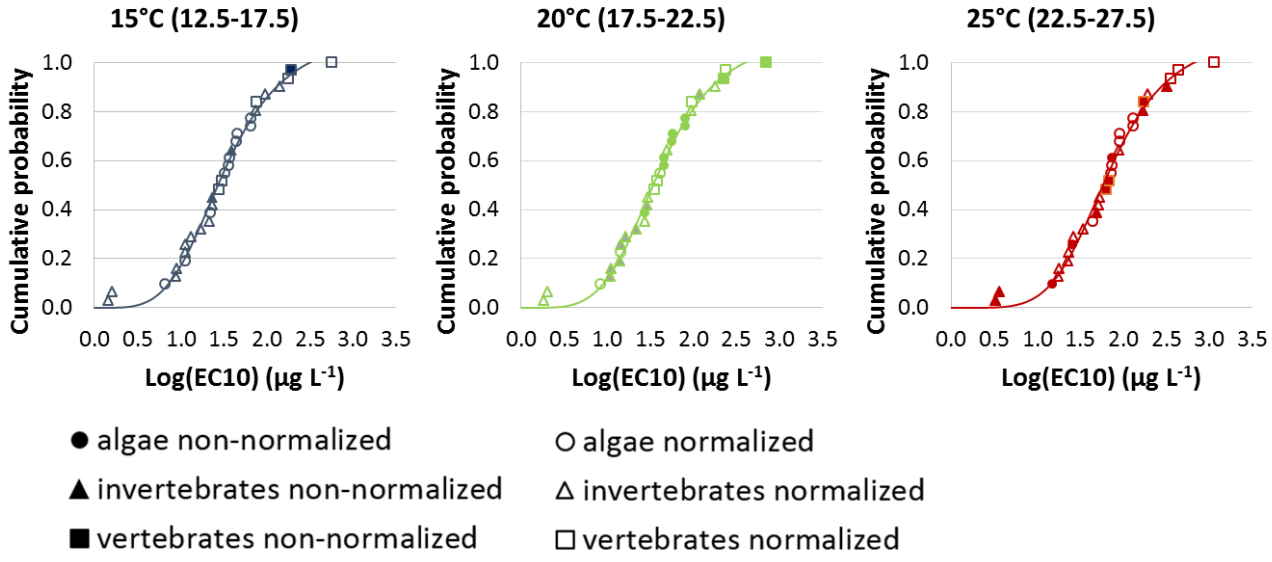

Figure 6.1. Normalized species sensitivity distribution (SSD) curves for the temperature classes 12.5°C-17.5°C, 17.5-22.5°C and 22.5-27.5°C. A logistic distribution was fitted on log-transformed data.

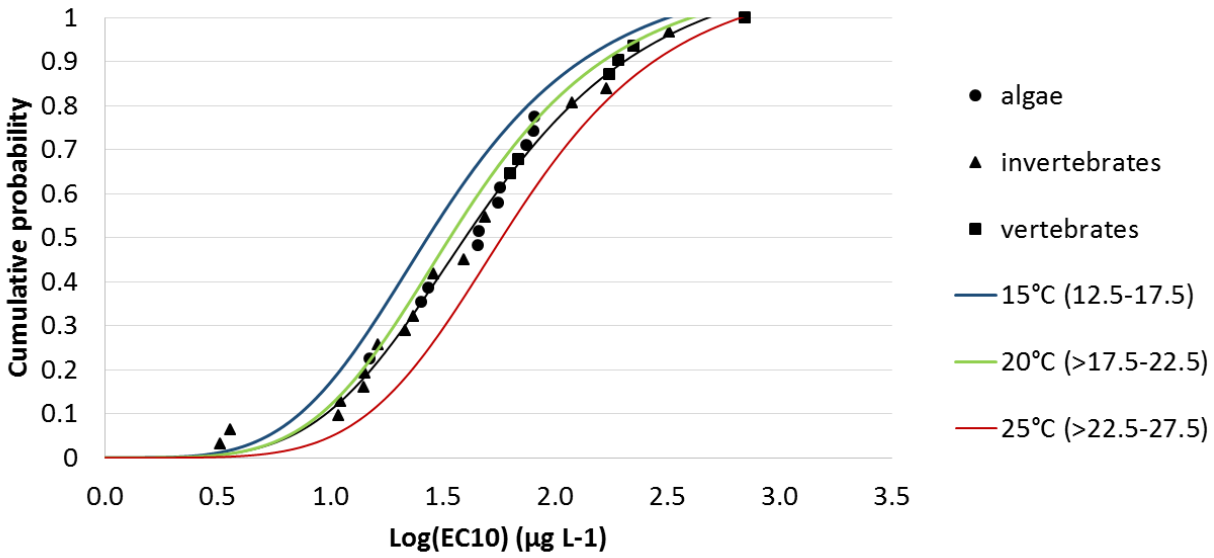

Figure 6.2. Normalized species sensitivity distribution (SSD) curves for the temperature classes 12.5°C-17.5°C, 17.5-22.5°C and 22.5-27.5°C and the non-normalized SSD curve. A logistic distribution was fitted on log-transformed data.

This thesis did not answer all the questions about the effect of temperature on chemical toxicity. Many questions remain. The effect of temperature on other type of chemical stressors as pesticides and pharmaceutical products on apical endpoints assessed in organisms in homeostatic balance with the environment temperature is still unknown and how that is translated to population-level as well. Also, constant temperature as it was tested in this thesis is not a realistic scenario since temperature fluctuates along the day and along the year. Nevertheless, what was clear in this thesis was that temperature had a significant effect on metal toxicity to *D. magna* and that acclimated organisms at higher temperatures were less sensitive to metal toxicity than acclimated organisms to lower temperature. Given these results and the importance of temperature for ectothermic organisms that represent the majority of the species in aquatic ecosystems it seems necessary to consider temperature as a factor in the chemical management context of ERA.

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Summary

Temperature influences the physiological processes of ectothermic organisms. Therefore it can interact with chemical toxicity. However, ecological risk assessment (ERA) is based on standard tests performed at a standard temperature. Thus, the potential influence of temperature on chemical toxicity is not taken into account. Furthermore, standard tests rely on apical endpoints (e.g. survival and reproduction of single individuals) measured within a single generation. Hence population-level effects and the effects from multigenerational exposures in the field are also disregarded in ERA. Therefore, the focus of this thesis was to explore the effect of temperature on metal toxicity to *Daphnia magna*. Several approaches were followed from single-generation and multigenerational tests assessing apical endpoints to a population experiment. The research of the present thesis also investigated the effects of temperature on metal toxicokinetic processes.

In chapter 2, the effect of temperature on chronic metal toxicity was investigated. Life table experiments were performed with Cu, Zn and Ni at 15, 20 and 25°C. General linear modeling indicated that chronic Cu, Zn and Ni toxicity to *D. magna* were all significantly affected by temperature. When averaged across four clones, the results suggest that chronic metal toxicity to *D. magna* was higher at 15°C than at 20°C, which is the temperature used in standard toxicity tests. At 15°C, the 21-d 50% effect concentration for reproduction (EC50) of Cu, Zn and Ni was 1.4, 1.1 and 1.3 times lower than at 20°C, respectively. At 25°C, chronic Cu and Zn toxicity did not change in comparison with 20°C, but chronic Ni toxicity was lower (21-d EC50 of Ni at 25°C was 1.6 times higher than at 20°C). The same trends were observed for Cu and Ni when the 21-d EC10 and EC20 were considered as the effect estimator, but not for Zn, which warns against extrapolating temperature effects on chemical toxicity across effect sizes. Overall though, chronic metal toxicity was generally highest at the lowest temperature investigated (15°C), which is in contrast with the usually observed higher acute metal toxicity at higher temperature. Furthermore, the effect of temperature on chronic Ni toxicity depended significantly on the clone. This warns against extrapolating results about effect of temperature on chemical toxicity from single clone studies to the population-level.

In ectothermic organism, the increase of temperature is usually associated with the increase of metal uptake rate constants (k_u) and with the increase of acute metal toxicity. However, the results of chapter 2 showed that chronic Ni toxicity to *D. magna* decreased with the increase of temperature (15°C>20°C>25°C). In chapter 3, the effect of temperature on Ni uptake and elimination in four *D. magna* clones (pre-acclimated, i.e. in homeostatic balance with environmental temperature) was investigated. Also, two assumptions associated with a simple one-compartment accumulation kinetics model were investigated: whether the k_u are constant over exposure duration; whether the elimination rate constant (k_e) change when *D. magna* is transferred to a non-contaminated medium. Nickel uptake and elimination in *D. magna* were both affected by temperature when averaged over the four clones. The k_u and k_e were lower at 25°C than at 20°C and they were similar between 20 and 15°C. These results suggest that the general idea that metal uptake increases with the increase of temperature may not be applicable when organisms physiologically adjust to the environmental temperature. Nickel uptake was significantly increased over time i.e. the k_u after 2 d of Ni exposure was higher than the initial k_u by 2.3, 1.5 and 1.7-fold at 15, 20 and 25°C, respectively. The Ni k_e of *D. magna* decreased when the external Ni exposure stopped. Therefore, future toxicokinetic studies should consider that k_u may depend on the physiological state of the organism, which may change through metal exposure, and that k_e may differ between the presence and the absence of the chemical stressor.

In chapter 4, the effect of Ni on *D. magna* reproduction at 15, 20 and 25°C along four generations was investigated. Multigenerational Ni effects on *D. magna* reproduction depended on the magnitude of the effect in the first generation (F0) and showed very different patterns at different temperatures. At low effect level concentrations (<EC10 of F0), chronic Ni toxicity at 15 and 20°C did not increase along four generations, and the increase of Ni toxicity at 25°C observed in F1 and F2 in some Ni treatments did not persist into F3, where complete recovery of reproduction was observed. At higher effect level concentrations, the multigenerational Ni effects depended on the test temperature. In F0, Ni toxicity was 6.5-fold lower at 25°C than at 15°C (based on EC50). However, the temperature effect on Ni toxicity was not explained by differences in Ni accumulation: at lower temperature lower internal Ni concentrations in *D. magna* were necessary to induce

the same Ni toxicity than at higher temperature. Overall, our results indicate that low single-generation chronic effect concentrations of Ni to *D. magna* (here EC10) are also protective in a long-term, multigenerational context.

In chapter 5, it was investigated whether the effect of temperature on chronic Ni toxicity to *D. magna* assessed on apical endpoints can be extrapolated to the population-level. The results showed no consistent Ni effects on total *D. magna* population abundance at 15, 20 and 25ºC although the Ni concentrations tested were previously found (chapter 4) to significantly reduce the apical reproduction. This result supports the idea that ERA should not extrapolate *as such* from apical endpoints to population-level. A Dynamic Energy Budget individual-based model (DEB-IBM) was calibrated using apical Ni toxicity data at 15, 20 and 25°C. The goal was to investigate whether the calibrated DEB-IBM would be able to predict the unexpected absence of effects at population-level. To predict the population response to Ni the model parameters (i.e. the physiological modes of action (PMoA)) that are affected by Ni were identified. The model with the PMoA with an effect on growth costs was able to predict the Ni effect on the apical endpoints size and reproduction. The present study showed that the calibrated DEB-IBM correctly predicted the unexpected absence of Ni effect to a *D. magna* population. Furthermore, the extrapolated EC50 values suggest that the effect of temperature on Ni toxicity to a *D. magna* population was smaller (the extrapolated EC50 is 1.9-fold higher at 25°C than at 15°C) than on Ni toxicity to *D. magna* apical reproduction (the EC50 is 6.5-fold higher at 25°C than at 15°C, previous study). These results show that the DEB-IBM can help to replace population experiments by in *silico* simulations and to optimize the experimental design of population studies.

Overall, this thesis showed that temperature has a significant effect on metal toxicity to *D. magna* and that organisms acclimated to at higher temperatures were less sensitive to metal toxicity than organisms acclimated to lower temperature. Given these results and the importance of temperature for ectothermic organisms, which represent the majority of the species in aquatic ecosystems, it seems necessary to consider temperature as a factor in the chemical management context of ERA.

Samenvatting

Temperatuur heeft een grote impact op fysiologische processen van ectotherme organismen waardoor het kan interageren met chemische toxiciteit. Echter, ecologische risico-evaluatie (ERA) is gebaseerd op standaardtesten die worden uitgevoerd bij een standaardtemperatuur. Als gevolg wordt de potentiële invloed van temperatuur op chemische toxiciteit niet in rekening gebracht. Bovendien zijn standaardtesten gebaseerd op apicale eindpunten (bijv. overleving en reproductie van individuele organismen) die worden gemeten binnen eenzelfde generatie. Populatie-effecten en effecten van blootstelling over meerdere generaties in het veld worden dus buiten beschouwing gelaten in ERA. Bijgevolg ligt de focus van deze thesis dan ook op het effect van temperatuur op metaaltoxiciteit voor *Daphnia magna*. Verschillende benaderingen werden hiervoor toegepast, van single generatie naar multi-generatie testen voor apicale eindpunten, tot populatie-experimenten. Het onderzoek in deze thesis toont ook het effect van temperatuur op toxico-kinetische processen van metalen aan.

In hoofdstuk 2 werd het effect van temperatuur op chronische metaaltoxiciteit onderzocht, en of dit effect verschilde tussen vier verschillende *D. magna* klonen. Life table experimenten zijn uitgevoerd met Cu, Zn, en Ni bij 15, 20 en 25°C. General linear modeling toonde aan dat chronische Cu, Zn and Ni toxiciteit bij *D. magna* significant werd beïnvloedt door de temperatuur. Uitgemiddeld over de klonen toonden de resultaten aan dat chronische metaaltoxiciteit in *D. magna* hoger was bij 15°C dan bij 20°C, de temperatuur bij standaardtesten. Bij 15°C was de 21-d 50% effect concentratie voor reproductie (EC50) voor Cu, Zn en Ni respectievelijk een factor 1.4, 1.1 en 1.3 lager dan bij 20°C. Bij 25°C was er geen verschil voor chronische Cu en Zn toxiciteit vergeleken met 20°C, maar de chronische Ni toxiciteit was wel lager (21-d EC50 voor Ni bij 25°C was een factor 1.6 hoger dan bij 20°C). Een identieke trend was waargenomen voor Cu en Ni wanneer de 21-d EC10 en EC20 werden beschouwd als estimators, maar niet voor Zn, wat waarschuwt over het extrapoleren van temperatuureffecten voor chemische toxiciteit voor effect concentraties. Al m*et al* was de chronische metaaltoxiciteit het grootst bij lagere temperaturen (15°C), wat in contrast staat met de hogere acute metaaltoxiciteit die geobserveerd wordt bij hogere temperaturen. Bovendien verschilde het effect van temperatuur op chronische Ni toxiciteit significant tussen de *D. magna* klonen.

Dit waarschuwt over het extrapolleren van het temperatuureffect op chemische toxiciteit van individuele klonen naar het populatieniveau.

In ectotherme organismen wordt een toename in temperatuur geassocieerd met een hogere metaal opnameconstante (k_u) en met een toename van de acute metaaltoxiciteit. De resultaten uit hoofdstuk 2 tonen echter aan dat chronische Ni toxiciteit in *D. magna* daalde bij stijgende temperatuur (15°C>20°C>25°C). In hoofdstuk 3 werd het temperatuureffect op Ni opname en eliminatie in vier *D. magna* klonen (ge-pre-acclimatiseerd, d.w.z. in homeostatische balans met de omgevingstemperatuur) onderzocht. Daarnaast werden twee assumpties geassocieerd met één-compartimentskinetiek experimenten onderzocht: of de k_u constant is gedurende blootstelling; en of de eliminatieconstante (k_e) verandert als *D. magna* wordt overgedragen naar een niet-gecontamineerd medium. Ni opname en eliminatie in *D. magna* werd beïnvloed door de temperatuur. De k_u en k_e waren lager bij 25 dan bij 20°C, en waren vergelijkbaar bij 20 en 15°C. Daarnaast werden ook verschillende temperatuurafhankelijke patronen ontdekt in k_u tussen de klonen. Deze resultaten toonden aan dat h*et al*gemeen idee dat metaalopname toeneemt met toenemende temperatuur ni*et al*tijd toepasbaar is wanneer organismen in homeostatische balans zijn met de omgevingstemperatuur. De Ni opnameconstante was significant toegenomen over tijd, d.w.z. de k_u na 2-d blootstelling aan Ni was hoger dan de initiële k_u, met een factor 2.3, 1.5 en 1.7 bij respectievelijke 15, 20 en 25°C. De Ni k_e van *D. magna* daalde wanneer de Ni blootstelling stopte. Daarom zouden toekomstige toxico-kinetische studies rekening moeten houden met het feit dat k_u afhankelijk kan zijn van de fysiologische toestand van het organisme die kan veranderen naargelang de chemische blootstelling, en dat k_e kan veranderen in aan- of afwezigheid van een chemische stressor.

In hoofdstuk 4 is het effect van Ni op *D. magna* reproductie bij 15, 20 en 25°C over vier generaties onderzocht. Multi-generatie effecten van Ni op *D. magna* reproductie waren afhankelijk van de grootte van het effect op de eerste generatie (F0) en toonden verschillende patronen bij de geteste temperaturen. Bij lage effect concentraties (<EC10 bij F0), toonde chronische Ni toxiciteit bij 15 en 20°C geen significante toename over de vier generaties, terwijl de toename in Ni toxiciteit bij 25°C bij F1 en F2 in een paar van de Ni treatments niet aanhield tot in F3, waar

compleet herstel van de reproductie was waargenomen. Bij hogere effect concentraties was het multi-generatie effect van Ni afhankelijk van de geteste temperatuur. In F0 was Ni toxiciteit een factor 6.5 lager bij 25°C dan bij 15°C (gebaseerd op de EC50), alhoewel het temperatuureffect op Ni toxiciteit niet kon verklaard worden door verschillen in Ni accumulatie. Bij lagere temperaturen waren er lagere interne Ni concentraties nodig om hetzelfde Ni toxiciteitseffect te induceren als bij hogere temperaturen. Over h*et al*gemeen tonen de resultaten aan dat lage single-generatie chronische effect concentraties van Ni voor *D. magna* (EC10 waarden) ook beschermend op lange termijn in een multi-generatie context.

In hoofdstuk 5 werd onderzocht of het effect van temperatuur op chronische Ni toxiciteit voor *D. magna* op apicale eindpunten kan geëxtrapoleerd worden naar het populatieniveau. De resultaten tonen geen consistent Ni effect op totale *D. magna* populatiedensiteit bij 15, 20 en 25°C, hoewel de geteste Ni concentraties eerder significante effecten toonden op individuele reproductie. Dit resultaat ondersteunt het idee dat ERA apicale eindpunten niet zou mogen extrapoleren naar het populatieniveau. Een Dynamic Energy Budget en Individual-Based Model (DEB-IBM) werd gekalibreerd gebruik makende van apicale Ni toxiciteitsdata bij 15, 20 en 25°C. Het doel was om te kijken of het gekalibreerde DEB-IBM de onverwachte afwezigheid van effecten op populatieniveau kon voorspellen. Om populatie-effecten door Ni te voorspellen werden model parameters geïdentificeerd die beïnvloed worden door Ni (d.w.z. de modes of action (PMoA)). Het model met de PMoA die de kosten voor groei beïnvloedt, was in staat het Ni effect op de apicale eindpunten groei en reproductie het best te fitten aan de observaties. Het gekalibreerde DEB-IBM voorspelde correct de onverwachte aanwezigheid van Ni effecten op een *D. magna* populatie. Bovendien tonen geëxtrapoleerde EC50 waarden aan dat het temperatuureffect op Ni toxiciteit voor een *D. magna* populatie kleiner was (de geëxtrapoleerde EC50 was een factor 1.9 hoger bij 25°C dan bij 15°C) dan de Ni toxiciteit voor *D. magna* apicale reproductie (de EC50 was een factor 6.5 hoger bij 25°C dan bij 15°C). Deze resultaten toonden aan dat DEB-IBM kan helpen bij optimaal design van experimenten met populatiestudies, of zelfs populatie-experimenten kan vervangen door middel van *in silico* simulaties.

Over h*et al*gemeen toonde deze thesis aan dat temperatuur een significant effect heeft op metaaltoxiciteit voor *D. magna*, en dat geacclimatiseerde organismen bij hogere temperaturen minder gevoelig waren voor metaaltoxiciteit dan geacclimatiseerde organismen bij lagere temperatuur. Gezien deze resultaten en het belang van temperatuur voor ectotherme organismen, die het grootste deel van de species in aquatische ecosystemen uitmaken, lijkt het dan ook noodzakelijk om temperatuur als factor mee te nemen in ERA.

Curriculum Vitae

Personal information

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Education

July 2012 Master degree in Applied Biology, Ecotoxicology and Toxicology at the University of Aveiro. Dissertation: Mechanism of toxicity in *Folsomia candida*.

July 2011 Degree in Biology at the University of Aveiro. Final project: Multigeneration effects of cadmium on the springtail *Folsomia candida*.

Professional experience

2013 – 2017 PhD student (Joint-PhD) at Laboratory of Environmental Toxicology and Aquatic Ecology, Environmental Toxicology Unit (Ghent University) and at Systemic Physiological and Ecotoxicological Research (University of Antwerp), supervised by Dr. Karel De Schamphelaere and by Dr. Ronny Blust.

2011 – 2012 Scientific Research Grant in the Project ref. PTDC/AACAMB/098112/2008, Bias-to-soil - Biomass ash: Characteristics in relation to its origin, treatment and application to soil. University of Aveiro, supervised by Dr. Mónica João de Barros Amorim.

Additional professional education

April 2017 Specialist course of “qPCR” followed at Biogazelle, Ghent, Belgium.

September 2011 Advanced course of “Practical Approach to Ecotoxicogenomics” at the Biology department, University of Aveiro, Portugal.

September 2010 Advanced course of “Topics on Environmental Mixture Toxicity” at the Biology department, University of Aveiro, Portugal.

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Pereira CMS, Janssen C, Blust R, and De Schamphelaere KAC (2016). Effect of temperature on nickel bioaccumulation and chronic toxicity *to Daphnia magna*. SETAC Europe 26th Annual Meeting in Nantes, France.

Pereira CMS, Janssen C, Blust R, and De Schamphelaere KAC (2015). Does chronic toxicity of copper and zinc to *Daphnia magna* depend on temperature? SETAC Europe 25th Annual Meeting, Barcelona, Spain.

Pereira CMS, Gomes SIL, Novais SC, Soares AMVM and Amorim MJB (2012). Multi-generational exposure of *Folsomia candida* to cadmium: survival, reproduction and metallothionein gene effects. SETAC Europe 22nd Annual Meeting, Berlin, Germany.

Poster presentations

Pereira CMS, Janssen C, Blust R, and De Schamphelaere KAC (2016). Effect of temperature on nickel biodynamics in *Daphnia magna* determinate with a stable isotope experiment. SETAC Europe 26th Annual Meeting in Nantes, France.

Pereira CMS, Gomes SIL, Novais SC, Soares AMVM and Amorim MJB (2011). Multi-generational exposure of *Folsomia candida* to cadmium: survival, reproduction and

metallothionein expression. Poster spotlight, SETAC Europe 21st Annual Meeting, Milan, Italy.

Appendix A

Supplementary material for Chapter 2

Table A1. Mean (± standard deviation) of temperature (T), dissolved oxygen concentration (O_2), the dissolved organic carbon (DOC) and the concentrations of Na, Mg , K and Ca along the Cu, Zn and Ni life table experiments.

Life table experiment	T (°C)	O_2 (mg L^{-1})	DOC (mg L^{-1})	Na (mg L^{-1})	Mg (mg L^{-1})	K (mg L^{-1})	Ca (mg L^{-1})
Cu and Zn	14.07 ±0.8	9.03 ± 0.12	3.27 ± 0.42	20.91 ± 0.21	9.99 ± 0.21	3.10 ± 0.07	56.54 ± 2.41
	20.17 ± 0.84	8.90 ± 0.00					
	24.69 ± 0.21	8.21 ± 0.08					
Ni	14.84 ± 0.44	9.73 ± 0.29	4.56 ± 1.41	20.50 ± 1.25	9.92 ± 0.58	3.02 ± 0.19	56.51 ± 2.74
	19.64 ± 0.55	9.08 ± 0.10					
	24.75 ± 0.25	8.47 ± 0.16					

Table A2. Summary table of the metal chemical analysis and of the pH values registered on the life table experiments with Cu, Zn and Ni at the different temperatures. All measurements are given as averages in the new medium and old medium after renewal. Mean ± standard desviation is reported.

Life table experiment	Nominal Concentrations (µg L^{-1})	Dissolved concentrations (µg L^{-1})	pH (15°C)	pH (20°C)	pH (25°C)
Cu	8	2.50 ± 0.29	7.15 ± 0.25	7.17 ± 0.27	7.11 ± 0.11
	56	16.00 ± 2.17	7.44 ± 0.30	7.46 ± 0.28	7.47 ± 0.22
	100	26.49 ± 2.17	7.50 ± 0.30	7.53 ± 0.27	7.50 ± 0.22
	140	26.49 ± 2.62	7.56 ± 0.33	7.58 ± 0.31	7.55 ± 0.16
	180	47.67 ± 9.61	7.63 ± 0.37	7.58 ± 0.32	7.57 ± 0.18
	360	79.44 ± 4.73	7.64 ± 0.41	7.56 ± 0.29	7.50 ± 0.25
Zn	13	n.d.	7.15 ± 0.25	7.17 ± 0.27	7.13 ± 0.30
	160	60.80 ± 22.29	7.17 ± 0.24	7.20 ± 0.25	7.15 ± 0.30
	260	115.62 ± 33.23	7.19 ± 0.29	7.23 ± 0.27	7.26 ± 0.29
	360	133.27 ± 26.43	7.33 ± 0.33	7.33 ± 0.27	7.33 ± 0.23
	560	219.98 ± 26.78	7.40 ± 0.40	7.38 ± 0.30	7.42 ± 0.22
	760	302.74 ± 30.12	7.62 ± 0.49	7.43 ± 0.27	7.44 ± 0.24
Ni	0	0.14 ± 0.04	7.53 ± 0.39	7.41 ± 0.19	7.41 ± 0.21
	50	26.06 ± 0.49	7.68 ± 0.47	7.47 ± 0.20	7.51 ± 0.36
	100	85.75 ± 3.11	7.72 ± 0.49	7.53 ± 0.25	7.53 ± 0.37
	200	128.22 ± 9.13	7.82 ± 0.56	7.63 ± 0.33	7.63 ± 0.41
	250	158.11 ± 14.40	7.89 ± 0.62	7.64 ± 0.32	7.67 ± 0.42
	300	196.20 ± 17.98	7.84 ± 0.58	7.69 ± 0.34	7.71 ± 0.42

[n.d.: not determined, the analysis of the Zn concentration in the control treatment indicated contamination from the filters used to take samples for dissolved metal concentration and, therefore, in this case the nominal concentration was used for the data analysis.]

Table A3. Control performance of each individual *Daphnia magna* clone of the Cu, Zn and Ni life table experiments at 15°C, 20°C and 25°C.

		Control mortality (%)			Survival time (d)			Number of offspring per individual female (mean ± standard deviation)			Coefficient of variation of number of offspring per individual female (%)		
Life table experiment	Clone	15°C	20 °C	25°C	15°C	20 °C	25°C	15°C	20 °C	25°C	15°C	20 °C	25°C
Cu and Zn	A	0.0	0.0	0.0	21	21	21	45.8±14.7	101.1±24.6	100.4±23.9	32	24	24
	B	0.0	0.0	12.5	21	21	20	31.4±11.2	99.3±15.0	106.6±26.5	36	15	25
	C	0.0	0.0	0.0	21	21	21	40.6±13.2	141.4±18.6	163.4±13.7	33	13	8
	D	0.0	0.0	16.7	21	21	20	16.8±7.7	41.3±16.3	53.8±22.0	46	39	41
Ni	A	0.0	0.0	50.0	21	21	16.8	45.0±16.8	46.5±12.8	13±14.8	37	28	114
	B	0.0	0.0	25.0	21	21	19	8.3±3.0	28.7±4.5	46.5±32.4	36	16	70
	C	0.0	0.0	0.0	21	21	21	49.8±13.8	80.5±11.0	115.8±32.3	28	14	28
	D	0.0	33.3	0.0	21	19.3	21	5.3±2.1	32.7±19.0	67.0±21.9	40	58	33

Table A4. Summary of Akaike information criterion (AIC) and the Akaike weight (*w)* of the 46 models for predict $\sqrt{R_0}$ (number of offspring per individual female), as function of temperature (T) and clone applied to each metal (Me (µg L^{-1})). The selected models for the Cu, Zn and Ni data set were M2, M7 and M4, respectively.

		Cu		*Zn*		*Ni*	
Model number	Model equation	AIC	*w*	AIC	*W*	AIC	*w*
M1	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\ Me + \beta_2(T, clone)\ Me^2 + \beta_3(T, clone)\ Me^3$	1436.09	0.03	1417.00	0.00	1218.58	0.00
M2	$\boldsymbol{\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)\ Me + \beta_2(T, clone)\ Me^2 + \beta_3(T, clone)\ Me^3}$	**1429.76**	**0.61**	1408.83	0.14	1214.73	0.02
M3	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\ Me + \beta_2(clone)\ Me^2 + \beta_3(T, clone)\ Me^3$	1433.76	0.08	1410.52	0.06	1210.32	0.22
M4	$\boldsymbol{\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\ Me + \beta_2(T, clone)\ Me^2 + \beta_3(clone)\ Me^3}$	1436.35	0.02	1409.83	0.08	**1208.36**	**0.59**
M5	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)\ Me + \beta_2(clone)\ Me^2 + \beta_3(T, clone)\ Me^3$	1434.15	0.07	1409.42	0.10	1213.52	0.04
M6	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\ Me + \beta_2(clone)\ Me^2 + \beta_3(clone)\ Me^3$	1452.75	0.00	1411.01	0.05	1227.08	0.00
M7	$\boldsymbol{\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)\ Me + \beta_2(T, clone)\ Me^2 + \beta_3(clone)\ Me^3}$	1439.81	0.00	**1407.55**	**0.27**	1217.89	0.01
M9	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T)\ Me + \beta_2(T, clone)\ Me^2 + \beta_3(T, clone)\ Me^3$	1436.48	0.02	1409.91	0.08	1225.12	0.00
M10	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\ Me + \beta_2(T)\ Me^2 + \beta_3(T, clone)\ Me^3$	1447.75	0.00	1419.10	0.00	1219.00	0.00
M11	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\ Me + \beta_2(T, clone)\ Me^2 + \beta_3(clone)\ Me^3$	1451.88	0.00	1420.79	0.00	1216.30	0.01
M12	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T)\ Me + \beta_2(T)\ Me^2 + \beta_3(clone)\ Me^3$	1458.35	0.00	1434.35	0.00	1233.21	0.00
M13	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\ Me + \beta_2(T, clone)\ Me^2 + \beta_3(T, clone)\ Me^3$	1453.99	0.00	1413.81	0.01	1234.52	0.00
M14	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T)\ Me + \beta_2(T, clone)\ Me^2 + \beta_3(T)\ Me^3$	1455.16	0.00	1423.41	0.00	1233.49	0.00
M15	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T)\ Me + \beta_2(T)\ Me^2 + \beta_3(T)\ Me^3$	1473.58	0.00	1455.77	0.00	1248.03	0.00
M16	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\ Me + \beta_2(T, clone)\ Me^2 + \beta_3(T, clone)\ Me^3$	1432.61	0.15	1411.10	0.04	1222.06	0.00
M17	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\ Me + \beta_2(clone)\ Me^2 + \beta_3(T, clone)\ Me^3$	1436.52	0.02	1411.19	0.04	1220.13	0.00
M18	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\ Me + \beta_2(T, clone)\ Me^2 + \beta_3(clone)\ Me^3$	1442.03	0.00	1409.38	0.11	1224.23	0.00

*Table continues on page 159.

Continuation of Table A4. Summary of Akaike information criterion (AIC) and the Akaike weight (*w)* of the 46 models for predict $\sqrt{R_0}$ (number of offspring per individual female), as function of temperature (T) and clone applied to each metal (Me (µg L^{-1})). The selected models for Cu, Zn and Ni data set were M2, M7 and M4, respectively.

		Cu		*Zn*		*Ni*	
Model number	Model equation	AIC	*w*	AIC	*w*	AIC	*w*
M19	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\, Me + \beta_2(clone)\, Me^2 + \beta_3(clone)\, Me^3$	1467.00	0.00	1433.71	0.00	1245.87	0.00
M20	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1 Me + \beta_2(T)\, Me^2 + \beta_3(T, clone)\, Me^3$	1454.47	0.00	1434.78	0.00	1230.15	0.00
M21	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\, Me + \beta_2(T, clone)\, Me^2 + \beta_3(T)\, Me^3$	1451.27	0.00	1424.00	0.00	1230.41	0.00
M22	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\, Me + \beta_2(T)\, Me^2 + \beta_3(T)\, Me^3$	1469.71	0.00	1455.88	0.00	1244.91	0.00
M23	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\, Me + \beta_2\, Me^2 + \beta_3(T, clone)\, Me^3$	1444.21	0.00	1422.25	0.00	1215.26	0.02
M24	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)\, Me + \beta_2\, Me^2 + \beta_3(T, clone)\, Me^3$	1443.84	0.00	1420.24	0.00	1217.44	0.01
M25	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\, Me + \beta_2\, Me^2 + \beta_3(clone)\, Me^3$	1461.59	0.00	1421.86	0.00	1230.40	0.00
M26	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)\, Me + \beta_2 Me^2 + \beta_3(clone)\, Me^3$	1473.38	0.00	1441.76	0.00	1243.53	0.00
M27	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T)\, Me + \beta_2\, Me^2 + \beta_3(T, clone)\, Me^3$	1454.77	0.00	1436.71	0.00	1229.45	0.00
M28	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\, Me + \beta_2\, Me^2 + \beta_3(T)\, Me^3$	1450.39	0.00	1416.64	0.00	1230.75	0.00
M29	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T)\, Me + \beta_2\, Me^2 + \beta_3(T)\, Me^3$	1470.01	0.00	1457.54	0.00	1244.27	0.00
M30	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\, Me + \beta_2(T, clone)\, Me^2 + \beta_3 Me^3$	1449.39	0.00	1423.46	0.00	1212.87	0.06
M31	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)\, Me + \beta_2(T, clone)\, Me^2 + \beta_3\, Me^3$	1451.77	0.00	1420.29	0.00	1220.99	0.00
M32	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\, Me + \beta_2(clone)\, Me^2 + \beta_3 Me^3$	1464.04	0.00	1423.64	0.00	1229.82	0.00
M33	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)\, Me + \beta_2(clone)\, Me^2 + \beta_3\, Me^3$	1475.64	0.00	1443.36	0.00	1242.88	0.00
M34	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)\, Me + \beta_2(T, clone)\, Me^2 + \beta_3\, Me^3$	1452.53	0.00	1425.63	0.00	1229.95	0.00
M35	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\, Me + \beta_2(T)\, Me^2 + \beta_3 Me^3$	1451.35	0.00	1416.23	0.00	1231.00	0.00

* Table continues on page 160.

Continuation of Table A4. Summary of Akaike information criterion (AIC) and the Akaike weight (*w)* of the 46 models for predict $\sqrt{R_0}$ (number of offspring per individual female), as function of temperature (T) and clone applied to each metal (Me (µg L^{-1})). The selected models for Cu, Zn and Ni data set were M2, M7 and M4, respectively.

		Cu		Zn		Ni	
Model number	Model equation	AIC	w	AIC	w	AIC	w
M36	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T)\, Me + \beta_2(T)\, Me^2 + \beta_3\, Me^3$	1470.89	0.00	1457.17	0.00	1244.45	0.00
M37	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1 Me + \beta_2 Me^2 + \beta_3(T, clone)\, Me^3$	1456.85	0.00	1435.78	0.00	1233.54	0.00
M38	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\, Me + \beta_2\, Me^2 + \beta_3(clone)\, Me^3$	1483.94	0.00	1454.91	0.00	1256.13	0.00
M39	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\, Me + \beta_2\, Me^2 + \beta_3(T)\, Me^3$	1471.28	0.00	1455.99	0.00	1247.71	0.00
M40	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\, Me + \beta_2(T, clone)\, Me^2 + \beta_3\, Me^3$	1459.52	0.00	1424.47	0.00	1235.26	0.00
M41	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1 Me + \beta_2(clone)\, Me^2 + \beta_3\, Me^3$	1481.72	0.00	1446.34	0.00	1254.40	0.00
M42	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\, Me + \beta_2(T)\, Me^2 + \beta_3\, Me^3$	1476.40	0.00	1455.39	0.00	1249.06	0.00
M43	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\, Me + \beta_2\, Me^2 + \beta_3\, Me^3$	1471.17	0.00	1419.77	0.00	1239.62	0.00
M44	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)\, Me + \beta_2\, Me^2 + \beta_3\, Me^3$	1481.54	0.00	1439.28	0.00	1251.19	0.00
M45	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T)\, Me + \beta_2\, Me^2 + \beta_3\, Me^3$	1487.77	0.00	1459.22	0.00	1252.13	0.00
M46	$\sqrt{R_0} = \beta_0(T, clone) + \beta_1\, Me + \beta_2\, Me^2 + \beta_3 Me^3$	1502.56	0.00	1477.93	0.00	1260.62	0.00

Table A5. Optimal third order linear model for Cu, Zn and Ni data set according to the Akaike weights with respective equations and adjusted explained variation (R^2).Where β_0 is the interface, T is temperature and β_1, β_2, β_3 are the regression coefficients of the gradient, curvature and aberrancy (β_3), respectively.

	Cu	Zn	Ni
Optimal model	Equation 4: $\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)Cu + \beta_2(T, clone)\ Cu^2 + \beta_3(T, clone)\ Cu^3$	Equation 5: $\sqrt{R_0} = \beta_0(T, clone) + \beta_1(clone)\ Zn + \beta_2(T, clone)\ Zn^2 + \beta_3(clone)\ Zn^3$	Equation 6: $\sqrt{R_0} = \beta_0(T, clone) + \beta_1(T, clone)\ Ni + \beta_2(T, clone)\ Ni^2 + \beta_3(clone)\ Ni^3$
Adjusted R^2	0.79	0.77	0.71

Table A6. Summary of the optimal third order linear model for Cu data set that corresponds to model 2 in table A3, the parameter estimates, standard error (SE) and significance (*p* value).

Predictor	Parameter estimate	SE	*p* value
(Intercept)	6.828E+00	7.567E-01	0.000
clone B	-1.491E+00	1.070E+00	0.165
clone C	-1.284E+00	1.070E+00	0.231
clone D	-4.215E+00	1.137E+00	0.000
20°C	2.631E+00	8.603E-01	0.002
25°C	2.911E+00	8.603E-01	0.001
Cu	8.655E-02	8.571E-02	0.313
Cu^2	-8.237E-03	2.999E-03	0.006
Cu^3	7.651E-05	2.646E-05	0.004
$clone\ B \times\ 20°C$	2.083E+00	1.217E+00	0.088
$clone\ C \times\ 20°C$	3.371E+00	1.217E+00	0.006
$clone\ D \times\ 20°C$	9.221E-01	1.266E+00	0.467
$clone\ B \times\ 25°C$	2.318E+00	1.217E+00	0.058
$clone\ C \times\ 25°C$	2.990E+00	1.217E+00	0.015
$clone\ D \times\ 25°C$	1.151E+00	1.298E+00	0.376
$clone\ B\ \times\ Cu$	-1.377E-01	1.212E-01	0.257
$clone\ C\ \times\ Cu$	1.967E-01	1.212E-01	0.106
$clone\ D\ \times\ Cu$	-2.460E-02	1.250E-01	0.844
$clone\ B\ \times\ Cu^2$	9.214E-03	4.241E-03	0.031
$clone\ C \times\ Cu^2$	-5.189E-03	4.241E-03	0.222
$clone\ D \times Cu^2$	4.187E-03	4.402E-03	0.342
$20°C \times\ Cu^2$	5.046E-03	1.600E-03	0.002
$25°C \times\ Cu^2$	4.922E-03	1.600E-03	0.002
$clone\ B\ \times\ Cu^3$	-9.149E-05	3.742E-05	0.015
$clone\ C\ \times\ Cu^3$	3.670E-05	3.742E-05	0.328
$clone\ D\ \times\ Cu^3$	-3.929E-05	3.910E-05	0.316
$20°C\ \times\ Cu^3$	-6.897E-05	1.988E-05	0.001
$25°C\ \times\ Cu^3$	-6.845E-05	1.988E-05	0.001
$clone\ B \times\ 20°C \times\ Cu^2$	-3.088E-03	2.262E-03	0.173

* Table continues on page 163.

Continuation of Table A6. Summary of the optimal third order linear model for Cu data set that corresponds to model 2 in table A3, the parameter estimates, standard error (SE) and significance (*p* value).

Predictor	Parameter estimate	SE	*p* value
$clone\ C \times\ 20℃ \times\ Cu^2$	-6.139E-03	2.262E-03	0.007
$clone\ D \times\ 20℃ \times\ Cu^2$	-6.673E-03	2.355E-03	0.005
$clone\ B \times\ 25℃ \times\ Cu^2$	-3.557E-03	2.262E-03	0.117
$clone\ C \times\ 25℃ \times\ Cu^2$	-3.934E-03	2.262E-03	0.083
$clone\ D \times\ 25℃ \times\ Cu^2$	-4.145E-03	2.382E-03	0.083
$clone\ B \times\ 20℃ \times\ Cu^3$	3.464E-05	2.812E-05	0.219
$clone\ C \times\ 20℃ \times\ Cu^3$	7.048E-05	2.812E-05	0.013
$clone\ D \times\ 20℃ \times\ Cu^3$	8.086E-05	2.926E-05	0.006
$clone\ B \times\ 25℃ \times\ Cu^3$	4.106E-05	2.812E-05	0.145
$clone\ C \times\ 25℃ \times\ Cu^3$	4.414E-05	2.812E-05	0.118
$clone\ D \times\ 25℃ \times\ Cu^3$	4.905E-05	2.954E-05	0.098
Adjusted R^2: 0.786			

Table A7. Summary of the optimal third order linear model for Zn data set that corresponds to model 7 in table A3, the parameter estimates, standard error (SE) and significance (*p* value).

Predictor	Parameter estimate	SE	*p* value
(Intercept)	6.221E+00	7.021E-01	0.000
clone B	-1.697E+00	9.930E-01	0.089
clone C	-2.253E-01	9.930E-01	0.821
clone D	-2.456E+00	1.059E+00	0.021
20°C	3.257E+00	6.957E-01	0.000
25°C	3.674E+00	6.957E-01	0.000
Zn	2.390E-02	2.079E-02	0.251
$\boldsymbol{Zn^2}$	-5.185E-04	1.747E-04	0.003
$\boldsymbol{Zn^3}$	1.229E-06	3.799E-07	0.001
$\boldsymbol{clone\ B \times 20°C}$	2.593E+00	9.838E-01	0.009
$\boldsymbol{clone\ C \times 20°C}$	2.169E+00	9.838E-01	0.028
$\boldsymbol{clone\ D \times 20°C}$	-3.661E-01	1.033E+00	0.723
$\boldsymbol{clone\ B \times 25°C}$	2.383E+00	9.838E-01	0.016
$\boldsymbol{clone\ C \times 25°C}$	2.379E+00	9.838E-01	0.016
$\boldsymbol{clone\ D \times 25°C}$	-3.923E-01	1.053E+00	0.710
$\boldsymbol{clone\ B \times Zn}$	-1.322E-02	2.940E-02	0.653
$\boldsymbol{clone\ C \times Zn}$	2.365E-02	2.940E-02	0.422
$\boldsymbol{clone\ D \times Zn}$	-5.660E-02	3.067E-02	0.066
$\boldsymbol{clone\ B \times Zn^2}$	2.358E-04	2.471E-04	0.341
$\boldsymbol{clone\ C \times Zn^2}$	-2.465E-04	2.471E-04	0.319
$\boldsymbol{clone\ D \times Zn^2}$	7.113E-04	2.561E-04	0.006
$\boldsymbol{20°C \times Zn^2}$	-4.052E-05	1.745E-05	0.021
$\boldsymbol{25°C \times Zn^2}$	-1.777E-05	1.745E-05	0.309
$\boldsymbol{clone\ B \times Zn^3}$	-5.944E-07	5.373E-07	0.269
$\boldsymbol{clone\ C \times Zn^3}$	5.849E-07	5.373E-07	0.277
$\boldsymbol{clone\ D \times Zn^3}$	-1.635E-06	5.549E-07	0.003
$\boldsymbol{clone\ B \times 20°C \times Zn^2}$	-1.998E-05	2.468E-05	0.419
$\boldsymbol{clone\ C \times 20°C \times Zn^2}$	-3.063E-05	2.468E-05	0.216
$\boldsymbol{clone\ D \times 20°C \times Zn^2}$	4.712E-06	2.571E-05	0.855
$\boldsymbol{clone\ B \times 25°C \times Zn^2}$	-2.327E-06	2.468E-05	0.925

* Table continues on page 165.

Continuation of Table A7. Summary of the optimal third order linear model for Zn data set that corresponds to model 7 in table A3, the parameter estimates, standard error (SE) and significance (*p* value).

Predictor	Parameter estimate	SE	*p* value
$\boldsymbol{clone\ C \times 25℃ \times Zn^2}$	-3.611E-05	2.468E-05	0.145
$\boldsymbol{clone\ D \times 25℃ \times Zn^2}$	-4.883E-06	2.593E-05	0.851
Adjusted R^2: 0.773			

Table A8. Summary of the optimal third order linear model for Ni data set that corresponds to model 4 in table A3, the parameter estimates, standard error (SE) and significance (*p* value).

Predictor	Parameter estimate	SE	*p* value
(Intercept)	5.343E+00	8.404E-01	0.000
clone B	-2.526E+00	1.200E+00	0.036
clone C	1.894E+00	1.188E+00	0.112
clone D	-2.715E+00	1.259E+00	0.032
20°C	1.889E+00	1.139E+00	0.099
25°C	-2.315E+00	1.139E+00	0.043
Ni	8.650E-03	2.828E-02	0.760
Ni^2	-4.115E-04	2.531E-04	0.105
Ni^3	1.272E-06	6.883E-07	0.066
$clone\ B \times 20°C$	8.112E-01	1.667E+00	0.627
$clone\ C \times 20°C$	1.078E+00	1.611E+00	0.504
$clone\ D \times 20°C$	3.792E-01	1.712E+00	0.825
$clone\ B \times 25°C$	6.357E+00	1.622E+00	0.000
$clone\ C \times 25°C$	5.897E+00	1.611E+00	0.000
$clone\ D \times 25°C$	7.750E+00	1.655E+00	0.000
$clone\ B \times Ni$	2.311E-02	4.011E-02	0.565
$clone\ C \times Ni$	-6.066E-02	3.999E-02	0.131
$clone\ D \times Ni$	-6.049E-02	4.126E-02	0.144
$20°C \times Ni$	-1.719E-02	2.728E-02	0.529
$25°C \times Ni$	1.002E-03	2.728E-02	0.971
$clone\ B \times Ni^2$	-1.813E-05	3.603E-04	0.960
$clone\ C \times Ni^2$	6.020E-04	3.579E-04	0.094
$clone\ D \times Ni^2$	8.825E-04	3.646E-04	0.016
$20°C \times Ni^2$	4.772E-05	1.172E-04	0.684
$25°C \times Ni^2$	2.418E-05	1.172E-04	0.837
$clone\ B \times Ni^3$	-2.221E-07	9.807E-07	0.821
$clone\ C \times Ni^3$	-1.622E-06	9.735E-07	0.097
$clone\ D \times Ni^3$	-2.593E-06	9.850E-07	0.009
$clone\ B \times 20°C \times Ni$	2.066E-02	3.916E-02	0.598
$clone\ C \times 20°C \times Ni$	6.715E-03	3.858E-02	0.862

* Table continues on page 167.

Continuation of Table A8. Summary of the optimal third order linear model for Ni data set that corresponds to model 4 in table A3, the parameter estimates, standard error (SE) and significance (*p* value).

Predictor	Parameter estimate	SE	*p* value
$\boldsymbol{clone\ D \times 20℃ \times Ni}$	-3.577E-02	3.972E-02	0.369
$\boldsymbol{clone\ B \times 25℃ \times Ni}$	3.525E-02	3.860E-02	0.362
$\boldsymbol{clone\ C \times 25℃ \times Ni}$	-1.772E-02	3.858E-02	0.647
$\boldsymbol{clone\ D \times 25℃ \times Ni}$	7.055E-02	3.915E-02	0.073
$\boldsymbol{clone\ B \times 20℃ \times Ni^2}$	-1.249E-04	1.673E-04	0.456
$\boldsymbol{clone\ C \times 20℃ \times Ni^2}$	-3.418E-05	1.657E-04	0.837
$\boldsymbol{clone\ D \times 20℃ \times Ni^2}$	1.520E-04	1.689E-04	0.369
$\boldsymbol{clone\ B \times 25℃ \times Ni^2}$	-2.575E-04	1.657E-04	0.122
$\boldsymbol{clone\ C \times 25℃ \times Ni^2}$	-3.403E-05	1.657E-04	0.838
$\boldsymbol{clone\ D \times 25℃ \times Ni^2}$	-4.355E-04	1.673E-04	0.010
Adjusted R^2: 0.708			

Appendix A

Table A9. The 21 day effective concentrations (ECx) for reproduction (R_0: number of offspring per individual female) (+/- standard error) of Cu, Zn and Ni at 15°C, 20°C (standard temperature recommended by OECD guideline) and 25°C, calculated for each individual *Daphnia magna* clone. n.d.: not determined.

		Cu (µg L^{-1})			Zn (µg L^{-1})			Ni (µg L^{-1})		
Clone	T (°C)	EC10	EC20	EC50	EC10	EC20	EC50	EC10	EC20	EC50
A	15.0	19.3 ± 6.0	22.8 ± 5.1	29.2 ± 4.0	111.8 ± 10.8	114.4 ± 5.6	118.9 ± 12.1	21.4 ± 10.8	30.1 ± 11.5	53.5 ± 12.6
	20.0	43.8 ± 6.0	45.2 ± 10.1	47.5 ± 1.0	114.2 ± 8.9	119.3 ± 6.8	128.6 ± 3.8	36.5 ± 9.4	46.6 ± 7.8	70.6 ± 11.2
	25.0	45.5 ± 15.8	46.3 ± 9.0	47.4 ± 1.7	97.4 ± 18.6	115.1 ± 15.1	153.1 ± 15.2	55.8 ± 85.5	59.7 ± 64.2	67.0 ± 20.6
B	15.0	9.4 ± 14.7	16.1 ± 17.7	36.1 ± 17.0	99.4 ± n.d.	101.7 ± n.d.	105.7 ± n.d.	73.0 ± 79.4	79.5 ± 82.2	108.3 ± 113.8
	20.0	46.2 ± 4.1	48.7 ± 3.3	52.8 ± 13.1	120.4 ± 12.2	126.3 ± 7.0	137.2 ± 5.5	82.5 ± 29.6	90.6 ± 30.3	125.3 ± 39.5
	25.0	45.3 ± 6.1	46.4 ± 3.4	48.1 ± 1.2	108.4 ± 28.0	138.3 ± 26.0	209.8 ± 19.3	86.4 ± 14.4	95.2 ± 15.0	134.3± 20.8
C	15.0	26.7 ± 11.1	30.6 ± 9.1	37.5 ± 5.2	106.5 ±13.8	111.9 ± 10.6	121.8 ± 7.6	24.2 ± 22.2	32.8 ± 20.6	55.3 ± 19.4
	20.0	36.7 ± 2.9	39.3 ± 2.3	43.6 ± 1.4	118.4 ± 6.1	122.0 ± 4.8	128.5 ± 2.4	32.5 ±10.1	45.7 ± 11.1	81.5 ± 15.7
	25.0	22.0 ± 4.7	28.3 ± 4.0	41.6 ± 2.2	53.2 ±10.0	71.6 ± 9.9	119.0 ± 8.5	22.0 ± 7.5	32.1 ± 8.4	61.5 ± 10.8
D	15.0	8.1 ± 29.9	11.9 ± 32.6	23.0 ± 33.2	9.4 ±109.5	21.2 ± 190.4	84.5 ± 392.0	7.4 ± 52.6	14.0 ± 76.5	41.5 ± 133.6
	20.0	36.0 ± n.d.	36.6 ± n.d.	37.7 ± n.d.	81.5 ± 54.9	100.9 ± 48.8	145.3 ± 30.8	6.9 ± 13.4	10.0 ± 15.3	19.3 ± 15.9
	25.0	43.9 ± 12.5	44.7 ± 9.8	46.2 ± 4.9	6.3 ± 23.3	15.6 ± 45.3	72.6 ±117.8	154.6 ± 10.8	163.5 ± 8.5	179.9 ± 4.7

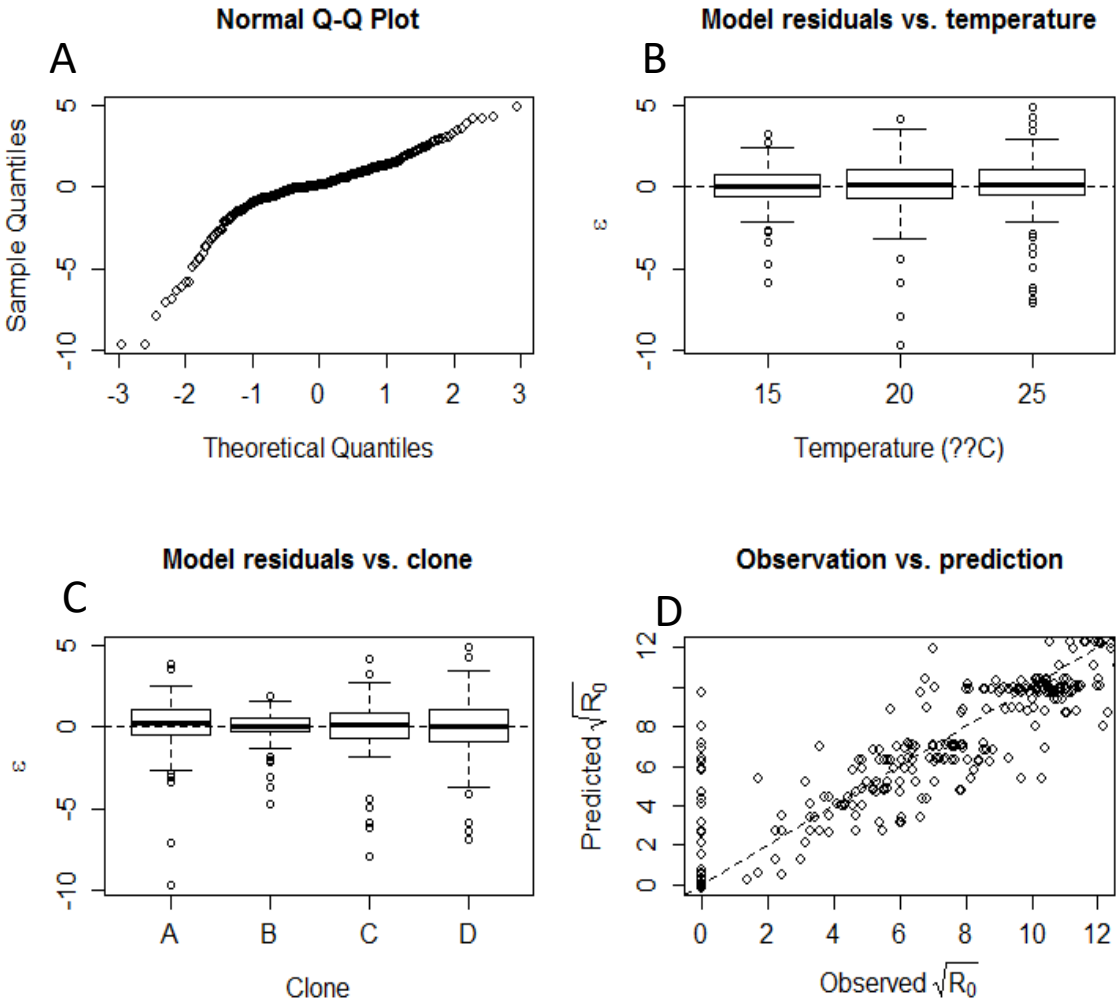

Figure A1. Model validation graphs for the optimal third order linear model for Cu data set (Table A4). Panel A shows QQ plot for normality. In panel B and C residuals of $\sqrt{R_0}$ (number of offspring per individual female) are plotted versus temperature and clone, respectively. Plot D *shows* the predicted $\sqrt{R_0}$ versus the observed $\sqrt{R_0}$.

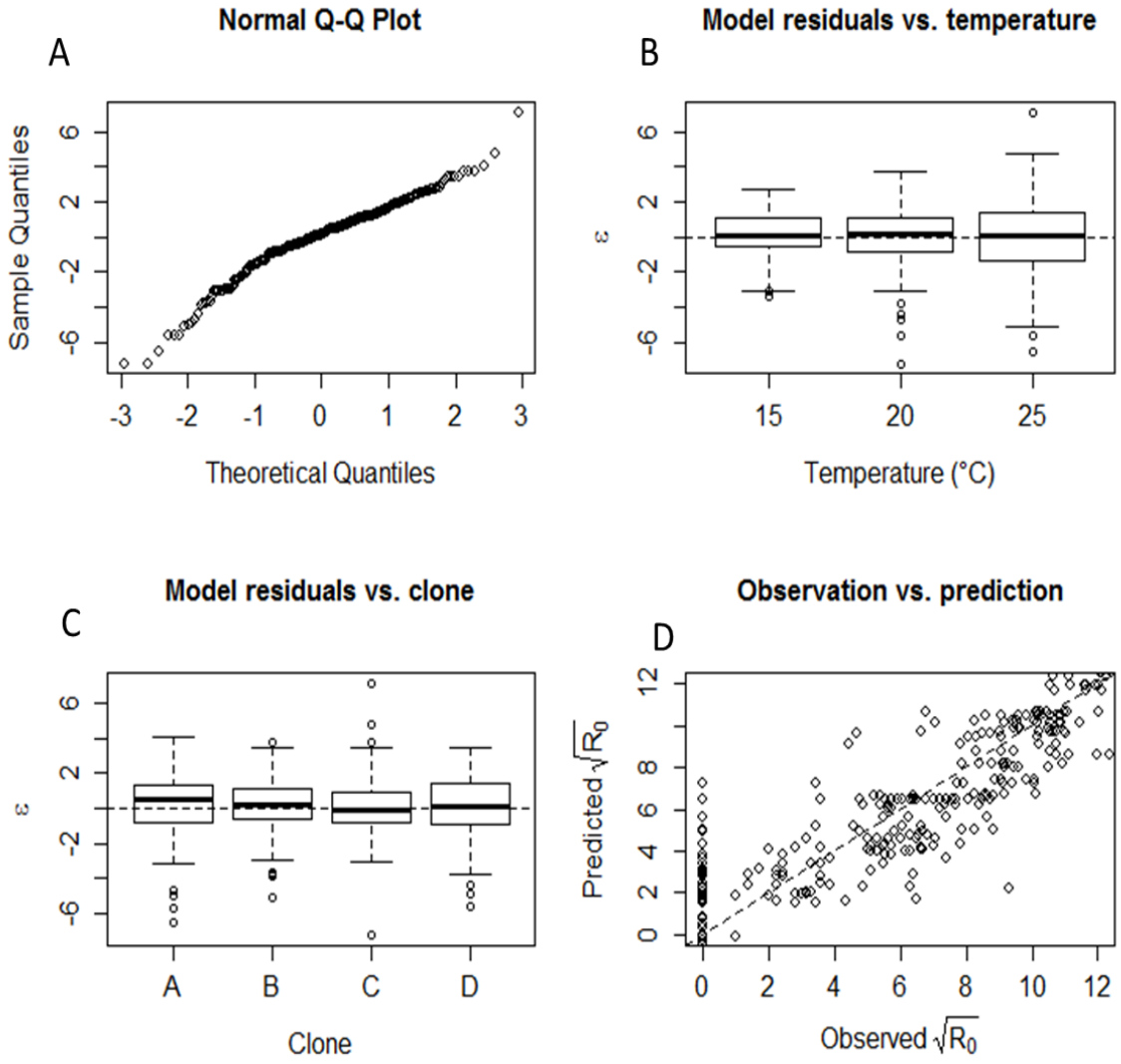

Figure A2. Model validation graphs for the optimal third order linear model for Zn data set (Table A5). Panel A shows QQ plot for normality. Panel B and C residuals of $\sqrt{R_0}$ (number of offspring per individual female) are plotted versus temperature and clone. Plot D shows the predicted $\sqrt{R_0}$ versus the observed $\sqrt{R_0}$.

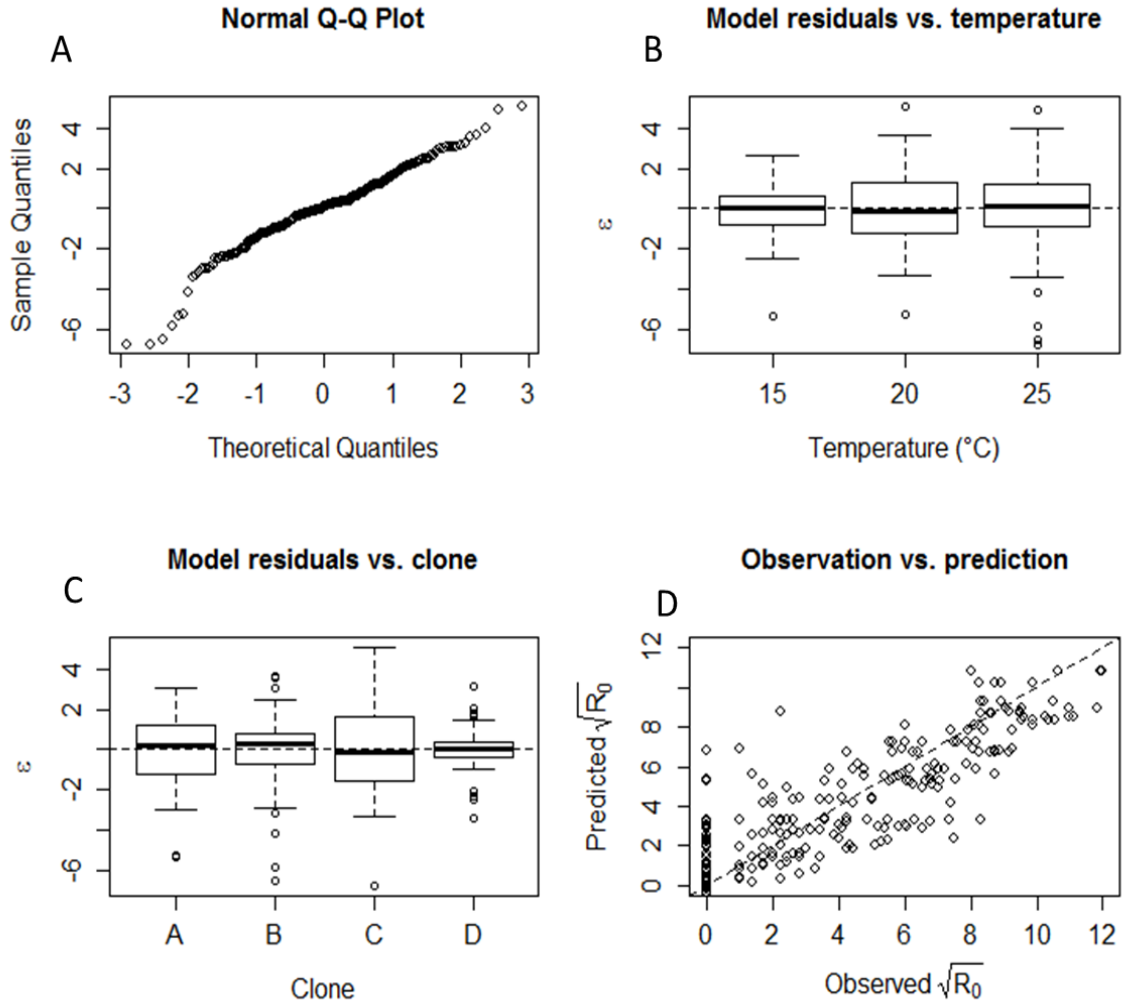

Figure A3. Model validation graphs for the optimal third order linear model for Ni data set (Table A6). Panel A shows QQ plot for normality. Panel B and C residuals $\sqrt{R_0}$ (number of offspring per individual female) are plotted versus temperature and clone. Plot D shows the predicted $\sqrt{R_0}$ versus the observed $\sqrt{R_0}$.

Appendix B

Supplementary material for Chapter 3

Table B1. The reference material, the detection limits and the quantification limits of all chemical analysis are given. The analytical instruments used are the High Resolution Inductively Coupled Plasma - Mass Spectrometry (HR-ICP-MS) (Thermo Scientific Element 2 XR) the iCAP 7000 Series Inductively Coupled Plasma Optical-Emission Spectrometry (ICP-OES) (Thermo Scientific) and the Total Organic Carbon L series CPH (TOC-L CPH) (Shimadzu, Duisburg, Germany).

Type of sample	Analytical instrument	Reference material	Substance	Low quantification limit
Water samples	HR-ICP-MS	SRM1640a	^{60}Ni	0.001 µg L^{-1}
			^{62}Ni	0.001 µg L^{-1}
	ICP-OES	TM-28.4 (lot 0815); TMDA-70.2 (lot 0815); CRANBERRY-05 (lot 0815) [Environment Canada]	Na	100.0 µg L^{-1}
			K	100.0 µg L^{-1}
			Ca	50.0 µg L^{-1}
			Mg	50.0 µg L^{-1}
	TOC-L CPH	CRANBERRY-05 (lot 0815) [Environment Canada]	Dissolved organic carbon	1.5 mg L^{-1}
Daphnia digested samples	HR-ICP-MS	SRM1640a	^{60}Ni	4 µg g^{-1}
			^{62}Ni	19 µg g^{-1}

Table B2. Summary table of total organic carbon (TOC) and dissolved organic carbon (DOC) concentrations and of the pH values registered on the experiment of Ni uptake and elimination by *Daphnia magna* at 15, 20 and 25°C. Mean and standard desviation (SD) is reported. n.d.: not determinate. The Ni treatments 56 and 73 µg Ni L^{-1} (dissolved concentration) are represented by 1[Ni] and 2[Ni], respectively. Nat.: natural isotope ratio.

Part I								
Test solution		T°C	TOC (mg L^{-1})		DOC (mg L^{-1})		pH	
			Mean	SD	Mean	SD	mean	SD
1[^{62}Ni]		15	5.74	0.51	3.38	0.54	7.56	0.18
		20	5.24	0.97	2.97	0.16	7.66	0.16
		25	5.76	0.44	3.34	0.34	7.54	0.07
2[^{62}Ni]		15	5.54	0.50	3.23	0.52	7.56	0.19
		20	5.22	0.56	3.44	1.22	7.65	0.16
		25	5.57	0.48	3.31	0.49	7.57	0.13
Control		15	5.34	0.22	3.49	0.82	7.47	0.16
		20	4.91	1.12	4.48	1.30	7.85	0.06
		25	5.89	0.69	3.46	0.16	7.53	0.09
Part II								
Test solution		T°C	TOC (mg L^{-1})		DOC (mg L^{-1})		pH	
0-48h	48-72h		Mean	SD	Mean	SD	mean	SD
1[^{62}Ni]	1[^{Nat}Ni]	15	5.92	0.35	4.18	1.35	7.62	0.17
		20	4.54	1.50	3.24	0.32	7.75	0.05
		25	5.01	1.07	4.34	0.80	7.43	0.14
2[^{62}Ni]	2[^{Nat}Ni]	15	4.99	1.44	3.10	0.12	7.58	0.14
		20	5.46	0.42	3.17	0.25	7.76	0.05
		25	5.66	0.23	3.67	0.53	7.46	0.12
1[^{62}Ni]	Control	15	4.76	n.d.	4.25	1.77	7.51	0.07
		20	5.53	0.06	3.34	n.d.	7.78	n.d.
		25	n.d.	n.d.	n.d.	n.d.	7.71	0.11
2[^{62}Ni]	Control	15	4.48	1.53	3.22	0.26	7.51	0.06
		20	4.96	n.d.	2.95	n.d.	7.78	n.d.
		25	n.d.	n.d.	n.d.	n.d.	7.72	0.12

Table B3. Nickel water concentrations (µg L^{-1}) registered on the Ni uptake and elimination experiment with four *Daphnia magna* clones at 15, 20 and 25°C. The Ni treatments 56 and 73 µg Ni L^{-1} (total nominal concentrations: 70 and 100 µg Ni L^{-1}) are represented by 1[Ni] and 2[Ni], respectively. The concentrations of the isotopes ^{60}Ni and ^{62}Ni are reported. In part I of the experiment, the ^{total}Ni concentrations were calculated based on the enriched isotope composition (98.02% of ^{62}Ni). In part II, the ^{total}Ni concentrations were calculated based on the natural isotope ratio (26.22% of ^{60}Ni). All values are given as averages (Av) in the new medium and old medium. Standard deviation (SD) is reported. d.l.: detection limit. Nat.: natural isotope ratio.

Part I											
Test solution		Total concentrations					Dissolved concentrations				
		^{60}Ni		^{62}Ni		^{total}Ni	^{60}Ni		^{62}Ni		^{total}Ni
		Av	SD	Av	SD	(^{62}Ni /0. 9802)	Av	SD	Av	SD	(^{62}Ni /0. 9802)
Control		0.1	0.1	<d.l.	<d.l.	0.0	0.1	0.1	<d.l.	<d.l.	0.0
1[^{62}Ni]		0.7	0.1	69.4	5.6	70.8	0.6	0.1	55.7	3.3	56.8
2[^{62}Ni]		1.0	0.2	98.2	8.7	100.2	0.7	0.2	72.5	13.1	73.7
Part II											
Test solution		Total					dissolved				
		^{60}Ni		^{62}Ni		^{total}Ni	^{60}Ni		^{62}Ni		^{total}Ni
0-48h	48-72h	Av	SD	Av	SD	(^{60}Ni/0.2622)	Av	SD	Av	SD	(^{60}Ni/0.2622)
1[^{62}Ni]]	1[^{Nat}Ni]	15.7	3.9	3.6	1.2	59.9	12.6	2.8	3.0	1.1	48.1
2[^{62}Ni]]	2[^{Nat}Ni]	24.8	4.1	6.1	1.5	94.7	18.2	5.0	4.1	1.4	69.5
1[^{62}Ni]	Control	0.1	0.0	1.7	1.0	0.2	0.1	0.1	1.5	0.3	0.3
2[^{62}Ni]	Control	0.1	0.0	2.5	0.7	0.3	0.1	0.1	2.6	1.4	0.3

Table B4. Uptake rate constants (k_u) (L g h^{-1}) estimated with all time points from 0-72h (ku,I) (Part I of the experiment) and also at the first day of the exposure (0-24h) ($k_{u,init}$), after 2d of exposure (48-72h) ($k_{u,48h}$) (Part II of the experiment) for the four *Daphnia magna* clones at 15, 20 and 25°C. Standard error (SE), lower (L) and upper (U) confidence limits (CL) and significance (*p*) are indicated. If negative values were obtained, 0 is indicated in the table. n.d.: not determined due to no sufficient biomass for analysis.

T(°C)	Clone	$k_{u,init}$	SE	*p*	L CL	U CL	$k_{u,48h}$	SE	*p*	L CL	U CL	$k_{u,I}$	SE	*p*	L CL	U CL
15	A	1.44	0.16	<0.001	1.10	1.79	0.99	0.37	>0.05	0.00	2.17	1.76	0.21	<0.001	1.29	2.23
	B	0.46	0.05	<0.001	0.36	0.56	0.41	0.04	<0.001	0.31	0.51	0.55	0.04	<0.001	0.47	0.62
	C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	D	0.22	0.03	<0.001	0.16	0.28	1.17	0.28	<0.01	0.58	1.75	0.52	0.07	<0.001	0.37	0.68
20	A	1.53	0.12	<0.001	1.28	1.77	2.07	0.19	<0.001	1.66	2.47	1.80	0.13	<0.001	1.54	2.06
	B	1.09	0.07	<0.001	0.95	1.24	1.12	0.07	<0.001	0.97	1.28	1.03	0.08	<0.001	0.87	1.19
	C	0.95	0.04	<0.001	0.86	1.04	1.19	0.11	<0.001	0.96	1.41	1.14	0.10	<0.001	0.93	1.35
	D	0.39	0.09	<0.01	0.20	0.58	0.87	0.05	<0.001	0.73	1.01	0.94	0.15	<0.001	0.61	1.28
25	A	0.53	0.03	<0.001	0.46	0.59	0.68	0.07	<0.001	0.54	0.82	0.56	0.03	<0.001	0.50	0.62
	B	0.17	0.02	<0.001	0.13	0.21	0.40	0.06	<0.001	0.28	0.53	0.24	0.02	<0.001	0.20	0.28
	C	0.34	0.04	<0.001	0.26	0.41	0.74	0.20	<0.01	0.31	1.17	0.40	0.03	<0.001	0.33	0.47
	D	0.75	0.10	<0.001	0.54	0.95	0.83	0.06	<0.001	0.70	0.96	0.72	0.06	<0.001	0.59	0.85

Table B5. Elimination rate constants (k_e) (h^{-1}) of Ni estimated for the four *Daphnia magna* clones at 15, 20 and 25°C in the presence and absence of Ni. After 48h of exposure to ^{62}Ni, *D. magna* were transferred to a medium spiked with Ni with a natural isotope ratio (presence) or to a control medium (absence). Standard error (SE), lower (L) and upper (U) confidence limits (CL) and significance (*p*) are indicated. n.d.: not determined due to no sufficient biomass for analysis.

T (°C)	Clone	$k_{e,presence}$	SE	*p*	L CL	U CL	$k_{e,absence}$	SE	*p*	L CL	U CL
15	A	0.50	0.09	<0.05	0.13	0.87	0.38	0.04	<0.001	0.29	0.47
	B	0.31	0.01	<0.001	0.28	0.34	0.35	0.05	<0.001	0.24	0.45
	C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	D	0.33	0.15	<0.05	0.01	0.65	0.22	0.02	<0.001	0.18	0.26
20	A	0.37	0.05	<0.001	0.26	0.48	0.34	0.03	<0.001	0.27	0.41
	B	0.34	0.06	<0.001	0.22	0.47	0.34	0.06	<0.001	0.22	0.46
	C	0.34	0.06	<0.001	0.21	0.47	0.29	0.04	<0.001	0.21	0.37
	D	0.26	0.03	<0.01	0.17	0.34	n.d.	n.d.	n.d.	n.d.	n.d.
25	A	0.21	0.03	<0.001	0.15	0.27	0.21	0.02	<0.001	0.18	0.25
	B	0.24	0.04	<0.001	0.16	0.32	0.11	0.01	<0.001	0.09	0.13
	C	0.30	0.06	<0.001	0.18	0.42	0.17	0.02	<0.001	0.13	0.21
	D	0.26	0.04	<0.001	0.19	0.34	0.26	0.03	<0.001	0.20	0.31

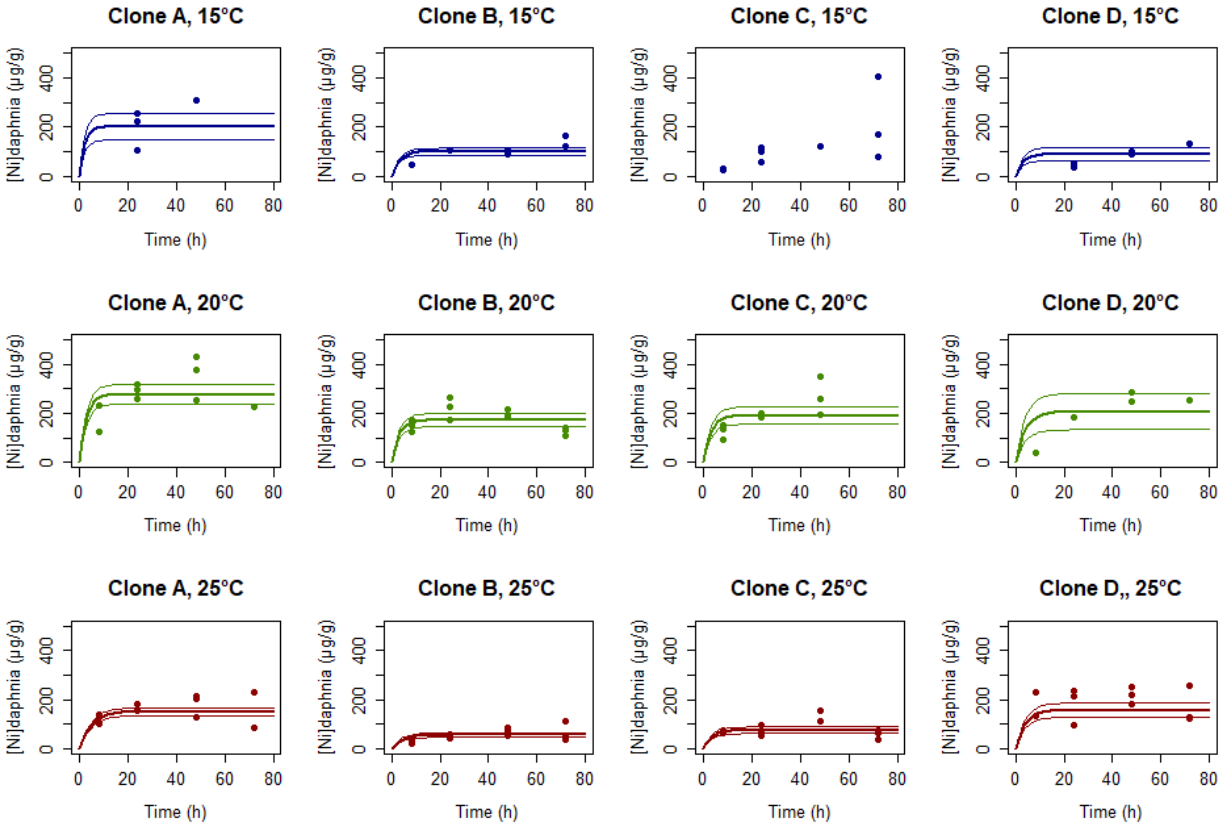

Figure B1. Internal Ni concentrations ($[Ni]_{daphnia}$) (µg g^{-1}) in four *Daphnia magna* clones at 15, 20 and 25°C exposed to 56 µg $^{62}Ni\ L^{-1}$ during 72h. The uptake rate constants were estimated based on the two Ni treatments, i.e. 56 and 73 µg $^{62}Ni\ L^{-1}$. Lines represent the estimated uptake curves and respective confidence limits. Dots represent the observed values. The non-significant estimated uptake curves were not plotted. All samples with a total body mass below than the quantification limit (i.e. 104 µg) were excluded from the analysis and from the plots.

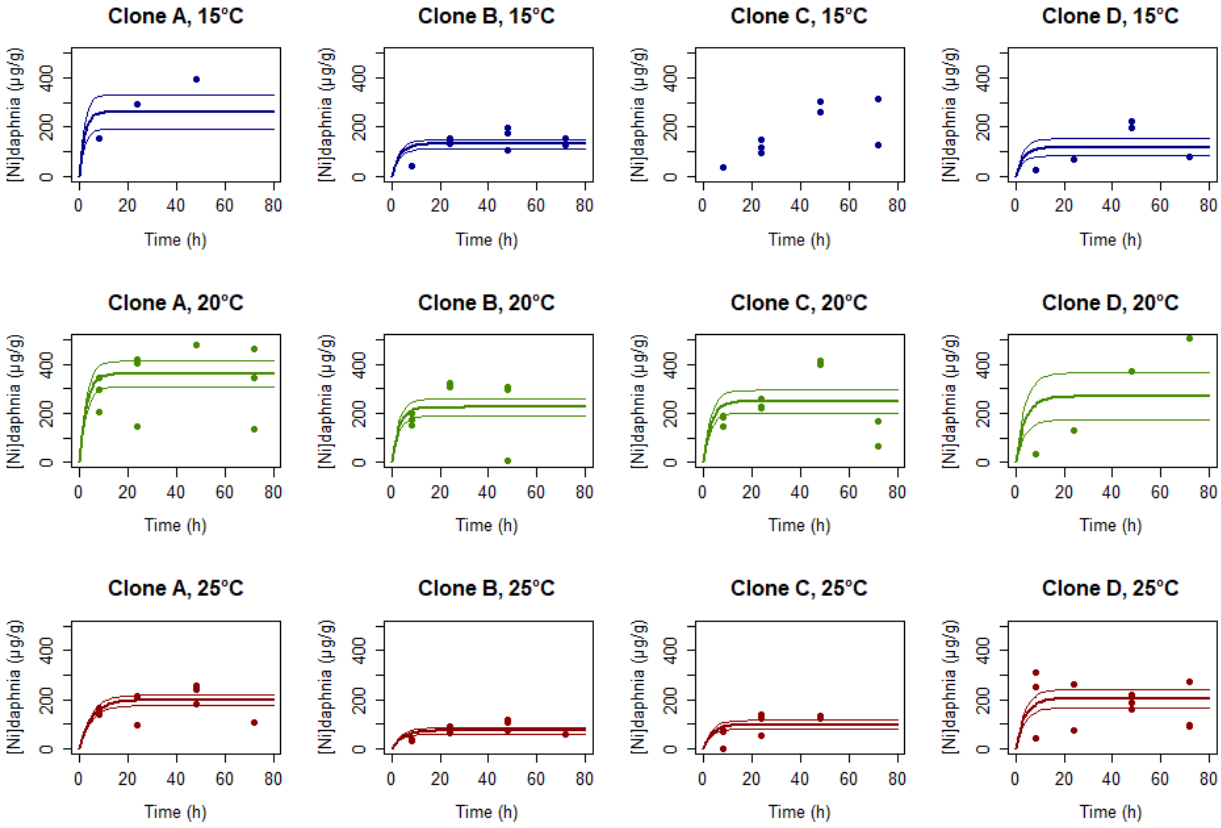

Figure B2. Internal Ni concentrations ($[Ni]_{daphnia}$) (µg g^{-1}) in four *Daphnia magna* clones at 15, 20 and 25°C exposed to 73 µg $^{62}Ni\ L^{-1}$ during 72h. The uptake rate constants were estimated based on the two Ni treatments, i.e. 56 and 73 µg $^{62}Ni\ L^{-1}$. Lines represent the estimated uptake curves and respective confidence limits. Dots represent the observed values. The non-significant estimated uptake curves were not plotted. All samples with a total body mass below than the quantification limit (i.e. 104 µg) were excluded from the analysis and from the plots.

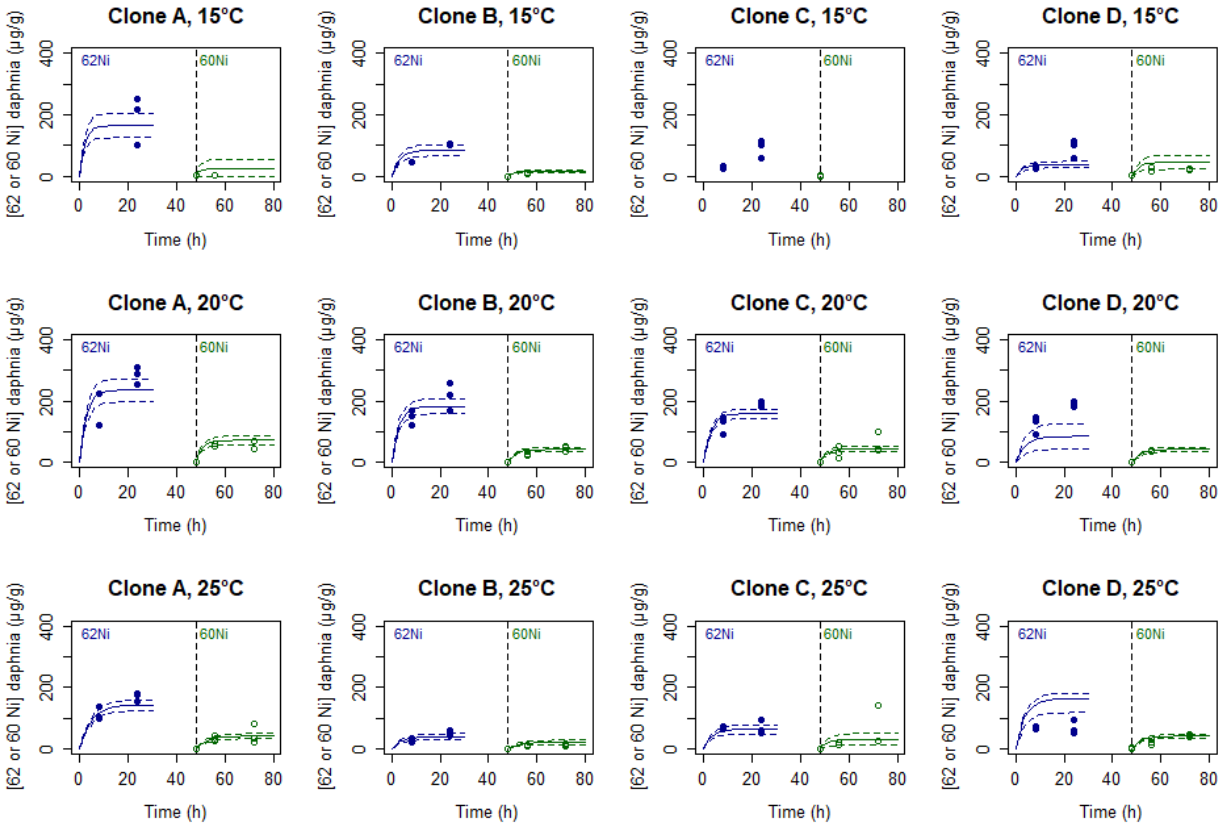

Figure B3. The uptake curves estimated at the first day of Ni exposure (0-24h, initial) (^{62}Ni) and after 48h (48-72h) (^{Nat}Ni) (Nat.: natural isotope ratio) in four *Daphnia magna* clones at 15, 20 and 25°C. The parameters to estimate the elimination curves were based on both Ni treatment, i.e., 56 and 73 µg L^{-1}. The water dissolved Ni concentrations were 56 µg ^{62}Ni L^{-1} and 13 µg ^{60}Ni L^{-1} in part I of the experiment (0-24h) and in part II (48-72h), respectively. The estimated uptake curves and the confidence limits are represented by lines. The observed values are represented by dots. The non-significant estimated uptake curves were not plotted. All samples with a total body mass below than the quantification limit (i.e. 104 µg) were excluded from the analysis and from the plots.

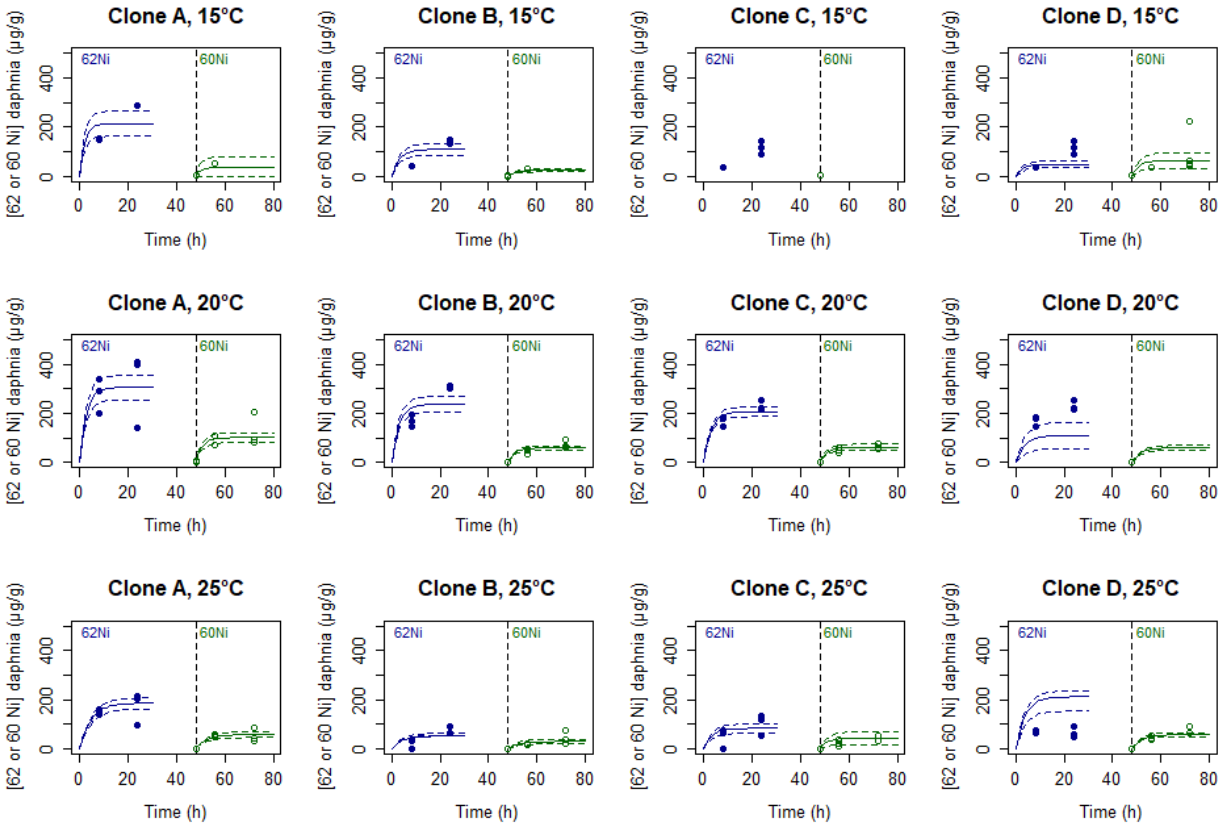

Figure B4. The uptake curves estimated at the first day of Ni exposure (0-24h, initial) (^{62}Ni) and after 48h (48-72h) (^{Nat}Ni) (Nat.: natural isotope ratio) in four *Daphnia magna* clones at 15, 20 and 25°C. The parameters to estimate the elimination curves were based on both Ni treatment, i.e., 56 and 73 µg L^{-1}. The water dissolved Ni concentrations were 73 µg ^{62}Ni L^{-1} and 18 µg ^{60}Ni L^{-1} in part I of the experiment (0-24h) and in part II (48-72h), respectively. The estimated uptake curves and the confidence limits are represented by lines. The observed values are represented by dots. The non-significant estimated uptake curves were not plotted. All samples with a total body mass below than the quantification limit (i.e. 104 µg) were excluded from the analysis and from the plots.

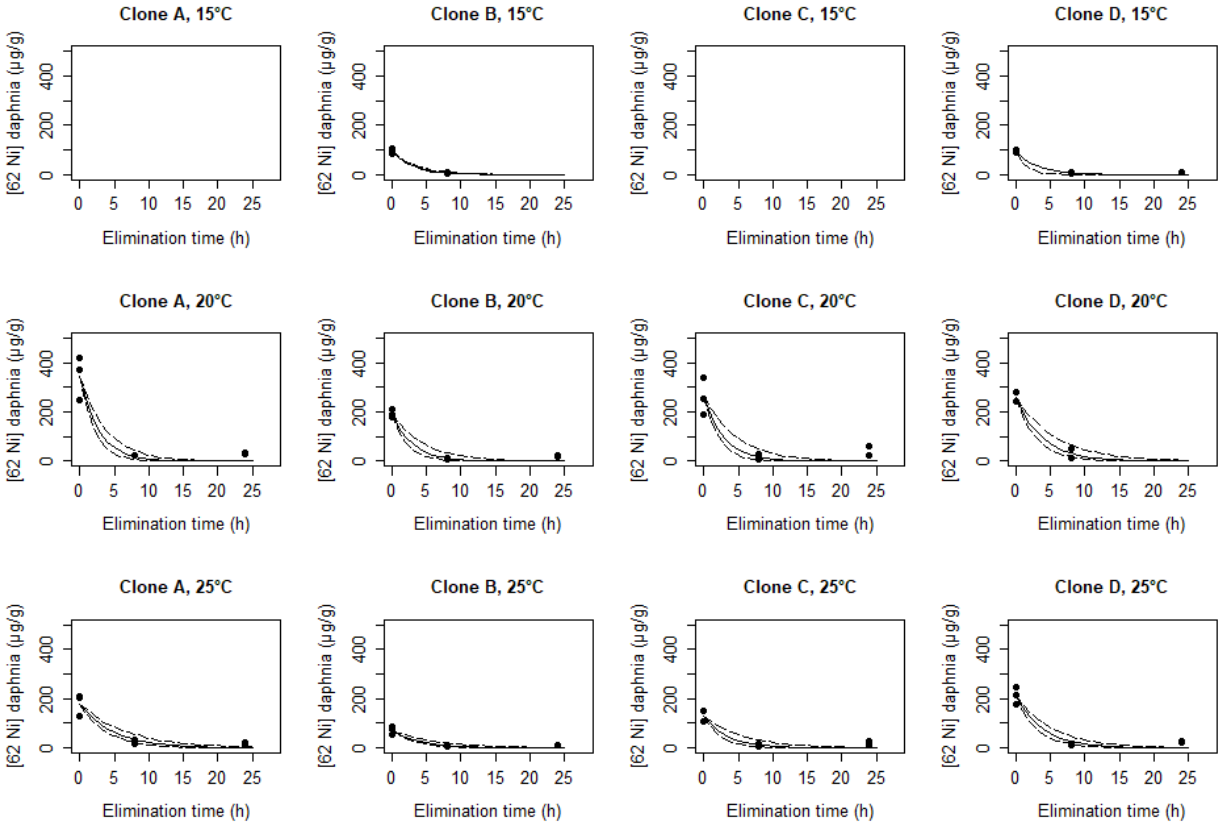

Figure B5. After 48h of exposure to ^{62}Ni, the elimination of Ni in four *Daphnia magna* clones at 15, 20 and 25°C was explored in the presence of Ni with a natural isotope ratio. The fitted elimination curves of Ni in *D. magna* and the respective confidence limits are represented by lines. The parameters to estimate the elimination curves were based on both Ni treatment, i.e., 56 and 73 µg L^{-1}. The observed values for the treatment 56 µg Ni L^{-1} are plotted and represented by dots. The non-significant estimated elimination curves were not plotted. All samples with a total body mass below than the quantification limit (i.e. 104 µg) were excluded from the analysis and from the plots.

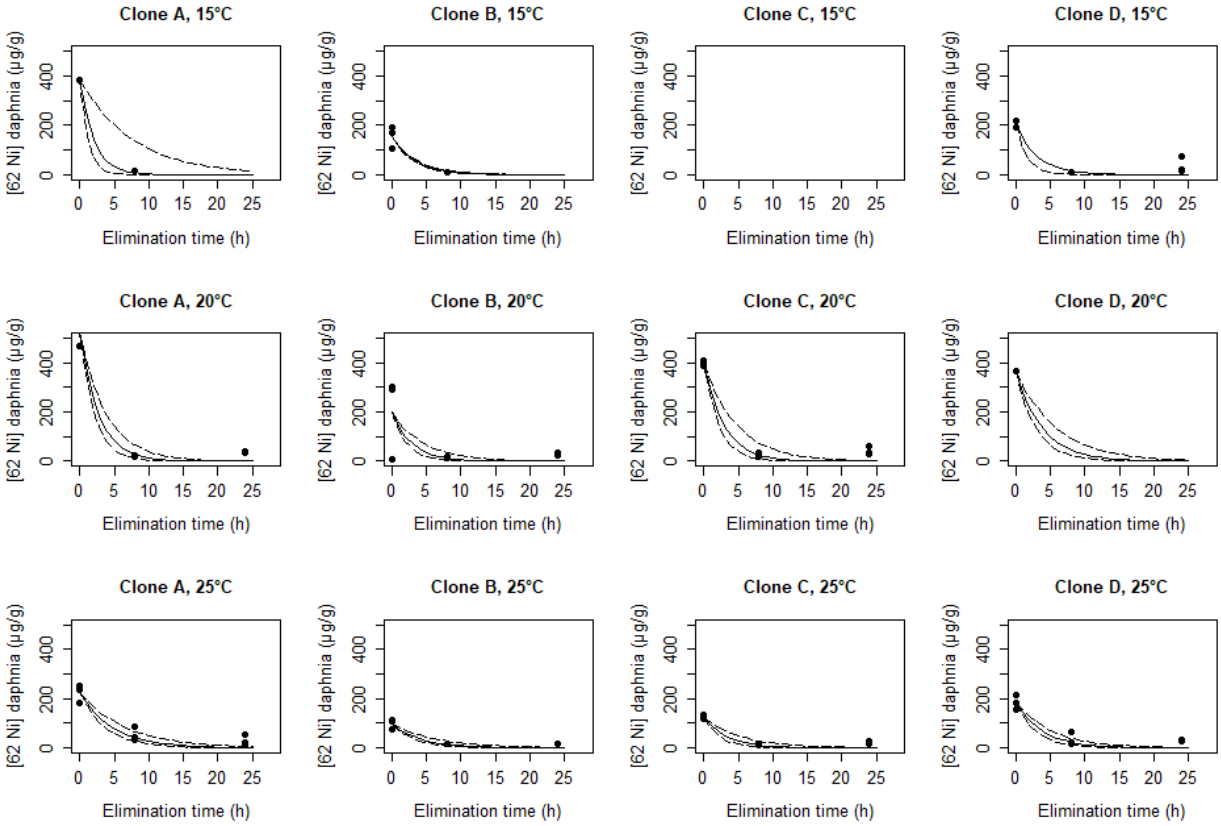

Figure B6. After 48h of exposure to ^{62}Ni, the elimination of Ni in four *Daphnia magna* clones at 15°C, 20°C and 25°C was explored in the presence of Ni with a natural isotope ratio. The fitted elimination curves of Ni in *D. magna* and the respective confidence limits are represented by lines. The parameters to estimate the elimination curves were based on both Ni treatment, i.e., 56 and 73 µg L^{-1}. The observed values for the treatment 73 µg Ni L^{-1} are plotted and represented by dots. All samples with a total body mass below than the quantification limit (i.e. 104 µg) were excluded from the analysis and from the plots.

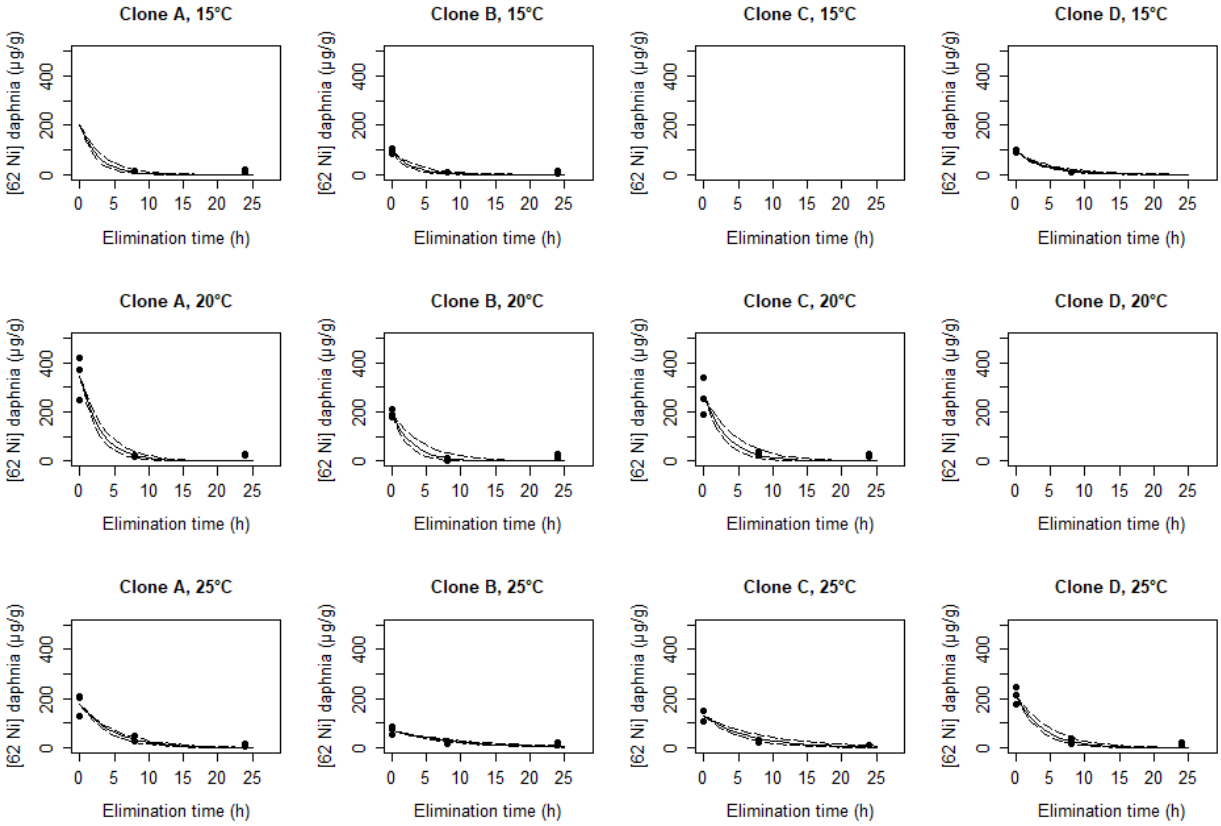

Figure B7. After 48h of exposure to ^{62}Ni, the elimination of Ni in four *Daphnia magna* clones at 15°C, 20°C and 25°C was explored in the absence of Ni (control medium). The fitted elimination curves of Ni in *D. magna* and the respective confidence limits are represented by lines. The parameters to estimate the elimination curves were based on both Ni treatment, i.e., 56 and 73 µg L^{-1}. The observed values for the treatment 56 µg Ni L^{-1} are plotted and represented by dots. All samples with a total body mass below than the quantification limit (i.e. 104 µg) were excluded from the analysis and from the plots.

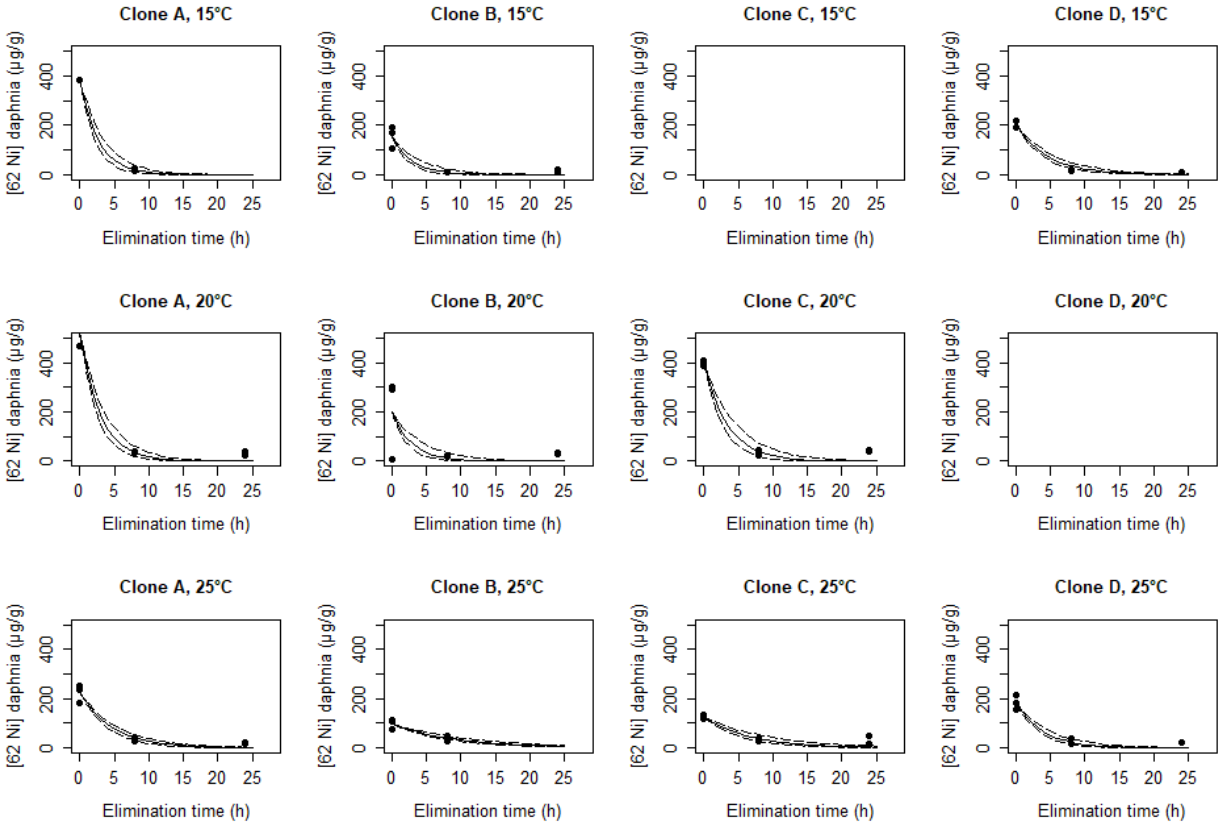

Figure B8. After 48h of exposure to ^{62}Ni, the elimination of Ni in four *Daphnia magna* clones at 15°C, 20°C and 25°C was explored in the absence of Ni (control medium). The fitted elimination curves of Ni in *D. magna* and the respective confidence limits are represented by lines. The parameters to estimate the elimination curves were based on both Ni treatment, i.e., 56 and 73 µg L^{-1}. The observed values for the treatment 73 µg Ni L^{-1} are plotted and represented by dots. All samples with a total body mass below than the quantification limit (i.e. 104 µg) were excluded from the analysis and from the plots.

Appendix C

Supplementary material for Chapter 4

Table C1. Recoveries (%) (mean) and the coefficient of variation (%) of Ni, Fe, Cu, Zn, Ca, K, Na and Mg measured in the reference tissue (mussel tissue 2977, NIST) used as quality control in the metal body digestions of *Daphnia magna* are shown. The procedure blanks (mean) and standard deviation (SD) are shown.

	Ni	Fe	Cu	Zn	Ca	K	Na	Mg
Recovery (%)	86.21	66.21	137.80	97.03	100.39	97.68	98.11	8.34
CV (%)	25.42	14.54	23.91	9.42	9.56	9.96	10.24	11.3
procedure blanks	0.24 µg L^{-1}	2.68 µg L^{-1}	1.43 µg L^{-1}	3.15 µg L^{-1}	0.06 mg L^{-1}	0.02 mg L^{-1}	0.01 mg L^{-1}	2.47 µg L^{-1}
procedure blanks (SD)	0.68	2.83	0.99	2.56	0.05	0.04	0.02	3.88

Table C2. The reference material, the detection limits and the quantification limits of all chemical analysis are given. The analytical instruments used are the iCAP 7000 Series Inductively Coupled Plasma Optical-Emission Spectrometry (ICP-OES) (Thermo Scientific), the Total Organic Carbon L series CPH (TOC-L CPH) (Shimadzu, Duisburg, Germany) and the Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS Furnace Autosampler, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Type of sample	Analytical instrument	Reference material	Substance	Detection limit	Low quantification limit
Water samples	ICP-OES	TM-28.4 (lot 0815); TMDA-70.2 (lot 0815); CRANBERRY-05 (lot 0815) [Environment Canada]	Ni	1.2 µg L^{-1}	4.0 µg L^{-1}
			Cu	2.0 µg L^{-1}	4.0 µg L^{-1}
			Zn	0.5 µg L^{-1}	2.0 µg L^{-1}
			Fe	17.0 µg L^{-1}	56.0 µg L^{-1}
			Na	30.0 µg L^{-1}	100.0 µg L^{-1}
			K	30.0 µg L^{-1}	100.0 µg L^{-1}
			Ca	15.0 µg L^{-1}	50.0 µg L^{-1}
			Mg	15.0 µg L^{-1}	50.0µg L^{-1}
	TOC-L CPH	CRANBERRY-05 (lot 0815) [Environment Canada]	DOC	0.5 mg L^{-1}	1.5 mg L^{-1}
Daphnia digested samples	ICP-OES	TM-28.4 (lot 0815); TMDA-70.2 (lot 0815); CRANBERRY-05 (lot 0815) [Environment Canada]	Cu	2.1 µg g^{-1}	7.1 µg g^{-1}
			Zn	9.0 µg g^{-1}	31.0 µg g^{-1}
			Fe	27.0 µg g^{-1}	94.0 µg g^{-1}
			Na	1.6 mg g^{-1}	5.5 mg g^{-1}
			K	0.5 mg g^{-1}	1.7 mg g^{-1}
			Ca	6.0 mg g^{-1}	19.0 mg g^{-1}
			Mg	120.0 µg g^{-1}	400.0 µg g^{-1}
	GFAAS Furnace Autosampler	TM-28.4, lot 0815 [Environment Canada]	Ni	0.2 µg g^{-1}	0.7 µg g^{-1}

Table C3. The effective concentrations (ECx) (+/- standard error) of Ni (µg L^{-1}) at 15, 20 (the standard temperature) and 25°C calculated with the total reproduction (% of control) expressed as number of offspring per individual *Daphnia magna* female produced until the organisms in control treatment released the 5th brood (*Rep5*) or the 3rd brood (*Rep3*). The best fitted dose response model used to estimate the ECx values is shown. n.d.: not determined.

T (°C)	*Rep5* (% of control)			*Rep3* (% of control)			Model
	EC10	EC20	EC50	EC10	EC20	EC50	
15	n.d.	n.d.	17.3 ± 2.2	n.d.	n.d.	12.9 ± 1.8	3-parameter Weibull
20	18.8 ± 5.7	32.6 ± 7.2	75.1 ± 8.9	24.4 ± 7.8	40.0 ± 9.1	82.1 ± 9.5	3-parameter Weibull
25	93.3 ± 12.4	105.2 ± 9.7	129.1 ± 9.4	98.5 ± 13.6	113.1 ± 12.1	143.2 ± 12.6	3-parameter log-logistic

Table C4. Temperature, dissolved oxygen concentration (O_2), dissolved organic carbon (DOC) concentration registered during the multigenerational Nitoxicity test to *Daphnia magna* along four generations (F0, F1, F2, F3) at 15, 20 and 25°C. Mean and standard deviation across all Ni treatments are reported. *a* is the 1st experiment at 25°C and *b* is the 2nd experiment performed at 25°C.

T (°C)	Generation	T (average) (°C)	O_2 (mg L^{-1})	DOC (mg L^{-1})
15	F0	14.88±0.76	9.72±0.73	3.74±0.82
	F1	14.75±0.72		
	F2	14.88±0.77		
	F3	14.83±1.04		
20	F0	19.78±0.46	8.87±0.70	3.40±0.48
	F1	19.70±0.56		
	F2	19.77±0.44		
	F3	19.73±0.56		
25*a*	F0	24.87±0.65	8.42±0.46	3.38±0.45
	F1	25.01±0.54		
	F2	25.05±0.51		
	F3	24.73±0.51		
25*b*	F0	24.87±0.65	8.25±0.48	3.5±0.52
	F1	25.01±0.54		
	F2	25.05±0.51		
	F3	24.73±0.51		

Table C5. pH values registered in the multigenerational Ni toxicity test to *Daphnia magna* along four generations (F0, F1, F2, F3) at 15, 20 and 25°C. All values are given as averages of the new medium and old medium. Nickel expressed as nominal (Nom Ni) concentration (µg L^{-1}). Mean and standard desviation (SD) are reported. *a* is the 1st experiment at 25°C and *b* is the 2nd experiment performed at 25°C. n.a.: not applicable because some Ni treatments did not continue to the following generations due to the lack of reproduction.

		F0		F1		F2		F3	
T (°C)	Nom Ni	Mean	SD	Mean	SD	Mean	SD	Mean	SD
15	0	8.06	0.31	8.05	0.26	8.02	0.28	8.27	0.49
	12	8.10	0.34	8.07	0.30	7.99	0.29	7.98	0.28
	25	8.12	0.36	8.07	0.30	8.01	0.26	8.00	0.27
	50	8.11	0.35	8.10	0.33	8.07	0.31	8.32	0.50
	100	8.13	0.35	8.08	0.31	8.03	0.31	n.a.	n.a.
	200	8.14	0.35	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	300	8.16	0.35	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
20	0	8.07	0.25	7.90	0.16	8.08	0.19	8.12	0.35
	12	8.14	0.30	7.93	0.25	8.12	0.24	8.06	0.26
	25	8.13	0.31	7.93	0.25	8.07	0.18	8.02	0.19
	50	8.15	0.34	8.04	0.39	n.a.	n.a.	n.a.	n.a.
	100	8.15	0.32	7.96	0.16	n.a.	n.a.	n.a.	n.a.
	200	8.16	0.35	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	300	8.14	0.36	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
25*a*	0	8.01	0.25	7.88	0.03	7.87	0.07	7.97	0.06
	12	8.04	0.23	7.89	0.04	7.90	0.11	7.94	0.07
	25	8.04	0.25	7.86	0.07	7.91	0.09	7.94	0.08
	50	8.05	0.27	7.87	0.07	7.91	0.09	8.03	0.04
	100	8.03	0.24	7.88	0.08	7.87	0.08	7.98	0.04
	200	8.08	0.25	7.90	0.03	7.95	0.07	n.a.	n.a.
	300	8.09	0.27	7.89	0.00	n.a.	n.a.	n.a.	n.a.
25*b*	0	7.91	0.08	7.87	0.08	7.84	0.01	7.88	0.12
	12	7.96	0.09	7.95	0.06	7.90	0.02	7.93	0.10
	25	7.95	0.08	7.95	0.05	7.86	0.06	7.93	0.11
	50	7.93	0.12	7.97	0.06	7.90	0.03	7.96	0.13
	100	7.95	0.08	8.02	0.07	7.92	0.01	7.97	0.11
	200	8.10	0.14	7.90	0.15	7.95	0.02	8.02	0.23
	300	8.04	0.05	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table C6. Water Na, Mg, K, Ca, Fe, Cu and Zn concentration registered on the multigenerational Ni toxicity test to *Daphnia magna* along four generations (G) (F0, F1, F2, F3) at 15, 20 and 25°C. T is temperature (°C), *a* is the 1st experiment at 25°C and *b* is the 2nd experiment performed at 25°C. Mean and standard deviation are reported.

T	G	Na (mg L^{-1})	Mg (mg L^{-1})	K (mg L^{-1})	Ca (mg L^{-1})	Fe (µg L^{-1})	Cu (µg L^{-1})	Zn (µg L^{-1})
15	F0	20.1±0.8	8.5±0.4	3.1±0.2	57.6±3.4	273.8±38.8	7.6±1.3	12.6±4.7
	F1	21.1±0.8	9.2±0.4	3.3±0.1	62.6±2.3	282.1±76.2	10.5±3.4	16.3±7.5
	F2	21.5±1.0	9.6±0.4	3.4±0.2	62.2±4.1	255.6±16.2	13.1±1.7	20.2±5.1
	F3	23.3±2.8	10.2±1.1	3.9±0.5	64.0±6.3	226.6±39.9	10.3±1.8	15.3±4.3
20	F0	20.4±0.8	8.5±0.4	3.1±0.2	57.2±3.5	268.9±46.4	7.7±1.7	18.8±14.0
	F1	19.3±0.4	8.6±0.5	3.0 ±0.3	56.5±4.7	281.3±19.1	7.6±0.9	14.1±16.8
	F2	19.8±0.2	8.3±0.3	3.3±0.1	60.8±2.1	301.8±10.9	7.4±1.0	10.3±2.4
	F3	22.1±1.3	9.8±0.4	3.5±0.2	65.7±3.6	254.5±66.6	11.9±3.9	17.7±8.1
25*a*	F0	20.6±0.3	8.3±0.1	3.1±0.1	57.0±0.8	280.3±8.6	7.6±0.8	18.0±5.8
	F1	19.1±0.2	8.7±0.7	3.0±0.2	55.2±4.8	274.9±8.2	7.9±1.1	18.1±20.5
	F2	19.2±0.1	8.4±0.1	2.8±0.0	54.0±0.5	277.9±6.1	8.0±1.2	10.5±3.0
	F3	20.9±1.2	9.0±0.7	3.3±0.1	62.7±3.0	280.7±50.3	8.0±1.3	12.6±3.9
25*b*	F0	20.2±0.8	8.4±0.4	3.3±0.1	61.7±2.3	294.7±13.4	7.5±0.8	13.1±7.1
	F1	22.5±1.1	9.9±0.5	3.5±0.2	66.6±3.3	262.6±55.7	11.0±3.6	17.6±5.4
	F2	22.8±0.6	10.0±0.2	3.5±0.1	67.6±1.5	270.6±10.7	13.8±1.3	20.0±2.1
	F3	21.7±1.4	9.6±0.6	3.4±0.2	63.7±5.6	260.6±25.2	13.8±1.1	20.2±2.3

Table C7. Water Ni concentrations (µg L^{-1}) registered on the multigenerational toxicity test along four generations (F0, F1, F2, F3) at 15, 20 and 25°C. All values are given as averages in the new medium and old medium after renewal. Mean ± standard desviation (SD) is reported. *a* is the 1st experiment at 25°C and *b* is the 2nd experiment performed at 25°C. d.l.: detection limit. n.a.: not applicable because some Ni treatments did not continue to the following generations due to the lack of reproduction.

		F0		F1		F2		F3	
T(°C)	Ni nominal	Ni total	Ni dissolved	Ni total	Ni dissolved	Ni total	Ni dissolved	Ni total	Ni dissolved
15	0	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	12	10.0±1.4	4.2±1.6	17.6±5.0	9.0±3.1	22.2±0.9	12.1±1.3	19.9±4.1	12.9±2.1
	25	21.8±2.0	10.9±2.5	33. 6±9.3	18.9±6.3	42.8±1.1	25.5±2.4	42.9±8.4	30.1±8.8
	50	49.2±1.6	26.0±3.0	70.9±20.6	42.2±13.2	88.8±2.8	55.6±3.6	87.0±1.7	54.4±11.5
	100	99.7±10.3	55.4±5.5	98.4±3.5	62.6±6.5	128.5±32.5	85.6±26.1	n.a.	n.a.
	200	199.9±19.5	126.4±17.6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	300	297.4±16.1	193.8±22.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
20	0	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	12	9.1±0.6	3.3±1.1	9.6±1.9	3.6±1.8	12.3±0.0	8.7±5.3	19.7±4.8	10.0±3.2
	25	20.4±1.2	8.9±1.7	20.4±3.5	10.4±2.6	24.6±0.8	13.0±1.1	37.8±9.7	19.7±7.2
	50	50.0±1.6	23.2±3.6	47.9±2.6	24.7±4.3	n.a.	n.a.	n.a.	n.a.
	100	106.5±9.4	54.2±6.9	90.4±1.1	49.5±7.6	n.a.	n.a.	n.a.	n.a.
	200	204.7±19.3	125.8±21.7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	300	300.4±12.5	193.4±28.9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

*Table continues on page 198.

Continuation of Table C7. Nickel concentrations (µg L^{-1}) registered on the multigenerational toxicity test along four generations (F0, F1, F2, F3) at 15, 20 and 25°C. All values are given as averages in the new medium and old medium after renewal. Mean ± standard desviation (SD) is reported. *a* is the 1st experiment at 25°C and *b* is the 2nd experiment performed at 25°C. d.l.: detection limit. n.a.: not applicable because some Ni treatments did not continue to the following generations due to the lack of reproduction.

		F0		F1		F2		F3	
T(°C)	Ni nominal	Ni total	Ni dissolved	Ni total	Ni dissolved	Ni total	Ni dissolved	Ni total	Ni dissolved
25*a*	0	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	12	8.8±0.4	3.6±0.6	8.6±0.1	3.0±1.8	11.1±1.8	4.0±1.8	12.5±0.4	6.5±0.7
	25	20.8±0.3	9.2±0.9	18.5±0.3	9.0±2.6	22.6±3.2	9.9±2.1	24.4±0.6	14.6±1.4
	50	51.0±0.3	25.8±3.7	45.9±1.0	25.4±8.6	48.4±2.5	24.2±2.3	50.4±0.7	28.5±2.5
	100	111.0±0.5	57.7±6.6	89.5±0.8	49.9±6.5	91.7±1.2	51.5±4.0	96.1±3.5	57.5±6.4
	200	223.1±1.0	148.7±36.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	300	310.6±0.7	183.6±19.5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
25*b*	0	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.	< d.l.
	12	12.3±0.4	5.8±1.1	18.4±5.8	9.7±4.4	23.2±1.0	13.0±1.7	22.8±1.2	11.6±1.7
	25	24.8±0.8	16.7±7.5	34.6±10.6	20.9±8.7	44.3±1.0	28.0±3.0	43.5±1.3	26.3±1.5
	50	50.2±0.5	26.6±2.8	73.1±21.8	43.2±18.8	93.3±1.5	60.9±5.9	89.8±3.3	56.6±5.0
	100	92.4±0.7	56.0±7.1	103.2±2.9	66.5±15.9	104.4±1.7	72.7±8.5	127.8±28.5	80.6±21.4
	200	207.0±10.0	120.3±10.8	203.8±22.4	122.6±22.8	181.6±0.2	121.5±13.2	163.8±13.4	118.6±7.5
	300	292.3±2.0	186.4±12.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table C8. Control performance of *Daphnia magna* on the multigenerational Ni toxicity test along four generations (G)(F0, F1, F2, F3) at 15, 20 and 25°C. Reproduction (*Rep)* is expressed as the number of offspring per individual female (mean ± standard deviation), CV is the coefficient of variation of number of offspring per individual female (%). *a* is the 1st experiment and *b* is the 2nd experiment performed at 25°C. At 25°C daphnids released the 5th brood before 21-d therefore the *Rep* until 21-d is not available (n.a.).

T (°C)	G	Control mortality (%) (21-d)	Control mortality (%) (at 3rd brood)	Control mortality (%) (at 5th brood)	*Rep* until 3rd brood	*Rep* until 5th brood	*Rep* until 21-d	CV (%) (*Rep* until 3rd brood)	CV (%) (*Rep* until 5td brood)	CV (%) (*Rep* until 21-d)
15	F0	0.0	0.0	0.0	58.1±15.1	105.2±21.0	12.7±4.6	25.9	19.9	36.1
	F1	0.0	0.0	6.7	68.8±13.3	119.9±37.7	21.8±12.1	19.4	31.5	55.7
	F2	0.0	0.0	7.1	37.1±17.4	118.4±35.6	6.3±3.1	47.1	30.1	48.6
	F3	0.0	0.0	0.0	83.8±11.1	138.5±19.7	32.9±13.0	13.2	14.2	39.6
20	F0	0.0	0.0	0.0	32.9±11.8	68.4±27.1	58.9±21.3	36.0	39.7	36.2
	F1	0.0	0.0	0.0	27.2±9.9	63.5±20.8	46.8±14.1	36.5	32.8	30.2
	F2	6.7	6.7	6.7	34.7±14.4	63.3±21.0	57.9±19.2	41.5	33.2	33.2
	F3	0.0	0.0	0.0	33.8±8.0	68.1±18.3	58.7±16.2	23.8	26.8	27.6
25*a*	F0	n.a.	6.7	13.3	21.9±11.6	62.5±34.1	n.a.	52.9	54.6	n.a.
	F1	n.a.	18.8	18.8	19.7±10.5	44.0±23.5	n.a.	53.3	53.5	n.a.
	F2	n.a.	0.0	6.7	31.0±10.8	65.7±14.3	n.a.	34.7	21.8	n.a.
	F3	n.a.	6.7	6.7	24.3±6.5	43.9±15.0	n.a.	26.5	34.3	n.a.
25*b*	F0	n.a.	0.0	6.7	24.5±11.3	44.7±17.5	n.a.	46.0	39.2	n.a.
	F1	n.a.	6.7	6.7	19.8±10.5	65.4±28.8	n.a.	52.9	44.0	n.a.
	F2	n.a.	0.0	0.0	29.9±8.7	53.1±10.8	n.a.	28.9	20.4	n.a.
	F3	n.a.	0.0	0.0	23.9±10.5	50.1±22.3	n.a.	44.1	44.6	n.a.

Table C9. Length of the neonates (<24h) released at the end of each *Daphnia magna* generation (F0, F1, F2 and F3) exposed to Niat 15, 20 (standard temperature) and 25°C. Length expressed as the mean (µm) and standard deviation (SD). Nickel concentration (µg L^{-1}) expressed as nominal and temperature as T (°C). The significant *p* values are marked with bold letters. Within each generation and each temperature a pairwise Wilcoxon Rank Sum Tests were performed and the Benjamini & Hochberg (1995) correction method was used to adjust the *p* values for multiple comparisons. *Results of the 2nd experiment performed at 25°C which is reported in the manuscript.

		F0			F1			F2			F3		
T	Ni	mean	sd	*p*	mean	sd	*p*	mean	sd	*p*	mean	sd	*p*
15	0	784.0	42.9		820.0	49.7		837.2	38.8		846.9	41.5	
	12	811.0	44.3	>0.05	776.4	47.1	>0.05	786.7	71.6	**<0.05**	839.6	66.0	>0.05
	25	756.3	85.2	>0.05	807.9	29.7	>0.05	842.2	23.5	>0.05	838.2	48.0	>0.05
	50	792.7	48.7	>0.05	816.1	48.1	>0.05	785.6	75.2	**<0.05**			
	100	834.9	38.9	>0.05	813.5	28.0	>0.05	806.4	29.9	**<0.05**			
20	0	834.4	57.4		840.4	43.6		821.3	48.5		820.6	38.0	
	12	831.7	48.7	>0.05	803.8	53.5	>0.05	818.4	51.6	>0.05	801.5	35.7	>0.05
	25	799.2	47.5	>0.05	778.1	54.7	**<0.05**	823.8	47.7	>0.05	797.6	48.0	>0.05
	50	795.2	47.5	>0.05									
	100	800.2	53.4	>0.05									
25*	0	847.5	48.7		796.2	46.2		876.2	46.8		956.2	103.9	
	12	795.1	30.8	**<0.05**	812.2	51.1	>0.05	867.2	30.6	>0.05	894.5	68.8	>0.05
	25	854.8	66.8	>0.05	837.1	31.1	**<0.05**	838.6	35.9	>0.05	817.6	44.1	**<0.05**
	50	846.8	53.2	>0.05	861.0	30.6	**<0.05**	896.9	28.2	>0.05	980.5	134.7	>0.05
	100	855.4	119.7	>0.05	846.2	18.0	**<0.05**	982.0	101.7	**<0.05**	818.8	8.1	**<0.05**
	200	1044.8	44.7	**<0.05**	844.1	31.6	**<0.05**	871.0	13.3	>0.05	931.4	97.6	>0.05

Table C10. Internal Ni concentrations *Daphnia magna* measured along four generations (F0, F1, F2, F3) of exposure at 15, 20 (standard temperature) and 25°C. Internal Ni concentrations (µg g^{-1}) are expressed as mean and standard deviation (SD). Nickel water concentrations (µg L^{-1}) are expressed as nominal (nom) and dissolved (dis). Temperature is expressed as T (°C). The Ni treatments bellow the effect concentrations that reduced reproduction in 50% (EC50) calculated for F0 at 15°C and EC10 calculated for F0 at 20, and 25°C are shaded. The percentage of Ni effect on *D. magna* reproduction in relation to control treatment (*Rep* %) is shown.

			F0				F1				F2				F3			
T(°C)	Ni nom	ECx F0	Ni dis	mean	sd	*Rep* %	Ni dis	mean	sd	*Rep*%	Ni dis	mean	sd	*Rep*%	Ni dis	mean	sd	*Rep*%
15	0	18.9	0	0.1	0.1		0	0.3	0.3		0	0.0	0.0		0	0.1	0.0	
	12		4	0.7	0.4	31	9	5.5	0.2	15	12	2.4	0.2	-10	13	1.4	0.3	0
	25		11	1.5	0.3	37	19	7.6	1.7	25	25	4.0	0.9	-10	30	2.8	1.2	13
	50		26	3.3	0.5	54	42	12.6	4.6	39	56	7.3	0.9	16	54	4.2	0.7	65
	100		55	6.8	1.2	73	63	11.0	6.4	4	86	14.2	4.6	4				
20	0	13.5	0	0.3	0.1		0	0.8	1.1		0	0.0	0.0		0	0.9	0.9	
	12		3	0.7	0.1	-10	4	0.9	0.2	0	9	3.1	1.9	-33	10	5.0	0.2	-19
	25		9	1.9	0.3	-2	10	1.6	0.7	20	13	4.1	0.1	-18	20	5.9	0.8	8
	50		23	6.5	2.1	23	25	3.3	1.6	97								
	100		54	5.3	3.3	36												
	200		126	6.8	0	70												
25	0	88.1	0	0.2	0.1		0	0.2	0.2		0	0.4	0.4		0	0.5	0.5	
	12		6	4.2	2.0	-2	10	4.4	1.9	49	13	3.1	1.8	31	12	3.1	1.5	-34
	25		17	7.4	1.8	-9	21	6.0	1.9	-2	28	5.6	1.6	4	26	6.9	4.3	-47
	50		27	6.8	0.8	-27	43	12.5	0.6	35	61	12.2	4.7	-30	57	9.4	2.4	-28
	100		56	12.3	2.3	-35	66	16.0	3.1	77	73	4.8	4.2	20	81	19.8	8.9	-10
	200		120	24.4	6.0	40	123	17.2	3.2	47	121	14.3	4.7	28	119	25.6	10.1	58

Table C11. The internal Ni concentrations in *Daphnia magna* exposed to Ni at 15, 20 and 25°C during four generation (F0, F1, F2 and F3) were described with Monod equation using the nlstool package in R software. The maximum $[Ni]_{daphnia}$ ($ß_{max}$) (µg g^{-1}) and the half saturation constant (µg L^{-1}) with corresponding 95% confidence intervals (CL) and significance (*p* value) are indicated. If negative values were obtained, 0 is indicated in the table. Temperature is expressed as T (°C) and generation as G. n.d.: not determined.

T (°C)	G	ßmax	SE	t value	*p*	Lower CL	Upper CL	Ks	SE	t value	*P*	Lower CL	Upper CL
15	F0	68.1	93.9	0.7	>0.05	0.0	274.8	497.9	749.1	0.7	>0.05	0.0	2146.5
	F1	14.7	3.6	4.,1	<0.01	6.8	22.5	15.3	11.1	1.4	>0.05	0.0	39.5
	F2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	F3	11.0	6.7	1.6	>0.05	0.0	25.9	88.4	79.4	1.1	>0.05	0.0	265.3
20	F0	7.9	1.9	4.3	<0.01	3.9	11.9	16.2	10.8	1.5	>0.05	0.0	39.4
	F1	8.2	8.7	0.9	>0.05	0.0	28.3	36.9	58.4	0.6	>0.05	0.0	171.6
	F2	11.6	21.3	0.5	>0.05	0.0	62.0	24.1	64.8	0.4	>0.05	0.0	177.4
	F3	7.2	2.6	2.8	<0.05	0.7	13.8	4.3	6.4	0.7	>0.05	0.0	20.8
25	F0	64.5	28.5	2.3	<0.05	3.8	125.3	204.1	129.3	1.6	>0.05	0.0	479.6
	F1	25.3	3.5	7.3	<0.001	17.8	32.7	49.6	15.6	3.2	<0.01	16.3	83.0
	F2	25.1	16.3	1.5	>0.05	0.0	59.6	111.9	124.5	0.9	>0.05	0.0	374.7
	F3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

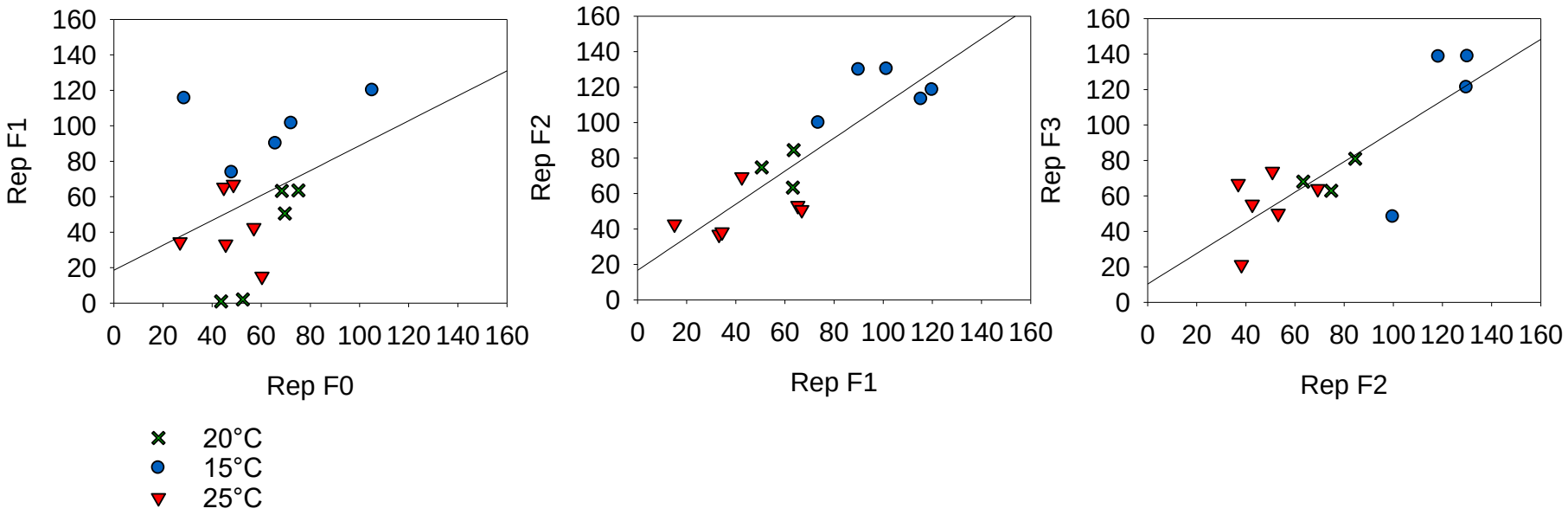

Figure C1. Plots of reproduction, expressed as number of offspring per individual female produced until the organisms in control treatment released the 5th brood (*Rep*), of generation F0 vs. F1, F1 vs. F2 and F2 vs. F3.

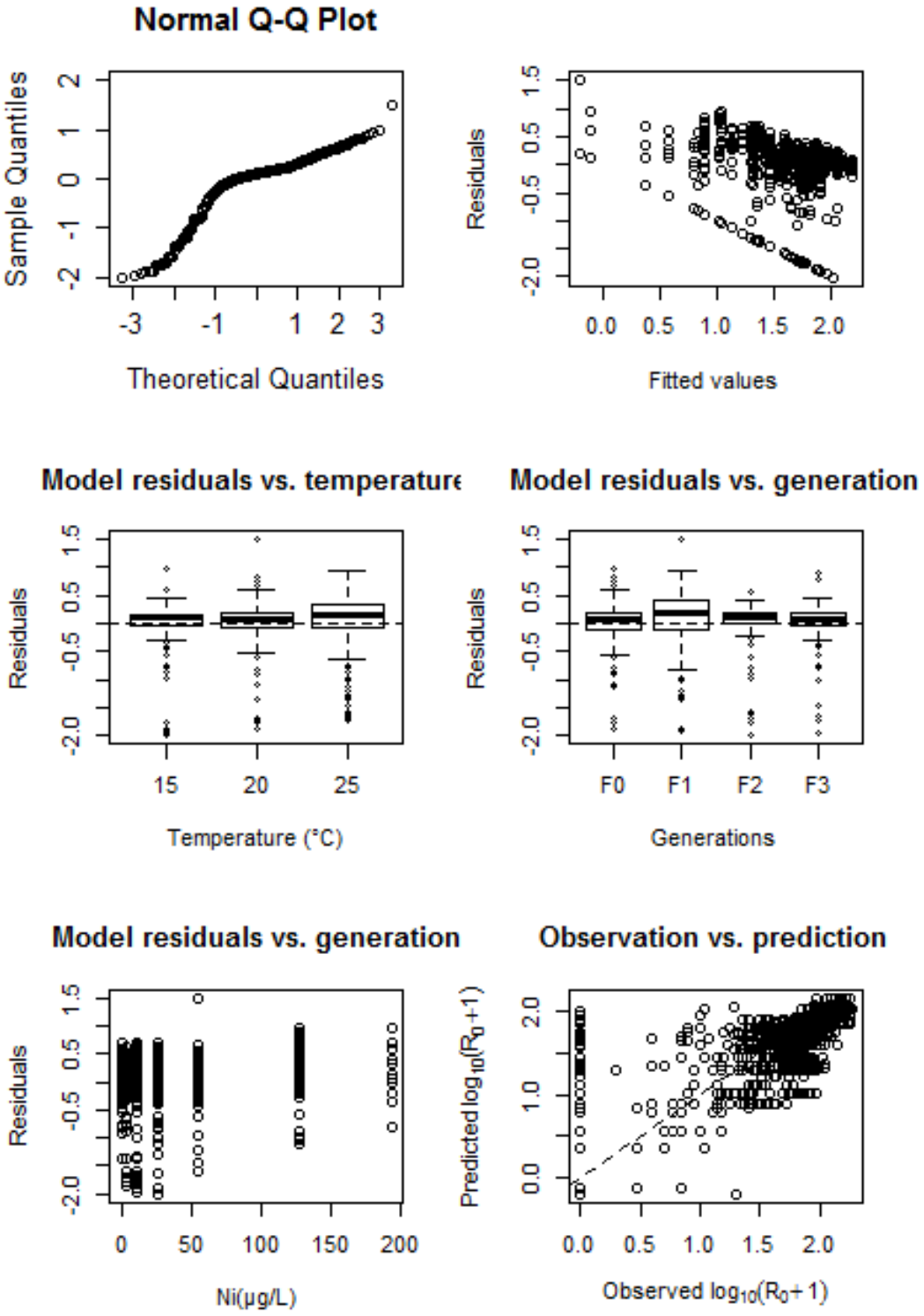

Figure C2. Model validation graphs the generalized estimation equation that predict the Ni effect on reproduction as a function of temperature (factorial variable) and generations (Ni × Temperature × Generation) with an auto-regressive correlation structure between generations. Reproduction expressed as $\log_{10}$-transformed number of offspring per individual female produced until the organisms in control treatment released the 5th brood plus 1 ($\log_{10}$ (*Rep5*+1)). Panel A shows QQ plot and panel B shows a plot of the residuals vs. fitted values for normality. In panel C, D and E residuals of log10 (*Rep5*+1) are plotted versus temperature, generation and Ni. Plot F shows the predicted $\log_{10}$ (R_0 5th brood+1) versus the observed $\log_{10}$ (*Rep5*+1).

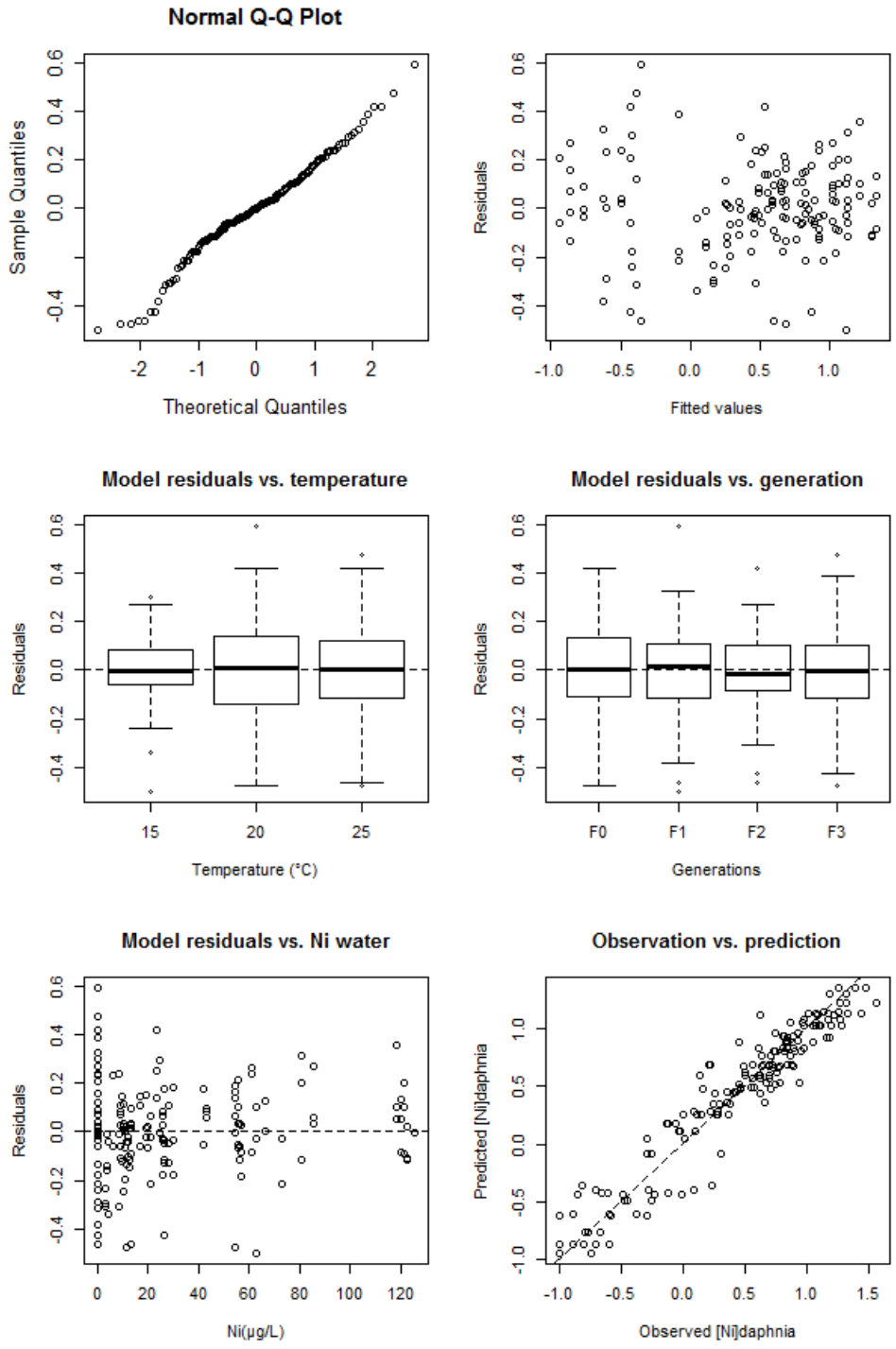

Figure C3. Model validation graphs of the generalized estimation equation analysis applied to the internal Ni concentrations in *Daphnia magna* ($[Ni]_{daphnia}$) with a three way-interaction between temperature (factorial variable), water Ni concentrations (continuous variable) and generation (factorial variable) with an auto-regressive correlation structure between generation. The $[Ni]_{daphnia}$ and the water Ni concentration were $\log_{10}$ (x+0.1) transformed. Panel A shows QQ plot and panel B shows a plot of the residuals vs. fitted values for normality. In panel C, D and E, the residuals of log10 ($[Ni]_{daphnia}$ +0.1) are plotted versus temperature, generation and Ni. Plot F shows the predicted log10 ($[Ni]_{daphnia}$ +0.1) versus the observed log10 ($[Ni]_{daphnia}$ +0.1).

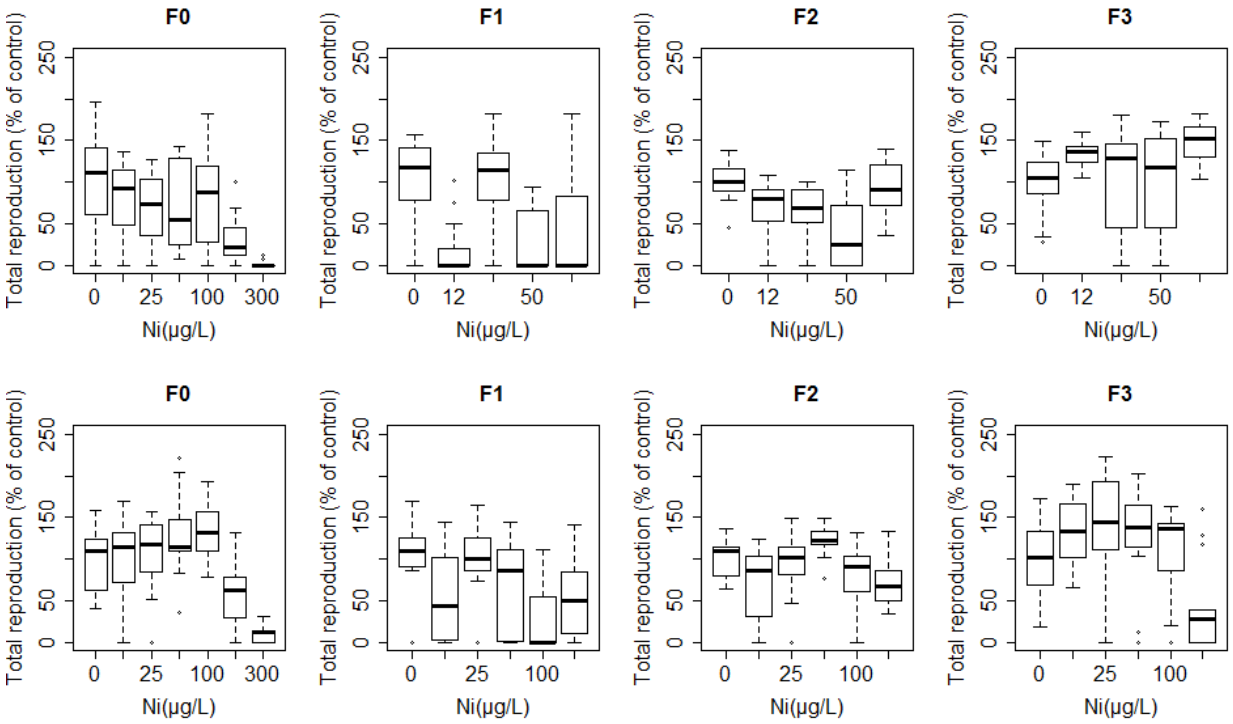

Figure C4. Box plots of the 1st and 2nd multigenerational Ni toxicity tests along four generations (F0, F1, F2, F3) at 25°C. Total reproduction (relative to the control) expressed as the number of offspring per individual female until the organisms in control treatment released the 5th brood. Nickel expressed as dissolved concentrations (µg L^{-1}).

Appendix D

Supplementary material for Chapter 5

Table D1. The reference material, the detection limits and the quantification limits of all chemical analysis are given. The analytical instruments used are the iCAP 7000 Series Inductively Coupled Plasma Optical-Emission Spectrometry (ICP-OES) (Thermo Scientific) and the Total Organic Carbon L series CPH (TOC-L CPH) (Shimadzu, Duisburg, Germany).

Analytical instrument	Reference material	Substance	Detection limit	Low quantification limit
ICP-OES	TM-28.4 (lot 0815); TMDA-70.2 (lot 0815); CRANBERRY-05 (lot 0815) [Environment Canada]	Ni	1.2 µg L^{-1}	4.0 µg L^{-1}
		Cu	2.0 µg L^{-1}	4.0 µg L^{-1}
		Zn	0.5 µg L^{-1}	2.0 µg L^{-1}
		Fe	17.0 µg L^{-1}	56.0 µg L^{-1}
		Na	30.0 µg L^{-1}	100.0 µg L^{-1}
		K	30.0 µg L^{-1}	100.0 µg L^{-1}
		Ca	15.0 µg L^{-1}	50.0 µg L^{-1}
		Mg	15.0 µg L^{-1}	50.0 µg L^{-1}
TOC-L CPH	CRANBERRY-05 (lot 0815) [Environment Canada]	Dissolved organic carbon	0.5 mg L^{-1}	1.5 mg L^{-1}

Table D2. The Ni concentrations, the dissolved organic carbon (DOC) and pH registered on the *Daphnia magna* population experiment at 15°C, 20°C and 25°C. All values are given as averages (Av) (± standard desviation (SD)) in the new medium and old medium after renewal. d.l.: detection limit. At 15°C, the reported values correspond until day 21.

Temperature (°C)	Ni nominal (µg L^{-1})	Ni total (µg L^{-1})		Ni dissolved (µg L^{-1})		DOC (mg L^{-1})		pH	
		Av	SD	Av	SD	Av	SD	Av	SD
15	0	<d.l.	<d.l.	<d.l.	<d.l.	2.79	0.42	7.79	0.07
	12	10.7	0.6	7.9	0.8	3.26	0.78	7.78	0.08
	100	105.4	3.6	82.3	8.2	3.40	0.52	7.79	0.07
20	0	<d.l.	<d.l.	<d.l.	<d.l.	3.56	1.25	7.85	0.13
	12	6.8	5.3	3.3	3.4	3.46	1.40	8.10	0.16
	100	85.1	18.4	62.3	12.0	3.40	1.03	8.11	0.18
25	0	<d.l.	<d.l.	<d.l.	<d.l.	3.53	1.03	7.91	0.08
	12	11.8	0.9	7.5	1.1	3.54	1.02	7.94	0.07
	100	110.2	5.5	79.6	8.1	3.72	1.19	7.93	0.06

Table D3. Sodium, K, Ca, Mg, Fe, Cu and Zn concentrations in the population experiment performed with *Daphnia magna* exposed to Ni along 9 weeks at 15, 20 and 25°C. T is temperature (°C), average (Av) and standard deviation (SD) are reported.

Temperature (°C)	Ni nominal (µg L^{-1})	Na (mg L^{-1})		K (mg L^{-1})		Ca (mg L^{-1})		Mg (mg L^{-1})		Fe (µg L^{-1})		Cu (µg L^{-1})		Zn (µg L^{-1})	
		Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD
15	0	18 .7	1 .2	3 .1	0 .1	55 .3	1 .4	8 .3	0 .2	256 .6	29 .8	8 .0	0 .3	13 .6	9 .7
	12	24 .4	1 .6	3 .2	0 .1	56 .4	1 .4	8 .5	0 .2	241 .0	18 .8	7 .1	0 .4	9 .3	3 .0
	100	20 .6	1 .8	3 .0	0 .1	56 .2	1 .9	8 .4	0 .2	238 .4	23 .5	7 .5	0 .6	8 .9	2 .0
20	0	23 .7	10 .1	3 .4	1 .4	66 .5	30 .4	9 .2	2 .6	217 .8	18 .8	7 .5	1 .5	11 .5	15 .6
	12	40 .2	19 .1	5 .0	1 .8	99 .6	41 .2	12 .5	3 .8	206 .6	55 .6	8 .7	1 .3	10 .8	8 .4
	100	40 .7	19 .8	5 .0	1 .9	100 .8	41 .9	12 .6	3 .8	205 .7	54 .6	8 .1	1 .1	11 .2	10 .1
25	0	20 .6	2 .9	3 .1	0 .5	58 .4	7 .2	8 .4	0 .7	217 .1	11 .3	7 .2	0 .6	7 .2	6 .4
	12	23 .5	3 .9	3 .4	0 .3	64 .5	6 .7	9 .1	0 .7	253 .1	15 .4	8 .3	0 .7	7 .8	2 .8
	100	22 .0	3 .1	3 .3	0 .3	62 .6	7 .5	8 .9	0 .8	245 .7	26 .8	8 .2	1 .8	14 .3	19 .2

Table D4. Results of the estimation of the effect parameters for the physiological modes of actions (PMoAs) for the life cycle experiment where *Daphnia magna* was exposed to Ni. Effect concentration that reduce reproduction in 50% (EC50) and slope (β) are reported with average (Av) and standard desviation (SD). The effect concentrations were determined with the *drc* package in R with the log-logistic dose-response model with 3 parameters (Equation D1)

(Eq D1)

$$y = \frac{d}{1 + \exp(\beta(\log(x) - \log(e))}$$

where y is the predicted reproduction or the predicted size (length), d is the value of the maximal response, β is the slope parameter, x is the dissolved Ni concentration (µg L^{-1}) and *e* is the threshold concentration that causes 50% effect. Results are based on the 100 best fits (based on sum of squared errors (SSE)) of 10 000 simulation runs. Model predictions were fitted against the experimental data of the *D. magna* adults size and reproduction. n.d.: not determinate; Inf.: infinite.

PMoA	Temperature (°C)	EC50 (µg L^{-1})	β	SSE
Reproduction costs (= embryo)	15	n.d.	n.d.	Inf.
	20	n.d.	n.d.	Inf.
	25	n.d.	n.d.	Inf.
General maintenance (both structural and maturity)	15	31.22±1.37	0.030±0.002	2.85
	20	34.62±1.27	0.034±0.004	4.35
	25	33.77±1.23	0.028±0.003	6.89
Assimilation	15	31.70±1.36	0.036±0.003	3.08
	20	33.71±1.35	0.037±0.004	5.52
	25	33.68±1.33	0.035±0.003	7.67
Growth costs	15	16.18±0.96	0.056±0.002	1.60
	20	27.65±1.34	0.028±0.002	1.61
	25	27.65±1.31	0.029±0.002	2.08
Maintenance (only structural)	15	30.30±1.52	0.034±0.003	2.84
	20	32.71±1.47	0.034±0.003	4.40
	25	32.95±1.44	0.033±0.003	6.98
Maintenance (only maturity)	15	n.d.	n.d.	Inf.
	20	n.d.	n.d.	Inf.
	25	n.d.	n.d.	Inf.

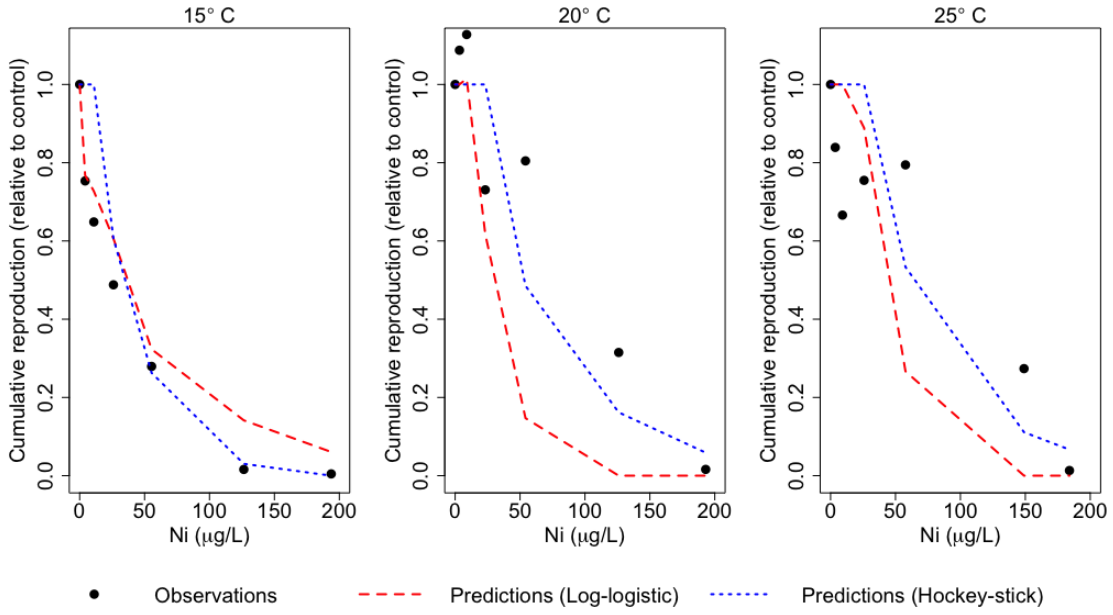

Figure D1. Calibration of the DEB-IBM for *Daphnia magna* of the physiological modes of action with an effect on growth costs (an increase in costs for growth), were plotted versus the observation from the apical Ni toxicity data set from chapter 4. To describe the relationship between the environmental stressor concentration (in this case Ni) and the level of stress on *D. magna* two models were used: the hockey-stick and the log-logistic. Each graph shows *D. magna* reproduction (cumulative reproduction relative to control) as a function of the Ni concentration at 15, 20 and 25°C. Lines represent predictions and dots observations.

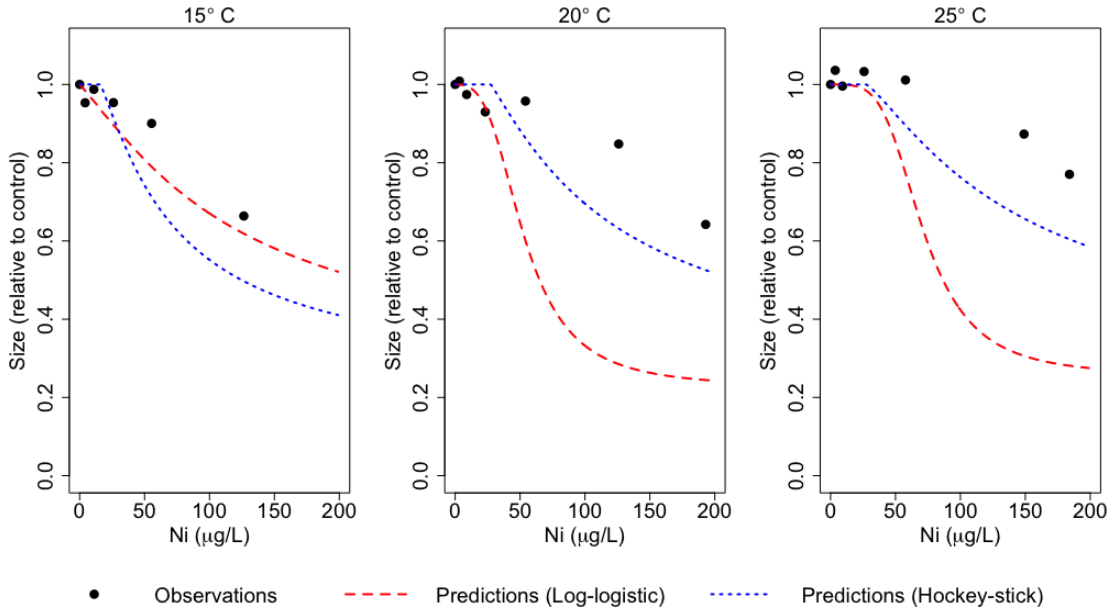

Figure D2. Calibration of the DEB-IBM for *Daphnia magna* of the physiological modes of action with an effect on growth costs (an increase in costs for growth), *w*ere plotted versus the observation from the apical Ni toxicity data set from chapter 4. To describe the relationship between the environmental stressor concentration (in this case Ni) and the level of stress on *D. magna* two models were used the hockey-stick and the log-logistic. Each graph shows *D. magna* size (length, relative to control) as a function of the Ni concentration at 15, 20 and 25°C. Lines represent predictions and dots observations.