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The combined effects of metal mixtures and natural stressors on aquatic invertebrates: Relating changes in metal accumulation to altered behavior and ecological effects

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De gecombineerde effecten van metaalmengsels en natuurlijke stressoren op aquatische ongewervelden: de relatie tussen veranderingen in metaalaccumulatie, gedrag en ecologische effecten

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Chapter 1.

General introduction

1.1. Metals in the aquatic environment

Metals are naturally present in the environment (Duffus, 2002). However, over the past centuries metal pollution has become a severe problem due to anthropogenic activities, endangering the health of humans and ecosystems. Activities such as large-scale metal mining, smelting, fossil-fuel combustion, municipal waste incineration and the use of metal containing pesticides and fertilizers have increased the emission rates of metals dramatically (Callender, 2003).

Metals, being elements, are not degraded by natural processes and are thus persistent. They can be divided into two groups: essential and non-essential metals. Non-essential metals, including cadmium and lead, are not required by organisms and need to be detoxified or excreted. We should note that cadmium has been shown to play a metabolic role in carbonic anhydrase in certain oceanic diatoms (Cullen et al., 1999), but is generally considered as non-essential for most organisms. Essential metals, on the other hand, are required for diverse biological functions of an organism, whose absence produces specific deficiency symptoms relieved only by that metal (Duffus, 2002). Copper, for example, is a functional part of several proteins responsible for, amongst others, electron transfer, energy production and regulation of transcription (e.g., haemocyanin, cytochrome c oxidase, Spl7; Festa and Thiele, 2011). Zinc is a key component of many enzymes including carbonic anhydrase (Rainbow, 2002). However, essential metals can become toxic when a threshold concentration is exceeded.

Aquatic organisms accumulate metals through diffusion (passive or using specific carrier proteins or channels) or ingestion (Rainbow and Black, 2005). In freshwater ecosystems, metals occur as a variety of organic and inorganic complexes. Generally, the uptake by organisms is related to the free metal ion activity (FIA). Metal speciation and bioavailability are influenced by a competition for metal ions between the biotic ligand and the other aqueous ligands, and the competition for the biotic ligand between the toxic metal ion and the other metal cations in solution (Di Toro et al.,

2001). Thus, environmental factors such as dissolved organic carbon, alkalinity and pH can change the uptake of metals (Bervoets and Blust, 1999; Erickson et al., 1996). For example, Ca^{2+} is known to compete for the same toxic action or transport sites as Cd^{2+} (Bervoets et al., 1995). Once taken up by organisms, these metals can move up the food chain resulting in biomagnification.

In contaminated aquatic ecosystems, metals often occur as mixtures. Interactions between metals can affect several processes that are important for the resultant toxicity. Firstly, one metal can change the bioavailability of the other: the ion speciation and competition for binding sites to organic matter in soil, sediments and the water phase can change free ion availability (Posthuma et al., 1997). Secondly, a metal can affect the uptake and the internal transport of others by, for example, competing for biological ligands or transport proteins (Niyogi et al., 2015). Lastly, metals can affect each other's metabolization rate (Cedergreen, 2014). Several models exist to predict mixture effects: the concentration addition model (CA) for chemicals with a similar mode of action and the independent action model (IA) for chemicals with dissimilar modes of action, both using the hazard of the individual chemicals (Altenburger et al., 2000). In the field of ecotoxicology, these models have been questioned. Several studies show more complex deviation patterns, namely dose-ratio dependent and dose level-dependent deviations (Banks et al., 2003; Jonker et al., 2005). It is possible for the same chemical to have a different mode of action dependent on the dose (McCarty and Borgert, 2006), which could, for example, result in different conclusions between chronic and acute studies of the same chemical mixtures. Additionally, the observed interactions can differ between different species (Norwood et al., 2003). Last, for many chemicals the modes of action are unknown. Cedergreen et al. (2008) used data of 98 different chemical mixtures to test the accuracy of both IA and CA. The analyses showed that neither of the models proved to be significantly better and that half of the experiments could not be correctly described with these two models. They concluded that the knowledge of the mode of action alone is insufficient to predict mixture toxicity effects on an organism level and

that secondary modes of action, uptake kinetics, transportation, metabolism, compartmentation and excretion of the chemicals are not considered by the models. Yet, all these factors can have potentially large impacts on the joint effects (Borgert et al., 2004). Considering these factors, accurate mixture modelling remains one of the biggest challenges in the field of ecotoxicology.

These metal interactions can result in synergistic effects ("more than additive", when the combined effects are greater than predicted by the concentration addition and independent action models), additive and antagonistic toxic effects ("less than additive", when the combined effects are smaller than predicted). In a review of metal mixture effects on aquatic biota by Norwood et al. (2003), the frequency with which antagonism, additivity and synergism was reported was respectively 43, 27 and 29%. Antagonism was thus the most prevalent interaction. Also a review by Vijver et al. (2011) found 51% of the responses to be antagonistic. Additionally, Cedergreen (2014) determined the frequency of synergy in 21 metal mixture studies and only found an occurrence of 3%, concluding that synergistic interactions between chemicals are rare and often occur at higher concentrations (in the mg/L range) than the concentrations normally found in metal polluted waters.

1.2. Natural stressors in the aquatic environment

Besides chemical stressors, organisms have to cope with a range of natural stressors, such as drought, oxygen depletion, pH and food fluctuations, parasitism, cold, heat, and predator stress. Both biotic and abiotic stressors can influence the physiology of organisms, which could result in an increased sensitivity to environmental stressors. In this thesis, we will discuss the effects of temperature stress as an abiotic stressor, and predator stress as a biotic stressor. Sokolova and Lannig (2008) found that temperature can severely affect the aerobic metabolic regulation of aquatic ectotherms (the energy demand, oxygen supply and mitochondrial function), resulting in an increased susceptibility to metal toxicity. Numerous studies can be found that observed a positive correlation between metal uptake and temperature. For example,

Baines et al. (2005) studied the mollusk *Mytilus edulis* and found an increased uptake of Cd, Co and Zn when temperature increased with 10 °C. Also Heugens et al. (2003) found an increased Cd accumulation at higher temperatures for *Daphnia magna*. Both temperature and chemical stress can result in lower mitochondrial efficiency, higher maintenance costs and an impaired oxygen uptake and delivery to the tissues, which can eventually lead to hypoxemia and energy deficiency. Therefore, exposure to one of these stressors may result in an increased sensitivity to the other.

Also predator stress, both visual cues and chemical cues, such as the predator's kairomones or the alarm cues of other prey animals, can increase the toxicity of chemicals. Slos and Stoks (2008) studied the damselfly larvae *Enallagma cyathigerum* in the presence of a predator and observed a higher oxygen consumption and a growth reduction, due to a decreased food assimilation efficiency. They linked this to a fight-or-flight response, which can induce glycolysis and lipolysis (McPeek et al., 2001). Furthermore, they found an increase of the stress protein hsp70, which was possibly induced as a response to the accelerated cellular metabolism due to predator stress and the costs necessary to maintain homeostasis. Janssens and Stoks (2013) investigated the effects of predator cues combined with pesticide stress for the same damselfly species. Both stressors reduced growth rate in an additive way and interacted synergistically for antioxidant defense and oxidative damage. Even more disturbing are the results of Relyea (2003) who studied the combined effects of predation and the pesticide carbaryl on six amphibian species and found that apparently safe concentrations became deadly when combined with predatory stress. For bullfrogs it even became up to 46 times more lethal. He hypothesized this synergy might be the result of the inhibition of acetylcholine esterase by both stressors. It should be noted that synergistic effects in nature might be weaker than when observed in the laboratory as stress levels fluctuate more rapidly or prey animals can move away from areas with high predator risk or chemical levels. On the other hand, because more than these two stressors are present in aquatic ecosystems, which could interact in an additive or synergistic way as well, they might be even more important

in nature. Interactions among multiple stressors can generate complex effects on ecosystems. Therefore, adequate environment risk assessment is essential.

1.3. Environmental monitoring and assessment

Ecological risk assessment of chemicals in the EU aims to protect ecosystems by deriving maximum concentrations which are not expected to cause negative ecological effects (EP, 2008; EP, 2013). These are called environmental quality standards (EQS). However, EQS are determined using classic laboratory toxicity tests. In these tests, the impact of a single chemical on test organisms is tested under constant, optimal conditions. This way, performance in the control treatments is optimized and the effects of the chemical are isolated. As discussed above, this fails to establish the actual impact of chemicals in nature. Heugens et al. (2001) found that, dependent on the effect parameter used, the toxicity under laboratory and field conditions differed with a factor of 2.6 to 130. The bioavailability in the field is usually lower than in the laboratory and organisms may adapt to chemical stress (Aiken et al., 2011; Klerks and Weis, 1987). However, throughout their lifetime, organisms cope with suboptimal conditions, facing environmental stressors, both biotic and abiotic. Toxicants rarely occur alone in aquatic ecosystems and mixture effects of other chemicals can influence the toxicity of each single chemical, of which synergistic interactions are of the greatest concern. These classic tests are also executed on the individual level, the representation of which for the entire population, community or ecosystem is questionable (Ferson et al., 1996). Additionally, in these tests the focus in many cases lies on mortality (determining the LC_{50}, the concentration that causes 50% of the test animals to die), while sublethal effects can severely impair fitness and are known to play a considerable role at much lower and therefore environmentally more relevant concentrations (Fleeger et al., 2003).

To account for this discrepancy between laboratory and field conditions, safety or uncertainty factors are used to extrapolate the results. Yet, the choice of these factors is based on little ecological evidence (Chapman et al., 1998). A possible advantage of

the safety factor is to reduce the probability of underestimating risk, as the current number and intensity of stressors on aquatic ecosystems are unprecedented in history (climate change, loss of biodiversity and habitat, anthropogenic pollution). For instance, the Living Planet Index shows a decline of 81% between 1970 and 2012 of freshwater populations (WWF, 2016). However, arbitrary safety factors might as well cause overly protective measures in risk assessment. While one might argue that the underestimation of risk is more dangerous, overestimation, on the other hand, is also economically undesirable as sometimes high costs are necessary to meet a 'good' environmental quality.

Because it is important to gather more knowledge about the way stressors interact, multiple stressor tests are gaining recognition in the field of ecotoxicology (Heugens et al., 2001; Sih et al., 2004). Jackson et al. (2016) performed a meta-analysis of multiple stressors on both chemical-natural stressor and natural-natural stressor pairs in freshwater ecosystems and found antagonistic interactions more frequently (41%) than synergistic (28%) and additive interactions (16%). However, Holmstrup et al. (2010) reviewed over 150 papers that studied the interactions between chemical and natural stressors, such as heat, cold, drought and oxygen depletion and found more than 50% of the studies to report synergism. Antagonism was less frequently reported. In conclusion, for a better protection of our environment it would be better for ecological monitoring to consider all stressors and their interactions at play.

1.4. Sublethal endpoints

The most common sublethal endpoints include growth, reproduction and behavior. Behavior can be described as the cumulative interaction of biotic and abiotic factors that represents the animal's response to internal (physiological) and external (environmental) factors that relates one animal to another (Grue et al., 2002). This makes it a very relevant endpoint in toxicity assessments. In the literature, nearly all metals have been reported to cause behavioral effects in aquatic organisms. These effects include altered orientation, behavioral avoidance, changed mate guarding

behavior, and reduced activities and swimming speeds (Boyd et al., 2002). Lopes et al. (2004) found that populations of *Daphnia longispina* avoided copper-exposed test chambers. Olfactory-mediated behaviors can also be impaired by metals, affecting the ability to detect chemosensory cues. Krång and Ekerholm (2006), for example, studied the effects of copper on male shore crabs and found a reduced pheromone perception. Pestana et al. (2007) observed significant reductions in feeding rate for two freshwater crustaceans when exposed to sublethal levels of cadmium and zinc.

Direct effects of toxicants, such as a reduced reproduction or increased mortality, reduce the abundance of a species. However, indirect effects that change biological interactions may also lead to an increased or decreased abundance of organisms and thereby change the community composition. For instance, chemicals can alter predator-prey relationships, resulting in trophic cascades. Firstly, metals could reduce predation rates (Ham et al., 1995). Strong top-down effects can be observed when a predator or grazer is more sensitive to the contaminant than its prey. Jak et al. (1996) found that toxic effects on zooplankton resulted in reduced grazing and consequentially caused elevated phytoplankton biomass. Secondly, bottom-up effects are observed when the susceptibility of a prey species to predation is increased or decreased (Taylor et al., 1995; Schulz and Dabrowski, 2001). There could also be an effect on the competition between species, when one species is more sensitive than the other. In conclusion, behavioral effects can cause significant changes in the ecosystem. Therefore, in the laboratory experiments of this dissertation, we studied the feeding rate and activity of our test organism *Asellus aquaticus*.

Besides these behavioral endpoints, we investigated the growth rate and the oxygen consumption rate as well. Metal toxicity can result in reduced growth rates by allocation of energy to defense mechanisms (Maltby, 1999). Also a lower respiration rate is often observed, as metals can disrupt the gill function of crustaceans (Spicer and Weber, 1991). Natural stressors might enhance this metal toxicity. For example, predator stress can reduce growth rate as well (Slos and Stoks, 2008). On the other hand, they can also cause the opposite effect of metals, e.g., predator cues and

increased temperatures are known to increase the oxygen consumption of aquatic organisms (Slos and Stoks, 2008; Sokolova and Lannig, 2008).

1.5. Micro- and mesocosms

To bridge the gap between field and laboratory conditions, experiments can be performed in a micro- or mesocosm, i.e. a controllable semi-natural system (with algae, invertebrates, macrophytes…) that can be indoor or outdoor. Microcosms comprise all ecosystems up to 1 m^3 while mesocosm are from 1 up to even 500 m^3 (Boyle and Fairchild, 1997; Spivak et al., 2011). Such systems can be artificial ponds or rivers and make experiments under more ecologically-relevant scenarios possible. They allow for some control, as different species can be introduced in relative abundances based on field conditions. Furthermore, they can be exposed to a desired amount of metals and specific metal mixtures. Still, a more natural situation is created as ecological processes, such as photosynthesis, respiration and decomposition, take place. Additionally, the treatments can be exposed to varying weather conditions and exchange of species is possible.

There are some disadvantages to the use of micro-and mesocosms, such as the high variability between replicates that increases over time (Figure 1), caused by factors such as complex interactions between organisms (e.g., predation, competition). Furthermore, as micro- and mesocosms are large and require large amounts of water and/or sediment, they create relatively large amounts of waste. Moreover, they can be expensive and are difficult to replicate (Jokiel et al., 2008). However, they provide greater ecological realism than single species tests as they can be conducted at a larger spatiotemporal scale and the effects of chemicals can be studied on individuals, populations and the whole aquatic invertebrate community.

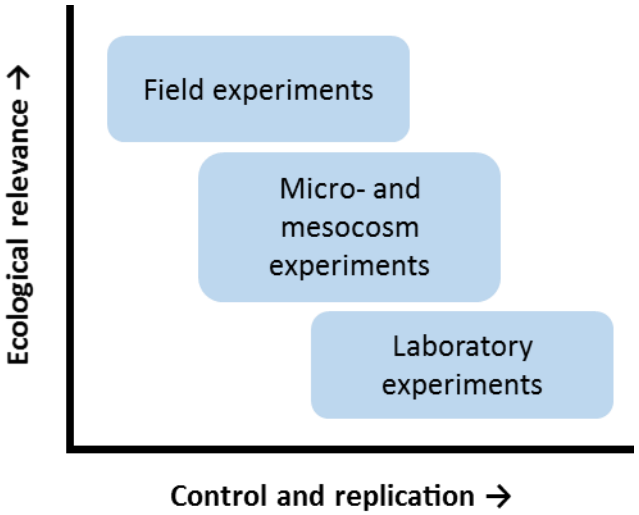

Figure 1: A schematic representation of the relationships between the ecological relevance and the replicability of field, mesocosm and laboratory experiment.

Only multispecies experiments can provide demonstrations of e.g. indirect trophic-level effects, compensatory shifts within a trophic level, or responses to chemicals within the context of seasonal patterns that modify water chemistry and birth and death rates of populations (Taub, 1997).

1.6. Aim and outline of the thesis

The aim of this study was to investigate the combined effects of metal mixtures and the natural stressors, temperature and predation pressure, on sublethal endpoints of the aquatic invertebrate *Asellus aquaticus*. Additionally, effects on a whole aquatic community were assessed in small artificial ecosystems. It was anticipated that by combining metal mixtures with natural stressors and assessing the effects on different levels of biological organization, the present thesis may contribute to the development of environmentally-relevant risk assessment.

After a general introduction about chemical and natural stressors in the aquatic environment and the problematics of current environmental risk assessment in **Chapter 1,** we investigated the toxicity of the metals Cd, Cu and Pb as single stressors (**Chapter 2**). This experiment was conducted on the freshwater isopod *A. aquaticus* to

determine the LC_{50}s, LC_{20}s and LC_{10}s for these animals. Moreover, the change of these LC_xs was plotted over time and the incipient LC_x (the concentration below which x% of exposed individuals will live indefinitely relative to the lethal effects of the toxicant) was calculated in order to determine the ideal exposure duration of future experiments.

The first part of this thesis consisted of laboratory experiments. This way we could study effects on the individual level under controlled conditions. In **Chapter 3**, we focused on both the acute and sublethal mixture effects of these metals. By exposing *A. aquaticus* to the single metals as well as the binary and tertiary mixtures, we learned more about the uptake rate, the mortality rate, the growth and the energy reserves (glycogen, lipid and protein reserves). In **Chapters 4 and 5**, we expanded on this research by adding a natural stressor, respectively predator and temperature stress. Environmentally-relevant concentrations were chosen to study sublethal effects. We linked the effects on respiration rate, accumulation, growth rate, and, as behavioral parameters, feeding rate and activity to the metal body concentrations and the free ion activities (FIA) in the water. Interesting interactions between both the metals as well as the metals and the natural stressors were discovered.

The second part of this thesis concerned a microcosm experiment (**Chapter 6**). To expand our study from the individual level to the population and the community level, an experiment was carried out in a mesocosm facility. We investigated the effects of the single metals Cd, Cu and Pb and their tertiary mixture on a simplified macroinvertebrate community. In each bucket, we placed *Asellus aquaticus*, *Daphnia magna, Chironomus riparius* (midge larvae), *Physella acuta* (Mollusca), *Elodea nuttallii* (macrophytes) and *Raphidocelis subcapitata* (algae). The effects of the metal mixtures and natural stressors were examined after 4 and 8 weeks, on the individual level (total metal accumulation, shoot and root length), the population level (species abundances, biomass) and the community structure (Shannon-Wiener index).

Finally, we summarized and discussed the general results of this thesis in **Chapter 7**. In our conclusion, we offered some future perspectives for this research area and environmental risk assessment in general.

Chapter 2.

How LC_x changes over time: Toxicity of Cd, Cu and Pb to the freshwater isopod *Asellus aquaticus*

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Abstract

Metal pollution is a serious threat to environmental health. While the aquatic isopod *Asellus aquaticus* L. (Isopoda) is an important decomposer of freshwater ecosystems, very little research has reported its long-term or incipient lethal concentrations for metals. Moreover, the lethal concentrations (LC_{50}s) that can be found in the literature are often based on unmeasured concentrations, which could lead to a severe underestimation of the actual toxicity. In the present exploratory study, the 1-, 4-, 7-, and 14-d LC_{10}, LC_{20}, and LC_{50} values of copper, cadmium, and lead for adult *A. aquaticus* were determined. The LC_x values were calculated using the nominal concentrations, the effective concentrations, and the free ion activities. Incipient lethal values were determined as well. In general, surprisingly lower lethal concentrations were found than in other studies. Furthermore, the present study shows that lethal concentrations based on free ion activities were generally much lower than nominal and effective concentrations. Although almost all LC values were higher than the environmental quality standards (EQS), the Cu 14-d LC_{10} and several (incipient) Pb LC_{10} and LC_{20} values, all calculated with free ion activities, were lower than the EQS. We conclude that lethal or effective concentrations based on free ion activities provide a more environmentally-realistic basis for policy making.

2.1. Introduction

Metals endanger aquatic life, with anthropogenic pollution playing an important role by introducing metals into nature through, for example, emission at smelting activities, domestic wastewater, and metal-containing pesticides and fertilizers. These trace metals can be divided into two groups. The first group is composed of the non-essential metals, which disrupt normal metabolic processes. Cadmium, for example, is known to compete with zinc and calcium, inhibiting uptake (Nath et al., 1984; Qiu et al., 2005; Rainbow and Black, 2005), whereas lead disrupts calcium and sodium homeostasis (Grosell et al., 2006). The second group is the essential metals, which are necessary for diverse biological functions (e.g., Cu). However, essential metals can become toxic as well when a certain threshold is exceeded, which varies depending on the water chemistry, the species, body mass, etc. (Erickson et al., 1996; Grosell et al., 2002). For instance, while many proteins are copper dependent, Cu can also inhibit sodium uptake and influence chloride absorption (Grosell et al., 2002; Grosell and Wood, 2002). Therefore, it is important to know at which concentration these metals affect the health of aquatic organisms.

The crustacean *Asellus aquaticus* L. (Isopoda) is an epibenthic detritivore and can be found throughout the northern hemisphere (Bloor, 2010). These isopods are an important component of freshwater ecosystems because they shred leaf material and so provide energy for other organisms, but they are also a food source for both invertebrate predators and fish (Bundschuh et al., 2012; Rask and Hiisivuori, 1985). *Asellus aquaticus* has a 1-yr life cycle and is often used as a test species in ex situ and in situ metal toxicity studies (Bloor, 2010; De Jonge et al., 2010; Migliore and De Nicola Giudici, 1990). This isopod is considered to be relatively tolerant to organically polluted waters (Aston and Milner, 1980; Bloor, 2010), but can be sensitive to trace metals (Migliore and De Nicola Giudici, 1990). Yet, very few studies have investigated long-term lethal effects of metals on *A. aquaticus* (De Nicola Giudici et al., 1987; De Nicola Giudici et al., 1988). Some studies have reported median lethal concentrations (LC_{50})

based on unmeasured concentrations, which could lead to large deviations from the real-life situation. Martin and Holdich (1986), for example, calculated LC values based on nominal concentrations. However, they observed significant precipitation of several metals and emphasized the need to consider this factor in interpreting their results. Sometimes measured concentrations were used (Ham et al., 1995; Van Ginneken et al., 2015), but to our knowledge no studies have used free ion activities of dissolved metals to determine LC values. Because free ions can bind to biological receptors and therefore represent the chemical reactivity, they are generally considered one of the best predictors of toxic effects (Renner, 1997). In fact, the free ion activity model (FIAM) or the biotic ligand model (BLM), an extension of the FIAM, are commonly used for the determination of LC_{50}s (Di Toro et al., 2001; Santore et al., 2001). The US Environmental Protection Agency (EPA) has incorporated the BLM into its aquatic life freshwater quality criteria for copper and is investigating its use for other metals (US EPA, 2007).

In the present study, we determined changes in LC_{10}s, LC_{20}s, and LC_{50}s over time for Cd, Cu, and Pb in adult *A. aquaticus* L. The LC values were calculated using the nominal concentrations, the effective concentrations, and the free ion activities. The aim of the present study was to compare these data with LC values found in the literature, which are mostly based on nominal values and could therefore lead to an underestimation of the actual impact of these metals. In addition, no LC_{10} or LC_{20} values for any metal can be found for *A. aquaticus* in either the USEPA ECOTOX knowledge base (US EPA, 2016) or the literature. Chronic effects such as growth or behavior are more vulnerable endpoints, and thus the LC_{10} and LC_{20} values are important in their evaluation.

2.2. Material and Methods

2.2.1. Test animals

Individuals of *A. aquaticus* were taken from the Laakbeek in Lille, Belgium (51° 13’ 51.5” N 4° 50’ 52.4” E; basin of the Scheldt River) with a pond net (500-µm mesh, 200 x 300-mm frame, and 500-mm bag depth) fitted to a 1.5-m handle. Lille is a small town

in Flanders, located in a region of extensive agriculture. Metal concentrations of the Laakbeek were measured in 2014 before the start of this experiment. We found very low concentrations of copper (4.58 ± 0.21 µg/L), cadmium, and lead (both <0.1 µg/L). The isopods were acclimated for at least 2 weeks in 20-L glass aquaria filled with moderately hard water (US EPA, 2002) under controlled light conditions (16:8 h light:dark photoperiod) and temperature (15 ± 1 °C) in a climate chamber, model WT15'/+5DU-WB (Weiss Technik). Exposures occurred in the same chamber under the same conditions. As food for *A. aquaticus*, we dried alder leaves (*Alnus glutinosa*) and submerged them in a bucket of aerated water from the Laakbeek, where they were conditioned for at least 6 d (Bloor, 2010). During acclimation, the organisms were fed ad libitum.

2.2.2. Experimental design

During a 14-d laboratory experiment *A. aquaticus* was exposed in polypropylene containers to 5 concentrations of Cd, Cu and Pb (Table 1). Stock solutions were prepared in US EPA moderately hard water (US EPA, 2002) with analytical grade salts of cadmium chloride hydrate ($CdCl_2.H_2O$), copper chloride dihydrate ($CuCl_2.2H_2O$) and lead nitrate ($Pb(NO_3)_2$). All metals were purchased from Merck. Stock solutions were further diluted to reach the desired concentrations. Next, 500 mL was poured into each polypropylene container and a net (1 mm mesh) was placed on the bottom of each container to ease the removal of the isopods. A control treatment with moderately hard water was added. Experiments were executed in triplicate. The containers were left to equilibrate for 24 h.

Ten individuals of *A. aquaticus* (size range: 7–9 mm) were introduced into each container. General water characteristics were monitored daily (240 measurements): T = 15 ± 1 °C; dissolved oxygen = 8.68 ± 0.03 mg/L; pH = 7.72 ± 0.03; electrical conductivity = 321 ± 2 µS/cm; and dissolved organic carbon (DOC) = 5.94 ± 0.13 mg/L. Because no food was provided in the containers, the animals were taken out of the containers after 7 d using the net and fed for 4 h with the conditioned alder leaves to

avoid cannibalism (Bloor, 2010). Every day, mortality was assessed and dead animals (no response to gentle prodding) were removed from the solution.

At days 0, 1, 4, 7, and 14, water samples (50 mL) were taken from all containers with a syringe and filtered through a 0.20-mm filter (Chromafil). At days 0 and 1, the metal concentrations were determined for only one of the replicates because no large variation was expected. At days 4, 7, and 14, samples of all replicates were analyzed. After taking the water samples, 50 mL of medium of the respective metal concentrations was added again. Part of the filtered water was acidified to pH 2 with HNO_3 (69%) to quantify DOC using a TOC analyzer (TOC-VCPH, Shimadzu). Next, the remaining filtered water was acidified to 1% with trace-metal–grade HNO_3 (69%) to determine the trace metals and major ions with an inductively coupled plasma–optic emission spectrometer (ThermoScientific, ICAP 6300 Duo). Standard reference material 1640a (National Institute of Standards and Technology) was used throughout the analyses to assess the instrument performance. Concentrations of the major ions were 16.8 ± 0.1 mg/L Ca; 3.12 ± 0.06 mg/L K; 13.5 ± 0.1 mg/L Mg; and 29.3 ± 0.2 mg/L Na. Water samples were stored at –20 °C until measured. As can be seen in Table 1, the copper and lead salts caused solubility issues that led to lower than expected effective concentrations. The free ion activities for the different metals were computed using the Windermere Humic Aqueous Model 6.0.13 (Natural Environment Research Council) in which 100% of the DOC was entered as fulvic acids (Christensen et al., 1999). Part of the DOC, however, may have been present as humic acids and other hydrophobic or hydrophilic fractions. Therefore, this might not incorporate all effects of DOC composition on metal toxicity (Leenheer and Croué., 2003).

Mortality data were corrected according to the following formula (Schneider-Orelli, 1947): Corrected % = ((% mortality in treated asellids - % mortality in control asellids)/(100 - % mortality in control asellids))×100.

Table 1: Overview of nominal concentrations, effective concentrations and free ion activities (FIAs) on day 0; control values are the means of 3 determinations (± SE).

Treatment	Nominal (µg/L)	Effective (µg/L)			FIAs (µg/L)		
	Metal	Cd	Cu	Pb	Cd	Cu	Pb
Control	<0.1	<0.1	1.74 ± 0.21	<0.1	<0.1	<0.1	<0.1
Cd A	0.1	<0.1	2.21	<0.1	<0.1	<0.1	<0.1
Cd B	1	1.02	1.70	<0.1	0.33	<0.1	<0.1
Cd C	10	11.3	2.00	<0.1	4.41	<0.1	<0.1
Cd D	60	69.9	1.44	<0.1	34.9	<0.1	<0.1
Cd E	180	182	1.41	<0.1	103	<0.1	<0.1
Cu A	50	<0.1	17.8	<0.1	<0.1	<0.1	<0.1
Cu B	500	<0.1	360	<0.1	<0.1	44.7	<0.1
Cu C	1000	<0.1	816	<0.1	<0.1	213	<0.1
Cu D	5000	<0.1	2496	<0.1	<0.1	877	<0.1
Cu E	50 000	<0.1	20350	<0.1	<0.1	8790	<0.1
Pb A	20	<0.1	1.78	0.71	<0.1	<0.1	<0.1
Pb B	200	<0.1	1.91	25.6	<0.1	<0.1	<0.1
Pb C	2000	<0.1	1.70	110	<0.1	<0.1	0.36
Pb D	20 000	<0.1	15.0	358	<0.1	<0.1	4.67
Pb E	200 000	<0.1	17.8	37616	<0.1	6.94	18982

The LC_{10}, LC_{20}, and LC_{50} values were calculated for the nominal, effective, and free ion concentrations of the metals using the package drc (Ritz and Streibig, 2005) of the statistical program R 3.1.2 (R Development Core Team, 2011). We used the concentrations measured on days 0, 1, 4, 7, and 14 to determine their respective LC values. The changes in all LC_xs were plotted in time. These toxicity curves approached asymptotes (parallel to the time axis), which are called the incipient LC_x values. This is the concentration below which 100 – x% would live indefinitely relative to the lethal effects of the toxicant (Moriarty, 2003) and can be used, for instance, to determine the ideal exposure duration of these isopods. The incipient LC_xs were determined using the one-phase decay equation in GraphPad Prism 7.00 for Windows (GraphPad Software).

2.3. Results

No isopods in the control treatment group died during the first 96 h. Mortality reached 6.67% after 7 d and 13.3% after 14 d. Because of the long exposure duration, this can be considered a chronic toxicity study, which is only invalid if mortality in the control treatments exceeds 20% (Cooney, 2003). Cumulative survival data of the different exposure treatments are shown in Figure 1. While several treatments of Cd and Cu were lethal, for Pb only the highest concentration caused severe mortality. This could be explained by the low effective concentrations and free ion activities compared with the nominal concentrations (Table 1). Consequently, estimations of LC values for Pb were less reliable, and no standard error could be calculated in some cases.

The LC_{10} versus time profile of the three metals showed a steep decline until day 4, after which the LC_{10}s slowly decreased until a plateau was reached (Figure 2). The 1-, 4-, 7, and 14-d LC_{10}s for Cd were 177, 6.64, 3.49, and 0.91 µg/L for the nominal concentrations; 183, 5.83, 3.43, and 0.80 µg/L for the effective concentrations; and 131, 4.87, 4.99, and 1.31 µg/L for the free ion activities.

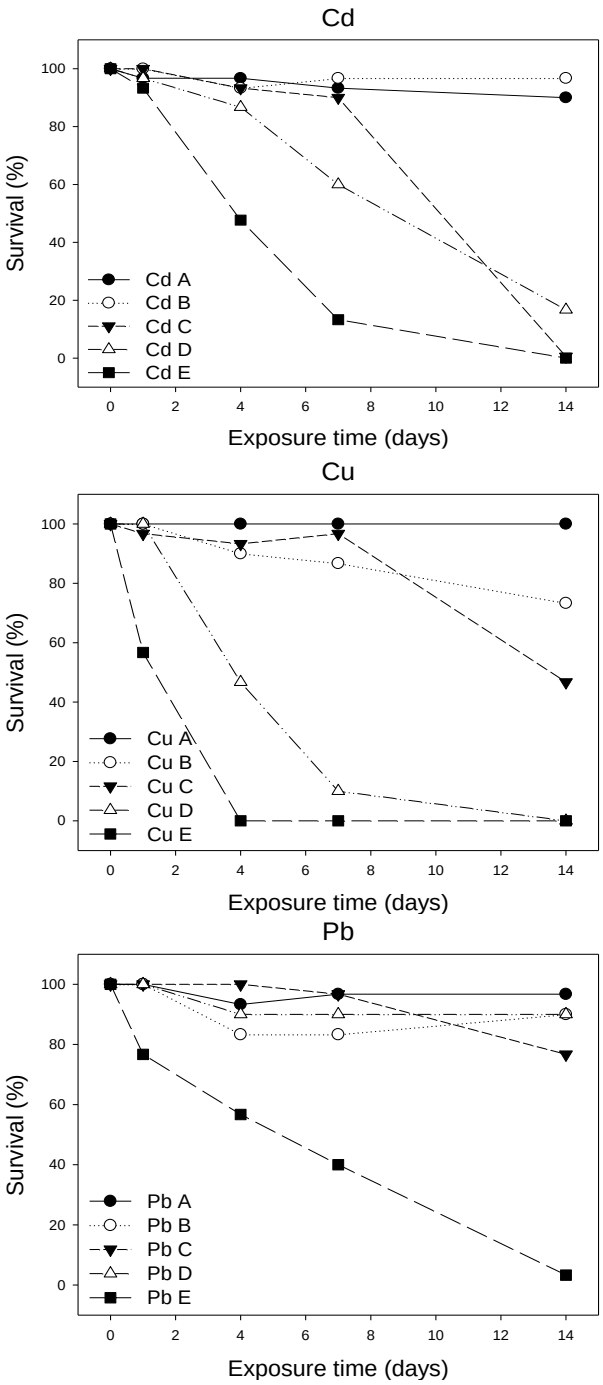

Figure 1: Survival of *A. aquaticus* as a function of days of metal exposure. Observed mortalities were corrected with the Schneider-Orelli formula (N = 480).

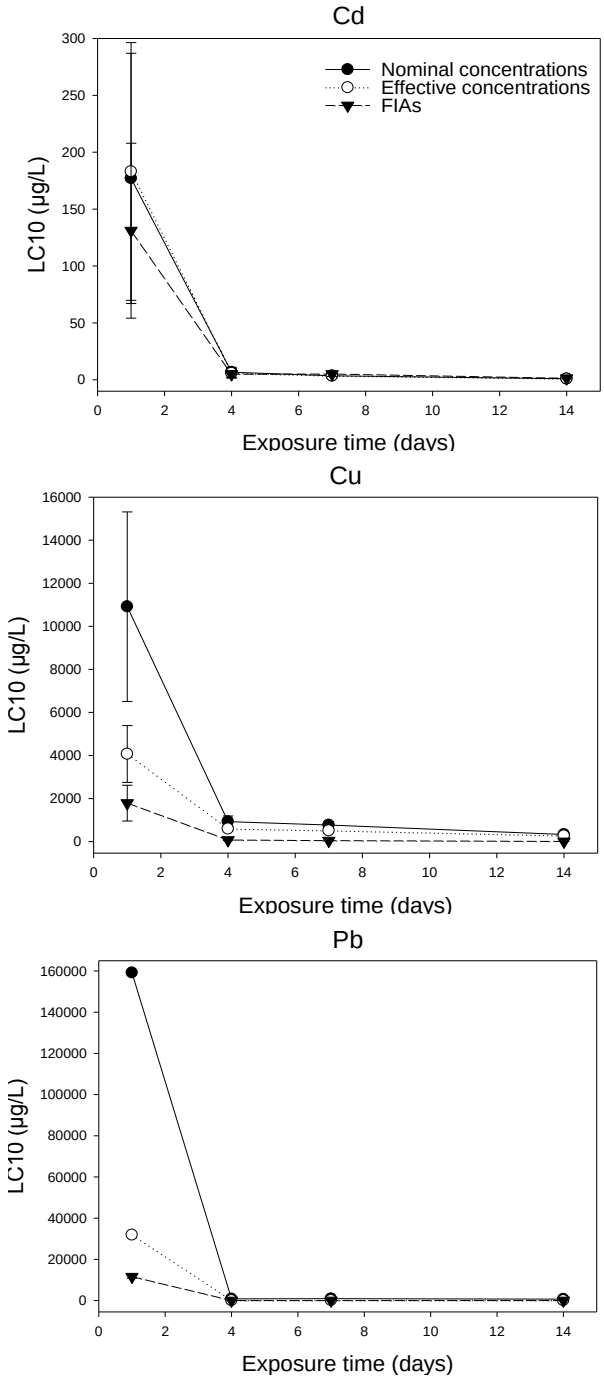

Figure 2: Comparison of LC_{10}s (± SE) calculated with the nominal concentrations, effective concentrations and free ion activities (FIAs) for Cd, Cu and Pb (N = 4 time points).

The 1-, 4-, 7-, and 14-d LC_{10}s of copper were 10914, 932, 773, and 332 µg/L for the nominal concentrations; 4070, 593, 512, and 271 µg/L for the effective concentrations; and 1788, 71.4, 44.8, and 6.16 µg/L for the free ion activities. Lastly, we calculated the following 1-, 4-, 7-, and 14-d LC_{10}s for Pb: 159 157, 820, 932, and 677 µg/L for the nominal concentrations; 31 887, 54.6, 97.4, and 49.7 µg/L for the effective concentrations; and 11 556, 0.06, 0.07, and 0.04 µg/L for the free ion activities. The LC_{20}s and LC_{50}s for Cd, Cu, and Pb all followed the same decline as can be seen for the LC_{10}s (Table 2). A clear difference was found between the nominal concentrations of Cu and Pb resulting in the highest LC_xs, while the free ion activities produced the lowest LC_xs. For example, the Cu 14-d LC_{50} of 853 µg/L based on the nominal concentrations is more than 30 times higher than the LC_{50} for the free ion activities. For Cd, on the other hand, the LC_xs of nominal concentrations, effective concentrations, and even the free ion activities after day 1, were similar.

Estimations of the incipient LC values were determined and again, calculating the incipient concentrations of Pb proved difficult (Table 3). In general, incipient LC values calculated with the free ion activities were lower. Almost all incipient concentrations were reached at 14 d. Only the incipient Cd LC_{50} of 5.27 µg/L calculated with free ion activity was not reached at day 14, but was very similar to the LC_{50} of 6.38 µg/L.

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Table 2: Overview of the LC_{20}s and LC_{50}s for each exposure time (± SE), calculated with the nominal concentrations, effective concentrations and free ion activities (FIAs) (N = 480). NA = "Not Available", could not be calculated by R.

		Nominal concentrations (µg/L)		Effective concentrations (µg/L)		FIAs (µg/L)	
Metal	**Day**	**LC_{20}**	**LC_{50}**	**LC_{20}**	**LC_{50}**	**LC_{20}**	**LC_{50}**
Cd	**1**	634 ± 386	5617 ± 4880	670 ± 395	6149 ± 5076	256 ± 233	803 ± 1288
	4	29.6 ± 13.4	380 ± 256	28.3 ± 13.3	420 ± 314	17.4 ± 7.3	153 ± 93
	7	9.83 ± 3.81	57.6 ± 16.9	10.1 ± 4.2	64.1 ± 19.3	9.88 ± 3.07	31.8 ± 6.7
	14	2.25 ± 0.85	10.5 ± 2.9	2.06 ± 0.85	10.4 ± 2.9	2.35 ± 0.72	6.38 ± 1.35
Cu	**1**	21174 ± 6341	65744 ± 6341	6853 ± 1655	16703 ± 4334	3751 ± 1281	13319 ± 4985
	4	1613 ± 357	4123 ± 825	800 ± 94	1333 ± 148	120 ± 24	291 ± 57
	7	1124 ± 194	2133 ± 338	662 ± 82	1026 ± 84	69.6 ± 14.9	147 ± 21
	14	471 ± 80	853 ± 103	351 ± 49	547 ± 48	10.6 ± 3.2	26.5 ± 4.8
Pb	**1**	191276 ± 47506	261902 ± NA	36246 ± NA	45122 ± NA	23686 ± 10418	80785 ± NA
	4	10538 ± 7379	829640 ± 646300	767 ± 556	70263 ± 62668	2.76 ± 3.30	2002 ± 1218
	7	7504 ± 4604	265170 ± 206020	602 ± 326	13562 ± 9330	1.93 ± 2.02	600 ± 383
	14	2733 ± 1320	29679 ± 12252	130 ± 43	677 ± 255	0.31 ± 0.22	9.13 ± 7.39

Table 3: Overview of the incipient LC_{10}s, LC_{20}s and LC_{50}s (± SE), calculated with the nominal concentrations, effective concentrations and free ion activities (FIAs) (N = 4 time points). NA = "Not Available", could not be calculated by R.

	Nominal concentrations (µg/L)			Effective concentrations (µg/L)			FIAs (µg/L)		
Metal	**LC_{10}**	**LC_{20}**	**LC_{50}**	**LC_{10}**	**LC_{20}**	**LC_{50}**	**LC_{10}**	**LC_{20}**	**LC_{50}**
Cd	2.11 ± 1.26	5.43 ± 3.46	21.7 ± 12.9	2.05 ± 1.31	5.56 ± 3.76	23.4 ± 15.0	3.11 ± 1.85	5.65 ± 3.66	5.27 ± 0.93
Cu	536 ± 222	765 ± 322	1408 ± 608	377 ± 122	491 ± 156	767 ± 238	24.3 ± 19.2	38.5 ± 29.3	84.4 ± 59.8
Pb	805 ± 128	4959 ± 2347	NA	67.21 ± NA	361 ± 236	NA	0.05 ± 0.01	1.12 ± 0.81	280 ± 283

2.4. Discussion

The present study was conducted to establish more accurate LC values for *A. aquaticus*. No studies could be found that calculated LC_{50} values based on free ion activities. Moreover, neither LC_{10} nor LC_{20} values were available in the literature, making it difficult to infer metal concentrations for chronic toxicity testing.

Because no LC_{10}s or LC_{20}s were found, only LC_{50} values can be compared (Table 4). Cadmium LC_{50}s in the present study are generally much lower than values found in the literature. Braginsky and Shcherban (1979) found a more than 10-fold higher 1-d LC_{50} (78 450 µg/L). The 4-d LC_{50} determined by Martin and Holdich (1986) was 1320 µg/L. This is three times higher than the one we calculated based on the effective concentrations. However, the metal concentrations were not measured in either study. Green et al. (1986) used measured concentrations and found a 4-d LC_{50} of 600 µg/L, similar to the 4-d LC_{50} of the present study. A study by Van Ginneken et al. (2015), set in the same conditions as the present study except for a higher exposure temperature, found a 10-d LC_{50} of 34.9 µg/L, comparable to our 7-d LC_{50}. The 15-d LC_{50} of 61 µg/L reported by Ham et al. (1995), in contrast, is most similar to the 7-d LC_{50} of the present study, even though Cd concentrations were also measured. For Cu, Martin and Holdich (1986) found a 4-d LC_{50} of 9210 µg/L, which is 2 times higher than the nominal 4-d LC_{50} of the present study and 7 times higher than the 4-d LC_{50} based on effective concentrations. De Nicola Giudici et al. (1988) found a very similar 7-d LC_{50} of 1000 µg/L, although this was established for juveniles, which are known to be generally more sensitive than adults to metals (Migliore and De Nicola Giudici, 1990; Naylor et al., 1990). For adult females however, they found a 14-d LC_{50} value of 5000 µg/L, which is 5-fold higher than the nominal 14-d LC_{50} of the present study. Yet again, metal concentrations of these studies were unmeasured. However, the 10-d LC_{50} of 2195 µg/L calculated by Van Ginneken et al. (2015) is also 4 times higher than the 14-d LC_{50} of the present study, even though both values were calculated using measured concentrations. Lead LC_{50}s for *A. aquaticus* in the literature are scarce. Van Ginneken

et al. (2015) found a 10-d LC_{50} of 443 µg/L calculated with the measured concentrations, which is comparable to the 14-d LC_{50} in the present study (677 µg/L for the effective concentrations). The Pb 4-d LC_{50} of the effective concentrations is similar to the 4-d LC_{50} of 64 100 µg/L determined by Martin and Holdich (1986).

The LC_{50}s of the present study were generally lower than the values found in the literature. This could be explained by interpopulation differences in sensitivity. It is possible that the animals from some studies were sampled from (heavily) metal-contaminated sites, and therefore were less sensitive than the isopods used in the present study because of genetic adaptation (Brown, 1976; Klerks and Weis, 1987). Still, the LC_{50} values of these studies differed greatly from the present study and are possibly not explained by the former reason alone. We also observed that many of the discussed studies used unmeasured metal concentrations. It is very likely that these were higher than the actual concentrations because of complex forming and precipitation. A similar pattern can be seen for the Cu and Pb data in the present study. When examining the lethal concentrations based on free ion activities, we found even lower values than for the nominal or measured concentrations, again especially for Cu and Pb. This can be explained by WHAM 6, which assumes a larger affinity of Cu and Pb to dissolved organic matter than Cd (Neagoe et al., 2012). The results in the present study demonstrate that, although often described as a tolerant species, these isopods are more sensitive than would be expected from the available LC_{50} data in the literature.

In the present study, mortality was used as an endpoint, but this requires high contaminant concentrations. In the natural environment, exposure to lower concentrations of metals will result in the manifestation of various sublethal effects, which can have large implications for the health of the entire ecosystem. For example, metals can cause oxidative stress in cells, which is linked to many fitness-related traits, such as growth, ability to combat disease and foraging behavior (Blockwell et al., 1998; Monaghan et al., 2009; Pestana et al., 2007). This could cause reduced reproduction rates, reduced detritus-processing rates and an increased risk of death, which may in

turn lead to an altered ecosystem structure and function (Maltby et al., 2002; Monaghan et al., 2009). Further study is required to investigate the sublethal effects of these LC values.

Other limitations of the present exploratory study need to be acknowledged, most notably that no metal body concentrations or metabolically available metals were measured (Rainbow, 2002; Wallace et al., 2003). Because part of the accumulated metals is biologically detoxified by metallothioneins and metal-rich granules, metabolically available metal could be considered a more reliable indicator of metal toxicity than body concentrations or free ion activities. A better understanding of subcellular partitioning is thus imperative to improve predictions of metal toxicity in nature (Wang and Rainbow, 2007).

Incipient LC values were determined as well. Because these are not influenced by time of exposure, they predict the effects of long-term exposure more accurately than short-term LC_xs (Pesch et al., 1979). Furthermore, the time at which acute lethality ceases can differ between different species and even contaminants. Therefore, knowing the incipient values allows for a meaningful comparison (Sprague, 1969). Other researchers, on the other hand, argue that these can only be compared with similar LC values and that ecological significance in terms of population effects is often unclear (Moriarty, 2003; Traas and van Leeuwen, 2007). Despite these valid arguments, the incipient concentrations of the present study are still useful for the design of future experiments, for example, to explore to which concentrations these isopods can be exposed when one is examining chronic effects or to investigate when a longer exposure time will not lead to any more discernible changes in effect, thus determining the optimal duration for future experiments. Because almost all incipient concentrations were reached at 14 d, we established that *A. aquaticus* should be exposed to Cu, Cd and Pb for longer than 7 d and preferably 14 d.

Table 4: Summary of LC_{50}s and experimental details: measured metal concentrations, length of *Asellus aquaticus*, temperature, pH & water hardness.

Metal	Endpoint	LC (µg/L)	Measured	Length (mm)	T°	pH	Hardness	References
Cd	4-day LC_{50}	1320	No	7	13	6.75	Soft	Martin and Holdich (1986)
	10-day LC_{50}	34.9	Yes	9.43 ± 0.17	20	8.04	Moderately hard	Van Ginneken et al. (2015)
	15-day LC_{50}	61	Yes	4 - 6	16	6.9	Moderately hard	Ham et al. (1995)
	1-day LC_{50}	78450	No	-	15	-	-	Braginsky and Shcherban (1979)
	4-day LC_{50}	600	Yes	8 - 10	11	7.83	Moderately hard	Green et al. (1986)
Cu	7-day LC_{50}	1000	No	1	18	7.2	Very hard	De Nicola Giudici et al. (1988)
	14-day LC_{50}	5000	No	6 - 8	18	7.2	Very hard	De Nicola Giudici et al. (1988)
	4-day LC_{50}	9210	No	7	13	6.75	Soft	Martin and Holdich (1986)
	10-day LC_{50}	2195	Yes	9.43 ± 0.17	20	8.04	Moderately hard	Van Ginneken et al. (2015)
Pb	4-day LC_{50}	64100	No	7	13	6.75	Soft	Martin and Holdich (1986)
	10-day LC_{50}	443	Yes	9.43 ± 0.17	20	8.04	Moderately hard	Van Ginneken et al. (2015)

All incipient values were higher than the environmental quality standards (EQS) except for the Pb LC_{10} and LC_{20} of the free ion activities, which were lower than its EQS (for Flanders, Belgium: Cu = 7 µg/L (VLAREM II, 2015); for Europe: Cd = 0.15 µg/L (EP, 2008); and for Pb = 1.2 µg/L (EP, 2013)). The Cu 14-d LC_{10} calculated with free ion activities was below its EQS as well. It is important to note that the EQS for Cu and Cd are based on dissolved metal concentrations. Although water hardness was taken into account for cadmium, other factors such as pH or dissolved organic matter were not considered. For some metals like Pb and Ni, however, an $EQS_{bioavailable}$ was developed, which refers to the bioavailable fraction of the dissolved concentration as determined by the physicochemical characteristics of the water (EP, 2013). While the Pb 4-, 7-, and 14-d LC_{10} and 14-d LC_{20} values were also lower than the EQS, these values must be interpreted carefully because mortality in most treatments was very low.

The present study shows that lethal or effective concentrations based on free ion activities could be considerably lower than values calculated using theoretical concentrations. While free ion activity plays an important role, it is not the sole factor determining toxicity. Interactions with the biotic ligand, i.e. the site of toxic action, should also be considered. Some improvements to the EQS for a few metals have already been made by the development of an $EQS_{bioavailable}$ (EP, 2013). However, it is imperative that further effort be made to incorporate the BLM concept or other bioavailability models in environmental policy making.

2.5. Acknowledgments

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Chapter 3.

Uptake and toxicity of Cd, Cu and Pb mixtures in the isopod *Asellus aquaticus* from waterborne exposure

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Abstract

The present study evaluated interactive effects of waterborne Cd, Cu and Pb mixtures on metal uptake rates in the isopod *Asellus aquaticus* and related this to mixture toxicity. Secondly, it was assessed whether observed mixture effects were better related to isopod body concentrations compared to exposure concentrations. Isopods were exposed for 10 days to single, binary and tertiary mixtures including five different concentrations of Cd (0.107 to 277 µg/L), Cu (3.35 to 2117 µg/L) and Pb (0.782 to 443 µg/L). Mortality was assessed every day while isopod body concentrations, growth (biomass) and energy reserves (glycogen, lipid and protein reserves) were assessed at the end of the experiment. Synergistic interactions of combined Cd and Pb exposure on Cd and Pb uptake as well as on growth rates and mortality rates were observed. No mixture interactions of Cu on Cd or Pb uptake (and vice versa), nor on toxicity endpoints were observed. All toxicity endpoints were related to body concentrations. However, mixture effects disappeared when growth and mortality rates were expressed on body concentrations instead of exposure concentrations. By combining information of mixture effects on metal uptake with mixture toxicity data, the present study provides more insight in the way metal mixtures interfere with aquatic organisms and how they can induce toxic effects.

3.1. Introduction

Metal pollution still poses a threat to aquatic ecosystems all over the world and managing ecological risks of metal-contaminated systems remains an important challenge. In contaminated environments trace metals most often occur in different mixtures, in which metals can strongly interfere with each other, producing antagonistic, synergistic or additive toxic effects (Norwood et al., 2003; Borgmann et al., 2008).

Besides effects on metal speciation in the medium (Norwood et al., 2013), exposure to metal mixtures encloses different possible biochemical interactions. Firstly, metal mixtures can interact at biotic ligands resulting in inhibition or increase of metal uptake and bioaccumulation (Norwood et al., 2007; Borgmann et al., 2008; Komjarova and Blust, 2008). Secondly, metal mixtures can induce toxicity responses, both directly at the site of toxic action and indirectly by interfering with internal pathways (e.g. detoxification processes) (Norwood et al., 2003; Vijver et al., 2011). Recent reviews on effects of metal mixtures have shown a large variety in combined effects, which can differ depending on the metal combinations and their concentrations, the used species and the measured endpoints (Weltje, 1998; Norwood et al., 2003; Vijver et al., 2011). For example, the study of Franklin et al. (2002) observed additive responses for bioaccumulation, but synergistic responses for growth after exposing the freshwater alga *Chlorella* sp. to Cd–Cu mixtures. The latter illustrates the need for metal mixture studies combining interactions at metal uptake and bioaccumulation with toxicity data, in order to reveal at which level interactions of metal mixtures are occurring. To date, only few studies have related metal mixture influences on uptake and accumulation to toxicity effects, especially for aquatic invertebrates (but see Franklin et al., 2002 and Abboud and Wilkinson, 2013 for algae; and Birceanu et al., 2008 for fish). Furthermore, mortality has been most frequently used as an endpoint to study metal mixture interactions, which is rather drastic and requires high exposure concentrations, while sublethal endpoints such as growth remain only poorly documented (Vijver et al., 2011).

The aim of the present study was first to evaluate interactive effects of waterborne Cd, Cu and Pb mixtures on metal uptake rates in the isopod *Asellus aquaticus* (L.) and relate these to mixture effects on sublethal endpoints as well as mortality. Secondly, it was assessed whether observed mixture effects were better related to exposure concentrations or to measured isopod body concentrations. Recently, Norwood et al. (2013) developed an effects addition model based on body concentrations to predict chronic mortality of metal mixtures in the amphipod *Hyalella azteca*. In fact, invertebrate body concentrations have been shown to be a useful predictor of metal mixture effects since they account for metal interactions that can occur at biotic ligands and they integrate multiple routes of exposures as well as pulse exposure events (Borgmann et al., 2008; Norwood et al., 2013; De Jonge et al., 2013). Therefore, we hypothesize that body concentrations will be a better predictor of mixture effects than exposure concentrations. Cadmium, Cu and Pb were chosen since they generally differ in uptake mechanism for aquatic organisms; i.e. Cd^{2+} is known to be a Ca^{2+} uptake inhibitor (Niyogi and Wood, 2004; Rainbow and Black, 2005), Cu^{2+} is a known Na^{+} uptake inhibitor (De Schamphelaere and Janssen, 2002; Grosell and Wood, 2002) and Pb^{2+} has been observed to inhibit both Ca^{2+} and Na^{+} uptake pathways (Rogers and Wood, 2004; Rogers et al., 2005). The isopod crustacean *A. aquaticus* is an important decomposer of organic material and can be found worldwide in diverse freshwater ecosystems of the temperate region. *Asellus aquaticus* goes through five marsupial stages of development. As they moult, their width and length increase (Bloor, 2010). Sexual maturity is reached in 46-60 days at 15°C when body length is 3.5 to 4.0 mm (Marcus et al., 1978). This species is known to accumulate trace metals both from waterborne and dietary sources (van Hattum et al., 1989; De Jonge et al., 2012) and has been frequently used in ecotoxicological assays (e.g. van Hattum et al., 1989; Bloor and Banks, 2006; De Jonge et al., 2012).

3.2. Material and Methods

3.2.1. Experimental design and water chemistry

A 10-day lab experiment was executed using the isopod *A. aquaticus*. All exposures occurred in acid-washed (1% HCl) polypropylene containers (125 mL) under controlled temperature (20 ± 1 °C) and light conditions (photoperiod of 16 h light and 8 h dark) in a climate chamber type WT15'/+5DU-WB (Weiss Technik, Reiskirchen-Lindenstruth, Germany).

Exposure concentrations were based on single metal toxicity data of *A. aquaticus* or related species from literature data, i.e. for Cd: 10-day LC_{50} of 54 µg/L (*A. aquaticus*; Ham et al., 1995), for Cu: 4-day LC_{50} of 650 µg/L (*A. meridianus*; Brown, 1976) and for Pb: 2-day LC_{50} of 280 µg/L (*A. meridianus*; Brown, 1976). Stock solutions of each metal were freshly prepared with analytical grade salts of cadmium chloride hydrate ($CdCl_2 \cdot H_2O$), copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) and lead chloride ($PbCl_2$) (all metal salts were purchased from VWRInt., Leuven, Belgium). Ultra-pure water (Milli-Q; Millipore, MA, USA) was used for the preparation of the stock solutions. Starting from the stock solution, for each metal five single metal exposure concentrations were prepared in 100 mL reconstituted freshwater (EPA medium-hard water; US EPA, 2002), resulting in total dissolved concentrations (analytically verified, see below) ranging from 24.9 to 277 µg/L for Cd, 151 to 2117 µg/L for Cu and 15.1 to 443 µg/L for Pb (Table 1). Maximal concentrations used in the present study are relatively high, although they can be encountered in metal-contaminated surface waters (e.g. Bryan and Gibbs, 1983; Bervoets et al., 2005; Stockdale et al., 2010; De Jonge et al., 2010, 2013). Mixture treatments were prepared by taking 50% of the single metal concentrations for the binary mixtures (Cd + Cu; Cd + Pb and Cu + Pb) (ray design with 1:1 concentration ratio) and 25% of the single metal concentrations for the tertiary mixture (Cd + Cu + Pb), resulting in four mixture treatments per metal (one single, two binary and one tertiary), each consisting of five different concentrations and one control.

Table 1: Overview of (a) exposure concentrations of the single metal treatments; (b) exposure concentrations of the binary and tertiary mixtures; and (c) water chemistry at the beginning of the experiment (day 0). Trace metal concentrations (Cd, Cu and Pb) of the control samples are presented as means (± standard error) of four determinations. Water chemistry measurements are presented as means (± standard error) of 72 (T, O_2, pH and EC; measured per exposure container) and three determinations (Ca, K, Mg, Na and DOC; measured in exposure medium before metal spiking), respectively. Water hardness (expressed as mg/L $CaCO_3$) was calculated according to the formula: 2.5 Ca + 4.1 Mg.

(a)

Treatment	Cd (µg/L)	Cu (µg/L)	Pb (µg/L)
Control	0.107 ± 0.034	3.35 ± 0.61	0.782 ± 0.264
Cd 1	24.9	4.61	<0.1
Cd 2	47.3	2.33	<0.1
Cd 3	87.1	2.35	<0.1
Cd 4	155	2.38	<0.1
Cd 5	277	1.31	<0.1
Cu 1	<0.1	151	<0.1
Cu 2	<0.1	296	<0.1
Cu 3	<0.1	904	<0.1
Cu 4	<0.1	1,481	<0.1
Cu 5	<0.1	2,117	<0.1
Pb 1	<0.1	2.42	15.1
Pb 2	<0.1	2.66	31.1
Pb 3	<0.1	2.55	74.7
Pb 4	<0.1	2.57	203
Pb 5	<0.1	5.12	443

(b)

Treatment	Cd (µg/L)	Cu (µg/L)	Pb (µg/L)
Cd + Cu 1	13.1	77.7	<0.1
Cd + Cu 2	24.5	148	<0.1
Cd + Cu 3	51.9	555	<0.1
Cd + Cu 4	76.6	1,005	<0.1
Cd + Cu 5	128	1,602	<0.1
Cd + Pb 1	13.5	4.66	10.7
Cd + Pb 2	28.9	6.23	21.7
Cd + Pb 3	57.2	4.55	49.1
Cd + Pb 4	69.6	4.44	84.6
Cd + Pb 5	123	5.20	250
Cu + Pb 1	<0.1	86.9	9.39
Cu + Pb 2	<0.1	168	14.7
Cu + Pb 3	<0.1	459	34.6
Cu + Pb 4	<0.1	1,128	104
Cu + Pb 5	<0.1	1,615	219
Cd + Cu + Pb 1	6.04	50.8	4.28
Cd + Cu + Pb 2	11.9	102	3.62
Cd + Cu + Pb 3	34.0	461	21.2
Cd + Cu + Pb 4	42.1	718	62.9
Cd + Cu + Pb 5	64.1	996	121

(c)

T (°C)	20 ± 1
Dissolved oxygen (mg/L)	8.23 ± 0.02
pH	8.04 ± 0.03
Electrical conductivity (µS/cm)	295 ± 5
DOC (mg/L)	0.83 ± 0.03
Ca (mg/L)	27.3 ± 1.2
K (mg/L)	3.09 ± 0.20
Mg (mg/L)	12.0 ± 2.4
Na (mg/L)	48.3 ± 4.6
Hardness (mg/L)	117 ± 10

Individuals of *A. aquaticus* were purchased from Blades Biological Ltd (Edenbridge, Kent, UK). Prior to exposure, all organisms were acclimatized for four weeks in acid-washed aquaria containing medium-hard EPA water and ad libitum amounts of food (alder leaves, *Alnus glutinosa*). At the start of the experiment, three individuals of equal length (9.43 ± 0.17 mm) were introduced in each container filled with 100 mL test medium. Organisms were separated by a three-part polypropylene septum to avoid cannibalism (Bloor and Banks, 2006). No food was provided throughout the experiment. Experiments were executed in duplicate. Mortality and growth were assessed for all six organisms from both replicate chambers per treatment. Metal body concentrations and energy reserves, however, were each assessed for three organisms from different replicate chambers. General water characteristics (dissolved oxygen, pH and electrical conductivity) in all experimental containers were monitored every working day. At the beginning (day 0) and end (day 10) of the experiment, water samples were taken from all containers with a syringe (10 mL), filtered through a 0.20 µm cellulose acetate filter (Schleicher & Schuell MicroScience GmbH, Dassel, Germany) and acidified to 1% HNO_3. Trace metals (total dissolved Cd, Cu, Pb and major ions Na, K, Mg and Ca) were analyzed using an inductively coupled plasma optic emission spectrometer (ICP-OES; Thermo scientific, ICAP 6300 Duo, Waltham, MA, USA). Dissolved organic carbon (DOC) was quantified using a TOC-analyzer (TOC-VCPH, Shimadzu Corporation, Kyoto, Japan). Analytical results regarding exposure concentrations and water chemistry are presented in Table 1.

3.2.2. Determination of body concentrations

Metal body concentrations and sublethal toxicity endpoints (growth and energy reserves) were measured at the end of the experiment (day 10). However, due to early mortality in both the Cd single and Cd+ Pb treatments, Cd and Pb body concentrations, growth and energy reserves were determined in animals that had died after seven days in the 87.1 and 155 µg/L single Cd concentration and the 57.2 µg/L Cd + 49.1 µg/L Pb concentration, and after four days in the 277 µg/L Cd concentration and both the 69.6

µg/L Cd + 84.6 µg/L Pb and the 123 µg/L Cd + 250 µg/L Pb concentration. Per treatment and concentration three individual organisms were rinsed with Milli-Q, wiped dry and placed in polypropylene vials. Five empty vials were included to be used as process blank samples together with five samples of certified reference mussel material (CRM278R) of the Community Bureau of Reference (European Union, Brussels, Belgium). Samples were dried for 48 h at 60 °C in a laboratory furnace. Next, they were cooled down under vacuum conditions using a desiccator and they were weighed on a Sartorius SE2 ultra microbalance (accuracy of 0.1 µg). Afterwards, samples were digested for 1 h at 110 °C in a trace-metal-grade HNO_3 (69%) — high-purity H_2O_2 (29%) solution (5:1; v/v) using a hot block (Environmental Express, Charleston, SC, USA). Digested samples were diluted three times and total Cd, Cu and Pb body concentrations were measured using a quadrupole inductively coupled plasma mass spectrometer (ICP-MS; 7700×, Agilent Technologies, Santa Clara, CA, USA). Metal concentrations in the process blank samples were all below quantification limits (< 0.1 µg/L) and the recoveries from the reference samples were consistently within 10% of the certified values for all three metals. Isopod body concentrations are expressed as µg/g dry weight. The dry weight/wet weight ratio was found to be 5.7 ± 0.5%. To obtain metal uptake rate constants and to compare them between the mixture treatments, the experimental data were fitted to a linear function using SigmaPlot (Version 12.0; Systat, Chicago, IL, USA): $[Me]_{body} = k_u [Me]_{water} t + [Me]_{bodybackground.}$, where $[Me]_{body}$ and $[Me]_{water}$ represent the metal concentrations in the isopods (µg/g dw) and in the water (µg/L) respectively, k_u is the uptake rate constant (L/g/day), t is exposure time (days) and $[Me]_{bodybackground}$ is the metal concentration (µg/g dw) already present in the organism at the start of exposure (background concentration) (Komjarova and Blust, 2008).

3.2.3. Toxicity endpoint assessment

The following toxicity endpoints were assessed: mortality after 10 days, mortality rate, growth and energy reserves (glycogen, lipid and protein reserves). Mortality

(expressed as % from total) was checked on seven time points (day 0, 1, 4, 7, 8, 9 and 10). Animals that were completely immobile and did not respond to prodding were considered dead and were removed from the test solution. Mortality rate (%/day) per exposure concentration was calculated as the linear fit of % mortality versus time (days) per concentration. Concentration-dependent mortality relationships for each of the three metals were calculated by regressing the daily mortality rates (i.e. the slope of the cumulative mortality percentage vs. time) versus the exposure-water concentration or the whole-body concentration of the metal (expressed in units of % L/µg/day or % g/µg/day). When possible, LC_{50} values (µg/L) were calculated, representing the concentration at which 50% mortality was observed.

To quantify isopod growth, pictures of all the exposed organisms were taken using a digital camera, which was placed perpendicular to the exposed organisms. Pictures were taken at day 0 and day 10, unless mortality occurred before the end of the experiment, which was the case for the single Cd and the binary Cd + Pb treatment (see results on growth and mortality). Isopod length (mm) was measured directly from the pictures using the image processing software ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA). Measured lengths were converted to dry weight (mg) by using the formula of Graça et al. (1993), who experimentally determined the following length-dry weight relationship for *A. aquaticus* (R^2 = 0.944; n = 97): ln(dry weight) = 2.71 ln(length) − 4.58. Finally, growth was expressed as the difference in dry weight (mg) between individuals at day 10 and day 0. Concentration-dependent growth relationships for each of the three metals were calculated by regressing the daily growth rates versus the exposure-water concentration or the whole-body concentration of the metal and were expressed as (mg dw)(L)(μg^{-1} metal)(day^{-1}) or (mg dw)^2(μg^{-1} metal)(day^{-1}).

To determine glycogen, lipid and protein concentrations, tissues of three individual isopods were homogenized on ice in 800 µL Milli-Q. Total glycogen content was determined with Anthrone reagent (Roe and Dailey, 1966). Absorption was measured

at 630 nm with bovine liver glycogen (Merck, Darmstadt, Germany) as standard. Total lipids were extracted following the method of Bligh and Dyer (1959). Absorbance was measured at 405 nm using tripalmitin (Sigma, Saint Louis, MO, USA) as standard. The total protein content was determined using Bradford's reagent (five times diluted; Bio-Rad, Belgium) (Bradford, 1976). The absorbance was measured at 590 nm using bovine serum albumin (BSA; Sigma) as standard. Concentration-dependent glycogen, lipid and protein storage/decrease relationships for each of the three metals were calculated by regressing the daily glycogen, lipid or protein storage/decrease rates versus the exposure-water concentration or the whole-body concentration of the metal (expressed as (mg glycogen/lipid/protein)(L)(g^{-1} ww)(μg^{-1} metal)(day^{-1}) or (mg glycogen/lipid/protein)(g dw)(g^{-1} ww)(μg^{-1} metal)(day^{-1})).

3.2.4. Statistics and mixture effects analyses

All data were tested for normality with the Shapiro–Wilk test and for homoscedasticity by the Levene median test. In case of a non-normal distribution, data were log-transformed before further statistical analysis. All endpoints (bioaccumulation, mortality, growth and energy reserves) were analyzed using two-way analysis of covariance (ANCOVA), with exposure or body concentrations taken as continuous variable and mixture treatment as categorical variable. Effects of combined metal exposures on metal uptake rates as well as on toxicity endpoints (calculated mortality rates, growth rates and glycogen/lipid/protein storage/decrease rates) were assessed using two different approaches: i.e. 1) interpretation of the interaction term and differences between slopes in the ANCOVA model, and 2) using the conceptual model of Independent Action (IA) to validate observed mixture effects. This mixture model assumes different modes of actions of the pollutants assessed. According to the model of IA, mixture effects are calculated using the formula: $E_{mix} = 1 - \prod^{i} (1 - E_i)$, where E_{mix} represents the metal mixture effect and E_i represents the effect of each metal alone (Bliss, 1939; Meyer et al., 2015). Significant differences between calculated and observed mixture effects were tested using a

Student's t-test. If observed effects were significantly stronger compared to the predicted effects, the interaction was considered as synergistic, whereas an antagonistic interaction was indicated by a significantly weaker observed effect compared to the predicted one. ANCOVA was executed using the package aov of the statistical software R (version 2.15.2). Tukey's HSD tests were performed to assess differences in slopes between mixture treatments using the package lm (R development core team, 2011). To estimate LC_{50} values, dose–response curves were fitted using a four-parameter log-logistic model. This was performed using SigmaPlot version 11.0 (Systat Software, Inc., San Jose California USA), which was also used for all other statistical analyses. Statistical significance was assumed for $p < 0.05$.

3.3. Results

3.3.1. Metal uptake rates

Cadmium body concentrations ranged from 3.48 ± 1.43 µg/g dw (control) to 821 ± 273 µg/g dw (Cd + Pb treatment) (Fig. 1). Following the results of the ANCOVA, Cd body concentrations significantly increased with increasing exposure concentration and a significant interaction between Cd exposure and treatment was found ($F = 12.0$; $p < 0.001$), indicating differences in slopes (k_u) of the linear regression between $[Cd]_{dissolved}$ and $[Cd]_{body}$ between the mixture treatments (Table 2). Calculated k_u appeared to be significantly higher in the Cd + Pb treatment compared to all other Cd treatments (Cd + Pb vs Cd single: $t = 5.49$; $p < 0.001$). The latter was supported by significantly greater uptake than predicted by the IA model ($t = -2.52$; $p = 0.031$).

Copper body concentrations ranged from 153 ± 49 µg/g dw (control) to 2188 ± 872 µg/g dw (Cu + Pb treatment) (Fig. 1). Copper body concentrations significantly increased with increasing exposure concentration. However, no significant differences in k_u between mixture treatments were observed (Table 2).

Lead body concentrations ranged from 3.24 ± 1.16 µg/g dw (control) to 2456 ± 309 µg/g dw (Pb + Cd treatment) (Fig. 1). Lead body concentrations significantly increased

with increasing exposure concentration and the calculated Pb k_u was significantly higher in the Pb + Cd treatment compared to all other Pb-treatments (significant ANCOVA interaction: $F = 22.7$; $p < 0.001$; Pb vs Pb + Cd: $t = -6.69$; $p < 0.001$) (Table 2). The latter was supported by significantly greater uptake than predicted by the IA model ($t = -4.45$; $p = 0.001$).

Table 2: Metal uptake rates from water per mixture treatment. Average k_u values and standard errors (n = 6) are presented, together with model diagnostics (R^2 for the ANCOVA model). Superscript letters indicate statistical differences ($p < 0.05$) between metal uptake rates.

Treatment	Mixture with	k_u (L g^{-1} d^{-1})	R^2
Cd	-	0.255 ± 0.022[a]	92.8%
	Cu	0.147 ± 0.028[a]	
	Pb	0.729 ± 0.187[b]	
	Cu + Pb	0.325 ± 0.096[a]	
Cu	-	0.071 ± 0.010[a]	71.1%
	Cd	0.055 ± 0.029[a]	
	Pb	0.107 ± 0.014[a]	
	Cd + Pb	0.078 ± 0.061[a]	
Pb	-	0.481 ± 0.032[a]	96.5%
	Cu	0.225 ± 0.033[a]	
	Cd	1.003 ± 0.113[b]	
	Cu + Cd	0.328 ± 0.083[a]	

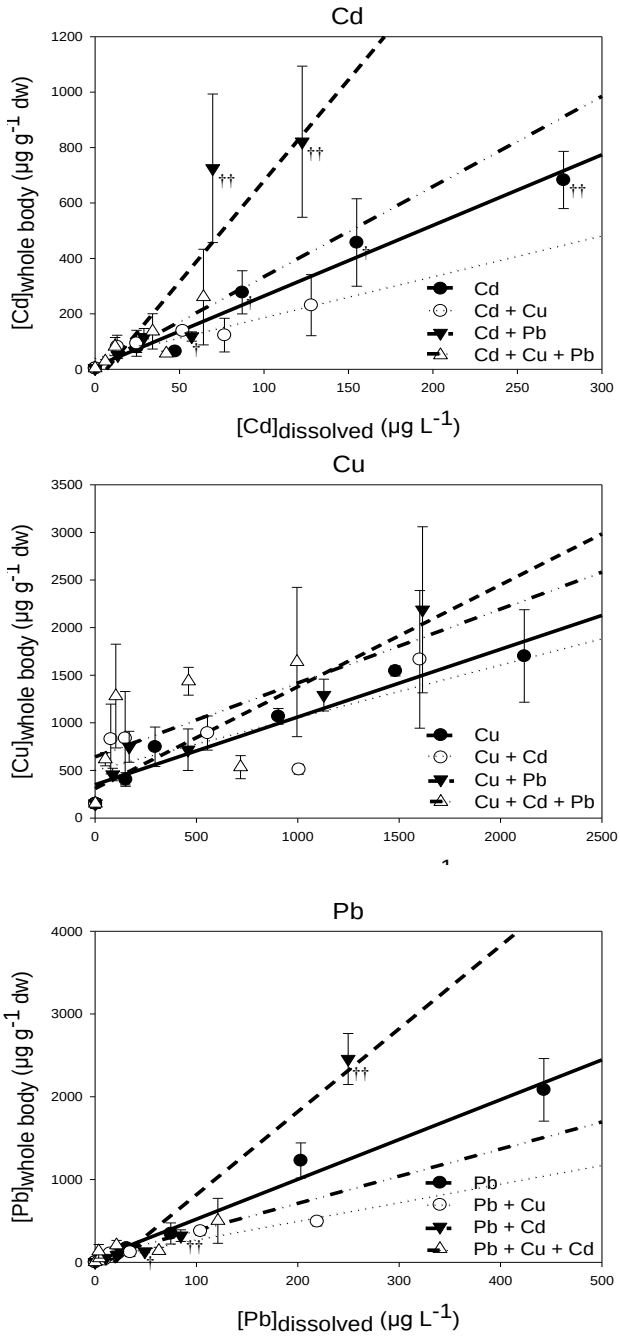


Figure 1: Metal body burdens of *A. aquaticus* after 10 days in function of exposure concentration (total dissolved) per mixture treatment. Average whole-body concentrations (n = 3) and standard errors are presented. †: Body concentrations were measured after seven days; ††: Body concentrations were measured after four days. Slopes (k_u) per treatment and model diagnostics are presented in Table 2.

3.3.2. Toxicity endpoints

3.3.2.1. Mortality

After 10 days of exposure, 100% mortality was only observed in the single Cd and Cd + Pb treatments. Maximum mortality observed in the other treatments was 83.3% for the Cd + Cu, 66.7% for the single Cu, 50% for the single Pb and 33.3% for the Cu + Pb and the tertiary treatments (see also Fig. S1). Only for Cd and Cu, significant LC_{50} values could be calculated: i.e. 34.9 ± 7.3 µg/L for Cd (R^2 = 0.978; p = 0.041) and 2195 ± 13 µg/L for Cu (R^2 = 0.824; p < 0.001). For Pb, LC_{50} values can be set at 443 ± 27 µg/L, since 50% mortality was observed at this concentration. Mortality rates based on % mortality per day of the different concentrations per treatment ranged from 0 (observed in several treatments including the control) to 12.2 ± 1.8 %/day (Cd + Pb) (Fig. 2; see also Fig. S2).

For all metals mortality rates significantly increased with increasing exposure concentration (Fig. 2). Only for Pb a significant ANCOVA interaction (F = 5.84; p = 0.007) was found, indicating significantly higher mortality rates in the Pb + Cd treatment compared to the single Pb (t = −2.44; p = 0.027). However, no significant interaction was found when compared to the Pb + Cu treatment and the tertiary mixture. The difference in mortality rate between the single Pb and Pb + Cd treatment was supported by significantly higher mortality than predicted by the IA model (t = −2.46; p = 0.034). No significant ANCOVA interactions were observed for the Cd (F = 2.75; p = 0.077) and Cu (F = 1.96; p = 0.161) treatments (Table 3).

Table 3: Mortality rates of *A. aquaticus* per mixture treatment. Mortality rates were calculated based on total dissolved metal concentrations (% L μg^{-1} d^{-1}; Fig.2) and based on body concentrations (% g μg^{-1} d^{-1}). Average mortality rates and standard errors are presented (n = 6 per mixture treatment), together with model diagnostics (R^2 for the ANCOVA model). Superscript letters indicate statistical differences between mortality rates.

Treatment	Mixture with	Mortality rate (% L μg^{-1} d^{-1})	R^2	Mortality rate (% g μg^{-1} d^{-1})	R^2
Cd	-	0.026 ± 0.015[a]	70.3%	0.012 ± 0.005[a]	58.2%
	Cu	0.064 ± 0.019[a]		0.012 ± 0.007[a]	
	Pb	0.109 ± 0.036[a]		0.037 ± 0.015[a]	
	Cu + Pb	0.038 ± 0.021[a]		0.007 ± 0.007[a]	
Cu	-	0.002 ± 0.001[a]	69.0%	0.003 ± 0.002[a]	41.2%
	Cd	0.001 ± 0.001[a]		0.001 ± 0.001[a]	
	Pb	0.005 ± 0.001[a]		0.003 ± 0.003[a]	
	Cd + Pb	0.002 ± 0.001[a]		0.001 ± 0.001[a]	
Pb	-	0.008 ± 0.004[a]	71.1%	0.001 ± 0.001[a]	71.9%
	Cu	0.010 ± 0.008[a]		0.004 ± 0.003[a]	
	Cd	0.040 ± 0.024[b]		0.003 ± 0.003[a]	
	Cu + Cd	0.018 ± 0.011[ab]		0.004 ± 0.003[a]	

Mortality rates expressed as a function of body concentrations significantly increased with increasing Cd and Pb exposure. However, this was not observed for Cu. No significant mixture effects were observed for any of the treatments. Models expressing mortality rates as a function of body concentration were generally less strong (lower R^2-values) compared to those based on water concentrations, except for Pb (Table 3).

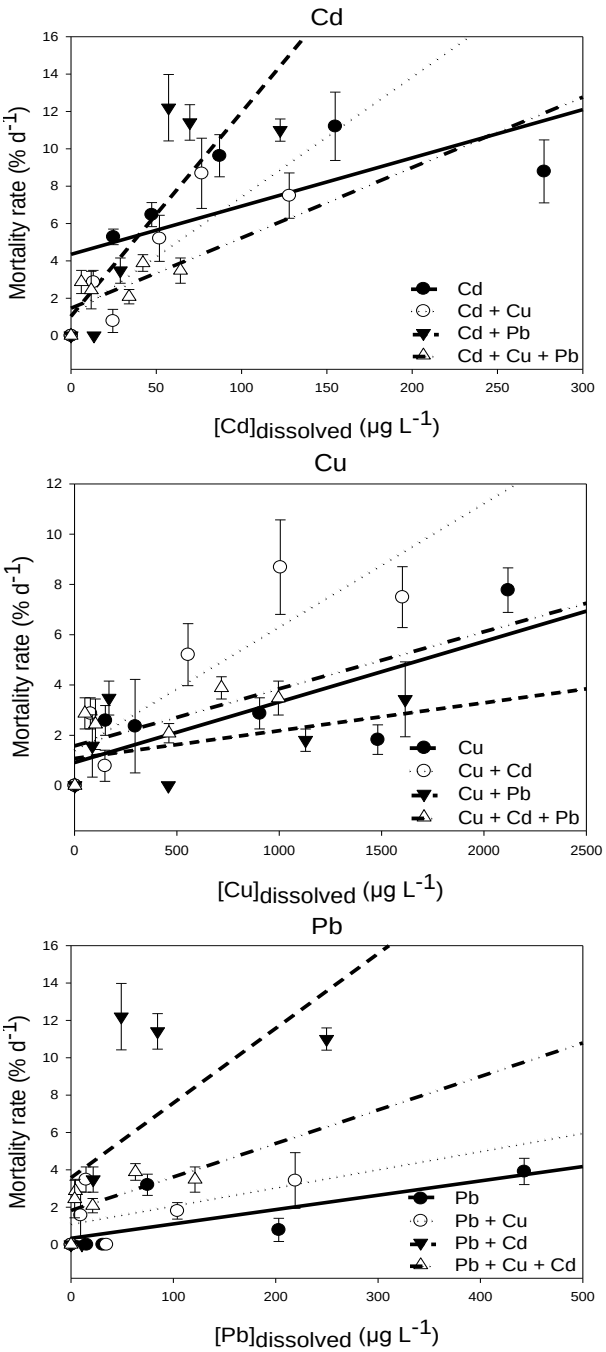


Figure 2: Mortality rates of *A. aquaticus* in function of exposure concentration (total dissolved) per mixture treatment. Average mortality rate (n = 7 time points) and standard errors are presented. Slopes (mortality rates) per treatment and model diagnostics are presented in Table 3.

3.3.2.2. Growth and energy reserves

Absolute growth varied from 2.02 ± 0.10 mg (Cu single) to −2.27 ± 0.06mg (Cd + Pb) (Fig. 3; see also Fig. S3). Growth significantly decreased with increasing Cd exposure and a significant ANCOVA interaction ($F = 4.87$; $p = 0.016$) was found, indicating a significant lower growth rate (thus stronger growth decrease) in the Cd + Pb treatment compared to the single Cd treatment (single Cd vs Cd + Pb: $t = -3.59$; $p = 0.003$), but not compared to the other Cd treatments. The difference in growth rate between the single Cd and Cd + Pb treatment was supported by a significantly lower growth rate than predicted by the IA model ($t = 2.95$; $p = 0.015$). Based on $[Cd]_{body}$, significant differences between growth rates could not be found (Table 4).

Table 4: Growth rates of *A. aquaticus* per mixture treatment. Growth rates were calculated based on total dissolved metal concentrations ((mg dw)(L)(μg^{-1} metal)(d^{-1}); Fig. 3) and based on body concentrations ((mg dw)^2(μg^{-1} metal)(d^{-1})). Average growth rates and standard errors are presented (n = 6 per mixture treatment), together with model diagnostics (R^2 for the ANCOVA model). Superscript letters indicate statistical differences between growth rates.

Metal	Mixture with	Growth rate ((mg dw)(L) (μg^{-1} metal)(d^{-1}))	R^2	Growth rate ((mg dw)^2 (μg^{-1} metal)(d^{-1}))	R^2
Cd	-	-0.56 ± 0.17^{a}	80.6%	-0.21 ± 0.07^{a}	75.2%
	Cu	-1.31 ± 0.37^{ab}		-0.80 ± 0.24^{a}	
	Pb	-2.23 ± 0.54^{b}		-0.31 ± 0.08^{a}	
	Cu + Pb	-1.50 ± 0.61^{ab}		-0.20 ± 0.24^{a}	
Cu	-	-0.07 ± 0.05^{a}	44.7%	-0.10 ± 0.06^{a}	41.4%
	Cd	-0.10 ± 0.03^{a}		-0.12 ± 0.04^{a}	
	Pb	0.001 ± 0.07^{a}		0.01 ± 0.06^{a}	
	Cd + Pb	-0.09 ± 0.40^{a}		-0.02 ± 0.04^{a}	
Pb	-	0.11 ± 0.13^{a}	56.4%	0.02 ± 0.03^{a}	53.0%
	Cu	-0.03 ± 0.5^{a}		-0.01 ± 0.21^{a}	
	Cd	-1.1 ± 0.22^{b}		-0.11 ± 0.02^{a}	
	Cu + Cd	-0.61 ± 0.39^{ab}		-0.12 ± 0.12^{a}	

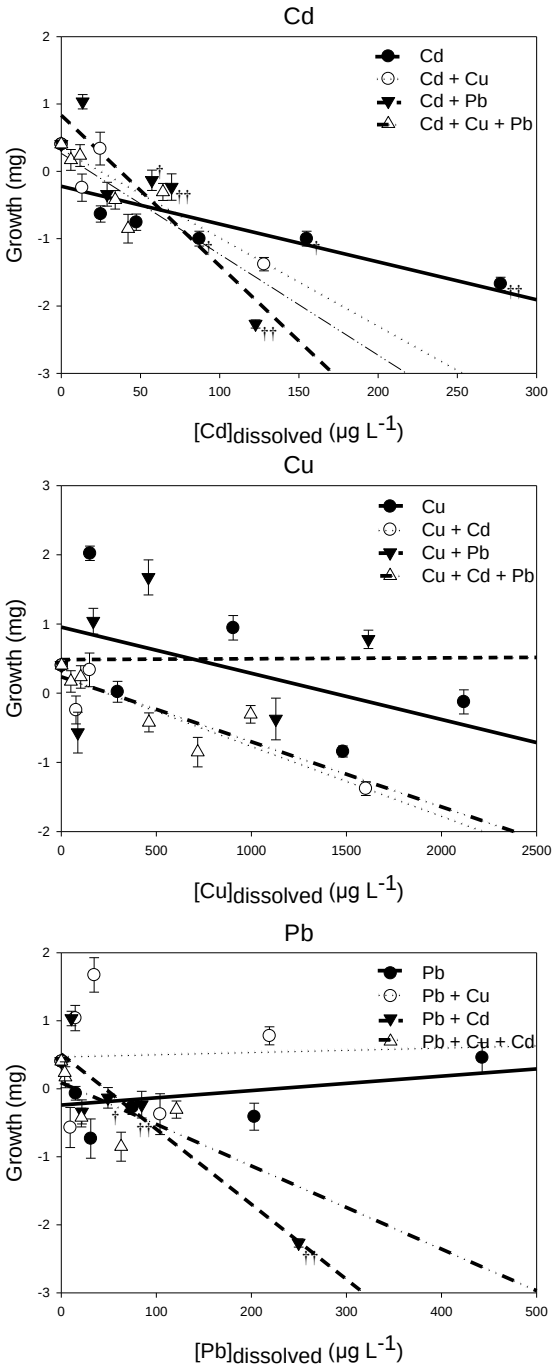


Figure 3: Growth of *A. aquaticus* after 10 days in function of exposure concentration (total dissolved) per mixture treatment. Average growth (n = 6) and standard errors are presented. †: Growth was measured after seven days; ††: Growth was measured after four days. Slopes (growth rates) per treatment and model diagnostics are presented in Table 4.

For Cu, growth did not significantly change with increasing water nor body concentration (Table 4). For Pb, growth significantly decreased with increasing exposure concentration and a significant ANCOVA interaction ($F = 4.60$; $p = 0.017$) was found, indicating a significant lower growth rate in the Pb + Cd treatment compared to the single Pb ($t = 3.58$; $p = 0.003$) and Pb + Cu ($t = 2.58$; $p = 0.020$) treatments; however, not when compared to the tertiary mixture. The difference in growth rate between the Pb single and Pb + Cd treatment was supported by significantly lower growth rate than predicted by the IA model ($t = 4.74$; $p = 0.001$). Based on $[Pb]_{body}$, significant differences between growth rates disappeared and models expressing growth as a function of body concentration were generally less strong (lower R^2) compared to those based on water concentrations (Table 4).

Glycogen concentrations ranged from 7.79 (single Cu treatment) to 0.91 mg/g ww (single Cd treatment) (Fig. 4; see also Fig. S4). For all metals, glycogen concentrations significantly decreased with increasing exposure concentration. However, no significant interactions between exposure concentration and mixture treatment were observed for any of the metals, both expressed on water concentrations and on body concentrations, indicating no significant differences in glycogen storage rates between the mixture treatments. Models expressing glycogen concentrations as a function of body concentrations were generally less strong (lower R^2) compared to those based on water concentrations, except for the model of Pb (Table 5).

Lipid concentrations ranged from 34.7 to 7.8 mg/g ww (both in Cd + Pb treatment) and protein concentrations ranged from 1.46 (Cd + Cu + Pb) to 0.17 (single Cd treatment) mg/g ww (results not shown). Lipid concentrations significantly decreased with increasing Cd ($t = -2.16$; $p = 0.044$) and Pb ($t = -2.65$; $p = 0.017$) exposure concentrations. However, for both metals no significant interaction with treatment was observed. For Cu, lipid concentrations did not significantly decrease with increasing exposure concentration. Protein concentrations did not significantly decrease with increasing Cd and Pb exposure, while they did significantly decrease

with increasing Cu ($t = -2.34$; $p = 0.031$), but without a significant interaction with treatment.

3.3.2.3. Relation between k_u and toxicity endpoints

Plotting calculated Cd k_u against isopod growth rate, mortality rate as well as glycogen storage rate revealed strong relations (R^2 ranging from 0.727 to 0.749), both positive (with mortality rate) and negative (with growth rate and glycogen storage rate; Fig. 5). These were not significant at the $p < 0.05$ level (however, they were significant at the $p < 0.10$ level), which is most likely due to the limited sample size (n = 5 treatments). 5). A very strong and significant positive relation ($R^2 = 0.906$; $p = 0.013$) was observed between glycogen storage rate and growth (Fig. 6). Observed relations between Cu and Pb k_u and toxicity endpoints were less strong (R^2 ranging from 0.169 to 0.778) compared to those for Cd and were not significant (not even at the $p < 0.10$ level; results not shown).

Table 5: Glycogen storage rates of *A. aquaticus* per mixture treatment. Glycogen storage rates were calculated based on total dissolved metal concentrations ((mg glycogen)(L)(g^{-1} ww)(μg^{-1} metal)(d^{-1})) (Fig. 4) and based on body concentrations ((mg glycogen)(g dw)(g^{-1} ww)(μg^{-1} metal)(d^{-1})). Average glycogen storage rates and standard errors are presented (n = 6 per mixture treatment), together with model diagnostics (R^2 for the ANCOVA model). None of the metal combinations differed significantly within a given metal, when the storage rates were regressed against dissolved metal concentration or against whole-body metal concentration.

Metal	Mixture with	Glycogen storage rate (log(mg glyc)(L) (g^{-1} ww) (μg^{-1} metal)(d^{-1}))	R^2	Glycogen storage rate (log(mg glyc)(g dw) (g^{-1} ww) (μg^{-1} metal)(d^{-1}))	R^2
Cd	-	-0.00017 ± 0.00007	74.8%	-0.00062 ± 0.00031	70.0%
	Cu	-0.00027 ± 0.00018		-0.00146 ± 0.00115	
	Pb	-0.00054 ± 0.00019		-0.00064 ± 0.00041	
	Cu + Pb	-0.00048 ± 0.00032		-0.00693 ± 0.00094	
Cu	-	-0.00002 ± 0.00001	56.5%	-0.00010 ± 0.00018	47.4%
	Cd	-0.00002 ± 0.00001		-0.00020 ± 0.00014	
	Pb	-0.00003 ± 0.00002		-0.00020 ± 0.00017	
	Cd + Pb	-0.00003 ± 0.00001		-0.00019 ± 0.00018	
Pb	-	-0.00004 ± 0.00008	62.2%	0.00013 ± 0.00010	62.3%
	Cu	-0.00013 ± 0.00010		-0.00029 ± 0.00032	
	Cd	-0.00024 ± 0.00007		-0.00008 ± 0.00007	
	Cu + Cd	-0.00022 ± 0.00015		-0.00037 ± 0.00036	

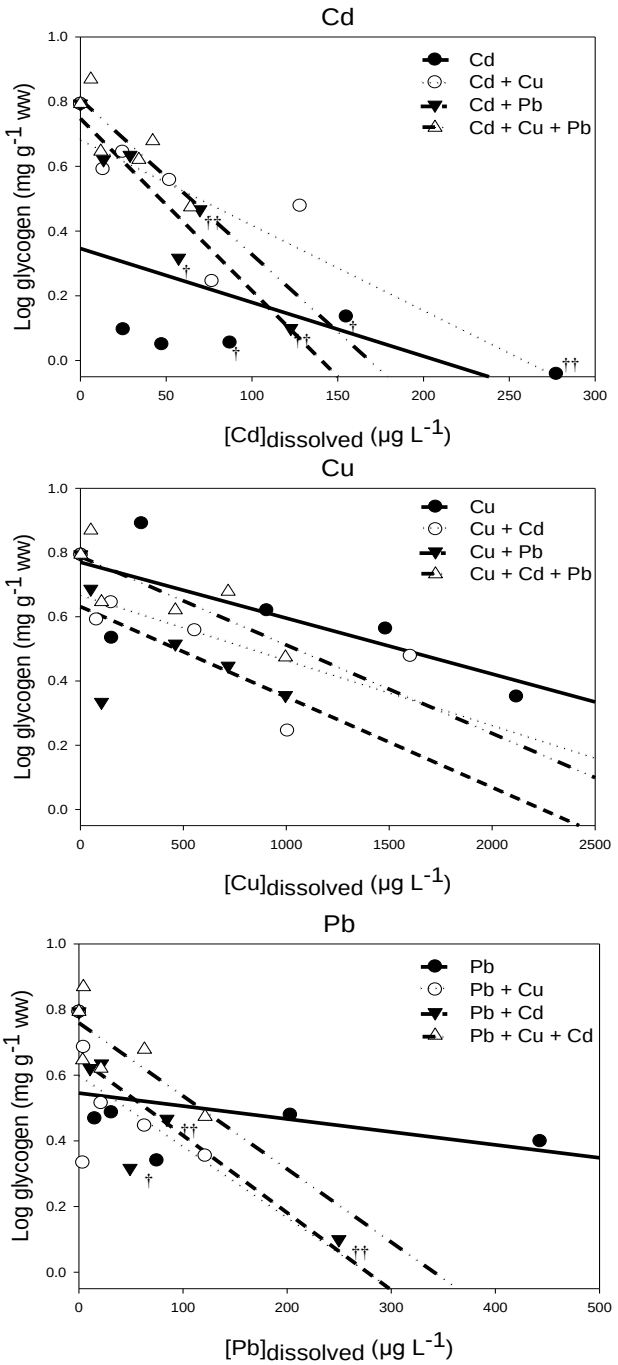


Figure 4: Glycogen concentrations in *A. aquaticus* after 10 days in function of exposure concentration (total dissolved) and per mixture treatment. Glycogen concentrations are the result of one measurement on a pool of three individuals. †: Glycogen was measured after seven days; ††: Glycogen was measured after four days. Slopes (glycogen storage rates) per treatment and model diagnostics are presented in Table 5.

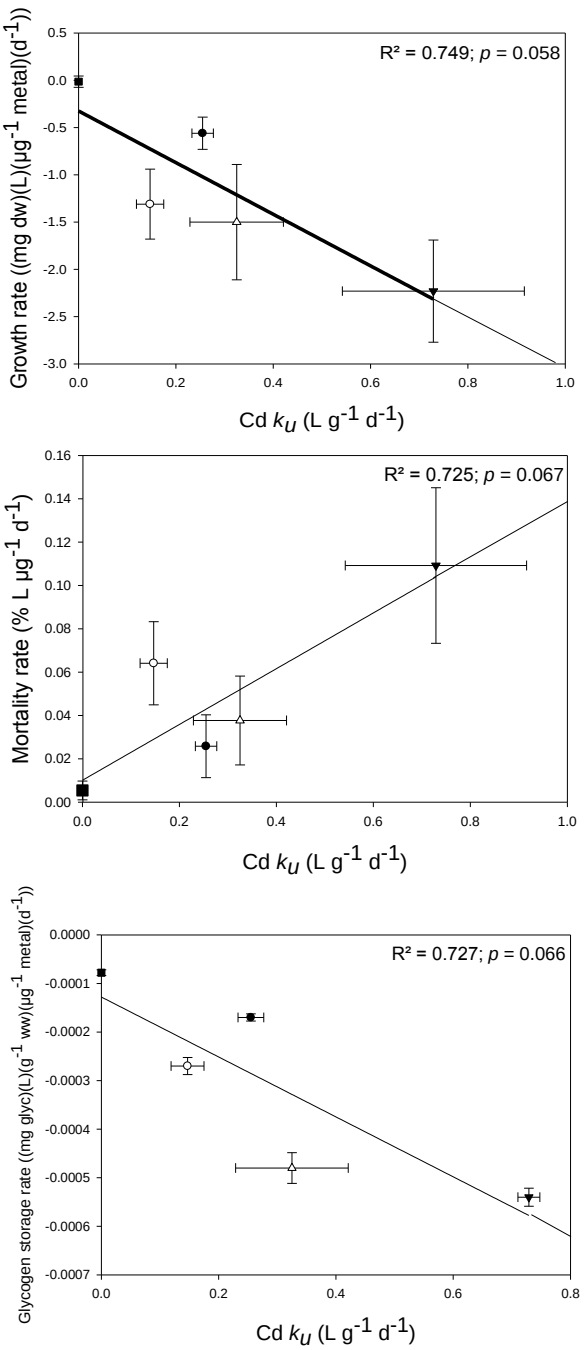


Figure 5: Relationship between Cd k_u and growth rate, mortality rate and glycogen storage rate. Average values and standard errors are presented per mixture treatment; Black circles: Cd; White circles: Cd + Cu; Black triangles: Cd + Pb; White triangles: Cd + Cu + Pb; Black squares: Cu + Pb. The slopes plotted for the Cu + Pb points are the average of the Cu + Pb and Pb + Cu sloped listed in Tables 3, 4, and 5; and the uptake rate for Cu + Pb is plotted as zero, because no Cd was added to those exposure waters.

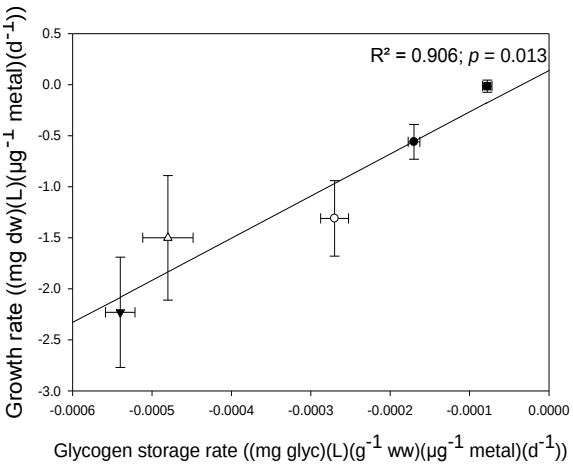


Figure 6: Relationship between glycogen storage rate and growth rate. Average values and standard errors are presented per mixture treatment; Black circle: Cd; White circle: Cd + Cu; Black triangle: Cd + Pb; White triangle: Cd + Cu + Pb; Black square: Cu + Pb.

3.4. Discussion

3.4.1. Metal uptake and bioaccumulation

We observed linear relationships which were linked to relatively high R^2, though curvilinear relationships were expected. This is possibly due to the relatively short time span of the experiment. Cadmium uptake rates (k_u) for *A. aquaticus* calculated from the linear regression between body concentrations and dissolved metal concentrations varied from 0.147 to 0.729 L/g/day and were generally higher than Cd k_u calculated for the crustacean *Daphnia magna* (k_u from 0.020 to 0.102 L/g/day) exposed to mixtures of Cd, Cu, Ni, Zn and Pb (Komjarova and Blust, 2008). Furthermore, our Cd uptake rates fall within the range of k_u calculated for other aquatic organisms such as the freshwater snail *Lymnaea stagnalis* (k_u from 0.39 to 0.98 L/g/day) (Croteau and Luoma, 2007) and the clam *Ruditapes decussatus* (k_u of 0.103 L/g/day) (Serafim and Bebianno, 2010). Calculated Pb k_u varied from 0.225 to 1.003 L/g/day, which were generally higher than the calculated k_u for Pb uptake in zebrafish gills (*Danio rerio*) (k_u from 0.004 to 0.250 L/g/day) exposed to mixtures of Cd, Cu, Ni, Zn and Pb (Komjarova and Blust, 2009).

Synergistic effects of Pb on Cd uptake and vice versa were observed. Combined exposure to Cd + Pb increased Cd k_u by a factor of three, while Cd addition increased Pb k_u by a factor of two. For both metals, the synergistic effects on k_u disappeared when Cu was added to the medium (in the tertiary mixture). Birceanu et al. (2008), however, found antagonistic interactions between Cd and Pb binding to the gill of *Oncorhynchus mykiss* in soft, moderately acidic water ([Ca] < 4 mg/L; pH 6) using comparable Pb (104 µg/L) and lower Cd concentrations (11.2 µg/L). Rogers and Wood (2004) demonstrated that competitive interactions between Cd and Pb are more important at very high Pb (~1035 µg/L) and low Cd (~1.12 µg/L) concentrations. Competitive inhibition and the resulting antagonistic effects of combined Cd + Pb exposure on both Cd and Pb uptake could be expected since both Cd and Pb compete for Ca^{2+} channel binding sites in aquatic organisms, including crustaceans (Niyogi and Wood, 2004; Rogers and Wood, 2004; Rainbow and Black, 2005). However, synergistic interactions have also been reported in literature. Winter et al. (2012) observed increased Cd-gill binding in rainbow trout (*O. mykiss*) at Pb concentrations comparable to those of the present study (155 – 311 µg/L). Using stable isotope analysis, Komjarova and Blust (2008) observed higher Pb uptake rates with increasing Cd exposure (22.4 µg/L) in *D. magna*. However, no effect of Pb on Cd uptake was observed. Applying the same approach to zebrafish, increased Pb uptake in the gills after Cd addition (at 5.6 µg/L, but not at 22.4 µg/L) was observed together with an increased Cd uptake after Pb addition (at 20.7 µg/L), but a decreased Cd uptake was found after the addition of 44.8 µg Pb/L (Komjarova and Blust, 2009). Since both Cd and Pb inhibit Ca^{2+} uptake, the organism can upregulate the activity of these channels to counteract disturbances in ion homeostasis and, as a result, also increase metal uptake (Rainbow, 1997). Additionally, both Cd and Pb most likely possess more binding sites, which could explain the observed synergistic effects as well (Birceanu et al., 2008). Lead can also interfere with Na^+ and Cl^- influx, possibly by inhibiting intracellular carbonic anhydrase and thereby reducing intracellular H^+, which is needed to drive Na^+ uptake (Rogers et al., 2003, 2005). Birceanu et al. (2008) observed that ionic

disturbances (Na^+ and Ca^{2+} influx) in *O. mykiss* were synergistic after exposure to Cd + Pb mixtures. The latter study hypothesized that Pb induced effects on Na^+ influx were strengthened by Cd, due to the Cd-induced inhibition of Na^+/K^+ ATPase activity, which is a well-known toxic effect of Cd exposure in fish as well as aquatic invertebrates (Postel et al., 1998; Atli and Canli, 2007; Birceanu et al., 2008).

Together with observations from literature, our results suggest that in Cd + Pb mixtures both metals can act synergistically at biotic ligands, but that the interactions are highly dependent on the exposure concentration and the chemistry of the water (in the present study: medium-hard water, low organic carbon). Abiotic factors, such as water hardness and alkalinity, the concentration of organic/inorganic ligands and pH can affect the toxicity of metals (Wang, 1987). For example, cations like Ca^{2+} and H^+ can compete with metals for biotic ligands, as can other metals, and as a result decrease toxicity (Di Toro et al., 2001). Metals can also bind to inorganic and organic ligands in the water, thereby decreasing the free ion activity and thus uptake (Weng et al., 2002).

Relations between dissolved Cu and body concentrations were generally less strong (lower model R^2) compared to those for Cd and Pb, which is probably due to the essential nature of Cu. Copper body concentrations can be homeostatically regulated within a certain environmental range (Rainbow, 2002). Calculated k_u (0.055 to 0.107 L/g/day) fall within the same range as those calculated for *R. decussatus* (0.053 L/g/day) (Serafim and Bebianno, 2010) and *D. magna* (0.010 to 0.250 L/g/day) (Komjarova and Blust, 2008), but are lower when compared to those calculated for *L. stagnalis* (0.55 to 0.79 L/g/day) (Croteau and Luoma, 2007). In the present study, no significant effect of Cu on Cd or Pb k_u or vice versa was observed. Since Cu and Cd both have different uptake mechanisms (Cu via Na^+ channels, Cd via Ca^{2+} channels) no direct competitive inhibition should be expected (De Schamphelaere and Janssen, 2002; Grosell and Wood, 2002; Niyogi and Wood, 2004; Rainbow and Black, 2005). Kraak et al. (1993) did not observe interactions between Cd and Cu on metal accumulation rates in zebra mussels (*Dreissena polymorpha*) exposed to Cd + Cu mixtures. Similarly, no effect of Cd on Cu k_u was observed in *D. rerio*, although Cd k_u decreased in the presence

of Cu (only at 25.4 µg/L) (Komjarova and Blust, 2009). Komjarova and Blust (2008) observed lower Cd k_u after Cu addition and vice versa in *D. magna* and explained their findings by indirect competition for a common uptake site (e.g. Fe^{2+} transporter DMT1; Bury et al., 2003; Garrick et al., 2006). The study of Pelgrom et al. (1995) indicated that Cu + Cd interactions on metal uptake and accumulation in tilapia (*Oreochromis mossambicus*) were more pronounced at lower exposure concentrations (19.1 µg/L Cu and 4.48 µg/L Cd). With respect to Cu and Pb, direct competitive inhibition could be expected since both metals have (partly) similar uptake mechanisms, i.e. via Na^+ channels (Rogers et al., 2003, 2005). In our study, Pb k_u lowered by a factor of two in the presence of Cu. However, the difference was not significant. Similarly, Cu addition did not affect Pb k_u in *D. magna* at low exposure concentrations (max. 15.9 µg/L Cu and 51.8 µg/L Pb) (Komjarova and Blust, 2008). In contrast, Pb uptake in the gill of *D. rerio* increased at 6.35 µg/L Cu, but strongly decreased at 25.4 µg/L Cu (Komjarova and Blust, 2009).

3.4.2. Toxicity endpoints

The calculated Cd LC_{50} of 34.9 ± 7.25 µg/L for single metal exposure is in agreement with the LC_{50} of 54 µg/L (38.4 to 75.5 µg/L) for *A. aquaticus* observed by Ham et al. (1995) (hardness of 87 mg/L $CaCO_3$). Copper and Pb LC_{50}s are higher (two and three times, respectively) compared to the values calculated by Brown (1976) for *A. meridianus*. However, the latter study used soft water (hardness of 25 mg/L $CaCO_3$) compared to the medium-hard water used in the present study (hardness of 117 mg/L $CaCO_3$).

Synergistic effects between Cd and Pb on growth and mortality rates were observed. Growth reduction rates of *A. aquaticus* in the Cd + Pb mixtures were a factor 4 higher compared to those for the single Cd treatment, while they were 10 times higher in the Pb + Cd treatment compared to the single Pb treatment. Mortality rates in the Pb + Cd treatment were five times higher compared to the single Pb treatment. Also the study of Wu et al. (2012) observed that at soil Cd concentrations around 0.1 µg/g the

addition of Pb could increase DNA damage in the earthworm *Eisenia fetida*. Furthermore, the study of Borgmann (1980) observed additive effects of Cd + Pb mixtures on growth kinetics in freshwater copepods. In the present study, strong relations were observed between Cd k_u and (sub)lethal endpoints (growth rate, mortality rate and glycogen storage rate). In general, the strongest toxic effects were observed in the treatments with highest Cd k_u (Cd + Pb treatment). In the present study, the increased Cd uptake and accumulation resulted in fast decreasing glycogen reserves, which, on its turn, was strongly related to growth reduction. De Jonge et al. (2012) also observed fast reductions in glycogen concentrations in *A. aquaticus* during an increasing influx of multiple metal ions. In the present study, the glycogen storage rates in the Cd + Pb and Pb + Cd treatments were three and six times higher compared to the single metal treatments, although the differences were not statistically significant.

Corresponding to the results of Cu uptake, no significant mixture interactions of Cu on Cd and/or Pb toxicity or vice versa were observed. Mortality (0.002 ± 0.001% L/µg/day), growth (−0.07 ± 0.05 (mg dw)(L)(μg^{-1} metal)(day^{-1})) and glycogen storage rates (−0.00002 ± 0.00001 (mg glycogen)(L)(g^{-1} ww)(μg^{-1} metal)(day^{-1})) were very consistent between the different Cu treatments. A study of Borgmann (1980) only observed additive effects of binary Cu + Cd and Cu + Pb mixtures on copepod growth. The study of Finlayson and Verrue (1982) also found that mixture effects of combined Cd and Cu mixtures on Chinook salmon (*Oncorhynchus tshawytscha*) were additive. Nevertheless, based on recent metal mixture reviews additivity is not the most common effect for Cu + Cd mixtures, since it was observed in only 35% (Norwood et al., 2003), 15% (Vijver et al., 2011) and 8% (Weltje, 1998) of the cases, respectively.

3.4.3. Using body concentrations to predict mixture toxicity

Isopod body concentrations were generally significantly related to observed (sub)lethal effects. In fact, metal toxicity in aquatic invertebrates has often been strongly related to bioaccumulation since it accounts for metal interactions that can

occur at biotic ligands and it integrates multiple routes of exposures (Borgmann et al., 2004, 2008; De Jonge et al., 2012). Using saturation models, Borgmann et al. (2004) found that metal accumulation in the amphipod *H. azteca* related well to chronic toxicity. The study of De Jonge et al. (2012) observed significant relations between *A. aquaticus* body concentrations and energy availability (sum of glycogen, lipid and protein reserves) during exposure to sediment-bound metal mixtures including Cd, Cu and Pb. Van Praet et al. (2014) also found significant relations between accumulated metal mixtures and glutathione-S-transferase activity (GST) as well as energy reserves in field-captured damselfly larvae (*Ischnura elegans*).

Despite strong relations between isopod body concentrations and toxicity, model R^2 were almost always lower compared to the ANCOVA based on dissolved metal concentrations (except for mortality and glycogen storage rates for Pb). Moreover, mixture effects disappeared when toxicity endpoints (growth rates and mortality rates) were expressed on body concentrations instead of water concentrations. This suggests that interactive effects between these metals took place outside the organism, with one metal affecting the availability of the other. This is commonly observed for metal ions, where ion speciation and competition for binding sites to organic matter in the water phase can change free ion availability (Cedergreen, 2014). Nevertheless, metal bioaccumulation in aquatic organisms has been suggested as a reliable predictor of metal mixture toxicity, both on organismal level (Borgmann et al., 2004, 2008; Abboud and Wilkinson, 2013; Norwood et al., 2013) and on community level (De Jonge et al., 2013). Furthermore, metabolically available metal has also been suggested as an even better predictor of metal mixture toxicity than metal body concentrations and would be interesting to include in future research (Vijver et al., 2004).

3.5. Conclusions

The present study revealed synergistic interactions of combined Cd and Pb exposure on both Cd and Pb uptake in the isopod *A. aquaticus*, when based on dissolved metal

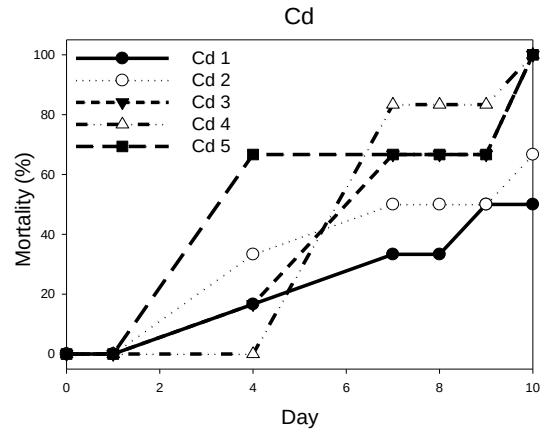
concentrations. Furthermore, synergistic effects of combined Cd and Pb exposure on growth rates and mortality rates were observed. No mixture interactions of Cu on Cd or Pb uptake (or vice versa), nor on toxicity endpoints were observed. Body concentrations in *A. aquaticus* were related to all toxicity endpoints. However, mixture effects disappeared when growth and mortality rates were expressed on body concentrations instead of exposure concentrations.

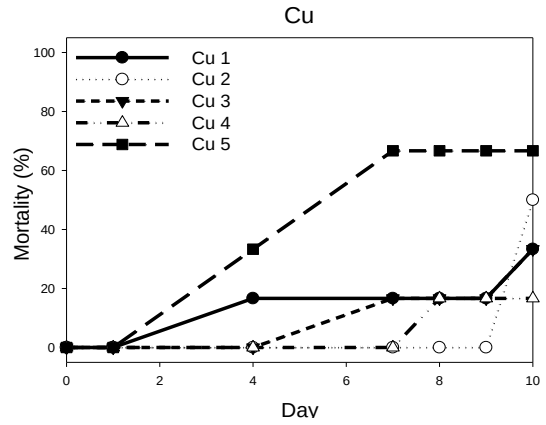
By combining information of mixture effects on metal uptake with mixture toxicity data, the present study could reveal more insights in the way metal mixtures interfere with aquatic organisms and how they can induce toxic effects. This type of information is of great value to understand the toxicity of metal mixtures and to improve current risk assessment of metal mixtures in the field.

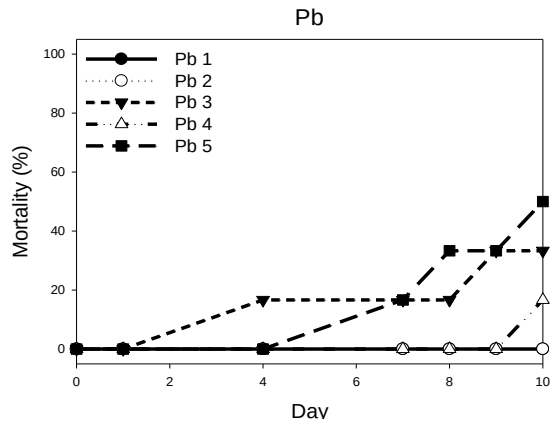
3.6. Acknowledgments

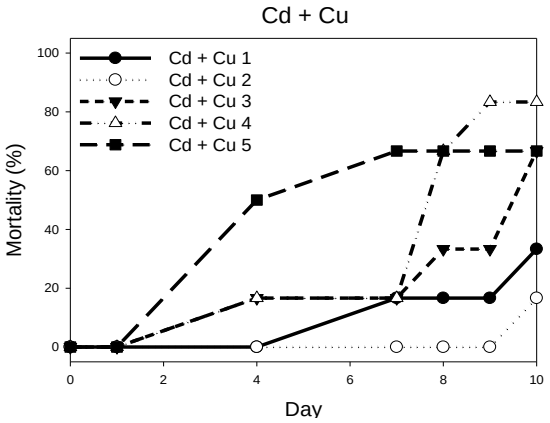
We would like to thank Francesc Perarnau Bayo and Mohamed Karem for their assistance in the lab. Steven Joosen is acknowledged for performing the metal analyses. Marjolein Van Ginneken is funded by a doctoral grant from the Research Foundation Flanders (FWO). Maarten De Jonge is funded by a post-doctoral grant (FWO) (Grant nr. 12B12113N).

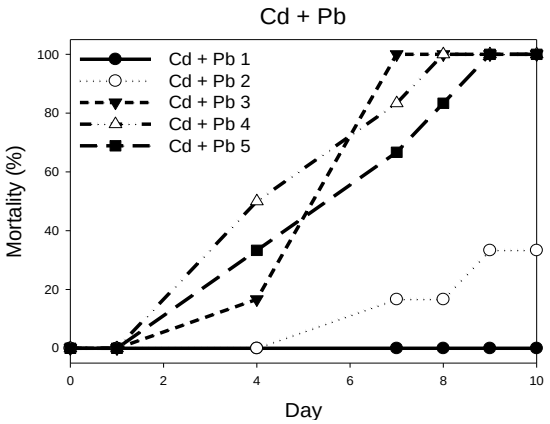
3.7. Supplementary material

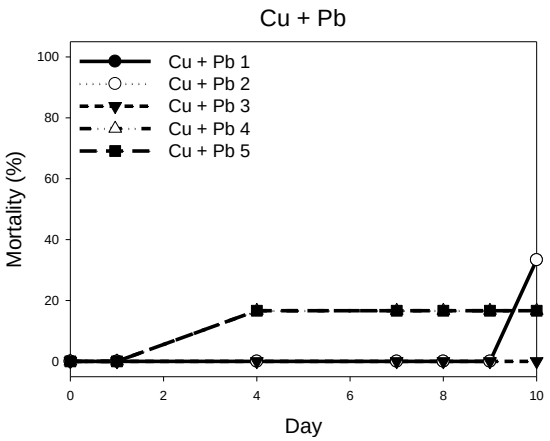







Cd + Cu
Cd + Cu 1
Cd + Cu 2
Cd + Cu 3
Cd + Cu 4
Cd + Cu 5
Mortality (%)
Day
0
20
40
60
80
100
0
2
4
6
8
10


Cd + Pb
Cd + Pb 1
Cd + Pb 2
Cd + Pb 3
Cd + Pb 4
Cd + Pb 5
Mortality (%)
Day
0
20
40
60
80
100
0
2
4
6
8
10


Cu + Pb
Cu + Pb 1
Cu + Pb 2
Cu + Pb 3
Cu + Pb 4
Cu + Pb 5
Mortality (%)
Day
0
20
40
60
80
100
0
2
4
6
8
10

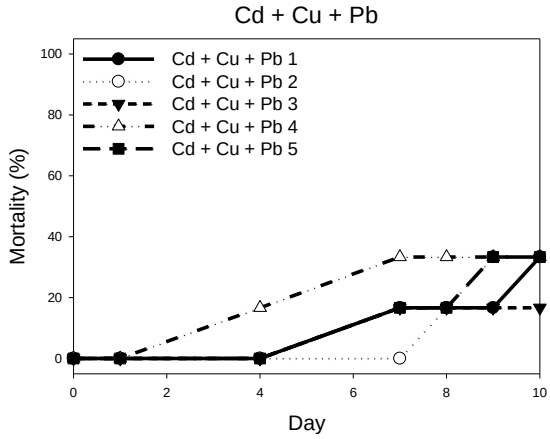


Figure S1: Mortality of *A. aquaticus* in function of the time exposed to the different water metal concentrations (total dissolved) (n = 7 time points).

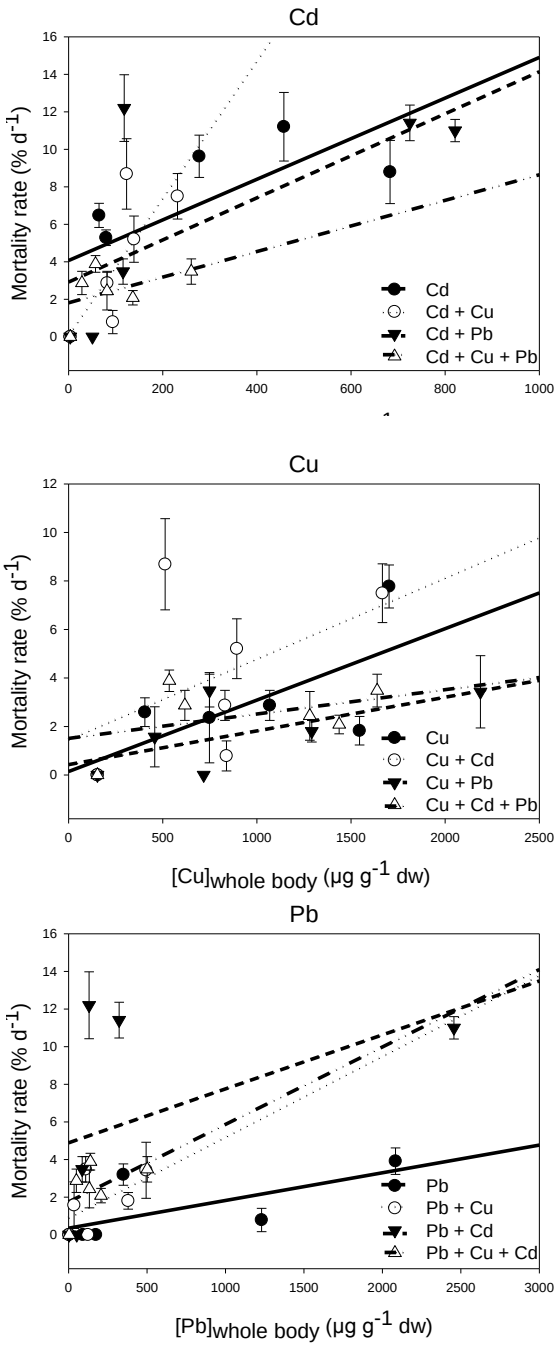


Figure S2: Mortality rate of *A. aquaticus* in function of the whole-body metal concentration and per mixture treatment. Average mortality rate (n = 7 time points) and standard errors are presented. Slopes (mortality rates) per treatment and model diagnostics are presented in Table 3.

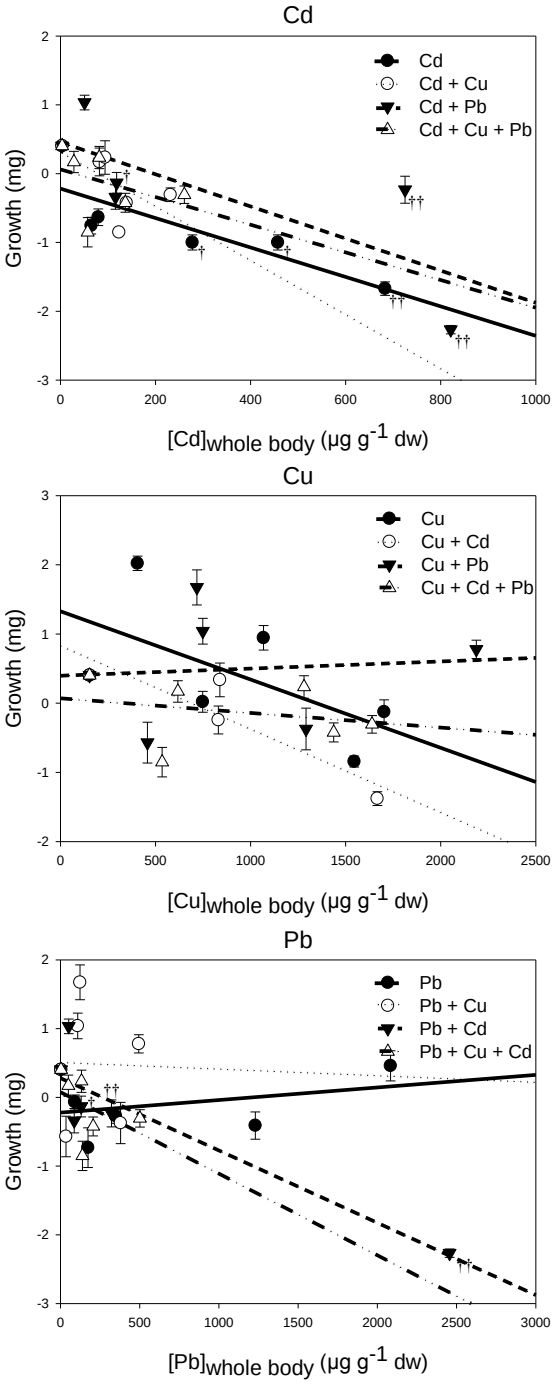


Figure S3: Growth of *A. aquaticus* after 10 days in function of the whole-body metal concentration and per mixture treatment. Average growth (n = 6) and standard errors are presented. †: Growth was measured after seven days; ††: Growth was measured after four days. Slopes (growth rates) per treatment and model diagnostics are presented in Table 4.

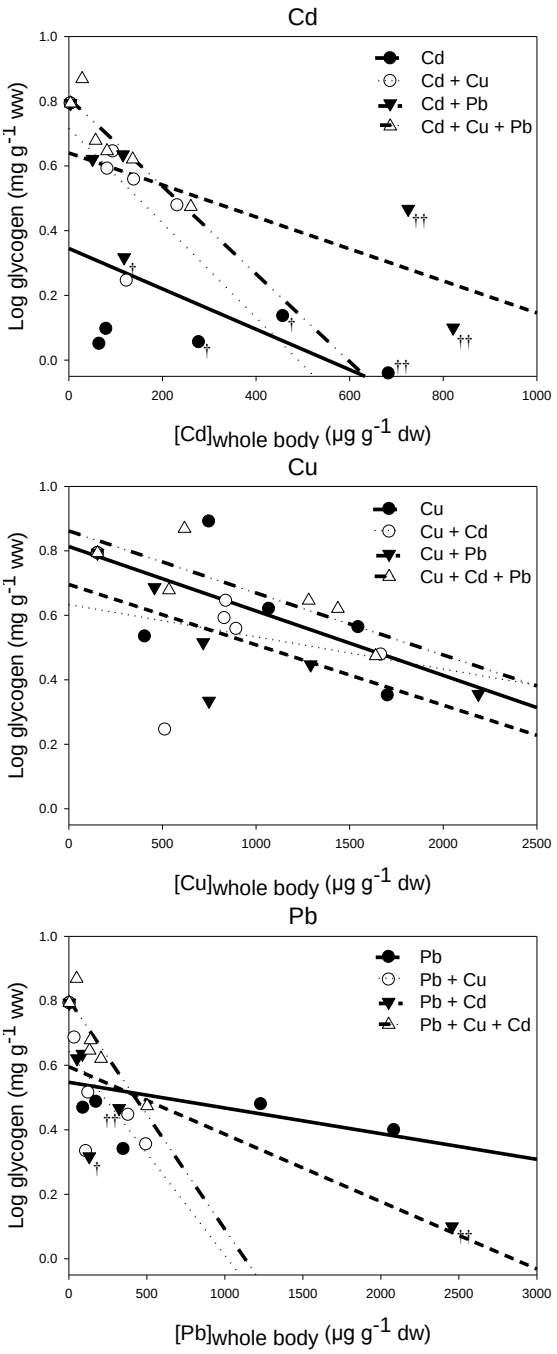


Figure S4: Glycogen concentrations in *A. aquaticus* after 10 days in function of the whole-body metal concentration and per mixture treatment. Glycogen concentrations are the result of one measurement on a pool of three individuals. †: Glycogen was measured after seven days; ††: Glycogen was measured after four days. Slopes (glycogen storage rates) per treatment and model diagnostics are presented in Table 5.

Chapter 4.

Combined effects of metal mixtures and predator stress on the freshwater isopod *Asellus aquaticus*

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Abstract

Predator stress has been demonstrated to change the toxicity of pollutants by inducing energetically-costly behavioral and physiological stress responses. While the combined effects of predator cues and pesticides are well documented, the interaction of predator stress with metals is a topic that has remained largely unexplored. In this laboratory experiment, the freshwater isopod *Asellus aquaticus* was exposed to predator cues and mixtures of Cd, Cu and Pb. We examined the effects on growth, respiration and, as behavioral parameters, feeding rate and activity. These were linked to the free ion activities (FIAs) in the water and the metal body concentrations. The findings revealed that Cu accumulation significantly reduced the response of isopods exposed to predator cues. Predator-stressed isopods with high Cu body burdens had a higher (feeding) activity than isopods with lower Cu concentrations, making themselves more susceptible to predation. Furthermore, we found a concentration-dependent interaction of the Cd + Pb mixtures on the feeding rate and a lower feeding rate for Cd and Pb predator exposed asellids. As several interactions were found between metals and predator stress, it demonstrates the importance of investigating how organisms and whole ecosystems respond to multiple stressors. A better understanding of these interactions will undoubtedly improve risk assessment and management.

4.1. Introduction

Classic toxicity tests study the impact of a single chemical on test organisms. Generally, the organisms are exposed without additional stressors and while the studied endpoints depend on the test organism, the focus still often lies on mortality. However, in contaminated ecosystems, multiple chemicals are often simultaneously present, resulting in synergistic, additive or antagonistic interactions (Cedergreen, 2014). Sublethal (behavioral) effects may be induced, which could have an important influence on the ecosystem, altering species abundances and diversity (Fleeger et al., 2003). Lastly, organisms rarely live under optimal conditions in natural ecosystems. Coping with predators, fluctuations in food availability or other natural stressors could affect the uptake or toxicity of chemicals as well (Folt et al., 1999; Heugens et al., 2001). Considerable effort is necessary to shed light on the possible effects and interactions of multiple stressors.

The present study examined the combined effects of predator stress and metal mixtures of Cd, Cu and Pb on *Asellus aquaticus* L. This freshwater isopod is an important detritivore and can be found throughout Europe and North America (Bloor, 2010). For the mixtures, metals were chosen with dissimilar modes of actions. While copper is known to interfere with the uptake pathway of Na^+ (De Schamphelaere and Janssen, 2002), cadmium can directly compete with Ca^{2+} for uptake. Lead, on the other hand, can disrupt both Na^+ and Ca^{2+} homeostasis (Komjarova and Blust, 2008; Rogers et al., 2003). Van Ginneken et al. (2015) already reported synergistic interactions on the mortality and growth rate of *A. aquaticus* exposed to Cd and Pb mixtures.

Previous research has shown that predator cues can alter the uptake and toxicity of chemicals, due to costly behavioral and physiological stress responses. For instance, the presence of a predator can cause the prey's oxygen consumption to increase, leading to a higher uptake rate of pollutants (Pestana et al., 2009). Additionally, it can cause oxidative damage (Slos and Stoks, 2008). When the pesticide carbaryl was combined with predator cues, Relyea (2003) found an increased lethality for two out

of six amphibian species. Schulz and Dabrowski (2001) observed a synergistic interaction of fish cues with the organophosphate insecticide azinphosmethyl (AZP) and the pyrethroid insecticide fenvalerate (FV) for mayfly nymphs, resulting in an increased mortality rate. For *Ceriodaphnia dubia*, Qin et al. (2011) demonstrated that predator stress influenced the toxicity of several pesticides differently. While predator cues interacted antagonistically with bifenthrin and thiacloprid, it acted synergistically with fipronil. Thus, interaction patterns differ among pollutants.

Although some research has been done on pesticides, less attention has been paid to the interaction of predator cues with metals. Qin et al. (2015) reported an antagonistic effect of predator stress on the acute toxicity of silver nanoparticles to daphnids. Additionally, there are a few articles concerning the effects of metals on predator-prey interactions (Clements, 1999; Kiffney, 1996). Lefcort et al. (2000), for example, reported that snails from heavy metal-polluted environments failed to exhibit antipredator behaviors in contrast to snails from reference lakes. These non-lethal effects of metals and predator cues could significantly change species interactions and community structure (Trussell et al., 2003). Still, knowledge is lacking. For fish, there are indications that certain metals such as Cu and Cd can affect the responses to olfactory cues, leading to possible disturbances in communication, growth and reproduction (Lürling and Scheffer, 2007). Yet, little is known about these effects on Crustacea nor on the effects of various metals combined.

As metal pollution is a widespread problem, it is important to comprehend how metals interact with other environmental stressors to estimate the actual effects of these pollutants in nature. Using environmentally realistic metal concentrations, we determined the following sublethal (fitness-related) endpoints of *A. aquaticus*: growth, respiration and, as behavioral parameters, feeding rate and activity. These endpoints were linked to the free ion activities (FIAs) in the water and the metal body concentrations. Additionally, we investigated the interactions of these metals with predator stress for each endpoint. We hypothesized that exposure of *A. aquaticus* to predator cues would cause an increased respiration rate and therefore an increased

metal accumulation, leading to a lower growth rate compared to the isopods without this additional stressor. Although the presence of predator cues generally leads to a lower activity (Stoks et al., 2005), defense mechanisms against metals are energetically costly and could be fueled by an enhanced food uptake. Moreover, metals could disrupt the transfer of chemical information, such as predator cues (Lürling and Scheffer, 2007). Therefore, we expected to find an increase in feeding rate and activity for isopods exposed to metals both with and without predator cues.

4.2. Material and Methods

4.2.1. Experimental set-up

A ten-day lab experiment was conducted on the aquatic sowbug *Asellus aquaticus*. The isopods were collected in the autumn of 2015 from a stream, the Laakbeek, in Lille, Belgium. In the laboratory, they were kept for minimally two weeks in a climate chamber type WT15'/+5DU-WB (Weiss Technik, Reiskirchen-Lindenstruth, Germany) at constant temperature (15 ± 1 °C) and light conditions (16:8 h light:dark photoperiod) in 20 L glass aquaria filled with reconstituted medium-hard freshwater (US EPA, 2002). Alder leaves were conditioned by drying them for 72 h and rehydrating them in water from the Laakbeek for a week. These leaves were provided ad libitum as food (Bloor, 2010). After minimally two weeks of acclimation, the experiment was performed under the same conditions.

Asellus aquaticus was exposed to a combination of predator cues and metal mixtures of Cd, Cu and Pb. Three concentrations for each metal were used: "L" = low, "M" = medium and "H" = high concentrations, which are equal to the EQS, 10 x EQS, and 100 x EQS (EP, 2008; VLAREM, 2015). We exposed the isopods to the single metals as well as to their binary and tertiary mixtures (Table 1). Four control treatments were added as well. The stable isotopes ^{65}Cu, ^{116}Cd and ^{204}Pb (CortecNet, Voisins-Le-Bretonneux, France) were added to moderately hard water (Table 1; US EPA, 2002) to prepare the metal concentrations. The nominal concentrations for Cd were: L = 1.29 10^{-3} µmol/L, M = 0.013 µmol/L and H = 0.129 µmol/L; for Cu: L = 0.108 µmol/L, M = 1.08 µmol/L and

H = 10.8 µmol/L; and for Pb: L = 0.035 µmol/L, M = 0.353 µmol/L and H = 3.53 µmol/L. Sublethal metal concentrations were chosen based on the mortality data presented in Van Ginneken et al. (2017).

Table 1: Overview of the dissolved metal concentrations at day 0. BMQL = Below method quantification limit. BMQL of ^{116}Cd = 0.647 10^{-3} µmol/L; BMQL of ^{65}Cu = 0.015 10^{-3} µmol/L; and ^{204}Pb = 0.005 10^{-3} µmol/L.

	^{116}Cd (µmol/L)	^{65}Cu (µmol/L)	^{204}Pb (µmol/L)
Controls	BMQL	6.61 10^{-3} ± 2.08 10^{-3}	0.427 10^{-3} ± 0.078 10^{-3}
Cd L	1.78 10^{-3} ± 0.34 10^{-3}	1.57 10^{-3} ± 0.19 10^{-3}	0.628 10^{-3} ± 0.020 10^{-3}
Cd M	12.1 10^{-3} ± 0.3 10^{-3}	2.50 10^{-3} ± 0.32 10^{-3}	0.711 10^{-3} ± 0.034 10^{-3}
Cd H	0.179 ± 0.022	5.04 10^{-3} ± 0.03 10^{-3}	BMQL
Cu L	BMQL	0.110 ± 0.002	0.417 10^{-3} ± 0.088 10^{-3}
Cu M	1.25 10^{-3} ± 0.22 10^{-3}	1.22 ± 0.13	0.721 10^{-3} ± 0.059 10^{-3}
Cu H	BMQL	23.3 ± 2.0	BMQL
Pb L	BMQL	1.97 10^{-3} ± 0.12 10^{-3}	23.2 10^{-3} ± 0.6 10^{-3}
Pb M	1.54 10^{-3} ± 0.18 10^{-3}	1.68 10^{-3} ± 0.25 10^{-3}	0.276 ± 0.002
Pb H	BMQL	8.35 10^{-3} ± 1.20 10^{-3}	3.08 ± 0.66
Cd + Cu L	1.54 10^{-3} ± 0.06 10^{-3}	0.133 ± 0.092 10^{-3}	0.476 10^{-3} ± 0.034 10^{-3}
Cd + Cu M	13.2 10^{-3} ± 0.5 10^{-3}	1.23 ± 0.09	0.515 10^{-3} ± 0.069 10^{-3}
Cd + Cu H	0.259 ± 0.022	17.2 ± 1.0	0.059 10^{-3} ± 0.015 10^{-3}
Cd + Pb L	1.38 10^{-3} ± 0.66 10^{-3}	0.847 10^{-3} ± 0.308 10^{-3}	5.25 10^{-3} ± 0.040 10^{-3}
Cd + Pb M	10.6 10^{-3} ± 0.2 10^{-3}	0.462 10^{-3} ± 0.231 10^{-3}	80.9 10^{-3} ± 1.0 10^{-3}
Cd + Pb H	0.286 ± 0.002	13.6 10^{-3} ± 0.6 10^{-3}	4.08 ± 0.16
Cu + Pb L	BMQL	0.118 ± 0.004	23.1 10^{-3} ± 0.3 10^{-3}
Cu + Pb M	1.41 10^{-3} ± 0.29 10^{-3}	0.995 ± 0.011	0.119 ± 0.008
Cu + Pb H	BMQL	18.8 ± 0.9	4.34 ± 0.17
Cd + Cu + Pb L	BMQL	74.1 10^{-3} ± 3.1 10^{-3}	4.85 10^{-3} ± 0.42 10^{-3}
Cd + Cu + Pb M	11.5 10^{-3} ± 0.3 10^{-3}	0.935 ± 0.014	88.7 10^{-3} ± 1.6 10^{-3}
Cd + Cu + Pb H	0.294 ± 0.019	18.4 ± 1.8	3.87 ± 0.66

When necessary, the pH was adjusted to 7.8 with 1 N NaOH. Next, 100 mL of this medium was added to acid-washed (1% HCl) polypropylene containers (125 mL). Ten

replicates per treatment were made, after which the solutions were left for 24 h to equilibrate. In each container, we placed one individual of *A. aquaticus* together with two alder leaf discs (*Alnus glutinosa*, d=16 mm, 22.0 ± 0.1 mg) that had been dried, weighed and 'conditioned' for six days in water from the Laakbeek in Lille (Bloor and Banks, 2006). Each isopod was photographed at the start of the experiment after which the length was determined using ImageJ 1.48 v. (U.S. National Institutes of Health, Maryland, USA). Using the following formula determined by Graça et al. (1993), the dry weights (dw) of the isopods were calculated: ln(dw) = 2.71 ln(length) − 4.58.

All metal treatments were combined with two predator treatments in which cues of both invertebrate and vertebrate predators were absent or present. Predators were caught in the same river as *A. aquaticus* to ensure predator recognition (Harris et al., 2013). The damselfly larvae *Calopteryx splendens* was used as the invertebrate predator. One larva was put in each container filled with 300 mL medium-hard water (US EPA, 2002) for 72 h (Janssens and Stoks, 2013). Additionally, one adult individual of the three-spined stickleback *Gasterosteus aculeatus* and the ninespine stickleback *Pungitius pungitius* were used for fish cues and were placed in a bucket with 8 L water for 24 h (based on Harris et al., 2013). After removing these animals, equal parts of these cues were mixed ((0.125 fish+1.67 damselfly larvae)/L). This stimulus water was frozen at −80 °C until needed. It retains its activity for at least two months (Pettersson et al., 2000). To add conspecific alarm cues, prior to use one *A. aquaticus* per 20 mL cue medium was homogenized. One mL of this predator cue mixture was added daily to all predator treatments. To compensate for this additional volume, 1 mL of medium-hard water was added to the non-predator stress treatments. Additionally, non-predator stress containers were taped, so that only predator treated isopods could see each other. Because *A. aquaticus* is cannibalistic, this ensured additional visual cues. General water characteristics (pH, dissolved oxygen and electrical conductivity) and mortality were monitored daily (Table 2). Immobile animals were considered dead and were removed from the solution.

At the end of the experiment, the animals were placed in medium-hard water without stressors. After acclimating for 15 min, they were filmed for 30 min. Videos were processed with the tracking program Lolitrack v.4 (Loligo Systems, Tjele, Denmark) that calculated the active time (%).

Table 2: Water chemistry measurements, presented as means (± SE) of 72 water samples at day 0. Water hardness was calculated according to the formula: 2.5 Ca + 4.1 Mg.

T (°C)	15 ± 1
pH	7.89 ± 0.02
EC (µS/cm)	404 ± 16
O_2 (mg/L)	9.34 ± 0.07
DOC (mg/L)	1.25 ± 0.07
Ca (mg/L)	15.1 ± 0.3
K (mg/L)	2.16 ± 0.04
Mg (mg/L)	13.6 ± 0.3
Na (mg/L)	37.5 ± 2.3
Water hardness (mg/L $CaCO_3$)	93.6 ± 2.2

Next, they were placed in glass chambers with oxygen minisensors in which oxygen concentrations (mg/L) were measured for 4 h using the programs WitroxView v.1.0.2 and Fibsoft v.1.0 (Loligo Systems, Tjele, Denmark). Respiration rates (RR) were calculated as follows: $RR=((O_1 - O_2)*(V - V_A))/(m_A*t)$, where O_1 is the oxygen concentration in the respiration chamber at the start of the experiment (mg/L), O_2 is the oxygen concentration in the respiration chamber at the end of the experiment (mg/L), V is the volume of the empty respiration chamber (mL), V_A is the volume of the isopod (assuming 1 mg isopod=0.001 mL) (mL), m_A is the mass of the isopod (mg wet weight) and t is the duration of the respiration experiment (h). Additionally, the growth rate (GR) and feeding rate (FR; Bloor and Banks, 2006) of each animal were determined as follows: $GR = (m_2 - m_1)/10$, where m_1 is the dry weight of the animal at day 0 (mg) and m_2 is the dry weight of the animal at day 10 (mg); and $FR = (L_1 * C_L) - L_2/m_2*10$, where L_1 is the dry weight of the *Alnus* discs initially supplied and L_2 is the dry weight

of leaf material remaining after 10 days (mg), m_2 is the dry weight of the animal at day 10 (mg) and C_L is the leaf weight change correction factor given by: $C_L = \Sigma(C_2/C_1)/N$, where C_1 in the initial dry weight of control leaves (mg), C_2 is the dry weight of the control leaves after 10 days (mg) and N is the number of control leaves. There were six control leaves per metal treatment. Finally, the animals were rinsed with Milli-Q water, wiped dry and placed in a −20 °C freezer awaiting further analysis.

4.2.2. Metal and DOC analysis

At day 0 and 10, water samples (50 mL) were taken from the containers with a syringe and filtered through a 0.20 µm filter (Chromafil, Macherey-Nagel, Düren, Germany). Next, one part was acidified to 1% HNO_3 to determine the major ions (Ca, K, Mg and Na) with an inductively coupled plasma optic emission spectrometer (ICP-OES; Thermo scientific, ICAP 6300 Duo, Waltham, MA, USA) and the trace metals (^{116}Cd, ^{65}Cu and ^{204}Pb) with a high resolution inductively coupled plasma mass spectrometer (HR-ICP-MS, Element XR, Thermo Scientific, Finnigan element 2, Bremen, Germany). The remaining water sample was acidified to pH 2 with 2N HCl and was used to quantify dissolved organic carbon with a TOC-analyzer (TOC-VCPH Shimadzu Corporation, Kyoto, Japan).

To measure the metal body concentrations of the isopods, they were dried for 72 h at 60 °C in a laboratory furnace. They were cooled in a desiccator after which they were weighed on a Sartorius SE2 ultra microbalance (accuracy of 1 µg). Three process blank samples and three samples of certified reference mussel material (SRM2976) of the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) were included. Next, all samples were digested in a solution of trace-metal-grade HNO_3 (69%) and high-purity H_2O_2 (29%) (3:1) for 1 h at 125 °C in a hot block (Environmental Express, Charleston, SC, USA). They were diluted to 3% HNO_3 and metal concentrations were measured using the HR-ICP-MS (Element XR, Thermo Scientific, Finnigan element 2, Bremen, Germany). Isopod body concentrations were expressed as µmol/g dw. Metal concentrations in the blank samples were all below quantification limits (< 0.075

µg/L for ^{116}Cd, < 0.001 µg/L for ^{65}Cu and ^{204}Pb) and the recoveries from the reference samples were consistently within 10% of the certified values for all three metals. The Windermere Humic Aqueous Model 6.0.13 (Natural Environment Research Council) was used to compute the free ion activities (FIAs) for the different metals. Dissolved metal concentrations below the detection limit were entered as the detection limit itself and 100% of the DOC was entered as fulvic acids.

4.2.3. Statistical analyses

Statistical analyses (ANCOVAs) were performed in R version 3.4.2, with metal concentration as a continuous variable and metal and predator treatment as categorical variables. Controls were added for each metal treatment. All models were first made with metal body concentrations and later compared to the model with free ion activities. The metal concentrations were log-transformed (log_{10}) to normalize distributions. Diagnostic plots were used to test the data for normality and homoscedasticity. We assessed the effects and interactions of the stressors by interpreting the interaction term and differences between slopes. If a significant difference was found ($p < 0.05$), a Tukey HSD test was conducted. Possible differences between the control treatments with and without predator stress were tested with a two-sample t-test or the Wilcoxon rank sum test. Graphs were made in Sigmaplot (Version 11.0; Systat, Chicago, IL, USA). Given the substantial amount of data, only graphs with significant trends or differences are shown. The animals that died before the end of the experiment were not included in the analyses.

4.3. Results

4.3.1. General

Mortality for the control treatments varied between 0 and 10% for the isopods that were not exposed to predator stress and between 10 and 40% for the predator exposed isopods. Other mortality rates did not exceed 20%, except for the Cd + Pb L treatment (30%) exposed to predator stress. No significant difference in mortality rate

was found between the treatments with and without predator cues. For growth rate, feeding rate, activity and respiration rate, substantial variations were observed between isopods from the same treatment, resulting in relatively low R^2. No significant three-way interactions were found.

4.3.2. Metal accumulation

Cadmium FIAs varied between 0.155 10^{-3} ± 0.009 10^{-3} µmol/L (Cd + Cu L without predator stress) and 78.0 10^{-3} ± 4.9 10^{-3} µmol/L (Cd + Cu + Pb H without predator stress; Table S1). Cadmium body burdens ranged from 1.75 10^{-3} ± 0.20 10^{-3} µmol/g dw (Cd + Pb L with predator stress) to 0.432 ± 0.057 µmol/g dw (Cd H with predator stress; Table S2). In general, we found a positive correlation between body concentration and FIA_{water} ($F = 394$, $p < 0.001$). Additionally, there was a significant interaction between body burden and metal treatment ($F = 4.64$, $p = 0.003$), indicating significant differences in slopes between the mixture treatments (Table 3, Fig. 1A). The single Cd treatment had a significantly larger slope than Cd + Cu ($t = -3.20$, $p = 0.001$) and Cd + Cu + Pb ($t = -2.73$, $p = 0.007$), suggesting an antagonistic interaction between those metals on Cd uptake. Also Cd + Cu and Cd + Pb had a significantly different slope ($t = 2.45$, $p = 0.015$). No interaction was found between body burden and predator treatment. Although the ANCOVA model indicated significant differences between the isopods with and without predator exposure, a Tukey HSD test revealed no significance.

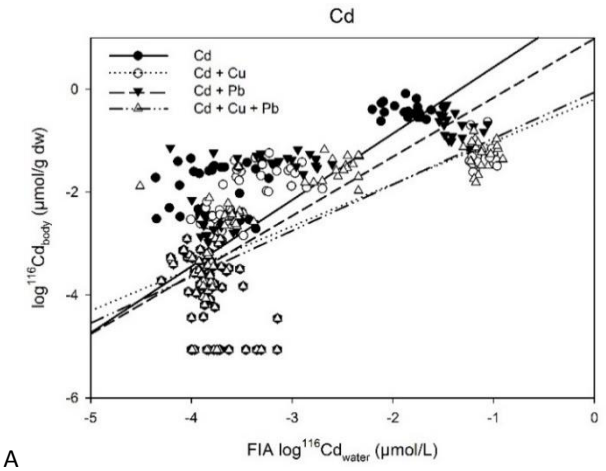
Copper FIAs varied between 0.188 10^{-6} ± 0.082 10^{-6} µmol/L (Cu + Cd + Pb L without predator stress) and 0.169 ± 0.008 µmol/L (Cu + Cd + Pb H with predator stress; Table S3), while body concentrations varied between 0.753 ± 0.074 µmol/g dw (Cu L without predator stress) to 8.26 ± 1.32 µmol/g dw (Cu H without predator stress; Table S4). A positive correlation between FIA_{water} and the Cu body burdens was found ($F = 897$, $p < 0.001$), but no significant differences in slopes were found between the metal treatments. However, there was an interaction between Cu exposure and predator treatment ($F = 8.19$, $p = 0.004$), resulting in a significantly larger slope of Cu

accumulation for *Asellus* exposed to predator stress (Fig. 1B). However, the difference in slopes between the treatments with and without predator cues is small.

Table 3: Slopes for metal accumulation, calculated based on FIA_{water}, after 10 days per metal and predator treatment (for Cd: N = 287; for Cu: N = 293; for Pb: N = 292). Average slopes and standard errors are presented, together with R^2 values for the ANCOVA model. Superscript lower case letters indicate statistical differences between treatments.

Metal	Mixture with	$Slope_{accumulation}$ (log(µmol/g dw). $log(µmol/L)^{-1}$)	Predator stress	$Slope_{accumulation}$ (log(µmol/g dw). $log(µmol/L)^{-1}$)	R^2
Cd	-	1.29 ± 0.13 [a]	Without	0.922 ± 0.063 [a]	47.0%
	Cu	0.821 ± 0.087 [b]	With	1.05 ± 0.079 [a]	
	Pb	1.15 ± 0.10 [a]			
	Cu + Pb	0.898 ± 0.076 [b]			
Cu	-	0.113 ± 0.008 [a]	Without	$98.1\ 10^{-3} \pm 5.0\ 10^{-3}$ [a]	65.3%
	Cd	$96.0\ 10^{-3} \pm 6.6\ 10^{-3}$ [a]	With	0.118 ± 0.005 [b]	
	Pb	0.106 ± 0.008 [a]			
	Cd + Pb	0.104 ± 0.006 [a]			
Pb	-	0.738 ± 0.043 [a]	Without	0.655 ± 0.021 [a]	78.6%
	Cd	0.729 ± 0.036 [a]	With	0.684 ± 0.025 [a]	
	Cu	0.632 ± 0.027 [b]			
	Cd + Cu	0.623 ± 0.026 [b]			

For Pb, FIAs ranged from $24.8\ 10^{-9} \pm 6.6\ 10^{-9}$ µmol/L (Pb L without predator stress) to $10.5\ 10^{-3} \pm 1.1\ 10^{-3}$ µmol/L (Pb + Cd + Cu H without predator stress; Table S5) and body concentrations from $27.8\ 10^{-3} \pm 8.7\ 10^{-3}$ µmol/g dw (Pb + Cu L without predator stress) to 6.33 ± 1.51 µmol/g dw (Pb + Cd H without predator stress; Table S6). Again, body burden increased when FIA_{water} increased ($F = 1750$, $p < 0.001$). While there was no interaction of body concentrations with predator stress, we did find an interaction with metal treatment ($F = 3.82$, $p = 0.01$) (Table 3).



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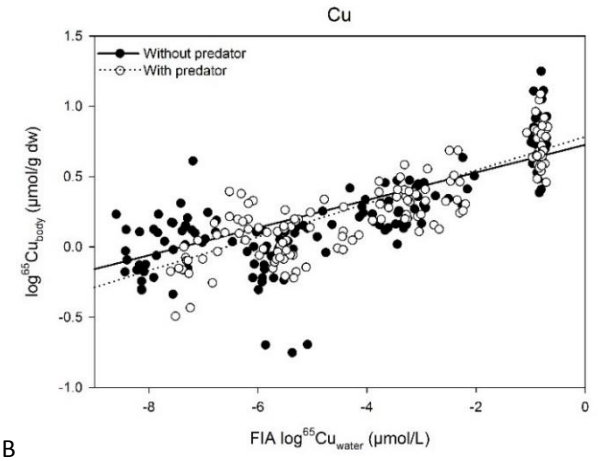

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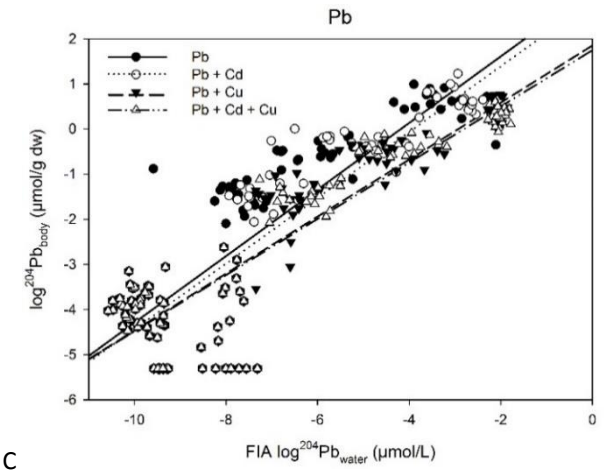


C

Figure 1: Metal body concentrations after 10 days in function of the free ion activities (FIAs): A) Cd metal treatments (N = 287); B) Cu predator treatments (N = 293); and C) Pb metal treatments (N = 292).

Significantly larger slopes for the single Pb and Pb + Cd treatment were found than for Pb + Cu ($t = -2.39$, $p = 0.017$ and $t = -2.16$, $p = 0.03$, respectively) and the tertiary mixture ($t = -2.60$, $p = 0.009$ and $t = -2.38$, $p = 0.018$, respectively), which indicates an antagonistic interaction for the latter metal mixtures (Fig. 1C). The ANCOVA model indicated a significant difference in Pb body burden between the treatments with and without predator stress, but this was not confirmed by a Tukey HSD test.

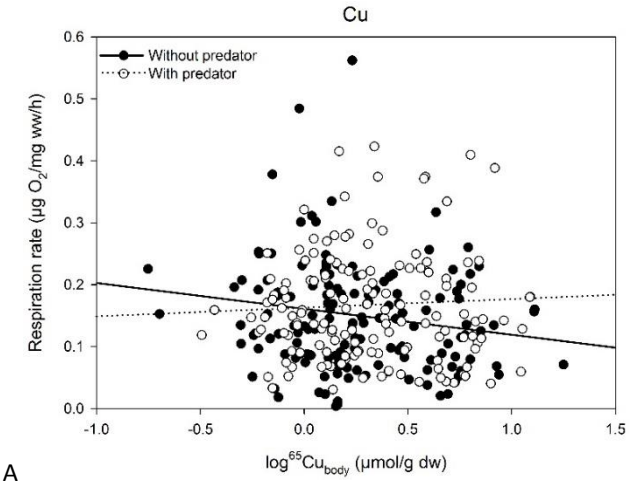
4.3.3. Respiration rate

Respiration rates varied from 0.106 ± 0.022 (Cu M without predator stress) to 0.311 ± 0.056 µg O_2/mg ww/h (Cd L without predator stress) (Table S7). The ANCOVA model comparing respiration rates to metal body concentrations revealed no significant differences between the Cd treatments (Table 4). For Cu and Pb, only a significant difference in respiration was found between the treatments with and without predator stress ($F = 4.07$, $p = 0.044$ and $F = 4.04$, $p = 0.045$, respectively). Copper isopods exposed to predation stress respired 0.018 µg O_2/mg ww/h or 12% more (Fig. 2A).

For Pb, the respiration rate was 0.017 O_2/mg ww/h or 11% higher (Fig. 2B). We investigated whether there was a stronger link with the metal FIAs in the water, but no significances were found for the three metals. Furthermore, no significant differences were observed between the control treatments with and without predator cues.

Table 4: Slopes for respiration rates, calculated based on body concentrations, after 10 days (N = 293). Average slopes and standard errors are presented, together with R^2 values for the ANCOVA model. Superscript lower-case letters indicate statistical differences between treatments.

Metal	Mixture with	$Slope_{respiration\ rate}$ ($\mu g\ O_2.mg\ ww^{-1}.h^{-1}.\ log(\mu mol/g\ dw)^{-1}$)	Predator stress	$Slope_{respiration\ rate}$ ($\mu g\ O_2.mg\ ww^{-1}.h^{-1}.\ log(\mu mol/g\ dw)^{-1}$)	R^2
Cd	**-**	$0.418\ 10^{-3} \pm 6.853\ 10^{-3}$ [a]	Without	$-7.04\ 10^{-3} \pm 5.03\ 10^{-3}$ [a]	2.87%
	Cu	$-9.35\ 10^{-3} \pm 6.39\ 10^{-3}$ [a]	With	$-0.489\ 10^{-3} \pm 4.451\ 10^{-3}$ [a]	
	Pb	$2.00\ 10^{-3} \pm 7.02\ 10^{-3}$ [a]			
	Cu + Pb	$-10.2\ 10^{-3} \pm 6.4\ 10^{-3}$ [a]			
Cu	-	$-17.1\ 10^{-3} \pm 22.4\ 10^{-3}$ [a]	Without	$-31.1\ 10^{-3} \pm 17.2\ 10^{-3}$ [a]	2.21%
	Cd	$-20.0\ 10^{-3} \pm 25.4\ 10^{-3}$ [a]	With	$14.0\ 10^{-3} \pm 16.8\ 10^{-3}$ [a]	
	Pb	$3.49\ 10^{-3} \pm 24.52\ 10^{-3}$ [a]			
	Cd + Pb	$-2.02\ 10^{-3} \pm 25.43\ 10^{-3}$ [a]			
Pb	-	$-2.46\ 10^{-3} \pm 4.04\ 10^{-3}$ [a]	Without	$-4.78\ 10^{-3} \pm 2.86\ 10^{-3}$ [a]	2.25%
	Cd	$2.72\ 10^{-3} \pm 4.69\ 10^{-3}$ [a]	With	$0.324\ 10^{-3} \pm 3.088\ 10^{-3}$ [a]	
	Cu	$-3.29\ 10^{-3} \pm 4.14\ 10^{-3}$ [a]			
	Cd + Cu	$-5.78\ 10^{-3} \pm 3.99\ 10^{-3}$ [a]			



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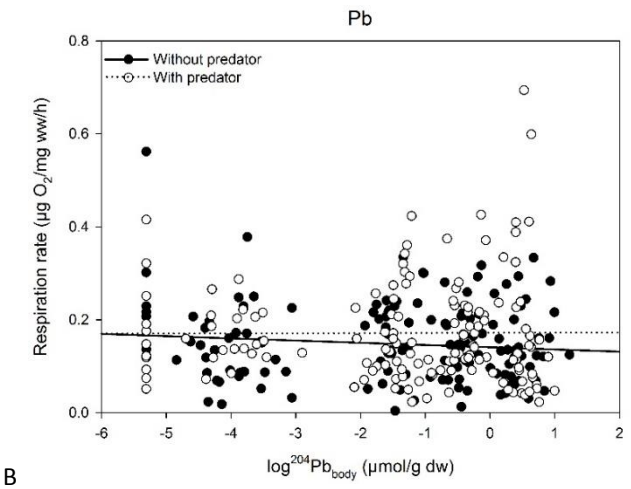


B

Figure 2: Respiration rates after 10 days in function of the metal body concentrations: A) Cu predator treatments (N = 287); and B) Pb predator treatments (N = 285).

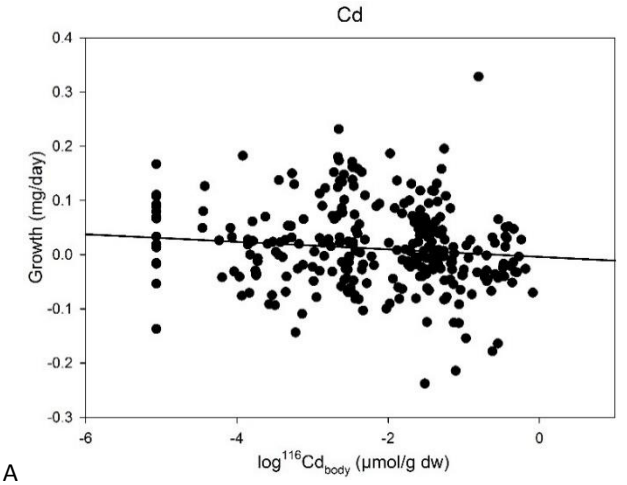
4.3.4. Growth rate

The highest average growth rate found for the three metals was 0.106 ± 0.028 mg/day (Cd + Cu + Pb L with predator stress), the lowest was $-62.5\ 10^{-3} \pm 19.2\ 10^{-3}$ mg/day (Cu H without predator stress) (Table S8). For the control treatments, no significant difference in growth rate was found between isopods with and without predator stress. A negative correlation ($F = 7.02$, $p = 0.008$) was found between Cd body burden and growth, but no interactions with metal or predator treatment were detected (Table 5, Fig. 3A).

Growth significantly decreased with increasing Cu body burden as well ($F = 43.2$, $p < 0.001$). There were no interactions of Cu body burden with metal treatment, but there was an interaction with predator treatment (Fig. 3B), resulting in a significantly lower slope for the growth rate of isopods exposed to predator stress ($t = -2.08$, $p = 0.038$).

Table 5: Slopes for growth rates, calculated based on body concentrations, after 10 days per predator treatment (N = 293). Average slopes and standard errors are presented, together with R^2 values for the ANCOVA model. Superscript lower-case letters indicate statistical differences between treatments.

Metal	Mixture with	$Slope_{growth\ rate}$ (mg dw.day^{-1}. log(µmol/g dw)$^{-1}$)	Predator stress	$Slope_{growth\ rate}$ (mg dw.day^{1}. log(µmol/g dw)$^{-1}$)	R^2
Cd	-	$-5.21\ 10^{-3} \pm 4.64\ 10^{-3}$ a	Without	$-12.1\ 10^{-3} \pm 3.7\ 10^{-3}$ a	4.18%
	Cu	$-13.4\ 10^{-3} \pm 5.1\ 10^{-3}$ a	With	$-2.62\ 10^{-3} \pm 3.80\ 10^{-3}$ a	
	Pb	$-4.93\ 10^{-3} \pm 5.26\ 10^{-3}$ a			
	Cu + Pb	$-5.64\ 10^{-3} \pm 6.31\ 10^{-3}$ a			
Cu	-	$-94.1\ 10^{-3} \pm 19.4\ 10^{-3}$ a	Without	$-49.8\ 10^{-3} \pm 13.8\ 10^{-3}$ a	12.1%
	Cd	$-46.0\ 10^{-3} \pm 21.5\ 10^{-3}$ a	With	$-93.4\ 10^{-3} \pm 16.2\ 10^{-3}$ b	
	Pb	$-61.2\ 10^{-3} \pm 19.7\ 10^{-3}$ a			
	Cd + Pb	$-74.9\ 10^{-3} \pm 23.7\ 10^{-3}$ a			
Pb	-	$-3.67\ 10^{-3} \pm 3.37\ 10^{-3}$ a	Without	$-1.10\ 10^{-3} \pm 2.40\ 10^{-3}$ a	2.75%
	Cd	$-1.31\ 10^{-3} \pm 3.51\ 10^{-3}$ a	With	$-6.04\ 10^{-3} \pm 2.65\ 10^{-3}$ a	
	Cu	$-7.73\ 10^{-3} \pm 3.47\ 10^{-3}$ a			
	Cd + Cu	$-1.45\ 10^{-3} \pm 3.92\ 10^{-3}$ a			



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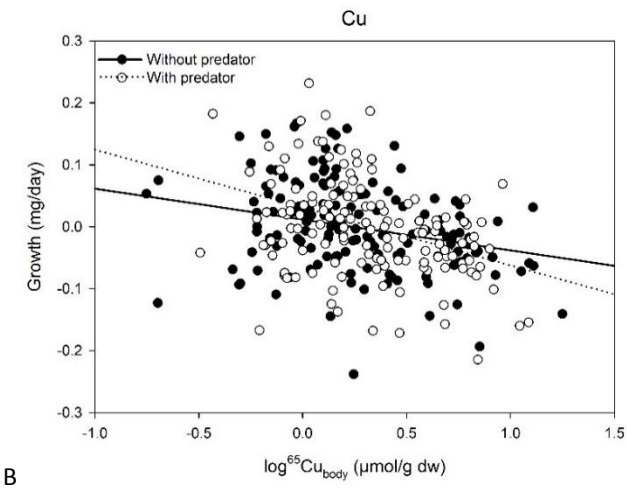

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Figure 3: Growth rates after 10 days in function of the metal body concentrations: A) Cd (N = 287); and B) Cu predator treatments (N = 293).

For Pb, no significant correlation or interactions with metal or predator treatment were found between body burden and growth rate. We found similar results for the Cd and Cu FIA_{water} model. For Pb, however, there was a significant negative correlation between growth rate and Pb FIA_{water} ($F=12.0$, $p< 0.001$).

4.3.5. Feeding rate

We found a significant difference between the feeding rates for the control treatments with and without predator cues: isopods that were not exposed to predator cues fed

0.035 mg/mg/day or 84% more than predator exposed isopods (W = 843, p = 0.005). Feeding rates ranged from 0 ± 0 (Cu + Pb H) to 0.358 ± 0.088 mg/mg/day (Cd + Pb M without predator stress) (Table S9).

While for Cd no significant relation was found for body concentrations and feeding rate, there was a significant interaction between Cd body burden and metal treatment (F = 4.48, p = 0.004) (Table 6, Fig. 4A). The slope of feeding rate for Cd + Pb was significantly higher than for the other treatments, caused by a significant increase in feeding rate for *Asellus* exposed to the Cd + Pb M concentrations (for Cd: t = 2.78, p = 0.006; for Cd + Cu: t = 3.34, p < 0.001; for Cd + Cu + Pb: t = 2.50, p = 0.013). There was no significant difference in slopes between the predator treatments, but isopods that were not exposed to predator stress generally fed 0.028 mg/mg/day or 53% more than those with predator stress (F = 16.3, p < 0.001) (Fig. 4B). We found similar significant effects for the Cd FIA_{water} model. However, here we did find a significant negative correlation between Cd FIA_{water} concentrations and feeding rate (F = 35.5, p < 0.001). Furthermore, we also observed other significant differences in slopes between metal treatments. The slope of Cd + Cu + Pb was less negative (0.001 ± 0.007) than the slopes for the other metal treatments (for Cd: −0.037 ± 0.009, t = −3.26, p = 0.001; for Cd + Cu: −0.024 ± 0.004, t = −2.53, p = 0.012; for Cd + Pb: −0.032 ± 0.008, t = −3.13, p = 0.002).

For Cu, we found a significant negative correlation between body burden and feeding rate (F = 44.3, p < 0.001). Only Cu + Cd + Pb had a significantly larger slope than the other treatments (for Cu: t = −2.54, p = 0.011; for Cu + Cd: t = −3.07, p = 0.002; for Cu + Pb: t = −2.39, p = 0.017), suggesting an antagonistic interaction between the three metals (Fig. 4C). Isopods that were not exposed to predator stress fed 0.023 mg/g/day or 49% more (Fig. 4D) and the slope of their feeding rate decreased more with increasing body burden (t = 2.00, p = 0.046) compared to *Asellus* treatments with predator cues. Again, the same significances were observed for the Cu FIA_{water} model.

Only the significant difference in slopes between Cu + Cd and Cu + Cd + Pb was not found.

Table 6: Slopes for feeding rates, calculated based on body concentrations, after 10 days per metal or predator treatment (for Cd: N = 287; for Cu: N = 293; for Pb: N = 292). Average slopes and standard errors are presented, together with R^2 values for the ANCOVA model. Superscript lower-case letters indicate statistical differences between treatments.

Metal	Mixture with	$Slope_{feeding\ rate}$ ($mg.mg^{-1}.day^{-1}.log(\mu mol/g\ dw)^{-1}$)	Predator stress	$Slope_{feeding\ rate}$ ($mg.mg^{-1}.day^{-1}.log(\mu mol/g)^{-1}$)	R^2
Cd	-	$-0.462\ 10^{-3} \pm 4.298\ 10^{-3\ a}$	Without	$1.50\ 10^{-3} \pm 4.60\ 10^{-3\ a}$	10.2%
	Cu	$-5.77\ 10^{-3} \pm 3.85\ 10^{-3\ a}$	With	$5.75\ 10^{-3} \pm 2.71\ 10^{-3\ a}$	
	Pb	$19.1\ 10^{-3} \pm 7.8\ 10^{-3\ b}$			
	Cu + Pb	$0.202\ 10^{-3} \pm 3.617\ 10^{-3\ a}$			
Cu	-	$-58.4\ 10^{-3} \pm 14.0\ 10^{-3\ a}$	Without	$-60.3\ 10^{-3} \pm 10.6\ 10^{-3\ a}$	14.9%
	Cd	$-72.4\ 10^{-3} \pm 15.0\ 10^{-3\ a}$	With	$-28.5\ 10^{-3} \pm 9.7\ 10^{-3\ b}$	
	Pb	$-56.6\ 10^{-3} \pm 15.6\ 10^{-3\ a}$			
	Cd + Pb	$-3.84\ 10^{-3} \pm 14.26\ 10^{-3\ b}$			
Pb	-	$1.75\ 10^{-3} \pm 2.97\ 10^{-3\ a}$	Without	$-0.136\ 10^{-3} \pm 2.95\ 10^{-3\ a}$	9.93%
	Cd	$11.7\ 10^{-3} \pm 5.2\ 10^{-3\ b}$	With	$4.24\ 10^{-3} \pm 1.87\ 10^{-3\ a}$	
	Cu	$-3.92\ 10^{-3} \pm 2.81\ 10^{-3\ a}$			
	Cd + Cu	$-2.76\ 10^{-3} \pm 2.24\ 10^{-3\ a}$			

Lead feeding rates showed no significant decrease in feeding rate with increasing body concentration, but we found a significantly larger slope for Pb + Cd than for the other treatments (for Pb: $t = -2.08$, $p = 0.038$; for Pb + Cu: $t = -3.19$, $p = 0.002$; for Pb + Cd + Cu: $t = -2.91$, $p = 0.004$) (Fig. 4E). Furthermore, asellids that were not exposed to predator stress again had a significantly higher feeding rate (Fig. 4F): they fed 0.032 mg/mg/day or 59% more ($F = 20.4$, $p < 0.001$). The FIA_{water} model only showed the significant difference in feeding rates between isopods with and without predator stress ($F = 19.7$, $p < 0.001$).

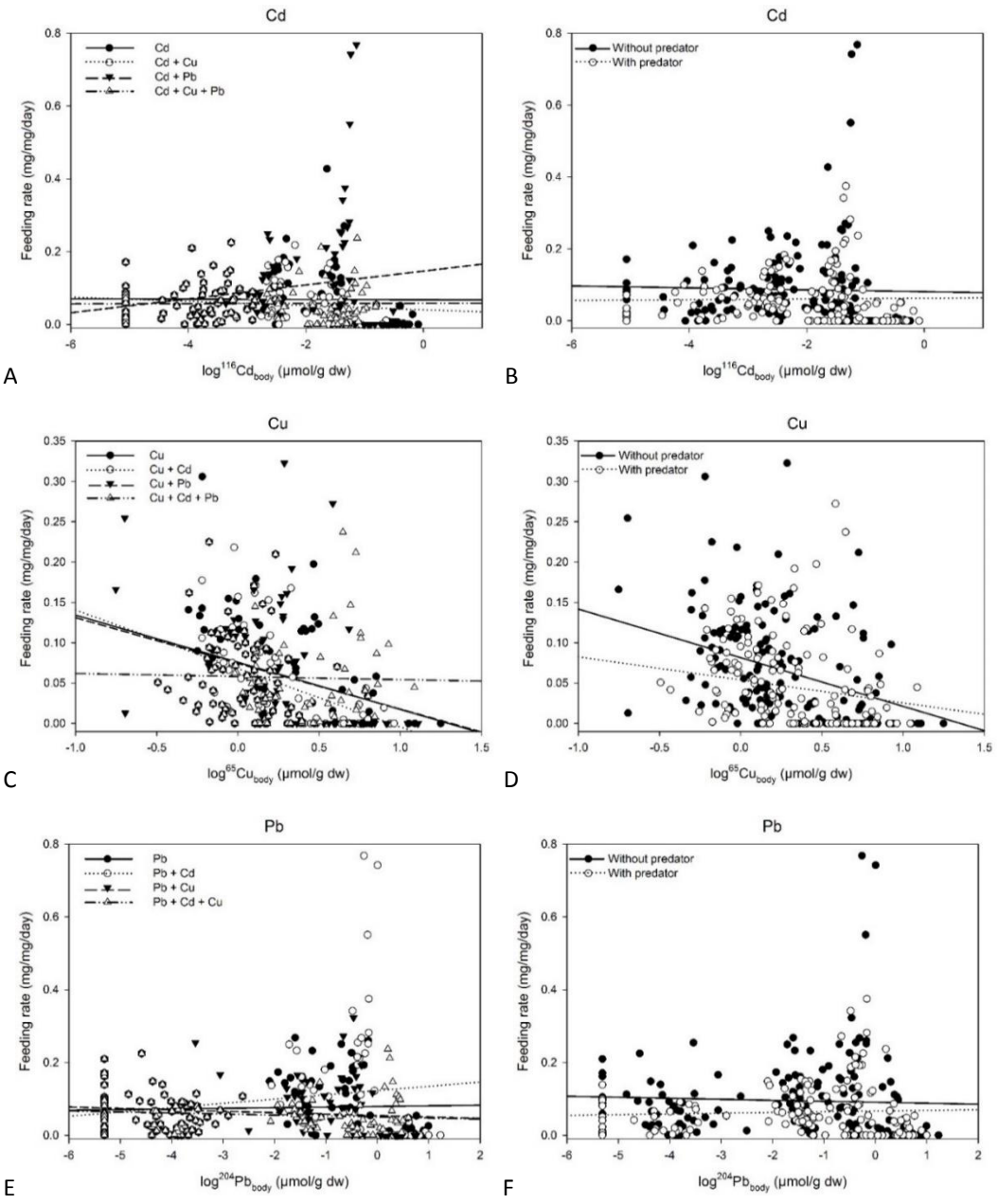


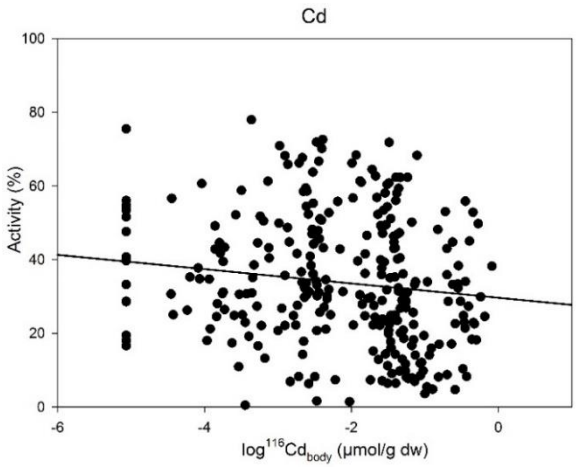
Figure 4: Feeding rates after 10 days in function of the metal body concentrations: A-F) metal and predator treatments for the three metals (for Cd: N = 287; for Cu: N = 293; and for Pb: N = 292).

4.3.6. Activity

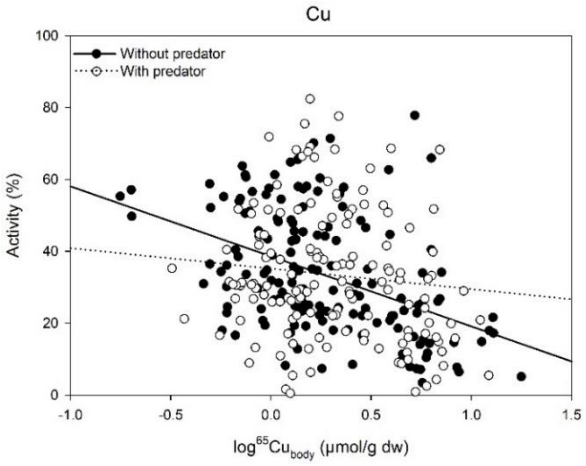
No significant difference in activity was found between the controls with and without predator exposure. Activity ranged from 15.7 ± 2.8 (Cd + Pb H without predator stress) to 53.1 ± 2.6% (Cd M without predator stress) (Table S10). For the three metals, activity decreased with increasing body burden (for Cd: $F = 12.9$, $p < 0.001$; for Cu: $F = 37.8$, $p < 0.001$; and for Pb: $F = 17.8$, $p < 0.001$) (Table 7, Fig. 5). Also for Cu, the slope of activity for isopods with predator stress decreased less than for the isopods without predator exposure ($t = 3.16$, $p = 0.002$). No other significant interactions were observed. For the FIA_{water} models, only significant negative correlations between FIA_{water} and activity were found (for Cd: $F = 32.7$, $p < 0.001$; for Cu: $F = 22.9$, $p < 0.001$; and for Pb: $F = 17.9$, $p < 0.001$).

Table 7: Slopes for activities, calculated based on body concentrations, after 10 days per predator treatment (N = 293). Average slopes and standard errors are presented, together with R^2 values for the ANCOVA model. Superscript lower-case letters indicate statistical differences between treatments.

Metal	Mixture with	$Slope_{activity}$ (%.log(µmol/g dw)$^{-1}$)	Predator stress	$Slope_{activity}$ (%.log(µmol/g dw)$^{-1}$)	R^2
Cd	-	-0.253 ± 0.995 [a]	Without	-0.767 ± 0.787 [a]	6.23%
	Cu	-2.81 ± 1.10 [a]	With	-3.04 ± 0.77 [a]	
	Pb	-2.21 ± 1.12 [a]			
	Cu + Pb	-3.34 ± 1.20 [a]			
Cu	-	-5.73 ± 4.32 [a]	Without	-19.5 ± 2.65 [a]	10.8%
	Cd	-17.3 ± 4.5 [a]	With	-5.70 ± 3.56 [b]	
	Pb	-16.3 ± 4.1 [a]			
	Cd + Pb	-15.0 ± 4.7 [a]			
Pb	-	-1.06 ± 0.70 [a]	Without	-1.74 ± 0.49 [a]	4.45%
	Cd	-1.16 ± 0.75 [a]	With	-1.33 ± 0.55 [a]	
	Cu	-1.96 ± 0.74 [a]			
	Pb	-2.04 ± 0.75 [a]			



A



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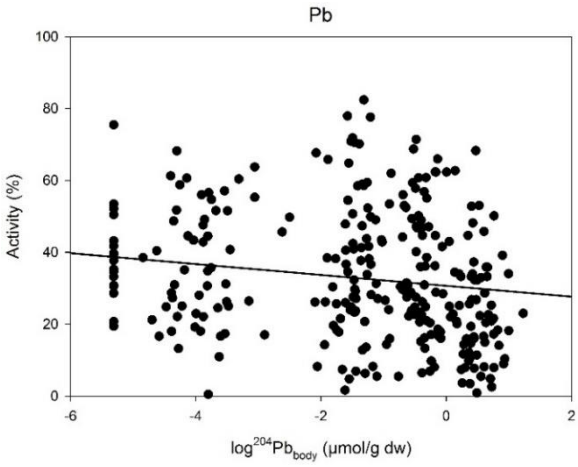

C

Figure 5: Activities after 10 days in function of the metal body concentrations: A) Cd (N = 287); B) Cu predator treatments (N = 293); and C) Pb (N = 292).

4.4. Discussion

We observed changes caused by either metal or predator stress for all endpoints. In several cases, the effects of predator stress were affected by the accumulated metals, thus providing strong evidence for stressor interactions. For the control treatments we only found a significant difference in feeding rate between asellids with and without predator exposure, which was expected as prey will avoid movement to reduce the risk to be detected by the perceived predator. We also expected a significantly lower activity for the predator exposed isopods, but this was not observed. The animals were transferred to water without predator cues for filming. Therefore, this is most likely caused due to the absence of the stressor.

Correlations between metal body burdens and FIAs resulted in relatively high R^2. Part of the variability can be explained by binding of the metals to the leaf discs, resulting in a secondary uptake route through ingestion. Consistent with the finding of our previous study (Van Ginneken et al., 2015), the present study observed for the Cd and Pb treatments significantly higher body concentrations for the single metals and Pb + Cd than for the other mixtures. Their slopes decreased approximately by a factor of 1.3. Synergistic interactions of Cd and Pb have already been reported and are most likely caused by combined disturbances of Ca^{2+} and Na^{+} homeostasis (Birceanu et al., 2008). Though the slopes of the Cd and Pb mixture were similar to the ones for the single metals. Therefore, it is more logical that Cd + Cu, Pb + Cu and Cd + Cu + Pb interacted antagonistically. An et al. (2004) also found a decreased toxicity of a tertiary mixture of Cd, Cu and Pb for *Cucumis sativus*. Both Cu and Pb are known to compete for the same uptake sites, namely via Na^{+} channels, so an antagonistic interaction is expected. Cadmium, however, has a different mode of action and enters via Ca^{2+} channels. Barata et al. (2006) demonstrated a less than additive toxicity of mixtures of Cd and Cu for *Daphnia magna* and interpreted their findings as the result of both metals being metallothionein inducers. Another explanation could be that these metals share a common uptake site and that this inhibited their uptake. Komjarova and Blust (2008) suggested Divalent Metal Transporter 1 (DMT1), an Fe^{2+} transporter.

Although no effects of predator stress were seen for Cd and Pb accumulation, we did find a significantly higher slope for Cu accumulation. The difference is small, but could be explained by the increased respiration rate we observed for isopods exposed to predator cues. An increased respiration rate was also found for Pb, but this did not result in an altered slope for Pb accumulation. An increase in oxygen consumption when exposed to predator cues is often observed (Beckerman et al., 2007; Slos and Stoks, 2008). However, we did not see an increase for Cd. A possible explanation could be that costs associated with repair processes were masked by reducing other metabolic costs, e.g. associated with locomotion (Knops et al., 2001). Furthermore, no studies could be found that investigated the effect of predator cues on accumulation, but e.g., Qin et al. (2011) found significantly lower LC values for *Ceriodaphnia dubia* when exposed to fipronil and predator cues, which could be the result of an increased body concentration. On the other hand, Qin et al. (2015) found that predator stress and silver nanoparticles interacted antagonistically for the LC_{50}s of *Daphnia magna*, indicating that effects can vary depending on factors such as the predator-prey model and the chemicals involved (Qin et al., 2011).

For Cd, a negative correlation was found between body burden and growth rate, but feeding rate did not decrease significantly (when linked to the body concentrations). This lower food conversion efficiency has been reported before. After exposing *Enallagma cyathigerum* to predator stress, Janssens and Stoks (2013) reported significantly lower growth rates, while food intake for pesticide treatments even increased. Again, this can be caused by a trade-off: by converting less food into biomass and investing more in defense mechanisms and strategies (Preisser et al., 2005).

In the literature, a reduced feeding rate is most often reported after exposure to pollutants (Bloor and Banks, 2006; Maltby et al., 2002). Yet, for the medium mixture of Cd and Pb, a very steep increase was observed for feeding rate, resulting in a slope that was increased by a factor of 6 minimally. For the highest concentrations of this mixture, feeding rates again decreased drastically. This mixture of cadmium and lead

did result in a high accumulation for both metals, but no concentration-dependence was observed nor were any other effects on endpoints. Van Ginneken et al. (2015) found a synergistic interaction resulting in a higher mortality rate and a lower growth rate. However, the concentrations of Cd used in that study were in general much higher (0.120 – 1.09 µmol/L) than the ones used in the present study. So, the interactions of these two metals did not only depend on the endpoint, but also seemed to depend on the exposure concentrations. Concentration-dependent interactions have been reported before for metals (Jonker et al., 2005). Sharma et al. (1999) studied the effects of Cd, Cu and Zn on the root growth of the plant *Silene vulgaris* and found non-additive or antagonistic responses for low concentrations. However, if one of the components in the mixture exceeded a certain level of toxicity, synergistic interactions were observed. It is possible that the medium concentrations we used triggered a range of energetically costly defense mechanisms, which had to be fuelled by a higher feeding rate, but that after some critical point these mechanisms (metallothioneins, antioxidant enzymes) became too costly to maintain. Further investigations are required to evaluate the effects on biochemical markers. When Cu was added to a mixture of Cd and Pb, there was no longer a significant increase in feeding rate. This is reflected in the accumulation data, where the tertiary mixture produced an antagonistic effect on the uptake of Cd and Pb. This antagonistic interaction was also observed for the feeding rates of Cu, where negative slopes for the single Cu treatment and the binary mixtures were at least a factor of 14 lower compared to the feeding rate of the tertiary mixture.

A lower foraging rate reduces the risk of being detected by a predator. This is evidenced by the Cd and Pb feeding rates of the predator exposed asellids. Yet, no significant differences in growth rate between the predator treatments for Cd and Pb were found, which could be an indication of a higher predator-induced growth efficiency. Normally, as explained above, lower growth efficiencies are reported. However, this higher growth efficiency for *Asellus* could be advantageous for two reasons. Firstly, their reproductive success is positively correlated with size (Bloor,

2010). The presence of predator cues could have triggered the isopods to invest more in reproduction. However, *A. aquaticus* forms precopula pairs, which swim more slowly and are more visible. This makes them far more likely to be consumed by a predator. Dunn et al. (2008), for example, observed that pair formation for *Gammarus duebeni* declined when predator cues were present. Thus, it is more likely that growth efficiency increased due to another reason. An alternative explanation is that larger isopods move faster and have more chance to escape than smaller individuals as swimming speed could be positively correlated to length or body weight (Eroukhmanoff and Svensson, 2009; Takeuchi and Watanabe, 1998).

Although we observed a negative correlation between activity and Cu body burden, we found that the slope for predator exposed isopods decreased less (by a factor of 3.4), meaning that Cu and predator stress acted antagonistically. The same can be seen for feeding rate, where the negative slope was also increased by a factor of 2 in the presence of a predator. As (foraging) activity increased for predator exposed isopods when Cu body burdens were increased, they were most likely more susceptible to predation. Clements et al. (1989) also observed that two species of caddisflies were significantly predated more in streams dosed with 0.094 µmol/L copper. Also for crustaceans, copper can impair responses to female odors or food (Krång and Ekerholm, 2006; Sherba et al., 2000). McIntyre et al. (2012) found that juvenile coho salmon (*Oncorhynchus kisutch*) exposed to low levels of copper (< 0.315 µmol/L) were unresponsive to conspecific alarm cues, therefore being more vulnerable to predatory attacks. For fish, Cu is known to inhibit physiological responsiveness, and even cause cell death, of olfactory receptor neurons (Baldwin et al., 2003; Hansen et al., 1999). The less sensitive chemoreceptor cells of Crustacea are possibly similarly affected (Olsén, 2010). Scott et al. (2003) found that Cd could also disrupt predator avoidance behaviors in juvenile rainbow trout, but this was not found in the present study. While the mechanisms of toxicity of these metals remain largely unclear, they are believed to interfere predominantly with the internal signaling pathways (Lürling and Scheffer, 2007).

A limitation of our study that needs to be acknowledged is the high variation between individuals, resulting in relatively low R^2. Despite these low R^2, a remarkable number of clear trends and significant differences between treatments became apparent. We found a lower growth rate for Cd, a decreased feeding rate for Cd and Pb predator stress treatments and a higher respiration rate for Cu and Pb predator stress treatments. Additionally, we also demonstrated that predator cues affected the slopes for the accumulation, growth rate, feeding rate and activity of the Cu treatments. It remains an open question whether these, sometimes small, differences could cause severe changes to the ecosystem. Yet, we must consider that *Asellus aquaticus* is an important decomposer. There is evidence that high accumulated concentrations of Cu cause a disruption of olfactory responses. As a result, they seem less able to detect predator cues and most likely will also struggle with finding food and mating partners. Additionally, it was demonstrated that cadmium and lead can interact synergistically for mortality and growth rate (Van Ginneken et al., 2015) and in the present study we found that they interact in a concentration-dependent manner for feeding rate as well. Consequently, these metals and their interactions with predator cues could cause serious alterations to the food web. Together these findings show that it is essential to consider the interactions of abiotic and biotic stressors in environmental risk assessment.

4.5. Acknowledgments

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4.6. Supplementary material

Table S1: Average ^{116}Cd FIAs (± SE) after 10 days for all metal and predator treatments (N = 287).

Metal	Concentration	^{116}Cd FIAs (µmol/L)	
		Without predator stress	**With predator stress**
Controls		$0.147\ 10^{-3} \pm 0.017\ 10^{-3}$	$0.216\ 10^{-3} \pm 0.026\ 10^{-3}$
Cd	L	$0.164\ 10^{-3} \pm 0.043\ 10^{-3}$	$0.198\ 10^{-3} \pm 0.043\ 10^{-3}$
	M	$0.190\ 10^{-3} \pm 0.060\ 10^{-3}$	$0.293\ 10^{-3} \pm 0.069\ 10^{-3}$
	H	$11.7\ 10^{-3} \pm 1.7\ 10^{-3}$	$18.0\ 10^{-3} \pm 2.0\ 10^{-3}$
Cd + Cu	L	$0.155\ 10^{-3} \pm 0.009\ 10^{-3}$	$0.216\ 10^{-3} \pm 0.017\ 10^{-3}$
	M	$0.664\ 10^{-3} \pm 0.112\ 10^{-3}$	$0.811\ 10^{-3} \pm 0.190\ 10^{-3}$
	H	$75.0\ 10^{-3} \pm 5.7\ 10^{-3}$	$73.2\ 10^{-3} \pm 5.3\ 10^{-3}$
Cd + Pb	L	$0.164\ 10^{-3} \pm 0.026\ 10^{-3}$	$0.224\ 10^{-3} \pm 0.035\ 10^{-3}$
	M	$0.742\ 10^{-3} \pm 0.181\ 10^{-3}$	$1.29\ 10^{-3} \pm 0.022\ 10^{-3}$
	H	$42.4\ 10^{-3} \pm 6.0\ 10^{-3}$	$46.0\ 10^{-3} \pm 5.9\ 10^{-3}$
Cd + Cu + Pb	L	$0.242\ 10^{-3} \pm 0.017\ 10^{-3}$	$0.293\ 10^{-3} \pm 0.017\ 10^{-3}$
	M	$2.61\ 10^{-3} \pm 0.56\ 10^{-3}$	$3.36\ 10^{-3} \pm 0.32\ 10^{-3}$
	H	$78.0\ 10^{-3} \pm 4.9\ 10^{-3}$	$77.5\ 10^{-3} \pm 8.0\ 10^{-3}$

Table S2: Average ^{116}Cd body burdens (± SE) after 10 days for all metal and predator treatments (N = 287).

Metal	Concentration	^{116}Cd body concentrations (µmol/g dw)	
		Without predator stress	**With predator stress**
Controls		$1.26\ 10^{-3} \pm 0.79\ 10^{-3}$	$0.276\ 10^{-3} \pm 0.060\ 10^{-3}$
Cd	L	$4.50\ 10^{-3} \pm 0.76\ 10^{-3}$	$3.42\ 10^{-3} \pm 0.35\ 10^{-3}$
	M	$31.1\ 10^{-3} \pm 4.1\ 10^{-3}$	$28.1\ 10^{-3} \pm 1.6\ 10^{-3}$
	H	0.397 ± 0.036	0.432 ± 0.057
Cd + Cu	L	$3.49\ 10^{-3} \pm 0.423\ 10^{-3}$	$4.01\ 10^{-3} \pm 1.33\ 10^{-3}$
	M	$27.4\ 10^{-3} \pm 4.7\ 10^{-3}$	$24.1\ 10^{-3} \pm 4.0\ 10^{-3}$
	H	$53.0\ 10^{-3} \pm 6.0\ 10^{-3}$	$79.5\ 10^{-3} \pm 23.5\ 10^{-3}$
Cd + Pb	L	$2.41\ 10^{-3} \pm 0.62\ 10^{-3}$	$1.75\ 10^{-3} \pm 0.20\ 10^{-3}$
	M	$48.7\ 10^{-3} \pm 5.5\ 10^{-3}$	$42.6\ 10^{-3} \pm 2.9\ 10^{-3}$
	H	0.228 ± 0.041	0.217 ± 0.069
Cd + Cu + Pb	L	$4.37\ 10^{-3} \pm 0.60\ 10^{-3}$	$2.90\ 10^{-3} \pm 0.24\ 10^{-3}$
	M	$33.2\ 10^{-3} \pm 7.2\ 10^{-3}$	$28.6\ 10^{-3} \pm 4.3\ 10^{-3}$
	H	$50.4\ 10^{-3} \pm 8.7\ 10^{-3}$	$77.0\ 10^{-3} \pm 12.3\ 10^{-3}$

Table S3: Average ^{65}Cu FIAs (± SE) after 10 days for all metal and predator treatments (N = 293).

Metal	Concentration	^{65}Cu FIAs (µmol/L)	
		Without predator stress	**With predator stress**
Controls		$0.157\ 10^{-6} \pm 0.120\ 10^{-6}$	$0.348\ 10^{-6} \pm 0.063\ 10^{-6}$
Cu	L	$1.77\ 10^{-6} \pm 0.32\ 10^{-6}$	$2.94\ 10^{-6} \pm 0.60\ 10^{-6}$
	M	$0.293\ 10^{-3} \pm 0.062\ 10^{-3}$	$0.493\ 10^{-3} \pm 0.154\ 10^{-3}$
	H	0.146 ± 0.007	0.149 ± 0.011
Cu + Cd	L	$1.22\ 10^{-6} \pm 0.23\ 10^{-6}$	$3.31\ 10^{-6} \pm 0.62\ 10^{-6}$
	M	$0.339\ 10^{-3} \pm 0.092\ 10^{-3}$	$0.616\ 10^{-3} \pm 0.108\ 10^{-3}$
	H	0.139 ± 0.005	0.139 ± 0.006
Cu + Pb	L	$0.209\ 10^{-6} \pm 0.017\ 10^{-6}$	$5.54\ 10^{-6} \pm 1.51\ 10^{-6}$
	M	$1.59\ 10^{-3} \pm 0.52\ 10^{-3}$	$2.05\ 10^{-3} \pm 0.57\ 10^{-3}$
	H	0.147 ± 0.009	0.165 ± 0.006
Cu + Cd + Pb	L	$0.188\ 10^{-6} \pm 0.082\ 10^{-6}$	$0.313\ 10^{-6} \pm 0.071\ 10^{-6}$
	M	$3.13\ 10^{-3} \pm 1.28\ 10^{-3}$	$3.63\ 10^{-3} \pm 0.68\ 10^{-3}$
	H	0.154 ± 0.011	0.169 ± 0.008

Table S4: Average ^{65}Cu body burdens (± SE) after 10 days for all metal and predator treatments (N = 293).

Metal	Concentration	^{65}Cu body concentrations (µmol/g dw)	
		Without predator stress	With predator stress
Controls		1.18 ± 0.10	1.18 ± 0.10
Cu	L	0.753 ± 0.074	0.818 ± 0.083
	M	2.00 ± 0.25	2.31 ± 0.17
	H	8.26 ± 1.32	5.24 ± 0.45
Cu + Cd	L	0.887 ± 0.069	1.05 ± 0.09
	M	1.86 ± 0.18	1.91 ± 0.12
	H	4.77 ± 0.60	5.31 ± 0.63
Cu + Pb	L	0.904 ± 0.165	1.37 ± 0.15
	M	1.97 ± 0.08	2.51 ± 0.34
	H	6.96 ± 1.08	6.18 ± 0.77
Cu + Cd + Pb	L	1.36 ± 0.10	1.28 ± 0.10
	M	2.74 ± 0.31	2.70 ± 0.35
	H	5.48 ± 0.23	6.36 ± 0.75

Table S5: Average ^{204}Pb FIAs (± SE) after 10 days for all metal and predator treatments (N = 292).

Metal	Concentration	^{204}Pb FIAs (µmol/L)	
		Without predator stress	**With predator stress**
Controls		$2.63\ 10^{-9} \pm 0.91\ 10^{-9}$	$5.49\ 10^{-9} \pm 1.91\ 10^{-9}$
Pb	L	$24.8\ 10^{-9} \pm 6.6\ 10^{-9}$	$25.4\ 10^{-9} \pm 6.2\ 10^{-9}$
	M	$1.82\ 10^{-6} \pm 0.93\ 10^{-6}$	$2.44\ 10^{-6} \pm 0.72\ 10^{-6}$
	H	$1.59\ 10^{-3} \pm 0.84\ 10^{-3}$	$1.73\ 10^{-3} \pm 0.81\ 10^{-3}$
Pb + Cd	L	$0.165\ 10^{-6} \pm 0.082\ 10^{-6}$	$0.119\ 10^{-6} \pm 0.065\ 10^{-6}$
	M	$0.015\ 10^{-3} \pm 0.010\ 10^{-3}$	$0.025\ 10^{-3} \pm 0.010\ 10^{-3}$
	H	$1.02\ 10^{-3} \pm 0.29\ 10^{-3}$	$2.88\ 10^{-3} \pm 0.85\ 10^{-3}$
Pb + Cu	L	$0.310\ 10^{-6} \pm 0.098\ 10^{-6}$	$0.268\ 10^{-6} \pm 0.080\ 10^{-6}$
	M	$0.074\ 10^{-3} \pm 0.039\ 10^{-3}$	$0.123\ 10^{-3} \pm 0.059\ 10^{-3}$
	H	$9.46\ 10^{-3} \pm 0.83\ 10^{-3}$	$8.82\ 10^{-3} \pm 0.64\ 10^{-3}$
Pb + Cd + Cu	L	$0.976\ 10^{-6} \pm 0.343\ 10^{-6}$	$1.26\ 10^{-6} \pm 0.37\ 10^{-6}$
	M	$0.181\ 10^{-3} \pm 0.088\ 10^{-3}$	$0.123\ 10^{-3} \pm 0.044\ 10^{-3}$
	H	$10.5\ 10^{-3} \pm 1.1\ 10^{-3}$	$8.68\ 10^{-3} \pm 0.78\ 10^{-3}$

Table S6: Average ^{204}Pb body burdens (± SE) after 10 days for all metal and predator treatments (N = 292).

Metal	Concentration	^{204}Pb body concentrations (µmol/g dw)	
		Without predator stress	**With predator stress**
Controls		$0.201\ 10^{-3} \pm 0.064\ 10^{-3}$	$0.132\ 10^{-3} \pm 0.044\ 10^{-3}$
Pb	L	$35.9\ 10^{-3} \pm 6.4\ 10^{-3}$	$34.4\ 10^{-3} \pm 5.8\ 10^{-3}$
	M	0.320 ± 0.053	0.308 ± 0.067
	H	4.16 ± 0.92	4.91 ± 0.55
Pb + Cd	L	$34.9\ 10^{-3} \pm 0.8\ 10^{-3}$	$43.1\ 10^{-3} \pm 7.6\ 10^{-3}$
	M	0.564 ± 0.064	0.484 ± 0.072
	H	6.33 ± 1.51	3.99 ± 0.829
Pb + Cu	L	$27.8\ 10^{-3} \pm 8.7\ 10^{-3}$	$41.7\ 10^{-3} \pm 8.5\ 10^{-3}$
	M	0.287 ± 0.029	0.366 ± 0.074
	H	2.88 ± 0.50	3.00 ± 0.47
Pb + Cd + Cu	L	$45.4\ 10^{-3} \pm 7.2\ 10^{-3}$	$34.8\ 10^{-3} \pm 7.1\ 10^{-3}$
	M	0.444 ± 0.071	0.433 ± 0.068
	H	1.86 ± 0.18	2.52 ± 0.30

Table S7: Average respiration rates (± SE) after 10 days for all metal and predator treatments (N = 439).

Metal	Concentration	Respiration rate (µg O_2/mg ww/h)	
		Without predator stress	**With predator stress**
Controls		0.162 ± 0.017	0.161± 0.014
Cd	L	0.311 ± 0.056	0.232 ± 0.051
	M	0.233 ± 0.038	0.249 ± 0.030
	H	0.135 ± 0.024	0.157 ± 0.031
Cu	L	0.164 ± 0.026	0.141 ± 0.027
	M	0.106 ± 0.022	0.152 ± 0.030
	H	0.130 ± 0.027	0.147 ± 0.021
Pb	L	0.172 ± 0.017	0.140 ± 0.035
	M	0.149 ± 0.027	0.202 ± 0.034
	H	0.182 ± 0.035	0.116 ± 0.017
Cd + Cu	L	0.189 ± 0.039	0.187 ± 0.022
	M	0.149 ± 0.026	0.163 ± 0.031
	H	0.121 ± 0.020	0.144 ± 0.019
Cd + Pb	L	0.204 ± 0.032	0.168 ± 0.035
	M	0.192 ± 0.020	0.167 ± 0.022
	H	0.205 ± 0.069	0.170 ± 0.062
Cu + Pb	L	0.214 ± 0.037	0.143 ± 0.041
	M	0.162 ± 0.028	0.143 ± 0.015
	H	0.147 ± 0.023	0.161 ± 0.043
Cd + Cu + Pb	L	0.130 ± 0.030	0.140 ± 0.027
	M	0.176 ± 0.031	0.161 ± 0.023
	H	0.118 ± 0.021	0.158 ± 0.041

Table S8: Average growth rates (± SE) after 10 days for all metal and predator treatments (N = 453).

Metal	Concentration	Growth rate (mg/day)	
		Without predator stress	**With predator stress**
Controls		$5.44\ 10^{-3} \pm 13.37\ 10^{-3}$	$33.2\ 10^{-3} \pm 15.6\ 10^{-3}$
Cd	L	$-14.9\ 10^{-3} \pm 23.1\ 10^{-3}$	$7.30\ 10^{-3} \pm 16.54\ 10^{-3}$
	M	$34.8\ 10^{-3} \pm 19.4\ 10^{-3}$	$5.12\ 10^{-3} \pm 21.77\ 10^{-3}$
	H	$-5.28\ 10^{-3} \pm 21.13\ 10^{-3}$	$3.07\ 10^{-3} \pm 14.69\ 10^{-3}$
Cu	L	$42.2\ 10^{-3} \pm 19.5\ 10^{-3}$	$10.1\ 10^{-3} \pm 28.3\ 10^{-3}$
	M	$-42.6\ 10^{-3} \pm 26.0\ 10^{-3}$	$-47.3\ 10^{-3} \pm 18.8\ 10^{-3}$
	H	$-62.5\ 10^{-3} \pm 19.2\ 10^{-3}$	$-49.2\ 10^{-3} \pm 8.8\ 10^{-3}$
Pb	L	$45.3\ 10^{-3} \pm 12.7\ 10^{-3}$	$47.9\ 10^{-3} \pm 20.5\ 10^{-3}$
	M	$63.7\ 10^{-3} \pm 11.3\ 10^{-3}$	$26.8\ 10^{-3} \pm 21.7\ 10^{-3}$
	H	$-48.5\ 10^{-3} \pm 13.7\ 10^{-3}$	$-36.9\ 10^{-3} \pm 12.1\ 10^{-3}$
Cd + Cu	L	$3.24\ 10^{-3} \pm 13.61\ 10^{-3}$	$-40.8\ 10^{-3} \pm 15.9\ 10^{-3}$
	M	$-2.73\ 10^{-3} \pm 17.07\ 10^{-3}$	$2.52\ 10^{-3} \pm 16.09\ 10^{-3}$
	H	$-30.6\ 10^{-3} \pm 15.1\ 10^{-3}$	$-19.2\ 10^{-3} \pm 17.5\ 10^{-3}$
Cd + Pb	L	$73.1\ 10^{-3} \pm 21.6\ 10^{-3}$	$42.2\ 10^{-3} \pm 26.7\ 10^{-3}$
	M	$82.2\ 10^{-3} \pm 15.8\ 10^{-3}$	$45.7\ 10^{-3} \pm 25.9\ 10^{-3}$
	H	$-30.8\ 10^{-3} \pm 9.5\ 10^{-3}$	$-46.9\ 10^{-3} \pm 16.9\ 10^{-3}$
Cu + Pb	L	$42.8\ 10^{-3} \pm 20.0\ 10^{-3}$	$-11.7\ 10^{-3} \pm 27.8\ 10^{-3}$
	M	$-4.11\ 10^{-3} \pm 16.73\ 10^{-3}$	$3.10\ 10^{-3} \pm 28.2\ 10^{-3}$
	H	$-35.4\ 10^{-3} \pm 13.6\ 10^{-3}$	$-39.8\ 10^{-3} \pm 19.2\ 10^{-3}$
Cd + Cu + Pb	L	$97.0\ 10^{-3} \pm 29.8\ 10^{-3}$	0.106 ± 0.028
	M	$58.4\ 10^{-3} \pm 23.6\ 10^{-3}$	$27.8\ 10^{-3} \pm 27.1\ 10^{-3}$
	H	$-9.93\ 10^{-3} \pm 11.51\ 10^{-3}$	$-51.0\ 10^{-3} \pm 24.77\ 10^{-3}$

Table S9: Average feeding rates (± SE) after 10 days for all metal and predator treatments (N = 453).

Metal	Concentration	Feeding rate (mg/mg/day)	
		Without predator stress	**With predator stress**
Controls		$76.8\ 10^{-3} \pm 8.9\ 10^{-3}$	$41.8\ 10^{-3} \pm 6.7\ 10^{-3}$
Cd	L	0.111 ± 0.022	$95.2\ 10^{-3} \pm 22.3\ 10^{-3}$
	M	0.168 ± 0.045	0.105 ± 0.021
	H	$6.32\ 10^{-3} \pm 5.65\ 10^{-3}$	$8.92\ 10^{-3} \pm 5.40\ 10^{-3}$
Cu	L	0.130 ± 0.025	0.124 ± 0.012
	M	$87.5\ 10^{-3} \pm 13.5\ 10^{-3}$	$78.8\ 10^{-3} \pm 20.0\ 10^{-3}$
	H	$18.4\ 10^{-3} \pm 7.8\ 10^{-3}$	$10.4\ 10^{-3} \pm 4.5\ 10^{-3}$
Pb	L	0.147 ± 0.023	$80.2\ 10^{-3} \pm 19.1\ 10^{-3}$
	M	0.177 ± 0.024	0.106 ± 0.018
	H	$6.81\ 10^{-3} \pm 3.17\ 10^{-3}$	$22.5\ 10^{-3} \pm 7.0\ 10^{-3}$
Cd + Cu	L	0.111 ± 0.021	$79.8\ 10^{-3} \pm 18.3\ 10^{-3}$
	M	$39.5\ 10^{-3} \pm 9.4\ 10^{-3}$	$72.0\ 10^{-3} \pm 20.7\ 10^{-3}$
	H	$3.99\ 10^{-3} \pm 2.33\ 10^{-3}$	$8.43\ 10^{-3} \pm 4.47\ 10^{-3}$
Cd + Pb	L	0.135 ± 0.026	$91.6\ 10^{-3} \pm 15.5\ 10^{-3}$
	M	0.358 ± 0.088	0.194 ± 0.037
	H	$1.09\ 10^{-3} \pm 1.09\ 10^{-3}$	$4.19\ 10^{-3} \pm 4.07\ 10^{-3}$
Cu + Pb	L	0.111 ± 0.023	$71.4\ 10^{-3} \pm 17.3\ 10^{-3}$
	M	0.111 ± 0.029	0.107 ± 0.026
	H	0 ± 0	0 ± 0
Cd + Cu + Pb	L	$53.3\ 10^{-3} \pm 16.2\ 10^{-3}$	$55.4\ 10^{-3} \pm 6.8\ 10^{-3}$
	M	$23.3\ 10^{-3} \pm 5.9\ 10^{-3}$	$18.4\ 10^{-3} \pm 9.4\ 10^{-3}$
	H	$89.4\ 10^{-3} \pm 21.0\ 10^{-3}$	$63.9\ 10^{-3} \pm 20.3\ 10^{-3}$

Table S10: Average activities (± SE) after 10 days for all metal and predator treatments (N = 453).

Metal	Concentration	Activity (%)	
		Without predator stress	**With predator stress**
Controls		37.2 ± 2.3	35.4 ± 3.1
Cd	L	36.4 ± 7.3	44.7 ± 5.4
	M	53.1 ± 2.6	38.4 ± 5.9
	H	35.5 ± 4.8	29.2 ± 4.23
Cu	L	41.9 ± 4.3	33.5 ± 5.3
	M	36.4 ± 6.2	47.3 ± 5.2
	H	28.4 ± 7.7	39.0 ± 6.3
Pb	L	38.3 ± 4.7	25.4 ± 5.6
	M	38.2 ± 4.9	41.7 ± 6.8
	H	23.2 ± 3.3	30.8 ± 5.3
Cd + Cu	L	39.6 ± 3.0	29.8 ± 2.3
	M	43.3 ± 4.8	40.1 ± 6.6
	H	19.0 ± 3.8	18.6 ± 2.7
Cd + Pb	L	40.4 ± 7.6	41.9 ± 7.8
	M	41.5 ± 7.7	32.1 ± 6.2
	H	15.7 ± 2.8	33.6 ± 4.2
Cu + Pb	L	41.1 ± 5.7	42.6 ± 6.5
	M	43.3 ± 5.4	25.4 ± 4.7
	H	19.6 ± 3.5	21.7 ± 5.3
Cd + Cu + Pb	L	34.1 ± 7.8	38.8 ± 8.3
	M	27.3 ± 3.3	31.4 ± 4.1
	H	22.6 ± 5.3	25.9 ± 6.3

Chapter 5.

The impact of temperature on metal mixture stress: Sublethal effects on the freshwater isopod *Asellus aquaticus*

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Abstract

Chemical and natural factors have been demonstrated to interact and potentially change the toxicity of the individual stressors. Yet, while there exists a multitude of papers studying the temperature-dependent toxicity of single chemicals, little research exists on the impact of temperature on chemical mixtures. This paper investigated the effect of temperature on environmentally-relevant mixtures of Cd, Cu and Pb. We linked the effects on respiration, growth, feeding rate and activity of *Asellus aquaticus* to the free ion activities, as a measure for the bioavailability of the metals, and the body concentrations. We observed interactions of temperature and metal body concentrations on all sublethal endpoints, except activity. Mixture effects on accumulation and feeding rate were observed as well and even an interaction between metal body burden, mixture and temperature treatment was revealed for the feeding rate of Pb exposed isopods. This research adds to a growing body of evidence that the current chemical-based monitoring is insufficient to estimate chemical toxicity in aquatic ecosystems and must, therefore, be complemented with effect-based tools.

5.1. Introduction

The Intergovernmental Panel on Climate Change predicts that global air temperatures will further rise between 0.3 and 4.8 °C by 2100, mainly attributable to human activities (IPCC, 2014). This increase will be coupled to an elevation of water temperatures, the largest of which are for the USA, Europe and eastern China (Van Vliet et al., 2013). Water data from the Rhine (Lobith, the Netherlands) show that the number of days of temperature exceedances above 20 °C quadrupled in the past 100 years, increasing from 22 to 85 days (CBS et al., 2017). Not only an increase in air temperature, but other human influences such as discharges of wastewater treatment plants and land use shifts could result in a temperature rise of aquatic ecosystems. So far, this warming has resulted in range shifts, changes in productivity and species composition (Heino et al., 2009).

Elevated water temperatures have important ecological consequences. First, a higher water temperature lowers the level of dissolved oxygen. Secondly, although temperature only has a minimal effect on metal speciation and thus the bioavailable fraction (Bervoets and Blust, 1999), it does increase the metabolic rate of animals, resulting in a higher oxygen demand and respiration rate (Sokolova and Lannig, 2008). This potentiates the toxicity of micropollutants including metals. Due to these higher metabolic costs, it can also influence the feeding rate and the growth (Sanford, 2002). Foraging rate is usually positively correlated with temperature (De Block and Stoks, 2003), but a decline is observed when the optimal temperature is exceeded (Nowicki et al., 2012). This is generally linked to a reduced food conversion efficiency. By influencing the feeding rate, temperature alters the uptake of micropollutants through ingestion. Additionally, an increase in temperature can change other physical factors, such as membrane permeability, partition coefficients or diffusion rates, and thus the subcellular partitioning of metals. This may result in a reduction of biologically detoxified metals (in metal-rich granules and heat-stable proteins) and more storage in the metal-sensitive fractions (Wallace et al., 2003). Lastly, temperature variations can trigger several defense mechanisms (e.g., heat-shock proteins), possibly affecting

the sensitivity of an organism (Feder and Hofmann, 1999). A review of 151 studies by Heugens et al. (2001) showed that in approximately 25 % of the cases a negative or no temperature effect on toxicity was reported. As uptake rates of metals may increase, so can elimination and detoxification rates. For example, after exposure to Cu, Boeckman and Bidwell (2006) saw no temperature effect on mortality for *Daphnia pulex*. However, the majority of the studies (more than 70 %) concluded that the toxicity of pollutants increased when temperature increased (Jacobson et al., 1997; Larrain et al., 1998).

The combination of these stressors has increasingly attracted the attention of ecotoxicologists. As water temperatures will continue to rise, the impact of pollution will most likely become more deleterious, but also more uncertain. Little research has described the effects of temperature on the toxicity of mixtures. Fonte et al. (2016) studied the effects of an antibiotic, microplastics and a temperature rise on the common goby and found an interaction between all three stressors for lipid peroxidation. Also for Crustacea, interactive effects have been found. Nieto et al. (2016) found an interaction for respiration rate between temperature and pharmaceuticals. In aquatic ecosystems, organisms are exposed to numerous chemicals. As mixtures can act in a synergistic, additive or antagonistic way, depending on the species, the type and even the concentrations of the chemicals (Norwood et al., 2003), it is of great interest to investigate if temperature can change these interactive effects.

In the present study, *Asellus aquaticus* was exposed to metal mixtures of Cd, Cu and Pb at 15 and 20 °C. This freshwater isopod can be found in the Northern hemisphere and is an important epibenthic detritivore (Bloor, 2010). Moreover, this species is often used in both *ex situ* and *in situ* toxicity tests (Bloor and Banks, 2006; Maltby, 1995; Naylor et al., 1990). The metals Cd, Cu and Pb were chosen as they have a dissimilar mode of action. Cadmium disturbs Ca^{2+} homeostasis, copper Na^{+} homeostasis and Pb both (Paquin et al., 2002; Rogers et al., 2003). We chose 15 °C as the control temperature (Bloor, 2010) and 20 °C as the increased temperature, both

lie within the water temperature range in Belgium. A small exploratory experiment was previously conducted, in which *A. aquaticus* was exposed to uncontaminated water at 15 °C and 20 °C, and both temperatures resulted in a mortality lower than 20 % (unpublished data). In the present study, we linked the respiration rate, the growth rate, the feeding rate and the activity of *A. aquaticus* to the metal body concentrations and the free ion activities (FIAs) in the water. We hypothesized that metal accumulation significantly increased at the higher temperature and that this could be linked to a higher respiration rate. Furthermore, we expected that these elevated body burdens would result in a higher feeding rate and activity, but a lower growth rate.

5.2. Material and Methods

5.2.1. Macroinvertebrate sampling

The freshwater isopod *Asellus aquaticus* was collected with a pond net (500 µm mesh, 200 x 300 mm frame and 500 mm bag depth) in the autumn of 2016 (T_{water} = 16.2 °C, pH = 7.8, O_2 = 10.2 mg/L, EC = 607 µS/cm) and the spring of 2017 (T_{water} = 16.8 °C, pH = 7.5, O_2 = 7.1 mg/L, EC = 536 µS/cm) from the Laakbeek in Lille, Belgium (a river in the Scheldt basin). The Laakbeek is located in a region of extensive agriculture. Metal concentrations in water from the Laakbeek were measured and found to be below environmental quality standards (EQS): Cu = 2.35 ± 0.76 µg/L; Cd < 0.01 µg/L; and Pb < 0.1 µg/L. At the lab, they were acclimated for minimally one week in two climate chambers type WT15'/+5DU-WB (Weiss Technik, Reiskirchen-Lindenstruth, Germany) at constant light conditions (16:8 h light:dark photoperiod) and temperature. Half of the isopods was kept in a climate chamber at 15 ± 1 °C, the other half at 20 ± 1 °C. They were placed in 20 L glass aquaria filled with medium-hard freshwater (pH = 7.94 ± 0.03; US EPA, 2002) and were fed *ad libitum* with alder leaves (*Alnus glutinosa*), that were 'conditioned' by placing them in a dry oven for 72 h and then rehydrating them in water from the Laakbeek for one week (Bloor, 2010).

5.2.2. Experimental design

Asellus aquaticus (average length = 7.96 ± 0.03 mm) was exposed for 10 days to metal mixtures of Cd, Cu and Pb at two different temperatures, i.e. 15 ± 1 °C and 20 ± 1 °C, the temperatures to which they were acclimated. We studied the effects of the single metals as well as the binary and tertiary mixtures (Table 1). Additionally, we added four control treatments. We chose three environmentally-relevant concentrations per metal: a low ("L"), medium ("M") and high ("H") concentration, which are equal to the EQS, 10 x EQS, and 25 x EQS (EP, 2008; VLAREM, 2015). For Cd, this was 1.29 10^{-3} µmol/L, 12.9 10^{-3} µmol/L and 32.4 10^{-3} µmol/L; for Cu, 0.108 µmol/L, 1.08 µmol/L and 2.70 µmol/L; and for Pb, 35.3 10^{-3} µmol/L, 0.353 µmol/L and 0.882 µmol/L. Because the exposure concentrations we chose were low and trace amounts of metals could be found in both the animals and the leaves used as a food source before the start of the experiment, we used the stable isotopes ^{65}Cu, ^{116}Cd and ^{204}Pb to track newly accumulated metal during this experiment (CortecNet, Voisins-Le-Bretonneux, France). To prepare the exposure concentrations, the isotopes were added to moderately hard water (Table 1; US EPA, 2002). When necessary, we used 1 N NaOH to adjust the pH to 7.8. Next, we poured 100 mL of this medium into acid-washed (minimally 24h in 1% HCl) polypropylene containers. All containers were taped at the start of the experiment as *Asellus aquaticus* is cannibalistic and visual cues could cause additional (predator) stress. Ten replicates per treatment were made, which were left for 24 h to equilibrate.

One isopod was placed in each container together with two alder leaf discs (*Alnus glutinosa*, d = 16 mm, 21.6 ± 0.1 mg dw) that had been dried, weighed and 'conditioned' for six days in water from the Laakbeek in Lille (Bloor and Banks, 2006). We analyzed the length of each isopod by photographing them at the start of the experiment. After measuring with the program ImageJ 1.48v. (U.S. National Institutes of Health, Maryland, USA), we calculated the dry weight as follows (Graça et al., 1993): ln(dw) = 2.71 ln(length) – 4.58. We monitored the general water characteristics (pH =

7.94 ± 0.01, EC = 328 ± 2 µS/cm and O_2 = 9.72 ± 0.04 mg/L at day 0) and mortality daily. When animals did not respond to gentle prodding, they were considered dead and were not included in the analyses (Ashauer et al., 2011).

Table 1: Summary of the dissolved metal concentrations at day 0. BMQL = Below method quantification limit. BMQL of ^{116}Cd = 8.63 10^{-6} µmol/L; BMQL of ^{65}Cu = 15.4 10^{-6} µmol/L; and ^{204}Pb = 4.90 10^{-6} µmol/L.

	^{116}Cd (µmol/L)	^{65}Cu (µmol/L)	^{204}Pb (µmol/L)
Controls	30.6 10^{-6} ± 7.76 10^{-6}	5.50 10^{-3} ± 0.50 10^{-3}	0.150 10^{-3} ± 0.032 10^{-3}
Cd L	1.24 10^{-3} ± 0.05 10^{-3}	4.01 10^{-3} ± 0.17 10^{-3}	0.338 10^{-3} ± 0
Cd M	14.2 10^{-3} ± 0.6 10^{-3}	8.12 10^{-3} ± 0.50 10^{-3}	BMQL
Cd H	43.6 10^{-3} ± 3.3 10^{-3}	6.16 10^{-3} ± 0.48 10^{-3}	0.285 10^{-3} ± 0.280 10^{-3}
Cu L	11.5 10^{-6} ± 2.9 10^{-6}	66.3 10^{-3} ± 5.8 10^{-3}	0.339 10^{-3} ± 0.001 10^{-3}
Cu M	21.6 10^{-6} ± 5.3 10^{-6}	0.937 ± 0.062	20.4 10^{-6} ± 15.5 10^{-6}
Cu H	21.6 10^{-6} ± 5.3 10^{-6}	3.04 ± 0.12	BMQL
Pb L	41.7 10^{-6} ± 18.7 10^{-6}	3.72 10^{-3} ± 0.26 10^{-3}	5.66 10^{-3} ± 0.68 10^{-3}
Pb M	28.8 10^{-6} ± 4.3 10^{-6}	11.1 10^{-3} ± 0.4 10^{-3}	0.325 ± 0.052
Pb H	46.0 10^{-6} ± 5.3 10^{-6}	6.48 10^{-3} ± 0.59 10^{-3}	0.831 ± 0.062
Cd + Cu L	1.19 10^{-3} ± 0.13 10^{-3}	59.1 10^{-3} ± 7.4 10^{-3}	0.339 10^{-3} ± 0.001 10^{-3}
Cd + Cu M	19.0 10^{-3} ± 0.8 10^{-3}	0.959 ± 0.079	0.105 10^{-3} ± 0.031 10^{-3}
Cd + Cu H	55.4 10^{-3} ± 1.1 10^{-3}	3.24 ± 0.06	0.586 10^{-3} ± 0.041 10^{-3}
Cd + Pb L	1.21 10^{-3} ± 0.09 10^{-3}	5.01 10^{-3} ± 0.43 10^{-3}	3.77 10^{-3} ± 0.47 10^{-3}
Cd + Pb M	22.3 10^{-3} ± 0.3 10^{-3}	12.6 10^{-3} ± 1.1 10^{-3}	0.334 ± 0.031
Cd + Pb H	36.5 10^{-3} ± 4.8 10^{-3}	6.01 10^{-3} ± 1.86 10^{-3}	0.724 ± 0.168
Cu + Pb L	80.5 10^{-6} ± 24.3 10^{-6}	68.6 10^{-3} ± 9.0 10^{-3}	6.28 10^{-3} ± 0.71 10^{-3}
Cu + Pb M	34.5 10^{-6} ± 4.5 10^{-6}	1.41 ± 0.04	0.301 ± 0.026
Cu + Pb H	47.5 10^{-6} ± 13.7 10^{-6}	2.35 ± 0.35	0.640 ± 0.131
Cd + Cu + Pb L	1.33 10^{-3} ± 0.1 10^{-3}	69.9 10^{-3} ± 8.3 10^{-3}	6.72 10^{-3} ± 1.27 10^{-3}
Cd + Cu + Pb M	22.8 10^{-3} ± 0.5 10^{-3}	1.40 ± 0.04	0.331 ± 0.018
Cd + Cu + Pb H	32.2 10^{-3} ± 2.8 10^{-3}	1.94 ± 0.20	0.534 ± 0.057

At day 10, the animals were placed in a petri dish with medium-hard water in their respective climate chambers, where they were filmed for 30 minutes after an

acclimation period of 15 minutes. Using the behavioral tracking program Lolitrack v.4 (Loligo Systems, Tjele, Denmark) the active time (%) was calculated. Afterward, we measured their oxygen consumption for 4 hours. The asellids were placed in glass chambers with oxygen mini sensors where the oxygen concentration was logged using the programs WitroxView v.1.0.2 and Fibsoft v.1.0 (Loligo Systems, Tjele, Denmark). Respiration rates (RR) were calculated using the following formula: $RR = ((O_1 – O_2)*(V – V_A))/(m_A*t)$, where O_1 is the oxygen concentration in the respiration chamber at the start of the experiment (mg/L), O_2 is the oxygen concentration in the respiration chamber at the end of the experiment (mg/L), V is the volume of the empty respiration chamber (mL), V_A is the volume of the isopod (assuming 1 mg isopod = 0.001 mL) (mL), m_A is the mass of the isopod (mg wet weight) and t is the duration of the respiration experiment (h). We used two different sets of respiration chambers. The volumes of the empty chambers were 14.7 ± 0.0 and 17.4 ± 0.1 mL. Oxygen levels decreased with 0.144 ± 0.005 mg O_2/h and the average oxygen concentration that was measured after 4 hours of respiration was 9.05 ± 0.05 mg/L O_2. Additionally, the growth rate (GR) of each animal was determined as follows: $GR = (m_2 – m_1)/10$, where m_1 is the dry weight of the animal at day 0 (mg) and m_2 is the dry weight of the animal at day 10 (mg). Lastly, we calculated the feeding rate (FR) as well: $FR = (L_1 * C_L) – L_2/m_2*10$, where L_1 is the dry weight of the *Alnus* discs initially supplied and L_2 is the dry weight of leaf material remaining after 10 days (mg) and C_L is the leaf weight change correction factor given by: $C_L = \Sigma(C_2/C_1)/N$, where C_1 in the initial dry weight of control leaves (mg), C_2 is the dry weight of the control leaves after 10 days (mg) and N is the number of control leaves (Bloor and Banks, 2006). There were six control leaves per metal and temperature treatment. After respiration measurements, the animals were removed, rinsed with Milli-Q and stored in a -20 °C freezer.

5.2.3. Metal and DOC analysis

At the start and end of the experiment (day 0 and day 10), 50 mL of water was taken from the containers with a syringe and filtered through a 0.20 µm filter (Chromafil,

Macherey-Nagel, Düren, Germany). A part was acidified to 1% HNO_3 to quantify the trace metals (^{116}Cd, ^{65}Cu, ^{204}Pb) using a high resolution inductively coupled plasma mass spectrometer (HR-ICP-MS, Element XR, Thermo Scientific, Finnigan element 2, Bremen, Germany). The major ion concentrations, on the other hand, were measured using an inductively coupled plasma optic emission spectrometer (ICP-OES; Thermo scientific, ICAP 6300 Duo, Waltham, MA, USA; Table 2). Next, dissolved organic carbon concentrations were measured in the other part of the water sample with a TOC-analyzer (TOC-VCPH, Shimadzu Corporation, Kyoto, Japan) after acifidication to pH 2 by adding 2N HCl.

Table 2: Overview of the water chemistry measurements, presented as means (± SE) of 50 water samples at day 0. Water hardness was calculated according to the formula: 2.5 Ca + 4.1 Mg.

DOC (mg/L)	Ca (mg/L)	K (mg/L)	Mg (mg/L)	Na (mg/L)	Water hardness (mg/L $CaCO_3$)
1.09 ± 0.05	12.5 ± 0.2	2.19 ± 0.04	11.9 ± 0.3	23.0 ± 0.6	81.0 ± 1.9

The weight of the leaves and isopods was determined after drying for 72 h at 60 °C in a laboratory furnace. After cooling in a desiccator, the isopods were weighed on a Sartorius SE2 ultra microbalance (accuracy of 1 µg). Next, they were placed in a hot block (Environmental Express, Charleston, SC, USA) for 1h at 125 °C in a mixture of trace-metal-grade HNO_3 (69%) and high-purity H_2O_2 (29%) (3:1). Milli-Q water was added to dilute to 3% HNO_3. Metal concentrations were measured with a high resolution inductively coupled plasma mass spectrometer (HR-ICP-MS, Element XR, Thermo Scientific, Finnigan element 2, Bremen, Germany) and were expressed as µmol/g dw. Additionally, we digested three process blank samples and three samples of certified reference mussel material (SRM2796) of the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). The metal concentrations in the blank samples were all below quantification limits (<0.001 µg/L for ^{116}Cd, ^{65}Cu and ^{204}Pb) and the recoveries from the reference samples were consistently within 10 % of the certified values for all three metals. Lastly, the FIAs were calculated with the

Windermere Humic Aqueous Model 6.0.13 (Natural Environment Research Council) using the dissolved metal concentrations and the DOC at day 10 (100 % entered as fulvic acids). As several metal concentrations were found to be below the detection limit, we entered them as the detection limit itself.

5.2.4. Statistical analyses

We compared the treatments using ANCOVAs, which were performed in R version 3.4.2. Metal concentrations were entered as a continuous variable and metal and temperature treatment as categorical variables. Data from the control treatments were included for each metal treatment. All data were tested for normality and homoscedasticity by using diagnostic plots. First, the models were made using the metal body concentrations and later compared to the FIA_{water} model. We log-transformed (log_{10}) all metal concentrations to normalize distributions. The effects and interactions of the stressors were assessed by interpreting the interaction term and the differences between slopes. If a p-value below 0.05 was found, we conducted a Tukey HSD test. Possible differences between the mortality rates at the two temperature treatments were tested with the Wilcoxon rank sum test. Graphs were constructed of all significant factors using Sigmaplot version 11.0 (Systat, Chicago, IL, USA).

5.3. Results

5.3.1. General

Mortality for the control treatments varied between 0 and 10 % for the isopods at 15 °C and between 0 and 30 % for the isopods at 20 °C (Table S1). The highest mortality rate for all metal treatments was 70 % for the Cd + Pb L treatment at 20 °C. We found that the mortality rate for all the 20 °C treatments was a factor 8 higher than for the 15 °C treatments (22.0 ± 3.8 % compared to 2.80 ± 1.08 %; $V = 3$, $p < 0.001$). No significant three-way interactions between the body concentrations, metal and temperature treatment were found, except for feeding rate (Pb treatments).

5.3.2. Metal accumulation

For Cd, FIAs varied between 1.37 10^{-6} ± 0.31 10^{-6} (Cd + Pb L at 20 °C) and 12.0 10^{-3} ± 0.3 10^{-3} µmol /L (Cd + Cu + Pb H at 15 °C), the body burdens between 2.45 10^{-3} ± 0.28 10^{-3} (Cd + Cu + Pb H at 15 °C) and 86.0 10^{-3} ± 15.9 10^{-3} µmol/g dw (Cd H at 20 °C; Tables S2-S3). The Cd FIAs were positively correlated to the metal body concentrations ($F = 1487$, $p < 0.001$). Moreover, there was a significant interaction between the Cd FIAs and metal treatment ($F = 7.26$, $p < 0.001$), indicating differences in slopes (Table 3; Figure 1A). We found that the slopes of Cd + Cu and the tertiary mixture were significantly lower than for the single Cd (for Cd + Cu: $t = -4.016$, $p < 0.001$; and for Cd + Cu + Pb: $t = -3.69$, $p < 0.001$) and Cd + Pb treatment (for Cd + Cu: $t = -2.72$, $p = 0.007$; and for Cd + Cu + Pb: $t = -2.34$, $p = 0.020$). The ANCOVA model also indicated a difference in Cd body concentration between the temperature treatments, but a Tukey HSD test revealed no significance.

Copper FIAs ranged from 1.38 10^{-6} ± 0.19 10^{-6} (Cu + Cd L at 20 °C) to 24.3 10^{-3} ± 2.7 10^{-3} µmol/L (Cu + Pb H at 15 °C) and the body burdens varied between 1.63 ± 0.09 (Cu L at 15 °C) and 4.31 ± 0.38 µmol/g dw (Cu + Cd + Pb M at 15 °C; Tables S4-S5). We observed a significant positive correlation between Cu FIA and body burden ($F = 282$, $p < 0.001$). Furthermore, Cu accumulation was 0.085 µmol/g dw, or 3.48 %, higher for the 20 °C treatments ($F = 5.66$, $p = 0.018$) than for the 15 °C treatments (Figure 1B). No other significant differences or interactions were found.

The FIAs of Pb varied between 0.130 10^{-6} ± 0.026 10^{-6} (Pb L at 20 °C) and 1.52 10^{-3} ± 0.45 10^{-3} µmol/L (Pb + Cd + Cu H at 15 °C), the body concentration between 44.3 10^{-3} ± 8.1 10^{-3} (Pb L at 15 °C) and 1.22 ± 0.13 µmol/g dw (Pb H at 20 °C; Tables S6-S7). Lead body concentrations were positively correlated with the FIAs ($F = 5547$, $p < 0.001$). Additionally, we discovered an interaction of the Pb FIAs with the metal treatments ($F = 6.97$, $p < 0.001$; Figure 1C). The slope of Cu + Pb was significantly lower than for Pb ($t = 2.92$, $p = 0.004$) and Cd + Pb ($t = 4.43$, $p < 0.001$). Also, the slope of Cu + Cd + Pb was significantly lower than Cd + Pb ($t = 2.78$, $p = 0.006$). Furthermore, we found an

interaction between the FIAs and the temperature treatments (Figure 1D). The warmer treatment had a higher slope ($F = 6.26$, $p = 0.013$).

Table 3: Slopes for metal accumulation, calculated based on FIA_{water}, after 10 days per metal and temperature treatment (for Cd and Cu: N = 272; and for Pb: N = 279). Average slopes and standard errors are presented, together with R^2 values for the ANCOVA model. Superscript lower case letters indicate statistical differences between treatments.

Metal	Mixture with	$Slope_{accumulation}$ (log(µmol/g dw). log(µmol/L)$^{-1}$)	T°	$Slope_{accumulation}$ (log(µmol/g dw). log(µmol/L)$^{-1}$)	R^2
Cd	-	0.698 ± 0.038 ^{a}	15 °C	0.558 ± 0.020 ^{a}	76.6 %
	Cu	0.516 ± 0.029 ^{b}	20 °C	0.609 ± 0.024 ^{a}	
	Pb	0.632 ± 0.030 ^{a}			
	Cu + Pb	0.537 ± 0.025 ^{b}			
Cu	-	62.6 10^{-3} ± 7.4 10^{-3} ^{a}	15 °C	57.8 10^{-3} ± 5.3 10^{-3} ^{a}	38.9 %
	Cd	50.6 10^{-3} ± 7.1 10^{-3} ^{a}	20°C	65.6 10^{-3} ± 4.8 10^{-3} ^{a}	
	Pb	56.4 10^{-3} ± 7.5 10^{-3} ^{a}			
	Cd + Pb	71.1 10^{-3} ± 6.9 10^{-3} ^{a}			
Pb	-	0.538 ± 0.014 ^{a}	15 °C	0.506 ± 0.010 ^{a}	92.2 %
	Cd	0.567 ± 0.018 ^{a}	20 °C	0.539 ± 0.010 ^{b}	
	Cu	0.482 ± 0.013 ^{b}			
	Cd + Cu	0.512 ± 0.013 ^{b}			

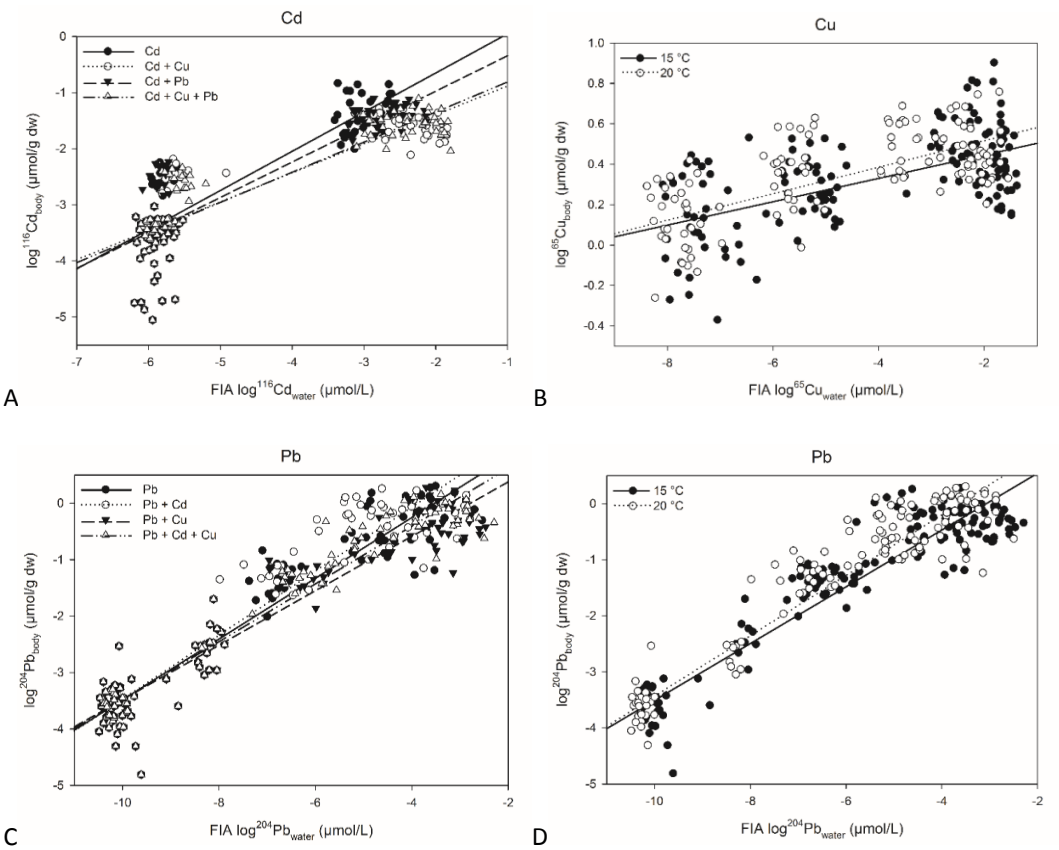


Figure 1: Significant relationships between the free ion activities (FIAs) and the metal body concentrations after 10 days: A) Cd metal treatments (N = 272); B) Cu temperature treatments (N = 272); and C-D) Pb metal and temperature treatments (N = 279).

5.3.3. Respiration rate

Respiration rates ranged from 65.4 10^{-3} ± 16.3 10^{-3} (Cu H at 15 °C) to 0.265 ± 0.078 µg O_2/mg ww/h (Cd + Cu M at 20 °C; Table S8). No difference in respiration rate between the two temperature control treatments was observed.

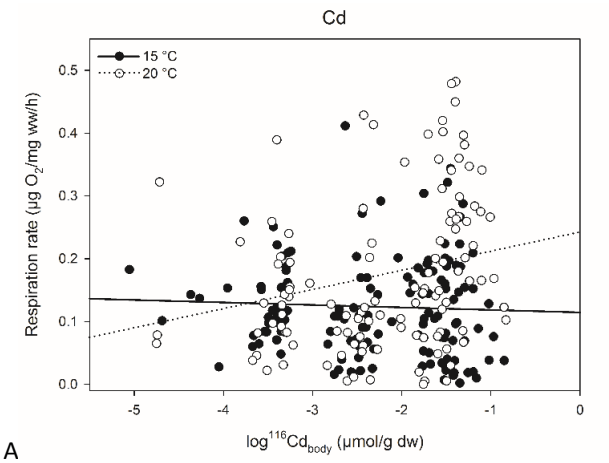
For Cd, isopods from the 20 °C treatments respired on average 50.7 10^{-3} µg O_2/mg ww/h or 41.7% more ($F = 13.5$, $p < 0.001$). Additionally, we found an interaction between body burdens and temperature ($F = 16.5$, $p < 0.001$). For the 15 °C treatments, the slope was slightly declining (Table 4; Figure 2A). For the 20 °C treatments, on the

other hand, the respiration rate was positively correlated with the accumulated cadmium ($t = 4.062$, $p < 0.001$).

Table 4: Slopes for respiration rates, calculated based on body concentrations, after 10 days per metal and temperature treatment (for Cd: N = 251, Cu: N = 247, Pb: N = 255). Average slopes and standard errors are presented, together with R^2 values for the ANCOVA model.

Metal	Mixture with	$Slope_{respiration\ rate}$ ($\mu g\ O_2.mg\ ww^{-1}.h^{-1}.\ log(\mu mol/g\ dw)^{-1}$)	T°	$Slope_{respiration\ rate}$ ($\mu g\ O_2.mg\ ww^{-1}.h^{-1}.\ log(\mu mol/g\ dw)^{-1}$)	R^2
Cd	-	$-5.86\ 10^{-3} \pm 8.32\ 10^{-3}$ a	15 °C	$-3.94\ 10^{-3} \pm 4.57\ 10^{-3}$ a	10.8 %
	Cu	$17.2\ 10^{-3} \pm 9.5\ 10^{-3}$ a	20 °C	$30.5\ 10^{-3} \pm 7.5\ 10^{-3}$ b	
	Pb	$18.2\ 10^{-3} \pm 8.3\ 10^{-3}$ a			
	Cu + Pb	$22.5\ 10^{-3} \pm 8.6\ 10^{-3}$ a			
Cu	-	$-33.0\ 10^{-3} \pm 31.1\ 10^{-3}$ a	15 °C	$-19.5\ 10^{-3} \pm 17.1\ 10^{-3}$ a	7.63 %
	Cd	$22.5\ 10^{-3} \pm 4.1\ 10^{-3}$ a	20 °C	$85.9\ 10^{-3} \pm 32.0\ 10^{-3}$ b	
	Pb	$19.0\ 10^{-3} \pm 32.0\ 10^{-3}$ a			
	Cd + Pb	$68.8\ 10^{-3} \pm 33.7\ 10^{-3}$ a			
Pb	-	$12.5\ 10^{-3} \pm 5.8\ 10^{-3}$ a	15°C	$-0.492\ 10^{-3} \pm 3.130\ 10^{-3}$ a	10.3 %
	Cd	$11.7\ 10^{-3} \pm 5.7\ 10^{-3}$ a	20 °C	$20.2\ 10^{-3} \pm 4.7\ 10^{-3}$ b	
	Cu	$1.10\ 10^{-3} \pm 5.42\ 10^{-3}$ a			
	Cd + Cu	$12.9\ 10^{-3} \pm 5.8\ 10^{-3}$ a			

For Cu and Pb, there was also a significant interaction between the body concentrations and temperature, indicating significantly different slopes (for Cu: $F = 9.54$, $p = 0.002$; for Pb: $F = 14.1$, $p < 0.001$). Similar to Cd, there was a negative correlation between body concentration and respiration rate at 15 °C, while the slope for the 20 °C treatments was positive (Figure 2B and 2C). This resulted in isopods of the warmer Cu treatments respiring $47.8\ 10^{-3}$ µg O_2/mg ww/h or 39.4% more ($F = 14.6$, $p < 0.001$) and for the warmer Pb treatments $55.8\ 10^{-3}$ µg O_2/mg ww/h or 44.9 % more ($F = 18.1$, $p < 0.001$). For all FIA_{water} models, the same significances were found.



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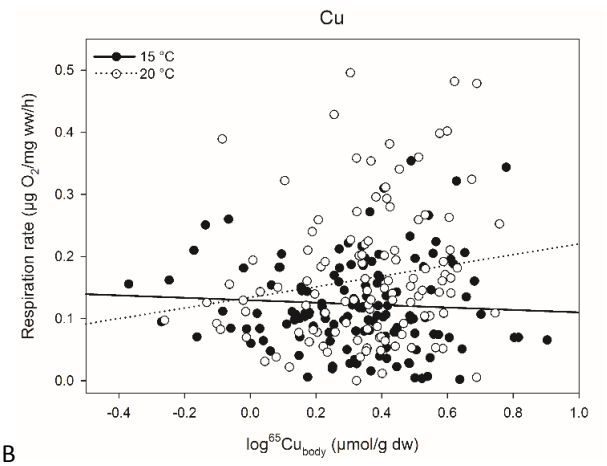

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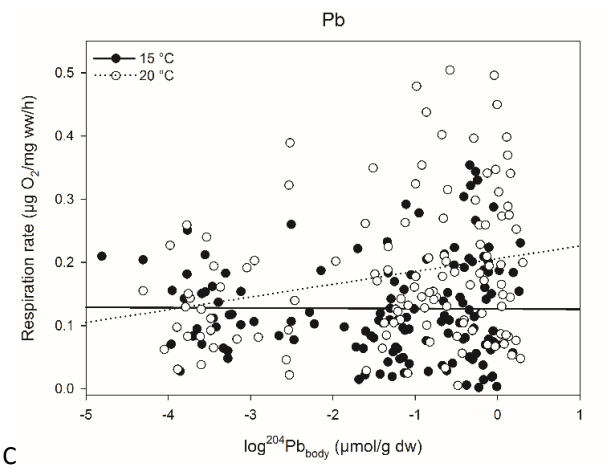

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Figure 2: Significant relationships between the metal body concentrations and the respiration rates after 10 days: temperature treatments of A) Cd (N = 251); B) Cu (N = 247); and C) Pb (N = 255).

5.3.4. Growth rate

Growth rates varied between -49.4 10^{-3} ± 18.8 10^{-3} (Cu H at 15 °C) and 88.7 10^{-3} ± 12.6 10^{-3} mg/day (Pb L at 15 °C; Table S9). Control asellids at 20 °C grew 11.2 10^{-3} ± 9.4 10^{-3} mg/day while controls at 15 °C lost 18.0 10^{-3} ± 9.710^{-3} mg/day ($F = 4.63$, $p = 0.035$).

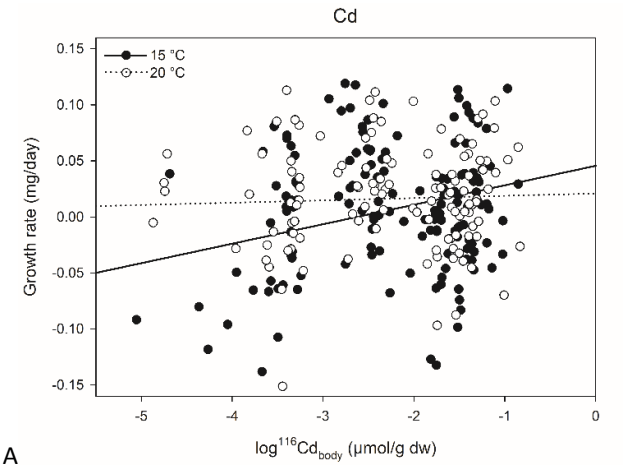
Growth rates correlated positively with Cd body concentrations ($F = 16.8$, $p < 0.001$). We found that isopods from the 20 °C treatments grew 11.2 10^{-3} mg/day (or 162 %) more than at 15 °C ($F = 15.04$, $p < 0.001$). However, we also discovered an interaction between Cd body concentrations and temperature (Table 5; Figure 3A), showing that the slope for growth for the 20 °C treatments was less steep ($F = 10.0$, $p = 0.002$). Using the FIA_{water} model, only the significant difference in growth between the temperature treatments was observed.

For Cu, we found a positive correlation between the body concentrations and the growth rate ($F = 6.95$, $p = 0.009$). No interactions were found, but isopods of the 20 °C treatments had a growth rate that was on average 0.006 mg/day (or 80.8 %) higher than the isopods of the 15 °C treatments ($F = 8.61$, $p = 0.004$; Figure 3B).

Lead body concentrations correlated positively with the growth rates ($F = 14.6$, $p < 0.001$). However, they interacted with temperature ($F = 4.56$, $p = 0.033$). The slope of growth rate for isopods of the 20 °C treatment was less positive than for the 15 °C treatment (Figure 3C). Yet, asellids of the warmer treatment did grow 109% or 6.92 10^{-3} mg/day more ($F = 11.3$, $p < 0.001$). For the Cu and Pb FIA_{water} model, the same significances were found.

Table 5: Slopes for growth rates, calculated based on body concentrations, after 10 days per metal and temperature treatment (for Cd and Cu: N = 272; for Pb: N = 279). Average slopes and standard errors are presented, together with R^2 values for the ANCOVA model. Superscipt lower case letters indicate statistical differences between treatments.

Metal	Mixture with	$Slope_{growth\ rate}$ (mg dw.day^{-1}. log(µmol/g dw)$^{-1}$)	T°	$Slope_{growth\ rate}$ (mg dw.day^{-1}. log(µmol/g dw)$^{-1}$)	R^2
Cd	-	$8.67\ 10^{-3} \pm 4.70\ 10^{-3\ a}$	15 °C	$17.4\ 10^{-3} \pm 3.5\ 10^{-3\ a}$	8.66 %
	Cu	$14.7\ 10^{-3} \pm 5.1\ 10^{-3\ a}$	20 °C	$2.05\ 10^{-3} \pm 3.30\ 10^{-3\ b}$	
	Pb	$10.5\ 10^{-3} \pm 4.9\ 10^{-3\ a}$			
	Cu + Pb	$6.45\ 10^{-3} \pm 5.17\ 10^{-3\ a}$			
Cu	-	$4.06\ 10^{-3} \pm 23.6\ 10^{-3\ a}$	15 °C	$22.9\ 10^{-3} \pm 14.5\ 10^{-3\ a}$	4.52 %
	Cd	$55.9\ 10^{-3} \pm 22.4\ 10^{-3\ a}$	20 °C	$33.4\ 10^{-3} \pm 15.2\ 10^{-3\ a}$	
	Pb	$36.4\ 10^{-3} \pm 18.9\ 10^{-3\ a}$			
	Cu + Pb	$15.2\ 10^{-3} \pm 20.7\ 10^{-3\ a}$			
Pb	-	$4.76\ 10^{-3} \pm 3.76\ 10^{-3\ a}$	15 °C	$10.2\ 10^{-3} \pm 2.5\ 10^{-3\ a}$	6.28 %
	Cd	$8.03\ 10^{-3} \pm 3.42\ 10^{-3\ a}$	20 °C	$2.73\ 10^{-3} \pm 2.26\ 10^{-3\ b}$	
	Cu	$7.70\ 10^{-3} \pm 3.16\ 10^{-3\ a}$			
	Cd + Cu	$5.55\ 10^{-3} \pm 3.49\ 10^{-3\ a}$			



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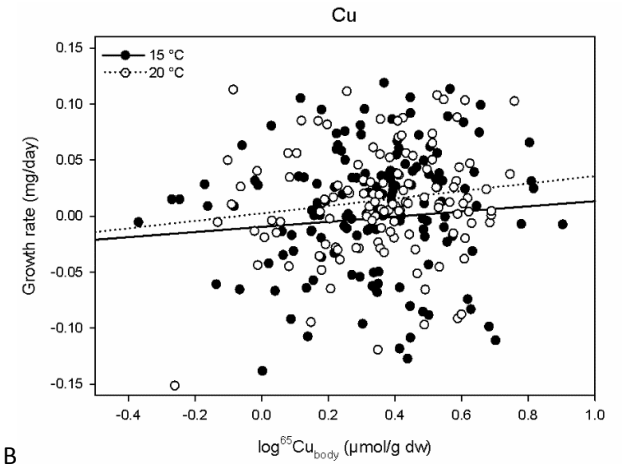

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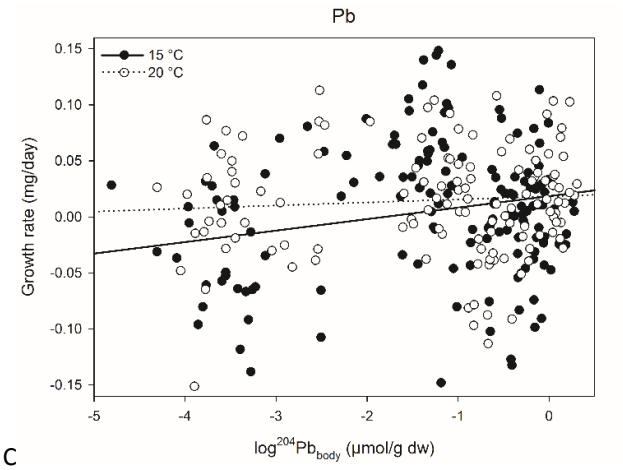

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Figure 3: Significant relationships between the metal body concentrations and the growth rates after 10 days: temperature treatments of A) Cd (N = 272); B) Cu (N = 272); and C) Pb (N = 279).

5.3.5. Feeding rate

Feeding rates varied between 8.69 10^{-3} ± 5.34 10^{-3} (Cd + Cu + Pb H 15 °C) and 0.286 ± 0.044 mg/mg/day (Cd + Pb H 20 °C; Table S10). Asellids from the control treatments at 20 °C fed on average 0.0371 mg/mg/day or 64.8 % more than from the controls at 15 °C ($F = 10.2$, $p = 0.002$).

For the cadmium treatments, we found a significantly higher feeding rate for the 20 °C isopods ($F = 103$, $p < 0.001$). They ate 64.8 10^{-3} mg/mg/day or 126 % more than the isopods at 15 °C. Furthermore, we discovered an interaction of Cd body burdens with metal treatment ($F = 5.93$, $p > 0.001$) as well as temperature treatment ($F = 20.0$, $p < 0.001$; Table 6; Figure 4A and 4B). While the slope of feeding rate was negative for the isopods of the 15 °C treatments, the slope for the 20 °C was positive ($t = 4.47$, $p < 0.001$). For the metal treatments, the slopes of the Cd + Cu and the Cd + Pb treatment were higher than the single Cd (for Cd + Cu: $t = -2.18$, $p = 0.030$; for Cd + Pb: $t = -3.55$, $p < 0.001$) and the tertiary mixture (for Cd + Cu: $t = -2.19$, $p = 0.029$; for Cd + Pb: $t = -3.55$, $p < 0.001$). For the FIA_{water} ANCOVA model, the same significances were observed, but only Cd + Pb had a different slope than the other three metal treatments (for Cd: $t = -3.16$, $p = 0.002$; for Cd + Cu: $t = -3.35$, $p < 0.001$; and for Cd + Cu + Pb: $t = -4.96$, $p < 0.001$).

We found a significant positive correlation between Cu body concentrations and feeding rate ($F = 5.60$, $p = 0.018$). Furthermore, we observed that isopods of the 20 °C treatments fed on average 0.063 mg/mg/day or 136 % more than isopods of the colder treatments ($F = 86.9$, $p < 0.001$; Figure 4C). For the FIA_{water} model, we found a significant interaction between the FIAs and temperature, indicating a significant difference between the slopes of the temperature treatments ($F = 4.02$, $p = 0.046$). There was a negative correlation between FIA and feeding rate (-4.98 10^{-3} ± 1.54 10^{-3} $mg.mg^{-1}.day^{-1}.log(\mu mol/L)^{-1}$) at 15 °C. The slope of feeding rate for the 20 °C treatments was slightly higher, but still negative (-0.317 10^{-3} ± 2.324 10^{-3} $mg.mg^{-1}.day^{-1}.log(\mu mol/L)^{-1}$).

Table 6: Slopes for feeding rates, calculated based on body concentrations, after 10 days per metal and temperature treatment (for Cd and Cu: N = 272; and for Pb: N = 279). Average slopes and standard errors are presented, together with R^2 values for the ANCOVA model. Superscript lower case letters indicate statistical differences between treatments.

Metal	Mixture with	$Slope_{feeding\ rate}$ ($mg.mg^{-1}.day^{-1}.log(\mu mol/g\ dw)^{-1}$)	T°	$Slope_{feeding\ rate}$ ($mg.mg^{-1}.day^{-1}.log(\mu mol/g)^{-1}$)	R^2
Cd	-	$-4.09\ 10^{-3} \pm 4.78\ 10^{-3\ a}$	15 °C	$-3.26\ 10^{-3} \pm 2.67\ 10^{-3\ a}$	26.8 %
	Cu	$11.9\ 10^{-3} \pm 6.0\ 10^{-3\ b}$	20 °C	$19.6\ 10^{-3} \pm 4.89\ 10^{-3\ b}$	
	Pb	$21.8\ 10^{-3} \pm 6.5\ 10^{-3\ b}$			
	Cu + Pb	$-1.74\ 10^{-3} \pm 6.01\ 10^{-3\ a}$			
Cu	-	$20.8\ 10^{-3} \pm 25.7\ 10^{-3\ a}$	15 °C	$10.1\ 10^{-3} \pm 10.5\ 10^{-3\ a}$	19.3 %
	Cd	$68.0\ 10^{-3} \pm 25.8\ 10^{-3\ a}$	20 °C	$48.5\ 10^{-3} \pm 23.1\ 10^{-3\ a}$	
	Pb	$26.4\ 10^{-3} \pm 25.7\ 10^{-3\ a}$			
	Cd + Pb	$5.41\ 10^{-3} \pm 24.0\ 10^{-3\ a}$			
Pb	-	$1.10\ 10^{-3} \pm 3.56\ 10^{-3\ a}$	15 °C	$-3.09\ 10^{-3} \pm 1.79\ 10^{-3\ a}$	22.8%
	Cd	$14.6\ 10^{-3} \pm 4.6\ 10^{-3\ b}$	20 °C	$5.34\ 10^{-3} \pm 3.55\ 10^{-3\ b}$	
	Cu	$-6.54\ 10^{-3} \pm 4.27\ 10^{-3\ a}$			
	Cd + Cu	$-3.28\ 10^{-3} \pm 4.06\ 10^{-3\ a}$			

For Pb, we found a three-way interaction between the body concentrations, metal and temperature treatment ($F = 2.84$, $p = 0.0375$). The slope Pb + Cd at 20 °C is significantly different from the slopes of the single Pb ($t = 2.53$, $p = 0.0118$) and Pb + Cu ($t = 2.52$, $p = 0.012$) treatments at 15 °C and vice versa. To ease the interpretation, we also checked the two-way interactions without the three-way interaction in our model. We found an interaction of Pb body burden with metal treatment ($F = 6.70$, $p < 0.001$; Figure 4D) as well as temperature treatment ($F = 5.77$, $p = 0.0167$; Figure 4E). Again, the slope of Pb + Cd is significantly higher than for the other metal treatments (for Pb: $t = -3.19$, $p = 0.002$; for Pb + Cu: $t = -4.23$, $p < 0.001$; and for Pb + Cd + Cu: $t = -3.35$, $p < 0.001$).

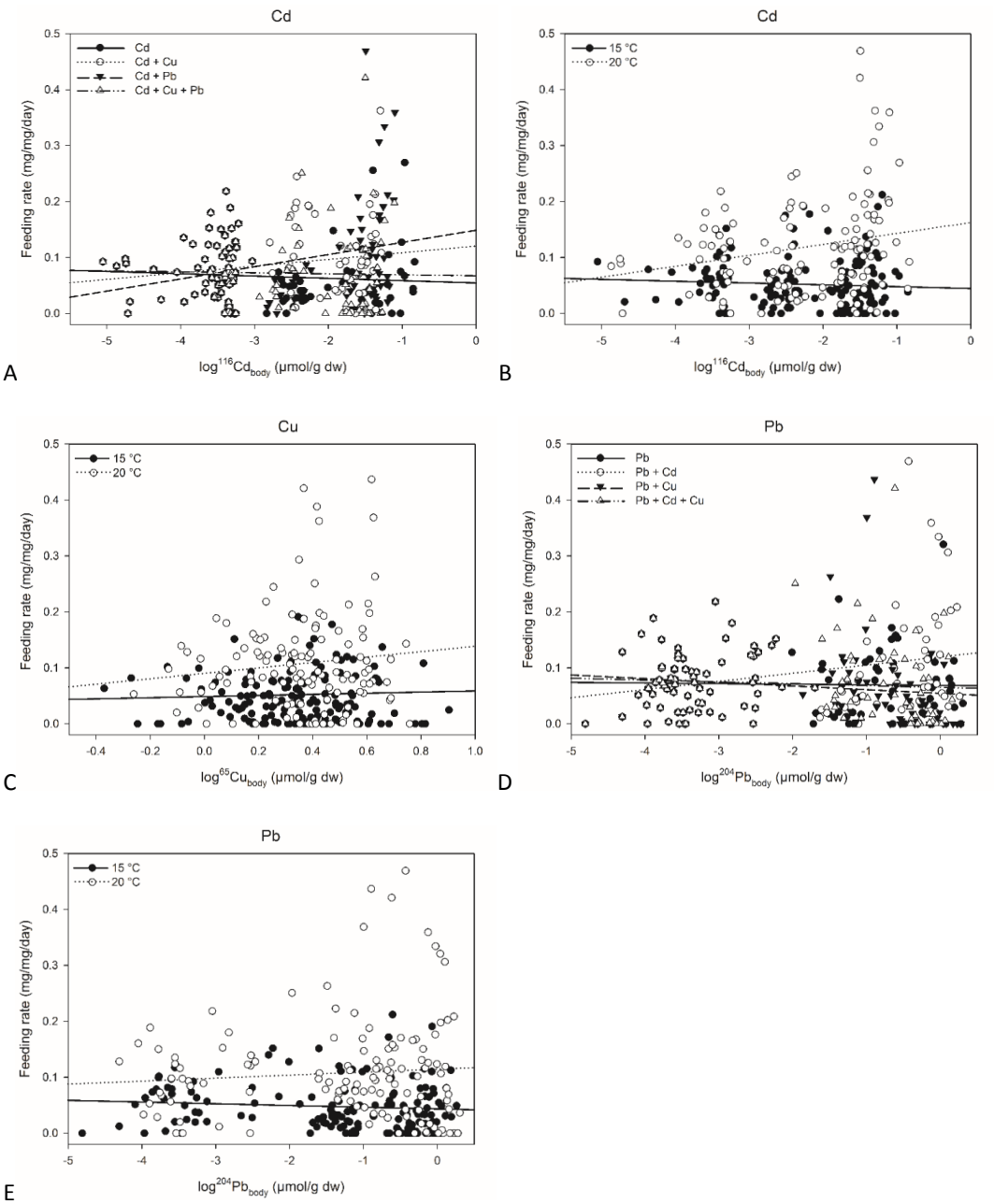

Figure 4: Significant relationships between the metal body concentrations and the feeding rates after 10 days: A-B) Cd metal and temperature treatments (N = 272); C) Cu temperature treatments (N = 272); and D-E) Pb metal and temperature treatments (N = 279).

Isopods at 20 °C ate 64.5 10^{-3} mg/mg/day or 144% more ($F = 92.5$, $p < 0.001$) and had a significantly higher (positive) slope than the feeding rate at 15 °C of which the slope was negatively correlated. The same differences were found with the FIA_{water} model.

5.3.6. Activity

Activities ranged from 17.2 ± 5.3 (Cd + Pb L at 20 °C) to 55.9 ± 6.2 % (Pb M at 20 °C; Table S11). No significant difference in activity was observed between the controls of the two temperature treatments. Also for the Cd and Cu treatments, no significant correlations or differences were found. For lead (Figure 5), we found a negative correlation between body concentration and activity (-1.28 ± 0.62 %.log(µg/g dw)$^{-1}$; $F = 4.32$, $p = 0.038$; $R^2 = 2.50$ %). However, this was not found with the FIA model.

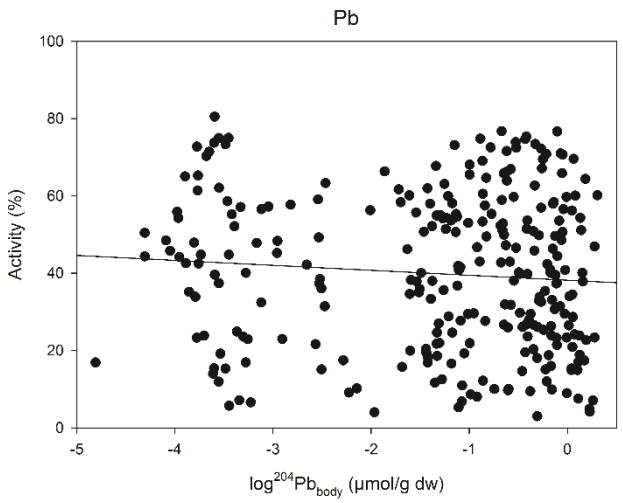

Figure 5: Significant relationship between the Pb body concentrations and the activities after 10 days (N = 279).

5.4. Discussion

In the present study, we investigated the effects of temperature on metal mixture stress. Temperature had a significant effect on all endpoints, except activity. We found several interactions with metal body burden and even a three-way interaction with mixture treatment for feeding rate, suggesting complex relations with this natural stressor.

For all metals at 15 °C, we found a declining respiration rate when the metal body concentrations increased. Interestingly, this relation inversed at 20 °C, resulting in a positive correlation. A positive effect of temperature on oxygen consumption was expected. Elevated temperatures cause an increase in metabolic rates, leading to a higher energy demand and thus respiration and/or feeding rates (Sokolova and Lannig, 2008). However, if this was the case, only a significant temperature effect would have been found. Interactions of temperature with (the concentration of) chemicals are less reported in the literature (Nieto et al., 2016). Khan et al. (2007) exposed earthworms to metals at different temperatures and observed an increase in oxygen consumption for Cd at 10-12 °C. This increase was even higher for 14-16 °C, also suggesting an interaction of Cd with temperature. A possible explanation might be found in the subcellular partitioning of the metals. If more metals were stored in metabolically-available fractions at 20 °C, thereby stimulating defense mechanisms, this would result in a higher respiration rate (Rainbow, 2007). Li et al. (2011) studied cadmium distribution in wheat roots and found that at a higher temperature the percentage of cadmium stored in metal-sensitive fractions (the organelles and heat-denatured proteins) increased, while the percentage of biologically-detoxified metals remained constant. Nevertheless, as defense mechanisms are costly and metals induce gill damage (Leung et al., 2000), it is unlikely that a higher respiration can be maintained. Therefore, we expect the respiration in the present study to stagnate and later decrease with increasing body concentrations.

As respiration rates were higher for the warmer treatments, we would expect the uptake rates and metal accumulation to be higher as well. This was observed for the Cu and Pb treatments. However, while we also expected a higher Cd uptake and accumulation (Cherkasov et al., 2007; Heugens et al., 2003), we did not find higher body concentrations. The lack of a temperature effect on Cd accumulation has been reported before (Cherkasov et al., 2006; Lannig et al., 2006). This might be explained by a temperature-induced change in membrane permeability (Hazel and Williams, 1990) or an increased elimination rate as observed for Cd in mayfly larvae by Odin et

al. (1995). Additionally, we saw a higher slope for accumulation for the single metals Pb and Cd and their mixture than for the other mixtures. The slopes were on average a factor of 1.2 higher. This was also observed by Van Ginneken et al. (2018) on the same species. This antagonistic interaction between Cu and Pb could be explained as both competing for uptake via Na^+ channels. However, when considering the tertiary mixture, we know that cadmium enters via Ca^{2+} channels. The inhibitory effects of Cu on Cd uptake were reported before by Komjarova and Blust (2008) and might be the result of competition for a common (unknown) uptake site, possibly Divalent Metal Ion Transporter 1. Yet, interestingly Cd and Pb did not affect Cu uptake, suggesting that copper outcompetes Cd and Pb for uptake (Franklin et al., 2002). This was also observed by Komjarova and Blust (2009). After exposing the zebrafish *Danio rerio* to mixtures of Cd, Cu, Ni, Pb and Zn, they found Cu uptake to be less affected by the presence of other metals than Cd and Pb.

As warmer temperatures cause metabolic rate to increase, leading to a higher energy demand, an increased feeding rate for all metal treatments at 20 °C was expected. Feeding rate for Cd and Pb treatments at 20 °C had a positive slope, while isopods of 15 °C treatments had a negatively correlated feeding rate. Considering this, it was interesting to find less increasing slopes for growth rate for Cd and Pb: they are lower by respectively a factor of 8 and 4. Thus, at lower body concentrations growth at 20 °C is higher. Yet, at higher body concentrations, the slopes of both temperature treatments meet, meaning there is no difference in growth anymore. We can conclude that there is most likely a lower food conversion efficiency. The energy gained from higher feeding rates is then less used for growth or locomotion, but is instead allocated to detoxification and cellular protection systems, such as metallothioneins (MT) and antioxidant enzymes (Sokolova et al., 2012). For copper, no decreased slope was found for growth rate for isopods at 20 °C. Correia et al. (2001) also found a higher MT synthesis and stimulated growth rates for the amphipod *Gammarus locusta* after sublethal Cu exposure. This might be explained by copper's role as an essential element.

In general, no negative growth rates were found, suggesting that the isopods could cope with the metal body concentrations, having the energy for both somatic growth and protection against metal damage. This positive correlation with body burden suggests a hormesis effect (i.e. when low levels of exposure to a toxic chemical that are harmful at high levels of exposure result in stimulatory effects on survival, growth, reproduction...). Hormesis is most likely an overcompensation response to a disruption in homeostasis, but there is no clear underlying mechanism (Calabrese, 1999). While not regularly reported, a review by Calabrese and Baldwin (1997) revealed hormetic effects in approximately 350 out of 4000 studies. We should note that hormesis on growth rate was not observed in Van Ginneken et al. (2018), but the use of higher exposure concentrations and thus higher metal accumulation could have masked this phenomenon.

In the present study, we observed that the feeding rate of Cd + Pb was higher than for the other mixtures. A concentration-dependent effect of the binary mixture Cd + Pb on feeding rate was already reported in the study of Van Ginneken et al. (2018) and was attributed to these specific concentrations triggering a range of energetically costly defense mechanisms. For the Cd treatments, we found a negative slope for the feeding rate of isopods at 15 °C, while the slope at 20 °C was positive. Nieto et al. (2016) also found that the ingestion rates of the shrimp *Atyaephyra desmarestii* increased when exposed at a warmer temperature. Besides affecting metabolic rates, it has been demonstrated that MT-synthesis is also temperature dependent, showing higher MT induction at higher temperatures (Van Cleef-Toedt et al., 2001; Leung et al., 2000). As defense mechanisms are costly, this could explain the positively correlated feeding rate. For Cu, we also found a higher feeding rate for Cu exposed isopods at 20 °C as well, which combined with the increased respiration rate led to a higher accumulation. Interestingly, we found a three-way interaction for Pb, meaning that the slopes differed depending on both the temperature and the mixture treatment. To our knowledge, an interaction between the concentration of the chemical, temperature and mixture treatment has not been reported before. Studies such as Ferreira et al.

(2016), Fonte et al. (2016) and Nieto et al. (2016) investigated the effects of pollutant mixtures and temperature on feeding rate, but none of them found a three-way interaction.

While it is difficult to derive concrete consequences of a future temperature rise, we observed increased feeding and respiration rates at a higher temperature and higher metal body concentrations. This suggests a larger energy demand is needed to protect all bodily functions and can probably not be maintained during longer metal exposure or higher temperatures (which is also indicated by the significantly higher mortality rate at 20 °C). Therefore, if no genetic adaptation occurs, future asellid populations will decrease and at an even faster rate in polluted ecosystems. Additionally, we found effects of metal mixtures on accumulation and feeding rates and we observed an interaction between temperature, mixture treatment and body burdens for the feeding rate of Pb treatments. Not only does this emphasize the importance of studying multiple stressor effects, it also demonstrates how the current environmental risk assessment is inadequate in predicting the toxicity of pollutants in ecosystems. As natural stressors are not included in the tests, environmental quality standards may be overprotective or perhaps not protective enough. We suggest shifting to, or complementing chemical-based monitoring with, effect-based tools *in situ* to identify ecological risks. Possible methods could be the use of biomarkers, i.e. indicators of contaminant stress at the molecular or individual level measured, or ecological methods, measuring changes observed at the population and/or community level (Connon et al., 2012; Wernersson et al., 2015). Ecotoxicological approaches need to evolve if we wish to understand the consequences of contaminants in nature and act on them.

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5.6. Supplementary material

Table S1: Average mortalities (± SE) after 10 days for all metal and temperature treatments (N = 500).

Metal	Concentration	Mortality (%)	
		15 °C	**20 °C**
Controls		2.5 ± 2.5	15 ± 6
Cd	L	0	0
	M	10	40
	H	0	20
Cu	L	0	10
	M	0	30
	H	0	40
Pb	L	0	30
	M	0	10
	H	0	0
Cd + Cu	L	0	30
	M	0	50
	H	0	20
Cd + Pb	L	10	70
	M	0	10
	H	10	30
Cu + Pb	L	20	50
	M	10	0
	H	0	0
Cd + Cu + Pb	L	0	40
	M	0	0
	H	0	10

Table S2: Average ^{116}Cd FIAs (± SE) after 10 days for all metal and temperature treatments (N = 272).

Metal	Concentration	^{116}Cd FIAs (µmol/L)	
		15 °C	**20 °C**
Controls		$1.65\ 10^{-6} \pm 0.086\ 10^{-6}$	$1.09\ 10^{-6} \pm 0.055\ 10^{-6}$
Cd	L	$2.07\ 10^{-6} \pm 0.15\ 10^{-6}$	$1.48\ 10^{-6} \pm 0.081\ 10^{-6}$
	M	$0.923\ 10^{-3} \pm 0.174\ 10^{-3}$	$0.562\ 10^{-3} \pm 0.052\ 10^{-3}$
	H	$1.71\ 10^{-3} \pm 0.18\ 10^{-3}$	$0.921\ 10^{-3} \pm 0.173\ 10^{-3}$
Cd + Cu	L	$2.50\ 10^{-6} \pm 0.19\ 10^{-6}$	$3.29\ 10^{-6} \pm 1.46\ 10^{-6}$
	M	$2.60\ 10^{-3} \pm 0.31\ 10^{-3}$	$1.33\ 10^{-3} \pm 0.23\ 10^{-3}$
	H	$10.2\ 10^{-3} \pm 1.2\ 10^{-3}$	$7.07\ 10^{-3} \pm 0.90\ 10^{-3}$
Cd + Pb	L	$1.57\ 10^{-6} \pm 0.10\ 10^{-6}$	$1.37\ 10^{-6} \pm 0.31\ 10^{-6}$
	M	$2.12\ 10^{-3} \pm 0.16\ 10^{-3}$	$0.759\ 10^{-3} \pm 0.055\ 10^{-3}$
	H	$5.18\ 10^{-3} \pm 0.56\ 10^{-3}$	$2.40\ 10^{-3} \pm 0.49\ 10^{-3}$
Cd + Cu + Pb	L	$2.94\ 10^{-6} \pm 0.25\ 10^{-6}$	$3.03\ 10^{-6} \pm 0.67\ 10^{-6}$
	M	$3.29\ 10^{-3} \pm 0.26\ 10^{-3}$	$1.37\ 10^{-3} \pm 0.14\ 10^{-3}$
	H	$12.0\ 10^{-3} \pm 0.3\ 10^{-3}$	$1.42\ 10^{-3} \pm 0.23\ 10^{-3}$

Table S3: Average ^{116}Cd body burdens (± SE) after 10 days for all metal and temperature treatments (N = 272).

Metal	Concentration	^{116}Cd body concentrations (µmol/g dw)	
		15 °C	**20 °C**
Controls		$0.334\ 10^{-3} \pm 0.026\ 10^{-3}$	$0.364\ 10^{-3} \pm 0.036\ 10^{-3}$
Cd	L	$3.70\ 10^{-3} \pm 0.28\ 10^{-3}$	$3.36\ 10^{-3} \pm 0.42\ 10^{-3}$
	M	$23.3\ 10^{-3} \pm 2.5\ 10^{-3}$	$20.3\ 10^{-3} \pm 5.2\ 10^{-3}$
	H	$75.3\ 10^{-3} \pm 10.7\ 10^{-3}$	$86.0\ 10^{-3} \pm 15.9\ 10^{-3}$
Cd + Cu	L	$3.77\ 10^{-3} \pm 0.48\ 10^{-3}$	$3.49\ 10^{-3} \pm 0.42\ 10^{-3}$
	M	$29.8\ 10^{-3} \pm 4.1\ 10^{-3}$	$36.4\ 10^{-3} \pm 5.5\ 10^{-3}$
	H	$21.8\ 10^{-3} \pm 2.3\ 10^{-3}$	$29.8\ 10^{-3} \pm 4.5\ 10^{-3}$
Cd + Pb	L	$3.33\ 10^{-3} \pm 0.55\ 10^{-3}$	$3.73\ 10^{-3} \pm 0.94\ 10^{-3}$
	M	$42.1\ 10^{-3} \pm 5.2\ 10^{-3}$	$32.7\ 10^{-3} \pm 4.2\ 10^{-3}$
	H	$46.1\ 10^{-3} \pm 5.01\ 10^{-3}$	$51.3\ 10^{-3} \pm 7.9\ 10^{-3}$
Cd + Cu + Pb	L	$2.45\ 10^{-3} \pm 0.28\ 10^{-3}$	$3.57\ 10^{-3} \pm 0.35\ 10^{-3}$
	M	$30.9\ 10^{-3} \pm 3.5\ 10^{-3}$	$25.0\ 10^{-3} \pm 3.4\ 10^{-3}$
	H	$27.1\ 10^{-3} \pm 4.1\ 10^{-3}$	$42.3\ 10^{-3} \pm 6.3\ 10^{-3}$

Table S4: Average ^{65}Cu FIAs (± SE) after 10 days for all metal and temperature treatments (N = 272).

Metal	Concentration	^{65}Cu FIAs (µmol/L)	
		15 °C	20 °C
Controls		$96.3\ 10^{-9} \pm 24.8\ 10^{-9}$	$23.3\ 10^{-9} \pm 4.0\ 10^{-9}$
Cu	L	$11.7\ 10^{-6} \pm 1.2\ 10^{-6}$	$4.86\ 10^{-6} \pm 1.01\ 10^{-6}$
	M	$4.30\ 10^{-3} \pm 0.75\ 10^{-3}$	$0.429\ 10^{-3} \pm 0.186\ 10^{-3}$
	H	$17.4\ 10^{-3} \pm 2.5\ 10^{-3}$	$4.81\ 10^{-3} \pm 1.03\ 10^{-3}$
Cu + Cd	L	$3.09\ 10^{-6} \pm 0.60\ 10^{-6}$	$1.38\ 10^{-6} \pm 0.19\ 10^{-6}$
	M	$11.8\ 10^{-3} \pm 2.2\ 10^{-3}$	$0.777\ 10^{-3} \pm 0.268\ 10^{-3}$
	H	$21.2\ 10^{-3} \pm 2.2\ 10^{-3}$	$11.7\ 10^{-3} \pm 1.7\ 10^{-3}$
Cu + Pb	L	$9.74\ 10^{-6} \pm 2.52\ 10^{-6}$	$4.06\ 10^{-6} \pm 0.92\ 10^{-6}$
	M	$6.21\ 10^{-3} \pm 1.51\ 10^{-3}$	$3.19\ 10^{-3} \pm 0.86\ 10^{-3}$
	H	$24.3\ 10^{-3} \pm 2.7\ 10^{-3}$	$9.44\ 10^{-3} \pm 1.94\ 10^{-3}$
Cu + Cd + Pb	L	$8.82\ 10^{-6}\ 2.38\ 10^{-6}$	$2.58\ 10^{-6}\ 0.62\ 10^{-6}$
	M	$3.70\ 10^{-3} \pm 0.78\ 10^{-3}$	$0.558\ 10^{-3} \pm 0.269\ 10^{-3}$
	H	$23.8\ 10^{-3} \pm 3.0\ 10^{-3}$	$12.8\ 10^{-3} \pm 2.9\ 10^{-3}$

Table S5: Average ^{65}Cu body burdens (± SE) after 10 days for all metal and temperature treatments (N = 272).

Metal	Concentration	^{65}Cu body concentrations (µmol/g dw)	
		15 °C	20 °C
Controls		1.52 ± 0.12	1.41 ± 0.09
Cu	L	1.63 ± 0.09	1.70 ± 0.18
	M	2.39 ± 0.14	2.40 ± 0.15
	H	4.08 ± 0.63	3.69 ± 0.71
Cu + Cd	L	2.45 ± 0.09	2.55 ± 0.26
	M	3.47 ± 0.39	3.26 ± 0.29
	H	2.10 ± 0.12	2.41 ± 0.18
Cu + Pb	L	2.69 ± 0.22	3.64 ± 0.32
	M	3.63 ± 0.45	3.75 ± 0.26
	H	1.97 ± 0.21	2.77 ± 0.37
Cu + Cd + Pb	L	1.63 ± 0.12	2.49 ± 0.24
	M	4.31 ± 0.38	3.63 ± 0.29
	H	2.84 ± 0.25	3.01 ± 0.20

Table S6: Average ^{204}Pb FIAs (± SE) after 10 days for all metal and temperature treatments (N = 279).

Metal	Concentration	^{204}Pb FIAs (µmol/L)	
		15 °C	**20 °C**
Controls		$2.16\ 10^{-9} \pm 0.62\ 10^{-9}$	$1.32\ 10^{-9} \pm 0.38\ 10^{-9}$
Pb	L	$0.223\ 10^{-6} \pm 0.041\ 10^{-6}$	$0.130\ 10^{-6} \pm 0.026\ 10^{-6}$
	M	$0.119\ 10^{-3} \pm 0.034\ 10^{-3}$	$20.1\ 10^{-6} \pm 4.7\ 10^{-6}$
	H	$0.708\ 10^{-3} \pm 0.305\ 10^{-3}$	$0.277\ 10^{-3} \pm 0.120\ 10^{-3}$
Pb + Cd	L	$0.227\ 10^{-6} \pm 0.068\ 10^{-6}$	$0.132\ 10^{-6} \pm 0.111\ 10^{-6}$
	M	$70.4\ 10^{-6} \pm 23.4\ 10^{-6}$	$9.61\ 10^{-6} \pm 3.41\ 10^{-6}$
	H	$0.557\ 10^{-3} \pm 0.199\ 10^{-3}$	$0.292\ 10^{-3} \pm 0.185\ 10^{-3}$
Pb + Cu	L	$0.979\ 10^{-6} \pm 0.217\ 10^{-6}$	$0.475\ 10^{-6} \pm 0.179\ 10^{-6}$
	M	$0.509\ 10^{-3} \pm 0.177\ 10^{-3}$	$0.273\ 10^{-3} \pm 0.099\ 10^{-3}$
	H	$1.52\ 10^{-3} \pm 0.32\ 10^{-3}$	$0.336\ 10^{-3} \pm 0.088\ 10^{-3}$
Pb + Cd + Cu	L	$1.06\ 10^{-6} \pm 0.30\ 10^{-6}$	$0.254\ 10^{-6} \pm 0.056\ 10^{-6}$
	M	$0.124\ 10^{-3} \pm 0.040\ 10^{-3}$	$38.7\ 10^{-6} \pm 31.5\ 10^{-6}$
	H	$1.52\ 10^{-3} \pm 0.45\ 10^{-3}$	$0.850\ 10^{-3} \pm 0.334\ 10^{-3}$

Table S7: Average ^{204}Pb body burdens (± SE) after 10 days for all metal and temperature treatments (N = 279).

Metal	Concentration	^{204}Pb body concentrations (µmol/g dw)	
		15 °C	**20 °C**
Controls		$1.59\ 10^{-3} \pm 0.57\ 10^{-3}$	$0.866\ 10^{-3} \pm 0.187\ 10^{-3}$
Pb	L	$44.3\ 10^{-3} \pm 8.1\ 10^{-3}$	$56.6\ 10^{-3} \pm 16.5\ 10^{-3}$
	M	0.211 ± 0.054	0.210 ± 0.020
	H	1.01 ± 0.15	1.22 ± 0.13
Pb + Cd	L	$49.3\ 10^{-3} \pm 7.5\ 10^{-3}$	$88.2\ 10^{-3} \pm 27.2\ 10^{-3}$
	M	0.728 ± 0.188	0.494 ± 0.087
	H	0.617 ± 0.080	1.06 ± 0.16
Pb + Cu	L	$49.9\ 10^{-3} \pm 6.8\ 10^{-3}$	$61.9\ 10^{-3} \pm 12.6\ 10^{-3}$
	M	0.315 ± 0.065	0.232 ± 0.046
	H	0.473 ± 0.079	1.15 ± 0.14
Pb + Cd + Cu	L	$46.8\ 10^{-3} \pm 5.6\ 10^{-3}$	$46.7\ 10^{-3} \pm 8.1\ 10^{-3}$
	M	0.544 ± 0.057	0.190 ± 0.032
	H	0.588 ± 0.068	0.916 ± 0.132

Table S8: Average respiration rates (± SE) after 10 days for all metal and temperature treatments (N = 388).

Metal	Concentration	Respiration rate (µg O_2/mg ww/h)	
		15 °C	**20 °C**
Controls		0.129 ± 0.010	0.138 ± 0.016
Cd	L	0.115 ± 0.039	$92.4\ 10^{-3} \pm 41.8\ 10^{-3}$
	M	0.107 ± 0.026	$72.4\ 10^{-3} \pm 46.6\ 10^{-3}$
	H	$81.5\ 10^{-3} \pm 20.8\ 10^{-3}$	0.202 ± 0.043
Cu	L	0.128 ± 0.016	0.146 ± 0.026
	M	0.139 ± 0.035	0.125 ± 0.039
	H	$65.4\ 10^{-3} \pm 16.3\ 10^{-3}$	0.143 ± 0.038
Pb	L	$69.0\ 10^{-3} \pm 12.0\ 10^{-3}$	0.166 ± 0.046
	M	0.163 ± 0.036	0.236 ± 0.053
	H	0.120 ± 0.021	0.198 ± 0.029
Cd + Cu	L	0.133 ± 0.033	0.153 ± 0.056
	M	0.113 ± 0.024	0.265 ± 0.078
	H	0.123 ± 0.019	0.222 ± 0.050
Cd + Pb	L	$98.7\ 10^{-3} \pm 27.8\ 10^{-3}$	0.106 ± 0.014
	M	0.159 ± 0.031	0.198 ± 0.036
	H	0.129 ± 0.036	0.233 ± 0.060
Cu + Pb	L	0.130 ± 0.025	0.164 ± 0.008
	M	0.146 ± 0.040	0.168 ± 0.035
	H	$84.7\ 10^{-3} \pm 16.0\ 10^{-3}$	0.157 ± 0.049
Cd + Cu + Pb	L	$87.5\ 10^{-3} \pm 16.7\ 10^{-3}$	0.138 ± 0.025
	M	0.144 ± 0.041	0.218 ± 0.048
	H	0.139 ± 0.027	0.243 ± 0.030

Table S9: Average growth rates (± SE) after 10 days for all metal and temperature treatments (N = 428).

Metal	Concentration	Growth rate (mg/day)	
		15 °C	**20 °C**
Controls		$-19.2\ 10^{-3} \pm 9.6\ 10^{-3}$	$11.2\ 10^{-3} \pm 9.4\ 10^{-3}$
Cd	L	$29.6\ 10^{-3} \pm 14.5\ 10^{-3}$	$30.5\ 10^{-3} \pm 7.2\ 10^{-3}$
	M	$0.953\ 10^{-3} \pm 14.112\ 10^{-3}$	$20.4\ 10^{-3} \pm 24.2\ 10^{-3}$
	H	$5.35\ 10^{-3} \pm 15.9\ 10^{-3}$	$10.3\ 10^{-3} \pm 16.8\ 10^{-3}$
Cu	L	$30.9\ 10^{-3} \pm 11.8\ 10^{-3}$	$-8.62\ 10^{-3} \pm 16.65\ 10^{-3}$
	M	$34.5\ 10^{-3} \pm 11.6\ 10^{-3}$	$25.3\ 10^{-3} \pm 14.1\ 10^{-3}$
	H	$-49.4\ 10^{-3} \pm 18.8\ 10^{-3}$	$-36.3\ 10^{-3} \pm 34.9\ 10^{-3}$
Pb	L	$88.7\ 10^{-3} \pm 12.6\ 10^{-3}$	$43.4\ 10^{-3} \pm 16.7\ 10^{-3}$
	M	$-38.2\ 10^{-3} \pm 22.0\ 10^{-3}$	$-28.1\ 10^{-3} \pm 19.5\ 10^{-3}$
	H	$-14.9\ 10^{-3} \pm 12.6\ 10^{-3}$	$21.2\ 10^{-3} \pm 10.3\ 10^{-3}$
Cd + Cu	L	$41.0\ 10^{-3} \pm 18.2\ 10^{-3}$	$48.0\ 10^{-3} \pm 14.2\ 10^{-3}$
	M	$31.1\ 10^{-3} \pm 16.9\ 10^{-3}$	$12.5\ 10^{-3} \pm 17.2\ 10^{-3}$
	H	$5.89\ 10^{-3} \pm 9.95\ 10^{-3}$	$16.8\ 10^{-3} \pm 14.3\ 10^{-3}$
Cd + Pb	L	$46.5\ 10^{-3} \pm 16.6\ 10^{-3}$	$15.9\ 10^{-3} \pm 27.3\ 10^{-3}$
	M	$-3.64\ 10^{-3} \pm 21.28\ 10^{-3}$	$-5.20\ 10^{-3} \pm 13.47\ 10^{-3}$
	H	$13.9\ 10^{-3} \pm 10.9\ 10^{-3}$	$53.1\ 10^{-3} \pm 11.1\ 10^{-3}$
Cu + Pb	L	$55.1\ 10^{-3} \pm 14.2\ 10^{-3}$	$21.1\ 10^{-3} \pm 13.6\ 10^{-3}$
	M	$12.7\ 10^{-3} \pm 15.6\ 10^{-3}$	$-0.254\ 10^{-3} \pm 17.709\ 10^{-3}$
	H	$5.79\ 10^{-3} \pm 7.06\ 10^{-3}$	$19.0\ 10^{-3} \pm 10.2\ 10^{-3}$
Cd + Cu + Pb	L	$33.9\ 10^{-3} \pm 13.4\ 10^{-3}$	$47.1\ 10^{-3} \pm 18.8\ 10^{-3}$
	M	$-32.9\ 10^{-3} \pm 19.6\ 10^{-3}$	$-18.2\ 10^{-3} \pm 13.3\ 10^{-3}$
	H	$16.7\ 10^{-3} \pm 16.9\ 10^{-3}$	$33.1\ 10^{-3} \pm 12.0\ 10^{-3}$

Table S10: Average feeding rates (± SE) after 10 days for all metal and temperature treatments (N = 428).

Metal	Concentration	Feeding rate (mg/mg/day)	
		15 °C	**20 °C**
Controls		$57.0\ 10^{-3} \pm 6.3\ 10^{-3}$	$94.4\ 10^{-3} \pm 9.9\ 10^{-3}$
Cd	L	$35.2\ 10^{-3} \pm 6.8\ 10^{-3}$	$34.8\ 10^{-3} \pm 6.6\ 10^{-3}$
	M	$48.8\ 10^{-3} \pm 17.1\ 10^{-3}$	$97.6\ 10^{-3} \pm 39.8\ 10^{-3}$
	H	$28.3\ 10^{-3} \pm 8.5\ 10^{-3}$	0.113 ± 0.025
Cu	L	$28.1\ 10^{-3} \pm 8.4\ 10^{-3}$	0.104 ± 0.012
	M	$29.2\ 10^{-3} \pm 8.3\ 10^{-3}$	0.172 ± 0.046
	H	$36.5\ 10^{-3} \pm 13.9\ 10^{-3}$	$73.0\ 10^{-3} \pm 23.7\ 10^{-3}$
Pb	L	$21.4\ 10^{-3} \pm 12.1\ 10^{-3}$	0.103 ± 0.021
	M	$56.5\ 10^{-3} \pm 20.6\ 10^{-3}$	0.101 ± 0.019
	H	$50.8\ 10^{-3} \pm 12.7\ 10^{-3}$	$78.0\ 10^{-3} \pm 29.5\ 10^{-3}$
Cd + Cu	L	0.107 ± 0.021	0.169 ± 0.020
	M	$80.0\ 10^{-3} \pm 11.8\ 10^{-3}$	0.139 ± 0.026
	H	$38.3\ 10^{-3} \pm 10.8\ 10^{-3}$	0.122 ± 0.040
Cd + Pb	L	$32.5\ 10^{-3} \pm 7.8\ 10^{-3}$	$60.7\ 10^{-3} \pm 15.2\ 10^{-3}$
	M	$42.8\ 10^{-3} \pm 4.9\ 10^{-3}$	0.137 ± 0.012
	H	$92.1\ 10^{-3} \pm 23.7\ 10^{-3}$	0.286 ± 0.044
Cu + Pb	L	$61.3\ 10^{-3} \pm 16.4\ 10^{-3}$	0.131 ± 0.038
	M	$19.3\ 10^{-3} \pm 7.1\ 10^{-3}$	0.131 ± 0.047
	H	$24.1\ 10^{-3} \pm 7.4\ 10^{-3}$	$28.4\ 10^{-3} \pm 15.3\ 10^{-3}$
Cd + Cu + Pb	L	$51.4\ 10^{-3} \pm 13.3\ 10^{-3}$	0.135 ± 0.027
	M	$37.0\ 10^{-3} \pm 12.6\ 10^{-3}$	0.133 ± 0.038
	H	$8.69\ 10^{-3} \pm 5.34\ 10^{-3}$	$75.2\ 10^{-3} \pm 25.9\ 10^{-3}$

Table S11: Average activities (± SE) after 10 days for all metal and temperature treatments (N = 428).

Metal	Concentration	Activity (%)	
		15 °C	**20 °C**
Controls		42.6 ± 3.4	41.2 ± 3.3
Cd	L	39.4 ± 6.6	48.4 ± 6.1
	M	51.1 ± 6.0	31.0 ± 8.7
	H	51.3 ± 4.1	48.9 ± 6.0
Cu	L	32.0 ± 6.3	38.8 ± 5.7
	M	38.0 ± 6.7	35.5 ± 8.9
	H	46.1 ± 5.5	41.9 ± 7.2
Pb	L	37.9 ± 6.3	38.6 ± 3.2
	M	39.1 ± 4.3	55.9 ± 6.2
	H	43.6 ± 6.2	35.3 ± 6.3
Cd + Cu	L	39.6 ± 9.4	28.0 ± 7.9
	M	50.6 ± 7.3	39.9 ± 12.5
	H	36.3 ± 4.5	34.0 ± 4.8
Cd + Pb	L	43.6 ± 4.0	17.2 ± 5.3
	M	36.2 ± 6.7	35.0 ± 7.7
	H	40.8 ± 7.0	27.1 ± 7.4
Cu + Pb	L	43.4 ± 8.1	41.4 ± 9.5
	M	52.3 ± 5.5	45.6 ± 7.9
	H	35.9 ± 6.2	37.4 ± 6.7
Cd + Cu + Pb	L	34.9 ± 7.1	26.9 ± 7.6
	M	44.9 ± 8.4	41.8 ± 6.3
	H	34.4 ± 4.3	48.7 ± 6.1

Chapter 6.

The effects of single metals and their mixture on a simplified freshwater community: a microcosm experiment

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Abstract

Evaluating ecological risks of metal contamination remains an important challenge. Current risk assessment focuses on the effects of single metals on individuals. However, the toxicity of metals can be influenced by other metals or natural stressors. Additionally, while the effects on individuals may be extensively investigated, the effects on populations and ecosystems are not always easily extrapolated. The present study, a microcosm experiment in a greenhouse, was designed to gain more insight into the effects of Cd, Cu and Pb and their mixture on populations and communities. Small ecosystems with several species of macroinvertebrates were exposed under semi-natural conditions. In each bucket, we placed *Asellus aquaticus* (Isopoda)*, Daphnia magna,* Chironominae (midge larvae), *Physella acuta* (Mollusca), *Elodea nuttallii* (macrophyte) and *Raphidocelis subcapitata* (alga). The effects of the metal mixtures were examined after 4 and 8 weeks, on the individual level (total metal accumulation), the population level (species abundances, algal biomass, shoot and root length) and the community structure (Shannon-Wiener index). After four and eight weeks, we found negative metal effects on plant growth. However, no effects were observed on invertebrate abundances nor any significant differences in Shannon-Wiener index were found, even though literature data reveal the induction of energetically costly defense mechanisms. These are possibly compensated by reducing other metabolic costs. As we found that some species appear to be more resilient than would be expected from the results of laboratory studies, more realistic metal exposure scenarios in micro- and mesocosm experiments could thus be a valuable addition to risk assessment.

6.1. Introduction

In the last century, anthropogenic activities causing metal emission in the environment, such as smelting and fossil-fuel combustion, have reached unprecedented proportions (Duruibe et al., 2007). Consequentially, much research focuses on the potential risks of metal pollution. To protect freshwater sites, the European Union (EU) has set environmental quality standards (EQS). These are maximum concentrations of a chemical which are expected not to cause any negative ecological effects (EP, 2008; EP, 2013). To establish an EQS, a chemical's toxicity is determined by exposing a test organism in the laboratory in constant and favorable conditions. While these laboratory tests provide data of the relative toxicity of chemicals, they do not necessarily reflect the effects of chemicals in aquatic ecosystems, where multiple stressors occur. Factors such as temperature fluctuations, food stress and inter-/intraspecific competition, can cause energetically costly stress responses and could, therefore, alter the organisms' vulnerability (Del Arco et al., 2015; Janssens and Stoks, 2013; Sokolova and Lannig, 2008). Not only natural stressors, but other metals can interact as well and cause synergistic, antagonistic and additive toxic effects (Norwood et al., 2003). Norwood et al. (2003) studied the effects of metal mixtures on aquatic biota and found that the frequency with which antagonism, additivity and synergism was reported was respectively 43, 27 and 29%.

Additionally, these laboratory tests are performed on the individual level, while the goal of environmental risk assessment is to protect higher levels of biological organization, such as populations, communities and ecosystems. The presence of multiple species with different sensitivities that can interact and influence each other should be considered in risk assessment as they might play an important role. For example, when the toxicity of a chemical is detrimental for one species leading to decreased population growth, another less sensitive species might be able to perform the same critical functions as the first one, which results in an undisturbed continuance of the polluted ecosystem (Walker, 1992). Alternatively, when a keystone species is affected, consequences for the ecosystem can be far-reaching. Aware of the

discrepancies between conditions in the laboratory and nature, the EU uses safety factors to extrapolate the results of laboratory tests. However, the choice of these factors is based on little ecological evidence and might cause over- or underprotective measures to be taken (Chapman et al., 1998).

Field experiments can be conducted to assess the effects of toxicants on (polluted) aquatic ecosystems. *In situ* studies are more ecologically-relevant as the interactions of populations and communities can be followed over time. However, these systems are complex. There is an increase in the uncertainty of results due to confounding factors like e.g. intra- and interspecific competition, predator stress or the physical properties of the sediment (Del Arco et al., 2015; Relyea, 2003; Solomon and Sibley, 2002). Additionally, field experiments are costly and responses of organisms are only detected after a contamination event has occurred. They are thus less useful to predict responses of contamination.

As another possibility to improve risk assessment, microcosm and mesocosm experiments can be performed. They are constructed to simulate parts of natural aquatic ecosystems, while certain factors can be controlled (Caquet et al., 2000). Disadvantages to the use of micro-and mesocosms include the relatively large amounts of waste and the high variability between replicates that increases over time, caused by factors such as complex interactions between organisms (e.g., competition; Jokiel et al., 2008). However, they provide greater ecological realism than single species tests as they can be conducted at a larger spatiotemporal scale and the effects of chemicals can be studied on the whole aquatic community.

In the present study, we describe a microcosm experiment in a greenhouse, which was designed to gain more insight into the effects of Cd, Cu and Pb under semi-natural conditions. Small freshwater ecosystems with several species of macroinvertebrates were exposed to these three metals and their mixture under semi-natural conditions. These metals have different modes of action. Copper can disrupt Na^+ homeostasis,

cadmium disrupts Ca^{2+} homeostasis and Pb disrupts both Na^{+} and Ca^{2+} uptake (Birceanu et al., 2008; De Schamphelaere and Janssen, 2002).

Several laboratory experiments, studying the effects of metal mixtures and natural stressors, were already performed on the aquatic sowbug *A. aquaticus* and links were made between metal body concentrations and relevant sublethal endpoints (Van Ginneken et al., 2015, 2018). In these studies, we found the tertiary mixture to act antagonistically for metal accumulation. Thus, uptake was higher in the single Cd and Pb treatments than in the tertiary mixture. This was not observed for Cu. In general, high metal body burdens were related to lower growth rates and activities. Therefore, we hypothesize that invertebrate populations will be affected more in the single Cd and Pb treatments compared to the mixture treatment, resulting in a lower ecosystem diversity.

6.2. Material and Methods

6.2.1. Organism collection and acclimation

All organisms were collected in September 2017. The freshwater isopod *Asellus aquaticus* and the submerged macrophyte *Elodea nuttallii* were taken from the Laakbeek (Basin of the Scheldt River) in Lille, Belgium. They were kept in the laboratory in a climate chamber type WT15'/+5DU-WB (Weiss Technik, Reiskirchen-Lindenstruth, Germany) at constant temperature (15 ± 1 °C) and light conditions (16:8 h light:dark photoperiod). *Asellus aquaticus* was fed with 'conditioned' alder leaves (*Alnus glutinosa*): leaves that had been dried and rehydrated for 6 days in water from the Laakbeek (Bloor, 2010). The water flea *Daphnia magna* (DAPHTOXKIT F magna, batch DM280815, MicroBioTests), of which we used the F_0 generation, was also bred in a climate chamber (at 20 ± 1°C and a 16:8 h light:dark photoperiod). They were fed with Spirulina Tabs (Sera). Additionally, the snail *Physella acuta* was bought from Aquaria Antwerp (Aartselaar, Belgium) and the midge larvae (Chironominae) were collected from the Diepe Beek, a small stream in Wommelgem, Belgium. All organisms were acclimated to the tap water (very hard: 196 mg/L $CaCO_3$, pH = 7.87 ± 0.04) used in this

experiment for minimally one week. Lastly, we used a culture of the algae *Raphidocelis subcapitata* from the laboratory of Environmental Toxicology and Aquatic Ecology, Environmental Toxicology Unit - GhEnToxLab (Ghent University).

6.2.2. Experimental set-up

This 8-week experiment was conducted in a 1000 m² greenhouse, from September to November 2017. We exposed a simplified macroinvertebrate community to the single metals Cd, Cu and Pb and to a mixture of the three metals in 30 L buckets, each filled with 10 L tap water and 3 kg Rhine Sand. The concentrations we used were 13.3 10^{-3} µmol/L for Cd, 1.10 µmol/L for Cu and 0.347 µmol/L for Pb. These are equal to 10 x EQS (EP, 2008; VLAREM, 2015) and were used in previous toxicity experiments (Van Ginneken et al., 2018, 2019). Stock solutions were prepared in acidified milli-Q (1 %, made with 69% trace-metal-grade HNO_3) with analytical grade salts of cadmium chloride hydrate ($CdCl_2.H_2O$, Alfa Aesar), copper chloride dihydrate ($CuCl_2.2H_2O$, Merck), and lead chloride ($PbCl_2$, Merck). Furthermore, a control treatment without metals was added as well. As a food source, we put in *Raphidocelis subcapitata* (100,000 cells/mL; Andreozzi et al., 2002) and conditioned *Alnus glutinosa* leaves (5 g dw + an extra 2.5 g dw in the second month). In total, there were six replicates per treatment, which were equally divided over three large ponds (diameter 2 m, water depth 1.2 m). They were placed randomly in each pond, after which the ponds were partially filled with water to buffer temperature fluctuations in the buckets.

At the start of the experiment, we placed 30 *Asellus aquaticus* (15 juveniles < 5 mm and 15 adults), 20 *Daphnia magna* (9-10 days old), 10 *Physella acuta* (size 2-5 mm) and 50 Chironominae larvae (3^{rd} - 4^{th} instar) in each bucket. Interspecific relationships can be found in Figure 1 (Bernot et al., 2005; Bloor, 2010; Chergui and Pattee, 1991; Cope and Winterbourn, 2004; De Schamphelaere et al., 2004; Ptatscheck et al., 2017; Van Hattum et al., 1989). These species abundances were tested previously (without metal exposure) in an exploratory experiment and resulted in population growth (unpublished data). Additionally, green stems and the apex of *E. nuttallii*, without a

trace of necrosis, were selected and a piece of 6 cm was cut off. Two of these pieces were planted (approximately 1 cm deep) in the sediment of all buckets. Next, a net was placed over the buckets to be able to check the emergence ratio of the Chironominae. The water characteristics (pH = 7.90 ± 0.02; $T_{month\ 1}$= 17.3 ± 0.1 °C and $T_{month\ 2}$ = 15.9 ± 0.3 °C; EC = 919 ± 12 µS.cm^{-1}; and DO = 7.34 ± 0.10 mg.L^{-1} O_2) and the emergence ratio were monitored daily. When the oxygen concentration dropped beneath 5 mg/L, O_2 concentrations were increased with an air pump. Further, half of the medium (with metals and algae) was renewed weekly and samples were taken before and after renewal for metal and DOC analysis. Additionally, we also determined chlorophyll *a*, as a proxy for algae biomass, by filtering 50 mL of water over round glass fiber filters (MN GF-3, 2.5 cm, Filter Service). These filters could then be stored for maximally two weeks at -20 °C. To quantify chlorophyll a, it was extracted using 90% aceton and measured spectrophotometrically, before and after acidification with HCl, at 750, 665, 645, 630 and 480 nm (Shimadzu UV-160).

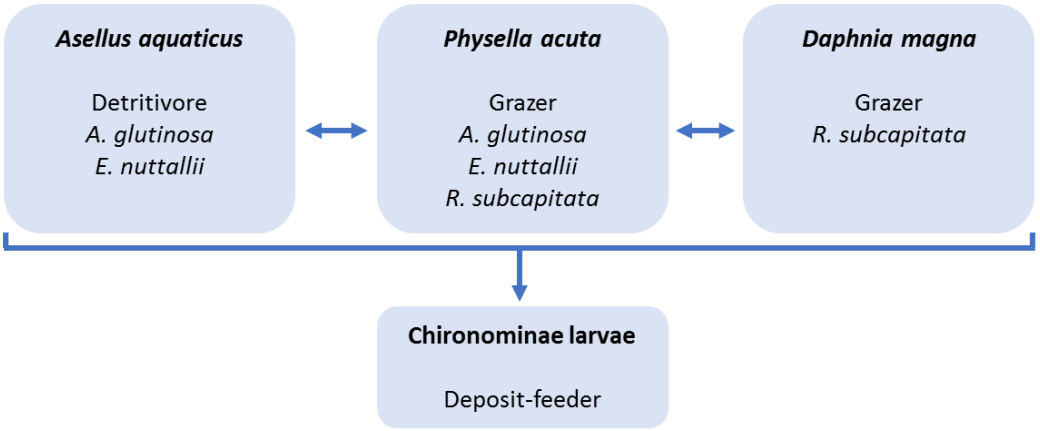


Figure 1: Community structure

After four and eight weeks, a replicate of each treatment was taken out of each pond, resulting in three replicates per sampling moment. In the laboratory, animals that were alive were counted, the shoots and the roots of the plants were measured (accuracy of 1 mm) and all were placed in a -20°C freezer awaiting further analysis. Species diversity and evenness were determined using the Shannon-Wiener index (Shannon,

1948). At the first sampling period after 4 weeks, daphnids were only counted in a water sample of 500 mL, which was then recalculated for 10 L. We did not measure metal accumulation for the Chironominae larvae, as almost none were found alive, nor in the adults.

6.2.3. Metal and DOC analysis

For the water samples, 60 mL of water was filtered through a 0.20 µm filter (Chromafil, Macherey-Nagel) and 10 mL was acidified to 1% HNO_3. Metal concentrations were measured using a quadrupole inductively coupled plasma mass spectrometer (ICP-MS; 7700×, Agilent Technologies), while major ion concentrations were determined with an inductively coupled plasma optic emission spectrometer (ICP-OES; Thermo scientific, ICAP 6300 Duo). The average major ion concentrations were: for Ca = 63.9 ± 0.3 mg/L; for K = 7.90 ± 0.06 mg/L; for Mg = 11.8 ± 0.1 mg/L; and for Na = 73.2 ± 0.8 mg/L. The dissolved metal concentrations were used to calculate the free metal ion activities (FIAs) as an estimation of the bioavailability, using the Windermere Humic Aqueous Model 6.0.13 (Natural Environment Research Council). For metal concentrations below the detection limit (< 0.1 µg/L for the metals; < 1 µg/L for the major ions), we entered the detection limit itself. The measured DOC was entered as fulvic acids. Additionally, the dissolved organic carbon concentration was measured in the remaining filtered water, after acidification to pH 2 using 2 N HCL, with a TOC-analyzer (TOC-VCPH, Shimadzu Corporation).

All organisms were dried for 72 h at 60 °C in a laboratory furnace. After cooling in a desiccator, they were weighed on a Sartorius SE2 ultra microbalance (accuracy of 1 µg). *Asellus aquaticus* were digested individually, while *D. magna* and *P. acuta* were pooled to +/- 100 organisms per sample and 3-4 per sample, respectively. Next, they were digested in a solution of trace-metal-grade HNO_3 (69%) and high-purity H_2O_2 (29%) (3:1 for *D. magna* and *A. aquaticus*, 6:1 for *P. acuta*) for 1 h at 125 °C in a hot block (Environmental Express, Charleston, SC, USA). Three blanks and three samples with certified reference material (SRM2976, National Institute of Standards and

Technology) were added as well. For the plant material, we digested the shoots and the roots separately. After adding 1 mL trace-metal-grade HNO_3 (69%) and 3 mL trace-metal-grade HCl (37%), the samples were microwaved using the Discover SP-D digester (CEM). All digested samples were diluted to 2% acid. Again, metal concentrations were measured with the ICP-MS (7700×, Agilent Technologies). Trace metal concentrations are expressed as µmol/g dw. *Daphnia magna* could not be weighed accurately and its concentrations are, therefore, expressed as µmol/individual.

6.2.4. Statistical analyses

Data were analyzed using linear mixed-effects models (packages 'lme4' and 'multcomp') in R v3.4.2. “Metal treatment” and “species” were fixed effects, while the ponds in which their respective buckets were placed were included as a random effect. A similar linear mixed-effects model was made for *E. nuttallii*, with “metal treatment” and the part of the plant (“shoot or root”) as fixed effects. When $p < 0.05$, we determined the effect to be significant. These mixed models account for non-independence of observations in the same pond. The data were tested for normality with histograms. Homoscedasticity was visually checked. Only significant differences between the controls and the metal treatments or the single metals and the tertiary mixture are shown.

6.3. Results

6.3.1. Metal and DOC concentrations

The dissolved metal concentrations did not differ significantly between the first and last four weeks, respectively weeks 0-4 and 4-8 (Table S1). For Cu and Pb, we found no significant difference in the concentrations of the single metal treatment and the tertiary treatment. However, the Cd concentration in the tertiary mixture was significantly lower than in the single Cd treatment ($p < 0.001$). Additionally, we found an average DOC concentration of 4.75 ± 0.10 mg/L for weeks 0-4 and 2.93 ± 0.06 mg/L for weeks 4-8.

The calculated FIAs of Cd and Cu were higher in the last four weeks compared to the first (for Cd: $F = 18.4$, $p < 0.001$; and for Cu: $F = 35.2$, $p < 0.001$; Table 1). Again, we found lower FIAs for Cd in the tertiary mixture than for single Cd ($p = 0.004$). For Pb, on the other hand, the concentrations were generally higher in the mixture ($p = 0.001$).

Table 1: Overview of the free ion activities (FIA) of 0-4 weeks and 4-8 weeks (N = 360).

	FIA Cd (µmol/L)	FIA Cu (µmol/L)	FIA Pb (µmol/L)
Week 0-4			
Controls	$0.434\ 10^{-3} \pm 0.010\ 10^{-3}$	$9.33\ 10^{-6} \pm 4.50\ 10^{-6}$	$58.3\ 10^{-9} \pm 8.5\ 10^{-9}$
Cd	$3.10\ 10^{-3} \pm 0.22\ 10^{-3}$	$0.143\ 10^{-3} \pm 0.139\ 10^{-3}$	$0.101\ 10^{-6} \pm 0.051\ 10^{-3}$
Cu	$0.608\ 10^{-3} \pm 0.047\ 10^{-3}$	$3.54\ 10^{-3} \pm 0.71\ 10^{-3}$	$1.29\ 10^{-6} \pm 0.20\ 10^{-6}$
Pb	$0.594\ 10^{-3} \pm 0.113\ 10^{-3}$	$20.5\ 10^{-6} \pm 6.4\ 10^{-3}$	$0.170\ 10^{-3} \pm 0.031\ 10^{-3}$
Cd + Cu + Pb	$2.95\ 10^{-3} \pm 0.31\ 10^{-3}$	$5.47\ 10^{-3} \pm 0.98\ 10^{-3}$	$0.629\ 10^{-3} \pm 0.130\ 10^{-3}$
Week 4-8			
Controls	$0.539\ 10^{-3} \pm 0.008\ 10^{-3}$	$21.2\ 10^{-6} \pm 7.2\ 10^{-6}$	$0.156\ 10^{-6} \pm 0.021\ 10^{-6}$
Cd	$4.49\ 10^{-3} \pm 0.29\ 10^{-3}$	$16.8\ 10^{-6} \pm 3.7\ 10^{-6}$	$0.143\ 10^{-6} \pm 0.017\ 10^{-3}$
Cu	$0.608\ 10^{-3} \pm 0.006\ 10^{-3}$	$7.57\ 10^{-3} \pm 1.53\ 10^{-3}$	$2.87\ 10^{-6} \pm 0.49\ 10^{-6}$
Pb	$0.567\ 10^{-3} \pm 0.018\ 10^{-3}$	$26.7\ 10^{-6} \pm 4.4\ 10^{-6}$	$0.200\ 10^{-3} \pm 0.040\ 10^{-3}$
Cd + Cu + Pb	$3.77\ 10^{-3} \pm 0.45\ 10^{-3}$	$11.0\ 10^{-3} \pm 2.2\ 10^{-3}$	$1.14\ 10^{-3} \pm 0.24\ 10^{-3}$

6.3.2. Accumulation

For *P. acuta*, we found a lower Cd body burden in the tertiary mixture than in the single Cd treatment ($p < 0.001$) after 4 and 8 weeks (Table 2). Although Cu accumulation was higher in the first four weeks ($p < 0.001$), after 8 weeks also a lower Cu burden was found in the tertiary mixture ($p < 0.001$). For the other invertebrates, no differences in accumulation were found between the metal treatments (Table S2-S3).

Furthermore, after 4 weeks, we observed a higher Pb concentration in the shoots for *E. nuttallii* in the tertiary mixture than in the single Pb treatment ($p < 0.001$; Table 3). Surprisingly, for the roots, the accumulated Pb was lower for the tertiary mixture ($p < 0.001$). After 8 weeks, these differences were no longer significant.

Table 2: Average accumulated Cd, Cu and Pb concentrations and abundances (± SE) after 4 and 8 weeks for *P. acuta* (N = 9 samples per treatment).

After week	Treatment	Cd (µmol/g dw)	Cu (µmol/g dw)	Pb (µmol/g dw)	#
4	Control	$19.7\ 10^{-3} \pm 3.0\ 10^{-3}$	2.59 ± 0.28	$10.7\ 10^{-3} \pm 1.4\ 10^{-3}$	10 ± 0
	Cd	0.141 ± 0.019	2.05 ± 0.18	$7.29\ 10^{-3} \pm 0.96\ 10^{-3}$	10 ± 0
	Cu	$12.7\ 10^{-3} \pm 1.1\ 10^{-3}$	16.6 ± 1.1	$12.2\ 10^{-3} \pm 1.1\ 10^{-3}$	10 ± 0
	Pb	$53.0\ 10^{-3} \pm 15.1\ 10^{-3}$	2.12 ± 0.25	0.600 ± 0.037	9 ± 1
	Cd + Cu + Pb	0.112 ± 0.009	19.3 ± 1.5	0.740 ± 0.053	10 ± 0
8	Control	$33.1\ 10^{-3} \pm 3.3\ 10^{-3}$	3.79 ± 0.43	$21.6\ 10^{-3} \pm 5.5\ 10^{-3}$	20 ± 10
	Cd	0.206 ± 0.012	4.49 ± 0.44	$14.1\ 10^{-3} \pm 1.4\ 10^{-3}$	30 ± 13
	Cu	$21.8\ 10^{-3} \pm 3.1\ 10^{-3}$	27.1 ± 2.9	$14.7\ 10^{-3} \pm 1.2\ 10^{-3}$	38 ± 10
	Pb	$33.7\ 10^{-3} \pm 2.5\ 10^{-3}$	3.77 ± 0.42	0.933 ± 0.062	31 ± 11
	Cd + Cu + Pb	0.169 ± 0.015	26.3 ± 1.6	1.02 ± 0.08	25 ± 8

6.3.3. Invertebrate abundances and plant growth: week 4

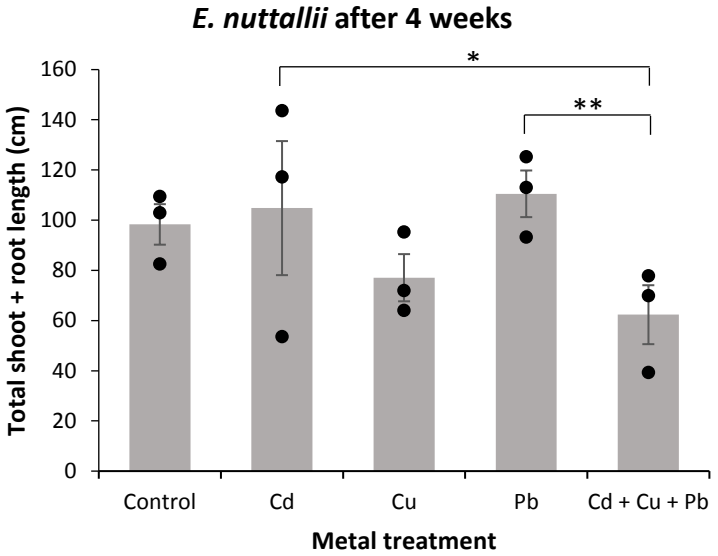
After four weeks, an effect of metal treatment on *E. nuttallii* was observed ($F = 4.32$, $p = 0.004$): shoots and roots were longer in the single Cd and Pb treatment than in the tertiary mixture ($p = 0.017$ and $p = 0.004$, respectively; Figure 2A; Table 3).

No effects of metal exposure were found on invertebrate abundances nor on chlorophyll a concentrations (Table S2-5). Also the Shannon-Wiener index revealed no significant differences between the different metal exposures (Table 4).

6.3.4. Invertebrate abundances and plant growth: week 8

After 8 weeks, roots and shoots of *E. nuttallii* were still longer in the single Cd treatments than the tertiary mixture ($p = 0.021$; Figure 2B; Table 3). We observed no other significant differences in invertebrate abundances nor in chlorophyll a concentrations between the metal treatments (Table S2-S5). Furthermore, we found that the Shannon-Wiener index did not significantly differ between the metal

treatments, but, in general, the index was higher after 8 weeks than after 4 weeks ($p < 0.001$; Table 4).



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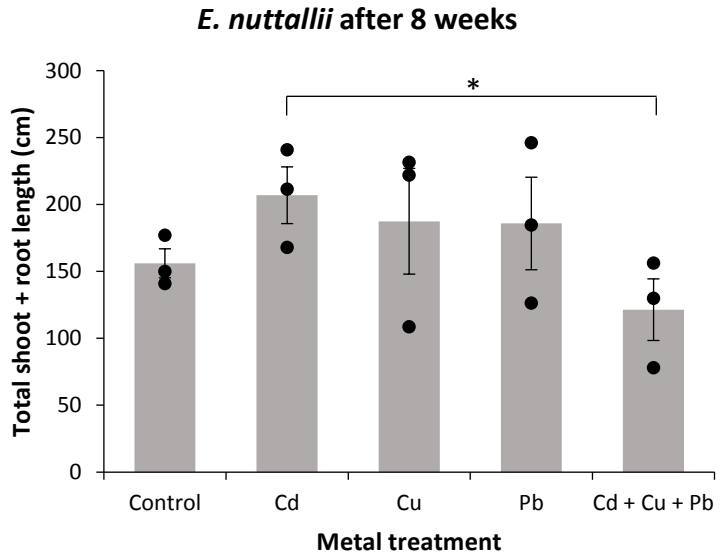

B

Figure 2: Average total lengths (± SE) of *E. nuttallii* per metal treatment A) after 4 weeks and B) after 8 weeks (* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$).

Table 3: Average accumulated Cd, Cu and Pb concentrations and lengths (± SE) after 4 and 8 weeks for *E. nuttallii*: (a) shoot; and (b) root (N = 3 samples per treatment).

(a)

After week	Treatment	Cd (µmol/g dw)	Cu (µmol/g dw)	Pb (µmol/g dw)	Length (cm)
4	Control	$27.6\ 10^{-3} \pm 1.8\ 10^{-3}$	0.703 ± 0.213	$20.6\ 10^{-3} \pm 7.3\ 10^{-3}$	41.2 ± 1.9
	Cd	0.196 ± 0.010	0.599 ± 0.109	$10.7\ 10^{-3} \pm 1.3\ 10^{-3}$	52.6 ± 10.3
	Cu	$26.6\ 10^{-3} \pm 5.1\ 10^{-3}$	2.67 ± 0.65	$12.8\ 10^{-3} \pm 2.4\ 10^{-3}$	46.2 ± 5.2
	Pb	$55.0\ 10^{-3} \pm 33.4\ 10^{-3}$	0.646 ± 0.312	0.812 ± 0.458	52.1 ± 4.3
	Cd + Cu + Pb	0.307 ± 0.054	2.98 ± 0.25	1.32 ± 0.26	35.6 ± 6.3
8	Control	$43.4\ 10^{-3} \pm 1.8\ 10^{-3}$	0.525 ± 0.103	$10.4\ 10^{-3} \pm 2.9\ 10^{-3}$	70.8 ± 3.7
	Cd	0.359 ± 0.029	0.450 ± 0.131	$8.15\ 10^{-3} \pm 1.21\ 10^{-3}$	88.1 ± 5.1
	Cu	$50.8\ 10^{-3} \pm 3.1\ 10^{-3}$	2.82 ± 0.37	$9.01\ 10^{-3} \pm 1.07\ 10^{-3}$	89.2 ± 11.8
	Pb	$43.3\ 10^{-3} \pm 2.9\ 10^{-3}$	0.318 ± 0.063	0.671 ± 0.218	88.7 ± 17.0
	Cd + Cu + Pb	0.556 ± 0.005	3.68 ± 0.35	1.13 ± 0.41	52.2 ± 4.4

(b)

After week	Treatment	Cd (µmol/g dw)	Cu (µmol/g dw)	Pb (µmol/g dw)	Length (cm)
4	Control	$22.7\ 10^{-3} \pm 3.1\ 10^{-3}$	0.487 ± 0.073	$70.7\ 10^{-3} \pm 8.1\ 10^{-3}$	57.1 ± 6.8
	Cd	$62.0\ 10^{-3} \pm 15.5\ 10^{-3}$	0.274 ± 0.045	$59.7\ 10^{-3} \pm 3.2\ 10^{-3}$	52.2 ± 16.9
	Cu	$12.0\ 10^{-3} \pm 2.9\ 10^{-3}$	0.865 ± 0.251	$48.4\ 10^{-3} \pm 5.9\ 10^{-3}$	30.9 ± 4.2
	Pb	$50.6\ 10^{-3} \pm 29.4\ 10^{-3}$	0.445 ± 0.193	0.313 ± 0.083	58.4 ± 6.5
	Cd + Cu + Pb	0.128 ± 0.018	1.28 ± 0.30	0.211 ± 0.070	26.7 ± 5.7
8	Control	$24.0\ 10^{-3} \pm 9.0\ 10^{-3}$	0.271 ± 0.052	$53.3\ 10^{-3} \pm 7.0\ 10^{-3}$	85.3 ± 10.7
	Cd	0.168 ± 0.027	0.351 ± 0.136	$46.5\ 10^{-3} \pm 2.9\ 10^{-3}$	119 ± 16
	Cu	$20.4\ 10^{-3} \pm 368\ 10^{-3}$	0.834 ± 0.368	$41.2\ 10^{-3} \pm 18.2\ 10^{-3}$	98.2 ± 28.0
	Pb	$30.1\ 10^{-3} \pm 16.8\ 10^{-3}$	0.362 ± 0.214	0.366 ± 0.111	97.1 ± 19.1
	Cd + Cu + Pb	0.249 ± 0.024	2.25 ± 0.60	0.569 ± 0.221	69.2 ± 21.5

Table 4: Average Shannon-Wiener indices (± SE) after 4 and 8 weeks.

After week	Treatment	*H'*
4	Control	0.174 ± 0.020
	Cd	0.572 ± 0.232
	Cu	0.198 ± 0.023
	Pb	0.383 ± 0.116
	Cd + Cu + Pb	0.233 ± 0.035
8	Control	0.886 ± 0.117
	Cd	1.06 ± 0.03
	Cu	1.11 ± 0.12
	Pb	0.940 ± 0.023
	Cd + Cu + Pb	0.980 ± 0.110

6.4. Discussion

After four and eight weeks, significant metal effects were observed on plant length. However, invertebrate numbers were not significantly different among the metal treatments. As a large variation in sensitivity to Cd, Cu and Pb exists (US EPA, 2018), each species is separately discussed below.

The waterflea *Daphnia magna* is widely used in toxicity tests and is sensitive to metals (Von der Ohe and Liess, 2004). For example, Barata et al. (2006) reported 48h-LC50 values of 5.44 µg/L Cd and 42.93 µg/L Cu and as 1d-EC50 (feeding) only 2.4 µg/L Cd and 5.5 µg/L Cu for *D. magna* of 4-5 days old. In the present study, no metal effects were observed, which is likely explained by acclimation. LeBlanc (1982) investigated the development of resistance of *D. magna* to copper and found that chronic Cu exposure of 30 µg/L led to a mortality of 50% of the first generation. However, survival improved with successive generations, with 80% surviving during the second generation exposure and 100% during the third. This result was attributed to non-genetic physiological adaptations. Stuhlbacher et al. (1992) studied Cd tolerance of *D. magna* and found that an elevated Cd tolerance was linked to an increase of

metallothionein-like proteins (MTLP), which play an essential role in protection against metal and oxidative stress. This energy demand caused by the induction of MTLPs and other repair and tolerance mechanisms is most likely compensated by an increased feeding rate or trade-offs with other costs, such as growth. For example, also Ward and Robinson (2009) studied Cd resistance in *D. magna* during 8 generations and reported no differences in life span, offspring production, or intrinsic rate of population increase (r). However, they did find that cadmium-adapted individuals were smaller. They also reported a decreased genetic diversity and increased sensitivity to other toxicants, which might threaten the long-term survival of the population.

For the Chironominae larvae, we observed an emergence of more than 50% in the controls, which is comparable to other studies (Grosell et al., 2006; Watts and Pascoe, 2000). Yet, no metal effects on emergence were observed. Similar as for *D. magna*, a study by Arambourou et al. (2013) reported that exposure to comparable Pb concentrations for *Chironomus riparius* caused an energetically-costly increase of metallothioneins and lipid peroxidation. Yet, a lower emergence was not observed in our present study. Again, most likely a trade-off took place and other metabolic costs were limited (Heinis et al., 1990; Villa et al., 2018). For Cd, Watts and Pascoe (2000) found both *C. riparius* and *C. tentans* to have a high tolerance with 10-day LC_{50} values of approximately 700 µg/L (hard water). Also Nebeker et al. (1984) found *C. tentans* not to be very sensitive to Cu, as adults emerged from fourth-instar larvae and pupae that survived 20-d exposure of up to 235 µg/L (soft water).

Physella acuta is known to be very tolerant to pollution (Mouthon and Charvet, 1999; Von der Ohe and Liess, 2004), which could explain the lack of metal effects on *P. acuta* abundance. However, when we studied the bioaccumulation data for *P. acuta*, we found a significantly lower Cd and Cu body burden for snails in the tertiary mixture compared to those in the single metal treatments. As cadmium interferes with Ca^{2+} uptake and Cu with Na^{+} uptake, no competitive effects are expected. However,

antagonistic interactions between Cu and Cd have been reported before (Kraak et al., 1993; Van Ginneken et al., 2018). These metals possibly share an unknown uptake site. One such ligand might be Divalent Metal Transporter 1 (DMT1), an Fe^{2+} transporter (Komjarova and Blust, 2008). A limitation to our study that needs to be acknowledged is the relatively short exposure duration. As differences in metal body burdens were detected between our single metal treatments and the mixture treatment, it is possible that a longer exposure duration would have revealed significant negative effects for *P. acuta* in the single Cu and Cd treatments.

Also for *Asellus aquaticus*, no significant effects on abundance were observed after four or eight weeks, even though similar metal body concentrations in a previous study by Van Ginneken et al. (2018) resulted in several negative effects such as lower activities and respiration rates, possibly indicating gill damage. Again, it might have been possible that acclimation and subsequent energy trade-offs took place. For instance, Pan and Wang (2012) studied the effects of metal exposure on the oyster *Crassostrea hongkongensis*. After oysters suffered from metal stress, they reported a decrease in dissolved uptake rate and a higher metal sequestration into non-toxic forms.

Lastly, we will discuss the effects on *E. nuttallii.* After the first four weeks, we observed a significantly lower growth for *E. nuttallii* in the tertiary mixture than for the single Cd and Pb treatment. We linked this to a significantly higher Pb concentration in the shoots. A lower Pb concentration was found in the roots, but a study on *Elodea canadensis* showed that 23% of metals accumulated by the roots can be translocated to the shoots (Fritioff and Greger, 2007). Lead can have detrimental effects on plants, affecting chloroplast membrane arrangement and the cell wall organization (Sergio et al., 2013). Although the difference was not significant, we also found a higher Cd concentration in both the roots and the shoots of the plants in the tertiary mixture. The negative effects of Cd on plants include inhibition of cell division and cell enlargement, interference with the division and the expansion of chloroplasts, and a

decreased photosynthetic activity (Dalla Vecchia et al., 2005; Koukal et al., 2003), which can explain the stunted growth observed in the present study. We did not find any effects of copper in the present study. Although Cu is an essential element, it can damage the photosynthetic apparatus of plants by interacting with certain enzymes when a certain threshold concentration is exceeded (Rabe et al., 1982). For example, Mal et al. (2002) found a significant effect of Cu on the growth of *Elodea canadensis,* but the lowest concentrations they used was 1 mg/L, which is much higher than in the present study. An et al. (2004) studied the effect of a mixture of Cd, Cu and Pb on *Cucumis sativus* and found, unlike this study, an antagonistic response of the tertiary mixture for accumulation and growth. However, they used higher exposure concentrations. Interactions often depend on the metal concentrations that are used and, additionally, they can vary among species (Norwood et al., 2003).

After eight weeks, the difference in growth between the tertiary mixture and the Pb treatment was not found anymore. A study of Sergio et al. (2013) on *Elodea canadensis* found Pb to be less toxic than Cd. Moreover, *Elodea nuttallii* did not seem to accumulate more Pb in the shoots after eight weeks than after four weeks, which can be explained by this species having a very efficient excretion rate (Larras et al., 2013). Yet, even if the Pb concentration did not change, an effect would still be expected after eight weeks. This lack of effect might be explained by subcellular compartmentalization. Larras et al. (2013) studied the tolerance of metals to *E. nuttallii* and found that over time more metals were stored in the cell walls and less were found in the cytosol, thus protecting the cellular machinery. Interestingly, the biomass of *R. subcapitata* was not significantly affected by the metals, although it is known to be a sensitive species of alga (Muyssen and Janssen, 2001; Van Sprang et al., 2009). This might be explained by the high variability between the replicates.

In conclusion, significant differences in plant growth between the different metal treatments were observed after four and eight weeks. However, no effects were observed on invertebrate abundances, even though some species had accumulated

body concentrations that were found to be damaging to the individual in previous laboratory studies (e.g. the energetically-costly induction of MT, decreased respiration rates and activities). Although some species thus appear to be more resilient than would be expected, caution must be taken with the interpretation of these results. It is possible that energy trade-offs caused by metal-induced defense mechanisms occurred, which might have long-term consequences. More realistic metal exposure scenarios in micro- and mesocosm experiments could be a valuable addition to risk assessment, but should ideally have a long exposure duration, which spans multiple generations, and be complemented with the study of sublethal endpoints (e.g., energy budget, MTLPs, reproduction and genetic diversity).

6.5. Acknowledgments

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6.6. Supplementary material

Table S1: Overview of the average dissolved metal concentrations of weeks 0-4 and weeks 4-8. BMQL = Below method quantification limit. BMQL of Cd = 0.890 10^{-3} µmol/L; BMQL of Cu = 1.57 10^{-3} µmol/L; and Pb = 0.483 10^{-3} µmol/L (N = 360).

	Cd (µmol/L)	Cu (µmol/L)	Pb (µmol/L)
Weeks 0-4			
Controls	BMQL	43.2 10^{-3} ± 2.9 10^{-3}	0.494 10^{-3} ± 0.011 10^{-3}
Cd	6.01 10^{-3} ± 0.33 10^{-3}	47.8 10^{-3} ± 11.5 10^{-3}	BMQL
Cu	0.982 10^{-3} ± 0.093 10^{-3}	0.451 ± 0.027	BMQL
Pb	1.07 10^{-3} ± 0.18 10^{-3}	41.0 10^{-3} ± 2.5 10^{-3}	94.0 10^{-3} ± 9.7 10^{-3}
Cd + Cu + Pb	4.39 10^{-3} ± 0.43 10^{-3}	0.452 ± 0.026	79.2 10^{-3} ± 8.7 10^{-3}
Weeks 4-8			
Controls	BMQL	43.8 10^{-3} ± 3.3 10^{-3}	BMQL
Cd	7.38 10^{-3} ± 0.41 10^{-3}	42.6 10^{-3} ± 2.4 10^{-3}	BMQL
Cu	BMQL	0.421 ± 0.041	BMQL
Pb	0.923 10^{-3} ± 0.020 10^{-3}	39.5 10^{-3} ± 0.8 10^{-3}	76.4 10^{-3} ± 10.8 10^{-3}
Cd + Cu + Pb	5.37 10^{-3} ± 0.61 10^{-3}	0.415 ± 0.041	73.3 10^{-3} ± 11.0 10^{-3}

Table S2: Average accumulated Cd, Cu and Pb concentrations and abundances (± SE) after 4 and 8 weeks for *D. magna* (N = 9 samples per treatment).

After week	Treatment	Cd (µmol/ind.)	Cu (µmol/ind.)	Pb (µmol/ind.)	Abundance
4	Control	$2.22\ 10^{-6} \pm 0.4\ 10^{-6}$	$14.5\ 10^{-6} \pm 2.7\ 10^{-6}$	$0.627\ 10^{-6} \pm 0.129\ 10^{-6}$	2807 ± 97
	Cd	$8.43\ 10^{-6} \pm 1.08\ 10^{-6}$	$13.5\ 10^{-6} \pm 2.2\ 10^{-6}$	$0.442\ 10^{-6} \pm 0.111\ 10^{-6}$	1393 ± 876
	Cu	$2.27\ 10^{-6} \pm 0.49\ 10^{-6}$	$61.6\ 10^{-6} \pm 8.1\ 10^{-6}$	$0.392\ 10^{-6} \pm 0.051\ 10^{-6}$	2860 ± 703
	Pb	$3.98\ 10^{-6} \pm 0.76\ 10^{-6}$	$8.68\ 10^{-6} \pm 0.69\ 10^{-6}$	$10.1\ 10^{-6} \pm 1.4\ 10^{-3}$	1320 ± 691
	Cd + Cu + Pb	$6.92\ 10^{-6} \pm 0.94\ 10^{-6}$	$65.3\ 10^{-6} \pm 7.3\ 10^{-6}$	$12.2\ 10^{-6} \pm 1.9\ 10^{-6}$	2487 ± 198
8	Control	$1.57\ 10^{-6}$	$26.2\ 10^{-6}$	$2.70\ 10^{-6}$	11 ± 11
	Cd	$4.94\ 10^{-6} \pm 1.88\ 10^{-6}$	$8.74\ 10^{-6} \pm 2.60\ 10^{-6}$	$0.655\ 10^{-6} \pm 0.188\ 10^{-6}$	90 ± 89
	Cu	$1.22\ 10^{-6} \pm 0.38\ 10^{-6}$	$81.4\ 10^{-6} \pm 0.82\ 10^{-6}$	$0.771\ 10^{-6} \pm 0.225\ 10^{-6}$	54 ± 30
	Pb	$2.15\ 10^{-6} \pm 0.74\ 10^{-6}$	$5.95\ 10^{-6} \pm 1.41\ 10^{-6}$	$10.9\ 10^{-6} \pm 3.35\ 10^{-6}$	230 ± 115
	Cd + Cu + Pb	$3.63\ 10^{-6} \pm 0.56\ 10^{-6}$	$66.7\ 10^{-6} \pm 10.7\ 10^{-6}$	$19.0\ 10^{-6} \pm 3.67\ 10^{-6}$	113 ± 61

Table S3: Average accumulated Cd, Cu and Pb concentrations and abundances (± SE) after 4 and 8 weeks for *A. aquaticus* (N = 9 samples per treatment).

After week	Treatment	Cd (µmol/g dw)	Cu (µmol/g dw)	Pb (µmol/g dw)	Abundance
4	Control	$4.88\ 10^{-3} \pm 0.46\ 10^{-3}$	3.29 ± 0.37	$7.65\ 10^{-3} \pm 5.09\ 10^{-3}$	56 ± 13
	Cd	$33.4\ 10^{-3} \pm 3.31\ 10^{-3}$	3.02 ± 0.49	$1.34\ 10^{-3} \pm 0.31\ 10^{-3}$	93 ± 16
	Cu	$4.99\ 10^{-3} \pm 0.47\ 10^{-3}$	6.63 ± 0.35	$2.31\ 10^{-3} \pm 0.74\ 10^{-3}$	72 ± 11
	Pb	$12.7\ 10^{-3} \pm 3.4\ 10^{-3}$	2.14 ± 0.16	$40.0\ 10^{-3} \pm 7.1\ 10^{-3}$	55 ± 14
	Cd + Cu + Pb	$39.7\ 10^{-3} \pm 4.4\ 10^{-3}$	7.59 ± 0.64	$47.2\ 10^{-3} \pm 9.6\ 10^{-3}$	98 ± 28
8	Control	$7.53\ 10^{-3} \pm 0.62\ 10^{-3}$	2.48 ± 0.32	$2.09\ 10^{-3} \pm 0.59\ 10^{-3}$	112 ± 19
	Cd	$46.6\ 10^{-3} \pm 4.7\ 10^{-3}$	2.57 ± 0.15	$1.92\ 10^{-3} \pm 0.43\ 10^{-3}$	60 ± 29
	Cu	$10.1\ 10^{-3} \pm 0.8\ 10^{-3}$	9.24 ± 0.45	$3.26\ 10^{-3} \pm 0.40\ 10^{-3}$	92 ± 25
	Pb	$6.58\ 10^{-3} \pm 0.55\ 10^{-3}$	2.26 ± 0.18	$56.7\ 10^{-3} \pm 10.1\ 10^{-3}$	130 ± 59
	Cd + Cu + Pb	$58.7\ 10^{-3} \pm 4.5\ 10^{-3}$	8.26 ± 0.46	$60.1\ 10^{-3} \pm 11.6\ 10^{-3}$	78 ± 25

Table S4: Average emerged Chironominae (± SE) after 4 and 8 weeks.

After week	Treatment	Abundance
4 & 8	Control	29 ± 1
	Cd	27 ± 4
	Cu	27 ± 4
	Pb	25 ± 5
	Cd + Cu + Pb	23 ± 1

Table S5: Average chlorophyll a concentrations (± SE), as an indication of *R. subcapitata* biomass, after 4 and 8 weeks (N = 48 and 24 samples per treatment, respectively).

After week	Treatment	Chl a (µg/L)
4	Control	6.69 ± 0.93
	Cd	7.98 ± 1.21
	Cu	8.84 ± 1.12
	Pb	7.33 ± 1.07
	Cd + Cu + Pb	8.42 ± 1.17
8	Control	20.2 ± 3.1
	Cd	29.5 ± 2.9
	Cu	30.3 ± 3.6
	Pb	21.3 ± 5.0
	Cd + Cu + Pb	19.0 ± 2.0

Chapter 7.

General discussion

Metals are posing a worldwide threat to aquatic ecosystems. In Flanders, the VMM (2017) reported that 10% of the studied sediments in lakes and riverbeds were (heavily) contaminated with trace metals. Also in surface waters, the EQS are regularly exceeded, e.g. cadmium and nickel were too high in 2.3% and 11.4% of the Flemish waterbodies, respectively. In contaminated environments trace metals most often occur in different mixtures, in which they can strongly interfere with each other, producing antagonistic, synergistic or additive toxic effects. Besides pollutants, natural stressors, such as fluctuating temperature, food shortages and predators, are present that might negatively affect organisms and alter metal toxicity. Yet, current environmental quality standards (EQS) are mainly based on laboratory tests under strictly controlled conditions in which test organisms are exposed to single compounds for a limited period of time. Moreover, in the setting of EQS, behavioral endpoints, which are more sensitive than mortality, have never been used. As a result, the current EQS for metals might result in under- or overprotecting the environment. The aim of this study was to investigate the combined effects of metal mixtures and the natural stressors, temperature and predation pressure, on sublethal endpoints of the aquatic invertebrate *Asellus aquaticus*. This isopod is an important decomposer in freshwater ecosystems in the northern hemisphere. Additionally, effects on a whole aquatic community were assessed in small artificial ecosystems. Combining metal mixtures with natural stressors and assessing the effects on different levels of biological organization contributes to the development of environmentally-relevant risk assessment.

7.1. Effects on the organism

7.1.1. Toxicity of the single metals

In Chapter 2 of this thesis, we investigated the toxicity of the single metals Cd, Cu and Pb to *A. aquaticus*. After studying the literature, we noticed that the available LC_{50} values for this species were all short-term (acute) values as well as mainly based on unmeasured concentrations, which could lead to a severe underestimation of the

actual impact of these metals. Furthermore, no EC or LC_{10}/LC_{20} values were available, which were necessary to estimate at which concentrations sublethal effects on the isopods occur. Therefore, an experiment was designed to determine the change of LC_{10}s, LC_{20}s and LC_{50}s over 14 days for Cd, Cu and Pb for *adult A. aquaticus*. Additionally, we calculated the incipient LC_x values. This is the concentration below which 100–x% would live indefinitely, which is thus not influenced by time of exposure. These LC_x values were calculated using the nominal concentrations, the effective concentrations and the free ion activities (FIAs).

In general, surprisingly lower lethal concentrations were found than in other studies. Furthermore, the present study showed that lethal concentrations based on free ion activities were generally much lower than nominal and effective concentrations. It can be concluded that caution must be taken when using mortality data available in the literature. Lastly, we used the incipient values to estimate the optimal exposure duration for future experiments, i.e. the duration at which no longer any discernible changes in effect were observed. This was determined to be longer than 7 days.

7.1.2. Metal interactions

In Chapters 3, 4 and 5, the effects of metal mixtures on *A. aquaticus* were studied. In Chapter 3, both lethal and sublethal endpoints were investigated, while Chapters 4 and 5 focused more on the sublethal effects. We discuss the metal-induced effects on each endpoint separately. A summary of all effects is given in Table 1. Generally, the R^2 we found in Chapters 4 and 5 were low, which means that only a limited amount of the observed variation could be explained by the variables metal concentration, mixture and natural stressor treatment. We could have possibly lowered intraspecific variation by e.g., selecting a single sex or only the second generation after breeding in the laboratory, but we could argue that this would have diminished the environmental relevance. Although we cannot make precise predictions based on our variables, our data did show significant trends.

We should note that we assumed linear relationships for our statistical analyses. These were tested using plots of residuals versus predicted values. For Chapters 4 and 5, only the accumulation graphs suggested parabolic curves would be better. We applied a nonlinear transformation to the $logFIA_{water}$ concentration data (^2, i.e. x-squared) and the same significant effects were found for all metals, except for Pb in the temperature stress experiment. We no longer found the interaction between temperature treatment and logFIA.

7.1.2.1. Accumulation

Generally, metal body concentrations increased when the dissolved water concentrations (Chapter 3) and free ion activities (Chapter 4 and 5) did. In Chapter 3, we observed that asellids accumulated more Cd and Pb in the binary Cd + Pb mixture than in the single metal treatments.

In Chapters 4 and 5, lower exposure concentrations were used and other interactions were observed. We no longer found a difference between the Cd and Pb uptake of isopods in the single metal treatments and the Cd + Pb mixture. However, we did find a significantly lower accumulation for Cd + Cu, Cu + Pb and the tertiary mixture, indicating an antagonistic effect for these mixtures. Both Cu and Pb are known to compete for the same uptake sites, namely via Na^+ channels, so an antagonistic interaction is expected. However, cadmium has a different mode of action and enters via Ca^{2+} channels. Barata et al. (2006) demonstrated a less than additive toxicity of mixtures of Cd and Cu for *Daphnia magna* and interpreted their findings as the result of both metals being metallothionein inducers. Another explanation could be that these metals share a common uptake site. Komjarova and Blust (2008) suggested Divalent Metal Transporter 1 (DMT1), an Fe^{2+} transporter. In conclusion, different metal interactions may be found depending on the metals present in the mixture and their exposure concentrations (Norwood et al., 2003).

7.1.2.2. Mortality

Mortality increased when the dissolved Cd, Cu and Pb concentrations increased (Chapter 3). The mortality rate was five times higher in the Pb + Cd mixture compared to the single Pb treatment. This was also found for Cd + Pb compared to the single Cd treatment, yet the difference was not significant. These effects can be explained by combined disturbances of both Ca^{2+} and Na^{+} homeostasis (Paquin et al., 2002; Rogers et al., 2003). This was only observed for the water concentration models and not for the body burden models, which indicates that interactive effects between these metals took place outside the organism, with one metal affecting the availability of the other. This is commonly observed for metal ions, where ion speciation and competition for binding sites to organic matter in the water phase can change free ion availability (Cedergreen, 2014). In Chapters 4 and 5, on the other hand, we used models with FIA_{water} concentrations, as a representation of the bioavailable fraction and, generally, the same significant effects and interactions were found in the FIA_{water} and the body burden models.

7.1.2.3. Respiration rate

In Chapters 4 and 5, we found a decrease in respiration rates when the metal body burdens increased (for all metals). In general, a decreased oxygen consumption in crustaceans is found after metal exposure (Chinni et al., 2002; Wu and Chen, 2004). Spicer and Weber (1991) studied the respiratory impairment in crustaceans due to metals and linked the effects to cytological damage of the respiratory system. They found a thickening of the branchial epithelium and profound changes in haemolymph flow pattern in the gill concomitant with increased vacuolization and reduced haemolymph spaces causing perfusion stagnation. No further metal interactions were detected.

Table 1: Summary of the mixture interactions observed in Chapter 3, 4 and 5 (indicated by superscript numbers). Significant covariances between the endpoints and the single metal concentrations are indicated as positive, negative or no covariance ("/"). Metal mixture interactions are illustrated with "=": no significant difference between the metal mixture and the single metal treatment; and "↑ or↓": interaction between mixture treatment and metal concentration, the slope for the respective metal mixture is significantly higher or lower than the slope for the single metal treatment.

Metal	Mixture with	Uptake rate	Mortality rate	Respiration rate	Growth rate	Energy reserves	Feeding rate	Activity
Cd	-	Positive [3,4,5]	Positive [3]	/ [4,5]	Negative [3,4] Positive [5]	Negative [3]	/ [4,5]	Negative [4] / [5]
	Cu	= [3] ↓ [4,5]	= [3]	= [4,5]	= [3,4,5]	= [3]	= [4] ↑ [5]	= [4,5]
	Pb	↑ [3] = [4,5]	= [3]	= [4,5]	↓ [3] = [4,5]	= [3]	↑ [4,5]	= [4,5]
	Cu + Pb	= [3] ↓ [4,5]	= [3]	= [4,5]	= [3,4,5]	= [3]	= [4,5]	= [4,5]
Cu	-	Positive [3,4,5]	Positive [3]	/ [4,5]	/ [3] Negative [4] Positive [5]	Negative [3]	Negative [4] Positive [5]	Negative [4] / [5]
	Cd	= [3,4,5]	= [3]	= [4,5]	= [3,4,5]	= [3]	= [4,5]	= [4,5]
	Pb	= [3,4,5]	= [3]	= [4,5]	= [3,4,5]	= [3]	= [4,5]	= [4,5]
	Cd + Pb	= [3,4,5]	= [3]	= [4,5]	= [3,4,5]	= [3]	↑ [4] = [5]	= [4,5]

Pb	-	Positive [3,4,5]	Positive [3]	/ [4,5]	Negative [3] / [4] Positive [5]	Negative [3]	/ [4,5]	Negative [4,5]
	Cd	↑ [3] = [4,5]	↑ [3]	= [4,5]	↓ [3] = [4,5]	= [3]	↑ [4,5]	= [4,5]
	Cu	= [3] ↓ [4,5]	= [3]	= [4,5]	= [3,4,5]	= [3]	= [4,5]	= [4,5]
	Cd + Cu	= [3] ↓ [4,5]	= [3]	= [4,5]	= [3,4,5]	= [3]	= [4,5]	= [4,5]

7.1.2.4. Growth rate and energy reserves

When metal exposure involved very high concentrations (higher than: for Cd, 32.4 10^{-3} µmol/L; for Cu, 2.70 µmol/L; and for Pb, 0.882 µmol/L), we found that growth rate decreased when metal body burdens increased (Chapter 3 and 4). In Chapter 3, we even found a significantly lower growth rate for isopods exposed to the Cd + Pb mixture compared to those exposed to the single Cd and Pb treatments, by minimally a factor of 4, which could be attributed to an increased Cd and Pb uptake for that binary mixture. We also studied the effect of metals on energy reserves. We found that metal exposure resulted in decreased glycogen concentrations for all three metals. Lipid concentrations were negatively affected by Cd and Pb, while protein content was decreased by Cu. This can most likely be attributed due to energy being allocated to defensive mechanisms, such as metallothioneins and antioxidant enzymes, to protect the body from metal damage (Valavanidis et al., 2006). It is not clear why the glycogen, lipid, and protein reserves are differently affected. According to Smolders et al. (2003), toxicant stress preferentially causes a depletion of the more readily available carbohydrate and lipid reserves instead of protein.

In Chapter 5, we used lower concentrations than in the previous chapters and positive slopes for growth rate were observed. This suggests that the isopods could cope with the metal body concentrations, having the energy for both somatic growth and protection against metal damage. This positive link with body burden suggests a hormesis effect (i.e. when low levels of exposure to a toxic chemical that are harmful at high levels of exposure result in stimulatory effects on survival, growth, reproduction...). Hormesis is most likely an overcompensation response to a disruption in homeostasis, but there is no clear underlying mechanism (Calabrese, 1999).

A limitation of our study that needs to be acknowledged is that we did not consider the effects of moulting during the experiments. As *A. aquaticus* moult, their width and length increases (Bloor, 2010). Therefore, we advise to first test for possible

confounding effects of ecdysis before using growth rate as an endpoint in future studies.

7.1.2.5. Feeding rate

There were no clear significant relationships found between the metal body concentrations and the feeding rates (Chapter 4 and 5). Yet, several significant interactions were observed between metal body burden and metal treatment. In Chapters 4 and 5, we found that the slope of feeding rate for the binary mixture Cd + Pb was significantly higher than for the single Cd and Pb treatments (by a factor of 6 minimally). The observed increase was concentration-dependent, as we found that feeding rates decreased drastically when exposure concentrations increased even more. Concentration-dependent interactions have been reported before for metals (Jonker et al., 2005). For instance, Sharma et al. (1999) studied the effects of Cd, Cu and Zn on the root growth of the plant *Silene vulgaris* and found non-additive or antagonistic responses for low concentrations. However, if one of the components in the mixture exceeded a certain level of toxicity, synergistic interactions were observed. We expect that the concentrations we used triggered a range of energetically costly defense mechanisms, which had to be fuelled by a higher feeding rate, but that after some critical point these mechanisms became too costly to maintain. Also the feeding rate of Cd + Cu was higher than for the single Cd treatment in Chapter 5, which can be explained as both disrupting homeostasis: Cu for Na^+ and Cd for Ca^{2+} (Paquin et al., 2002). Yet, this will most likely also decrease when a critical concentration is reached.

7.1.2.6. Activity

A negative effect on activity was found for all three metals in Chapter 4. A decreased activity might have serious ecological consequences as it can affect several essential biological processes, such as feeding, competition, upstream-migration, mating or seeking shelter. It can, for example, also reduce avoidance behavior. Also Gerhardt (1995) found a decreased locomotion for metal-exposed *Gammarus pulex*. A decreased swimming performance might be explained by resource allocation to other

physiological processes. Additionally, Salanki (1992) demonstrated that metals can affect neuronal functions by changing the permeability of neuronal membranes.

In Chapter 5, we only observed a negative effect on activity for Pb, which was not found for the FIA_{water} model. This might suggest a type I error. The exposure concentrations in Chapter 5 were likely not high enough to elicit a significant reduction in activity.

7.1.3. Effects of natural stressors

In Chapters 4 and 5, we studied the combined effects of metal mixtures and a natural stressor. We discuss the effects for each natural stressor separately.

7.1.3.1. Predator stress

In Chapter 4, we focused on the effects of predator cues on metal mixture stress (Table 2). We found a significantly lower feeding rate for predator-exposed asellids in the control treatments compared to asellids without predator cues, which is expected as prey animals will try to avoid detection by predators by lowering activity. Furthermore, we found a decreased feeding rate for Cd and Pb predator stress treatments and a higher respiration rate for Cu and Pb predator stress treatments. Surprisingly, no significant differences in growth rate between the predator treatments for Cd and Pb were found, which could be an indication of a higher predator-induced food conversion efficiency. This might be advantageous, because larger isopods move faster and have more chance to escape than smaller individuals, as swimming speed could be positively correlated to length or body weight (Eroukhmanoff and Svensson, 2009; Takeuchi and Watanabe, 1998). Additionally, we also demonstrated that predator cues affected the slope for Cu accumulation, resulting in a slightly higher slope. This can probably be linked to the higher respiration rate. As more Cu accumulated in predator exposed isopods, this might also explain the more negative slope for growth rate. Furthermore, we also observed an effect on the slopes for feeding rate and activity of the Cu treatments. When the isopods were exposed to predator cues, the slopes were less negative. This means that with higher Cu body concentrations predator-exposed

isopods fed and moved more than at lower Cu concentrations, making them more susceptible to predation. For fish, Cu is known to inhibit physiological responsiveness, and even cause cell death, of olfactory receptor neurons (Baldwin et al., 2003; Hansen et al., 1999). The less sensitive chemo-receptor cells of Crustacea are possibly similarly affected, making them more vulnerable to predatory attacks (Olsén, 2010). Also responses to female odors or food can be impaired (Krång and Ekerholm, 2006; Sherba et al., 2000).

Table 2: Summary of the predator stress effects observed in Chapter 4. Significant covariances between the endpoints and the metal body concentrations without predator stress are indicated as positive, negative or no covariance ("/"). Effects of predator stress are illustrated with "=": no significant difference between the treatments with and without predator stress; "+ or –": no interaction between predator stress treatment and metal body concentration, but there is a significant positive or negative effect; and "↑ or↓": interaction between predator stress treatment and metal body concentration, the slope for the predator-exposed treatments is significantly higher or lower than the slope for the treatments without predator stress.

Metal	Predator stress	Uptake rate	Respiration rate	Growth rate	Feeding rate	Activity
Cd	Without	Positive	/	Negative	/	Negative
	With	=	=	=	-	=
Cu	Without	Positive	/	Negative	Negative	Negative
	With	↑	+	↓	↑	↑
Pb	Without	Positive	/	/	/	Negative
	With	=	+	=	-	=

7.1.3.2. Temperature stress

We investigated the combined effects of temperature and metal mixtures in Chapter 5 (Table 3). When asellids were exposed at 15 °C, we found that respiration rate decreased when metal accumulation increased. However, at 20 °C this slope was positive. Additionally, we found higher feeding rates for isopods in the warmer treatments as well and, while isopods of the Cd and Pb treatments had a negatively correlated feeding rate at 15 °C, a positive slope for feeding rate was found at 20 °C. Elevated temperatures cause an increase in metabolic rates (Sokolova and Lannig,

2008). Yet, it was interesting to not only find a temperature effect, but also a stimulatory interaction effect of temperature with the accumulated metals. This might be explained by differential subcellular partitioning of the metals, resulting in altered toxicities. Li et al. (2011) demonstrated that Cd distribution in wheat plants changed at a higher temperature. More metals were stored in metal-sensitive fractions, thereby stimulating defense mechanisms, which would result in a higher energy demand (Rainbow, 2007).

Table 3: Summary of the temperature stress effects observed in Chapter 4. The observed covariances between the endpoints and the metal body concentrations at the control temperature of 15 °C are indicated as positive, negative or no covariance ("/"). Effects of temperature stress (20 °C) are illustrated with "=": no significant difference between the two temperature treatments; "+": no interaction between temperature stress treatment and metal body concentration, but there is a significant positive effect; and "↑": interaction between temperature stress treatment and metal body concentration, the slope for the 20 °C treatments is significantly higher than the slope for the 15 °C treatments.

Metal	Temperature	Uptake rate	Respiration rate	Growth rate	Feeding rate	Activity
Cd	15 °C	Positive	/	Positive	/	/
	20 °C	=	↑	↓	↑	=
Cu	15 °C	Positive	/	Positive	Positive	/
	20 °C	+	↑	+	+	=
Pb	15 °C	Positive	/	Positive	/	Negative
	20 °C	=	↑	↓	↑	=

The increased respiration and feeding rate most likely led to the significantly higher accumulation for Cu. Moreover, we observed less increasing slopes for the growth rates of Cd and Pb: they were lower by respectively a factor of 8 and 4. At lower body concentrations growth at 20 °C was higher, but at higher body concentrations, the slopes of both temperature treatments meet, meaning there is no difference in growth anymore.

As feeding rates were higher, but slopes for growth rate were lower, there is most likely a lower food conversion efficiency. The energy gained from higher feeding rates

is then less used for growth or locomotion, but is instead allocated to detoxification and cellular protection systems, such as metallothioneins (MT) and antioxidant enzymes (Sokolova et al., 2012).

7.2. Effects on populations and communities

We designed a microcosm experiment in a greenhouse to gain more insight into the effects of Cd, Cu and Pb and their mixture on populations and communities. Small ecosystems with several species of macroinvertebrates were exposed under semi-natural conditions. In each bucket, we placed *Asellus aquaticus* (Isopoda), *Daphnia magna*, Chironominae (midge larvae), *Physella acuta* (Mollusca), *Elodea nuttallii* (macrophyte) and *Raphidocelis subcapitata* (alga). The effects of the metal mixtures and natural stressors were examined after 4 and 8 weeks, on the individual level (total metal accumulation), the population level (species abundances, algal biomass, shoot and root length) and the community structure (Shannon-Wiener index). Significant differences in plant growth between the different metal treatments were observed after four and eight weeks. However, no effects were observed on invertebrate abundances, even though some species had accumulated body concentrations that were found to be damaging to the individual in previous laboratory studies (e.g. the energetically-costly induction of MTLPs, decreased respiration rates and activities). Although some species thus appear to be more resilient than would be expected, caution must be taken with the interpretation of these results. It is possible that energy trade-offs caused by metal-induced defense mechanisms occurred, which might have long-term consequences. We conclude that more realistic metal exposure scenarios in micro- and mesocosm experiments could be a valuable addition to risk assessment, but should ideally have a long exposure duration, which spans multiple generations, and be complemented with the study of sublethal endpoints (e.g., energy budget, MTLPs, reproduction and genetic diversity).

7.3. Conclusions and future perspectives

When metals are accumulated they can be present in organelles and heat-sensitive proteins, also called the metal-sensitive fractions, or can be biologically detoxified by either storing them in metal-rich granules or by binding them to metallothioneins (Wallace et al., 2003). As stated previously, it might be possible for stressors to change this subcellular partitioning. Further research focused on separating these fractions and measuring the metal concentrations would provide more insight in the animals' ability to cope with stress.

In this thesis it became clear that natural stressors, both biotic and abiotic factors, can change the toxicity of metals. Additionally, other metals can interact as well. It is very difficult to predict their combined effects. Depending on the exposure concentration and duration, the endpoint and the species, stressor interactions and effects can change. The Water Framework Directive does not take these additional stressors into account and, therefore, does not accurately assess the toxicity of metals in freshwater ecosystems. Furthermore, EQS are only available for a limited number of substances (EP, 2013), while millions of chemicals are known.

We advise complementing chemical-based monitoring with effect-based tools *in situ* to identify ecological risks. One such method could be the use of biomarkers, which are biological responses (biochemical, physiological, histological, or morphological) at the individual level or below, observed in field exposed organisms. They can be divided into two categories, namely general and specific biomarkers. General (integrative) biomarkers respond to several classes of toxic substances and frequently also to other types of stressors (e.g., lysosomal membrane stability). Specific biomarkers, on the other hand, respond primarily to only a few groups of substances (e.g., imposex, caused by organic tin compounds; Viarengo et al., 2007). Many biomarkers are already available (a list can be found in EC, 2014), but new are being developed using high-throughput molecular profiling techniques, called "omics" (genomics, proteomics, metabolomics,…). These techniques are used to study the collection of all genes or

gene products such as the genome, proteome or metabolome, respectively. They can thus help to identify toxic modes of action and develop molecular biomarkers that can be used as "early warning" signals to predict effects that at a later stage could have an impact on physiological level and further on at population level (Wernersson et al., 2015).

Ecological indicators can be used as well, measuring changes observed at the population and/or community level (Connon et al., 2012). These changes can be described in terms of structure (e.g., species composition and abundance) and function (e.g., ecosystem respiration, nutrient dynamics). The Species At Risk (SPEAR) bioindicator system, for example, is based on biological traits and provides information about effects occurring on community levels (EC, 2014).

We would specifically suggest monitoring ecosystems with caged organisms (e.g., bivalves) to obtain a high level of standardization. First, highly sensitive, low-cost general biomarkers can be used, which cover more substances and are therefore valuable in identifying areas of concern in environments exposed to complex exposures. Additionally, an ecological indicator can be determined for the species already present in the ecosystem. If a problem is detected (e.g., a critical value is exceeded), multiple specific biomarkers can be used to test and confirm responses due to certain types of substances that are present in elevated concentrations. With this information, the specific pollutants can be identified by chemical analyses. In conclusion, it is clear that ecotoxicological approaches need to evolve if we wish to improve environmental risk assessment.

Nederlandstalige samenvatting

Metaalvervuiling vormt een bedreiging voor ecosystemen wereldwijd. De milieukwaliteitsnormen voor metalen zijn nog steeds vooral gebaseerd op klassieke laboratoriumtesten, waarbij een organisme wordt blootgesteld aan slechts één chemische stof zonder extra stressors. Maar in de natuur worden organismen blootgesteld aan combinaties van vervuilende stoffen. Deze kunnen interageren en synergistische, antagonistische of additieve effecten veroorzaken. Verder hebben organismen in de natuur ook te maken met andere stressors zoals predators, te weinig voedsel, temperatuurschommelingen... De huidige milieukwaliteitsnormen zijn dus mogelijk te beschermend of juist niet beschermend genoeg. Ecologisch relevantere toxiciteitstesten zijn daarom een noodzaak.

In deze thesis bestudeerde ik de gecombineerde effecten van natuurlijke stressors en metaalmengsels van cadmium, koper en lood op aquatische ongewervelden. Het eerste deel van deze thesis bestaat uit laboratoriumexperimenten op de zoetwaterpissebed *Asellus aquaticus*. De effecten van deze metalen werden zo bestudeerd op organismaal niveau onder gecontroleerde omstandigheden. *Asellus aquaticus* is een epibenthische soort die kan teruggevonden worden in zoetwaterecosystemen op het noordelijk halfrond en leeft van organisch materiaal. In **hoofdstuk 2** werd de toxiciteit van koper, cadmium en lood apart onderzocht en de LC_{50}, LC_{20} en LC_{10} op basis van de nominale, effectieve en vrije ionconcentraties bepaald. Op basis van dit experiment werden de sublethale concentraties berekend. De LC-waardes op basis van effectieve en vrij ionconcentraties waren in deze studie veel lager dan in de literatuur, waar vaak gebruikt wordt gemaakt van ongemeten concentraties. Verder werden ook de oneindige effectconcentraties berekend: dit is de concentratie waarbij x% van de individuen zal blijven leven ongeacht de blootstellingstijd. Omdat bijna alle oneindige effectconcentraties bereikt waren op 14 dagen, werd besloten dat *A. aquaticus* minstens 7 dagen moest blootgesteld worden.

Hoofdstuk 3 richtte zich op de (sub)lethale effecten van metaalmengsels, nl. de metaalopname, mortaliteit, groei en energiereserves (glycogeen, lipiden en eiwitten). In **hoofdstukken 4 en 5** werd het onderzoek uitgebreid door het toevoegen van een natuuurlijke stressor, resp. predator- en temperatuurstress. In deze hoofdstukken werden sublethale effecten bestudeerd zoals de zuurstofconsumptie, groei, voedingssnelheid en gedrag. Deze eindpunten werden gelinkt aan zowel de geaccumuleerde metaalconcentraties als aan de vrije ionconcentraties in het water. Er werden metaalinteracties gevonden voor accumulatie, groei- en voedingssnelheid, maar afhankelijk van de concentraties veranderde het interactie-effect. Er was bv. een synergistisch effect voor Cd en Pb accumulatie voor het binaire mengsel Cd + Pb in hoofdstuk 3. In hoofdstukken 4 en 5 echter, werd deze interactie niet meer teruggevonden, maar zagen we een antagonistische interactie van Cu + Pb, Cu + Cd en Cd + Cu + Pb. Tevens zagen we ook dat natuurlijke stressors niet enkel een stimulerend of afzwakkend effect kunnen hebben op de toxiciteit van metalen, maar dat deze ook kunnen verschillen afhankelijk van het geaccumuleerde metaal. Koper bijvoorbeeld kan schade berokkenen aan olfactorische receptor neuronen, waardoor het organisme minder reageert op chemische prikkels. Indien een zoetwaterpissebed meer koper had opgenomen, reageerde deze minder op predator stress en werd de voedingssnelheid en activiteit verhoogd, waardoor er zo meer risico was om opgemerkt te worden door predators.

Het tweede deel van de thesis bestaat uit een microcosmosexperiment (**hoofdstuk 6**). Om mijn studie uit te breiden van het organismale niveau tot effecten op populaties en gemeenschappen, werd er een experiment uitgevoerd in een mesocosmos. De effecten van Cd, Cu en Pb en hun tertiair mengsel werden bestudeerd op een vereenvoudigde macroinvertebraatgemeenschap. In elke emmer zat *Asellus aquaticus* (Isopoda), de watervlo *Daphnia magna*, *Physella acuta* (Mollusca), Chironominae muggenlarven, *Elodea nuttallii* (macrophyt) en de alg *Raphidocelis subcapitata*. De effecten van de metaalmengsels werden onderzocht na 4 en 8 weken op individueel niveau (metaalaccumulatie, plantlengte), populatieniveau (soortabundanties,

biomassa) en gemeenschapsstructuur (Shannon-Wiener index). Er werden na 4 en 8 weken geen significante verschillen tussen invertebraatabundanties waargenomen, mogelijk door de hoge variatie tussen de replicaten of door energietrade-offs.

In **hoofdstuk 7** wordt alles samengevat. Er kan geconcludeerd worden dat natuurlijke stressoren de toxiciteit van metalen kunnen veranderen. Verder kunnen ook metalen interageren. Het is echter zeer moeilijk om hun gecombineerde effecten te voorspellen. Afhankelijk van de blootstellingsduur en -concentraties, het eindpunt en de soort kunnen stressorinteracties veranderen. Het huidige waterbeleid houdt geen rekening met additionele stressors. In deze thesis wordt aangeraden dat we evolueren naar monitoring van effecten *in situ*, in aanvulling van monitoring van chemische concentraties, om zo ecologische risico's te identificeren.

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CONFERENCE CONTRIBUTIONS

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