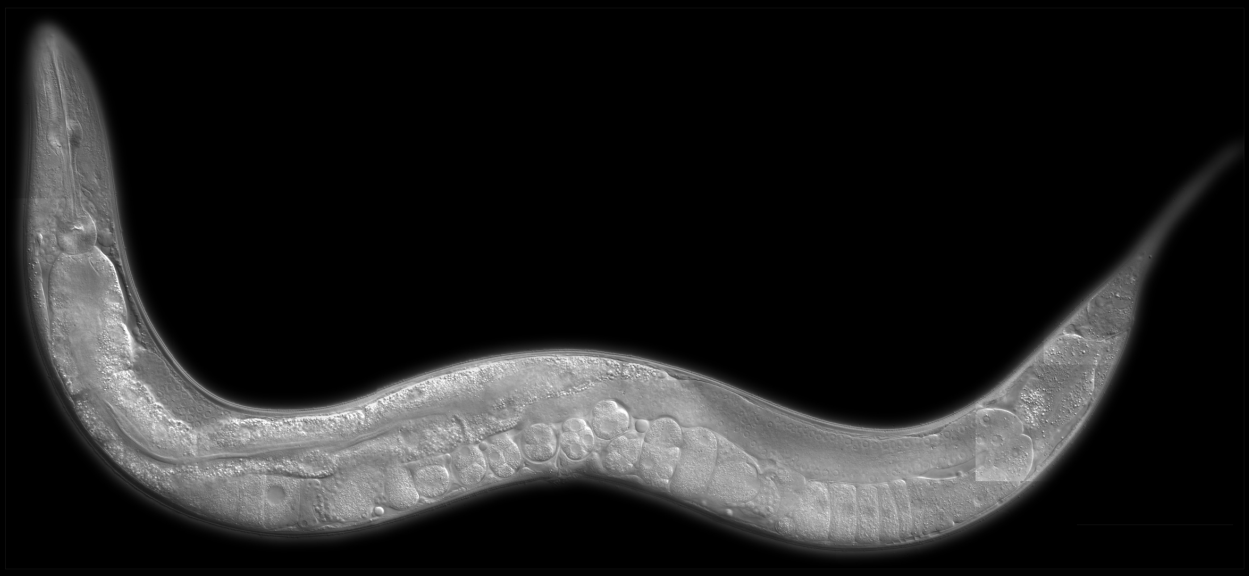
Toxic effects of single and combined metals at the individual and population level of *C. elegans*

Proefschrift voorgelegd tot het behalen van de graad van doctor in
de wetenschappen: biologie aan de Universiteit Antwerpen te verdedigen door

Sofie Moyson



Promotoren
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Faculteit Wetenschappen
Departement Biologie

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Te verdedigen door **Sofie Moyson**

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List of abbreviations

ACN	Acetonitrile
AGC	Automated gain control
CA	Concentration addition
CeMM	*C. elegans* maintenance medium
CI	Chemotactic index
CPE	Carboxypeptidase E
FA	Formic acid
FLP	FMRFamide-like peptides
HCD	High energy collision dissociation
IA	Independent action
ICP-MS	Inductively coupled plasma mass spectometry
ICP-OES	Inductively coupled plasma optical emission spectometry
L1-L4	Larval stage of *C. elegans*
LCx	Lethal concentration at which x% of the test organisms dies
NGM	Nematode growth medium
NLP	Neuropeptide-like protein peptides
PAM	Peptidyl-glycine α-amidating monooxygenase
TEAB	Triethyl ammonium bicarbonate

Contents

Chapter 1

General introduction

1.1. Metal pollution

Metal pollution is a worldwide, persistent and complex environmental problem. Contaminating metals occur in the soil, in the aquatic environment (in dissolved form or associated with particulate matter) and in the atmosphere (in particulates, aerosols and in some cases in gaseous form) (Luoma and Rainbow, 2008). Due to biochemical cycles metals can be transferred between the different environmental compartments (Järup, 2003; Luoma and Rainbow, 2008). Metals of natural origin result from weathering of rocks, volcanic eruptions, forest fires etc., while anthropogenic sources of metals include mining, melting of metallic ores, pesticide production, waste incineration plants, industrial production, dumping of sewage sludge and burning of fossil fuels (Moolenaar and Lexmond, 1999; Khan et al., 2008) (Fig. 1.1).

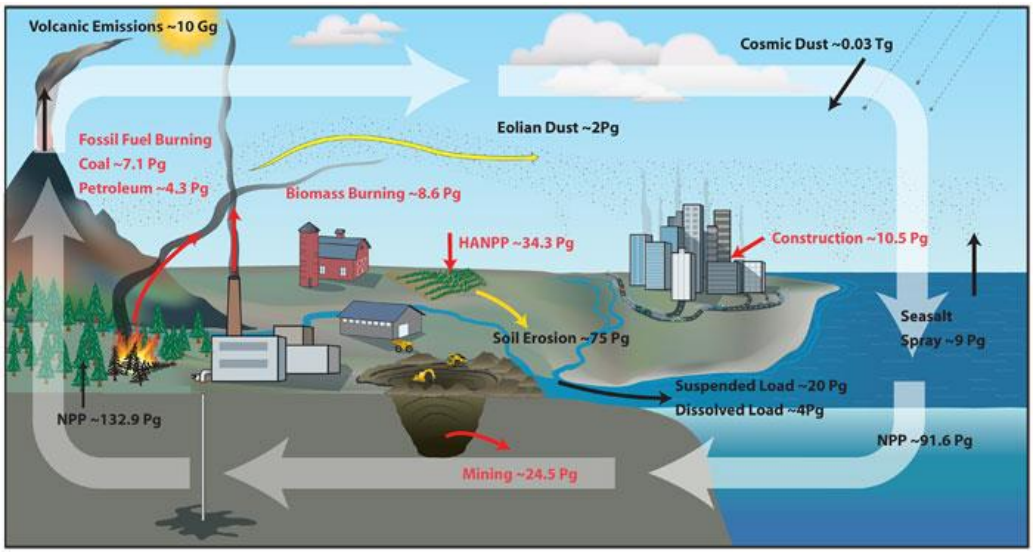

Figure 1.1. Transfer of metals by natural and anthropogenic sources (Pg=pictogram, Tg= teragram, Gp=gigagram, NPP=net primary production, HANNP=human apportionment of net primary production) (Sen and Peucker-Ehrenbrink, 2012).

Furthermore, metals are ubiquitous components of almost all products used by humans (e.g. batteries, jewellery, cars, household appliances, building sector, money). The number of anthropogenic sources of heavy metals has increased during the past

decades, leading to higher metal concentrations in aquatic environments, especially in mining and industrial areas (Peredney and Williams, 2000). Twenty years ago, natural emissions of metals into the atmosphere were estimated at $1x10^3$ ton Cd/year, $19x10^3$ ton Cu/year and $4x10^3$ ton Zn/year worldwide, whereas anthropogenic emissions were estimated at $7.5x10^3$ ton Cd/year, $56x10^3$ ton Cu/ year and $320x10^3$ ton Zn/year (Clark et al., 1997). In 2016, in Flanders, emissions from anthropogenic sources into the air were estimated at 1.4 ton Cd, 20.4 ton Cu and 39.7 ton Zn (VMM, 2018). Because of these dramatically increased metal emissions, ecological risks from metal contamination may occur increasingly. In addition, polluted surface water, ground water and soil can contaminate drinking water, fish, crops and life stock, and can therefore pose a risk to human health (Liu, 2003). In order to be taken up and to cause adverse effects in organisms, pollutants must be accessible to organisms and thus be bioavailable.

1.2. Bioavailability

Bioavailability is defined as the degree to which a metal is free for uptake into or adsorption onto an organism, expressed as a fraction of the total environmental concentration (Bervoets and Blust, 1999; Baker et al., 2003; Peakall and Burger, 2003, van Gestel, 2008). It is influenced by the metal chemistry, the interaction between the metal and the environment and species-specific physiological and ecological traits. Indeed, the distribution of an element over its physicochemical forms, i.e. metal speciation, plays an important role in bioavailability and is influenced by pH, DOC, water hardness, temperature, ionic strength, redox, interaction with organic matter and other complexants (e.g. bacteria), metal concentration, binding sites, etc. (Donkin and Williams, 1995; De Schampelaere and Janssen, 2002; Luoma and Rainbow, 2008). An increased metal bioavailability will cause a higher metal uptake in body tissue and will potentially result in an increased body accumulation of metals (Rainbow, 2002, 2007). The bioaccumulation of a metal can be modulated by the differential uptake

(waterborne and dietborne) and the transport and sequestration within an animal (Dallinger and Rainbow, 1993). It is important to take into account that the complexation of metal ions by organic matter lowers the aqueous free metal ion concentration and can reduce metal mobility. Although metals may occur in different forms, free metal ions are important to estimate free metal uptake and possible toxic effects (Jakob et al., 2017).

1.3. Toxicity

Once a metal has entered an organism, it can bind to a ligand for which it has affinity. However, if one metal substitutes another and thereby blocks the catalytic active site of a metalloprotein, adverse effects may occur (Luoma and Rainbow, 2008). Moreover, a metal may also bind elsewhere on a protein, preventing its normal metabolic action. As a response, the metal might be excreted or detoxified by strong metal binding proteins such as metallothioneins or by insoluble metalliferous granules (Luoma and Rainbow, 2008). Metal bioavailability, differences in uptake route, assimilation efficiency and detoxification mechanisms (e.g. metallothioneins, heat shock proteins, pumps, transporters, etc.) may all influence metal toxicity (Anderson et al., 2003; Rainbow, 2007; Martinez-Finley and Aschner, 2011; Roh et al., 2014). Hence, the characteristics of waterborne and dietborne exposures, organismal detoxification and excretion strategies ultimately determine the fate and effect of the metal.

When the metal uptake rate exceeds the combined rates of detoxification and excretion, a critical concentration of metabolically available metal can be accumulated and toxic effects may occur (Rainbow, 2002, 2007; Jacob et al., 2017).

Metabolically available metal accumulation can lead to serious health risks for diverse animals, including humans. It is well known that metals can cause neurotoxicity, alter neuronal excitability and impair chemosensation, and that they are involved in neurodegenerative diseases (Mathie et al., 2006; Wright and Baccarelli, 2007). Furthermore, free radicals and reactive oxygen species generated by metals can induce

protein and deoxyribonucleic oxidation and lipid peroxidation (Caito et al., 2012). They also disrupt cellular homeostasis by impairing DNA repair, inhibiting enzyme activity and affecting protein binding (Martinez-Finley and Aschner, 2011). Metal toxicity is species specific, which can be explained by inherent differences between the species and differences in test conditions and exposure time. A short-term exposure of 96 h covers almost the entire generation time of *Daphnia sp* and *C. elegans*, while for fish with a life span of a few years, 96 h is considered to be an acute exposure (Luoma and Rainbow, 2008).

Toxic effects also differ between essential and nonessential metals; in addition, an organism can be sensitive for a certain metal, but tolerant for another (Williams and Dusenbery, 1990). In contrast to non-essential metals (e.g. Cd), essential metals (e.g. Zn and Cu) play a role in important biological functions and have an optimal concentration range at which sufficient metal is available to meet metabolic requirements. However, when metal availability is deficient or in excess, adverse effects may occur. The presence of (non-)essential metals may disrupt normal biological processes, depending on their concentration, dynamic chemical speciation, bioavailability and biological species sensitivities. Essential metals are not necessarily less toxic than non-essential metals; the only difference is that the energetic costs to detoxify metal excess will only become significant when optimal metal concentrations are exceeded (Luoma and Rainbow, 2008).

Zn, Cu and Cd are 'transition metals' that belong to the middle block of the periodic table. Cd has a similar valency and ion radius as ionic Ca and to a lesser extent as the Zn ion; therefore Cd may exert its toxic action by displacing Ca and Zn in biological systems. As a result, Cd can, for example, cause cellular lesions and mitochondrial dysfunction, destabilize biomembranes and interact with thiol protein groups (Vallee and Ulmer, 1972; Popham and Webster, 1979; Goldstein and Czapski, 1986; Alt et al., 1990). Zn plays an important role in proteins involved in growth, reproduction, development and immune functions (Watanabe et al., 1997). At increased concentrations, Zn can become

toxic due to, inter alia, interference with Ca homeostasis (Hogstrand et al., 1996) and it can cause multiple biological defects affecting life span, reproduction, development, etc. (Wang et al., 2007; Chen et al., 2013). Finally, Cu functions as a catalytic cofactor in redox chemistry of enzymes and proteins, which are important for growth and development, while the proteins are involved in vital processes such as respiration, hormone production, neurotransmitter synthesis etc. However, above optimal concentrations, Cu can induce protein and DNA damage, free radical generation, cellular injury and structural impairment of essential metal binding sites (Vallee and Ulmer, 1972; Popham and Webster, 1979; Goldstein and Czapski, 1986; Alt et al., 1990).

To study the toxicity of single metals, several endpoints have been used, ranging from the molecular to the population level, including gene transcription, physiology, locomotion, feeding, lethality, growth and reproduction (Power and de Pomerai 1999; Anderson et al. 2003; Boyd et al. 2003; Boyd and Williams 2003; Calafato et al. 2008; Lagido et al. 2008; Höss et al. 2011; Song et al.2014; Moyson et al., 2016). Some of these endpoints are discussed below.

Mortality

Mortality is one of the most studied endpoints for both short-term and long-term toxicity tests. Mortality, however, is a crude effect in response to relatively high and therefore environmentally irrelevant concentrations, while at much lower concentrations the underlying sublethal effects may be expressed.

Behaviour

An organism can behave differently when encountering a metal polluted area. If possible, the organism will leave this area and move to a non-contaminated area. Therefore, chemosensation represents a complex and important response of the animal to its environment. An organism needs a well-functioning sensory system to survive e.g. to find a food source, to deal with environmental changes and pollution, to find a mate, to avoid predator attacks, etc. In response to metal contamination, an animal will change its locomotion to escape or to survive the contamination. Since locomotion is

coordinated by the nervous system and metals may excert neurotoxic effects, behavioural assays such as locomotion can be simple, sensitive and powerful tools for analysing metal toxicity. Metals are also known to affect reproductive behaviour (Roh et al., 2006; Cedergreen et al., 2016) and to adjust feeding behaviour by blocking food uptake and affecting gut structure (Popham and Webster, 1979; Höss et al., 2011). Locomotion and feeding are connected endpoints since animals have to move towards their food (Boyd at al., 2003).

Population effects

A change in the number of offspring is a common observed phenomenon after exposure to chemicals (e.g. Boyd and Williams, 2003; Boyd et al., 2011; Nørhave et al., 2012). This can be the result of changes in different reproductive stages such as oocyte and sperm formation, fertilization, development, egg-laying and hatching success. By studying the whole population, effects on both adults and the most sensitive stages of the life cycle (e.g. embryos and larvae) are taken into account. For the nematode *C. elegans* phenotypic responses to environmental stress (e.g. inadequate food quantity and quality, presence of chemicals) have been observed for adult lifespan, lifetime fecundity, timing of reproduction and body size (Álvarez et al., 2005; Roh et al., 2006; Lagido et al., 2009; Nørhave et al., 2012; Chun et al., 2017). Indeed, development of eggs, embryos and larvae and their recruitment in adult populations can be (seriously) affected by metal exposure (Luoma and Rainbow, 2008). Therefore, if toxicity affects reproduction, development and maturation of a species, it consequently will also affect the fate of the population. Depending on the reproductive strategy (gonochoric strategy or asexual (hermaphroditic) strategy), different population effects are observed (Álvarez et al., 2005). The intrinsic population growth rate integrates the entire age schedules of survival and fertility into a single measure, and it is thus a more useful and sensitive parameter than just lifespan, fecundity or body length. Although population studies can be very sensitive and possibly give more insight into metal toxicity, they are still scarce (except for *Daphnia sp*. and *Folsomia sp*.). This might be due to the fact that

some population effects only become apparent after several generations have been exposed. However, studying different generations can reveal interesting effects: it was observed that some metal effects were transferred from the P1 generation, living in a contaminated environment, to the F1 generation, living in a non-polluted area, while some defects disappeared (Wang et al., 2007).

Molecular changes

To maintain homeostasis, there are several molecular players: transcription factors, hormones, peptides, metabolites, etc. In addition, complex networks of transporter proteins, metal binding proteins and stress response mechanisms regulate the cellular metal content (Martinez-Finley et al., 2011). However, under non-optimal and toxic conditions, molecular changes may occur. Mechanistic insights (mode-of-action) into toxicity are needed to reveal why an organism appears to be more vulnerable to a toxic compound than another. To obtain functional insights, respective mutant or knock down lines can be used (e.g. Calafato et al., 2008; Ma et al.,2009; Roh et al., 2014; Kumar et al., 2016; Chun et al., 2017). Moreover, since molecular changes are likely to precede toxic outcomes on the cellular or organismal level, changes in molecular patterns (fingerprints) may serve as early warning systems for toxicity (Backhaus and Faust, 2012). Therefore, multi-endpoint approaches and pathways based on 'omics strategies', allow studying the function of identified molecular players and will become very important in toxicological risk assessment.

Combining effects at different functional levels

Metal effects can be studied at different organisational levels from molecular to population level and beyond. Combining effects at different levels is necessary to better understand the observed toxicity. However, the number of studies combining effects at different organisational levels is still small and therefore these studies are urgently required.

1.4. Metal mixtures

The increased release of metals to the (aquatic) environment and their ability to cause various adverse effects on species, possibly leading to changes in species distributions, taxa richness of the community and eventual loss of ecosystem functioning, make it necessary to investigate and regulate the potential risk of these chemicals. However, in contrast to the increasing understanding of the toxic effects of single metals, much less is known about their effects in mixtures, which frequently occur in the natural environment. Although a few studies have been performed with mixtures of Cu, Cd or Zn on earthworms (Weltje, 1998), *Enchytraeus sp*. (Lock and Janssen, 2002), *Folsomia candida* (van Gestel and Hensbergen, 1997) and *C. elegans* (Chu and Chow, 2002; Jonker, 2004ab; Lagido et al., 2009), metal mixture toxicity studies on soil invertebrates are still scarce. Their effects upon interaction can be additive, antagonistic or synergistic. Mixture toxicity can be assessed by multiple dose-response analysis of single compounds in comparison with their combinations. In the current literature, data are compared with predictions based on the concentration addition or independent action model (Martin et al., 2009; Jonker et al., 2004b, 2005). However, it is becoming increasingly clear that more complex deviation patterns e.g. dose-ratio dependent deviation, dose-level dependent deviation, and synergistic/antagonistic deviation, need to be addressed for predicting mixture toxicity and establishing a robust scientific basis in the setting of standards for environmental risk assessment and protection.

1.5. Environmental regulations

Several organisations have the task of assessing the potential risk of chemicals for the environment and human health. For example, the EPA (Environmental Protection Agency) is an agency in the Unites States that functions to protect human health and environments from environmental risks by developing and enhancing regulations. According to the EPA, ecological risk assessment consists of 3 stages: (i) problem definition i.e. identifying which entity is in danger and needs protection, (ii) analysis by

characterisation of exposures and ecological effects and (iii) risk characterisation consisting of risk estimation and risk description (EPA, 2013). The European community has introduced REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) to protect human health and the environment from exposure to chemicals. Therefore, REACH collects all toxicity data and information on all chemical products produced or imported in Europe, while the industries involved are responsible for providing information on the substances and for managing the potential risk (European Commission, 2007). To achieve these goals, the governments have formulated environmental quality standards for contaminants in the environment. The Flanders Environment Agency (VMM) covers three main areas (water, air, environment) and draws up the Flanders Environment Report using reliable and up-to-date information concerning the state of the environment in Flanders. Their reports serve as a scientific basis for the Flemish environmental policy, providing an outlook on the environment of tomorrow. Therefore, they use data from a network of several sampling sites in Flanders, where - among others - the physiochemical quality of water and/or sediment is monitored regularly. Dissolved Cd, Cu and Zn levels in freshwater range from 0.3 to 10 nmol/L (33.7 - 1124.1 ng/L), 8-80 nmol/L (0.5 - 5.1 µg/L) and 30-60 nmol/L (2 - 3.9 µg/L) respectively (Bervoets and Blust, 2003; Campbell et al., 2008). In contaminated areas Cd and Cu concentrations can exceed 200 nmol/L (22.5 µg/L) and 300 nmol/L (19.1 µg/L) respectively, while Zn concentrations can vary between 2000-3000 nmol/L (130 - 196 µg/L) or even reach up to 34000 nmol/L (2223.3 µg/L) (Bervoets and Blust, 2003). In 2015, most dissolved Cu and Cd concentrations in freshwater were of the same order of magnitude or slightly higher than mentioned above, while Zn levels were more spread. Furthermore, in contaminated areas, Cd levels did not exceed 200 nmol/L, while Cu concentration exceeded 300 nmol/L and dissolved Zn concentration exceeded 2500 nmol/L at least one time in approximately 0.5% and 2.7% of the sampling areas respectively (VMM unpublished data).

To assess metal concentrations in ground water more than 5000 sampling locations are being used, of which 12% exceeded the environmental standards (Table 1.1) (VMM,

2013). Cd and Zn ground water concentrations exceeded the norm in 1.5% of the sampling locations, while the Cu ground water norm was only exceeded at 3 sampling points in 2011 (VMM, 2013). Furthermore, the occurrence of metals in air and groundwater showed a declining trend between 2000 and 2010 (MIRA, 2013).

Table 1.1: Detection limits used by the VMM in 2011 and norms for metal concentrations in ground water and drinking water (µg/L)

Metal	Detection limit	Ground water norm	Drink water norm
Arsenic	5	20	10
Bohrium	100	1000	1000
Cadmium	0.5	5	5*
Chromium	5	50	50
Cobalt	5	n.a.	n.a.
Copper	5	100	2000**
Mercury	0.5	1	1
Lead	5	20	10***
Nickel	5	40	20
Zinc	10	500	5000****

* Target value: 3 µg/L; ** target value: 100 µg/L at entrance water distribution network and 1000 µg/L between water distribution network and domestic network; *** valid from 25.12.2013 and measured at the tap; **** target value at exit of water purification plant: 200 µg/L

However, the above threshold concentrations are based on single metals, while in natural environments metals (mainly) occur in mixed conditions. One of the major challenges in ecotoxicology is thus to gain insight into mixture toxicology in order to establish realistic environmental quality criteria for environmental risk assessment and protection. Furthermore, the threshold concentrations are mainly based on toxicity studies carried out under controlled laboratory conditions. In contrast, predicting

toxicities in the field is very complex due to rapidly changing environmental parameters (e.g. pH, temperature), bioavailability and the presence of multiple stressors (different chemicals, starvation, desiccation, etc.). Finally, the current approach to risk assessment is usually based on extrapolation of toxicity data obtained from the cellular and organismal level of relevant species (Fig. 1.2), since the study of the effects on a whole ecosystem with all different species and communities is very complex. Model species that are frequently used for risk assessment of metals are e.g. *Pseudokirchneriella subcapita, Daphnia magna, Caenorhabditis elegans* and *Danio rerio.* For the soil compartment *C. elegans* can serve as an excellent model organism, allowing toxicity assessments at the molecular, individual and population level to be combined.

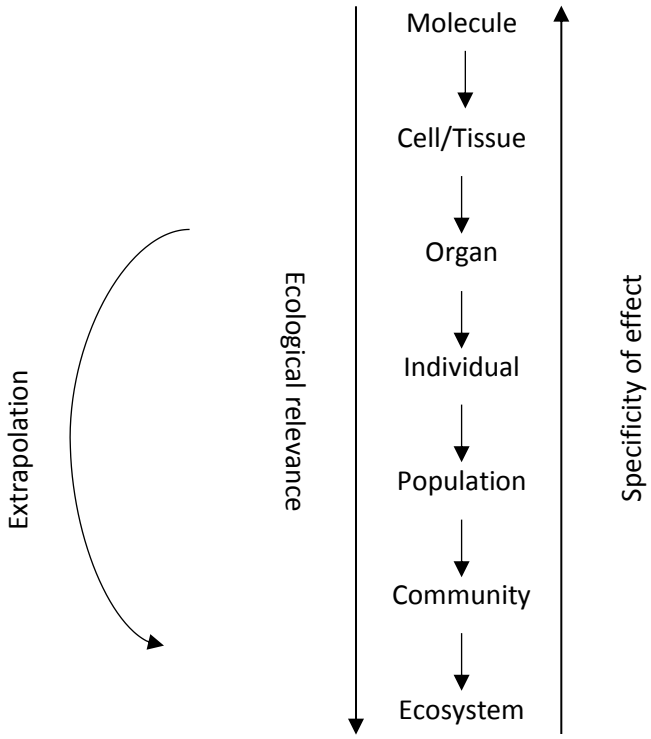

Figure 1.2. Overview of the risk assessment scheme, using different functional organisational levels. Toxicity data of the cellular and individual level are extrapolated to estimate the risk of the pollutant to the ecosystem.

1.6. Model organism *Caenorhabditis elegans*

Soil nematodes (e.g. *C. elegans*) live within the interstitial waters of soil particles and are thus in direct contact with dissolved contaminants. They have a considerable ecological value due to their major role in nutrient cycling and dynamics by feeding on microbes (mainly bacteria) and fungi. In addition, *C. elegans* (Fig. 1.3) is often used in laboratory toxicity tests.

Figure 1.3. *Caenorhabditis elegans* (WormAtlas, 2002-2018)

C. elegans, a rhabditid nematode, appeared in literature already in 1900 (Maupas, 1900), but Brenner (1974) was the first researcher to introduce *C. elegans* as an important experimental model organism. Since then, the nematodes have often been used as a unique model for fundamental neuroscience (e.g. Parkinson disease and Alzheimer disease), aging and (eco)toxicology, for example in studies on metal toxicity and metal homeostasis. By the use of these non-vertebrate model organisms, ethical issues are avoided since it fits the "3 R" principle (replacement with alternative methods, reduction of the use of laboratory animals and refinement of current techniques).

The majority of *C. elegans* populations consists of hermaphrodites. Males occur in 0.1% of the population and are more slender and slightly shorter than hermaphrodites (WormAtlas, 2002-2018). Although *C. elegans* has an anatomically simple body plan, a simple nervous system of 302 neuronal cells and a fairly simple anatomy of 959 somatic

cells, these nematodes exhibit diverse behaviours, such as chemotactic behaviour, oxygen sensing, social behaviour, etc. Furthermore, its rapid development of 3-4 days (Fig. 1.4), short life span of 2-3 weeks, small body size, rapid reproduction and large number of offspring per adult result in a fast subsequence of generations and large number of nematodes, leading to high-throughput screening for laboratory toxicity testing and standardization of experiments (Khanna et al. 1997; Leung et al. 2008; WormAtlas 2002-2018). Due to its life history traits and its known genome, this model organism can be used for studies ranging from the molecular (protein) level to the population level, which are urgently needed in toxicity studies.

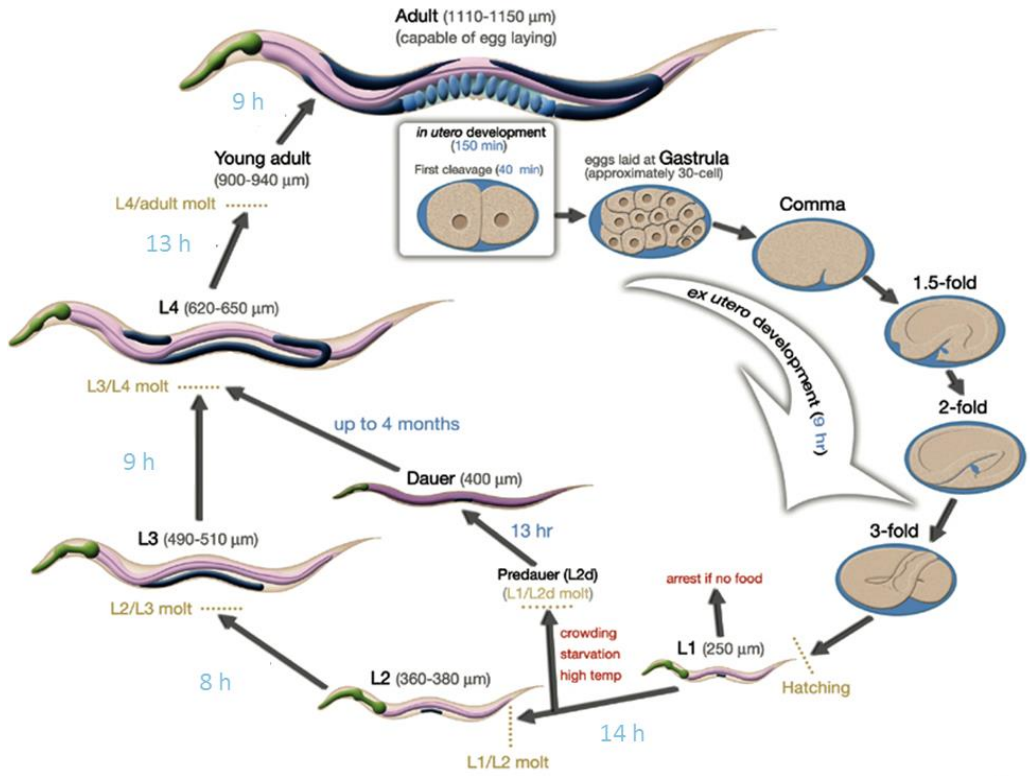

Figure 1.4. Life cycle of *C. elegans* at 20°C (WormAtlas, 2002-2018)

For the N2 strain of *C. elegans,* maintained at 20°C in hanging drops of semi-fluid culture medium, Muschiol et al. (2009) concluded that the minimum and mean generation time were 73 h and 90 h respectively. Furthermore, time from egg deposition to hatching was 7.3 h, age at first egg deposition 73 h, age at maximum egg-laying 108.2 h and lifespan was 16.7 days (Muschiol et al., 2009). Moreover, *C. elegans* is not expensive to maintain under laboratory conditions. To perform toxicity tests with *C. elegans,* different media have been used: soil, agar plates, sediment samples and liquid medium (Popham and Webster 1979; Dhawan et al. 2000; Korthals et al. 2000; Höss et al. 2001; Álvarez et al. 2005; Harvey et al. 2008; Muschiol et al. 2009; Sutphin and Kaerberlein 2008; Lira et al. 2011; Nørhave et al. 2001; Kumar et al. 2016). Although Nematode Growth Medium (NGM) agar plates are generally used for culturing *C. elegans*, they have some disadvantages, such as *C. elegans* crawling up the plate wall due to desiccation. In addition, due to solubility limitations, the bacterial lawn (*Escherichia coli* as feeding source) is located on top of the surface of the agar plates and nematodes reside on top of the agar, whereas bacteria, nematodes and compounds are more mixed in liquid medium (Williams and Dusenbery, 1990). Therefore, liquid medium (K-medium), representing the interstitial pore water within the soil, is more convenient for metal toxicity testing. Since *C. elegans* can survive in deionised water supplemented with NaCl and KCl, K-medium was used as test medium in this thesis to reduce the interaction of metals with ligands and thus preclude precipitation.

1.7. Objectives and thesis outline

Although metals (mainly) occur in mixtures in natural environments, metal effects are usually studied for each metal separately. Furthermore, the current regulations only take into account the occurrence of single metals. Metal mixture studies are therefore necessary to provide a solid scientific basis for setting standards for environmental protection and risk assessment. The first objective of this thesis was to gain insight into

the toxicity of single metals (Zn, Cu, Cd) and to investigate whether and how metal toxicity changes in mixtures.

Metal effects can be studied at different organisational levels from molecular to population level and beyond. Combining effects at different levels is necessary to better understand the observed toxicity. However, the number of studies combining effects at different organisational levels is still small. The life history traits of *C. elegans* and its known genome, make it possible to study toxicity effects at different organisational levels. As a second objective the effects on multiple endpoints ranging from the molecular level to the individual level and even to the population level of *C. elegans* are studied and compared with each other.

Thesis outline

In the **first part** of this thesis the sensitivity of *C. elegans* to the selected metals (Cu, Cd, Zn) is determined, therefore differences between exposure concentrations are measured and changes of the toxic effects over time are analysed. Furthermore, it is investigated whether and how these sensitivities are affected by each other in mixture exposure scenarios, with special attention to the ZnCd mixture. In this study different endpoints are assessed on the individual and population level.

Subsequently, the bioavailability and accumulation of these metals in the body of *C. elegans* are measured to link metal speciation and body burden to the observed toxic effects.

Finally, a last aim is to obtain mechanistic insights (mode-of-action) into single metal and mixture toxicity at the molecular level. Different omics approaches are used in an attempt to investigate whether single metal and mixture interaction effects, observed at the morphological, behavioural and physiological level could be explained (and predicted) by induced molecular changes.

Chapter 1 contains a general introduction on environmental pollution by metals, their bioavailability and their toxicity effects on organisms. Finally, the role and benefits of *C. elegans* as a laboratory test organism are described.

The toxic effects of Cu, Cd and Zn, both as single metals and in mixtures, are analysed in **chapter 2** by the study of lethality changes over time, based on dose-response curves. The LC20 value i.e. the lethal concentration that kills 20% of the test organisms, is used to measure locomotor behaviour (crawling speed on agar plates and thrashing behaviour in liquid medium) and to investigate the degree of modulating or impairing (chemo)sensory capacities. All mixtures consist of combinations of LC20 values of the corresponding single metals. In contrast to the short-term experiments (24 h and 48 h) in chapter 2, **chapter 3** deals with experiments at population level and individual level, conducted over several days. Chronic exposures are more likely to occur in natural aquatic ecosystems and are therefore more relevant. In the first part of this chapter, body length and time effect on population size (10-12 days) are analysed for exposures to the lethal concentrations LC5 and LC20 of Cu, Cd and Zn (as single metals and in combinations). In the second part, population size and mortality of nematodes, exposed to a range of LC concentrations of Zn in combination with an LC20 concentration of Cd is measured, to better understand the mitigating effect of Zn on Cd.

In **chapter 4**, it is tested whether the metals can be taken up by *C. elegans* and whether they compete with each other to enter the *C. elegans* body, by comparing the metal accumulation (mg metal/g wormpellet) of nematodes exposed for 24 h to single metals and to their mixtures. The effect of *E. coli* on the metal speciation was tested by measuring the free metal ion concentration of Zn, Cu and Cd and their mixtures for 12 days, both in the presence and absence of *E. coli* bacteria. The results of body burden and free metal concentration are combined as an attempt to understand the observed toxic effects of chapters 2 and 3.

Since insights into the molecular mechanisms can help to understand the metal toxicology, differential proteomics are performed in **chapter 5**. The aim of this study is

to get at least the picture of the biochemical pathways that are affected by exposure to LC10 of Cu and Cd (as single metals and in combination). Last but not least, an exploratory peptidomics study is performed to further unravel the peptidome of non-exposed mixed stage *C. elegans* nematodes.

Finally, data from the different chapters are integrated and discussed together in **chapter 6**. This chapter summarizes the main findings of this thesis and provides future research perspectives.

Chapter 2

Mixture effects of copper, cadmium and zinc on mortality and behaviour of *C. elegans*

Moyson S., Vissenberg K., Fransen E., Blust R., Husson SJ. Mixture effects of copper, cadmium and zinc on mortality and behaviour of *C. elegans.* Published in *Environmental Toxicology and Chemistry* (2018) 37 (1): 145-159.

Abstract

The toxic effects of zinc (Zn), copper (Cu), and cadmium (Cd), both as single metals and in combination, were examined in the nematode *Caenorhabditis elegans*. Metal effects on mortality were analysed in a time-dependent manner using different concentrations in K-medium. To investigate the effects on locomotion and chemosensation, concentrations inducing 20% mortality (LC20) were used. The results showed that Cu was more toxic to *C. elegans* than Cd and Zn, resulting in higher mortality and a more reduced locomotion. Mortality increased over time for all metals. When Cd was added to Cu, and vice versa, significant increases in toxicity were noted. Different interactions between the metals were observed for the mixtures ZnCd, ZnCu, CuCd, and ZnCuCd. Zinc seemed to have a neutral effect on Cd toxicity, while in combination with Cu, a similar additive effect was seen as for the CuCd combination. Binary and tertiary metal mixtures caused a strong decrease in locomotion, except for the ZnCd combination, where Zn seemed to have a neutral effect. After $LC20_{24\ h}$ exposure, reduced crawling speed (except for Zn) and reduced thrashing behaviour (except for Zn and the ZnCdmixture) were observed. Almost no significant effects were observed on chemosensation. Because the same trend of mixture effects was noted in locomotion and in mortality tests, locomotion can probably be considered a sensitive endpoint for metal toxicities.

Keywords: Mixtures, *Caenorhabditis elegans,* Metals, Behavioural toxicology, Mortality

2.1. Introduction

Metal accumulation in soil is caused by both industrial and natural sources. Moreover, the number of anthropogenic sources of heavy metals in soil has increased during the past decades, leading to higher metal concentrations in aquatic environments, especially in mining and industrial areas (Peredney and Williams, 2000). Exposure to these metals can lead to serious health hazards for diverse animals, including humans, and results in a persistent (eco)toxicological concern. It is well known that metals can cause neurotoxicity, alter neuronal excitability, and impair chemosensation, and that they are involved in neurodegenerative diseases (Mathie et al., 2006; Wright and Baccarelli, 2007). Furthermore, free radicals and reactive oxygen species generated by metals can induce protein and deoxyribonuclease (DNA) oxidation and lipid peroxidation (Caito et al., 2012). They also disrupt cellular homeostasis by impairing DNA repair, inhibiting enzyme activity, and affecting protein binding (Martinez-Finley and Ashner, 2011). In general, cadmium (Cd) replaces zinc (Zn) in various proteins and causes cellular lesions and mitochondrial dysfunction, for example, while an overload of the essential metal copper (Cu) can lead to protein damage, cellular injury, and structural impairment of essential metal binding sites (Valle and Ulmer, 1972; Popham and Webster, 1979; Goldstein and Czapski, 1986; Alt et al., 1990). An excess of Zn, another essential element, can cause multiple biological defects affecting life span, reproduction, development, and so on (Wang et al., 2007; Chen et al., 2013).

Soil nematodes, living within the interstitial waters of soil particles, are in direct contact with dissolved contaminants and play a major role in nutrient cycling and dynamics by feeding on bacteria and fungi. In these soil nematodes, a reduction in movement (Anderson et al., 2003; Boyd et al., 2003), feeding (Boyd and Williams, 2003; Boyd et al., 2003), reproduction (Jonker et al., 2004a; Wang et al., 2007; Höss et al., 2011), bioluminescence (Lagido et al., 2009), and growth, delay in egg laying (Popham and Webster, 1979; Power and de Pomerai, 1999), and increased generation time (Calafato et al., 2008) was noted after Zn, Cu, and/or Cd exposure. Compared with the nematodes

Pristionchus pacificus and *Panagrellus redivivus*, *Caenorhabditis elegans* shows an intermediate toxicity response to Cu in aquatic and soil tests, making *C. elegans* a representative of other rhabditid species for toxicity testing (Boyd and Williams, 2003). However, it is not yet clear whether the main uptake route for metals is from dietborne or water-borne exposure. Höss and co-workers (2011) suggested that the toxic effects are the result of "aqueous cadmium," taken up together with the bacteria, rather than bacterial-bound or total Cd concentrations. Bacteria may facilitate uptake of dissolved Cd by stimulating pharyngeal pumping; however, they may also adsorb significant amounts of Cd, thereby potentially lowering the Cd toxicity. Höss et al. (2011) could not exclude the possibility that food-associated Cd also had a partly toxic effect. Likewise, Offermann et al. (2009) found no straightforward relationship between internal response and Cd bioaccumulation. In contrast to Höss et al. (2011), dietary exposure was found to play the major role in Cd bioaccumulation and internal availability, whereas aqueous exposure was a less important contributor.

The bacterivorous nematode *C. elegans*, ubiquitous in soil, has a considerable ecological value and is of great importance in laboratory toxicity tests. Its rapid growth, small body size, and fast reproduction enhance high-throughput screening (Leung et al., 2008). Because mixture toxicity tests generate many samples in a semifactorial design, a rapid and simple manipulation of test organisms is needed. Furthermore, *C. elegans* can respond to aversive stimuli such as certain odorants (e.g., repellent 1-octanol), touch sensation, and a variety of toxic compounds, such as metals (Tobin and Bargmann, 2004) via a number of sensory neurons in its head and tail and via mechanosensory neurons distributed along its body (Ward et al., 1975). Because of its centralized nervous system consisting of only 302 neurons, *C. elegans* can be used for assessing behavioural changes. Although the *C. elegans* nervous system is simple, it shares all basic features with those of higher animals, including humans (Dhawan et al., 2000). Metal toxicity research with *C. elegans* has been performed in soil, on agar plates, in sediment samples, and in liquid medium, representing the interstitial porewater within the soil (Popham and Webster, 1979; Dhawan et al., 2000; Korthals et al., 2000; Höss et al.,

2001). However, in contrast to our increasing understanding of the toxic effects of single metals, much less is known about their effects on interaction, which frequently occurs in the natural environment. Because each metal may affect a variety of metabolic pathways, causing specific toxic effects (Chu and Chow, 2002), interactive effects of metals in mixtures can be additive, antagonistic, or synergistic, all resulting in different toxicity responses. Unfortunately, because of their complex relationships in biological systems, no consistent explanation for the effect of metals in interactions exists. Metal mixture toxicity studies with soil invertebrates are still scarce, although a few studies have been performed with mixtures of Cu, Cd, or Zn with earthworms (Weltje, 1998), *Enchytraeus sp*. (Lock and Janssen, 2002), *Folsomia candida* (Van Gestel and Hensbergen, 1997), and *C. elegans* (Jonker et al., 2004b; Lagido et al., 2009). Furthermore, unlike mortality, reproduction, body length, egg production, and stress protein production, locomotion has been less evaluated as a toxicological endpoint for both single metals and mixtures (Power and de Pomerai, 1999; Dhawan et al., 1999, 2000; Roh et al., 2006; Höss et al., 2011).

The aim of the present study was to gain insight into the sensitivity of *C. elegans* to the selected metals (Cu, Cd, Zn) and to investigate whether and how these sensitivities are affected by each other in mixture exposure scenarios. To assess different endpoints, we fully exploited the benefits of *C. elegans* as a unique model for both fundamental neuroscience and (eco) toxicology (Brenner, 1974). Young fed adult nematodes were exposed for 2, 8, 24, and 48 h to different concentrations of Cu, Cd, and Zn (as single metals and in combination) to analyse mortality effects over time, based on dose–response curves, and to gain insight into mixture toxicity. Locomotor behaviour was evaluated by measuring crawling speed on agar plates and thrashing behaviour in liquid medium. Furthermore, the degree of modulating or impairing (chemo)sensory capacities as a result of metal exposure was investigated. For the above locomotory and chemosensation tests, the effects of single metals and mixtures were assessed using the lethal concentration, 20% (LC20) of single metals. Behavioural responses were compared with mortality, and finally these behavioural responses were determined as

an endpoint for assessing toxic effects on *C. elegans*, exposed to single metals and their mixtures.

2.2. Materials and methods

2.2.1. *Caenorhabditis elegans* culture

The wild-type N2 strain of the nematode *C. elegans* was obtained from the Caenorhabditis Genetic Center (Minneapolis, MN, USA). The nematodes were maintained at 20°C on nematode growth medium agar plates, seeded with *Escherichia coli* (OP50 strain) as a food source. Synchronization of the nematodes was achieved by bleaching: a treatment of mixed stage nematodes with a hypochlorite solution (5N NaOH, 8% sodium hypochlorite) kills the nematodes that are not protected by an eggshell. Eggs were raised on OP50-seeded nematode growth medium plates. Young L4 nematodes were transferred to a 24-well plate filled with 900 mL K-medium (52mMNaCl, 32mM KCl, 5mg/mL cholesterol, pH 5.1) (Williams and Dusenbery, 1990), supplemented with *E. coli* OP50 (1.5 g/L), and under gentle shaking conditions (160 rpm) incubated at 20°C for 24 h.

2.2.2. Test media and experimental set up

Dilution series of Cd (0, 2.5, 5, 10, 100, 400, 800, and 1200 mg/L), Cu (0, 0.05, 0.5, 2.5, 25, 50, 100, and 200 mg/L), and Zn (0, 5, 10, 25, 50, 100, 200, and 400 mg/L) were made from $CdCl_2$.2.5H2O (Alfa Aesar), $CuCl_2$. $2H_2O$ (Merck) and $ZnCl_2$ (Alfa Aesar) in K-medium. Metal concentrations were verified (typically 93–108% recovery) by high resolution inductively coupled plasma mass spectrometry (Element XR, Thermo Fisher Scientific). The metal loading in our experiments, expressed in terms of mg/g of bacteria, is in line with reported metal contents of polluted soils that have been used for toxicity studies with *C. elegans* (Höss et al., 2009). Prior to toxicity testing, metal solutions were incubated with the bacterial suspensions for 12 h at 4°C to allow metal partitioning

between the aqueous phase and the bacteria. After the incubation period, 100 mL of test medium (K-medium containing metal concentration) or control (K-medium) was added to the 24-well plate, bringing the total volume of each well up to 1 mL. Three replicates were made for each concentration. For each sample of each experiment, the pH determined before (5.1 ± 0.1) and after the experiment (5.2 ± 0.3) was within an acceptable pH range for *C. elegans*, excluding its potential effect on the measured parameters.

2.2.3. Mortality tests

Approximately 10 nematodes/replicate were used for each of the following mortality tests.

2.2.3.1. Single metal

After 0, 2, 8, 24, and 48 h of exposure to either Cu, Cd, or Zn, the number of living nematodes was counted in each well using a stereomicroscope (Nikon AZ100). As mentioned in "Test media and experimental set up", 8 different concentrations of each metal were used. Nematodes that were not moving or did not respond to gentle plate shaking were considered to be dead. The critical metal concentration at which survival was significantly influenced was determined as well as the strength of the decrease of survival. Furthermore, LC10, LC20, and LC50 values were determined for each metal.

2.2.3.2. Metal mixture

For the mortality test of the CuCd mixture, all combinations of the concentrations used for single-metal mortality were tested, except for the 2 highest concentrations of both metals. The number of living nematodes was counted in each well after 0, 2, 8, 24, and 48 h of exposure using a stereomicroscope (Nikon AZ100). Nematodes that were not moving or did not respond to gentle plate shaking were considered to be dead. We

determined how survival, at a given concentration of the first metal, was changed by adding the second metal and whether there was an interaction between the 2 metals.

To examine whether metal mixtures led to higher toxicities compared with individual compounds and which interactive effects of metals occur in mixtures, the LC20 of metal A was added to the LC20 of metal B. This was determined for both LC20 of 24 h ($LC20_{24\ h}$) and for LC20 of 48 h ($LC20_{48\ h}$) of Cu, Cd, and Zn (Table 2.1). A mortality rate of approximately 40% for binary mixtures and approximately 60% for tertiary mixtures was expected if the metals would have a simple additive effect[1]. Deviations of these values may be the result of interactions between the 2 metals because of common cellular targets or uptake mechanisms. We defined a synergistic interaction as a combination of 2 metals having a significantly 25% higher mortality rate than expected (Chu and Chow, 2002). An antagonistic effect was suggested if the mortality rate was significantly 25% lower than predicted. Mortality rates of mixtures of $LC20_{24\ h}$ after 48 h of exposure were compared with the sum of the mortality rates of the corresponding single metals.

2.2.4. Locomotion

For the locomotor analyses, nematodes were exposed for 24 h to $LC20_{24\ h}$ and for 48 h to $LC20_{48\ h}$ of the 3 metals (Table 2.1). Mixtures of CuCd, ZnCu, ZnCd, and ZnCuCd, made by combinations of the LC20 concentrations of the single metals, were tested. For each treatment 3 replicates were used.

2.2.4.1. Average crawling speed on solid medium

After 24 and 48 h, approximately 20 nematodes were washed with K-medium and transferred to small nematode growth medium plates, seeded with *E. coli* (OP50 strain). After 30 min, nematode locomotion was recorded for 1 min (15 frames/s) by video tracking with a camera (Nikon DS-Ri1), attached to a stereomicroscope (Nikon AZ100).

1: For a thorough discussion about the addition of LC values, see Chapter 6, section 6.1 page 164.

Using the plugin wrMTrck in the image analysis program ImageJ, nematodes appeared as dark objects on a bright background. To avoid any other dark objects, a specific threshold was selected and subsequently the average crawling speed of each nematode was calculated. Dead nematodes were excluded from the analysis.

2.2.4.2. Thrashing behaviour in liquid medium

A thrash is defined as a change in the direction of midbody bending (Chen et al., 2013). After 24 and 48 h of metal exposure, the number of thrashes in 30 s was counted for each nematode in test medium. Therefore, their movement was recorded for 30 s (15 frames/s) by video tracking with a camera (Nikon) attached to a stereomicroscope (Nikon AZ100). The video was manually analysed. The number of full body bends was also quantified for each nematode exposed during 48 h to $LC20_{24\,h}$.

2.2.5. Chemosensation

For the chemosensory analyses, nematodes were exposed for 24 h to $LC20_{24\,h}$ and for 48 h to $LC20_{48\,h}$ of Cu and Cd (Table 2.1). The CuCd mixture, made by the combination of the LC20 concentrations of the single metals, was also tested.

2.2.5.1. Chemotactic index

For this test, nematodes were exposed in Falcon tubes instead of 24-well plates. After metal exposure, nematodes were washed with K-medium, and approximately 30 nematodes were transferred to a 5-cm agar plate seeded with OP50 *E. coli*. The plate was divided into quadrants. Opposite quadrants contained the test solution (1-octanol) or the control solution (K-medium). Nematodes and solutions were added according to the protocol of Margie et al. (2013).

After 60 min, the number of nematodes in each quadrant was counted under the microscope (Leica S8APO), and the chemotactic index (CI) was calculated as

$$\mathrm{CI} = \frac{\sum (\mathrm{T1+T2}) - \sum (\mathrm{C1+C2})}{\sum(\mathrm{T1+T2+C1+C2})}$$

where T1 and T2 refer to the number of nematodes in the test quadrants and C1 and C2 to the number of nematodes in the control areas. The closer the chemotactic index is to –1, the stronger the avoidance to the odorant. A positive chemotactic index suggests that nematodes are attracted to the odorant, while the compound is neutral to the animals when chemotactic index is close to zero. The test was conducted twice and for each treatment 6 replicates were used. Dead nematodes were excluded from the analysis.

2.2.5.2. Drop test

Nematodes were washed after the exposure period, and approximately 10 nematodes were transferred to a 9-cm agar plate. For each treatment 3 replicates were used. A small drop of 1-octanol was placed approximately 0.3 mm before the head of a moving nematode. Drops were delivered using 2-mL glass capillaries (Drummond Scientific). The reaction time between drop placing and the start of moving backward was measured for each living nematode. Therefore, these tests were performed by video tracking at 10 frames/s with a camera (iDS GigE uEye RE), attached to a microscope (Leica S8APO). The video was manually analysed with the software program VirtualDub 1.10.4.

2.2.6. Statistical analysis

Data were analysed with the statistical program R, Ver 2.13.1, with a 5% level of significance.

2.2.6.1. Mortality

To determine the critical concentration of each metal, 95% confidence intervals were made for the survival fractions at the different time points. Then, for each metal concentration and for each time point it was checked whether the 95% confidence interval included the fraction of 95% survival.

Linear mixed models were fitted to test the possible effects of exposure time and metal concentrations and their interaction on the fraction of surviving animals. In all models, the survival fraction was entered as a dependent variable. Exposure time and metal concentration, plus their interaction, were included as fixed effects. Observations with 100% survival (time=0 h and control group) were omitted from the analysis. Because the survival fraction was repeatedly measured over time within the same wells, observations from the same well were not independent. To account for this dependence, a random intercept term for well was added to the model. Significance of the interaction term was calculated using an F test with a Kenward–Roger correction for degrees of freedom. When the interaction between exposure time and metal concentration was significant, separate regression models were fitted for the different exposure times, and the slopes of the regression lines with 95% confidence intervals were calculated at different exposure times. Finally, LC10, LC20, and LC50 values (24 and 48 h) of the single metals were determined by fitting dose–response curves. The main effects of metal exposure, exposure time, and their interaction on LC values were analysed by a two-way analysis of variance (ANOVA). Subsequently, pairwise differences between the groups were calculated, with a Tukey honest significant difference correction for multiple hypothesis testing. To test for the interaction between Cu and Cd, linear regression models were fitted. At each exposure time, multiple linear regression models were fitted to investigate whether the decrease in survival fraction with increasing $\log_{10}$ [Cu concentration] was altered by the Cd concentration and vice versa. First, linear regression models were fitted with the $\log_{10}$ [Cu concentration] and the Cd concentration as independent variables, plus their interaction. The interaction

term in these models tests whether the effect of $\log_{10}$ [Cu concentration] is the same across all Cd concentrations. If this term was significant, separate models were fitted with $\log_{10}$ [Cu concentration] as independent variable, at each separate concentration of Cd. The above calculations were repeated swapping Cd and Cu concentration, specifying Cd concentration as the independent variable at a fixed value of the $\log_{10}$ [Cu concentration].

Separate models were fitted for each exposure time. Control observations were omitted from the analysis. Again, regression models were fitted to estimate the slopes of the regression lines with 95% confidence intervals at different exposure times.

Finally, dose–response curves were fitted using the 2- parameter log-logistic model to estimate LC10, LC20, and LC50 values. These curves were fitted using the drm() function (R core team, 2014) in the drc package (Ritz and Streibig, 2005) in R. A nonparametric ANOVA, Kruskal–Wallis test, followed by pairwise comparison testing with Bonferroni correction was performed to determine whether the mixtures of LC20 concentrations led to significant higher mortalities than the individual metals. To test which interactive effects occur in the mixtures, the observed mortality rates were compared with the expected values using the Wilcoxon test.

2.2.6.2. Locomotion

The main effects of metal exposure, exposure time, and their interaction on the average speed of the nematodes were analysed by a two-way ANOVA. Comparisons within treatment were carried out by a one-way ANOVA. Subsequently, a Tukey honest significant difference test was used to determine the differences between groups. For thrashing behaviour, the effects of metal exposure on $LC20_{24\ h}$ (measured at 24 and 48 h) and $LC20_{48\ h}$ were analysed separately. For the $LC20_{24\ h}$ values, the main effects of metal exposure and exposure time were analysed by two-way generalized linear model procedures (quasi-Poisson distribution). One-way generalized linear model procedures were used for $LC20_{48\ h}$ concentrations (quasi-Poisson distribution). Subsequently, a

Tukey honest significant difference test was used to determine the differences between groups.

2.2.6.3. Chemosensation

Absolute data of the quadrant tests were used and a reciprocal root transformation of data from the drop test was applied. The main effects of metal exposure on the chemosensory capacities of the nematodes were analysed by a one-way ANOVA separately for $LC20_{24\,h}$ and $LC20_{48\,h}$. Subsequently, a Tukey honest significant difference test was used to determine the differences between groups.

2.3. Results

2.3.1. Mortality

2.3.1.1. Single metals

For all metal concentrations, mortality rate increased along with the length of the exposure period (Supplemental Data, Fig. S2.1). Zinc-exposed nematodes showed a stepwise decrease of surviving fraction, which was significant for concentrations starting from 200 mg/L at 2 h, 50 mg/L at 8 h, 10 mg/L at 24 h, and 5 mg/L at 48 h (Fig. 2.1A). Nematodes exposed to Cu for 2 and 8 h had a significant lower survival from 25 mg/L onward, except for one measurement (50 mg/L after 2 h). After 24 h, survival was already declining from 2.5 mg/L, while after 48 h of exposure all Cu-exposed nematodes showed a lower survival fraction (Fig. 2.1B). Cadmium did not alter survival after 2 h of exposure, except for the 10 mg/L concentration. For the other time points, survival was significantly lower than control starting from 2.5 mg/L (Fig. 2.1C). For each metal, the interaction between exposure time and concentration was highly significant for the regression slopes ($P<0.001$).

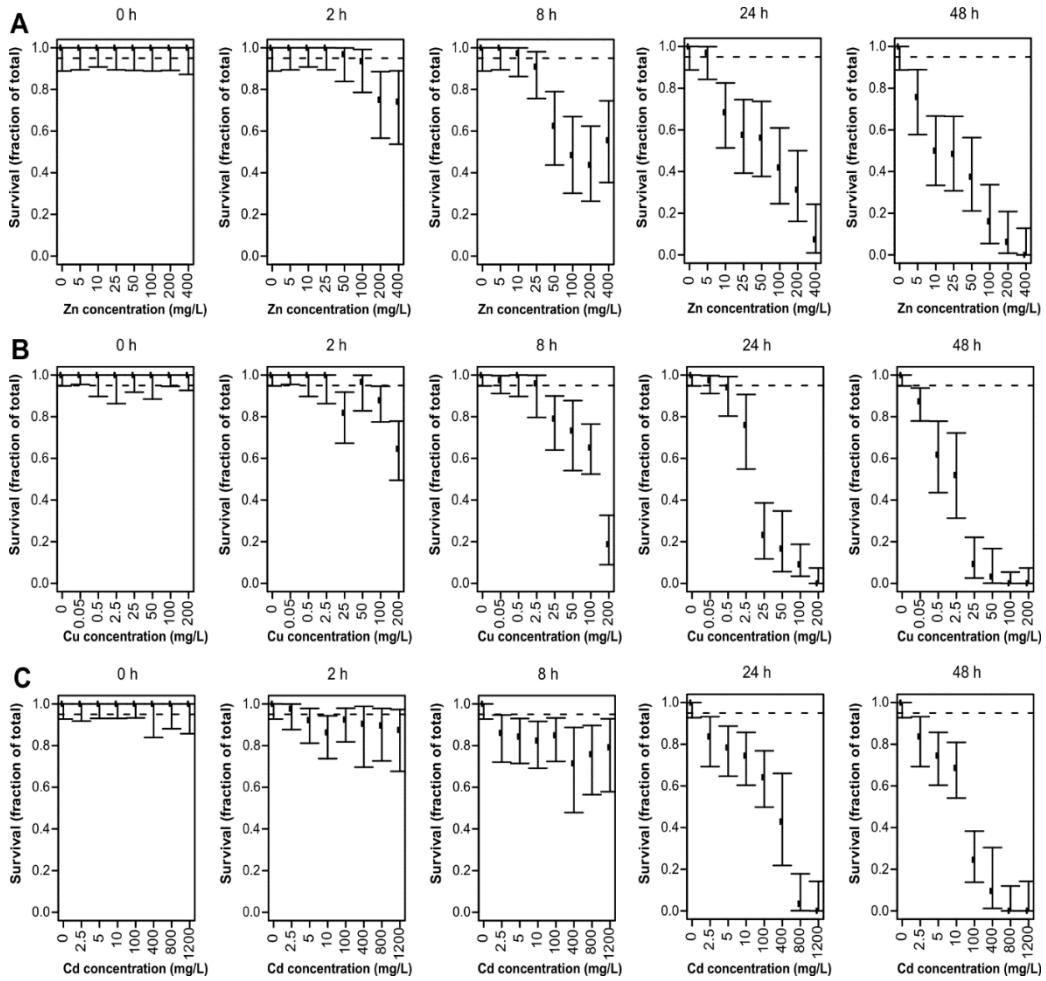

Figure 2.1: Concentration–response relationship for exposure to zinc (Zn, A), copper (Cu, B) and cadmium (Cd, C) at different exposure times. Dotted line denotes 95% survival. Survival proportions are shown with their 95% confidence intervals represented as error bars (n=3).

All regression coefficients were negative, meaning that the survival fraction declined with increasing concentration. Except for Cd at exposure times 2 and 8 h and for Cu at 2 h, a highly significant effect of metal exposure on the survival fraction was noted (P<0.001). For Zn, a highly significant effect of metal exposure on the fraction of survival was observed for each exposure time (P<0.001). The regression slope of Zn was always the steepest, although not significantly different from Cu at 24 h and from Cd from 24 h

onward. Cadmium had the least steep regression slope, but it was not significantly different from Cu (and from Zn from 24 h onwards); at 24 h Cu had a similar slope as Cd (Fig. 2.2 and Supplemental Data, Table S2.1).

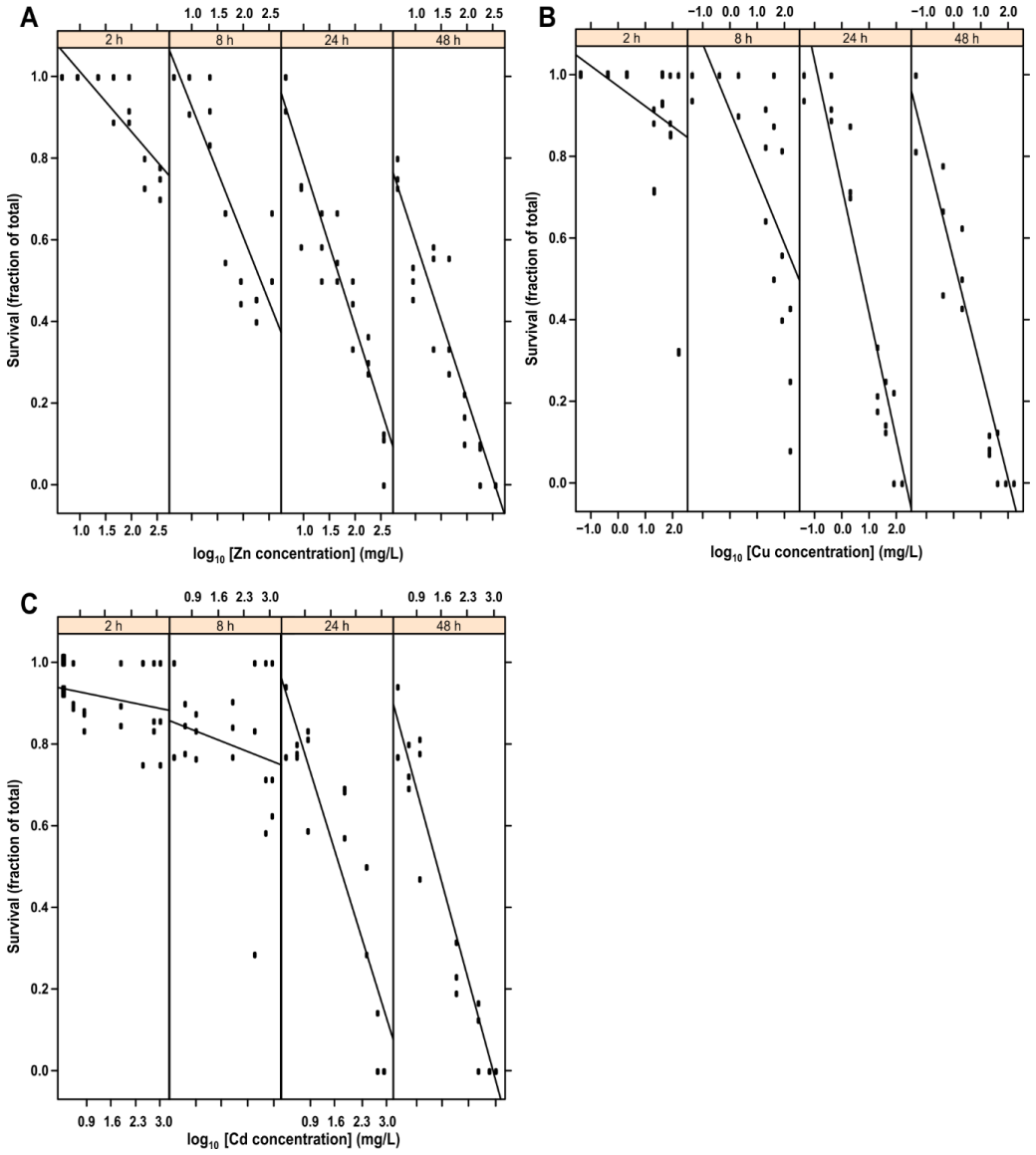

Figure 2.2: Scatterplots of the fraction of survival plotted against the $\log_{10}$ [metal concentration]. Solid lines represent the linear regression slopes of zinc (Zn, A), copper (Cu, B), and cadmium (Cd, C) at different exposure times (n=3).

Compared to Cd, the LC50 value of Cu was 91.5% lower after 24 h of exposure and even 95.8% lower after 48 h of exposure (P<0.001; Table 2.1). Compared to Zn, Cu had a LC50 which was 88.1% lower after 24 h and 94.6% lower after 48 h (P<0.001). The LC50 concentration of Zn was 28.7% (24 h) and 21.1% (48 h) lower than the LC50 of Cd, which was not significant. The lethal concentrations of the metals Zn, Cu, and Cd significantly decreased over time by 68.3, 85.7, and 71.3%, respectively (Table 2.1).

Table 2.1: Concentrations of Zn, Cu, and Cd, inducing a lethality of 10% (LC10), 20% (LC20) and 50 % (LC50) after 24 and 48 h of exposure.

		LC10		LC20		LC50	
	Time (h)	(mg/L)	(mM)	(mg/L)	(mM)	(mg/L)	(mM)
Zn	24	3.528 ± 1.488	0.054 ± 0.023	9.501 ± 2.841	0.145 ± 0.043	51.689 ± 9.746	0.791 ± 0.149
	48	1.481 ± 0.670	0.023 ± 0.010	3.596 ± 1.213	0.055 ± 0.019	16.380 ± 3.112	0.251 ± 0.048
Cu	24	0.523 ± 0.203	0.008 ± 0.003	1.299 ± 0.409	0.020 ± 0.006	6.146 ± 1.393	0.097 ± 0.022
	48	0.051 ± 0.020	0.001 ± 0.001	0.146 ± 0.047	0.002 ± 0.001	0.884 ± 0.221	0.014 ± 0.003
Cd	24	1.696 ± 0.802	0.015 ± 0.007	7.110 ± 2.315	0.063 ± 0.021	72.476 ± 18.681	0.644 ± 0.166
	48	1.600 ± 0.525	0.014 ± 0.005	4.121 ± 1.052	0.036 ± 0.009	20.765 ± 3.971	0.185 ± 0.035

2.3.1.2. Metal mixtures

For the CuCd mixture, all regression coefficients were negative, meaning that the fraction of survival declined with increasing metal concentrations. When Cd was added to Cu, a significant increase in toxicity was noticed 2 h after 10 mg/L Cd was added (P<0.05) and 8 h after 0 (P<0.05), 2.5 (P<0.01), 10 (P<0.05), and 400 mg/L (P<0.001) Cd was added to Cu. After 24 and 48 h, every Cd concentration that was combined with Cu induced a highly significant decrease in survival rate (P<0.001; 400 mg/L 48 h, P<0.01). The interaction between Cd concentration and $\log_{10}$ [Cu concentration] was significant after 8 h (P<0.01) and 48 h (P<0.001) (Fig. 2.3 and Supplemental Data, Fig. S2.2 and Table S2.2). Similarly, the decrease in survival fraction with increasing $\log_{10}$ [Cd concentration] was altered by the Cu concentration (Fig. 2.4 and Supplemental Data, Fig. S2.3 and Table S2.3). A significant effect on the regression slope was noted 2 h after 0.5 mg/L Cu (P<0.01) and 8 h after 25 mg/L Cu (P<0.001) were added to Cd. After 24 h of exposure, all combinations of Cd with 0 (P<0.001), 0.05 (P<0.01), 0.5 (P<0.001), 2.5 (P<0.001), 25 (P<0.01), or 50 (P<0.05) mg/L Cu caused a decrease in survival. When concentrations of 0 to 2.5 mg/L Cu were combined with Cd, an increase in mortality was observed after 48 h of exposure (P<0.001). A significant interaction between Cu concentration and $\log_{10}$ [Cd concentration] was seen after exposure periods of 8 (P<0.05), 24 (P<0.05) and 48 h (P<0.001). Curiously, the intercept on the y-axis was very different for the various regression lines. This means that in the absence of one metal, the presence of the other metal already had a strong effect on survival. All the regression slopes were similar when Cu or Cd was added to the other metal. Except for the combinations of the 2 highest concentrations of one metal with the other, a trend was seen where the regression slopes became steeper over time (Supplemental Data, Tables S2.2 and S2.3).

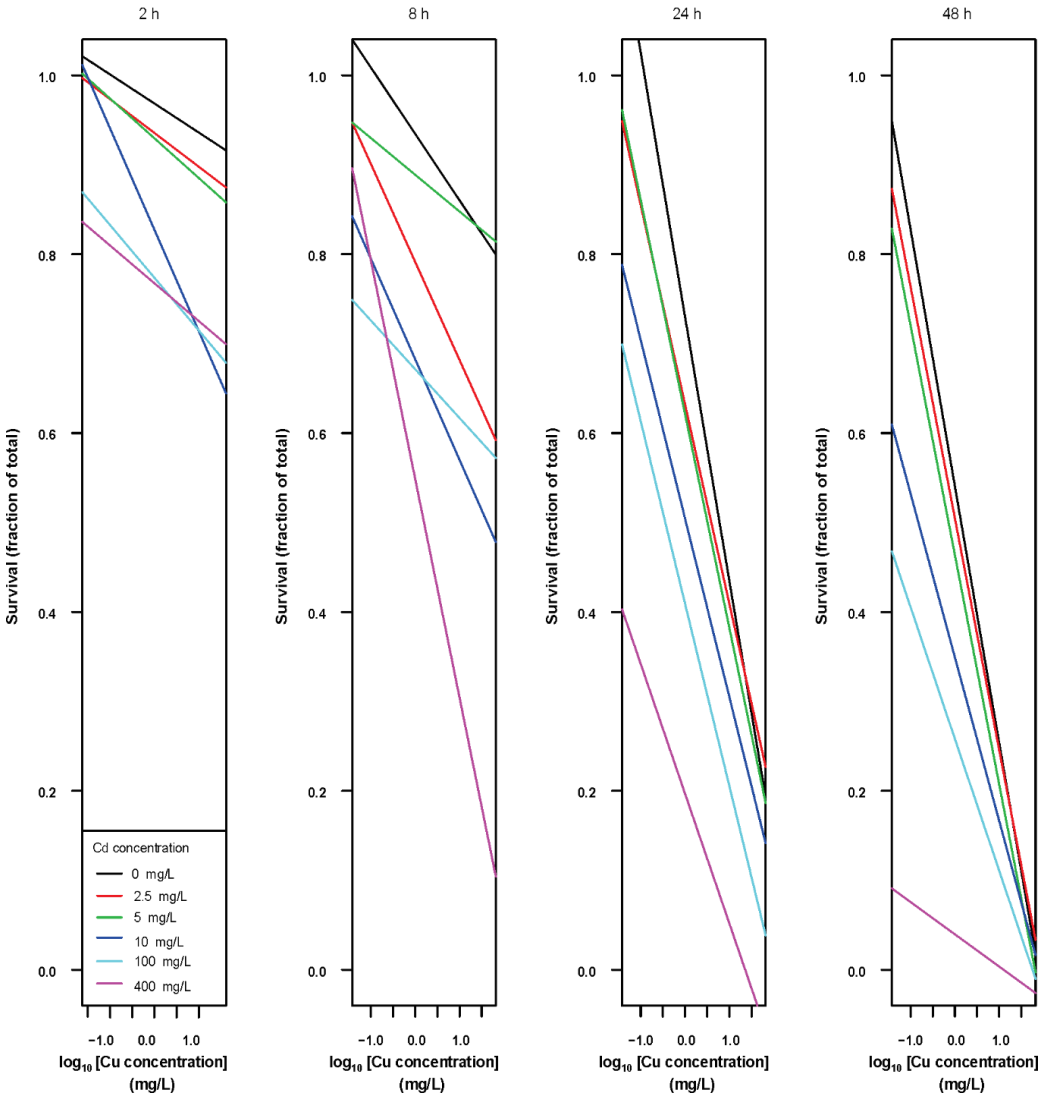

Figure 2.3: Regression slopes of the copper (Cu) and cadmium (Cd) mixtures at different exposure times. Graphs represent combinations when Cd was added to $\log_{10}$ [Cu concentrations] (n=3).

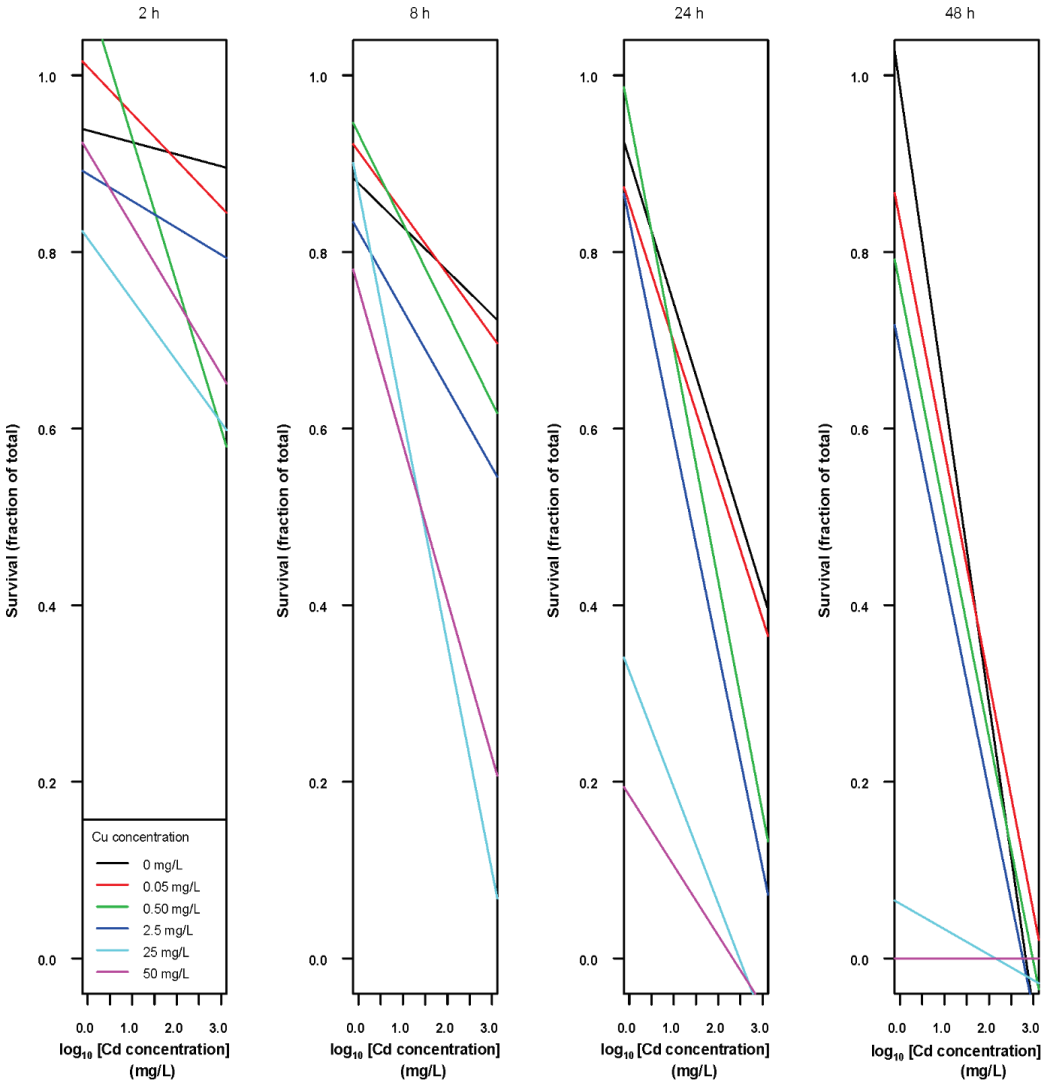

Figure 2.4: Regression slopes of the copper (Cu) and cadmium (Cd) mixtures at different exposure times. Graphs represent combinations when Cu was added to $\log_{10}$ [Cd concentrations] (n=3).

When Zn was added to Cu, mortality increased significantly (P<0.01; Fig. 2.5). The ZnCu mixture caused a higher mortality compared with Cu ($LC20_{24\ h}$ 24 h) and with both individual metals ($LC20_{48\ h}$ and $LC20_{24\ h}$ 48 h; P<0.05). However, Zn seemed to have a neutral effect on Cd, because no obvious increase in mortality was seen (Fig. 2.5). For both $LC20_{24\ h}$ (24 and 48 h) and $LC20_{48\ h}$, the observed mortality was 58.7 (16.5%), 45.3 (31.0%), and 56.3% (21.9%), respectively, lower than the expected mortality of 40% (P<0.05). In the CuCd mixture, a significantly higher mortality compared with the single metals was observed (P<0.05; $LC20_{24\ h}$ 24 and 48 h; Fig. 2.5), although only Cu had a significant lower mortality than the mixture of $LC20_{48\ h}$ of Cu and Cd (P<0.05). The mortality of the ZnCu and CuCd mixtures did not differ significantly from the expected value: mortality in these metal combinations suggests additive effects. The mortality rate of the tertiary ZnCuCd mixture was significantly higher compared with the 3 single metals (P<0.05; $LC20_{24\ h}$ 24 h and 48 h) and with Cu and Zn (LC2048 h; P<0.05). When the metals were combined in a tertiary mixture, an additive effect was seen after 24 h, but after 48 h (for both $LC20_{24\ h}$ and $LC20_{48\ h}$) this effect became antagonistic (P<0.05; Fig. 2.5).

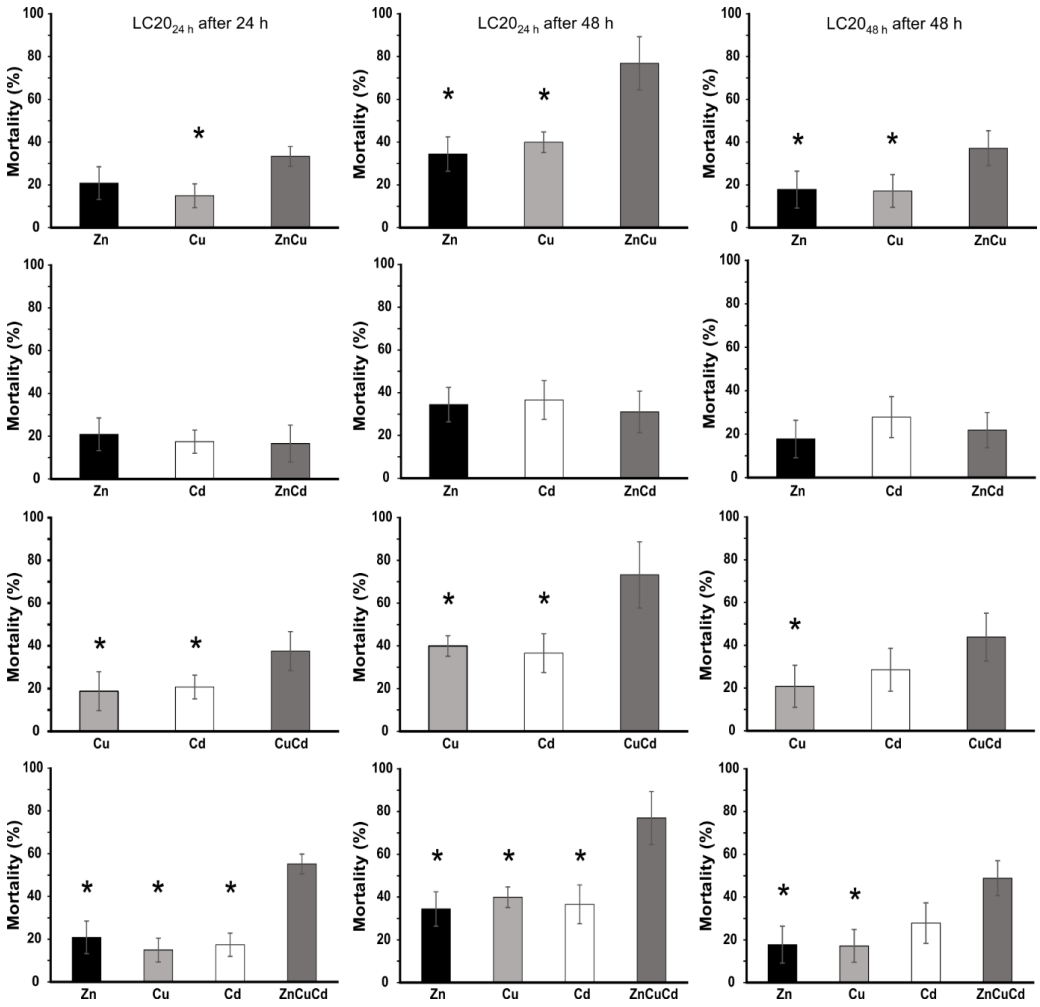

Figure 2.5: Mortality (%) of nematodes exposed to LC20 concentrations (20% lethality) of metals and their mixtures. Nematodes were exposed to the $LC20_{24\ h}$ concentrations for 24 h (left), 48 h (middle), or $LC20_{48\ h}$ for 48 h (right). Data are shown as mean ± standard deviation. The test was conducted twice with 3 technical replicates each. Asterisks (*) denote significant differences (P<0.05) compared with the mixture.

2.3.2. Locomotion

Metal exposure had a significant effect on the average speed and thrashing behaviour of *C. elegans* (P<0.001). A 24 h exposure to $LC20_{24\ h}$, induced a significant decrease in average crawling speed with 33.6%, 80.9%, 49.7%, 72.1%, 85.8% and 83.3% of nematodes exposed to Cu (P<0.05), Cd (P<0.001) and to the mixtures ZnCu (P<0.001), ZnCd (P<0.001), CuCd (P<0.001), and ZnCuCd (P<0.001) respectively (Fig. 2.6A). Exposure to Zn seemed to have no effect. Furthermore, the average crawling speed of nematodes exposed to the ZnCu and ZnCd mixture was lower than the ones treated with Zn (P<0.001), whereas exposure to the CuCd mixture caused a significantly lower speed than exposure to only Cu (P<0.001). The *C. elegans* exposed to the CuCd mixture made from $LC20_{48\ h}$ concentrations had 84.3, 84.6, and 83.3% lower average speeds than respectively non-exposed (P<0.01), Cd-exposed (P<0.05), and Cu-exposed nematodes (P<0.01; Fig. 2.6B).

Also in liquid medium, exposure to $LC20_{24\ h}$ (at 24 and 48 h) of Cu (P<0.001), Cd (P<0.01), and the mixtures ZnCu (P<0.01), CuCd (P<0.001), and ZnCuCd (P<0.01) resulted in a decrease in locomotion of *C. elegans* (Fig. 2.7A). The nematodes showed 86.3, 58.9, 96.7, 81.3, and 90.4% less thrashes, respectively, compared with the control. Exposure to ZnCu and ZnCuCd mixtures led to diminished thrashing behaviour compared with the Zn exposure (P<0.05). Thrashing behaviour of exposed nematodes to $LC20_{48\ h}$ concentrations of the mixtures ZnCu (P<0.01), CuCd (P<0.01), and ZnCuCd (P<0.01) decreased significantly with 84.1, 97.7, and 86.9%, respectively, compared with the control (Fig. 2.7B). Both CuCd (P<0.05) and ZnCuCd (P<0.01) exposure caused less thrashes than Cd exposure alone.

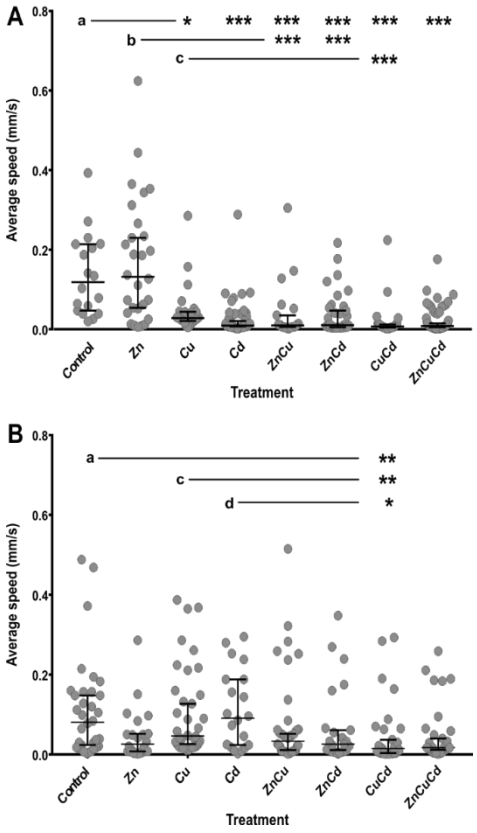

Figure 2.6: Average speed (mm/s) of nematodes exposed to LC20 concentrations (20% lethality) of metals and their mixtures. Nematodes were exposed to $LC20_{24\ h}$ (A) or to $LC20_{48\ h}$ (B) concentrations. Individual data are shown as well as the median and interquartile range (n=3). Asterisks denote significant differences (* P<0.05; ** P<0.01; *** P<0.001) compared with the corresponding control (a) and with the corresponding single metals Zn (b), Cu (c) and Cd (d).

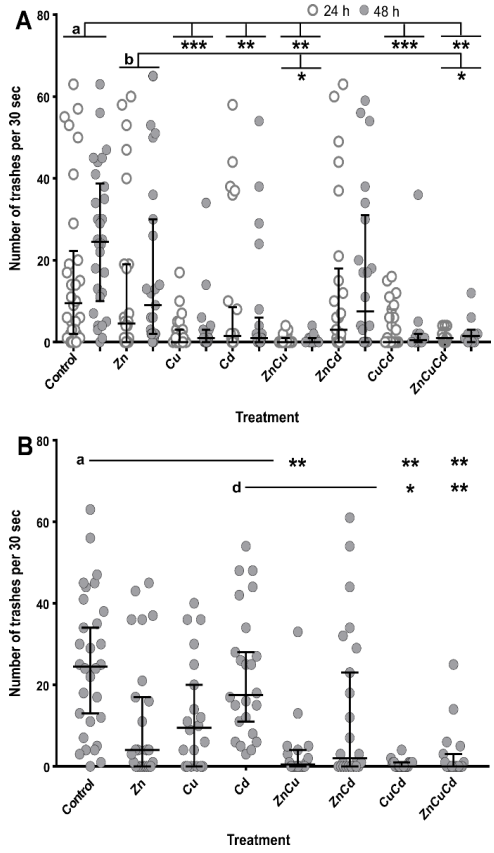

Figure 2.7: Thrashing behavior of nematodes exposed to LC20 concentrations (20% lethality) of metals and their mixtures. Nematodes were exposed to $LC20_{24\ h}$ (A) or to $LC20_{48\ h}$ (B) concentrations. Individual data are shown as well as the median and interquartile range (n=3). Asterisks denote significant differences (* P<0.05; ** P<0.01; *** P<0.001) compared with the corresponding control (a) and with the single metals Zn (b) and Cd (d).

2.3.3. Chemosensation

No significant effect of metal treatment on the chemotactic index was observed. However, a (nonsignificant) trend was seen, where Cu exposure slightly impaired the avoidance reaction to the repellent (Fig. 2.8). The error bars suggest very heterogeneous reactions of the population of nematodes studied.

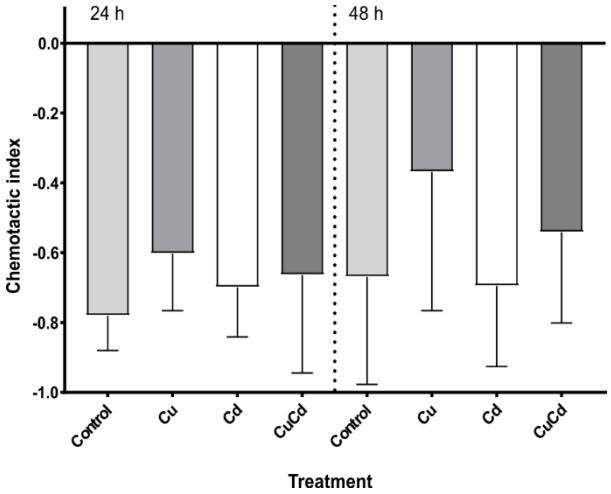

Figure 2.8: Chemotactic index of nematodes exposed to LC20 concentrations (20% lethality) of copper (Cu) and cadmium (Cd) and their mixture. Left: exposure to the $LC20_{24\ h}$ concentrations at 24 h. Right: Exposure to the $LC20_{48\ h}$ concentration at 48 h. Values are mean ± standard deviation. The test was conducted twice with 3 technical replicates each. No significant differences were observed.

For the drop tests, metal exposure only had a significant effect (P<0.01) in the group exposed to $LC20_{48\ h}$, where Cu exposed nematodes had a longer reaction time (P<0.01) compared with control (Fig. 2.9).

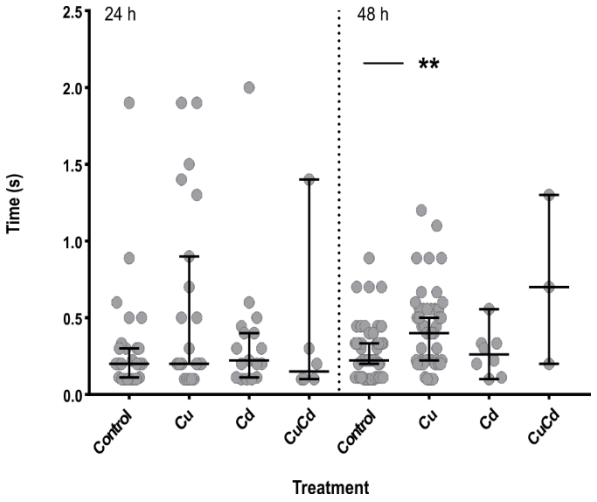

Figure 2.9: Reaction time (s) of nematodes exposed to LC20 concentrations (20% lethality) of metals and their mixtures. Exposure to the $LC20_{24\ h}$ concentrations (left) and to the $LC20_{48\ h}$ concentration (right). Individual data are shown as well as the median and 95% confidence intervals (n=3). Asterisks denote significant differences (* P<0.05; ** P<0.01; *** P<0.001) compared with the corresponding control.

2.4. Discussion

We studied the effects of Cu, Cd, and Zn exposure in a time dependent manner, using different concentrations. Toxic effects of single metals and mixtures on mortality, locomotion, and chemosensation of *C. elegans* were analysed. Mortality rate was the highest for Cu, and for all metals mortality increased over time. Different interactions were observed for ZnCd, ZnCu, CuCd, and ZnCuCd mixtures. Our study indicated that even at low concentrations, the locomotion, both on agar plates and in liquid medium, was disturbed, whereas almost no significant effect was observed on chemosensation.

Based on toxicant mass units of mg/L, Cd was the least toxic and Cu the most toxic to *C. elegans*, which is consistent with prior studies (Williams and Dusenbery, 1990; Freeman et al., 1998; Dhawan et al., 2000; Chu and Chow, 2002). When the data are converted to micromolar, the order of mortality was Cu > Cd > Zn. However, lethal concentrations of Cd and Zn were not significantly different. The LC50 values corresponded to earlier findings (Williams and Dusenbery, 1990; Chu and Chow, 2002). Differences in toxicity in previous studies may be the result of differences in methodology, the nature of the chemicals, the age of the nematodes tested, the *E. coli* density, the number and density of the animals, exposure time, life stage, temperature, and so forth. Furthermore, for all metals, the survival fraction decreased with increasing concentrations. At each time point, the regression slope of Zn was the most negative, although the 95% confidence intervals slightly overlapped with Cu after 24 h of exposure and with Cd from 24 h onwards. At 2 and 8 h after Zn addition, mortality seemed to increase faster than when Cu or Cd was added. Cadmium had the least steep regression slope until 8 h of exposure, but at 24 h the slope was similar to that of Cu and at 48 h the slope was intermediary between Cu and Zn. However, the 95% confidence intervals of Cd overlapped with those of Cu from 2 h onward. This finding suggests that a longer exposure time is needed for Cd to become even with or more toxic than the other metals, which is confirmed in the study of Williams and Dusenbery (1990), where Cd became more toxic than Cu and Zn when the exposure period was extended to 96 h; the LC50s were 0.06 mg/L (0.5 µM),

0.26 mg/L (4.1 µM), and 1 mg/L (15.3 µM), respectively. Moreover, in comparison with $LC50_{24\,h}$, $LC50_{48\,h}$ strongly decreased for all metals, representing the increased toxicity of metals for longer exposure periods. This decrease was also seen in earlier studies (Williams and Dusenbery, 1990; Kammenga et al., 1994; Cressman III and Williams, 1997). In the present study, Zn-exposed nematodes showed a stepwise decrease in survival over time. Copper exposure already caused an increasing mortality after 2 h, but after 24 h a huge drop in survival was noted. For Cd-exposed nematodes the decline in survival started after 8 h, but became more prominent after 24 h of exposure. The difference in declining rates suggests that uptake and elimination rates differ between metals (Kammenga et al., 1994). Again, after 48 h of exposure, Cd seemed less toxic to *C. elegans* than the other metals. However, for most aquatic organisms, Cd is the most toxic compound (Dhawan et al., 2000). When the $LC50_{96\,h}$ of *C. elegans* was compared with *Daphnia* species, the average of invertebrates and benthos (e.g., bristle worm, caddis fly), *C. elegans* was more sensitive than the average of invertebrates and more sensitive to Zn and Cd than benthic organisms. For Cu, this was the opposite: *C. elegans* was less sensitive than benthos, showing less sensitivity to both Cu and Zn than *Daphnia* sp., but sensitivity for Cd was similar (Williams and Dusenbery, 1990). In another study (Kammenga et al., 1994), 12 nematode species were compared in terms of sensitivity to Cd and pentachlorophenol. After an aquatic exposure of 72 h, *C. elegans* seemed to perceive only an intermediate effect of Cd and was insensitive to pentachlorophenol. Fast colonizers (e.g., *Diplogasteritus* sp.) were relatively more sensitive to Cd than slow colonizing species (e.g., *Aporcelaimellus obtusicaudatus*). It was suggested that the variety in ectodermal tissue among nematodes plays an important role in explaining the observed differences in acute toxicity data. The high level of Cd insensitivity of *C. elegans* suggests that these nematodes possess efficient defence mechanisms preventing Cd-related damage. Cellular detoxification systems (including glutathione, metallothioneins, heat shock proteins, pumps, and transporters) regulate intracellular metal levels by detoxifying and excretion of metals (Martinez-Finley and Aschner, 2011). This implies that a sensitive molecular response may contribute to resistance at the

organismal level, resulting in relatively high LC50 concentrations. It was also seen that *C. elegans* was less sensitive to Cd in K-medium (3000 mg/L NaCl, 2360 mg/L KCl, pH 5.5–6.0, total alkalinity of 0.1 mg/L as $CaCO_3$), compared with Recon (US Environmental Protection Agency moderately hard reconstituted reference water: 96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4.2H_2O$, 60 mg/L $MgSO_4$, 4 mg/L, pH 7.5–8.1, total alkalinity of 80 mg/L as $CaCO_3$) and moderately hard mineral water (Cressman III and Williams, 1997). Furthermore, Cd is reported to be predominantly present as chloro-complexes in K-medium (64%), whereas the concentration of the more biologically active free form was much lower. In Recon media, chloro-complexes are reported to be a minor fraction of the total Cd concentration (<1%) (Cressman III and Williams, 1997). Copper, on the other hand, is mostly present in the free-ion form (92%), which may explain the lower LC50 values (Freeman et al., 1998). The metal speciation and ensuing toxic effects are likely dependent on the composition and/or pH of soil or aquatic system. The lethal concentrations reported for Cu, Cd, and Zn in soil systems were much lower than those in aquatic solutions, which was explained by the presence of organic complexants (Höss et al., 2001). These organic complexants confound interpretation of data for soil systems: complexation of metal ions by organic matter lowers the free metal ion concentration in solution and can reduce metal mobility. It was suggested that *E. coli* functions both as a food organism and as a vector for contamination uptake (Höss et al., 2001). This might also be the case in our study.

Because all observed regression coefficients of metal mixtures were negative, the fraction of survival declined with increasing metal concentrations. When Cd was added to Cu and vice versa, significant increases in toxicity were observed, indicating a significant interaction whereby one metal caused a mortality increase when it was added to the other. All the regression slopes were similar, meaning that the toxicity in terms of mortality changed at similar rates when Cu or Cd were added to each other. However, when reproduction is considered as endpoint, more Cd in the mixture caused a decrease in toxicity, whereas more Cu increased the toxic effect on reproduction (Jonker et al., et al., 2004a). However, it is difficult to relate these observations to

underlying physiological mechanisms. It is possible that defence mechanisms play a role. All the regression slopes became steeper over time, unless the 2 highest concentrations of one metal were combined with each other. For these concentrations, the intercept on the y-axis was already much lower compared with the other concentrations, at which, nevertheless, a 48 h exposure caused a mortality of (almost) 100%.

The CuCd combination with $LC20_{24\,h}$ concentrations led to a significant higher mortality (after 24 and 48 h) compared with the single metals, whereas Cu had a significant lower mortality than the CuCd mixture of $LC20_{48\,h}$. Zinc seemed to have a neutral effect on Cd, because no obvious increase in toxicity was noticed, which is supported by earlier studies (Chu and Chow, 2002). For the ZnCu combination, a higher mortality compared with Cu ($LC20_{24\,h}$ 24 h) and with both metals ($LC20_{48\,h}$ and $LC20_{24\,h}$ 48 h) was noted. The CuCd and ZnCu mixtures did not differ significantly from the expected mortality, indicating that these metals appear to have an additive effect in combination. This corresponds to the findings of a 6-mo soil test with different nematode communities (Korthals et al., 2000), where an additive or less than additive effect of Cu and Zn was observed. However, in another study (Chu and Chow, 2002) the interaction between Cu and Cd and between Cu and Zn appeared to be synergistic. In that study the combination of LC20 of Cu and Cd even led to 100% mortality after 48 h. Furthermore, the mortality caused by the ZnCuCd mixture was significantly higher compared with the single compounds ($LC20_{24\,h}$ 24 and 48 h) and with Cu and Zn ($LC20_{48\,h}$). In this tertiary mixture, an additive effect was seen at 24 h, but after 48 h (for both $LC20_{24\,h}$ and $LC20_{48\,h}$) this effect became antagonistic. These interactive effects were also observed in a soil toxicity test, using a transgenic strain of *C. elegans*, carrying a stress-inducible β-galactosidase reporter, where the combination of Cu and Cd led to a larger response than Cd alone, whereas the ZnCd mixture caused a lower β-galactosidase activity than Cd alone (Calafato et al., 2008). They also tested for the internal metal content of *C. elegans* tissues, which was only fraction of the total metal content. Differences in uptake route, assimilation efficiency, bioavailability, and so on may result in various metal toxicities (Anderson et al., 2003; Rainbow, 2007). Zinc, Cu, and Cd may compete

for the entry into nematodes, eliciting different responses than for the individual metals. Another hypothetical explanation for the interactions is based on the stability indexes of metal ions and their binding sites (Nieboer and Fletcher, 1996), the covalent index and ionic index. Metals with a high ionic index are being displaced by metals with high covalent indexes for the binding sites. Copper and Cd have a similar ionic index, while Cu has the highest covalent index followed by Cd and Zn. This sequence corresponded to the observed toxicity in that study, when metal concentrations were expressed in mmol/L. Metals with a higher covalent index tend to have a synergistic effect, whereas metals with a low covalent index seem to have a variable impact (Chu and Chow, 2002). Furthermore, it was seen that Zn tends to neutralize the toxic effect of other metals with a low covalent index (e.g., Cd). Finally, it is possible that deviations from additivity are only seen under certain conditions: in the study of Jonker et al. (Jonker et al., 2004b), synergism was only observed at high dose levels (i.e., higher than LC50), while we only considered the combinations of LC20 concentrations in the present study. Furthermore, for the ZnCu and CuCd mixtures, additive effects were observed after 24 and 48 h of exposure, whereas the effects of the tertiary mixture changed from additive to antagonistic after 48 h of exposure, suggesting that a longer exposure period can induce other interactive effects. The differences in toxicity indicate the importance of testing mixtures and their individual metals simultaneously to obtain a reliable insight in the combined actions.

Many *C. elegans* behaviours such as locomotion and chemosensation are modulated by the presence of food (Hart and Chao, 2010). When nematodes are fed on a bacterial lawn, they switch between 2 different behaviour patterns. Approximately 80% of the time, the nematodes are moving slowly and staying in a restricted area (“dwelling”), but they can suddenly switch into rapid locomotion across the lawn (“roaming”). Besides speed lowering, the presence of a food source enhances avoidance responses to soluble repellents.

As in many animals, Cu and Cd have been found to affect movement in *C. elegans* (Dhawan et al., 2000; Boyd and Williams, 2003; De Boeck et al., 2006). In the study of Dhawan and co-workers (Dhawan et al., 2000), fed nematodes exposed to Cu and Cd for 24 h showed a change in movement at much lower concentrations than the concentration at which they showed mortality. The behavioural median effect concentration values for nematodes were between 20 and 50 times lower than the LC50 values. Behaviour was much more sensitive to Cd and Cu than mortality, whereas for nematodes exposed to Zn, the opposite was true. This was also observed in our study: nematodes exposed to $LC20_{24\ h}$ of Cu and Cd had a significantly lower crawling speed and showed a diminished thrashing behaviour compared with control, in contrast to nematodes exposed to Zn. The number of thrashes corresponds to the control finding of Li et al. (2013), and a similar effect for Cu (9531.9 mg/L (150 mM), 12 h) was observed in the study of Xing et al. (2009). However, in an earlier study using 163.5 mg/L (2.5 mM), 4904.3 mg/L (75 mM), and 13078.0 mg/L (200 mM) Zn (without food), *C. elegans* showed less body bends after 72 h compared with control (Wang et al., 2007), suggesting that a starvation period can severely increase the toxic effect of Zn on thrashing behaviour. The shorter exposure period and/or presence of food can be an explanation of why we did not see an effect of Zn on locomotion (yet).

In our study both locomotor responses of nematodes decreased more in binary and tertiary mixtures, which was in line with the findings of the mortality tests. After 24 h of exposure, the average speed in the ZnCu and ZnCd mixture was lower compared with Zn alone, whereas the average speed of the nematodes exposed to the CuCd mixture was significantly different from that of nematodes exposed to Cu alone. The *C. elegans* exposed to Cu had a slightly higher speed than in the ZnCu mixture, whereas Cd induced a similar speed as the CuCd mixture, but a slightly lower speed than in the ZnCd mixture. For thrashing behaviour, a trend was observed when nematodes exposed to ZnCu and ZnCuCd mixtures showed less thrashes compared with the single metals, whereas for nematodes exposed to the ZnCd mixture, thrashing behaviour was similar to that of

nematodes exposed to the single metals. This finding again suggests that Zn has a neutral effect on Cd and that Cu and Zn or Cd have additive effects on locomotion.

A 48 h exposure to $LC20_{48\,h}$ did not affect the movement of nematodes exposed to the single metals. When the nematodes were exposed to the CuCd mixture, their average speed decreased compared with both control and single compounds. A decrease in thrashing behaviour was observed after exposure to the CuCd, ZnCu, and tertiary mixtures.

To crawl forward in an unobstructed environment, *C. elegans* uses a sinusoidal-like locomotion, which is modulated by a number of processes including omega-turns, pirouettes, and gentle turns. This locomotion pattern is controlled by interneurons, which receive and integrate input signals from various chemo- and mechanosensory neurons (Bargmann, 2006). When *C. elegans* encounters a potentially harmful chemical, it avoids this compound by reversing its movement, which is mediated by sensory amphid neurons. These reversals are suppressed by antagonistic inputs from phasmid neurons (Hilliard et al., 2002). Hilliard and coworkers (2002) suggested that *C. elegans* utilizes a simple head–tail chemical sensory map for avoiding toxic compounds, whereas a more flexible temporal strategy that can locate signals in any direction is used for chemotaxis to food sources and mates. Chemosensation thus represents a complex and important response of the animal to its environment. In the present study, Cu-exposed nematodes showed a slightly altered chemotactic index, although this was not significant. In the study of Moore et al. (2014), the chemotactic index showed a huge variation. Furthermore, control nematodes started a backward motion within 1 s of the delivery of the drop, which corresponds to the findings of Hilliard et al. (2002). All animals reacted within 4 s, causing positive responses to each drop. The nonsignificant trend observed for the chemotactic index was significant in the drop test. Nematodes exposed to $LC20_{48\,h}$ of Cu needed significantly more time to start a backward movement, compared with control, although the reaction was still very fast and positive. This result suggests that an exposure to LC20 of Cu slightly affects the neurons. A longer exposure

period, absence of food, or higher concentrations may probably induce more significant results. Because movement of exposed nematodes was reduced, a slower or no avoidance response to octanol was expected, but almost no difference from control was observed: chemosensation still seemed strong enough to elicit chemotactic and fast avoidance response. In contrast to locomotion, chemosensation did not seem sensitive enough as an endpoint for assessing toxic effects on *C. elegans*.

2.5. Conclusions

The results of the present study showed that the toxicity of Cu was higher compared with Cd and Zn, resulting in higher mortality rates and reduced locomotion. Under mixed-metal exposure conditions, the interaction effects were dependent on the metal combinations employed. For both mortality and locomotion, toxicity was higher for the CuCd and ZnCu mixture than for the single-metal exposures. Zinc seemed to have a neutral effect on Cd, because no clear mortality increase or reduction in locomotion was observed. Because the same trend of mixture effects was noted in locomotion and in mortality tests, locomotion could probably be considered as a sensitive endpoint in metal toxicity studies. Moreover, for Cu and Cd, survival declined by 20% after 24 h of exposure, whereas locomotion had already decreased by 34 and 86%, respectively. Despite considerable research efforts in recent years, the effects of metal mixtures are not yet well understood. There is a notable paucity of information on mixed-metal effects in soil invertebrates such as *C. elegans*. In this regard we highlight that environmental quality standards are still based on single-metal toxicity and do not take into account the physiochemical conditions in the exposure medium. More research on metal mixture effects at different levels (molecular, survival, reproduction, population, etc.) is needed to elucidate the mechanistic basis of toxic effects. Such knowledge will establish a robust scientific basis in the setting of standards for environmental protection and environment risk assessment.

2.6. Acknowledgment

We thank V. Kayawe Mubiana and S. Joosen for analyzing metal concentrations. The present study was funded by the University Research Fund (BOF) and the Interuniversity Attraction Poles Programme–Belgian State–Belgian Science Policy [IUAP VI/33].

2.7. Supplemental tables and figures

Table S2.1: Slopes of the regression lines of Zn, Cu and Cd after 2 h, 8 h, 24 h and 48 h of exposure with their 95% confidence intervals. Asterisks (*) denote a significant effect (P<0.05) of a change in concentration on the survival fraction.

	Time (h)			
	2	8	24	48
Zn	-0.15 ± 0.04 *	-0.32 ± 0.08 *	-0.40 ± 0.06 *	-0.39 ± 0.06 *
Cu	-0.05 ± 0.05	-0.16 ± 0.07 *	-0.31 ± 0.04 *	-0.27 ± 0.03 *
Cd	-0.02 ± 0.03	-0.04 ± 0.07	-0.29 ± 0.06 *	-0.33 ± 0.04 *

Table S2.2: Slopes of the regression lines and corresponding 95% confidence intervals of the CuCd mixture after 2 h, 8 h, 24 h and 48 h of exposure. Fraction of survival as a function of $\log_{10}$ [Cu concentration], at different levels of Cd concentration. P-values (*P*<0.05; *P*<0.01; *P*<0.001) denote a significant effect of cadmium on the survival fraction with increasing $\log_{10}$ [Cu concentration].

	Time (h)											
	2			8			24			48		
Cd (mg/L)	Slope	*P*-value	R^2	Slope	*P*-value	R^2	Slope	*P*-value	R^2	Slope	*P*-value	R^2
0	-0.03 ± 0.03	ns	0.32	-0.07 ± 0.06	<0.05	0.77	-0.30 ± 0.06	<0.001	0.89	-0.29 ± 0.05	<0.001	0.98
2.5	-0.04 ± 0.04	ns	0.82	-0.11 ± 0.07	<0.05	0.73	-0.22 ± 0.08	<0.001	0.83	-0.26 ± 0.09	<0.001	0.87
5	-0.04 ± 0.05	ns	0.35	-0.04 ± 0.07	ns	0.29	-0.24 ± 0.09	<0.001	0.81	-0.26 ± 0.06	<0.001	0.92
10	-0.11 ± 0.07	<0.05	0.76	-0.11 ± 0.08	<0.05	0.64	-0.20 ± 0.08	<0.001	0.78	-0.18 ± 0.07	<0.001	0.72
100	-0.06 ± 0.09	ns	0.21	-0.05 ± 0.08	ns	0.31	-0.20 ± 0.07	<0.001	0.78	-0.15 ± 0.06	<0.001	0.91
400	-0.04 ± 0.10	ns	0.26	-0.24 ± 0.10	<0.001	0.91	-0.15 ± 0.05	<0.001	0.89	-0.04 ± 0.02	<0.01	0.58

Table S2.3: Slopes of the regression lines and corresponding 95% confidence intervals of the CuCd mixture after 2 h, 8 h, 24 h and 48 h of exposure. Fraction of survival as a function of log_{10} [Cd concentration], at different levels of Cu concentration. P-values (*P*<0.05; *P*<0.01; *P*<0.001) denote a significant effect of copper on the survival fraction with increasing log_{10} [Cd concentration].

	Time (h)											
	2			8			24			48		
Cu (mg/L)	Slope	*P*-value	R^2	Slope	*P*-value	R^2	Slope	*P*-value	R^2	Slope	*P*-value	R^2
0	-0.01 ± 0.05	ns	0.10	-0.05 ± 0.10	ns	0.60	-0.16 ± 0.06	<0.001	0.92	-0.35 ± 0.06	<0.001	0.99
0.05	-0.05 ± 0.08	ns	0.45	-0.07 ± 0.11	ns	0.33	-0.16 ± 0.07	<0.01	0.91	-0.26 ± 0.11	<0.001	0.88
0.5	-0.17 ± 0.09	<0.01	0.90	-0.10 ± 0.11	ns	0.97	-0.26 ± 0.11	<0.001	0.84	-0.26 ± 0.11	<0.001	0.87
2.5	-0.03 ± 0.11	ns	0.06	-0.09 ± 0.08	ns	0.61	-0.25 ± 0.09	<0.001	0.79	-0.25 ± 0.07	<0.001	0.95
25	-0.07 ± 0.11	ns	0.26	-0.25 ± 0.11	<0.001	0.86	-0.13 ± 0.07	<0.01	0.89	-0.03 ± 0.03	ns	0.54
50	-0.08 ± 0.10	ns	0.41	-0.18 ± 0.17	ns	0.29	-0.08 ± 0.06	<0.05	0.79	-0.00 ± 0.00	NA	NA

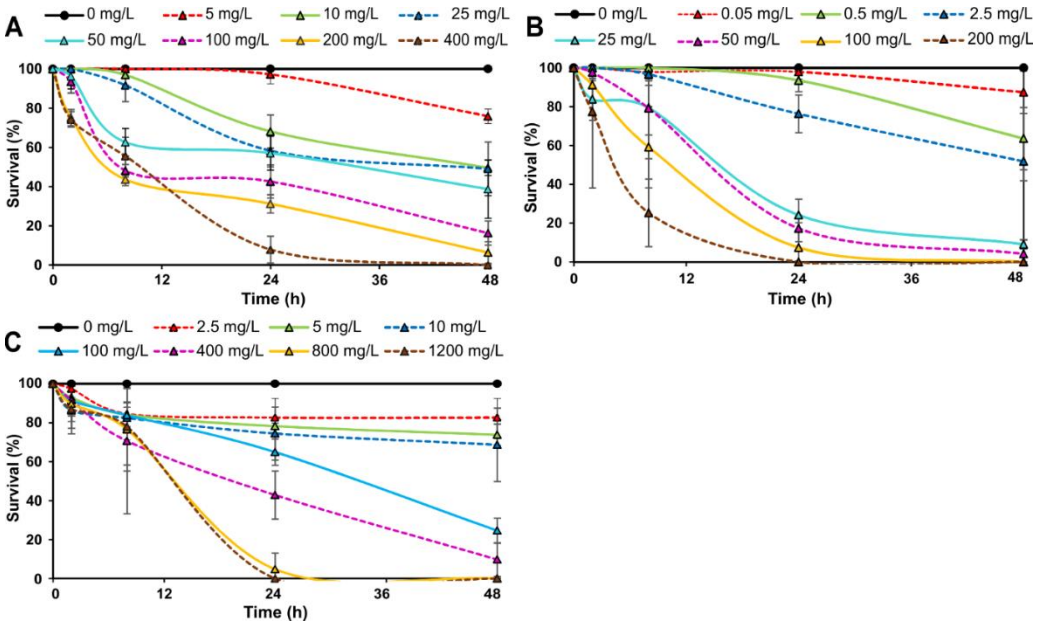

Figure S2.1. Concentration-response relationship for exposure to Zn (A), Cu (B) and Cd (C) at different exposure times. Data are expressed as mean ± SD.

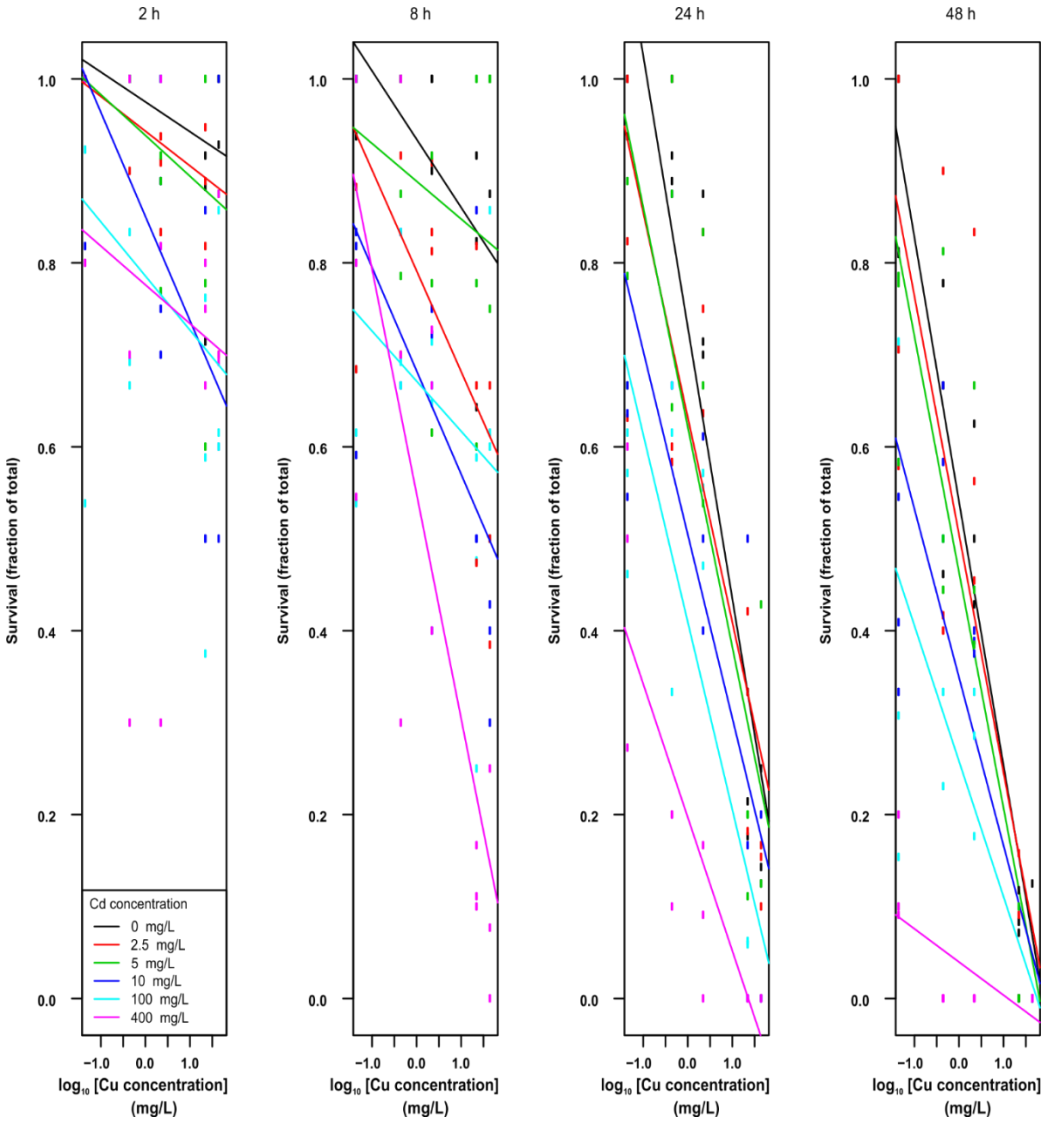

Figure S2.2. Survival fraction plotted versus the logarithm of the Cu concentration, with the different colors pointing at the different Cd concentrations. Solid lines, obtained via linear regression, represent the change in survival with increasing Cu concentration, at a given concentration of Cd. Survival fractions for mixtures without Cu were omitted from the graph.

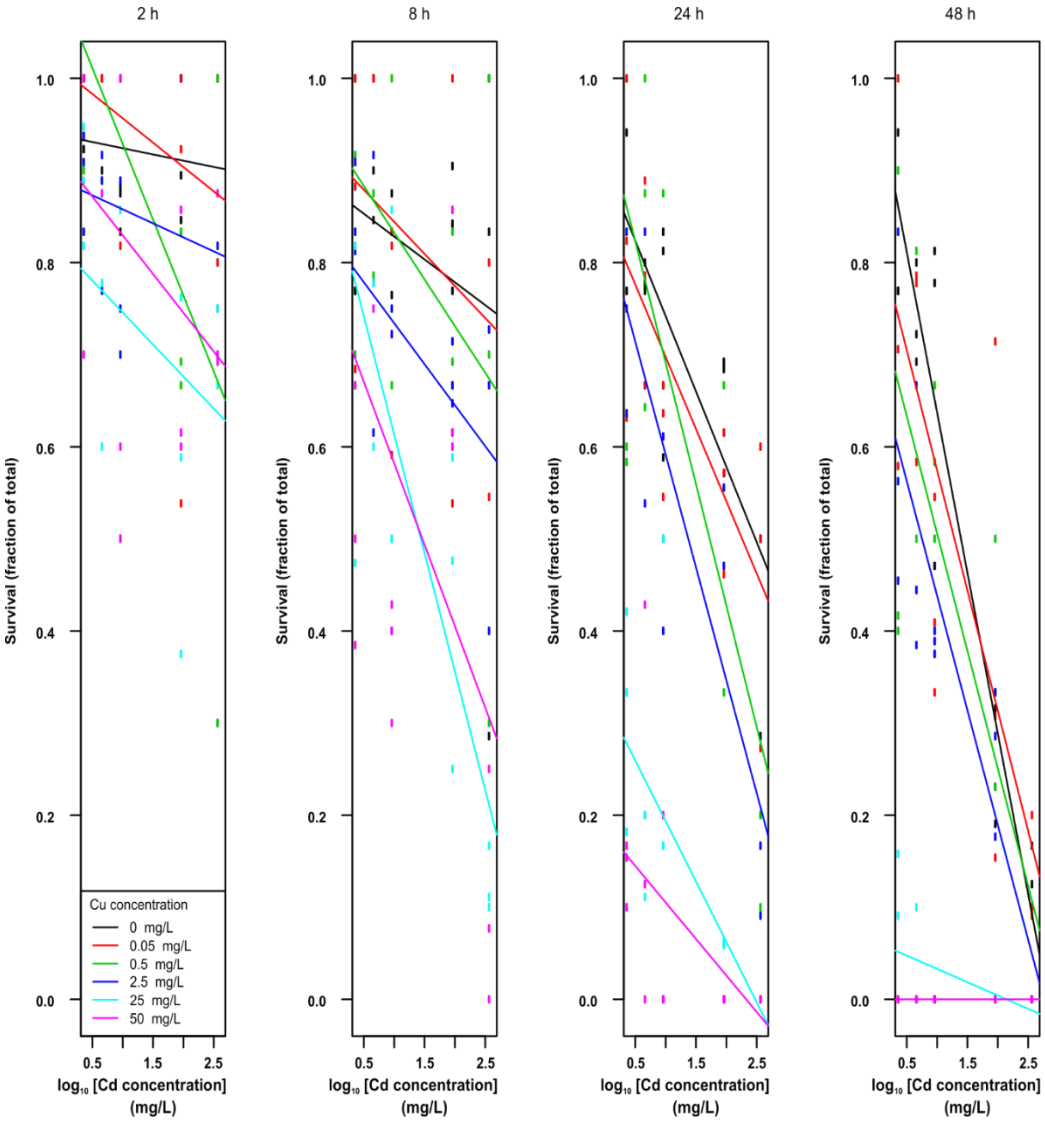

Figure S2.3. Survival fraction plotted versus the logarithm of the Cd concentration, with the different colors pointing at the different Cu concentrations. Solid lines, obtained via linear regression, represent the change in survival with increasing Cd concentration, at a given concentration of Cu. Survival fractions for mixtures without Cd were omitted from the graph.

Chapter 3

The effect of metal mixture composition on toxicity to *Caenorhabditis elegans* at individual and population levels

Moyson S., Town RM., Vissenberg K., Blust R., Husson SJ. The effect of metal mixture composition on toxicity to *C. elegans* at individual and population levels. In preparation for submission to *PLoS ONE*.

Abstract

The toxicity of zinc (Zn), copper (Cu), and cadmium (Cd) to the nematode *Caenorhabditis elegans* was characterised under single metal and mixture scenarios at different organisational levels. The effects on population size and body length were investigated at two concentrations corresponding to the 24 h LC5 and LC20 levels. Metal toxicity was dependent on metal concentration, exposure time and mixture composition. Populations exposed to LC20 levels of Cd, ZnCu, CuCd and ZnCuCd plummeted, while for all LC5 concentrations, population size continued to increase, albeit that single metals were less harmful than mixtures. Interestingly, the population size of nematodes exposed to the LC20 combination of Zn and Cd increased over time. Combinations of the LC20 of Cd with a range of Zn concentrations showed concentration dependent mitigating effects on population size and antagonistic effects on mortality. By combining effects at different organisational levels, more insight into metal toxicity was obtained. Metal effects were more evident on population size than on body length or mortality, suggesting that population size could be considered as a sensitive endpoint. This proposition is strengthened by the connection between dose-response at the transcriptomic level and effects at the individual and population levels.

Keywords: Population, Body length, Metals, Mixtures, *C. elegans*

3.1. Introduction

Metals of natural and anthropogenic origin can be mobilised in environmental compartments and thereby pose a toxicological risk. Essential metals (e.g. Zn, Cu) are needed for biological functions, while non-essential metals (e.g. Cd, Pb) may disrupt biological processes depending on their concentration, dynamic chemical speciation, bioavailability and biological species sensitivities. Exposure to nonessential metals and to a deficiency or an overload of essential metals can be detrimental to many animals. For ethical reasons, toxicological studies are preferably performed on non-vertebrate organisms, e.g. soil nematodes such as *C. elegans*.

C. elegans is a bacterivorous nematode that is ubiquitous in soil and fulfils an important ecological role. Furthermore, it has a short life cycle and can survive a wide range of pH and osmolarity, which facilitates its utility in high throughput toxicity testing (Khanna et al., 1997; Leung et al., 2008). It is a well characterised model organism that has found wide application in mechanistic studies on the toxicity of metals, organic compounds, and nanoparticles (Yu et al., 2017; Fueser et al., 2018; Wang et al., 2018). After a rapid development of 3-4 days through four larval stages (L1-L4), the adult hermaphrodite produces oocytes during a 4-day fertile period. The production amounts to 300 progeny via self-fertilisation, and to 1200-1400 after mating with a male. After the fertile period, the mature adults live for a further 10-15 days. When the environmental quality is not optimal, a developmentally arrested third larval stage, dauer larva, is formed (Harvey et al. 2008). These larvae (± 0.4 mm) have a 4 to 8 times longer lifespan and will only develop into L4 when the conditions become favourable. Likewise, adult lifespan, lifetime fecundity and body length vary according to the environmental quality (Harvey et al., 2008). Body length increases with life stage, progressing from ca. 370 µm at first molt, to 480 µm at second molt, 640 µm at third molt, 850 µm at fourth molt and to 1060 µm when egg laying starts (T = 20°C, wormatlas, 2002-2018). Adult *C. elegans* typically range from 1-1.5 mm in length (Boyd and Williams 2003). Males are more slender, slightly shorter (0.8 mm) and less present in the population (± 0.1%) than

hermaphrodites (wormatlas, 2002-2018). The highly predictable developmental features of *C. elegans* cause their growth responses to be good toxicological endpoints.

Due to the above mentioned life history traits and known genome, *C. elegans* has been used to study the toxic effects of single metals on various endpoints ranging from the molecular to the population level: e.g. a reduction of bioluminescence, movement, feeding, growth and reproduction, a delay in egg laying and an increased generation time were reported after Zn, Cu and/or Cd exposure at LC20-LC50 values (Power and de Pomerai, 1999; Anderson et al., 2003; Boyd et al., 2003; Boyd and Williams, 2003; Calafato et al., 2008; Lagido et al., 2009; Höss et al., 2011). Furthermore, metal exposure can influence protein levels and gene transcription in order to maintain homeostasis (Davis et al., 2009; Martinez-Finley and Aschner, 2011; Roh et al., 2014; Dietrich et al., 2016). However, there is a paucity of studies that consider the combined toxic effects at different organisational levels. Furthermore, in contrast to single metals, knowledge about metal mixture effects is still scarce. Metal mixture toxicity is typically evaluated in terms of the “concentration addition” (CA) model or the “independent action” (IA) model. In both cases, deviations from the expected toxicity are interpreted as being the result of interactions between the metals. The extent of deviation deemed to be significant, can be based on a statistical approach or simple judgement (ECETOC report, 2011). Interactive effects of metals can be additive, antagonistic or synergistic. The potential interplay between the biotic handling processes for different metal ions means that the nature and timescale of toxicity responses under mixture conditions does not follow straightforwardly from observations on single metal exposures. Testing mixtures and the corresponding individual metals simultaneously is thus necessary to obtain insight into the combined actions. Most studies on metal toxicity to *C. elegans* correspond to short exposure periods (24 h or 48 h) (e.g. Dhawan et al., 2000; Chu and Chow, 2002; Boyd et al., 2003).

In our previous study the effects of single metals and mixtures on the mortality and behaviour (locomotion and chemosensation) of *C. elegans* after short exposure times,

i.e. 24 h and 48 h, were described (Moyson et al., 2018). However, there is a paucity of information on the longer term effects of metals, even for single metal exposures. It is known that the adverse effects of Cd become more apparent after a few days of exposure (Williams and Dusenbery, 1990), which may influence its contribution to the toxicity of mixtures as a function of time. Furthermore, toxicity to individuals can result in e.g. mortality and decreased development, which will be reflected at the population level in lower abundance, altered distribution of the population etc. Population size can thus be considered as a holistic endpoint that integrates individual life histories and trans-generational effects. *C. elegans* is able to alter both its larval development and its reproductive strategy (e.g. lifetime fecundity and time of reproduction) in response to environmental stress (Harvey et al., 2008) and the influence of pollutants on the population growth of *C. elegans* has been proposed as a means to assess long-term environmental effects (Dutilleul et al., 2015; Yu et al., 2017). Herein we assess the utility of population size as a potential sensitive endpoint for metal toxicity. The aim of the present study is to gain insights into the sensitivity of *C. elegans* to selected metals (Cu, Cd, Zn) and to investigate whether and how these sensitivities are mutually affected in mixture exposure scenarios at the individual (body length) and population level (population size), and the time dependence thereof (10-12 days). To better understand the mitigating effect of Zn on Cd toxicity, mortality and population size of nematodes were studied after 24 h and 48 h of exposure to different LC concentrations of Zn, whether or not in combination with LC20 of Cd. The main objectives of our study were to (i) examine differences between single metal exposures, to (ii) analyse how toxicl effects change over time, to (iii) determine if the same responses are observed at different exposure concentrations, to (iv) identify which types of interactive effects occur in metal mixtures and the time evolution thereof, and finally to (v) link the observed effects of different endpoints from the same or different organisational level.

3.2. Materials and methods

3.2.1. *Caenorhabditis elegans* culture

Caenorhabditis elegans nematodes, wild type N2 strain, were obtained from the Caenorhabditis Genetic Centre, Minneapolis, USA. NGM agar plates, seeded with *Escherichia coli* (OP50 strain) as food source, were used for nematode maintenance at 20°C (Brenner, 1974). To obtain nematodes of the same age, bleaching was performed by adding a hypochlorite solution (5 N NaOH, 8% sodium hypochlorite) to mixed-stage *C. elegans*, thereby killing the nematodes that were not protected by an egg shell. Eggs were raised on NGM plates without food to obtain highly synchronous L1 larvae by arresting their development for 24 h.

3.2.2. Test media

Two concentrations of metals were studied in single and mixture scenarios. We chose concentrations corresponding to the 24 h LC5 and LC20 levels (Table 3.1), which were determined previously (Moyson et al., 2018) for the same life stage. For convenience, we subsequently use the terminology “LC5” and “LC20” to denote these concentrations, even though herein we studied effects at times beyond 24 h. Exposure media were prepared from $CdCl_2.2.5H_2O$ (Alfa Aesar), $CuCl_2.2H_2O$ (Merck A.G.) and $ZnCl_2$ (Alfa Aesar) in K-medium, supplemented with *E. coli* bacteria (1.5 - 1.7 g/L). The metal concentrations used are in line with reported metal contents of polluted soils that have been used for toxicity studies with *C. elegans* (Höss et al., 2009). The mixtures ZnCu, ZnCd, ZnCuCd, and CuCd were prepared by combining the corresponding LC concentrations of the individual metals. Metal concentrations were verified by ICP-OES (ICAP 6300 Duo, Thermo scientific) (95% - 115% recovery). Since exposure media contained *E. coli* bacteria, samples were first freeze-dried (Heto Powerdry LL 30000, Thermo Scientific), 250 µL concentrated nitric acid (TraceMetal Grade, Fisher Chemical) was added and all samples were digested at 110°C for 30 minutes using a heating plate

(HotBlock, Environmental Express). MilliQ water was added to make the total volume up to 10 mL.

To allow metal partitioning between the aqueous phase and the bacteria, prior to toxicity testing, metal solutions were first incubated with the bacterial suspensions for 12 h at 4°C. For each sample of each experiment, the determined pH before (5.1 ± 0.1), during and at the end of the experiment of 48 h (5.4 ± 0.3), 10 days (6.0 ± 0.3) and 12 days (7.2 ± 0.3) was within an acceptable pH range for *C. elegans*, thus excluding any potential effects of pH variation on the measured parameters. For each treatment three replicates were made.

Table 3.1: LC values of Zn, Cu and Cd after 24 h of exposure (NA = not applicable).

	LC2		LC5		LC20		LC40		LC60	
	(mg/L)	(mM)	(mg/L)	(mM)	(mg/L)	(mM)	(mg/L)	(mM)	(mg/L)	(mM)
Zn	0.445 ± 0.314	0.007 ± 0.005	1.416 ± 0.771	0.022 ± 0.012	9.501 ± 2.841	0.145 ± 0.043	31.495 ± 6.186	0.482 ± 0.095	84.823 ± 17.601	1.297 ± 0.269
Cu	NA	NA	0.226 ± 0.104	0.004 ± 0.002	1.299 ± 0.409	0.020 ± 0.006	NA	NA	NA	NA
Cd	NA	NA	0.474 ± 0.289	0.004 ± 0.003	7.110 ± 2.315	0.063 ± 0.021	NA	NA	NA	NA

3.2.3. Determination of population size and body length after exposure to LC5 and LC20

24 h after bleaching, the synchronous L1 nematodes were transferred to OP50 seeded NGM plates. After 60 h, approximately 10 adult nematodes were transferred to a 24-well plate filled with 1 mL K-medium (52 mM NaCl, 32 mM KCl, 5 µg/mL cholesterol, pH 5.1) (Mathie et al., 2006) or test medium (K-medium containing the test metal concentration(s)), supplemented with *E. coli* OP50 (1.5 – 1.7 g/L). The well plates were continuously shaken (160 rpm, 20°C). For all 8 treatments (i.e. control, Zn, Cu, Cd, ZnCu, ZnCd, CuCd and ZnCuCd), the population size (i.e. number of live larvae and adult nematodes) was determined over a period of 10 days when exposed to LC20 and over 12 days when exposed to LC5. During a long exposure period, multiple generations can be formed and thus the total population size provides insight into trans-generational effects. Every 36 h, medium with nematodes was transferred to a new well with fresh medium (metal stock with *E. coli*) (both in equal volumes) and was split in two, thereby reducing the number of nematodes by a factor of 64 at day 10 (Wang et al., 2007; Harvey et al., 2008). However, the number of nematodes in groups exposed to LC5 was exceptionally reduced by a factor of 4 at day 6 and the control, Zn and Cu group were reduced by a factor of 8 at that time. To be able to count the number of nematodes, the number in the control LC20 group was further reduced starting from day 6 onwards: 64x at day 6, 128x at 8 day, 512x at day 9, 2048x at day 10. Our protocol ensured that nutritional factors were sufficient throughout the exposures, i.e. changes in population size cannot be ascribed to a deficiency of bacteria. Every 24 h, the number of living nematodes was counted in each well by taking a picture and making a video of 10 seconds (15 fps) with a camera (Nikon DS-Ri1), attached to a stereomicroscope (Nikon AZ100). Nematodes that were not moving or did not respond to gentle plate shaking, were considered to be dead. These videos were also used for body length measurements. Body length differences between generations were very clear, enabling larvae from one generation to be easily distinguished from the larger subsequent life stages of the previous generation (Fig. S3.1). The body length of the post-larval stage

nematodes (not necessarily from the same life stage) was measured after 0, 5 (approximate time at which a second new generation can be formed) and 10 days of exposure and also after 12 days of LC5 exposure, using Image-J software. (https://imagej.nih.gov/ij). At each time point, 20 nematodes were counted in each of the three replicates.

3.2.4. Determination of population size and mortality in mixtures of Cd with different Zn concentrations

After 24 h the synchronous L1 nematodes were transferred to OP50 seeded NGM plates. 60 h later, approximately 10 adult nematodes were transferred to a 24-well plate filled with 1 mL K-medium (52 mM NaCl, 32 mM KCl, 5 µg/mL cholesterol, pH 5.1) (Mathie et al., 2006) or test medium (K-medium containing the test metal concentration(s)), supplemented with *E. coli* OP50 (1.5 g/L). The well plates were continuously shaken (160 revolutions per minute (rpm), 20°C). Nematodes were exposed to 24 h LC2, LC5, LC20, LC40 and LC60 of Zn (Moyson et al., 2018) with and without the presence of LC20 of Cd (Table 3.1). The population size (number of larvae and adults) was determined for each exposure over a period of 2 days. Nematodes were not transferred to a new well after 36 h, because of the short exposure period and the ability to correctly count the number of nematodes. Furthermore, after 0, 24, and 48 h of exposure, the number of live adult worms from the original population was counted in each well by the same procedure as described in Section 3.2.3.

3.2.5. Statistical analysis

Data were analysed with the statistical program R, Version 3.1.2., with a 5% level of significance. Normality was checked visually by histograms and by the Shapiro–Wilk test. The Bartlett test was used to verify the homogeneity of variances.

3.2.5.1. Body length after LC5 and LC20 exposure

To test the possible effects of exposure time, treatment and their interaction on the body length, linear mixed models were fitted. For LC5, a linear model with time as a continuous variable was fitted. Since the change over time was not linear for LC20, time was entered as a categorical variable. Body length was repeatedly measured over time; therefore, a random intercept term was added to the model to account for the non-independence between observations resulting from the same treatment. For LC5, regression lines that model body length vs. time were fitted for each treatment separately, and their slopes were estimated. The slopes of such lines for the single metals, the mixtures and between the mixture and corresponding single metals were compared with each other using multiple linear regression. The identification of significant differences in slopes between treatments was taken as an indication of differences in the time dependency of toxicity between treatments. A Dunnett test was carried out to compare the slopes of the treatments with the control. In case of LC20, no slopes were estimated since the change over time was not linear. Therefore, for each treatment separately, the difference in body length between the 3 time points was calculated with a one-way ANOVA with a Tukey correction for multiple comparison. Furthermore, for each day a one-way ANOVA followed by a Tukey post hoc test was fitted to determine the differences in body length between the single metals, the mixtures and between the mixture and corresponding single metals. Finally, at each day the different metal treatments were compared with the control by a Dunnett post hoc test.

3.2.5.2. Population size after exposure to LC5, LC20 and ZnCd mixtures

Differences in population size were analysed for LC5, LC20 and ZnCd mixtures separately. Multiple generations were generated in our study and some populations even crashed. Furthermore, different trends of population size were observed between treatments (exponential, linear, polynomial), which made it difficult to describe the population growth rate by a single number and to compare the population growth rates

between treatments and exposure concentrations. Therefore, for all combinations of treatments, three values that summarise effects on the *C. elegans* population size were compared, namely (i) the area under the population size vs. time curve, (ii) the maximum population size attained during the exposure, and (iii) the population size at the end point. For LC5 and LC20 treatments, the values were computed on the basis of the logarithm of the number of nematodes. Areas under the population size vs. time curve were calculated using the trapezoid rule, as implemented in the trapz function using the pracma package in R (Borchers, 2017). This area is the cumulative sum of all individuals of the population multiplied by the time they were alive and is thus an indication of the population size during the whole experiment. Since treatments can have the same area under the population size vs. time curve, while showing a very different shaped curve (e.g. one with a high peak followed by a population crash and one with a stable population), 2 more parameters (maximum and end population size) were measured to compare the patterns of population size between treatments. The control group of the LC20 experiment was visually strongly significantly different from the other treatments; since the focus was to compare differences between the various treatments, this control group was omitted from our analysis. One of the replicates of LC20 of Zn was also omitted, since it was an outlier according to diagnostic plots. Since the maximum population size of each LC5 and ZnCd mixture treatment was reached at the end of the experiment, only the end population size was analysed, except for the LC5 ZnCuCd mixture where the maximum was already reached at day 8. For each parameter value, a one-way ANOVA was fitted to test the effect of each treatment. To test if the magnitude of each value was different in the metal treatments compared to the control, a posthoc analysis with a Dunnett correction was carried out. Subsequently, a one-way ANOVA was fitted for each parameter to test whether the outcome was the same across treatments. If there was a significant difference between treatments, a posthoc analysis with Tukey correction for multiple testing was performed. In this way, the differences between the single metals, between the mixtures and between the mixture and corresponding single metals were analysed.

3.2.5.3. Mortality of ZnCd mixtures

To determine whether the mixtures of LC20 of Cd with different Zn concentrations led to significant higher mortalities than the individual metals, a nonparametric ANOVA, Kruskal–Wallis test, followed by posthoc analysis with Bonferroni correction for multiple testing was performed.

Mixture effects were assessed using the conceptual model of Concentration Addition (CA) and Independent Action (IA) (Jonker et al., 2005). In contrast to population size, full dose-response profiles are available to predict mixture effects on mortality according to the more advanced formulas of CA and IA (Nys et al., 2017; Moyson et al., 2018). CA assumes similar modes of action, while IA assumes different modes of action of the single metals. The expression for the CA model is given by:

$$1=\sum_{i=1}^{n}\frac{c_i}{EC50_i \times \left(\frac{y}{100-y}\right)^{1/\beta}}$$

and that for the IA model is:

$$y=100 \times \left[1-\prod_{i=1}^{n}\left(\frac{1}{1+\left(\frac{c_i}{EC50_i}\right)^{\beta}}\right)\right]$$

where c_i is the concentration of compound i, n is the number of compounds, y is the predicted effect of the mixture and β is the slope of the dose-response curve.

The observed mortalities were compared with the values expected on the basis of the CA model and the IA model, using the non-parametric Wilcoxon test, to analyse which interactive effects occur in the mixtures. Additive effects were observed if predicted mixture effects did not deviate from measured effect. If observed mixture effects were significantly higher than the predicted from both models the interaction was considered

as synergistic, whereas an antagonistic interaction was indicated by a significantly lower observed effect compared to the predicted one from both models.

3.3. Results and discussion

For clarity, we firstly discuss the effects of single metal exposures, followed by those of the various mixture exposures.

3.3.1. Population effects of single metals: metal treatment, concentration and time effects

3.3.1.1. Body length

At the start of the LC20 experiment, all nematodes had the same age (24 h since onset of L4 stage) and body length as the control (Fig. 3.1). As described in section 3.2.3, we did not follow the change in body length of particular individuals, but rather the average body length of the post-larval stage nematodes was determined and used as an indicator of effects at the population level.

The body length of LC20 exposed nematodes decreased over time, whilst that of the control organisms increased (P<0.001) (Fig. 3.1A; Table S3.1). After 5 days of exposure, the Cu and Cd exposed nematodes were 80.9% smaller than the control, while for Zn exposures they were 31.2% smaller (P<0.001) (Table S3.2). The maximum measured body length of Cu and Cd exposed nematodes corresponded to the length of L1 larvae, while Zn exposure resulted in a similar length as L4 larvae. At the end of the experiment (day 10), the difference in body length between the control and Cu exposed nematodes was similar to that of day 5, while the difference compared to Zn (+17.3%) was larger (P<0.001) (Table S3.3). Cu exposed nematodes had a similar maximum size as at day 5, while the maximum measured length of Zn exposed *C. elegans* was reduced to the size of L3 larvae at day 10. Since the nematodes exposed to Cd were dead at the end of the experiment, their body length was not measured. It is possible that exposure to single LC20 concentrations affected the development of *C. elegans*, resulting in smaller body

lengths. In contrast, the body length of LC5 exposed nematodes was not affected by any of the single metal treatments (Fig. 3.1B), which suggests that they reached adulthood.

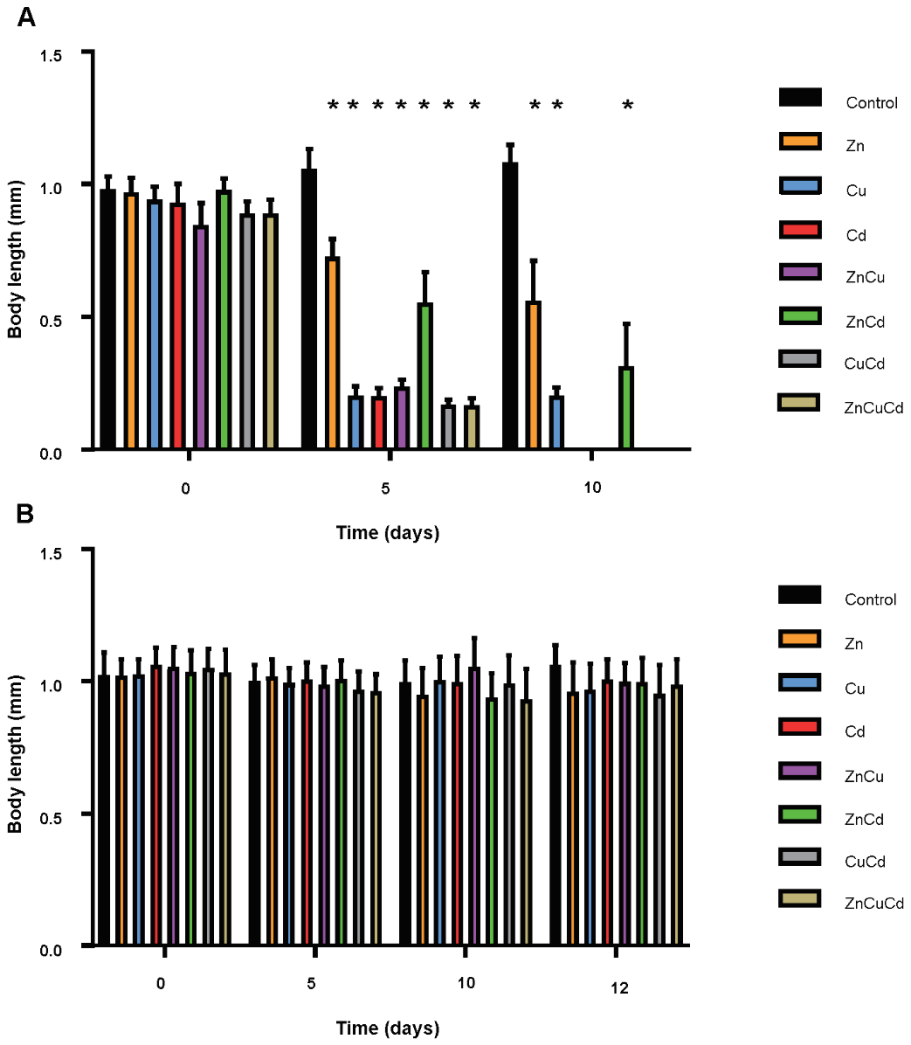

Figure 3.1: Body length (mm) of nematodes exposed to LC20 (A) and LC5 (B). Data are shown as mean ± standard deviation (n=3). Asterisks denote significant differences compared to the corresponding control.

These observations are in agreement with trends reported in earlier studies. For example, the EC50 for body length after an exposure of 24 h was 3.2 mg/L (0.05 mM)

for Cu, 15.1 mg/L (0.23 mM) for Zn, and 5.7 mg/L (0.05 mM) for Cd (Jiang et al., 2016). Others have observed a concentration dependent effect of Cd on developmental stages, e.g. exposure to 2.3 mg/L (20 µM) Cd decreased the number of adults and increased the proportion of nematodes in the L4 stage with a very small number of nematodes in the L3 stage (Lagido et al., 2009). Mixed populations of L4 and L3 larval stages were present after exposure to 3.4 mg/L (30 µM) Cd, while no nematodes developed further than L1-L2 larval stages when exposed to 11.2 mg/L (100 µM) Cd. In the case of Cu (on NGM plates), at the optimal Cu concentration of 0.13 mg/L (2 µM), *C. elegans* developed at day 3 into gravid adults, whereas at higher Cu concentrations nematodes maximally reached L3 larval or young adult's stage (Chun et al., 2017). Similarly, in our study nematodes exposed to Cu at LC5 (0.23 mg/L; *ca*. 4 µM) developed into adults, while at LC20 (1.3 mg/L; *ca.* 20 µM) they only reached the length of L1 larvae. In the case of Zn (in *C. elegans* maintenance medium (CeMM)) nematodes were not able to mature or reproduce in the absence of Zn; exposure to 0.07 – 0.65 mg/L (1-10 µM) caused impaired growth, and optimal growth was in the range of 1.96 – 65.39 mg/L (30 µM to 1 mM) (Davis et al., 2009). In the present study (in liquid medium) body length was not affected by 1.4 mg/L (22 µM) of Zn, but was reduced upon exposure to 9.5 mg/L (145 µM) Zn. Differences between studies in terms of the absolute concentrations at which effects were observed can be ascribed to the different test media and different protocols used, amount of bacteria, type of bacteria etc. These effects of single metal treatments on development of LC20 exposed nematodes are reflected in the observations of population size discussed below.

3.3.1.2. Population size

For control nematodes, the start of new generations was observed clearly with a population increase every 3-4 days. Nematodes exposed to LC20 concentrations of single metals and their mixtures did not show this pattern (Fig. 3.2A). Instead, it seems that only one new generation was formed, whereafter the population size started to decrease. Zn had a similar maximum population size as Cu, although this was already

reached at day 4 for Cu, while the population size of Zn slightly increased until day 8. Thereafter, population size decreased by 25.9% for Zn and by 77.8% for Cu exposure. At day 10, the population size of the Zn and Cu exposed nematodes was comparable. Following attainment of the maximum population size (day 3), the population of Cd exposed nematodes decreased dramatically until all were dead at day 10 (Fig. 3.2A). Thus, as the exposure time increased, the toxicity of Cd was increasingly manifested. Furthermore, Cd had a smaller area under the population size vs. time curve, smaller maximum and smaller end population size compared to Zn and Cu (Table S3.4-S3.6). Further information on the population size and area under the population size vs. time curve for the LC20 treatments is given in the Supplementary Material, Tables S3.4-S3.6. The crucial role of the exposure time on the relative magnitudes of the observed effects points to differences in the dynamics of uptake and biotic handling processes amongst the three metals (Rainbow, 2007).

When exposed to the lower LC5 concentration, Cd caused a lower area under the population size vs. time curve (58.9%, $P<0.05$) (Fig. 3.2B; Table S3.7), while Zn caused a lower maximum population size (78.3%, $P<0.01$) compared to the control group (Table S3.8). Interestingly, the population size in the Cu exposure did not differ from the control, suggesting that the chosen Cu concentration might approximate the essential Cu concentration (Chun et al., 2017). Nevertheless, upon extending the exposure time beyond day 10, the population size in the Cu exposure levelled off from day 11 onwards, whereas the control population further increased (Fig. 3.2B). This finding shows that studies with longer exposure times are necessary to reveal the potential toxicity of low Cu concentrations in the longer term. Population sizes in single metal exposures to Zn and Cd were similar until day 10, after which the population of Cd increased to a greater extent and approached that of Cu at day 12. In contrast to the LC20 exposures, the maximum population size of all single metal treatments was reached at day 12.

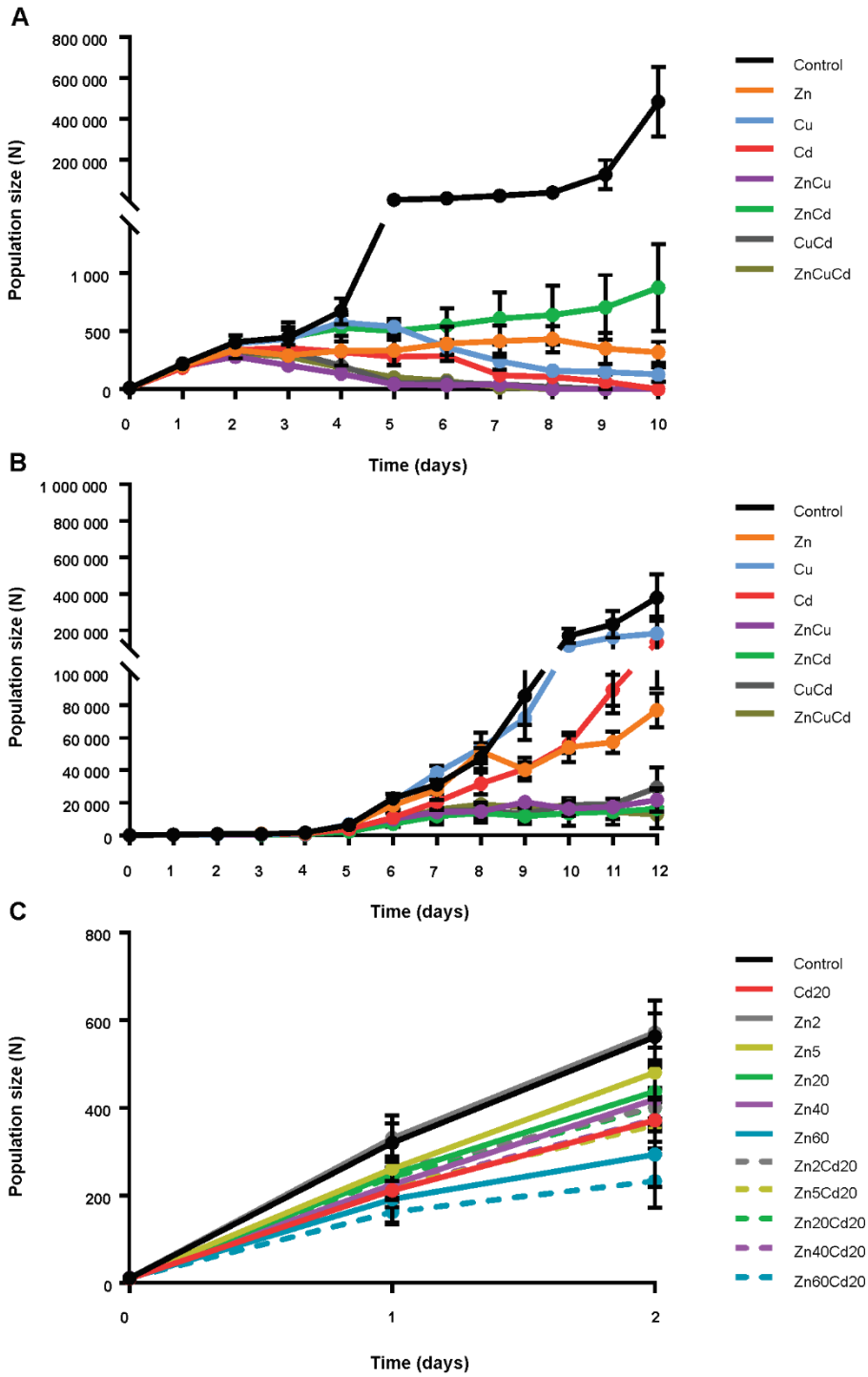

Figure 3.2: Population size (N) of nematodes exposed to LC20 (A), to LC5 (B) and to mixtures of LC20 of Cd with different LCs of Zn (C). Data are shown as mean ± standard deviation (n=3).

Several studies have reported the effects of single metal exposures on *C. elegans* at the population level which are in broad agreement with our findings (Álvarez et al., 2005; Lira et al., 2011).

3.3.1.3. Combination of effects on the population level with other organisational levels

For LC20 exposed nematodes, a decrease in population size was expected because exposed nematodes did not reach adulthood, which disrupted egg laying, causing the number of new larvae to decline. Furthermore, larval nematodes are more sensitive to metal toxicity than adults (Chu and Chow, 2002), which also contributes to the observed population size decrease. Exposure time was also found to be an important factor in determining the absolute and relative magnitude of toxic effects. Short-term 24 h or 48 h exposures typically show Cu to be more toxic than Zn or Cd to *C. elegans* (Williams and Dusenbery, 1990; Dhawan et al., 2000; Chu and Chow, 2002; Moyson et al., 2018). However, in the present study, the relative toxicity order of Cu and Cd was reversed as the exposure time increased. This effect has been reported by others on e.g. mortality (Williams and Dusenbery, 1990).

Although similar declining trends were observed for body length and population size of LC20 exposed nematodes, no effects on body length were observed after LC5 exposure, while population size was slightly affected. It seems that the reproductive characteristics (start of egg laying, number of eggs, time of hatching, hatching success, etc.) of the nematodes exposed to LC5 were affected, which is reflected in differences in population size. These observations indicate that population size is a more holistic and sensitive endpoint than body length alone.

The hypothesis that an affected development and reproduction may play an important role in the declining population sizes for LC5 and LC20 is confirmed in earlier studies. According to numerous publications, the fecundity and growth of *C. elegans* is reduced by Cd (Popham and Webster, 1979; Power and Pomerai, 1999; Höss et al., 2001, 2011).

Decreased fecundity is ascribed to a combination of slow growth and shorter lifespan, preventing development into adulthood, combined with a decrease in number of eggs produced of which fewer were fertilised (Nørhave et al., 2012). These results are in agreement with findings for isopods, *Daphnia magna* and mites (Popham and Webster, 1979; Kammenga et al., 2001; Álvarez et al., 2003; Heugens et al., 2006; Nørhave et al., 2012).

Although Cu is essential, in excess or deficiency it has detrimental effects on brood size and life span and causes an increase in generation time and impairment of development (manifested as reduced body size and growth) (Calafato et al., 2008; Song et al., 2014; Cedergreen et al., 2016; Chun et al., 2017; Fueser et al., 2018; Moyson et al., 2018). Since Cu affects reproduction and life-span, it consequently influences population growth. The reproduction EC50, i.e. the concentration of Cu required to reduce reproduction by 50% relative to control, was found to be 2.04 ± 0.19 mg/L (0.032 mM ± 0.003 mM) for *C. elegans* and 2.21 ± 0.93 mg/L (0.035 mM ± 0.015 mM) for *P. pacificus* (Boyd and Williams, 2003). Furthermore, the population growth at day 3 was maximal at low Cu concentrations (≤ 0.6 mg/L; 10 µM), but impaired at 9.5 mg/L (150 µM) Cu, causing smaller brood size and delayed development (Chun et al., 2017). These results are consistent with the present study: the population exposed to LC20 of Cu (20 µM), reached its maximum size at day 4 and thereafter decreased, which suggests that the reproductive traits were affected.

In the case of Zn, deficient or excess concentrations are known to have harmful effects on (population) growth, survival, development and reproduction of many animals, including *C. elegans*. (Eisler, 1977; Wang et al., 2007; Wang and Ezemaduka, 2014; Kumar et al., 2016). Besides population size, lifespan decrease has also been reported to be concentration dependent: a Zn concentration of 163.5 mg/L (2.5 µM) caused a lifespan shortening of 3 days, while exposure to 4904.3 mg/L (75 µM) or 13078 mg/L (200 µM) reduced lifespan by 4 days (Wang et al., 2007).

Overall, single metal treatments of Zn, Cu and Cd are all able to influence life span and several characteristics, which could explain the observed effects on body length and population size. In addition, hormesis (an adaptive response to low stress levels) might explain the relatively large population size after LC5 exposures in the present study (Calabrese and Baldwin, 2001).

3.3.2. Population effects of mixtures: metal treatment, concentration and time effects

3.3.2.1. Body length

The body length of nematodes exposed to LC20 combinations decreased over time (Fig. 3.1A, Table 3.S1). After 5 days of exposure, the ZnCu, CuCd and ZnCuCd exposed nematodes were 77.5 – 84.2% smaller than the control, while for ZnCd exposure they were 47.6% smaller (P<0.001) (Table S3.2). After exposure to ZnCd, the body length was much larger compared to the other mixtures and 175.6 % larger than of those exposed to Cd alone, while body length of nematodes exposed to Zn was 31.3% larger than of those exposed to ZnCd (P<0.001). This suggests that Zn reduced the toxic effect of Cd on the body length of *C. elegans*. In contrast, the body length of nematodes exposed to ZnCu was similar to that for exposures to Cu alone and much smaller than for Zn alone (P<0.001) (Table S3.2). Apparently Zn was not able to mitigate Cu toxicity, rather it seemed to increase the toxicity. The body length of nematodes exposed to Cu and Cd alone was on average 19.6% larger compared to CuCd (P<0.01). The tertiary mixture led to a smaller body length compared to any of the single metals. At the end of the experiment (day 10), the difference in body length between the control and ZnCd (+23.4%) was larger (P<0.001) than at day 5 (Table S3.3). Nematodes exposed to ZnCu, CuCd and ZnCuCd were dead, therefore their body length was not measured. The body length of Zn was 78.3% larger than ZnCd at day 10 (P<0.01) (Table S3.3). As discussed above for the single metals, exposure to combinations of LC20 concentrations might also affect the development of *C. elegans*, causing smaller body lengths. This was reflected at day 5 in the correspondence between the body length of ZnCu, CuCd and

ZnCuCd exposed nematodes and the length of L1 larvae, while the body length of nematodes exposed to ZnCd was similar to that of L3 larvae. At day 10, the body length of nematodes exposed to ZnCd was similar to the length at the first molt (L1 to L2 larvae) or of dauer larvae. These results indicate that nematodes exposed to LC20 mixtures were not able to reach adulthood, leading to a decline in population size.

In contrast, the body length of nematodes exposed to LC5 mixtures was similar to the control and the single metal exposures (Fig. 3.1B).

3.3.2.2. Population size

The maximum population size of nematodes exposed to ZnCu, CuCd and ZnCuCd combinations of LC20 concentrations was reached within 2-3 days and no nematodes survived until the end of the experiment at day 8-9 (Fig. 3.2A). These mixtures led to a lower area under the population size vs. time curve, a lower maximum population size and a decreased population size at day 10 of exposure as compared to at least one of the corresponding single metals (Table S3.4-S3.6). Interestingly, the nematodes exposed to ZnCd had a larger area under the population size vs. time curve and larger population size at day 10 compared to Cd alone, and a larger area under the curve, larger maximum and greater end population size than the other mixtures (Table S3.4-S3.6). The maximum population size of the ZnCd exposed nematodes was reached at day 10. Zn therefore seemed to have a mitigating effect on the toxicity of Cd.

In contrast to the LC20 treatments, the maximum population size of all combinations of LC5 treatments was reached at day 12, except for ZnCuCd where the maximum was reached at day 8. Nematodes exposed to the LC5 combinations ZnCu, ZnCd, CuCd and ZnCuCd (86.9%, $P<0.001$) had a lower area under the population size vs. time curve and lower end population size (94.8%, $P<0.001$) than the control group (Fig. 3.2B; Table S3.7-S3.8). Furthermore, all LC5 mixtures had a lower area under the population size vs. time curve and smaller end population as compared to the corresponding single metals (Fig. 3.2B, Table S3.7-S3.8). In contrast to the LC20 exposure, the population size in the LC5

ZnCd mixture had a similar pattern to that of the other mixtures. It seems that the LC5 concentration of Zn was too low to mitigate toxicity effects at the population level. For all LC5 mixtures, no deleterious declines in the number of living nematodes were noticed, rather the population size of mixtures even slightly increased over the course of the exposures (Fig. 3.2B).

The interactive effect of single metals on population size in ZnCu, CuCd and ZnCuCd exposures of both LC20 and LC5 concentrations sseemed to be either additive or synergistic. The differences in toxicity indicate the importance of simultaneous testing of mixtures and the corresponding single metals. Furthermore, it is important to take exposure time into account, since the nature of the interactive effects may evolve with time (Margerit et al., 2015). In our study, mixtures of LC20 did not differ from single metals in terms of when the maximum population size was reached (Fig. 3.2A). However, thereafter, the population size of mixtures decreased much faster compared to the corresponding single metals. Even for LC5, differences in population size between mixtures and single metals were more obvious from day 6 onwards (Fig. 2B). Furthermore, it is possible that additivity is only noticed under certain conditions. In our study the combination of Cu and Cd had a larger additive effect at LC5 than at LC20, while the combined additive effect of Cu and Zn at LC5 was similarly additive or slightly less additive than at LC20 (Fig. 3.2). However, in the study of Jonker et al. (2004) synergistic interaction effects of Zn and Cu on the population growth, after 1 week of exposure, were only observed at high dose levels (i.e. higher than LC50). In contrast, the interaction between Zn and Cd in the present study appeared to be antagonistic at LC20 concentrations and additive at LC5 concentrations.

3.3.2.3. Combination of effects on population level with other organisational levels

The toxic effects were again more evident for population size than for body length. For example, after exposure to the ZnCd mixture of LC20 values, body length was on average 196.6% larger as compared to the other mixtures, while population size was on

average 768.0% higher. The differences between mixtures and the corresponding single metal exposures were also more apparent for population size than for body length. At day 5, the population sizes of Cu and Cd exposed nematodes were on average 707.9% larger than of those exposed to the CuCd mixture, while their body length was on average only 19.6% larger (P<0.01).

Although metal mixture toxicity is typically evaluated in terms of the "concentration addition" (CA) model or the "independent action" (IA) model, these simplistic additive approaches have limitations (Chen et al., 2013; Crémazy et al. 2018; Nys et al., 2018). For example, mixture toxicity effects on the temporal evolution of the body length and population size of *C. elegans* are difficult to interpret with this simple framework. As noted above, the body length does not refer to a particular individual but rather represents the average body length of the post-larval stage nematodes of the population, which are not necessarily the same age or from the same life stage. These conditions, as well as the absence of full dose-response profiles, preclude use of the available expressions for the CA and IA models for analysing mixture effects. As a next step, additional experiments with different exposure concentrations are needed to fit dose-response curves as a function of exposure time, and to use this information to model and predict mixture effects at these organisational levels. Such studies would allow an evaluation of the ability of the CA and IA models to predict the temporal evolution of mixture effects. However, these models should be applied with some caution. Our results showed a different population size pattern for $LC20_{24\ h}$ and $LC5_{24\ h}$ exposures, which changed over time. Given these limitations, the relative toxicity effects exerted by mixtures on body length and population size were described and compared with those arising from the corresponding single metals. In most cases the interactive effects are evident from the data shown in the figures, e.g. antagonistic effect of Zn and Cd in LC20 mixtures (Fig 3.1 and 3.2).

Mixtures of LC5 combinations did not cause an effect on body length, while ZnCu, CuCd and ZnCuCd mixtures of LC20 concentrations seemed to cause either additive or

synergistic effects. These mixtures of both LC5 and LC20 concentrations also caused either additive or synergistic effects on population size. Others have reported that Zn and Cu have additive or antagonistic effects at the individual level (mortality, behaviour) and at the community level (number of nematodes in soil) (Korthals et al., 2000; Moyson et al., 2018), and that there are highly synergistic interactions between Cu and Cd, and between Cu and Zn at the individual level (mortality) (Chu and Chow, 2002). Such interactive effects were also observed at the molecular level in a transgenic strain of *C. elegans*, carrying a stress-inducible β-galactosidase reporter, where the combination of Cu and Cd in soil led to a larger response than Cd alone, whereas the ZnCd mixture caused a lower β-galactosidase activity than Cd alone (Calafato et al., 2008). This antagonistic effect was also observed in the present study for the LC20 combination on both body length and population size. Zn seemed thus to have a mitigating effect on Cd due to e.g. competition for Ca channels. Furthermore, this effect was also observed in our previous work at the individual level (mortality and locomotion) (Moyson et al., 2018) and in other studies at the molecular (β-galactosidase activity) and individual level (mortality, body burden, body length) (Chu and Chow, 2002; Power and de Pomerai, 1999).

3.3.2.4. Mitigating effect of Zn on Cd toxicity at the individual and population level

To better understand the mitigating effect of Zn, the population size and mortality of a range of LC concentrations of Zn in combination with a LC20 concentration of Cd was measured. All combinations at 24 h and 48 h, had a lower mortality than that predicted on the basis of the CA and IA models ($P<0.05$) , i.e. antagonistic effects are operative (Fig. 3.3, Tables S3.9 and S3.10). Furthermore, compared to the Cd20 exposure, Zn60Cd20 at both time points, caused a significantly higher mortality, while the mixture Zn2Cd20 at 48 h induced a lower mortality (Tables S3.9 and S3.10).

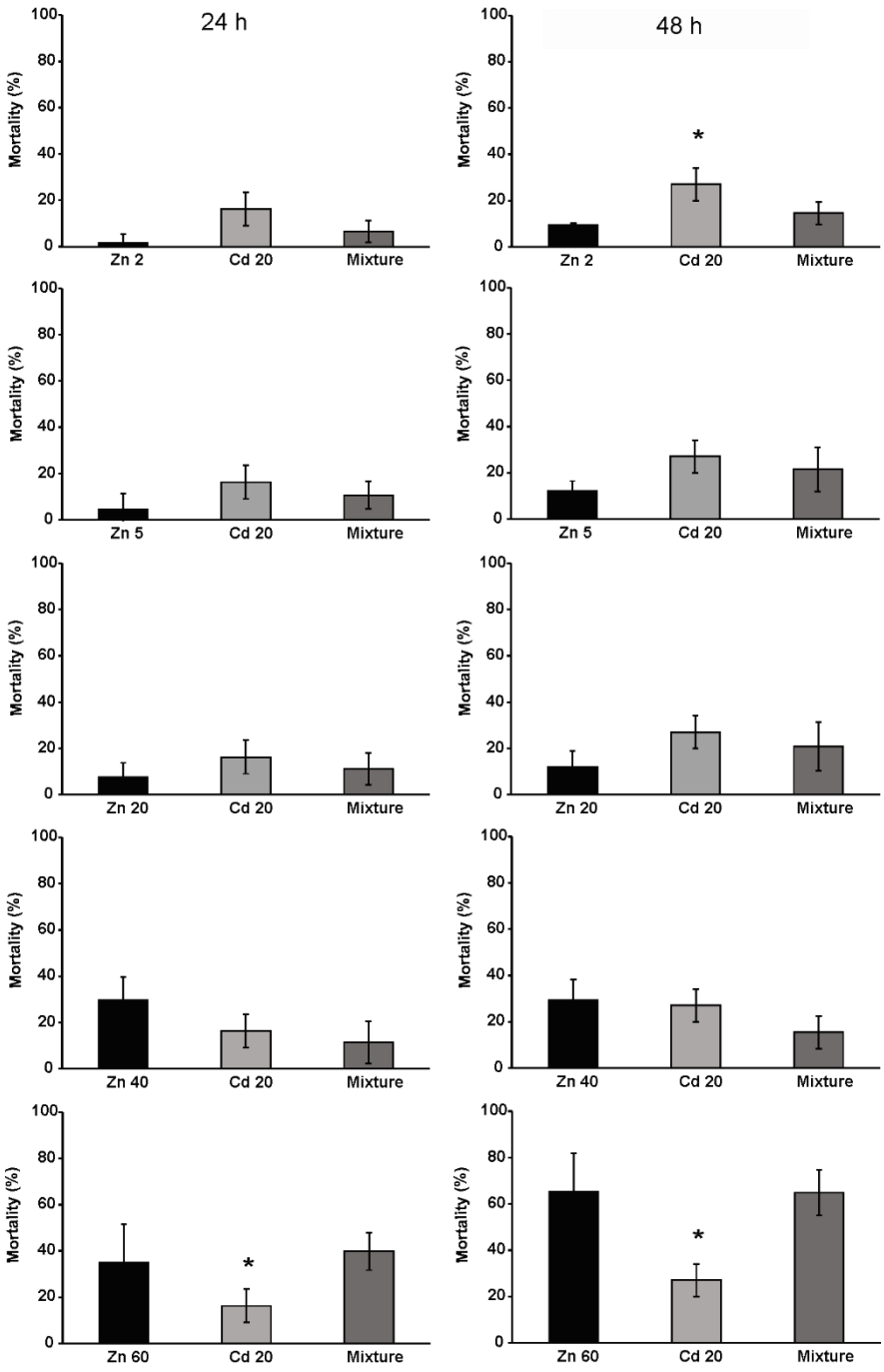

Figure 3.3: Mortality (%) of nematodes exposed to lethal concentrations LC2, LC5, LC20, LC40, LC60 of Zn and LC20 of Cd and their mixtures. Nematodes were exposed during 24 h (left) and 48 h (right). Data are shown as mean ± standard deviation (n=6). Asterisks (*) denote significant differences (P<0.05) compared to the mixture.

Although all ZnCd combinations had an antagonistic effect on the mortality, this effect seemed to be concentration dependent and the strongest antagonistic effect was observed for the Zn40Cd20 mixture. This concentration dependency of the mitigating effect of Zn was more pronounced for the population size. Population size for all treatments reached its maximum at the end of the experiment, day 2, even for the mixture Zn60Cd20 for which a high mortality was noted, but a concentration dependent relation was observed (Fig. 3.2C). When Cd20 was combined with Zn2 (P<0.001) or Zn5 (P<0.01), the population size decreased, resulting in smaller areas under the population size vs. time curve and smaller end population sizes compared to the corresponding Zn-only exposures (Tables S3.11 and S3.12). Furthermore, the combination of Zn60 with Cd20 resulted in a significantly smaller area under the population size vs. time graphs (P<0.05) and end population size (P<0.01) compared to Cd20 alone. In contrast, the mixtures Zn20Cd20 and Zn40Cd20 had a population size that was similar to the corresponding single metal exposures, suggesting that Zn concentrations in this range were best able to mitigate the toxicity of LC20 concentrations of Cd. Apparently, LC5 and LC2 concentrations of Zn were too low to protect the nematodes against the harmful effect of Cd, while LC60 of Zn alone already caused a population decline of 46.7%. The concentration dependence of Zn toxicity is apparent from data reported by Dietrich et al. (2016): in CeMM liquid medium, the population growth rate was maximal at Zn concentrations ranging from 30 – 500 µM, dose-dependent decreases were noticed for concentrations higher than 500 µM and the population growth rate was severely impaired at 2.5 mM Zn. In our study, the population size also decreased dose-dependently, especially LC60 (1.3 mM) caused a significant reduction (Fig. 3.2C). Population size was maximal at concentrations below 30 µM. This suggests that high Zn concentrations disturb Zn homeostasis and that excessive Zn can replace other physiologically important metal ions such as Cu and Mn or bind to ectopic protein sites (Dietrich et al., 2016). The concentration-dependent protective role of Zn on Cd toxicity has been reported in studies on a wide range of organisms including *Daphnia magna* (Attar and Maly, 1982; Meyer et al., 2015; Pérez and Hoang, 2017), freshwater bivalves

(Hemelraad et al., 1987), cladocera (Shaw et al., 2006) and rainbow trout (Dew et al., 2016).

Zn and Cd are both Group 12 metals that can bind to identical macromolecular structures via coordination with oxygen, nitrogen and sulphur functionalities, albeit with different affinity (Nieboer and Richardson, 1980; Brzoska and Moniuszko-Jakoniuk, 2001). In general, Cd can replace Zn in various proteins causing dysfunction and can compete with Zn as a cofactor. Cellular detoxification systems (including glutathione, metallothioneins, heat shock proteins, pumps and transporters) regulate intracellular metal levels by detoxifying and excreting metals (Martinez-Finley and Aschner, 2011). In *C. elegans* transmembrane Zn transporters belong to the CDF or ZIP family. ZIP proteins increase the concentration of cytoplasmic Zn by importing, while CDF proteins function to lower cytoplasmic Zn concentration by exporting across the plasma membrane (Roh et al., 2014; Dietrich et al., 2016). Amongst members of the CDF family, *cdf-1* seems to promote resistance to high Zn levels by promotion of Zn excretion and/or limiting Zn uptake, while *cdf-2* functions to promote Zn accumulation. High levels of Zn induce transcript levels of *cdf-2*, *ttm-1b*, *mtl-1* and *mtl-2*, the promoters of which contain a similar sequence, called HZA (Roh et al., 2014; Dietrich et al., 2016). It is suggested that HZA serves as an enhancer to mediate transcriptional activation in response to high Zn or Cd levels. In contrast, Cu does not affect gene expression, indicating that HZA does not mediate a response to all metals (Roh et al., 2014; Dietrich et al., 2016). In the study of Davis et al. (2009) a dose-response increase in *cdf-2* expression was noticed as a function of increasing Zn concentration, while *cdf-1* was most abundant at low Zn concentrations. We compared the fold-change in *cdf-2* expression reported by Davis et al. (2009) with our observed changes in mortality and population size. Figure 3.4 shows that beyond a certain concentration of around 1 mM (i.e. around the LC60 concentration of Zn in our study), no further increase in *cdf-2* expression was observed and this corresponds to the condition under which a decrease in the extent of antagonistic effects in the ZnCd mixtures was noted.

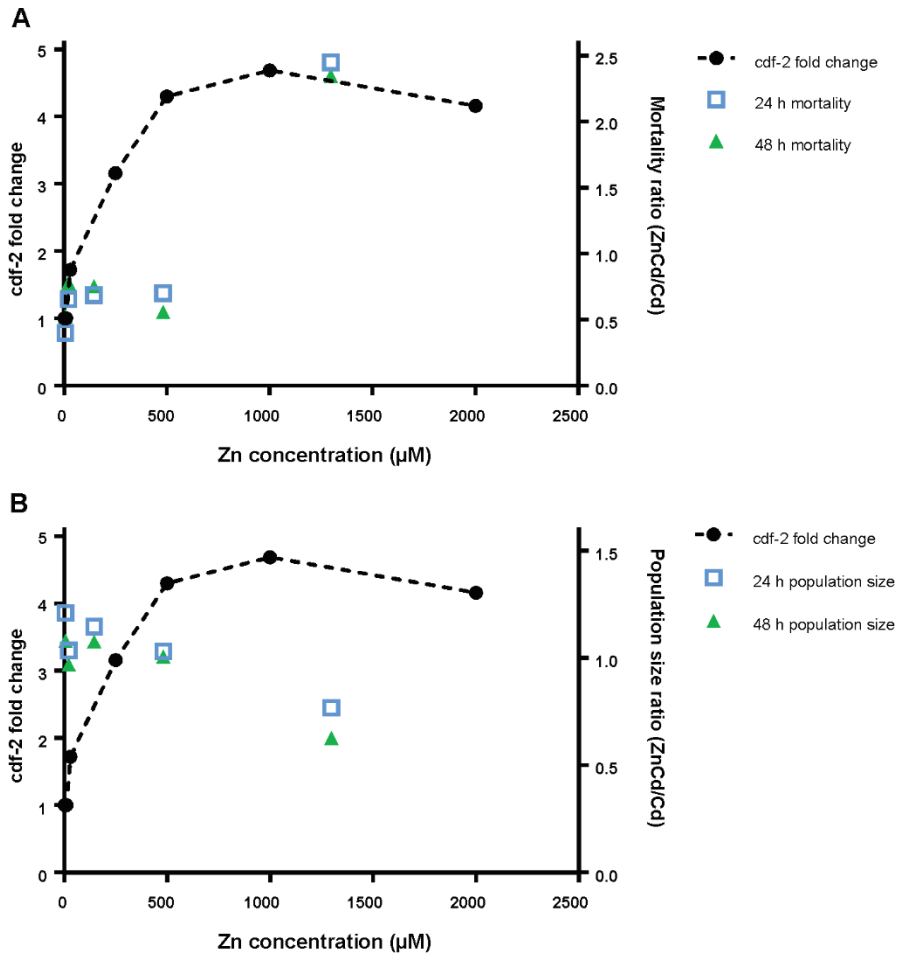

Figure 3.4. (A) Effect of Zn on the Cd20 level mortality after 24 h and 48 h (right-hand axis) as compared to the fold-change in level of *cdf-2* expression in Zn-only exposures (left hand axis; data from Davis et al., 2009). The mortality data are shown as the ratio of mortality in the ZnCd mixture to that in the corresponding Cd only exposure, i.e. a value greater than unity reflects greater toxicity in the mixture exposure. The fold-change in *cdf-2* expression is relative to that observed in Zn at 2 µM. (B) Effect of Zn on the Cd20 population size after 24 h and 48 h (right-hand axis) as compared to the fold-change in level of *cdf-2* expression in Zn-only exposures (left hand axis). The population data are shown as the ratio of the population size in the ZnCd mixture to that in the corresponding Cd only exposure, i.e. a value less than unity reflects greater toxicity in the mixture exposure.

It is possible that the Zn60Cd20 mixture reached or even exceeded the capacity of the system to cope. That is, above a certain Zn concentration there is no further increase in the ability to excrete, chelate or store Zn or Cd, and thus toxicity of both metals is manifested. At the other extreme, i.e. limiting Zn conditions, it has been reported that Cd can displace Zn and trigger a transcriptional response that results in reduced Zn availability (Roh et al., 2014; Dietrich et al., 2016). This process may explain aspects of the mode-of-action of Cd toxicity and its time dependence.

The overall toxicity observed at the individual and population levels is a consequence of the dynamic features of all the involved components (uptake, internal handling and elimination). The translation of exposure to accumulation and toxicity requires knowledge of the kinetics of all the contributing events. The HZA element appears to provide important insights into mechanistic links between the effects of Zn and Cd at the transcriptomic level and those at the individual and population level.

3.4. Conclusions

Our study confirmed that metal toxicity to *C. elegans* is concentration and time dependent, resulting in different trends for population size and body length. In turn, different trends were observed under single vs. mixed-metal exposure conditions. The nature and extent of the effects observed under mixture scenarios were also dependent on the metal combinations employed. Nevertheless, with the exception of ZnCd combinations, the toxicity of mixtures was always greater than that of the corresponding LC concentrations of single metals, and thus either additive or synergistic. The mitigating effect of Zn on Cd toxicity was found to be concentration dependent: e.g. LC20 Zn reduced the toxicity of LC20 Cd, whilst LC5 Zn had no effect.

A significant outcome of our study is the importance of studying effects on different endpoints and on different organisational levels, and the time dependence thereof. Toxic effects at one level, e.g. the molecular or individual, can help to better understand the observations at another organisational level, e.g. the population. Furthermore, it is

possible that at a certain time point no effects are seen at one level, but are evident at another. These findings underscore the need to monitor effects at multiple organisational levels. This study also highlighted the importance of long-term studies. For example, differences between LC20 treatments can already be observed for mortality and behaviour after 24 - 48 h (Moyson et al., 2018), while for population size differences became more evident after 3-4 days of exposure. This can be explained by the fact that population size integrates individual life histories and transgenerational effects, which take time to be reflected in the whole population. For example, if the development of nematodes of the new generation is affected, the effects will only be clearly visible 3-4 days later via the absence of a large increase in population size due to e.g. inability to lay eggs. Overall, metal effects were more evident on population size rather than body length or mortality, suggesting that population size is a sensitive endpoint for effects on long timescales.

Overall, it is evident that data on metal (mixture) effects at different levels (molecular, survival, reproduction, population, etc.) and exposure timescales is needed to elucidate the mechanistic basis of toxic effects. Unfortunately, environmental quality standards are still based on single metal toxicity at short exposure durations and do not take into account the chronic, multi-stressor exposure scenarios that are typical for environmental systems. The mechanistic links we observe herein between transcriptomic responses and mixed metal toxicity at the individual and population level may lead the way to development of a more comprehensive basis for environmental risk assessment.

3.5. Acknowledgements

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concentrations. The nematode strain used in this study was provided by the Caenorhabditis Genetic Center, which is funded by the National Institutes of Health Office of Infrastructure programs. Conflicts of interest: none to declare.

3.6. Supplemental data

3.6.1. Body length after LC20 exposure

Table S3.1: Body length changes over time during LC20 exposures. Percentages represent the increase in body length over time for control and decrease over time for metal treatments. Asterisks denote significant differences (*P<0.05; ** P<0.01; *** P<0.001) (ns= not significant; NA = not applicable).

	0-5 days	5-10 days	0-10 days
Control	7.7% ***	2.8% *	10.8% ***
Zn	25.1% ***	22.8% ***	42.2% ***
Cu	78.4% ***	ns	78.4% ***
Cd	78.4% ***	NA	NA
ZnCu	71.8% ***	NA	NA
ZnCd	43.4% ***	43.1% ***	67.8% ***
CuCd	81.0% ***	NA	NA
ZnCuCd	81.3% ***	NA	NA

Table S3.2: Between treatment comparison of the body length after 5 days of exposure to LC20 concentrations . Asterisks denote significant differences (*P<0.05; ** P<0.01; *** P<0.001) (ns= not significant; NA = not applicable).

	Control	Zn	Cu	Cd	ZnCu	ZnCd	CuCd	ZnCuCd
Control	NA	***	***	***	***	***	***	***
Zn	***	NA	***	***	***	***	NA	***
Cu	***	***	NA	ns	ns	NA	**	**
Cd	***	***	ns	NA	NA	***	**	**
ZnCu	***	***	ns	NA	NA	***	ns	ns
ZnCd	***	***	NA	***	***	NA	***	***
CuCd	***	NA	**	**	ns	***	NA	ns
ZnCuCd	***	***	**	**	ns	***	ns	NA

Table S3.3: Between treatment comparison of the body length after 10 days of exposure to LC20 concentrations. Asterisks denote significant differences (*P<0.05; ** P<0.01; *** P<0.001) (ns= not significant; NA = not applicable).

	Control	Zn	Cu	Cd	ZnCu	ZnCd	CuCd	ZnCuCd
Control	NA	***	***	NA	NA	***	NA	NA
Zn	***	NA	**	NA	NA	**	NA	NA
Cu	***	**	NA	NA	NA	NA	NA	NA
Cd	NA	NA	NA	NA	NA	NA	NA	NA
ZnCu	NA	NA	NA	NA	NA	NA	NA	NA
ZnCd	***	**	NA	NA	NA	NA	NA	NA
CuCd	NA	NA	NA	NA	NA	NA	NA	NA
ZnCuCd	NA	NA	NA	NA	NA	NA	NA	NA

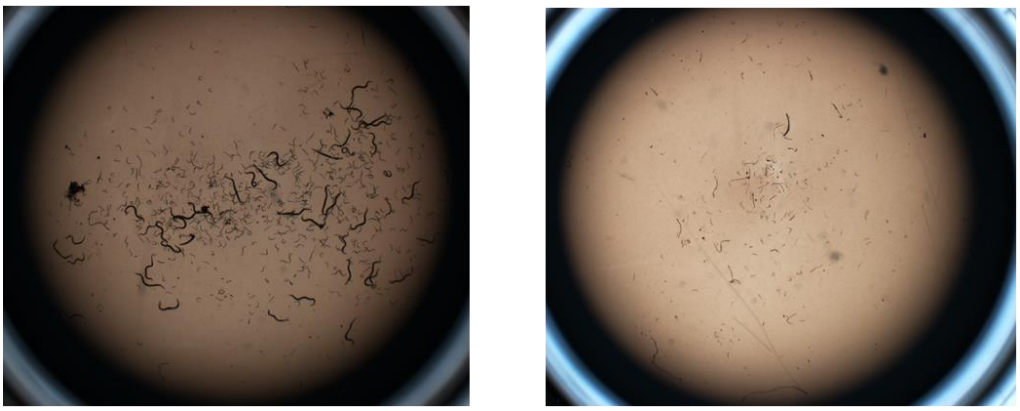

Figure S3.1: Pictures taken at day 5 of 1/8 of the population of nematodes exposed to control (left) and LC20 of Cu (right) (magnification 6x).

3.6.2. Population size after LC5 and LC20 exposure and in mixtures of LC20 of Cd with different Zn concentrations

3.6.2.1. LC20

Table S3.4: Between treatment comparison of the area under the population size vs. time curve of LC20 exposure. Asterisks denote significant differences (* $P<0.05$; ** $P<0.01$; *** $P<0.001$) (ns= not significant; NA = not applicable).

	Zn	Cu	Cd	ZnCu	ZnCd	CuCd	ZnCuCd
Zn	NA	ns	*	***	ns	NA	***
Cu	ns	NA	*	***	NA	***	***
Cd	*	*	NA	NA	**	**	***
ZnCu	***	***	NA	NA	***	ns	ns
ZnCd	ns	NA	**	***	NA	***	***
CuCd	NA	***	**	ns	***	NA	ns
ZnCuCd	***	***	***	ns	***	ns	NA

Table S3.5: Between treatment comparison of the maximum population size of LC20 exposure. Asterisks denote significant differences (* *P*<0.05; ***P*<0.01; *** *P*<0.001) (ns= not significant; NA = not applicable).

	Zn	Cu	Cd	ZnCu	ZnCd	CuCd	ZnCuCd
Zn	NA	ns	**	***	ns	NA	**
Cu	ns	NA	***	***	NA	***	***
Cd	**	***	NA	NA	ns	ns	ns
ZnCu	***	***	NA	NA	**	ns	ns
ZnCd	ns	NA	ns	**	NA	*	*
CuCd	NA	***	ns	ns	*	NA	ns
ZnCuCd	**	***	ns	ns	*	ns	NA

Table S3.6: Between treatment comparison of the end population size of LC20 exposure. Asterisks denote significant differences (* *P*<0.05; ** *P*<0.01; *** *P*<0.001) (ns= not significant; NA = not applicable).

	Zn	Cu	Cd	ZnCu	ZnCd	CuCd	ZnCuCd
Zn	NA	ns	***	***	ns	NA	***
Cu	ns	NA	***	***	NA	***	***
Cd	***	***	NA	NA	***	ns	ns
ZnCu	***	***	NA	NA	***	ns	ns
ZnCd	ns	NA	***	***	NA	***	***
CuCd	NA	***	ns	ns	***	NA	ns
ZnCuCd	***	***	ns	ns	***	ns	NA

3.6.2.2. LC5

Table S3.7: Between treatment comparison of the area under the population size vs. time curve of LC5 exposure. Asterisks denote significant differences (* $P<0.05$; ** $P<0.01$; *** $P<0.001$) (ns= not significant; NA = not applicable).

	Control	Zn	Cu	Cd	ZnCu	ZnCd	CuCd	ZnCuCd
Control	NA	ns	ns	*	***	***	***	***
Zn	ns	NA	ns	ns	**	*	NA	**
Cu	ns	ns	NA	ns	***	NA	**	***
Cd	*	ns	ns	NA	NA	*	*	**
ZnCu	***	**	***	NA	NA	ns	ns	ns
ZnCd	***	*	NA	*	ns	NA	ns	ns
CuCd	***	NA	**	*	ns	ns	NA	ns
ZnCuCd	***	**	***	**	ns	ns	ns	NA

Table S3.8: Between treatment comparison of the end population size of LC5 exposure. Asterisks denote significant differences (* *P*<0.05; ** *P*<0.01; *** *P*<0.001). (ns= not significant; NA = not applicable).

	Control	Zn	Cu	Cd	ZnCu	ZnCd	CuCd	ZnCuCd
Control	NA	**	ns	ns	***	***	***	***
Zn	**	NA	ns	ns	**	*	NA	***
Cu	ns	ns	NA	ns	***	NA	**	***
Cd	ns	ns	ns	NA	NA	**	**	***
ZnCu	***	**	***	NA	NA	ns	ns	ns
ZnCd	***	*	NA	**	ns	NA	ns	ns
CuCd	***	NA	**	**	ns	ns	NA	ns
ZnCuCd	***	***	***	***	ns	ns	ns	NA

3.6.3. ZnCd mixtures

3.6.3.1. Mortality

Table S3.9: Observed mortality after 24 h of exposure to ZnCd mixtures compared between treatments and with predictions from the CA and IA model. Asterisks denote significant differences (* P<0.05; ** P<0.01; ***P<0.001) (ns= not significant; NA = not applicable).

	Expected CA	Expected IA	Cd20	Zn2	Zn5	Zn20	Zn40	Zn60
Zn2Cd20	*	*	ns	ns	NA	NA	NA	NA
Zn5Cd20	*	*	ns	NA	ns	NA	NA	NA
Zn20Cd20	*	*	ns	NA	NA	ns	NA	NA
Zn40Cd20	*	*	ns	NA	NA	NA	ns	NA
Zn60Cd20	*	*	*	NA	NA	NA	NA	ns

Table S3.10: Observed mortality after 48 h of exposure to ZnCd mixtures compared between treatments and with predictions from the CA and IA model. Asterisks denote significant differences (* $P<0.05$; ** $P<0.01$; *** $P<0.001$) (ns= not significant; NA = not applicable).

	Expected CA	Expected IA	Cd20	Zn2	Zn5	Zn20	Zn40	Zn60
Zn2Cd20	*	*	*	ns	NA	NA	NA	NA
Zn5Cd20	*	*	ns	NA	ns	NA	NA	NA
Zn20Cd20	*	*	ns	NA	NA	ns	NA	NA
Zn40Cd20	*	*	ns	NA	NA	NA	ns	NA
Zn60Cd20	*	*	*	NA	NA	NA	NA	ns

3.6.3.2. Population size

Table S3.11: Between treatment comparison of the area under the population size vs. time curve of ZnCd mixture exposures. Asterisks denote significant differences (* P<0.05; ** P<0.01; *** P<0.001) (ns= not significant; NA = not applicable).

	Control	Cd20	Zn2	Zn5	Zn20	Zn40	Zn60	Zn2Cd20	Zn5Cd20	Zn20Cd20	Zn40Cd20	Zn60Cd20
Control	NA	***	ns	ns	**	***	***	**	***	***	***	***
Cd20	***	NA	***	ns	ns	ns	ns	ns	ns	ns	ns	*
Zn2	ns	***	NA	*	**	***	***	***	NA	NA	NA	NA
Zn5	ns	ns	*	NA	ns	ns	**	NA	**	NA	NA	NA
Zn20	**	ns	**	ns	NA	ns	*	NA	NA	ns	NA	NA
Zn40	***	ns	***	ns	ns	NA	ns	NA	NA	NA	ns	NA
Zn60	***	ns	***	**	*	ns	NA	NA	NA	NA	NA	ns
Zn2Cd20	**	ns	***	NA	NA	NA	NA	NA	ns	ns	ns	***
Zn5Cd20	***	ns	NA	**	NA	NA	NA	ns	NA	ns	ns	*
Zn20Cd20	***	ns	NA	NA	ns	NA	NA	ns	ns	NA	ns	***
Zn40Cd20	***	ns	NA	NA	NA	ns	NA	ns	ns	ns	NA	*
Zn60Cd20	***	*	NA	NA	NA	NA	ns	***	*	***	*	NA

Table S3.12: Between treatment comparison of the end population size of ZnCd mixture exposures. Asterisks denote significant differences (* *P*<0.05; ** *P*<0.01; *** *P*<0.001) (ns= not significant; NA = not applicable).

	Control	Cd20	Zn2	Zn5	Zn20	Zn40	Zn60	Zn2Cd20	Zn5Cd20	Zn20Cd20	Zn40Cd20	Zn60Cd20
Control	NA	***	ns	ns	**	***	***	***	***	***	***	***
Cd20	***	NA	***	ns	ns	ns	ns	ns	ns	ns	ns	**
Zn2	ns	***	NA	ns	*	**	***	***	NA	NA	NA	NA
Zn5	ns	ns	ns	NA	ns	ns	***	NA	**	NA	NA	NA
Zn20	**	ns	*	ns	NA	ns	**	NA	NA	ns	NA	NA
Zn40	***	ns	**	ns	ns	NA	*	NA	NA	NA	ns	NA
Zn60	***	ns	***	***	**	*	NA	NA	NA	NA	NA	ns
Zn2Cd20	***	ns	***	NA	NA	NA	NA	NA	ns	ns	ns	***
Zn5Cd20	***	ns	NA	**	NA	NA	NA	ns	NA	ns	ns	**
Zn20Cd20	***	ns	NA	NA	ns	NA	NA	ns	ns	NA	ns	***
Zn40Cd20	***	ns	NA	NA	NA	ns	NA	ns	ns	ns	NA	*
Zn60Cd20	***	**	NA	NA	NA	NA	ns	***	**	***	*	NA

Chapter 4

The interplay between chemical speciation and physiology determines the bioaccumulation and toxicity of Cu(II) and Cd(II) to *C. elegans*

Moyson S., Town RM., Joosen S., Husson SJ., Blust R. The interplay between chemical speciation and physiology determines the bioaccumulation and toxicity of Cu(II) and Cd(II) to *C. elegans.* Published in *Journal of Applied Toxicology* (2018): 1-12 (doi:10.1002/jat.3718).

Abstract

Using the well documented model organism *C. elegans*, a combined analysis of metal speciation in the exposure medium and body burdens of metals (Zn, Cu and Cd) was performed, and factors that are predictive of toxicological endpoints in single metal and mixed metal exposures were identified. Cu, and to a lesser extent Cd, is found to associate with *Escherichia coli* in the exposure medium (the food source for *C. elegans*) as evidenced by the observed decrease in both their dissolved and free metal ion concentrations. Together with a critical analysis of literature data, our results suggest that free metal ion concentrations and thus aqueous uptake routes are the best predictor of internal concentrations under all conditions considered, and of metal toxicity in single metal exposures. Additional factors are involved in determining the toxicity of metal mixtures. In general, the eventual adverse effects of metals on biota are expected to be a consequence of the interplay between chemical speciation in the exposure medium, the timescale of exposure, the exposure route, as well as the nature and timescale of the biotic handling pathways.

Keywords: Body burden, *E. coli*, Free metal ion, Metal speciation, Mixtures, Toxicity

4.1. Introduction

Soil nematodes such as *Caenorhabditis elegans* play a major role in nutrient cycling and dynamics by feeding on bacteria and fungi. As they live within the interstitial waters of soil particles, they are in direct contact with dissolved contaminants. Their abundance, ecological value, characteristics of life history and ease of cultivation and maintenance in the laboratory, make these nematodes excellent organisms for testing aquatic and soil toxicity (Hunt, 2016). Among others, metal toxicity effects on *C. elegans* have been studied using different exposure media and endpoints such as gene expression, reproduction, growth, mortality and locomotion (Popham and Webster, 1979; Power and de Pomerai, 1999; Höss et al., 2001; Boyd et al., 2003; Boyd and Williams, 2003; Höss et al., 2011; Hunt et al., 2013; Inokuchi et al., 2015). Soil-dwelling and benthic organisms are exposed to metals via dissolved and/or dietary routes. The significance of the exposure route will depend on metal speciation in the environmental compartment, as well as the physiology of the organism.

The physicochemical forms of metal ions, i.e. their chemical speciation, depends on the nature of the metal ion as well as the conditions in the medium, e.g. pH, DOC, water hardness, temperature, ionic strength, redox, interaction with organic matter and other complexants (e.g. bacteria), metal concentration, etc. It is typically assumed that free hydrated metal ions are bioavailable; other chemical species may also be accessible to organisms depending on the conditions at the medium/organism interface and the uptake route, e.g. dietary exposure (Jansen et al., 2002; van Leeuwen et al., 2005, 2017). Evidently, a higher concentration of bioavailable metal species in the exposure medium has the potential to cause a higher metal uptake in the body tissue and may result in an increased body burden (Rainbow, 2002, 2007) and/or greater toxicity. Thus, the total body burden of metals in invertebrates depends on the uptake route and bioaccumulation pattern. The bioaccumulation of a metal can be modulated by the differential uptake, transport and sequestration within an animal (Dallinger and Rainbow, 1993). However, there is no consensus on whether the main uptake route for

metals is caused by dietborne exposure or waterborne exposure, which will depend on the physiological features of the organism and the prevailing environmental conditions.

In the case of *C. elegans,* it is not straightforward to discriminate between waterborne and dietborne exposure because the pharyngeal pumping rate is strongly affected by the presence of particles, e.g. bacteria (Offerman et al., 2009; Dwyer and Aamodt, 2013). Furthermore, only a small food volume can be ingested and remains only for a short period (3-10 min) in the weakly acidic intestinal environment of *C. elegans* (pH *ca.* 4; Bender et al., 2013; Chauhan et al., 2013). The organismal detoxification, excretion strategies and characteristics of waterborne and dietborne exposures determine the fate of the metal. Toxicity may occur when the metal uptake rate exceeds the combined rates of detoxification and excretion such that a critical internal threshold is reached (Rainbow 2002, 2007; Adams et al., 2011). Furthermore, total metal concentrations in the exposure medium are generally a poor representation of the actual exposure conditions experienced by the organism. Depending on the exposure scenario, the total concentration of a metal will be distributed over a range of different physicochemical forms. For example, based on an arbitrary size cut-off, typically 0.45 µm or 0.2 µm, "particulate" (e.g. metal adsorbed to bacteria) versus "dissolved" metal species can be distinguished. Within the dissolved metal fraction, metals may be present as e.g. free hydrated ions and complexes with small organic molecules. The free metal ion is generally the bioreactive form (Morel and Morel-Laurens, 1983). Therefore, the concentration of the free metal ion as well as that of the complex forms that can readily dissociate to generate the free metal ion, are expected to be bioavailable (van Leeuwen et al., 2005). Toxicity arises from the deleterious effects exerted by metal ions within cells, thus internal metal concentrations (body burdens) and notably the subcellular distribution of metal species are anticipated to be better predictors of toxicity (Adams et al., 2011). In addition, environmental exposures typically involve mixtures of metal species, yet the processes which determine (eco)toxicological effects under such conditions remain poorly understood.

This work addresses knowledge gaps in the relationship between metal speciation and biouptake and provides insights into the factors governing bioaccumulation and ensuing toxic effects. The aim of the present work is to identify the metal species and uptake route (waterborne versus dietborne) that are the best predictors of body burdens and toxic effects in the soil nematode *C. elegans* under single metal and mixed metal exposure conditions. To achieve this goal, we present data on (i) metal speciation in the exposure medium, specifically the free metal ion concentrations and thereby the extent of metal association with *E. coli*, and (ii) the metal body burden (mg metal/g wormpellet) of *C. elegans*. The obtained results on chemical speciation and body burden are combined with a critical analysis of literature data on toxic effects.

4.2. Materials and methods

4.2.1. Free metal ions

Sublethal concentrations of Cu (LC5) and Cu, Cd and Zn (LC20) after a 24 h exposure (Table 4.1; Moyson et al., 2018) were prepared from 500 x stock solutions of $CuCl_2.2H_2O$ (AnalaR Normapur), $CdCl_2.2.5H_2O$ (Alfa Aesar) and $ZnCl_2$ (Alfa Aesar) in K-medium (52 mM NaCl, 32 mM KCl, 5 µg/mL cholesterol, pH 5.1). The mixtures ZnCu, ZnCd, CuCd and ZnCuCd were prepared by combining the LC20s of the corresponding single metals. The experiment was conducted in both the absence and presence of *E. coli* bacteria (1.5-1.7 g/L) and for each condition three replicates were made. The LC20 metal loading (mg/g bacteria) used in this experiment was in line with reported metal contents of polluted soils that have been used for toxicity studies with *C. elegans* (Höss et al., 2009).

Table 4.1: LC5 and LC20 values of Zn, Cu and Cd after 24 h of exposure (NA = not applicable).

	LC5		LC20	
	(mg/L)	(mM)	(mg/L)	(mM)
Zn	NA	NA	9.501 ± 2.841	0.145 ± 0.043
Cu	0.226 ± 0.104	0.004 ± 0.002	1.299 ± 0.409	0.020 ± 0.006
Cd	NA	NA	7.110 ± 2.315	0.063 ± 0.021

Free metal ion concentrations were measured for 12 days in K-medium in the absence and presence of *E. coli*, using Ion Selective Electrodes (ISEs) and an Ag/AgCl reference electrode (Metrohm), connected to a pH/ion meter (Metrohm). An ISE gives a direct measure of the free metal ion concentration; the decrease in free metal ion concentration upon addition of *E. coli* represents the extent to which the metal ions are associated with the bacteria. Measurements were performed in a climate chamber at *T* = 20°C, i.e. the same conditions as those used in the *C. elegans* exposures. The first measurement took place after 24 h, which corresponds to the time at which the body burden was determined (as described in section 4.2.2). Free Cd^{2+} and Cu^{2+} concentrations were determined in K-medium (data not shown). Free Cd^{2+} concentrations were also measured in Cd and the ZnCd mixture. Cu interfered with the response of the Cd ISE, thus It was not possible to measure the free Cd^{2+} concentration in the mixtures CuCd and ZnCuCd. However, Cd did not interfere with the response of the Cu ISE, therefore the free Cu^{2+} concentration was measured for LC5 Cu, LC20 Cu and the mixtures ZnCu, CuCd and ZnCuCd.

The calibration line for Cu and Cd was Nernstian, *i.e.* the slope of the log of the free metal ion concentration *vs*. *E* (mV) was always between 25 and 30 at *T* = 293.15 K. The pH of each replicate was measured at day 0, day 10 and day 12 and some replicates were randomly checked during the experiment. A fourth replicate was used for daily pH measurement and for sampling, both filtered filtered (0.2 µm supor polyethersulfone ion chromatography membrane in an acrodisc 13 mm syringe filter, Pall Corporation)

and non-filtered, to measure the proportion dissolved vs. total metal and to verify metal concentrations (91%-100.6% recovery) using HR-ICP-MS (Element XR, Thermo Scientific). Stock solution concentrations were verified by ICP-OES (ICAP 6300 Duo, Thermo Scientific). Therefore, samples containing *E. coli* bacteria were first freeze-dried (Heto Powerdry LL 30000, Thermo Scientific) and 250 µL nitric acid (TraceMetal Grade, Fisher Chemical) was added. All samples were digested at 110°C for 30 minutes, using a heating plate (HotBlock, Environmental Express). MilliQ water was added, making the total volume 10 mL. For each treatment without *E. coli*, the pH measured before (5.1 ± 0.1) was similar to the pH measured after the experiment (4.9 ± 0.1), while the pH of treatments with *E. coli* increased from 5.1 ± 0.0 to 5.9 ± 0.3. Similar pH values and increases were also noticed in an earlier study (unpublished data).

4.2.2. Body burden (internal concentration) measurement

4.2.2.1. *Caenorhabditis elegans* culture and synchronisation

Wild type *Caenorhabditis elegans* nematodes of the N2 strain were obtained from the Caenorhabditis Genetic Centre, Minneapolis, USA. Nematodes were maintained in optimal conditions on nematode growth medium (NGM) agar plates at 20°C, seeded with *Escherichia coli* (OP50 strain) as food source (Brenner, 1974). No dauer stage individuals were present in the parental generation. Synchronization of the nematodes was performed by bleaching, adding a hypochlorite solution (5 N NaOH, 8% sodium hypochlorite) to mixed-stage *C. elegans*, killing the nematodes that were not protected by an egg shell. Eggs were raised on OP50-seeded NGM agar plates.

4.2.2.2. Test media

LC20 concentrations of Zn, Cu and Cd after a 24 h exposure (Table 4.1) and determined in our previous study (Moyson et al., 2018), were made from $ZnCl_2$ (Alfa Aesar), $CuCl_2.2H_2O$ (AnalaR Normapur) and $CdCl_2.2.5H_2O$ (Alfa Aesar) in K-medium (52 mM NaCl, 32 mM KCl, 5 µg/mL cholesterol, pH 5.1), supplemented with *E. coli* bacteria (1.5

- 1.7 g/L). The mixtures ZnCu, ZnCd, CuCd and ZnCuCd were prepared by combining the corresponding LC20 concentrations. Stock solutions were made of tenfold higher concentrations. ICP-OES (ICAP 6300 Duo, Thermo Scientific) was used to verify metal concentrations of stock and exposure solutions (93% - 113% recovery). Metal solutions were incubated with the bacterial suspensions for 12 h at 4°C prior to toxicity testing, allowing metal partitioning between the aqueous phase and the bacteria. Since the determined average pH before (5.3 ± 0.2) and after the experiment (5.4 ± 0.4) was within an acceptable pH range for *C. elegans*, its potential effects on the measured parameters were excluded.

4.2.2.3. Body burden

Young L4 nematodes (24 h since the onset of the L4 stage) were washed several times and transferred to a NGM plate without food, to get rid of *E. coli* bacteria. Approximately 4.5 mg nematodes were transferred to 15 mL Falcon tubes (polypropylene) filled with 9 mL K-medium and 1 mL test medium (K-medium containing the test metal concentration(s)) or control (K-medium), supplemented with *E. coli* OP50 (1.5 - 1.7 g/L). During the experiment, the Falcon tubes were shaken continuously (160 rpm, 20°C). After 24 h of metal exposure to LC20, nematodes were washed three times with physiological water (9 g/L NaCl) to get rid of bacteria. Subsequently, *C. elegans* were killed slowly by gradually increasing the temperature: the low rate of temperature change ensured that the worms remained intact, thereby avoiding any potential loss of internal metal content. The dead nematodes were washed again. The physiological water with the nematodes was filtered using a 5 µM membrane filter paper (Whatman), which was placed in a plastic filter holder (Schleider and Schuell). The filter paper containing the nematodes was then plugged into a Falcon tube by the use of tweezers and 0.2 µL nitric acid was added. Overnight, the Falcon tubes were placed under a fume hood. The following day, these tubes were transferred to a hot block (110°C) for 30 minutes. After cooling down of the samples, MilliQ water was added, bringing the volume up to 4 mL. For each treatment three replicates were made. Body burdens

(internal concentrations) of Cu, Cd, Zn, Na, K, Ca, Mg and Fe were measured by a HR-ICP-MS (Element XR, Thermo Scientific).

4.2.3. Statistical analysis

Data were analysed with the statistical program R, Version 3.1.2., with a 5% level of significance. Normality was checked visually by histograms and by the Shapiro–Wilk test. The Bartlett test was used to verify the homogeneity of variances.

4.2.3.1. Free metal ions

Generalized mixed models were fitted to test the possible effects of exposure time, *E. coli* presence/absence, treatments and their interactions on the free metal (Cu, Cd) ion concentration and percentage. In all models, exposure time (days) was entered as a continuous variable. *E. coli* presence/absence and treatments, plus their interactions, were included as fixed effects. Because the free metal ion concentration was repeatedly measured within the same wells over time, observations from the same well were not independent. To account for this non-independence, a random intercept term for well was added to the model. Measurements of the control group and Zn group were omitted from the analysis, since the free metal ion concentration was always (close to) zero. Subsequently, for each metal treatment both with and without *E. coli*, a one-way ANCOVA analysis was fitted to determine the slope of the regression line and thus analyse the effect of time on the free metal ion concentration and percentage. When time did not have an influence on free metal ions, a one-way analysis of covariance (ANCOVA) was fitted for each treatment to analyse if the slopes of the regression lines of *E. coli* presence and absence differed. Likewise, the main effect of metal treatment on the slopes of the regression lines of the free metal ion concentration was analysed when - in both treatments involved - *E. coli* was present or absent. Thus, free metal concentration was compared for each treatment in the presence and absence of *E. coli*. For each *E. coli* condition, the following comparisons between treatments were made:

Cd vs. ZnCd, Cu LC5 vs. CuLC20, CuLC20 vs. ZnCu, CuLC20 vs. CuCd, CuLC20 vs. ZnCuCd, ZnCu vs. CuCd, ZnCu vs. ZnCuCd and CuCd vs. ZnCuCd.

To analyse the differences in dissolved Zn, Cd and Cu concentrations, data of the different time points were pooled. For each treatment, a one-way ANOVA analysis was conducted to determine the effect of *E. coli* presence on the dissolved metal concentration and percentage. Furthermore, in the same *E. coli* condition, comparisons between 2 treatments were carried out by one-way ANOVA analyses with treatment and *E. coli* condition as main effects. Per *E. coli* condition the same comparisons between Cu and Cd treatments were made as mentioned above for metal speciation. For dissolved Zn, following comparisons were made for each *E. coli* condition: Zn vs. ZnCu, Zn vs. ZnCd, Zn vs. ZnCuCd, ZnCu vs. ZnCd, ZnCu vs. ZnCuCd and ZnCd vs. ZnCuCd. If the requirements for ANOVA were not fulfilled, a log- transformation of data was applied.

4.2.3.2. Body burden

Because the requirements for ANOVA were not fulfilled, a log-transformation of data was applied. The main effect of metal treatment on the metal body burden of the nematodes was analysed by a one-way ANOVA. If there was a significant difference between treatments in uptake of Mg, Ca, K, Fe or Na, a posthoc analysis with Tukey correction was carried out to determine the differences between groups. For Cd, Cu and Zn uptake, metal exposed groups were compared with the control group using a Dunnett post hoc test. Subsequently, Tukey honest significant difference tests were used to determine the differences between groups exposed to the measured ion (e.g. for Cd uptake: Cd, ZnCd, CuCd and ZnCuCd with each other).

4.2.4. Relationship between exposure conditions, bioaccumulation and toxic effects

To identify the relationship between our results on chemical speciation in the exposure medium, and body burden (bioaccumulation) we computed the maximum expected

metal intake by waterborne and dietborne routes and compared this with the measured body burden. To establish links between the body burden and toxic effects, we performed a critical interpretation of two independent literature reports on (i) *cdr-1* gene expression as a function of Cd body burden (Offerman et al., 2009), and (ii) growth status as a function of Cu speciation in the exposure medium (Yu et al., 2012).

4.3. Results

4.3.1. Metal speciation in the exposure medium

In the present study, both the dissolved and free ion concentrations, expressed as absolute concentrations and as percentages of the total concentration, were measured for both Cd and Cu in single metal and metal mixture exposures. The dissolved Zn concentration and percentage was also measured in different treatments. To investigate the influence of *E. coli* bacteria as a potential metal complexant, the experiment was conducted over a range of 12 days, both in presence and absence of *E. coli*.

For all Cd and Cu treatments, both the free metal ion concentration and percentage remained stable over time, except for CuLC5 where *E. coli* presence caused a gradual decrease over time in the free Cu ion concentration and percentage, reaching an 88.7% reduction after 12 days ($P<0.001$) (Fig. 4.1, dashed gray line).

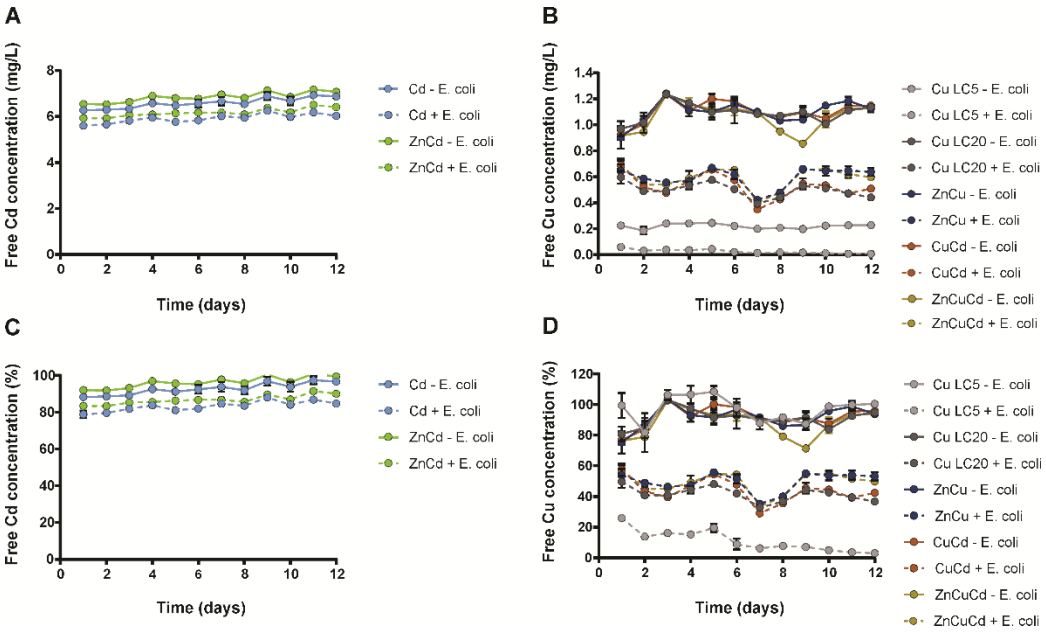

Figure 4.1: Free Cd ion concentration (mg/L (A); % (C)) of Cd and ZnCd treatment in the presence and absence of *E. coli*. Free Cu ion concentration ((mg/L) B; % (D)) of CuLC5, CuLC20, ZnCu, CuCd and ZnCuCd treatment in the presence and absence of *E.coli*. Data are shown as mean ± standard deviation (n=3).

4.3.1.1. Metal speciation in *E. coli* absence

In all treatments in the absence of *E. coli*, the ISE measurements indicate that practically all of the Cd and Cu was found to be in the free ion form (average 94.5% and 92%, respectively), (Fig. 4.1).

4.3.1.2. Metal speciation in *E. coli* presence

The presence of *E. coli* affected the speciation of both Cu and Cd (Fig. 4.1 and 4.2). The bacterial influence was greater for Cu than for Cd treatments, resulting in an average of 39.0% for the free Cu ion percentage, while the mean free Cd ion percentage was still 85.0%. The presence of *E. coli* caused a mean decrease of 10.0% in free Cd ion concentration and percentage in both Cd and ZnCd exposure, as compared to in the absence of bacteria (P<0.001). In the case of Cu, the presence of *E. coli* led to an even greater decrease in free ion concentration and percentage in all treatments (P<0.001)

by, on average, 88.7% for CuLC5, 54.4% for CuLC20, 45.9% for ZnCu, 52.3% for CuCd, and 44.2% for ZnCuCd. Again, the effect was more pronounced for the CuLC5 exposure.

Similar trends were found for dissolved metal concentrations, especially for Cu (Fig. 4.2). The concentrations of dissolved Cd did not differ between *E. coli* presence or absence, while presence of *E. coli*, in percentage terms, caused a significantly slightly lower concentration of dissolved Cd in the Cd treatment (4%, $P<0.05$) compared to *E. coli* absence (Fig. 4.2). Similarly, for Zn treatments no difference between *E. coli* conditions could be found, except for a smaller dissolved Zn concentration with *E. coli* than without bacteria (17%, $P<0.05$) (Fig. 4.3). This factor difference reflects the amount of Zn that is associated with the *E*. *coli*. In contrast, the presence of *E. coli* bacteria caused a reduction of the dissolved Cu concentration in all treatments; again the largest percentage decrease was noted for CuLC5 (Fig. 4.2). Compared to *E. coli* absence, the dissolved Cu concentration in the presence of *E. coli* decreased by 71.2% for CuLC5, 51.3% for CuLC20, 39.7% for ZnCu, 48.5% for CuCd and 44.7% for ZnCuCd ($P<0.001$). Slightly smaller reductions between *E. coli* conditions were observed for the percentage of dissolved Cu: 61% for CuLC5 ($P<0.001$), 34.8% for CuLC20 ($P<0.01$), 27.7% for ZnCu ($P<0.01$), 31.8% for CuCd ($P<0.01$) and 29.6% for ZnCuCd ($P<0.001$).

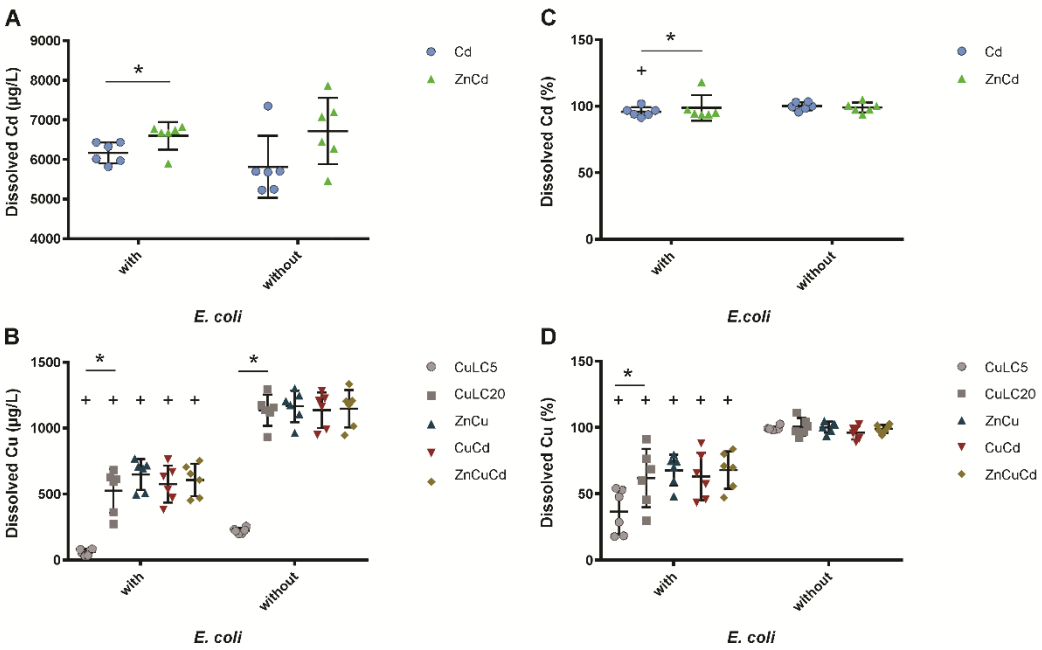

Figure 4.2: Dissolved Cd concentration (µg/L (A); % (C)) of Cd and ZnCd treatment in the presence and absence of *E. coli*. Dissolved Cu concentration (mg/L (B); % (D)) of CuLC5, CuLC20, ZnCu, CuCd and ZnCuCd treatment in the presence and absence of *E.coli*. Data are shown as mean of pooled data ± standard deviation. Symbols denote significant differences between metal treatment (*) and between *E. coli* conditions (+).

4.3.1.3. Concentration dependence of Cu speciation

Although the presence of *E. coli* had a significant effect on the free metal ion concentration of Cu, differences between LC5 and LC20 treatments were smaller. Independent of the presence of *E. coli*, dissolved Cu concentration differed between CuLC5 and CuLC20 (P<0.001), while dissolved Cu percentage only differed in *E. coli* presence (P<0.05) (Fig. 4.2). In the absence of *E. coli*, CuLC20 had on average a 6.2% lower percentage of free Cu ions than CuLC5 (P<0.01) (Fig. 4.1). However, in that condition free Cu concentration of CuLC20 was 5 times higher than of CuLC5 (P<0.001), which was expected from the higher total Cu exposure concentration. In the presence of *E. coli*, CuLC20 had a 20.1 times higher free Cu ion concentration (P<0.001), while its percentage of free Cu ions was on average 3.8 times higher than for CuLC5 (P<0.001)

(Fig. 4.1). The lowest Cu concentration (CuLC5) showed to have the strongest reduction in free Cu over time and at the end of the experiment almost all Cu was bound in *E. coli* presence (97%), while at CuLC20 exposure 54% of Cu was bound to *E. coli* bacteria (Fig. 4.1).

4.3.1.4. Metal speciation in mixtures

Differences in metal speciation between mixtures and single metals were smaller than those between individual metals (Fig. 4.1, 4.2, and 4.3). ZnCd had a 4.0% higher free Cd ion concentration and percentage than Cd exposure, both with and without *E. coli* (P<0.001) (Fig. 4.1). No difference in dissolved Cd concentration and percentage was noted between Cd and ZnCd in the absence of *E. coli*, while in *E. coli* presence a lower dissolved Cd concentration (7.5%) and percentage (3.9%) was observed for Cd than for ZnCd (P<0.05) (Fig. 4.2). Treatments with equal total Cu concentration did not differ in the absence of *E. coli*, but in *E. coli* presence, free Cu ion concentration and percentage of CuLC20 was slightly lower than in the case of ZnCu and ZnCuCd (15.9%, P<0.001) (Fig. 4.1). Moreover, a 12.6% higher free Cu ion concentration and percentage of ZnCu and ZnCuCd than in the case of CuCd was observed (P<0.01). Furthermore, in *E. coli* presence, the concentration and percentage of free Cu ions of CuCd was similar to that of CuLC20, while free Cu percentage of ZnCu was similar to that of ZnCuCd. In contrast, no difference in concentration or percentage of dissolved Cu was measured between mixtures and corresponding single metals (Fig. 4.2). In addition, no difference between treatments was noted for dissolved Zn concentration and percentage (Fig. 4.3).

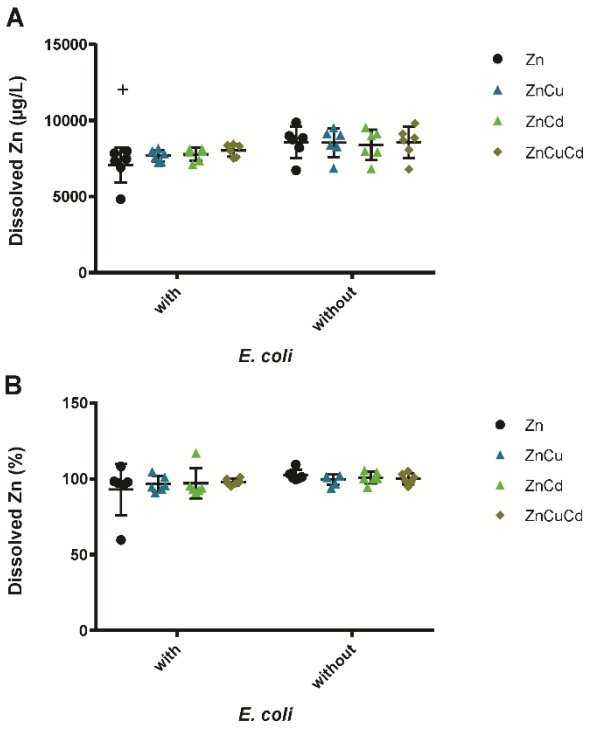

Figure 4.3: Dissolved Zn concentration (µg/L (A); % (B)) of Zn, ZnCu, ZnCd and ZnCuCd treatment in the presence and absence of *E.coli*. Data are shown as mean of pooled data ± standard deviation. Symbols denote significant differences between *E. coli* conditions (+).

4.3.2. Body burden

Nematodes accumulated significant amounts of metals in their bodies under all LC20 exposure conditions considered. As compared to the control, Cu body burdens in Cu (20.2x, $P<0.001$), ZnCu (16.4x, $P<0.001$), CuCd (20.4x, $P<0.001$) and ZnCuCd (12.2x, $P<0.01$) exposed nematodes was significantly higher (Fig. 4.4). Also Cd accumulation in Cd (24.7x, $P<0.001$), ZnCd (26.9x, $P<0.001$), CuCd (12.7x, $P<0.01$) and ZnCuCd (12.7x, $P<0.01$) exposed nematodes was significantly greater than that of the control. For Zn, the difference in body burden between metal exposed groups and the control was not significant due to the large standard deviation. Furthermore, no significant differences were found for accumulation of the major elements Na, K, Ca, Mg and Fe, under any of the exposure conditions.

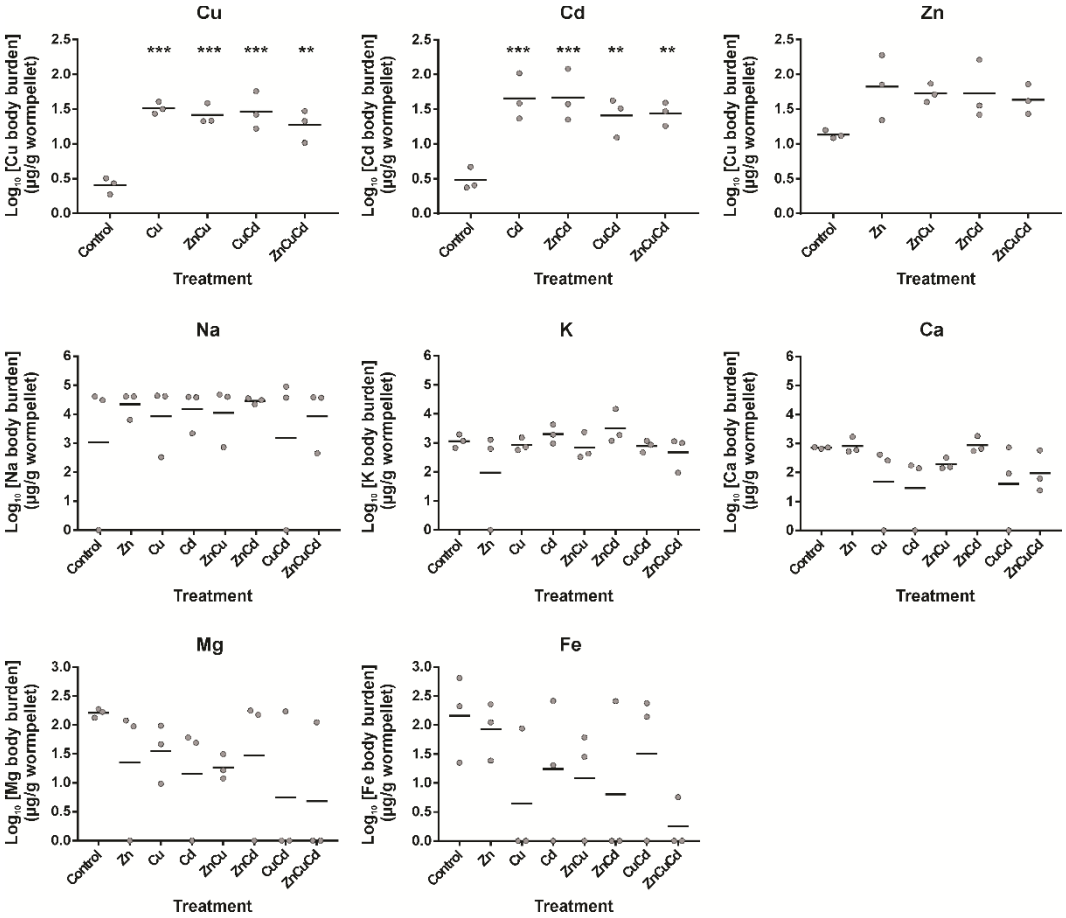

Figure 4.4. Body burden (internal concentration) of Cu, Cd, Zn, Na, K, Ca, Mg and Fe of nematodes exposed for 24 h to LC20 concentrations of metals and their mixtures. Replicates (n=3) are shown as well as the average. The body burden refers to the wet weight of the nematodes. Asterisks denote significant differences (**P*<0.05; ***P*<0.01; ****P*<0.001) compared to control.

4.4. Discussion

Our results on metal speciation in the exposure medium provide insights into the factors governing bioaccumulation by *C. elegans*, and the ensuing toxic effects. Each of these factors is discussed below.

4.4.1. Metal speciation in the exposure medium

In the absence of *E. coli* practically all of the Cd and Cu are found to be in the free ion form (Fig. 4.1). Using Visual MINTEQ, others have reported the percentage of free Cu^{2+} and Zn^{2+} to be 92% in K-medium (Freeman et al., 1998), in good agreement with our data, whilst the major Cd species are predicted to be chloro-complexes ($CdCl^{-}$) (64%) and free ions (20%) (Cressman III and Williams, 1997). The apparent discrepancy between our ISE measurements of Cd and the predictions of Visual MINTEQ are likely due to uncertainties in the stability constants used in the model. The stability of metal ion complexes with chloride is rather low, and the computed speciation is sensitive to the magnitude of the stability constant, *K*, employed. Visual MINTEQ uses a log *K* value of 0.3 for CuCl and 1.98 for CdCl. However, in aqueous media, log *K* values as high as *ca.* 1 have been reported for CuCl (Sato and Kato, 1977) and the IUPAC recommended value is 0.83 (Powell et al., 2007), while for CdCl, log *K* values as low as 0.5 have been reported (Simoes et al., 1981). As some reports of log *K* values for ZnCl are of order 0.5, it is possible that the majority of this metal is in the free ion form in the exposure media (Aparicio and Elizalde, 1996; de Robertis and de Stefano, 1998). The magnitude of the stability constants for these chloride species is comparable to that computed for an outer-sphere ion pair based purely on electrostatic considerations (Fuoss, 1958). In the presence of *E. coli*, the Cu and Cd speciation depends on the metal-to-bacteria ratio. Fig. 4.1 shows that at low Cu concentration, all Cu is bound to *E. coli*, while at higher Cu concentration a greater proportion of Cu is present in the form of free ions. Our results are in broad agreement with literature data on Cd-*E. coli* (Höss et al., 2011) and Cu-*E. coli* binding (Mullen et al., 1989; Fang et al., 2009), i.e. they lie on the same metal-binding isotherm.

4.4.2. Body burden

Our results indicate that, although less total Cu in the exposure medium was required to cause the same lethality of 20% as Cd and Zn (Moyson et al., 2018), this is not

reflected in a higher total body burden. Rather, in contrast, the total body burden of Cu was 40.7% lower compared to Cd, and 61.4% lower than Zn accumulation after single metal exposure (Fig. 4.5). Per liter of exposure medium, one gram of worms took up 0.8% of total Cd, 2.5% of total Cu and 0.9% of total Zn from the metal exposure medium (i.e. in presence of *E. coli*).

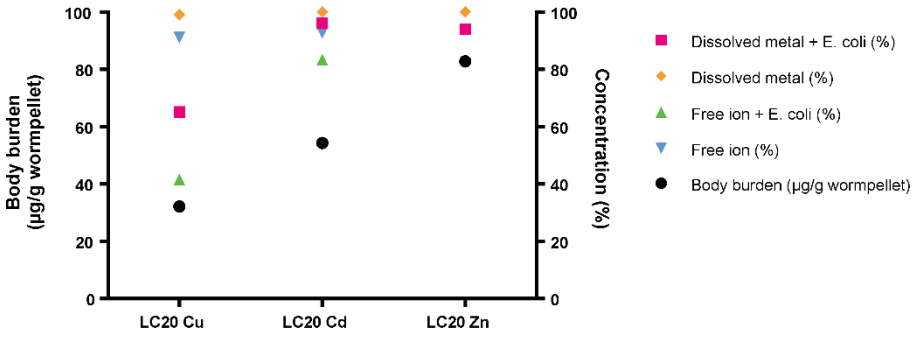

Figure 4.5. Dissolved and free metal ions (%) of Cu, Cd and Zn after exposure to their LC20 concentrations (right-hand axis) and body burden (internal metal concentration) of nematodes exposed to these LC20 concentrations (left-hand axis). The body burden refers to the wet weight of the nematodes.

The amount of metals accumulated by *C. elegans*, coupled with information on metal speciation in the exposure medium, provides insights into the relative contributions of waterborne and dietborne metals to the body burden. Considering the waterborne free metal ions, in the case of Cu (LC20) the concentration of free Cu^{2+} is $9x10^{-6}$ mol dm^{-3} and the surface area of the biointerface is approximately $6.6x10^{-7}$ m^2 (external and internal surface). The steady-state limiting diffusive supply flux of the free Cu^{2+} is given by Dc/δ (van Leeuwen et al., 2005) where D is the diffusion coefficient of Cu (*ca.* $7x10^{-10}$ m^2 s^{-1}; Li and Gregory, 1974), c is the concentration of Cu^{2+}, and δ is the thickness of the aqueous diffusion layer (*ca.* $5x10^{-4}$ m in unstirred solution; Levich, 1962), which yields a value of $1.3x10^{-8}$ mol m^{-2} s^{-1}, i.e. a total of $7.4x10^{-10}$ mol per worm after 24 h of exposure. If the entire supply flux of Cu^{2+} was accumulated by *C. elegans*, the ensuing body burden would be 4700 µg/g wet weight. The measured body burden is more than two orders of

magnitude lower than this, i.e. the free metal ion concentration alone is well able to satisfy the uptake demand of the organism. A parallel computation for Cd shows the same picture: the measured body burden in this case is approximately 1000 times lower than that computed on the basis of the supply flux of the free ion being the determinant of bioaccumulation. For assessment of the dietborne exposure the ingestion rate was estimated to be 10^5 *E. coli* per worm per day (Gomez-Amaro et al., 2015). In the case of Cu, the concentration of Cu that is associated with *E. coli* is $1.1x10^{-5}$ mol dm^{-3} at an *E. coli* concentration of 1.6 g (wet weight) dm^{-3}. The wet weight of an *E. coli* cell is *ca.* 10^{-12} g, and thus each *E. coli* contains $4.4x10^{-16}$ g Cu. Accordingly, ingestion of 10^5 *E. coli* by an individual nematode (with a wet weight of *ca.* 10^{-5} g) corresponds to an intake of $4.4x10^{-11}$ g of Cu, which is equivalent to 4.4 µg/g wet weight. This is a factor *ca*. 10 *lower* than the measured body burden. For the case of Cd, a parallel analysis also yields 4.4 µg/g wet weight based on ingestion of bacteria, which is a factor *ca*. 15 times lower than the measured body burden. These results imply that the free metal ion is the predominant contributor to bioaccumulation of both Cu and Cd.

In agreement with our results, Höss et al. (2011) suggested that the main Cd uptake route is from "aqueous Cd", taken up together with the bacteria, rather than from bacterial-bound Cd concentrations. The "aqueous Cd" corresponded to all forms of Cd remaining in solution after bacteria removal. Nevertheless, the outcome should be regarded with some caution because the pharyngeal pumping rate is sensitive to the presence of metal ions, albeit that typically a *lower* rate is observed (Jones and Candido, 1999), i.e. if anything, our calculations are an overestimation of the dietborne contribution to the body burden. The bioaccumulation pattern of Cu and Cd in the present study was also noticed in earlier studies. In these studies, a concentration dependent metal content was established for Cu and Cd, which was also time dependent for Cd (Offerman et al., 2009; Chun et al., 2017).

In the case of Zn, the body burden of *C. elegans* is reported to be largely proportional to the dietary Zn and can be moderately changed in response to that, although the

uptake route has not been established (Davis et al., 2009). Nematodes exposed to 32.7 mg/L (0.5 mM) Zn in the presence of bacteria had a 109% higher total Zn content, compared to control nematodes (Kumar et al., 2016). Baseline Zn content increased from 0.09 µg/mg in L3 stage to 0.1 µg/mg for 1-day-old adults and to 0.14 µg/mg for 5-day-old adults, indicating a moderate increase in zinc content with age (Kumar et al., 2016).

4.4.3. Relationship between exposure conditions, bioaccumulation, and toxic effects

Models for prediction of metal biouptake, e.g. the biotic ligand model and the free ion activity model, assume that only free metal ions are available for biouptake (Campbell, 1995; Brown and Markich, 2000; Paquin et al., 2002; Slaveykova and Wilkinson, 2005; Jakob et al., 2017), and attempts have been made to use metal ion characteristics as predictors of toxic effects (Renner, 1997; Tatara et al., 1997, 1998). Recent work with *C. elegans* showed that toxicological responses to metal ions were strongly time dependent (Moyson et al., submitted), yet the results reported herein show that the metal speciation in the exposure medium is largely invariant with time (Fig. 4.1). In agreement with our results, others have assumed that Cd speciation of a 48 h exposure would be comparable to that of 24 h (Cressman III and Williams, 1997; Freeman et al., 1998). Accordingly, the nature and time dependence of adverse effects also involves the nature and time dependence of biological processes, e.g. bio-uptake, bioaccumulation and defence mechanisms.

Another factor to consider in the case of *C. elegans*, is the differentiated role played by bacteria in metal toxicity. Bacteria are necessary as a food source to prevent effects of starvation, but their presence affects the rate of pharyngeal pumping and influences metal speciation. For example, our results show that the presence of *E. coli* has a larger influence on Cu speciation than on Cd speciation. The influence of bacteria on the results of toxicity testing has been observed in several studies (Williams and Dusenbery,

1988; Donkin and Dusenbery, 1993; Donkin and Williams 1995). The presence and density of bacteria (Höss et al., 2011; Win et al., 2013) as well as the bacterial species (Venette and Ferris, 1998) are reported to have an influence on the metal toxicity. For example, it was proposed that Cd availability decreases with increasing bacterial density, resulting in a lower Cd toxicity (Boyd et al., 2003; Offermann et al., 2009; Höss et al., 2011). However, no consensus exists about the interpretation of their data, so many hypotheses have been proposed. For example, the reduced Cd toxicity at higher bacterial densities might be explained by the Cd induced feeding inhibition (Höss et al., 2011). Metals may affect feeding behaviour by blocking pharyngeal pumping and affecting or damaging gut structure (Popham and Webster, 1979). For Cu, Zn and Cd, the EC50 for feeding was 3.32 mg/L (0.05 mM), 12.6 mg/L (0.19 mM) and 5.2 mg/L (0.05 mM) respectively (Jiang et al., 2016), which are comparable to the concentrations used in the present study. Another explanation might be that metals bound to the bacteria are less bioavailabile, resulting in reduced metal toxicity with increasing food densities (Boyd et al., 2003). Because a similar toxicity was observed if *C. elegans* was fed with live or dead bacteria, the potential binding of Cd to bacteria is thought to be passive (Anderson et al., 2001). Blériot and co-workers (2014) also determined the presence of a binding protein for Cu in *E. coli*. It was suggested that *E. coli* functions both as a food organism and as a vector for contamination uptake (Höss et al., 2001). Nevertheless, under the conditions used herein, metals bound to *E. coli* are not the major contributor to the body burden (see §4.4.2). Consistent with our findings, for the case of single metal ion exposure of *C. elegans*, data published by Offerman et al. (2009) reveal a consistent link between Cd body burden and toxic effect (*cdr-1* expression), irrespective of the composition of the exposure medium (different total Cd concentrations and different *E. coli* concentrations) and different exposure times (Fig. 4.6). The transcription of cadmium-responsive gene-1 (*cdr-1*) is induced only in response to Cd exposure for intracellular Cd defence.

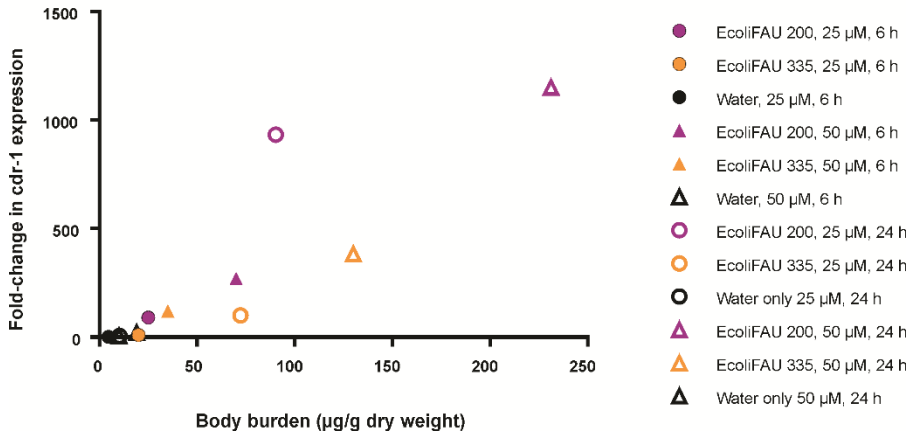

Figure 4.6: Relationship between *C. elegans* body burdens of Cd and *cdr-1* expression. The nematodes were exposed to different *E. coli* and Cd concentrations for different exposure times as indicated in the legend. (Data are obtained from Offerman et al., 2009).

This was in contrast with the findings for other organisms, e.g. *Daphnia magna* (De Schampelaere et al, 2004), where, body burden alone did not appear to be a good indicator of metal toxicity. Knowing the uptake mechanism can help us to better understand the earlier observed toxic effects. A couple of other studies have attempted to identify the relationships between waterborne vs. dietborne metals and toxicological endpoints for single metal exposures (Höss et al., 2011; Yu et al., 2012). Höss and co-workers (2011) performed 48 h - 96 h exposures to Cd concentrations in K-medium between 0 and 8 mg/L (0 – 0.07 mM) combined with *E. coli* at concentrations of 0-2000 formazin absorption units (FAU). By analysis of % inhibition of reproduction as a function of total, “aqueous”, and bacterial-bound Cd concentrations, Höss et al. (2011) found that the aqueous Cd was the best predictor of toxicity. We performed a similar analysis of the data published by Yu et al. (2012) on Cu toxicity to *C. elegans* under a range of waterborne and foodborne concentrations in K-medium. The results for the endpoint of growth status clearly show that the waterborne Cu is the best predictor of the eventual toxicity (Fig. 4.7). The results discussed in the preceding sections imply that the majority of the waterborne Cu is in the form of the free metal ion.

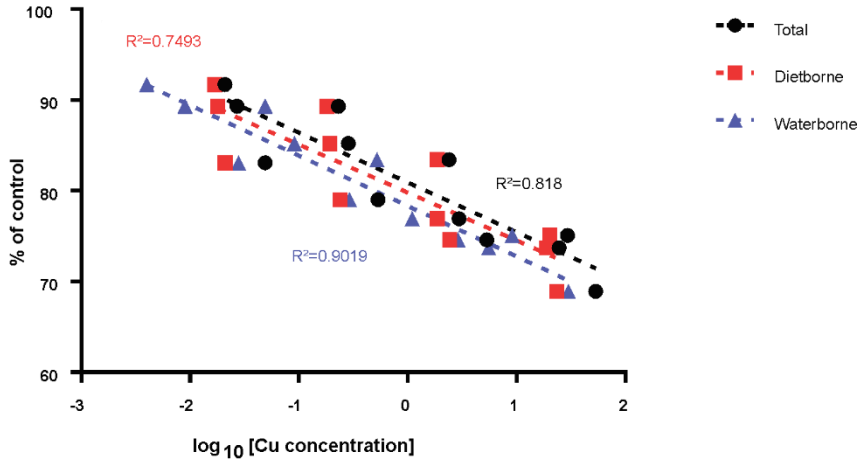

Figure 4.7: Growth status of *C. elegans* after exposure to Cu under a range of conditions. The various Cu concentrations (mg/L) correspond to the total concentration (black circles), the amount associated with the *E. coli* food (red squares) and the amount in the aqueous phase (blue triangles). (Data are obtained from Yu et al., 2012).

In the case of metal mixtures, body burden alone is not a straightforward indicator of toxic effects. Earlier described differences in toxicity between mixtures and their corresponding single metals (Moyson et al., 2018; Moyson et al., submitted) cannot be explained by the competition to enter the *C. elegans* body since body burdens were of similar value (Fig. 4.4). Furthermore, there were no differences in metal speciation found between mixtures and their corresponding single metals. That is, mixture toxicity effects can be unambiguously ascribed to biotic handling differences and not to differences in the exposure medium. It seems likely that the metal toxicity is a consequence of their different modes of action, as well as potential differences in subcellular compartmentalisation. Body accumulation in combination with intracellular speciation, i.e. the distribution of internalised metals over the tissues (e.g. gut, vesicles) in which metals are stored or detoxified, could provide important insights into metal toxicity. For example, transmembrane Zn transporters such as CDF-proteins may influence Zn homeostasis by mobilizing Zn. CDF-2 is involved in storage of Zn in vesicles of intestinal cells, while CDF-1 may transport Zn from these cells to the body cavity or

intestinal lumen to promote excretion (Davis et al., 2009; Dietrich et al., 2016). In our earlier study (Moyson et al., submitted) the potential role of *cdf-2* in mitigating Cd toxicity was identified, and thus it is possible that Cd is also stored in the gut granules, thereby rendering it biologically inactive. The same detoxification mechanism has been proposed for Cu (Chun et al., 2017), nevertheless, our observation that at comparable body burdens Cu is more toxic than Zn or Cd suggests that this process is less effective in case of Cu. In contrast to Cd and Zn, Cu is observed to be homogeneously distributed throughout the body of the nematodes (Jackson et al., 2005).

In addition, an organism's capacity to detoxify accumulated metals may be effectively reduced under mixture scenarios. Exposure to metal mixtures in soils, at concentrations less than 20 mg/L, has been reported to result in a decrease in body burden of each single metal, whilst at higher concentrations similar total accumulated metal levels for mixtures and corresponding single metals were observed (Power and de Pomerai, 1999). In the present study, the above mentioned limit was reached at lower concentrations probably due to the use of a different exposure medium, suggesting that lower exposure concentrations may result in differences in accumulation between single metals and mixture exposures. These findings suggest that the organism is able to regulate metal uptake below a certain threshold level. The applicable threshold will depend on the chemical speciation (bioavailability) in the exposure medium.

Finally, although free ion activity in the exposure medium is important, the nature and timescale of interactions with the external biointerface and subsequently intracellular sites of toxic action must also be taken into account in the interpretation (Duval, 2016; Duval et al., 2016).

4.5. Conclusions

Until now, there has been much discussion in the literature regarding the role of *E. coli* in determining toxic effects to *C. elegans*, yet the significance of dietborne or waterborne metal exposure routes had not been unambiguously determined. The

situation has been compounded by differences in methodology, nature of the metals, *E. coli* density, type of exposure medium, exposure time, pH, etc. between studies. Herein, a combined analysis of metal speciation in the exposure medium, body burdens of metals, and toxicological endpoints suggests that the free metal ion concentration in the exposure medium is the best predictor of the body burden and the ensuing toxicity to *C. elegans* under our conditions. In the case of metal mixtures, additional biotic handling processes also play a role. Although significant differences in population size, body length, mortality and behaviour of metal mixtures and corresponding single metals were observed in our previous studies conducted under the same experimental conditions (Moyson et al., 2018; Moyson et al., submitted), the present work reveals almost no differences between single and mixture treatments in terms of the internal (body burden), free and dissolved metal concentrations. These observations suggest that the differences in the toxicity of different metals (e.g. Cu *vs*. Cd), and in the toxicity of mixtures of metals, are largely due to differences in the nature and timescale of biotic handling mechanisms, i.e. assimilation efficiency, internal speciation and detoxification mechanisms such as production of metallothioneins and heat shock proteins, regulation of pumps, etc. (Anderson et al., 2003; Rainbow, 2007; Martinez-Finley and Aschner, 2011). Similar rationale has been used to explain differences in metal ion toxicity to other biological species (Niyogi et al., 2015). For example, in the case of fish, it was seen that Cu accumulation was correlated with the concentration of metallothioneins and Cu was bound with these proteins in gibel carp and common carp, while they were not correlated in rainbow trout, indicating the difference in metal tolerance (De Boeck et al., 2003). The external concentration, exposure time, presence and nature of organic complexants, uptake and elimination rates and internal storage capacity determine whether the metal body burden reaches steady-state within the experimental period. If the rate of metal uptake exceeds the combined rates of detoxification and excretion, a critical concentration of metabolically available metal can be accumulated, resulting in toxic effects (Rainbow, 2002, 2007; Adams et al., 2011; Jacob et al., 2017).

Acknowledgements

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Chapter 5

An introduction to peptidomics and proteomics

Abstract

Neuropeptides are present in all metazoan species. Due to the controlled action of processing enzymes, they can be derived from larger, inactive precursor proteins. These signalling molecules function as neurotransmitters or hormones in e.g. cell–cell communication to orchestrate a wide variety of physiological conditions and behaviours. Their peptide sequence together with possible post-translational modifications is important when studying their function. To identify and characterize the endogenously present peptide complement of a defined tissue or organism, a peptidomics approach is applied, while a proteomics approach is used to identify the proteome of a tissue or organism. The soil nematode *Caenorhabditis elegans* was the first multicellular organism to have its genome fully sequenced and extensive bioinformatics and genetic tools exist to facilitate (molecular) research on this organism (The *C.elegans* sequencing consortium, 1998) Furthermore, it has many advantages to make high throughput analysis possible such as its life history traits, ease of cultivation, low cost etc. In the first part of the current study, a differential proteomics study was applied to unravel the effects of Cu, Cd and their mixture on the proteome of *C. elegans*. In total, only 7 differential proteins were found: 1 for Cu exposed nematodes, 4 for Cd exposed nematodes and 2 for mixture exposed nematodes. This finding suggests that nematodes were still able to maintain protein homeostasis after 24 h of exposure to LC10 metal concentrations. In the last part of this study, non-exposed *C. elegans* nematodes were used for an exploratory peptidomics study as an attempt to further unravel the peptidome of *C. elegans*: 5 new NLP peptides from 5 different novel peptide precursors were discovered.

Key words: Differential proteomics, *C. elegans*, Metals, Precursor, Peptidomics

5.1. Differential proteomics

5.1.1. Introduction

Accumulation of metals in the environment can be of natural and industrial origin. Exposure to non-essential metals and an overload or deficiency of essential metals can cause serious health hazards for diverse animals including humans. Complex networks of transporter proteins, metal binding proteins and stress response mechanisms regulate the cellular metal content (Martinez-Finley et al., 2012). Different molecular players exist to maintain homeostasis: transcription factors, hormones, peptides, metabolites, etc. All metazoan organisms use diverse signalling molecules including small molecule neurotransmitters like acetholcholine, γ-aminobutyric acid, nitric oxide, excitatory amino acids such as glutamate and biogenic amines like octopamine, tyramine, serotonin and dopamine. Unfortunately, classical toxicological assays don't reveal why an organism appears to be more vulnerable to a toxic compound, hence, mechanistic insights into toxicity are needed.

Proteins are crucial in nearly all biological processes: catalysis, structural support and signal transmission. The proteome, PROTein complement of a genOME, describes the whole set of proteins coded by the genome of an organism at a given time point and in a given situation. The proteome represents the functional expression of information by characterising the type, functions and interactions of proteins. The proteome is thus not static, but will vary with e.g. developmental stage and environmental conditions. Proteomics studies digest proteins with proteases to identify the proteins in the sample. Protein quantification of the samples can be performed by metabolic labeling (e.g. SILAC), chemical labeling (e.g. TMT) or label-free strategies. To identify the proteome of a certain tissue, organism, etc. liquid chromatography and mass spectrometry techniques can be used. However, these techniques also have limitations. Proteins and peptides of high and moderate levels are detectible by MS techniques, while molecules of low levels are difficult to detect since they are not sufficiently present to provide

reasonable signals in the MS/MS analysis for identification, indicating a limited dynamic range.

The soil nematode, *Caenorhabditis elegans* was the first multicellular organism to have its genome fully sequenced and extensive bioinformatics and genetic tools exist to facilitate research on this organism (The *C.elegans* sequencing consortium, 1998). Its genome is around $1/30^{th}$ of the human genome size and encodes for over 22 000 proteins. Although *C. elegans* has a simple nervous system of 302 neuronal cells and a quite simple anatomy of 959 somatic cells, these nematodes exhibit diverse behaviours, like chemotactic behaviour, oxygen sensing, etc. Their rapid growth, small body size, fast reproduction and identical amount of cells enhance high throughput screening (Leung et al., 2008) and standardization of experiments. Compared to mammalian models, more compounds can be tested while reducing the costs, time and sacrifice of laboratory animals. Therefore, *C. elegans* is frequently used as a unique model for both fundamental neuroscience and (eco)toxicology (Brenner, 1974), for example in metal toxicity and metal homeostasis studies.

The aim of this study is to obtain mechanistic insights by differential proteomics at the molecular level or at least to get an idea of the biochemical pathways that are affected by Cu and Cd exposure. By the differential proteomics approach, the main proteins after the metal treatment are identified as well as the differentially expressed proteins compared to the control treatment. Therefore, young fed adult nematodes are exposed during 24 h to LC10 (metal concentration inducing 10% lethality) of Cu and Cd (as single metals and in combination) to analyse effects on their proteome and to gain mechanistic insight into mixture toxicity.

5.1.2. Materials and methods

5.1.2.1. *Caenorhabditis elegans* culture and materials

Caenorhabditis elegans nematodes (wild type N2 strain) were obtained from the Caenorhabditis Genetic Centre (Minneapolis, USA) and were maintained at 20°C on

nematode growth medium (NGM) agar plates, seeded with *Escherichia coli* (OP50 strain) as food source. To achieve nematodes of the same age, mixed-stage *C. elegans* were treated with a hypochlorite solution (5 N NaOH, 5% sodium hypochlorite), killing the nematodes that were not protected by an egg shell. Eggs were raised on 48 OP50-seeded NGM plates. Young L4 nematodes were transferred to 50-mL falcon tubes filled with 40.5 mL K-medium (52 mM NaCl, 32 mM KCl, 5 µg/mL cholesterol, pH 5.1) (Williams and Dusenbery, 1990), supplemented with *E. coli* OP50 (1.5 g/L). The worm pellet was incubated for 24 h at 20°C under gentle shaking conditions (160 revolutions per minute (rpm)).

5.1.2.2. Test media and materials

Nematodes were exposed for 24 h to LC10 of Cd (1.7 ± 0.8 mg/L; 0.015 ± 0.07 mM) and Cu (0.5 ± 0.2 mg/L; 0.008 ± 0.003 mM) and to their combination, made from $CdCl_2.2.5H_2O$ (Alfa Aesar) and $CuCl_2.2H_2O$ (Merk A.G.) in K-medium. LC10 values were determined in an earlier study (Moyson et al., 2018). Metal concentrations were measured (98.6 – 105.7% recovery) by High Resolution Inductively Coupled Plasma Mass Spectrometer ICP-MS (Element XR, Thermo Fisher Scientific). Prior to toxicity testing, metal solutions were incubated with the bacterial suspensions for 12 h at 4°C to allow metal partitioning between the aqueous phase and the bacteria. After the incubation period, 4.5 mL of test medium (metal solution) or control (K-medium) was added to falcon tubes, bringing the total volume of each falcon tube up to 45 mL. 3 replicates for each exposed group and 9 replicates for control were used. For each metal solution, pH was measured before (5.1 ± 0.1) and after the experiment (5.7 ± 0.3), which is an acceptable pH range for *C. elegans*.

Required products were purchased from different companies. Water and acetonitrile (ACN) (LC-MS grade) were purchased from Biosolve, TFA (HPLC grade) from VWR, trypsin gold MS kit from Promega and BCA protein assay kit from Pierce. Protease inhibitor, phosphatase inhibitor, RIPA buffer, *tris*(2-carboxyethyl)phosphine, iodoacetamine and hydroxylamine were ordered from Thermo Scientific. Triethyl

ammonium bicarbonate, acetone (LC-MS grade) and α-cyano-4-hydroxycinnamic acid were purchased from Sigma Aldrich.

5.1.2.3. Protein extraction and TMT labeling

After metal exposure, worms were washed with physiological water (5.6 g/L NaCl), which was repeated twice. Living worms were separated from dead individuals by adding a sucrose gradient, shaking and centrifugation (500 g, 1 min). Living worms were transferred to a new falcon tube and washed with physiological water (5.6 g/L NaCl). For protein extraction, the worm pellet was transferred to a low binding bullet tube filled with RIPA buffer, protease and phosphatase. The mixture was sonicated on ice and centrifuged (14 000 g, 4°C, 15 min.). Protein concentration was determined using the BCA Protein Assay Kit (Pierce). 40 µg protein was spiked with β-caseine. Next, acetone precipitation was performed by adding triethyl ammonium bicarbonate (TEAB), *tris*(2-carboxyethyl)phosphine, iodoacetamine and acetone to the protein sample. Thereafter, acetone was removed from the samples and the pellet was resolved in TEAB and trypsine for digestion. 5 µg protein was dried and resuspended in TEAB. Simultaneously, ACN was added to the labels (TMT Mass Tagging kits). For each TMT run, 3 control samples and 3 exposed samples of the same treatment were used (Fig. 5.1). In total 3 TMTsixplex runs were performed. Subsequently, labels were added to the samples and after incubation of 1 hour, hydroxylamine was added. Samples of control and of one metal treatment were combined in 1 low protein binding bullet tube. Finally, the samples were desalted in Pierce C18 spin columns (Thermo Scientific), and dried, using a speedvac concentrator.

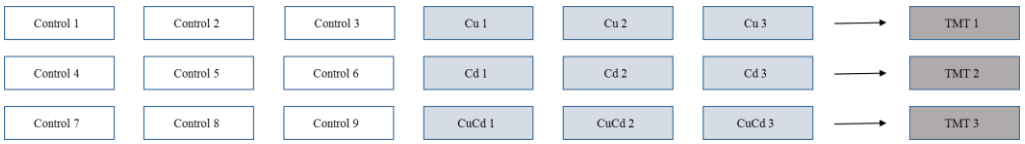

Figure 5.1: Scheme of the different TMTsixplex runs

5.1.2.4. MS-analysis

For Q exactive measurements, samples were resuspended in mobile phase A (3% ACN + 0.1% FA) and Glu-1-Fibrinopeptide-B. 1 µg protein was injected in the EASY-nlc 1000 system coupled to the Q Exactive Plus mass spectrometer (Thermo Scientific) and loaded on a nanoviper C18 trap column (Thermo Fisher Scientific), coupled in line with a nanoviper C18 analytical column (Thermo Fisher Scientific) for 1D separation with a flow rate of 0.3 µL/min. After 240 min the gradient was made. A full scan spectrum (350-1850 m/z, resolution 70 000) was followed by high energy collision dissociation (hcd). Top 5 of most abundant ions of the MS/MS spectra at any point during the gradient was used for the acquisition method. AGC target value was set at 3×10^6 ions and maximum injection time was 80 ms. The RAW data from the MS were analysed, using the software program 'Proteome Discover 2.1'. The Uniprot database, filtered for *Caenorhabditis elegans,* was used for peptide identification by Sequest and the SwissProt database was used by Mascot. The precursor mass tolerance was set at 10 ppm and the fragment mass tolerance at 0.5 Da. Trypsin was specified as the digesting enzyme and 2 missed cleavages were allowed. The results were filtered for confident peptide-to-spectrum matches (PSMs). Protein identification was set at a peptide false discovery rate of 1% using a target-decoy-based strategy. First ranked peptides with 2 or more than 2 peptides per protein, including 1 unique peptide were used. Oxidation (M) and phosphorylation (ST, Y) were set as dynamic modifications, while carbamidomethyl (C) and TMTsixplex (n-tem, K) were considered as a fixed modification.

5.1.2.5. Bioinformatical analysis

The bioinformatical analysis was performed in MATLAB version R2013a following the QC-QUAN method with PSMs files as input files, exported from Proteome Discoverer 2.1. These data were converted into a matrix of the reporter ion intensities of only the most confident PSMs of unique non-redundant modified peptides. Tukey boxplots were made to check if there was a systematic shift of the intensity distribution. To remove possible systematic and technical errors, data were normalized by CONSTANd

(CONstrained STANdardization) normalization (Maes et al., 2016). Therefore, the data were checked for their quality by principal component analysis and hierarchical clustering dendrogram with spearman correction, with the data assembled according to the biological subclasses. Subsequently, DE t.tests (Differential Expression) were performed for each protein with Benjamini-Hochberg correction. The p-value was adjusted to the number of t-tests. An adjusted p-value of <0.05 and a fold change of 1.5 upregulation or -1.5 downregulation was required for differential proteins. To check if the requirements were fulfilled, a volcano plot was made.

5.1.3. Results and discussion

A differential proteomics approach was applied to identify up- and downregulated proteins of nematodes exposed for 24 h to Cu, Cd and the mixture CuCd, compared to control. For TMT 1, TMT 2 and TMT 3, respectively, we were able to identify 814 proteins and 8968 PSMs, of which 538 had unique peptides, 546 proteins and 5149 PSMs, of which 336 had unique peptides, and 405 proteins and 3567 PSMs, of which 238 had unique peptides. For Cu exposed nematodes, only 1 differential protein was found: this aspartyl protease was upregulated. The gene *asp-1* has a function in aspartic-type endopeptidase activity, proteolysis and protein catabolic processes. For Cd exposed worms 3 differential proteins and an uncharacterized protein (gene *cele-zc247.1*) were identified, all of which were downregulated. The probable malate dehydrogenase mitochondrial protein (gene *mdh-2*) has catalytic functions, and plays a role in carbohydrate and malate metabolic processes, tricarboxylic acid cycle and L-malate dehydrogenase activity. The two heat shock proteins function in stress responses such as heat, unfolded protein binding and endoplasmic reticulum unfolded protein response. The gene *hsp-16.48* also plays a role in determining the adult lifespan. In nematodes exposed to the CuCd mixture, a heat shock 70 kDa protein (gene *hsp-3*), functioning in the ATPase activity, ATP binding during stress response and endoplasmic reticulum unfolded protein response, was upregulated. A 60S ribosomal protein (gene

rpl-36), which is a structural constituent of the ribosome and cytoplasmatic translation and plays a role in RNA binding, was also identified (Table 5.1). There was no overlap between groups.

Table 5.1: Identification of differential proteins by LC-MS/MS (n=3). Accession number, number of peptides, adjusted *P*-value and fold change (FC) are listed for each differential protein.

Accession number	Description	Number of peptides	Adjusted *P-value*	FC
Cu				
G5EEI4	ASpartyl Protease OS	7	0.003486509	2.334234
Cd				
G5EBP5	Uncharacterized protein OS	3	0.010449777	0.592523
O02640	Probable malate dehydrogenase. mitochondrial OS	14	0.000771192	0.617089
P02513	Heat shock protein Hsp-16.41 OS	2	0.014578368	0.344008
P34696	Heat shock protein Hsp-16.1/Hsp-16.11 OS	4	0.00241226	0.662994
CuCd				
P27420	Heat shock 70 kDa protein C OS	12	0.010970403	1.595372
P49181	60S ribosomal protein L36 OS	6	0.020458505	1.498367

Previous studies used the proteomics approach to get a better understanding of the reproduction, development, starvation, ageing, longevity or innate immunity of *C. elegans* (Klang et al., 2014; Husson et al., 2015; Larence et al., 2015). It was seen, for example, that the rate of appearance of newly synthesized proteins was high during the first 5 days of adulthood, then slowed down and increased again from day 11 onwards (Vukoti et al., 2015). However, there are not many proteomics studies on the effects of metal exposure, although cellular detoxification systems, including glutathione,

methallothioneins, heat shock proteins, pumps and transporters, are known to regulate intracellular metal levels by detoxifying and excretion of metals (Martinez-Finley and Aschner, 2011). Furthermore, Cd can affect gene expression by influencing signal transduction pathways by affecting the activities of protein kinase C, cAMP-dependent protein kinase and calmodulin (Liao and Freedman, 1998). Also in our study, 2 heat shock proteins for Cd exposure and 1 for exposure to the CuCd mixture were identified. Only HSP-70, in the CuCd mixture, was upregulated. The presence of these heat shock proteins suggests that the proteins are under stress. It is possible that more subtle differences between groups can be observed at a lower exposure concentration.

Larence et al. (2015) analysed approximately 45 000 unique peptides, of which more than 5500 protein groups were quantified in the extracts of fed and starved nematodes. More than 500 proteins were up- or downregulated due to starvation. In blood clams (*Tegillarca granosa)*, 365 out of 2318 proteins were identified as differential proteins after Cd exposure (Bao et al., 2016). In the present study, the amount of identified differential proteins was low and no clear pathway was affected, since they all have different functions. It is possible that the practical and technical settings in our study were not optimal and led to the low number of identifications. First, only 3 replicates for each metal treatment were compared with 3 control replicates. An increase in sample size might also increase the number of protein identifications. Second, whole nematodes (N = ± 500-600) were extracted without distinguishing between the cell types; it is possible that for some cell types effects can be seen on the proteome level, but not for other cell types, making it difficult to detect these effects when whole bodies are used. Third, the gradient was set at 240 min, while for TMT 1 the last identification was performed after 173 min. For TMT 2 and TMT 3, the retention time was even shorter: 153 and 134 min, respectively. This implies that the last hour no identifications were made. A less steep slope of the gradient would be more suitable to have a better separation of the peak counts. Fourth, the low number of identified proteins could be the result of an incomplete extraction of the protein. The use of another buffer, e.g. ammonium bicarbonate, might increase the number of identifications. Furthermore, a

setting with the top 5 of the most abundant ions was used. This setting is OK if there are fewer than 6 peaks at any point of the gradient. However, in the case of 6 or possibly more peaks, they will all be ignored now. So, a top 10 or even top 20 setting would possibly have increased the number of identified proteins. It would also have been interesting to filter the data for *E. coli* bacteria as an indication of possible contamination since these proteins may have overwhelmed the *C. elegans* proteins. However, the number of *E. coli* proteins in a test sample was low.

Metal exposure does not seem to have a clear effect on the proteome level of *C. elegans* in this study. Although the chosen concentration, LC10, was relatively high, we were not able to identify many differential proteins after 24 h of exposure. In contrast, reduction in feeding, reproduction, growth, movement, etc. was noted after metal exposure (Power and de Pomerai, 1999; Anderson et al., 2003; Boyd et al., 2003; Boyd and Williams, 2003; Moyson et al., 2018). We suggest that at this point, the nematodes were still able to maintain protein homeostasis. A possible explanation is that the new proteins are immediately synthesized to compensate for the deficiency caused by affected and degraded proteins, whereby the protein concentration remains (almost) constant. However, it is possible that at other levels, e.g. genome, metabolome, behaviour, etc. toxic effects are more pronounced. This also suggests that the proteomics approach is not yet an optimal tool for testing metal toxicity in *C. elegans*.

5.2. Peptidomics

5.2.1. Introduction

To maintain homeostasis, different molecular players exist: transcription factors, hormones, peptides, metabolites, etc. All metazoan organisms use a wide range of diverse signalling molecules including small molecule neurotransmitters like acetholcholine, γ-aminobutyric acid, nitric oxide, excitatory amino acids such as glutamate and biogenic amines like octopamine, tyramine, serotonin and dopamine. Besides these small signalling molecules, they also have a wide variety of bioactive peptides for signalling between neurons. These neuropeptides influence a wide range of physiological processes, including behaviour, by binding to cell surface receptors (e.g. G-protein coupled receptors). Larger inactive precursor proteins produce these bioactive peptides by the action of peptidases in the endoplasmatic reticulum and trans-Golgi vesicles. First, the *N*-terminal signal peptide is removed by a signal peptidase during translation of the protein. When they enter the vesicles, endopeptidases, including prohormone convertases, cleave the remaining precursor at defined cleavage sites to generate intermediates with *C*-terminal Lys and/or Arg residues. The endopeptidases cleave at pairs of basic amino acids (mainly KR and RR, while RK and KK are found in lower frequency) (Veenstra, 2000; Fricker et al., 2006). Subsequently, carboxypeptidase E (CPE) removes the basic residues at the carboxyterminus of the intermediates (Sapio and Fricker, 2014). Finally, every amino acid with a carboxyterminal glycine will be transformed into an amide by peptidyl-glycine α-amidating monooxygenase (PAM) (Eipper et al., 1993; Prigge et al., 2000). To study these bioactive peptides, a peptidomics approach is applied. In contrast to the proteomics approach, peptidomics studies do not use digestive enzymes to unravel the native peptide forms and their post-translational modifications. To identify the peptidome of a certain tissue, organism, etc. liquid chromatography and mass spectrometry techniques can be used. Mass spectrometry-based peptidomics methods are able to detect a large number of peptides in a single experiment and to identify post-

translational modifications. However, these techniques also have limitations. Proteins and peptides of high and moderate levels are detectible by MS techniques, while molecules of low levels are difficult to detect since they are not sufficiently present to provide reasonable signals in the MS/MS analysis for identification, indicating a limited dynamic range. In contrast to proteomics studies, this can be a problem for peptidomics where a breakdown of a small fraction of the major proteins can overwhelm the signals of the neuropeptides, which are present at low levels.

Caenorhabditis elegans was the first multicellular organism to have its genome fully sequenced and extensive bioinformatics and genetic tools exist to facilitate research on this organism (The *C.elegans* sequencing consortium, 1998). The genomic database predicts several *C. elegans* neuropeptide precursor genes, coding for around 250 peptides. It is not possible to predict if all these peptides are expressed or what their post translational modifications are. The neuropeptides of *C. elegans* can be subdivided into three major families based on their conserved motifs: FMRFamide-like peptide (*flp*) gene family, insulin-like peptides and neuropeptide-like protein (*nlp*) genes. In 2009, genetic, biochemical and in silico analysis revealed 33 *flp* genes, 45 *nlp* genes and 40 insuline-like peptides (Husson et al., 2009). Thereafter, a few more peptides have been discovered (e.g. Husson et al., 2015). Since new discovered peptides have a striking sequence similarity with those of invertebrates, the number of identified neuropeptide genes in *C. elegans* seems to be an underestimate of the total number (Husson et al., 2007). The fast reproduction of *C. elegans*, rapid growth, small body size and identical amount of cells improve the possibility of high throughput screening (Leung et al., 2008) and standardization of the experiments.

The first aim of this study is to further unravel the peptidome of non-exposed mixed stage *C. elegans* nematodes. Secondly, it is being investigated whether this omics approach can be used for metal toxicity tests. Since only a few differential proteins were detected, while clear effects were noticed on the behaviour of the nematodes, it is possible that the observed metal effects are reflected in the peptidome.

5.2.2. Materials and methods

5.2.2.1. *Caenorhabditis elegans* culture and materials

Wild type *Caenorhabditis elegans* (N2 Bristol variety) were raised at 20°C on nematode growth medium (NGM) agar plates, cultured with *Escherichia coli* (OP50 strain) as food source. The nematodes were obtained from the Caenorhabditis Genetic Centre (Minneapolis, USA). LC-MS grade and HPLC grade products were purchased from different companies. Water and Acetonitrile (ACN) (LC-MS grade) were purchased from Biosolve, methanol (LC-MS grade), acetic acid (HPLC grade) and α-cyano-4-hydroxycinnamic acid from Sigma-Aldrich, N-hexane, TFA (HPLC grade) and formic acid (FA) from VWR.

5.2.2.2. Peptide extraction

Mixed stage *C. elegans* nematodes were collected from 11 nematode growth agar plates (90 mm diameter) by washing with physiological water (9 g/L NaCl). Subsequently, the worms were shaken and centrifuged for 3 min at 400 g to get rid of the bacteria. The nematode pellet was placed in a 5 mL ice cold extraction solvent containing methanol/water/acetic acid (90/9/1, v/v/v %). Next, the nematodes were mixed with a vortexmixer, homogenized using a glass homogenizator and sonicated on ice using a bar sonicator (Branson Sonifier SLPe cell disruptor). After centrifugation at 13 000 rpm for 15 min, the pellet was discarded and the methanol was evaporated using a speedvac concentrator (Eppendorf 5301 concentrator centrifugal evaporator). The remaining aqueous residue was delipidated by adding an equal volume of n-hexane. After a 1 min centrifugation to remove the organic layer, and a 15 min Speedvac run to be sure the organic layer was removed, the aqueous solution was desalted by solid phase extraction using a Pierce C18 Spin Column (Thermo Scientific) according to the manufacturer's procedure. The obtained peptide sample was stored at 4 °C prior to analysis.

5.2.2.3. Peptidomics analysis by liquid chromatography and mass spectrometry

First, a sample aliquot was dried, 1.2 µL α-cyanomatrix (2mg/mL α-cyano-4-hydroxycinnamic acid in 50% ACN in pure water + 0.1% TFA) was added, and the sample was spin-down centrifuged. This was necessary for a sample quality check by 4800 MALDI TOF/TOF (Applied Biosystems/MDS SCIEX).

An Eksigent nanoLC-Ultra system connected to a Thermo Scientific LTQ-Orbitrap Velos mass spectrometer was used to perform LC-MS/MS analysis. Samples were dried and resuspended in 9 µL mobile phase A (2% ACN in pure water + 0.1% FA) and 0.2 µL Glu-1-Fibrinopeptide-B (2 fmol/1µL injection). 5 µl of the sample was loaded with an isocratic flow of 2% ACN in water with 0.1% FA and a flow rate of 5 µL/min on the trapping column (Pepmap C18, 300 µm x 20 mm, Dionex). After 2 min, the pre-column was placed online with the analytical capillary column (Pepmap C18, 75 µm x 150 mm nano column, Dionex), by switching the column-switching valve. During 45 min a linear gradient from 2% ACN in water and 0.1 % FA to 40% ACN in water and 0.1% FA was used to conduct separation. The flow rate was 350 nl/min. A data dependent MS/MS mode was used for the LTQ-Orbitrap Velos; a full scan spectrum (350-5000 m/z, resolution 60 000) was followed by a maximum of 10 collision-induced dissociation (CID) tandem mass spectra (100 to 2000 m/z). Peptide ions were selected as the 10 most intense peaks of the MS1 scan, while single charged ions were rejected. In the LTQ ion trap part of the mass spectrometer, CID scans were needed. The normalized collision energy used was 35% in CID and a dynamic exclusion list of 45 s was applied. The following settings were chosen: automated gain control (AGC) target value of 5×10^4 ions, maximum injection time of 100 ms and a minimum signal threshold of 500 counts. The RAW data from the MS were compared with the NCBI database filtered for the taxonomy of *C. elegans,* using Mascot in the software program 'Proteome Discover 1.3'. Peptide identification was set at a false discovery rate of 5% using a target-decoy-based strategy. The precursor mass tolerance was set at 10 ppm and the fragment mass tolerance at 0.5 Da. There was no digesting enzyme specified and 2 missed cleavages were allowed.

Carboxyterminal amidation, pyroglutamic acid and methionine oxidation were set as variable modifications. Furthermore, peptides had to meet the following requirements to correspond to endogenous peptides: the presence of a signal sequence, presence of cleavage sites amino- and carboxyterminally located from the peptide and presence of aminoterminally located glycine if there is a carboxyterminal amidation. The signal sequence of the signal peptide was identified by SignalP3.0. To check for sequence homologues with already known neuropeptides of different organisms, BlastP was used.

5.2.3. Results and discussion

Annotation of (neuro)peptide precursors can be quite challenging, even from well-annotated protein databases. Even if it is feasible, predicting the peptides that originate from that precursor can be difficult. Furthermore, the endogenous peptide content of a cell, tissue or organism is spatially and temporally dynamic, which has to be taken into account when monitoring peptide profiles. Different mechanisms exist to generate peptide diversity. Besides cell-specific expression of the respective precursor and its processing enzymes, the resulting mRNAs can be alternatively spliced and alternative proteolytic processing of the resulting precursor proteins can contribute to peptide diversity. Bioactive peptides often have a lower ion intensity and less predictable fragmentation compared to tryptic peptides, which may explain the relatively low number of bioactive peptides.

The LC-MS/MS approach allowed us to identify 51 endogenous peptides of non-exposed mixed stage *C. elegans* nematodes (Table 5.2). Some of these peptides were oxidized (m) and/or carboxyterminally or aminoterminally amidated.

No peptides were identified belonging to the **insulin-like peptide** group. These peptides can serve as hormones, growth factors and neurotransmitters, which play a role in the regulation of growth and development. They are e.g. important for the metabolism, growth, reproduction and ageing of insects (Wu and Brown, 2006).

19 of the identified neuropeptides belong to the **FMRFamide related peptides**, which is one of the most diverse and well-studied peptide group in invertebrates. These peptides have a non-polar hydrophobic amino acid (L, I, M, V) in the third position of the carboxyterminus. The FMRFamide peptide was first identified in the clam *Mercenaria mercenaria* (Price and Greenberg, 1977) and is an important neuropeptide in several phyla such as Hexapoda, Nematoda, Mollusca and Annelida (Oetken et al., 2004). In invertebrates, the FMRFamide-related peptides are known to affect e.g. blood pressure, heart rate, gut motility, feeding behaviour, locomotion and reproduction.

32 **neuropeptide-like protein peptides** could be identified, including 5 entirely novel peptides originating from 5 different and, till now, unknown *C. elegans* peptide precursors. These NLP peptides are considered antimicrobial (Pujol et al., 2008).

Table 5.2: Identification of *C. elegans* neuropeptides by LC-MS/MS (pooled data of 2 samples).

Protein precursor	Peptide sequence	Mass (Da)
FMRFamide-related (FLP) peptides		
FLP-1	SDPNFLRFamide	993.5093
	SADPNFLRFamide	1064.5485
	AAADPNFLRFamide	1119.5906
FLP-3	SADDSAPFGTMRfamide	1399.6197
	NPENDTPFGTmRFamide	1539.6861
FLP-5	qSSAEDADYLEKYQRIA	1968.9098
FLP-6	SSDMEVIGNEGVDGDAHDLF	2105.8799
	SSDmEVIGNEGVDGDAHDLF	2121.8746
FLP-9	SGYPLVIDDEEMRMD	1768.7638
	SGYPLVIDDEEMRmD	1784.7581
FLP-10	RVDPNAELLYLDQLLI	1884.0461
FLP-11	SPLDEEDFAPESPLqamide	1671.7588
FLP-13	ASSAPLIRFamide	959.5633
	APEASPFIRFamide	1132.6110
FLP-16	AVLPADYAYGVADEMSALPDSGSLFAEQRPS	3226.5082
FLP-18	DFDGAMPGVLRfamide	1322.6437
	DFDGAmPGVLRfamide	1338.6379
FLP-22	qVFDLDGQQLAGLEQNDARLMEQQV	2827.3357
FLP-24	VPSAGDmMVRfamide	1223.5795
	VPSAGDmmVRfamide	1239.5738
FLP-26	FRLPFQFFGANEDFNSGLT	2206.0420
	GGAGEPLAFSPDmLSLRFamide	1878.9391
F40F8.5	TVLDGDMGEPTEDQEDTNDHA	2287.8978
	TVLDGDmGEPTEDQEDTNDHA	2303.9063
Neuropeptide-like protein (NLP) peptides		
NLP-5	SVSQLNQYAGFDTLGGMGLamide	1955.9345
NLP-7	GSDIDDPRFFSGAFamide	1528.7024
NLP-9	TPIAEAQGAPEDVDDRRELE	2210.0505
NLP-11	SAPMASDYGNQFQMYNRLIDA	2391.0704
	SAPmASDYGNQFQMYNRLIDaamide	2406.0616
	SPAISPAYQFENAFGLSEALERAamide	2466.2181

NLP-14	ALDGLDGAGFGFD	1253.5630
	ALDGLDGSGFGFD	1269.5590
	ALNSLDGAGFGFE	1296.5975
	ALNSLDGQGFGFE	1353.6276
NLP-15	AFDSLAGSGFGAFN	1359.6175
	AFDSLAGSGFSGFD	1376.5950
	AFDSLAGQGFTGFE	1445.6448
	AFDSLAGSGFDNGFN	1517.6414
	AFDTVSTSGFDDFKL	1648.7719
NLP-21	GGARAFLTEm	1067.5070
	GGARAFSADVGDDY	1399.6005
NLP-35	AVVSGYDNIYQVLAPRF	1910.9858
NLP-39	VPNFQADNVPEAGGRV	1353.6276
	EVPNFQADNVPEAGGRV	1668.8234
NLP-40	APSAPAGLEEKL	1181.6299
NLP-42	SALLQPENNPEWNQLGWAw	2251.0738
NLP-43	SSAPVHYFEDSIAQEAGPSME	2251.9661
NLP-49	SPSMGLSLAEYMASPQGGDNFHFMPsamide	2756.1951
NLP-50	FMYEMEDSYPVSGELPK	2020.8742
	FMYEmDSYPVSGELPK	2037.8741
PDF-1	SNAELINGLIGMDLGKLSAVamide	2013.1021
	SNAELINGLIGmDLGKLSAVamide	2029.0971
	SNAELINGLLSMNLNKLSGAamide	2057.1053
	SNAELINGLLSmlLNKLSGAamide	2073.0980
	SPLLYRAPQYQmYDDVQFV	2348.1065
Newly characterized NLP peptides		
Y12A6A.2 (NLP-53)	NQGAGSVSLDSLASLPMLRyamide	2077.0601
C30H6.100 (NLP-54)	SLPIEYPDETVMYESRF	2074.9473
	SLPIEYPDETVmYESRF	2090.9410
F14F11.2 (NLP-55)	MYINPDYYYVEQLPTM	2038.9125
Y57G11C.45 (NLP-56)	SSIMTDDVEPPQLLTRQL	2043.0280
F08G12.8 (NLP-57)	SPIHGIWNNLPAPPQ	1639.8453

The novel peptides NQGAGSVSLDSLASLPMLRYamide, SLPIEYPDETVMYESRF, MYINPDYYYVEQLPTM, SSIMTDDVEPPQLLTRQL, SPIHGIWNNLPAPPQ originate from different precursors (Y12A6A.2, C30H6.10, F14F11.2, Y57G11C.45, F08G12.8), in which they are all flanked by conventional basic cleavage sites, or by a signal peptide at the aminoterminus (Table 5.3). We propose to annotate them as neuropeptide-like protein precursors NLP-53, NLP-54, NLP-55, NLP-56, and NLP-57 respectively. These novel precursors were published in WormBase, a freely accessible online database on the (systems) biology and genome of the nematode model organism *C. elegans*, with information about other related nematodes.

The NQGAGSVSLDSLASLPMLRYamide peptide of *C. elegans* is 100% identical to that of *Caenorhabditis briggsae, Caenorhabditis latens* and *Caenorhabditis remanei.* The SLPIEYADDSAIYENRF peptide of the hypothetical protein CRE_28712 of *C. remanei* is 65% identical to the novel SLPIEYPDETVMYESRF peptide of *C. elegans*. The MYINPDYYYVEQLPTMT peptide was found in *Caenorhabditis briggsae* and a 94 % identical peptide (MYVNPDYYYVEQLPTM) was identified in *C. remanei*. The RASILTDSPPPQILTRQL peptide from *C. briggsae* has a 71% sequence homology with the novel SSIMTDDVEPPQLLTRQL *C. elegans* peptide. The nematodes *C. briggsae* and *C. remanei* also have the SPIHGIWNNLPAPPQ peptide.

NLP-53 and NLP-56 are enriched in the ventral nerve cord and (dopaminergic) neurons, while NLP-56 is also enriched in the hypodermis. NLP-54 is enriched in the neurons, while NLP-57 is enriched both in neurons and in the intestine. NLP-55 is involved in locomotion, reproduction and embryonic development (WormBase).

Table 5.3: Novel *C. elegans* precursors from which peptides were identified by 1D LC MS/MS. The neuropeptide is shown in bold, the signal peptide is shown in italics and the cleaving sites are underlined.

Y12A6A.2 (NLP-53)

*MSSWLRTLFV FFIVLVVSTS A*LPSDYVRFL IQSARNHENS YYPQEEAGFM RVNR**NQGAGS VSLDSLASLP MLRYG**

C30H6.10 (NLP-54)

MMNYSVVLLL FALCSIVAAS PMWFDEEEPM NLRAFRVMPN QLDSIDASRR LLKRASMFNK RRGRELFGKR **SLPIEYPDET VMYESRF**RRR ANELFG

F14F11.2 (NLP-55)

MSCISMLFIL LVACLLVSNA **MYINPDYYYV EQLPTM**KKSG QLRALAGSRN CFFSPVNCII THDINSYRRL AKGSSYA

PY57G11C.45 (NLP-56)

*MPSPSSLLGS LLLVCAVLTI TSRA***SSIMTD DVEPPQLLTR QL**RSFPYSVS FYRMLGHDRQ LRPYYGVNDE VAALIDSMNS DNVANEDVFP TRPRRSDGLR GYACRFKFCR IYDA

F08G12.8 (NLP-57)

*MRFILGLLIA IVAFVAS***SPI HGIWNNLPAP PQ**KRVYGFYN YLPKEEDDRD KRNTILLLTP NEDYVE

Although its genome is sequenced in 1998 and approximately 350 neuropeptides have been predicted for *C. elegans*, only a part (about 200 neuropeptides) has been identified until now, due to the large efforts needed for tissue collection and purification strategies. We were able to identify 51 endogenous peptides using nanoscale liquid chromatography and tandem mass spectrometry, of which 46 peptides were previously sequenced. In contrast to the large effort, as in other studies, only a small number of novel peptides could be unravelled. For example, Husson et al. (2005) identified 60 endogenous peptides, 11 of which were novel, and they added only 3 more to the list in 2007. In contrast to bio-informatics, MS approach reveals posttranslational modifications such as amidation, aminoterminal truncation etc. Nevertheless, only 2 samples (out of 10) were successfully analysed (the results were combined in Table 5.2). Furthermore, only 12 out of 51 identified peptides were found in both experiments,

indicating a low reproducibility. Since whole nematodes (N = ± 500-600) were extracted and not just the central nervous system (which is impossible), it might be possible that some peptide concentrations were below the sensitivity of the mass spectrometer. Furthermore, only ions above a certain threshold were selected for fragmentation. Other ions, which might correspond to the theoretical masses of predicted neuropeptides, were not taken into account, because spectra might be of insufficient quality to confirm identity. It is also possible that some peptides were not properly extracted and it has to be noticed that not all peptides ionize with the same efficiency in the electrospray source. Because of the low success rate (only 2 successful experiments with peptide identification) and the time-consuming and costly laboratory work, this technique should be further optimised in order to be used for metal toxicity tests.

Chapter 6

General conclusions and future perspectives

Although metals (mainly) occur in mixtures in the natural environment, metal effects are usually studied for each metal separately and at each organisational level separately. The main objective of this thesis was to gain insight into the toxicity of single metals (Zn, Cu, Cd) and to investigate whether and how toxicity changed in mixture exposure scenarios. Therefore, both short-term and long-term studies were performed using different metal concentrations and multiple endpoints were studied, ranging from the molecular to the population level. As a second objective, the results obtained from different levels were linked together and discussed. Finally, the bioavailability and bioaccumulation of the metals were determined as an attempt to elucidate the observed toxic effects. For this metal toxicity study, the soil nematode *Caenorhabditis elegans* was used as model organism.

The metal concentrations used in this study were much higher than the ground water standards in Flanders, Belgium (Cd: 5 µg/L; Zn: 500 µg/L; Cu: 100 µg/L). For drinking water, however, the Flemish standards are less stringent (Cd: 5 µg/L; Zn: 5000 µg/L; Cu: 2000 µg/L), which is more in line with the LC20 of Zn (24 h: 9500 µg/L, 48 h: 3596 µg/L) and the LC20 of Cu (24 h: 1290 µg/L, 48 h: 146 µg/L). To investigate the effects of Cd, the test concentrations had to be higher than in the environmental regulations. However, it is difficult to compare these concentrations in terms of relevant exposure conditions, since exposure time, pH, food availability, temperature, etc. can influence toxicity. For example, under our conditions, with *E. coli* as food source, a significant fraction of the total metal concentration is expected to be associated with the bacteria i.e. the free metal ion concentration in the aqueous medium is expected to be a small fraction of the total concentration. In any case, the metal load in our experiments, expressed in terms of mg per g of bacteria, was in accordance with the reported metal content of contaminated soils used for toxicity studies with *C. elegans* (Höss et al., 2009).

6.1. Metal mixture toxicity

In contrast to the increasing understanding of the toxic effects of single metals, the number of studies concerning their effects upon interaction, remains low. Moreover, only single metals are used in environmental regulation, although metal mixtures often occur in the natural environment. Interactions between chemicals can occur in the environment affecting each other's bioavailability during the uptake by an organism and influencing the toxicokinetics of the chemicals during internal processing of the compounds (detoxification and biotransformation) and during the intoxication of the organisms (toxicodynamics). For example, Zn, Cu, and Cd can compete for entry into nematodes (e.g. competition for Ca channels between Cd and Zn), eliciting different responses than when the nematodes are exposed to the single metals. The potential interplay between the biotic handling processes for different metal ions means that the nature and timescale of toxicity responses under mixture conditions does not follow straightforwardly from observations on single metal exposures. Therefore, in order to gain insight into metal mixture toxicity, it is necessary to test mixtures and corresponding single metals simultaneously.

Mixture toxicity can be assessed by multiple dose-response analysis of single compounds in comparison with their combinations. In the current literature, data are compared with predictions based on the Concentration Addition (CA) or Independent Action (IA) model (Martin et al., 2009; Jonker et al., 2004b, 2005).

According to the model of CA, mixture effects are predicted using the formula:

$$1=\sum_{i=1}^{n}\frac{c_i}{EC50_i \times \left(\frac{y}{100-y}\right)^{1/\beta}}$$

For IA, the formula below is used to predict mixture effects:

$$y=100 \times [1- \prod_{i=1}^{n} \left(\frac{1}{1+\left(\frac{c_i}{EC50_i}\right)^{\beta}} \right)]$$

In the above formulas, c_i is the concentration of compound i, n is the number of compounds, y is the predicted effect of the mixture and β is the slope of the dose-response curve.

In both models, metals do not interact with each other: CA assumes a similar mode of action, while IA assumes a dissimilar mode of action. Deviations from the expected toxicity, calculated with both models, are interpreted as being the result of interactions between the metals, which can be additive, antagonistic (weakening) or synergistic (enforcing). However, it is becoming increasingly clear that more complex deviation patterns e.g. dose-ratio dependent deviation, dose-level dependent deviation, synergistic/antagonistic deviation, need to be addressed to predict mixture toxicity.

Our study provided insight into whether and how metal toxicity changes in mixture exposure scenarios by comparing mixture toxicity effects with the toxicity of the corresponding single metals (Zn, Cu, Cd) and by investigating the interaction effects. To achieve this, the mixture effects were assessed against the sum of the expected effects of exposure to LC20 concentrations of the single metals. The binary mixture effect was considered additive within a band of ± 25% of the expected LC40 value of the metal mixture. A synergistic action occurred when the expected value for the mixture was significantly exceeded by more than 25%; if the measured value was at least 25% significantly lower than the expected value; the interaction of the metals was considered antagonistic. However, the slopes of the dose-response curves of the metals were not the same: the studied metals therefore have a different mode of action, while contributing to the same biological endpoint. Therefore, it was recommended to use the Independent Action model instead of the Concentration Addition model to determine the interaction effects in the mixture. For binary mixtures, it is recommended

to use half of the lethal concentrations to assess the mixture effect against the expected LC20 value of the mixture:

$$\frac{LC20A}{2} + \frac{LC20B}{2} = LC20\ Mix$$

This corresponds, at 24 h, with a concentration of 0.65, 3.56 and 4.75 mg/L for Cu, Cd and Zn, respectively, while a respective mortality of 11.9, 14.5 and 12.5% is expected. At 48 h, this corresponds with 0.073 mg/L Cu, 2.06 mg/L Cd and 1.798 mg/L Zn with an expected mortality of 12.8%, 12.1% and 11.7% for Cu, Cd and Zn, respectively. Binary mixtures of these metal concentrations will cause a mortality of 20% if the metals act additively.

In the CuCd mixture study in Chapter 2 (Fig. 2.3 and 2.4), the combination of 0.5 mg/L Cu and 2.5 mg/L Cd, which approximates the LC20/2 concentrations at 24 h, resulted in an observed mortality of 27.2% after 24 h, while the predicted mortality based on the CA and IA models is 16.8 and 20.5% respectively. The observed mortality did not differ significantly from the values predicted according to both models, indicating an additive interactive effect of Cu and Cd.

To check whether the use of models would lead to new conclusions, a concise follow-up calculation is performed below, in which the mortality data obtained in chapters 2 and 3 were again assessed by comparing the observed mortality data with the predictions based on the IA model and CA model.

Reanalysis of the ***mortality*** data of LC20 combinations of Chapter 2 (Fig 2.4.) resulted in a significantly lower mortality for the ZnCd mixture after an exposure to the $LC20_{24\ h}$ after 24 h and 48 h, compared to the predictions of the CA and IA models. For ZnCu and CuCd the mortality after an exposure to $LC20_{24\ h}$ did not differ from the model values after 24 h and 48 h. For all mixtures, an exposure to $LC20_{48\ h}$ resulted after 48 h in similar mortalities as the predictions based on the models. In addition, the observed mortalities of the combinations of different Cu concentrations with different Cd concentrations

(Fig. 2.3. and 2.4.) were compared with predicted mortalities from the CA and IA model and visualised in Fig.6.1. At 24 h, data were positioned close to the line indicating additive effects, while, at 48 h, some combinations showed less than additive (slightly antagonistic) effects.

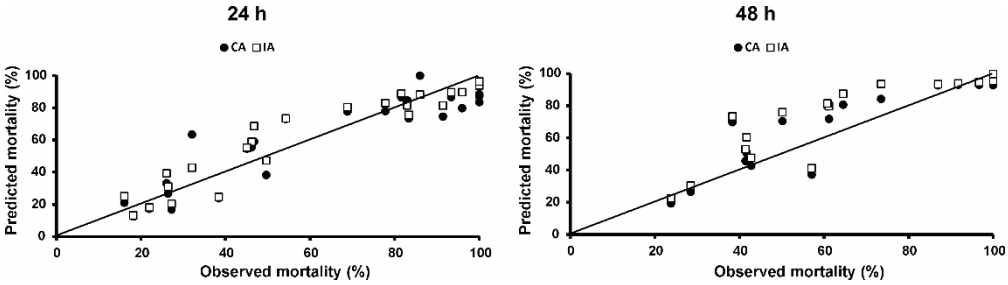

Figure 6.1: Comparison of observed mortality and predicted mortality based on the concentration addition (CA) and independent action (IA) model for CuCd combinations after 24 h (left) and 48 h (right). Line indicates additive effects, data above line indicate antagonistic effects, data below line indicate synergistic effects.

Mortality data from the different combinations of LC20 of Cd with LC2, LC5, LC20, LC40 and LC60 of Zn from chapter 3 (Fig.3.3) were also reassessed as described above. Both at 24 h and 48 h, a lower mortality than predicted on the basis of the CA and IA model was observed for all combinations, i.e. antagonistic effects are operative (Fig. 3.3, Tables S3.9 and S3.10).

However, the previous models can only be used for binary mixtures. Therefore, for the tertiary mixtures, another formula for the Independent Action is used, which is based on effects:

$$1 - E(mix) = (1 - E(A))(1 - E(B))(1 - E(C))$$

where E(mix) represents the probable metal mixture effect and E(A), E(B) and E(C) represent the effect of each metal separately. This equation implies that the probability of the absence of a mixture effect (1-E(mix)) is the product of the probabilities of not being affected by any of the single compounds. The mortality of ZnCuCd did not differ from the predicted mortality after an exposure to $LC20_{24\,h}$ for 24 h and $LC20_{48\,h}$ for 48 h,

while an exposure to $LC20_{24\ h}$ resulted in a lower mortality than predicted after 48 h of exposure.

For ***chemosensory and behavioural*** data (e.g. trashing behaviour, average crawling speed), full dose-response profiles are missing to interpret the interaction effects of mixtures by comparing the observed data with the predicted data according to the CA and IA model based on the exposure concentration, slope of dose-response curve and EC50. Therefore, observed effects were compared with the probable mixture effects predicted by the Independent Action model without taking into account exposure concentration and dose-response characteristics (Bliss, 1939), and using the simplified formula:

$$E(mix) = E(A) + E(B) - E(A)E(B)$$

where E(mix) represents the probable metal mixture effect and E(A) and E(B) represent the effect of each metal separately. This equation means that the cumulative effect of the mixture is the result of combining the probabilities that a compound at the concentration at which it is present in the mixture, exerts a toxic effect (ranging from 0 to 1).

For example, reassessment of the thrashing data of Chapter 2 (Fig. 2.7), indicated a synergistic effect for ZnCu after an exposure to $LC20_{24\ h}$ (pooled data from 24 h and 48 h) and $LC20_{48\ h}$. The mixtures CuCd and ZnCuCd acted each additive after an exposure to $LC20_{24\ h}$ (pooled data from 24 h and 48 h) and synergistic after an exposure to $LC20_{48\ h}$. The observed locomotory effect of ZnCd appeared to be additive after both $LC20_{24\ h}$ and $LC20_{48\ h}$ exposures, while antagonistic effects were expected. However, when calculations were made with the average value of 6 replicates instead of the individual data separately, the interaction of the $LC20_{24\ h}$ combination appeared to be antagonistic. The (non-significant) difference with the IA model might be due to the high individual variation i.e. some nematodes showed a similar trashing behaviour as control, while others showed almost no thrashing behaviour.

Interestingly, the originally described interaction effects on mortality and behaviour are not significantly different from the interaction effects reassessed on the basis of the CA and IA models. Furthermore, the predictions from these models were very similar, as can be seen in Fig. 6.1.

The small amount of metal mixture toxicity studies are usually carried out for a short period of time, and metal mixture studies for more than 1 week are rare. However, time dependency of single metal toxicity has already been observed: for example, Cd toxicity effects increase with increasing exposure time. Therefore, it would also be interesting to perform long-term toxicity studies on metal mixtures, because it is possible that interactive effects of the metals change over time.

Although metal mixture toxicity is typically evaluated using the CA and IA model, these approaches show some limitations (Chen et al., 2013; Crémazy et al. 2018; Nys et al., 2018). For *C. elegans* for example, mixture toxicity effects on the temporal evolution of ***body length*** and ***population size*** are much more difficult to interpret with these models. First, full dose-response profiles were absent, but more importantly, our studies were carried out in the long term implying that various life stages and ages had to be taken into account. Therefore, we could not use the CA or IA models for analysing mixture effects. Additional experiments with different exposure concentrations are needed to fit dose-response curves as a function of exposure time. That information can than be used to model and predict mixture effects at different organisational levels. As such, the suitability of the CA and IA models to predict the temporal evolution of mixture effects can be evaluated. However, these models should be applied with some caution. Our results showed a different population size pattern for $LC20_{24\ h}$ and $LC5_{24\ h}$ exposures, which changed over time. So, depending on the situation, the whole story can change because time and exposure concentration are not taken into account in the simplified formula of the IA model for effects. For example, the protective role of Zn on the Cd toxicity when LC20 concentrations were used, disappeared when using the combination of LC5 concentrations. If the simplified formulae of Independent Action would be used

for predicting mixture effects, only additive effects will be identified from day 5 onwards for all LC20 treatments, because for each metal treatment the population size differed more than 95% from control. This again shows the limitation of not taking time into account. However, the obtained results from the simplified formula of the IA model for short-term exposures of max. 4 days for population size or 2 days for thrashing behaviour seemed reasonable. However, it is better to use, if possible, the formulas taking EC50, exposure concentration, time and slope of dose-response curve into account.

6.2. Combination of different organisational levels

Toxic effects observed at one organisational level can help to better understand or explain the observations at another level. Furthermore, it is possible that at a certain moment it can be wrongly concluded that the chemical has no effect on the organism, because an unaffected level or undetectable variable is considered while clear effects can be noticed at another level. Unfortunately, the number of studies into different endpoints of various organisational levels, remains low. Therefore, in the present study, toxicity effects of metals on different endpoints of different organisational levels were investigated. Both toxicity effects of single and of combined metals were studied at the molecular, individual and population level. The results are discussed below.

6.2.1. Molecular level

In this study (chapter 5), only 7 differential proteins were identified after 24 h of exposure: 1 for Cu exposure, 4 for Cd exposure and 2 for CuCd exposure, although the concentration employed (LC10) was relatively high. Chosen practical (too small sample size) and technical settings might explain this low number, although it is also possible that nematodes were still able to maintain ***protein*** homeostasis. Therefore, this study couldn't provide a picture of the biochemical pathways that are affected by exposure to

LC10 of Cu and Cd (as single metals and in combination) and it was therefore not possible to identify fingerprints that can serve as early warning systems for toxicity.

In addition, there are not many proteomics studies on metal effects, suggesting that the proteomics approach is not yet an optimal tool for metal toxicity research in *C. elegans*. However, cellular detoxification systems, including glutathione, methallothioneins, heat shock proteins, pumps and transporters, are known to regulate intracellular metal levels by detoxifying and excretion of metals (Martinez-Finley and Aschner, 2011). For example, the transcription of the cadmium-responsive gene-1 (*cdr-1*) is induced only for intracellular Cd defence in response to Cd exposure. High levels of Zn induce transcript levels of *cdf-2*, *ttm-1b*, *mtl-1* and *mtl-2*, the promoters of which contain a similar sequence, called HZA (Roh et al., 2014; Dietrich et al., 2016). It is suggested that HZA serves as an enhancer to mediate transcriptional activation in response to high Zn or Cd levels. For systemic copper homeostasis in *C. elegans*, the intestinal copper exporter CUA-1 is required (Chun et al., 2017). In our study the identification of 2 heat shock proteins for Cd exposure and 1 for exposure to the CuCd mixture indicated the presence of detoxification systems. Only HSP-70 in the CuCd mixture was upregulated. The presence of these heat shock proteins suggests that the proteins are under stress. It is possible that with a lower exposure concentration more subtle differences between groups can be observed.

In contrast, many peptidomics studies on *C. elegans* exist (e.g. De Haes et al., 2015; Husson et al., 2007; Husson et al., 2010), although the peptidomics approach can be quite challenging by, for example, the annotation of (neuro)peptide precursors and the large efforts needed for tissue collection and purification strategies. The peptidomics approach, therefore, must first be further optimised before it can be used efficiently for metal toxicity tests. In the present study, 51 endogenous peptides of non-exposed mixed stage *C. elegans* nematodes were identified, including 5 novel neuropeptide-like protein peptides from 5 novel precursors. The bioactive peptides govern a diverse array of physiological processes and behaviours such as feeding, locomotion and reproduction. Knock-down of these peptides (e.g. FLP) already showed reduced

locomotion (Li and Kim, 2014). It would be interesting to check whether the parameters, affected at other levels, are reflected at the ***peptidome*** level.

6.2.2. Individual level

Toxic effects at the individual level were studied using different endpoints in chapters 2 (mortality, average crawling speed, thrashing behaviour and chemosensation) and 3 (mortality).

Because of the ease of evaluation, ***mortality*** is the most studied short-term endpoint for *C. elegans* responses to single metal exposures (e.g. Donkin and Williams, 1995; Boyd and Williams, 2003; Wang and Ezemaduka, 2014). The results in chapter 2 showed that for short-term exposures (2 h, 8 h, 24 h and 48 h) the mortality of Cu exposed nematodes was higher than in Cd and Zn exposure. The survival fraction decreased with increasing metal concentrations and exposure time, reflecting the increased toxicity of metals for longer exposure periods. For metal mixtures, the LC20s of constituent metals were combined. Interactive effects were dependent on the metal combinations employed and the exposure time. To determine these interactive effects, observed mortalities were compared with predicted mortalities on the basis of the CA and IA model. In ZnCu and CuCd the constituent metals acted additively at $LC20_{24\,h}$ (for 24 h and 48 h) and $LC20_{48\,h}$. The tertiary mixture ZnCuCd showed additive interaction effects at $LC20_{24\,h}$ (for 24 h) and $LC20_{48\,h}$, while antagonistic effects were observed at $LC20_{24\,h}$ (for 48 h). Interestingly, the ZnCd mixture showed antagonistic effects after an exposure to the $LC20_{24\,h}$ after 24 h and 48 h, but the observed mortality did not differ from model predictions for the $LC20_{48\,h}$ exposure (P= 0.06). To better understand the mitigating effect of Zn on Cd toxicity due to e.g. competition for Ca channels, the mortality of a range of LC concentrations of Zn in combination with the LC20 concentration of Cd was measured in chapter 3 for exposures of 24 h and 48 h. The mixture effects appeared to be concentration dependent. Compared to the Cd20 exposure, Zn60Cd20 caused a significantly higher mortality at both time points, while the mixture Zn2Cd20 induced a

lower mortality at 48 h (Tables S3.9 and S3.10). Furthermore, all combinations at 24 h and 48 h, had a lower mortality than predicted on the basis of the CA and IA model (P<0.05), i.e. antagonistic effects were again operative (Fig. 3.3, Tables S3.11 and S3.12).

However, unlike mortality, reproduction, body length and egg production, ***locomotion*** has been less evaluated as a toxicological endpoint for both single metals and metal mixtures (Power and de Pomerai, 1999; Dhawan et al., 1999; Dhawan et al., 2000; Roh et al., 2006; Höss et al., 2011). Our study showed that the toxicity of Cu was again higher compared to Cd and Zn, resulting in a more reduced locomotion of the nematodes. In addition, for Cu and Cd, survival of exposed nematodes declined by 20% after 24 h of exposure, whereas locomotion already decreased by 34% and 86%, respectively. In contrast, for Zn, survival was more affected than locomotion. Furthermore, the locomotor responses (crawling speed and thrashing behaviour) decreased more in the binary and tertiary mixtures, which was in line with the findings of the mortality tests. Since a similar trend of effects was noted in locomotion and in mortality tests, but mixture toxicity effects on locomotion were stronger than on mortality, locomotion may probably be considered as a sensitive sublethal endpoint in metal toxicity studies.

Locomotion is controlled by neural processes (via interneurons), which receive and integrate input signals from various chemo- and mechanosensory neurons, and is mediated by amphid and phasmid neurons when encountering a potentially harmful chemical. In addition, several studies using mutants have shown that FLP and NLP (e.g. NLP-49) neuropeptides can modulate the locomotion of *C. elegans* (Chang et al., 2015; Chew et al., 2018). For example, *flp-1* and *flp-18* play an important role in suppressing overexcitation of the locomotory circuit (Li and Kim, 2014), while the knock-down of *flp-9* induces less thrashes as compared to the wild-type *C. elegans* (Li and Kim, 2008). Although, in this study, locomotor behaviour was clearly affected by metal exposure, this was not reflected at the molecular level when exposed to a lower concentration (LC10). A possible explanation is that the new proteins are immediately synthesized to compensate for the deficiency caused by affected and degraded proteins, which means

that the protein concentration remains (almost) constant. Furthermore, *C. elegans* needs a proper neuronal functioning and an unaffected locomotor system to change direction in order to avoid harmful chemicals. Although locomotion was clearly affected, almost no difference from control was observed in both drop test and chemotactic index test: ***chemosensation*** seemed strong enough to elicit a chemotactic and rapid avoidance response. Unlike locomotion, chemosensation did not seem sensitive enough to be considered as an endpoint for assessing toxic effects on *C. elegans*.

6.2.3. Population level

Most metal toxicity studies with *C. elegans* are conducted with an exposure time of 24 h to 48 h (e.g. Power and de Pomerai, 1999; Dhawan et al. 2000; Chu and Chow 2002; Boyd et al. 2003; Haegerbaeumer et al., 2018), while some studies are performed for 3-4 days (Höss et al., 2001; Calafato et al., 2008; Harvey et al., 2008; Höss et al., 2009; Song et al., 2014) and only a few studies examined a certain endpoint for more than a week e.g. growth, population size and mainly mortality (Wang et al., 2007; Harvey et al., 2008; Davis et al., 2009; Muschiol et al., 2009; Lira et al., 2011; Nørhave et al., 2012; Kumar et al., 2016). However, such long-term studies may provide better insights into metal toxicity, because metal toxicity may increase with longer exposure time (Williams and Dusenbery, 1990). Therefore, in contrast to the short-term experiments (2 h, 8 h, 24 h and/or 48 h) carried out at the molecular and individual level, chapter 3 deals with experiments studying the interactive effects of the exposure to combinations of LC5 and LC20 of single metals at the population level for several days. The first measured endpoint, ***body length***, represents the average body length of the post-larval stage nematodes of the population (not necessarily from the same age or from the same life stage). The other endpoint, ***population size***, integrates life history traits and can thus be a useful and sensitive endpoint in long-term studies.

Metal toxicity indeed appeared to be concentration and time dependent, resulting in different trends for population size and body length. Although LC20 of Cu seemed to be

the most toxic in short-term exposures as observed in higher mortality and reduced behaviour (Williams and Dusenbery, 1990; Dhawan et al., 2000; Chu and Chow, 2002; Moyson et al., 2018), exposure for 10 days induced a less severe population decline than Cd exposure, and population size was similar to that of the control at LC5 concentration. For all single and mixed metals, the body length of LC20 exposed nematodes followed a similar trend as population size, while LC5 exposure induced slight effects on population size for single metals but no effects on body length were observed for both single and combined metals. Furthermore, populations exposed to Cd and LC20 mixtures plummeted but remained stable in mixtures of LC5 concentrations. For LC20, a decrease in population size was expected because exposed nematodes did not reach larval maturity, which disrupted egg laying, causing the number of new larvae to decline. Furthermore, fewer eggs developed into adults after LC20 exposure, suggesting that mortality rate was higher for young L1 worms than for adults (Chu and Chow, 2002). It is possible that the reproductive characteristics of the nematodes exposed to LC5, e.g. start of egg laying, number of eggs, time of hatching, hatching success, etc. were affected, which is reflected in differences in the population size of mixtures compared to control and corresponding single metals. This is supported by reports that *C. elegans* is able to alter both its larval development and its reproductive strategy in response to environmental stress (Álvarez et al., 2005; Harvey et al. 2008). According to numerous publications, fecundity, development and growth of *C. elegans* is reduced by Cd, Cu and Zn (Eisler 1977; Popham and Webster 1979; Power and Pomerai 1999; Höss et al. 2001; Wang et al. 2007; Calafato et al. 2008; Höss et al. 2011 Nørhave et al. 2012; Song et al. 2014; Wang and Ezemaduka 2014; Cedergreen et al. 2016; Kumar et al. 2016; Chun et al. 2017). Overall, metal effects were more evident on population size rather than on body length, suggesting that population size might be considered as a sensitive endpoint. For example, the population sizes in the Cu and Cd exposures were on average 707.9% larger than in the CuCd mixture, while body length was on average only 19.6% larger.

As discussed above, due to limitations of the models, mixture effects were not compared with predictions of the CA and IA models to identify the interaction types, but were described and compared with the effects of the corresponding single metals. The interactive effect of the single metals in ZnCu, CuCd and ZnCuCd mixtures on body length and population size, when exposed to LC20 concentrations, and on population size, when exposed to LC5 concentrations, was observed to be either additive or synergistic, which is in line with our findings at the individual level. Others reported that Zn and Cu have additive or less than additive (slightly antagonistic) effects at the community level (number of nematodes in soil) (Korthals et al., 2000), and that there are highly synergistic interactions between Cu and Cd, and between Cu and Zn at the individual level (mortality) (Chu and Chow, 2002). In addition, additive effects for the CuCd mixture were observed at the molecular level (β-galactosidase activity) (Power and de Pomerai, 1999). As for single metals, mixture toxicity appeared to be concentration and time dependent. In our study the combination of Cu and Cd had a larger additive effect at LC5 than at LC20, while the combined additive effect of Cu and Zn at LC5 was similarly additive or slightly less additive than at LC20 (Fig. 3.2). In contrast, for the entire experiment and for both endpoints, the interaction between Zn and Cd appeared to be antagonistic at LC20 concentrations and additive at LC5 concentrations. This effect was also observed in our previous study at the individual level (mortality and locomotion) (Moyson et al., 2018) and in other studies at the molecular (β-galactosidase activity) and individual level (mortality, body burden, body length) (Power and de Pomerai, 1999; Chu and Chow, 2002; Calafato et al.,2008).

To better understand the mitigating effect of Zn on Cd toxicity, a short-term study (24 h and 48 h) was performed to analyse the population size of exposures to mixtures of the LC20 concentration of Cd with a range of LC concentrations of Zn. The concentration dependency of the mitigating effect of Zn was more pronounced for the population size than for the mortality. After 48 h, the mixtures Zn20Cd20 and Zn40Cd20 showed antagonistic effects, which disappeared when using lower (LC2 and LC5) or higher (LC60) Zn concentrations. The observed concentration dependency of the antagonistic effect

of ZnCd mixtures at the individual and population level can be linked to findings of an earlier study at the molecular level (Davis et al., 2009). In *C. elegans* transmembrane Zn transporters of the CDF family function to lower cytoplasmic Zn concentration (Roh et al., 2014; Dietrich et al., 2016): *cdf-1* seems to promote Zn excretion and/or limiting Zn uptake, while *cdf-2* functions to promote Zn accumulation. Davies and co-workers (2009) found that *cdf-1* was most abundant at low Zn concentrations, while a dose-response increase in *cdf-2* expression was noticed as a function of increasing Zn concentration till a certain concentration of about 1 mM (i.e. around the LC60 concentration of Zn in our study). This concentration corresponds to the condition under which we observed a decrease in the extent of antagonistic effects in the ZnCd mixtures. It is possible that the Zn60Cd20 mixture reached or even exceeded the capacity of the system to cope, and thus toxicity of both metals is manifested.

6.2.4. General conclusions

A significant outcome of this study is the importance of studying effects on different endpoints and at different organisational levels, and the time dependence thereof. Toxic effects at one level, e.g. the molecular or individual, can help to better understand the observations at another organisational level, but are evident at another. These findings underscore the need to monitor effects at multiple organisational levels. This study also highlighted the importance of long-term studies. For example, differences between LC20 treatments can already be observed for mortality and behaviour after 24 - 48 h (Moyson et al., 2018), while for population size differences became more evident after 3-4 days of exposure. This can be explained by the fact that population size integrates individual life histories and transgenerational effects, which take time to be reflected in the entire population. For example, if the development of nematodes of the new generation is affected, the effects will only be clearly visible 3-4 days later via the absence of a large increase in population size due to e.g. inability to lay eggs.

Unfortunately, environmental quality standards are still based on single metal toxicity at short exposure durations and do not take into account the chronic, multi-stressor exposure scenarios that are typical for environmental systems. The mechanistic links we observed herein between transcriptomic responses and mixed metal toxicity at the individual and population level may lead the way to the development of a more comprehensive basis for environmental risk assessment.

6.3. Combination of metal speciation and physiology determines metal accumulation and toxicity

Several metal toxicity studies have been conducted with *C. elegans*, but the effects were not linked to the bioavailable concentrations, uptake mechanisms and internal handling of the metals (e.g. accumulation, detoxification, excretion). Furthermore, the metal uptake route of *C. elegans* remained unknown. Insights into the bioavailable metal concentrations (free metal ions) and organismal accumulation patterns may help to better understand the earlier observed toxic effects. In our study ***metal speciation*** data and physiological characteristics of *E. coli* and *C. elegans* were linked with ***body burden*** and earlier described toxicity effects to determine the ***uptake route*** of *C. elegans* and the metal species that are the best predictors of body burdens and toxic effects under single metal and mixed metal exposure conditions.

Our study showed that the percentage of free metal ions or dissolved metal was similar for the single metals in *E. coli* absence, while less free Cu^{2+} ions (39%) were available for *C. elegans* nematodes than Cd^{2+} and Zn^{2+} ions and more Cu, in percentage terms, was taken up by the nematodes in *E. coli* presence. Although time effects and differences between treatments were observed in previous chapters 2 and 3, conducted under the same experimental conditions, metal speciation appeared not to be time dependent and only slight differences between treatments were observed. However, metal speciation seemed to be concentration dependent, which was also observed for metal toxicity in chapters 2 and 3. Furthermore, the results of chapter 4 suggest that the free

metal ion concentration (i.e. waterborne uptake) in the exposure medium is the best predictor of the internal concentration of all treatments and of the ensuing toxicity of single metal exposures. Also in other studies, waterborne uptake seemed to be the best predictor for toxicity on reproduction and growth rate (Höss et al., 2011; Yu et al., 2012). Nevertheless, the outcome should be regarded with some caution. For example, the pharyngeal pumping rate is sensitive to the presence of metal ions, albeit that typically a lower rate is observed (Jones and Candido, 1999), i.e. if anything, our calculations are an overestimation of the dietborne contribution to the body burden. At least 10^3 *E.coli* per worm per day are needed for minimal population growth, while 10^5 *E. coli* per worm per day are necessary to sustain an optimal population growth (Venette and Ferris, 1998). Because the population size of control nematodes highly increased over time (chapter 3), sufficient *E. coli* were present, but the affected feeding behaviour probably caused an uptake of less than 10^5 *E. coli* bacteria per day per metal exposed worm. Furthermore, waterborne contribution was based on a 100% diffusion rate, which will be typically lower due to e.g. affected transporters, channels etc., and was thus an overestimation as well.

Finally, the availability of a compound in the exposure medium and its accumulation in the body, does not necessarily mean that the compound causes toxic effects. As a result, the observed differences in toxic effects between treatments (e.g. mixtures vs. corresponding single metals) may indicate biotic handling differences instead of differences in exposure concentrations. First, the metals have to be taken up, but during that phase metals can damage transporters, channels etc., can destabilize biomembranes, can compete with each other to enter the organism and, for example, Cd may displace Ca while entering the body. Second, after uptake metals can be accumulated in tissues, detoxified or their toxicological damage can be repaired. Therefore, It would be interesting to study the intracellular speciation, i.e. the distribution of internalised metals across the tissue (e.g. gut, vesicles) and thus to analyse the storage location, detoxification of metals and toxicological activities instead of the total metal accumulation. In our study, the total metal accumulation (mg metal/g

wormpellet) in nematodes exposed for 24 h to single metals and to their mixtures did not differ, while the toxic effects described in chapters 2 and 3 were different. It is thus possible that the total uptake does not give a correct insight into possible toxicity, while the local uptake does. It was previously observed that, depending on the concentration, Zn is stored in vesicles of intestinal cells or transported to the intestinal lumen for excretion (Davis et al., 2009; Dietrich et al., 2016). Due to the potential role of *cdf-2* in mitigating Cd toxicity, it is possible that Cd is also stored in the gut granules (Moyson et al., submitted). In contrast to Cd and Zn, Cu is observed to be homogeneously distributed throughout the body of the nematodes (Jackson et al., 2005). Due to this spatial difference, it would be interesting to analyse the intracellular speciation in mixture exposure conditions.

Therefore, it would be interesting to measure the uptake rate, which depends on the physico-chemical features of the compound, the affinity of the compound for water and the organism, the uptake of the compound and the capacity of the organism to eliminate this compound passively or actively (diffusion, biotransformation and excretion). This could provide more information since it is linked with the biologically active metal, while total body burden is linked to the bio-inactive metal. The dynamics of metal accumulation and toxicity are described by the BIMBAM (bio-inactive pool - bio-active pool) model. Metal uptake is function of the external availability, while toxicity is function of the internal availability. Toxic effects depend on the response time of the biological machinery, activation of detoxification processes, activation of metabolic pathways, and storage in organs/tissue and disturbance of the homeostasis (caused by overloaded BIM compartments). If the rate of metal uptake exceeds the combined rates of detoxification and excretion, a critical concentration of metabolically available metal can be accumulated, resulting in toxic effects (Rainbow, 2002, 2007; Adams et al., 2011; Jacob et al., 2017).

6.4. *C. elegans* as test organism

Because of their abundance, ecological value, characteristics of life history and ease of cultivation and maintenance in the laboratory, *C. elegans* nematodes are excellent organisms for high throughput testing for fundamental neuroscience (e.g. Parkinson disease and Alzheimer disease), aging and (eco)toxicology (Brenner, 1974; Khanna et al.,1997; Leung et al., 2008; Hunt, 2016). However, in a one-man *C. elegans* laboratory, the efficiency of high throughput testing was low. The time-consuming continuous cultivation of *C. elegans* and *E. coli* bacteria during the entire 4-year study formed an obstacle to the experiments. Moreover, the NGM agar needed for the cultivation of these organisms was prepared manually (3 g NaCl, 20 g agar, 2.5 g peptone, 975 mL MilliQ water, 25 mL KPO_4 buffer, 1mL 1M $CaCl_2$, 1mL 1M $MgSO_4$ and 5 mg/mL cholesterol in ethanol) and poured together in Petri dishes. In addition to the NGM plates for the maintenance of the *C. elegans* culture (3 plates every 3 days), extra plates were needed for the experiments. For example for the body burden study, 81 NGM agar plates with *C. elegans* and seeded with *E. coli* were needed to collect enough eggs. Thereafter, the synchronized eggs were raised (after bleaching) on 81 new NGM agar plates. This high number of agar plates was required to obtain approximately 4.5 mg of adults per treatment replicate, which is the amount of sample material needed to be able to detect metals with the ICP. In addition to the cultivation of sufficient test organisms, sufficient *E. coli* bacteria (harvested from an agar plate and grown in LB medium) had to be available and liquid exposure medium had to be prepared. Because the workload was too high to carry out the entire experiment at a single time, replicates of each treatment were exposed on 3 consecutive days. Furthermore, starting the experiments with adult nematodes in the morning, implied a synchronization of nematodes by bleaching 4 days earlier at night time. This practical issue made it impossible to measure body burden for 12 consecutive days, which would have been interesting for understanding population size effects and linking these results to metal speciation.

Furthermore, it is not recommended to split an experiment over several weeks, as *C. elegans* showed some variation in number of eggs laid and number of hatched eggs over time, although *C. elegans* were always kept under the same laboratory conditions. There also seemed to be a seasonal influence (better behavioural tests in winter than in summer)(unpublished data), although nematodes were always kept in an incubator and experiments were carried out in a climate chamber with constant conditions, so no explanation for this variation can be found. Because the results of the experiments, analysing the mortality of metal mixture exposures (Fig. 2.5), performed in July 2016 and repeated in October 2016, were not significantly different, they were combined. In contrast, the Zn, Cd and ZnCd mortality experiments (Fig. 3.3) carried out in May 2017, showed variation with the mortality results obtained in 2016 (Table 6.1). Although 6 replicates were used in May 2017, the standard deviations were - in terms of percentage - higher than for the 2 other experiments with only 3 replicates.

Table 6.1. Mortality data (%) after an exposure to $LC20_{24\ h}$ for 24 h and 48 h, obtained at 3 different times dates.

	July 2016		October 2016		May 2017	
	24 h	48 h	24 h	48 h	24 h	48 h
Zn	19.3 ± 8.9	32.2 ± 7.9	21.8 ± 6.6	35.6 ± 8.0	7.8 ± 6.0	12.0 ± 6.8
Cd	15.3 ± 2.1	36.9 ± 11.4	19.6 ± 6.7	36.3 ± 5.9	16.3 ± 7.2	27.0 ± 7.0
ZnCd	14.6 ± 11.2	30.9 ± 10.4	18.4 ± 3.9	31.6 ± 9.0	11.2 ± 7.0	20.9 ± 10.4

Another disadvantage of working with *C. elegans* is that a group of 500-600 whole nematodes is needed per treatment replicate to be able to measure proteins and internal metal concentrations, while for e.g. fish just 1 brain is sufficient. By only examining the brain, the number of neuropeptides will be less affected by the

abundance of other peptides. In our study, we could not make a difference between cell types, which might have caused the low number of identified differential proteins. In this regard, it would also have been interesting to test with e.g. fluorescence techniques where the metals are accumulated in the body (intestine, granules, etc.).

Furthermore, although *C. elegans* has been frequently used to study (single) metal toxicity effects using different exposure media (e.g. agar, soil, liquid medium) and different endpoints (e.g. gene expression, reproduction, growth, mortality and locomotion) (Popham and Webster, 1979; Power and de Pomerai, 1999; Höss et al., 2001; Boyd et al., 2003; Boyd and Williams, 2003; Höss et al., 2011; Hunt et al., 2013; Inokuchi et al., 2015), it seems that *C. elegans* is not very sensitive to metal exposures. It was clear, for example, that some individuals still had a similar locomotory behaviour as the control nematodes, while average locomotion was reduced (Fig. 2.6 and 2.7), However, in aquatic and soil tests, *Caenorhabditis elegans* showed an intermediate toxicity response to Cu compared to the nematodes *Pristionchus pacificus* and *Panagrellus redivivus*, making *C. elegans* a representative of other rhabditid species for toxicity testing (Boyd and Williams, 2003). Also for Cd, *C. elegans* seemed to perceive only an intermediate effect compared to 11 nematode species (Kammenga et al., 1994). Fast colonizing nematodes (e.g. *Diplogasteritus* sp.) were relatively more sensitive to Cd than slow colonizing species (e.g. *Aporcelaimellus obtusicaudatus*). *C. elegans* was more sensitive (lower $LC50_{96\ h}$) to Zn and Cd than the average of invertebrates and benthos (e.g. bristle worm, caddis fly), while benthic organisms were more sensitive to Cu than *C. elegans*. However, *C. elegans was* less sensitive to both Cu and Zn than *Daphnia* sp., but sensitivity to Cd was similar (Williams and Dusenbery, 1990). Furthermore, compared to *Asellus aquaticus*, *C. elegans* was more sensitive to Cu, but less sensitive to Cd (Van Ginneken et al., 2017). The high level of Cd insensitivity of *C. elegans* suggests that these nematodes possess efficient defence mechanisms (e.g. metallothioneins, heat shock proteins, pumps, and transporters) preventing metal damage (Martinez-Finley and Aschner, 2011). Due to the fact that *C. elegans* seemed to be relatively insensitive to metals, although high exposure concentrations were used, this nematode

species can function as a model organism to unravel the mechanisms of their resistance to metals by studying e.g. their detoxification (e.g. proteins, internal accumulation and storage in the body) and excretion mechanisms. Because *C. elegans* showed an intermediate response to metals compared to other organisms, it is better to use studies on more sensitive organisms than *C. elegans* (e.g. *Daphnia species*) for risk assessment to establish environmental standards.

6.5. Brief summary

In this thesis the toxic effects of single metals (Zn, Cu and Cd) were compared with those of their combinations on the soil nematode *C. elegans* and this for several endpoints at different organisational levels and for both short-term and long-term exposures .

Metal mixture toxicity

This study gave insight into metal mixture toxicity by comparing the obtained results with the effects of the corresponding single metals and, if possible, with predictions from the CA and IA model. Due to limitations of the models, we were not able to determine the temporal evolution of these mixtures (yet). As for single metals, toxicity of mixtures appeared to be time and concentration dependent. Most mixtures had a stronger toxic effect than the constituent single metals, resulting in additive or synergistic effects for the ZnCu, CuCd and ZnCuCd mixtures, while the mixture ZnCd acted antagonistically depending on the Zn concentrations used.

Combining effects at different organisational levels

Our results showed that results from single and combined metals were better understood when effects on different endpoints at different levels were combined. For example, similar trends were observed for different endpoints but they were more pronounced at one level than at another (e.g. concentration dependency of the antagonism between Zn and Cd was more clear at population level than at individual

level). Similarly, no metal effect was observed at the molecular level (proteome), although clear effects were noticed at the individual and population level. Some parameters also seemed to be more sensitive endpoints than other parameters measured under the same conditions. For example, metal toxicity was more pronounced in terms of locomotion than of mortality and chemosensation for short-term studies at the individual level, while it was more evident on population size than body length for long-term studies at the population level. The use of different exposure times also revealed that long-term studies are needed because toxicity may increase with a longer exposure time.

Combining metal speciation and physiology to determine metal accumulation and toxicity

Our findings showed that metals bound to *E. coli* bacteria were not the major contributor to body burden. Instead, free metal ions and hence the waterborne uptake route was identified as the best predictor for body burdens of both single metals and mixtures and for toxicity of single metals. Since there were no differences in body burden or metal speciation between metal mixtures and corresponding single metals, we were not able to link uptake route with metal mixture toxicity.

6.6. Future perspectives

In this thesis, the effects of single metals and mixtures were studied at different levels, ranging from the molecular to the population level. However, there are still many gaps in the knowledge about metal uptake, bioaccumulation, detoxification and sequestration within an animal, metal speciation and metal mixture effects. Therefore, some suggestions for future research are listed below:

- The metal (mixture) toxicities were only tested in one species, *C. elegans*. However, other species may respond differently to metal exposure, due to different uptake

mechanisms or internal processing. Studying metal mixture toxicity in different organisms could potentially increase our knowledge of interaction effects.

- All experiments were performed under controlled laboratory conditions such as constant temperature, light cycle and pH, although these conditions never occur in natural ecosystems.

- For all experiments, 3-6 technical replicates were used, but a larger sample size is recommended to make the analysis more powerful. However, for the population size experiments, working with more replicates is not possible since all population sizes were counted manually 3 times per treatment replicate per time point. So, in order to obtain population size data from a large sample size in a relatively short time, the counting of living nematodes must be automated. Because of the workload for growing enough nematodes and preparing agar plates (as described in 6.4), it was also not possible to increase the number of replicates in a test. Therefore, the experiment should be repeated over time.

- In order to clarify the mechanistic basis of toxic effects, more research into metal mixture effects is necessary. Knowledge of effects on multi endpoints (molecular, physiology, behaviour, reproduction, population, etc.) at different organisational levels can form an important scientific basis for setting standards for environmental protection and risk assessment. In that way, for example, the mechanistic links between transcriptomic responses and mixed metal toxicity at the individual and population level, observed in chapter 3, may lead the way to the development of a more comprehensive basis for environmental risk assessment. Especially for short-term studies, observed mixture toxicity may be compared (with some caution) with predictions based on the CA and IA model to better understand the interactions between metals and the resulting mixture toxicity. In contrast, additional long-term experiments are needed with different exposure concentrations to fit dose-response curves as a function of exposure time with the intention to evaluate the suitability

of the CA and IA models for predicting the temporal evolution of mixture effects before the models can be used to predict mixture effects over time.

- Although different concentrations were used in this thesis, it would also be interesting to determine the NOEC (No Observed Effect Concentration) level for different endpoints such as locomotion and population size.

- The present work highlights the importance of long-term studies. Therefore, different and longer exposure timescales are needed to clarify the mechanistic basis of toxic effects.

- For omics studies and body burdens, whole *C. elegans* nematodes were used. It would be interesting to focus on the molecular changes in a particular cell type, which was not possible (yet), and to study the accumulation and distribution of metals in tissues by e.g. fluorescence techniques.

- Despite the numerous metal studies on *C. elegans*, there is still no consensus on how these metals are taken up by the organism. Our results in combination with critical literature research have shown that free metal ions and therefore waterborne uptake routes are the best predictor of internal concentrations and toxicity effects of single metals. Therefore, additional experiments can be useful to confirm our hypothesis and to investigate additional factors responsible for metal mixture toxicity. Furthermore, the study of the uptake rate and the internal metal content instead of the total metal content of an organism in combination with the analysis of the tissues (e.g. gut, vesicles) in which metals are stored or detoxified, could provide important insights into metal toxicity.

- Especially for mixtures, our results suggest that not only free metal ion concentrations are responsible for body burdens and toxic effects. Therefore, more research is needed to understand the relationship between bioavailability, differences in uptake route, assimilation efficiency, internal speciation,

detoxification mechanisms (e.g. metallothioneins, heat shock proteins, pumps, etc.) and finally toxicity.

- Biomarkers are used for the discovery of pathogenic processes in medical applications and can also be useful in ecotoxicological studies. However, more time and more studies are needed to further optimise technical settings, practical work etc. before molecular patterns (fingerprints) can serve as early warning systems for ecotoxicity. It is also possible that other omics approaches (e.g. metabolomics) than proteomics are suitable for metal toxicity.

Chapter 7

Nederlandstalige samenvatting

Metalen kunnen door natuurlijke (bv. vulkaanuitbarstingen) of antropogene activiteiten (bv. mijnen) in de bodem, lucht en aquatische systemen terechtkomen. Door de toenemende menselijke activiteit is de aanwezigheid van metalen in het milieu de laatste decennia sterk toegenomen (Peredney and Williams, 2000). Omdat metalen persistent zijn, kunnen ze jarenlang aanwezig blijven in het milieu en biobeschikbaar worden voor organismen. Aangezien deze polluenten worden doorgegeven via de voedselketen, kan een blootstelling aan deze verontreinigende stoffen leiden tot ernstige gezondheidseffecten voor verschillende organismen, inclusief de mens.

De toxiciteit van metalen is afhankelijk van verschillende factoren zoals biobeschikbaarheid, opname- en detoxificatiemechanismen van organismen en excretie-efficiëntie (Rainbow, 2002, 2007; Jacob et al., 2017). Biobeschikbaarheid wordt gedefinieerd als de mate waarin een metaal vrij is voor opname in of adsorptie aan de membranen van een organisme en wordt uitgedrukt als de fractie van de concentratie waaraan het organisme is blootgesteld (Bervoets and Blust, 1999; Baker et al., 2003; Peakall and Burger, 2003; van Gestel, 2008). Metaalbiobeschikbaarheid wordt beïnvloed door talrijke factoren waaronder de metaalspeciatie (de verschillende fysicochemische vormen van een metaal), de interactie tussen het metaal en de omgeving, pH, DOC, temperatuur, interactie met organisch materiaal en andere complexen (bv. bacteriën), metaalconcentratie en soortspecifieke fysiologische en ecologische eigenschappen (Donkin and Williams, 1995; De Schampelaere and Janssen, 2002; Luoma and Rainbow, 2008). Al deze factoren bepalen of een metaal wordt opgenomen door een organisme, waarbij de metaal-bioaccumulatie in het organisme wordt gemoduleerd door differentiële opname (via water of dieet) en transport en opslag in specifieke organen of weefsels. Indien het evenwicht tussen opname en excretie verstoord is en de opgenomen concentratie de drempelwaarde overschrijdt, zullen er detoxificatiemechanismen (bv. pompen, transporters) ingesteld worden en kunnen toxische effecten optreden (Rainbow, 2002, 2007; Jacob et al., 2017).

In het algemeen kan metaalblootstelling verschillende pathologische effecten induceren zoals neurotoxiciteit, vorming van vrije radicalen, verstoring van cellulaire homeostase, enz. Hoewel essentiële metalen (bv. Zn, Cu) noodzakelijk zijn voor diverse biologische functies, kan een tekort of overdosis leiden tot schadelijke effecten. In het onderzoek naar de effecten van blootstelling aan enkelvoudige metalen (zowel essentiële als niet-essentiële metalen) werden reeds verschillende eindpunten bestudeerd van moleculair tot populatieniveau. Het aantal studies dat toxicologische effecten op verschillende organisatieniveaus combineert, is echter nog beperkt. Ook de toxiciteit van metaalmengsels werd nog weinig bestudeerd in tegenstelling tot de toenemende kennis over de effecten van enkelvoudige metalen. De interactieve effecten van metalen in mengsels kunnen additief, synergistisch (versterkend) of antagonistisch (verzwakkend) van aard zijn. Door de resultaten van metaalmengsels te vergelijken met die van de overeenkomstige enkelvoudige metalen, kan inzicht verkregen worden in de verschillende interacties.

Het hoofddoel van deze thesis was om inzicht te verwerven in de toxiciteit van enkelvoudige metalen (Zn, Cu en Cd) en te onderzoeken hoe deze toxiciteit verandert wanneer de enkelvoudige metalen gecombineerd worden in verschillende metaalmengsels. Hiervoor werden er meerdere eindpunten bestudeerd (apart en combineerd), gaande van het moleculair tot het populatie niveau. Er werden bovendien zowel korte termijn (24 h - 48 h) als lange termijn (10 - 12 dagen) studies uitgevoerd met verschillende blootstellingsconcentraties. Tot slot werden de biobeschikbaarheid en bioaccumulatie van de metalen bepaald om de geobserveerde toxische effecten te verklaren. Omwille van zijn goedkoop prijskaartje, snelle ontwikkeling, kleine lichaamslengte, snelle reproductie, korte levensduur en snelle opeenvolging van generaties, werd de bodemnematode *Caenorhabditis elegans* (Leung et al., 2008) gebruikt als testorganisme in deze doctoraatsstudie.

In **hoofdstuk 2** werden de korte termijn effecten van Zn, Cu and Cd (zowel apart als in combinatie) onderzocht op het individueel niveau (mortaliteit, gedrag en

chemosensatie) van *C. elegans*. De resultaten toonden aan dat Cu meer toxisch was dan Cd en Zn, wat geconcludeerd kon worden uit de hogere mortaliteit en sterker gereduceerde locomotie. De toxische effecten van metaalmengsels waren afhankelijk van de gebruikte metaalcombinaties. De mengsels CuCd, ZnCu en ZnCuCd veroorzaakten een hogere mortaliteit en sterker gereduceerde locomotie dan de overeenkomstige enkelvoudige metalen. In tegenstelling tot deze additieve en synergistische effecten, werd er een antagonistisch interactief effect waargenomen voor het ZnCd mengsel. Dit resultaat suggereert dat Zn het toxisch effect van Cd matigt. Er werden gelijkaardige trends waargenomen voor gedrag en mortaliteit, maar de verschillen waren meer uitgesproken voor gedrag. Hierdoor kan locomotie als een gevoelig subletaal eindpunt voorgesteld worden voor korte termijn studies (24 h of 48 h). Voor chemosensatie werden er enkel beperkte effecten waargenomen.

Lange termijn studies (10 tot 12 dagen) op het individuele en populatieniveau, beschreven in **hoofdstuk 3**, bevestigden dat metaaltoxiciteit zowel tijd- als concentratie afhankelijk is. Blootstelling aan de LC20 van Cu was bijvoorbeeld het meest toxisch na 24 h en 48 h, terwijl er na 10 dagen een sterkere populatie afname vastgesteld werd bij de LC20 van Cd. Bij een blootstelling aan de LC5 van Cu bleef de populatiegrootte echter gelijkaardig aan die van de controlegroep. Opnieuw werden er additieve of synergistische effecten waargenomen voor de mengsels ZnCu, CuCd en ZnCuCd. De resultaten toonden tevens aan dat het matigend effect van Zn op de Cd toxiciteit concentratie afhankelijk is: de Zn LC20 zorgde voor een daling van de toxiciteit, terwijl de Zn LC5 een te lage concentratie bleek om de toxiciteit van Cd te verminderen. Bovendien werd er eenzelfde trend voor populatiegrootte en lichaamsgrootte vastgesteld na een blootstelling aan LC20 concentraties. Een blootstelling aan LC5 concentraties zorgde voor minder uitgesproken effecten op de populatiegrootte, terwijl lichaamslengte niet werd beïnvloed. Tot slot bleek populatiegrootte een gevoeliger eindpunt te zijn dan lichaamslengte en was het concentratie-afhankelijk matigend effect van Zn op Cd toxiciteit meer uitgesproken voor populatiegrootte dan voor mortaliteit.

Vervolgens werd er in **hoofdstuk 4** onderzocht of de geobserveerde toxiciteit kan verklaard worden door verschillen in metaalaccumulatie (24 h) of metaalspeciatie (12 dagen). In een eerste deel werd er onderzocht of metalen worden opgenomen door *C. elegans* en of deze in competitie gaan om *C. elegans* binnen te dringen. In het tweede deel werd het effect van *E. coli* op de metaalspeciatie onderzocht door de concentratie aan vrije metaalionen en opgeloste metaalconcentraties te meten. Onze studie toonde aan dat, hoewel er minder Cu dan Cd en Zn nodig is om dezelfde 20% mortaliteit te veroorzaken, er minder vrije Cu ionen beschikbaar waren voor opname dan Cd en Zn ionen, maar dat er procentueel meer Cu werd opgenomen door de nematoden. Deze resultaten suggereren dat opnames via water en dus vrije metaalionen van belang zijn voor toxiciteit in *C. elegans*. Aangezien metaalspeciatie niet tijdsgebonden was en net als metaalaccumulatie niet verschilde tussen mengsels en overeenkomstige enkelvoudige metalen, kunnen er andere factoren belangrijk zijn in het voorspellen van toxiciteit van metaalmengsels.

Doordat inzichten in moleculaire mechanismen kunnen bijdragen tot het begrijpen van metaaltoxiciteit en moleculaire spelers aangewend kunnen worden om schadelijke effecten te voorspellen, werd er een differentiële proteomics studie uitgevoerd in **hoofdstuk 5**. Hoewel de gekozen concentratie, LC10, hoog genoeg was, konden er niet veel differentiële eiwitten geïdentificeerd worden na een blootstelling van 24 h. Aangezien er slechts 7 differentiële eiwitten werden gevonden (Cu:1; Cd:4; CuCd:2) konden er ook geen biomerkers voorgesteld worden. Het zou kunnen dat de proteomics benadering nog niet geschikt is voor het onderzoeken van metaaltoxiciteit op *C. elegans*, wat blijkt uit het lage aantal proteomics studies over metaaleffecten. Een andere mogelijke verklaring is dat *C. elegans* nog in staat was om zijn proteïne homeostase te bewaren. Tot slot werd er nog een peptidomics studie uitgevoerd om het peptidoom van niet-blootgestelde nematoden verder te ontrafelen. Er werden 51 endogene peptiden geïdentificeerd, waaronder 5 nieuwe 'neuropeptide-like protein peptides' (NQGAGSVSLDSLASLPMLRYamide, SLPIEYPDETVMYESRF, MYINPDYYYVEQLPTM, SSIMTDDVEPPQLLTRQL, SPIHGIWNNLPAPPQ) afkomstig van 5 nieuwe precursors (NLP-

53, NLP-54, NLP-55, NLP-56, and NLP-57), die gepubliceerd werden in de 'WormBase' databank.

We kunnen dus besluiten dat het onderzoek van metaalmengsels interessante inzichten biedt in de metaaltoxiciteit. Zo werden additieve, antagonistische en synergistische effecten waargenomen voor eindpunten gemeten op verschillende organisatieniveaus. Bovendien kunnen we concluderen dat metaaltoxiciteit tijds- en concentratieafhankelijk is voor zowel enkelvoudige metalen als mengsels, hoger is voor mengsels dan voor de overeenkomstige enkelvoudige metalen (behalve voor ZnCd, waarvan het antagonistisch effect afhangt van de Zn concentratie), meer uitgesproken is voor locomotie op korte termijn en voor populatiegrootte op lange termijn, voorspeld kan worden op basis van vrije metaalconcentraties van enkelvoudige metalen en tot slot nog niet gereflecteerd wordt op het proteoom niveau van *C. elegans*. De studie toonde ook aan dat bepaalde toxische effecten meer uitgesproken zijn op een bepaald organisatieniveau of eindpunt dan op een ander (vb. concentratieafhankelijk ZnCd antagonisme op populatiegrootte vs. mortaliteit), wat het belang van het bestuderen van verschillende eindpunten en organisatieniveaus duidelijk maakt. Het nut van lange termijn blootstelling werd duidelijk in hoofdstuk 3 voor het eindpunt populatiegrootte dat verschillende transgeneratieve effecten en effecten op de individuele levensgeschiedenis combineert, en waarbij duidelijke effecten pas merkbaar worden na 3-4 dagen wanneer een nieuwe generatie zou gevormd moeten worden. Meer studies zouden echter moeten volgen om de hiaten in de kennis van toxiciteit van metaalmengsels op te vullen, maar ook om metaalopname, bioaccumulatie, detoxificatie en opslag in specifieke organen en weefsels en metaalspeciatie beter te begrijpen. Aangezien de relatief hoge blootstellingsconcentraties in onze studie suggereren dat *C. elegans* weinig gevoelig is voor metaalblootstelling, is deze nematode misschien een interessant onderzoeksorganisme voor de studie naar resistentiemechanismen tegen metaaltoxiciteit.

Chapter 8

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Chapter 9

Curriculum vitae

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Education and professional experience

2014 - present	PhD student: *Toxic effects of single and combined metals at the individual and population level of C. elegans.* Promoters Prof. Dr. Ronny Blust, Dr. Steven J. Husson and Prof. Dr. Geert Baggerman University of Antwerp, Antwerp, Belgium
2013 - 2014	PhD student: *Scaling ecotoxicological effects across levels of organization: towards an ecologically relevant evaluation of mixed toxicity.* Promoter Prof. Dr. Gudrun De Boeck University of Antwerp, Antwerp, Belgium
2011 - 2013	Master of Science in Biology: Evolutionary and Behavioural Biology, great distinction University of Antwerp, Antwerp, Belgium
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Scientific communications

Papers

- **Moyson S**, Liew HJ, Diricx M, Sinha AK, Blust R, De Boeck G. 2015. The combined effect of hypoxia and nutritional status on metabolic and ionoregulatory responses of common carp (*Cyprinus carpio*). *Comparative Biochemistry and Physiology, Part A: Molecular and Integrative Physiology.* 179, 133-143.
- Husson S, **Moyson S**, Valkenborg D, Baggerman G, Mertens I. 2015. Proteomics applications in *Caenorhabditis elegans* research. *Biochemical and biophysical research communications* 468:4, 519-524.
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- **Moyson S**, Vissenberg K, Fransen E, Blust R, Husson SJ. 2018. Mixture effects of copper, cadmium and zinc on mortality and behavior of *C. elegans. Environmental Toxicology and Chemistry* 37:1, 145-149.
- **Moyson S,** Town RM, Vissenberg K, Blust R, Husson SJ. The effect of metal mixture composition on toxicity to *C. elegans* at individual and population levels. In preparation for submission to *PLoS ONE*.
- **Moyson S**, Town RM, Joosen S, Husson SJ, Blust R. 2018. The interplay between chemical speciation and physiology determines the bioaccumulation and toxicity of Cu^{2+} and Cd^{2+} to *C. elegans*. *Journal of Applied Toxicology*, 1-12**.**

Platform presentations

- **Moyson S**, Liew HJ, Diricx M, Sinha AK, Blust R, De Boeck G. 2014. The combined effect of hypoxia and nutritional status on metabolic and ionoregulatory responses of common carp (*Cyprinus carpio*). Annual Main Meeting 2014 Society of Experimental Biology, Manchester, UK. 1-4 July: 7.
- **Moyson S**, Baggerman G, Blust R, Husson S. 2017. Sensitivity of *Caenorhabditis elegans* to mixtures of zinc copper and cadmium. SETAC Europe 27th International Meeting, Brussels, BE. 7-11 May: 186.

Poster presentations

- Liew HJ, Fazio A, Van Dooren N, **Moyson S**, Delcroix A, Faggio C, De Boeck G. 2013. Kidney compensates ion losses in copper-exposed exercised shubunkin (*Carassius auratus auratus*). Annual Main Meeting 2013 Society of Experimental Biology, Valencia, Spain. 1-4 July: 95.
- **Moyson S**, Baggerman G, Blust R, Husson S. 2016. Modulatory toxicity effects of metal mixtures on chemosensation and mortality of *C. elegans*. SETAC Europe 26th International Meeting, Nantes, France. 22-26 May: 129.
- **Moyson S**, Baggerman G, Blust R, Husson SJ. 2016. Effects of copper, cadmium, zinc and their mixtures on survival and behaviour of the soil nematode *Caenorhabditis elegans*. BelTox Annual Meeting 2016, Louvain-La-Neuve, Belgium. 9 December: 22.
- **Moyson S**, Baggerman G, Blust R, Husson SJ. 2016. Effects of copper, cadmium, zinc and their mixtures on survival and behaviour of the soil nematode *Caenorhabditis elegans*. 23rd Zoology Congress, Antwerp, Belgium. 15-17 December: 147.
- **Moyson S**, Vissenberg K, Baggerman G, Blust R, Husson SJ. 2017. Mixtures of zinc, copper and cadmium cause different responses in *Caenorhabditis elegans*. Annual Main Meeting 2017 Society of Experimental Biology, Gothenburg, SWE. 3-6 July: 2.