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The interplay between chemical speciation and physiology determines the bioaccumulation and toxicity of Cu^{2+} and Cd^{2+} to *C. elegans*

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ABSTRACT

The metal body burden of the soil nematode *C. elegans* was determined after 24 h of exposure to LC20 values of Zn, Cu and Cd in liquid medium (supplemented with *E. coli*), both as single metals and as metal mixtures. Connections were identified between chemical speciation in the exposure medium (12 days), body burden, and the earlier described toxicological effects of metal exposure. Cu, and to a lesser extent Cd, was found to associate with *E. coli* as evidenced by the observed decrease in both their dissolved and free metal ion concentrations. Furthermore, binding of Cu to *E. coli* bacteria was dependent on the metal-to-bacteria ratio: at a low Cu concentration (CuLC5) almost all metal was bound, while at a higher Cu concentration (CuLC20) 46.0% remained in the free ion form. In contrast, the concentration of dissolved Zn was not affected by *E. coli*, implying negligible association of this metal ion with the bacteria. Together with a critical analysis of literature data, our results suggest that free metal ion concentrations and thus aqueous uptake routes are the best predictor of internal concentrations under all conditions considered, and of metal toxicity in single metal exposures. Additional factors are involved in determining the toxicity of metal mixtures. In general, the eventual adverse effects of metals on biota are expected to be a consequence of the interplay between chemical speciation in the exposure medium, the timescale of exposure, the exposure route, as well as the nature and timescale of the biotic handling pathways.

Short abstract

A greater bacterial influence was noticed for Cu than for Cd and Zn treatments. However, free metal ion concentrations appeared to be the best predictor of internal concentrations for all treatments, and of metal toxicity of single metal exposures. Furthermore, a concentration dependent Cu speciation was noticed. Since metal speciation and body burden did not differ between mixtures and corresponding single metals, additional factors must be involved in determining metal mixture toxicity.

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

1. INTRODUCTION

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

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

In the case of *C. elegans,* it is not straightforward to discriminate between waterborne and dietborne exposure because the pharyngeal pumping rate is strongly affected by the presence of particles, e.g. bacteria (Offerman et al., 2009; Dwyer and Aamodt, 2013). Furthermore, only a small food volume can be ingested and remains only for a short period (3-10 min) in the weakly acidic intestinal environment of *C. elegans* (pH *ca.* 4; Bender et al., 2013; Chauhan et al., 2013). The organismal detoxification, excretion strategies and characteristics of waterborne and dietborne exposures determine the fate of the metal. Toxicity may occur when the metal uptake rate exceeds the combined rates of detoxification and excretion such that a critical internal threshold is reached (Rainbow 2002, 2007; Adams et al., 2011). Furthermore, total metal concentrations in the exposure medium are generally a poor representation of the actual exposure conditions experienced by the organism due to e.g. metal adsorption by particles such as bacteria. Depending on the exposure scenario, dissolved metal concentrations, concentrations of readily dissociable ("labile") metal complexes or of free metal ions, or internal metal concentrations are anticipated to be better predictors of toxicity. In addition, environmental exposures typically involve mixtures of metal species, yet the processes which determine (eco)toxicological effects under such conditions remain poorly understood.

In the present study, the metal speciation in the exposure medium (also in absence of *E. coli*) and the ensuing metal body burden (mg metal/g wet weight wormpellet) were characterised for *C. elegans* exposed to single metals and their mixtures in the presence of *E. coli* bacteria.

The results, together with a critical analysis of literature data, were used to identify the metal species and/or uptake route that are the best predictors of toxicological effects.

2. MATERIALS AND METHODS

2.1. Free metal ions

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

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

The calibration line for Cu and Cd was Nernstian, *i.e.* the slope of the log of the free metal ion concentration *vs*. E (mV) was always between 25 and 30 at T = 293.15 K. The pH of each replicate was measured at day 0, day 10 and day 12 and some replicates were randomly checked during the experiment. A fourth replicate was used for daily pH measurement and for sampling, both filtered and non-filtered, to measure the proportion dissolved vs. total metal and to verify metal concentrations (91%-100.6% recovery) using HR-ICP-MS (Element XR, Thermo Scientific). Stock solution concentrations were verified by ICP-OES (ICAP 6300 Duo, Thermo Scientific). Therefore, samples containing *E. coli* bacteria were first freeze-dried (Heto Powerdry LL 30000, Thermo Scientific) and 250 µL nitric acid (TraceMetal Grade, Fisher Chemical) was added. All samples were digested at 110°C for 30 minutes, using a heating plate (HotBlock, Environmental Express). MilliQ water was added, making the total volume 10 mL. For each treatment without *E. coli*, the pH measured before (5.1 ± 0.1) was similar to the pH measured after the experiment (4.9 ± 0.1), while the pH of treatments with *E.coli* increased from 5.1 ± 0.0 to 5.9 ± 0.3. Similar pH values and increases were also noticed in an earlier study (Moyson et al., submitted).

2.2. Body burden

2.2.1. *Caenorhabditis elegans* culture and synchronization

Wild type *Caenorhabditis elegans* nematodes of the N2 strain were obtained from the Caenorhabditis Genetic Centre, Minneapolis, USA. Nematodes were maintained on nematode growth medium (NGM) agar plates at 20°C, seeded with *Escherichia coli* (OP50 strain) as food source (Brenner, 1974). Synchronization of the nematodes was performed by bleaching, adding a hypochlorite solution (5 N NaOH, 8% sodium hypochlorite) to mixed-stage *C.*

elegans, killing the nematodes that were not protected by an egg shell. Eggs were raised on OP50-seeded NGM agar plates.

2.2.2. Test media

LC20 concentrations of Zn, Cu and Cd after a 24 h exposure (Table 4.1) and determined in our previous study (Moyson et al., 2018), were made from $ZnCl_2$ (Alfa Aesar), $CuCl_2.2H_2O$ (AnalaR Normapur) and $CdCl_2.2.5H_2O$ (Alfa Aesar) in K-medium (52 mM NaCl, 32 mM KCl, 5 µg/mL cholesterol, pH 5.1), supplemented with *E. coli* bacteria (1.5 - 1.7 g/L). The mixtures ZnCu, ZnCd, CuCd and ZnCuCd were prepared by combining the corresponding LC20 concentrations. Stock solutions were made of tenfold higher concentrations. ICP-OES (ICAP 6300 Duo, Thermo Scientific) was used to verify metal concentrations of stock and exposure solutions (93% - 113% recovery). Metal solutions were incubated with the bacterial suspensions for 12 h at 4°C prior to toxicity testing, allowing metal partitioning between the aqueous phase and the bacteria. Since the determined average pH before (5.3 ± 0.2) and after the experiment (5.4 ± 0.4) was within an acceptable pH range for *C. elegans*, its potential effects on the measured parameters were excluded.

2.2.3. Internal concentration measurement

Young 24 h L4 nematodes were washed several times and transferred to a NGM plate without food, to get rid of *E.coli* bacteria. Approximately 4.5 mg nematodes were transferred to 15 mL Falcon tubes filled with 9 mL K-medium and 1 mL test medium (K-medium containing the test metal concentration(s)) or control (K-medium), supplemented with *E. coli* OP50 (1.5 - 1.7 g/L). During the experiment, the Falcon tubes were shaken continuously (160 rpm, 20°C). After 24 h of metal exposure to LC20, nematodes were washed three times with physiological water (9 g/L NaCl) to get rid of bacteria. Subsequently, *C. elegans* were killed slowly by gradually increasing the temperature. The dead nematodes were washed again. The physiological water with the nematodes was filtered using a 5 µM membrane filter paper (Whatman), which was placed in a plastic filter holder (Schleider and Schuell). The filter paper containing the nematodes was then plugged into a Falcon tube by the use of tweezers and 0.2 µL nitric acid was added. Overnight, the Falcon tubes were placed under a fume hood. The following day, these tubes were transferred to a hot block (110°C) for 30 minutes. After cooling down of the samples, MilliQ water was added, bringing the volume up to 4 mL. For each treatment three replicates were made. Internal concentrations of Cu, Cd, Zn, Na, K, Ca, Mg and Fe were measured by a HR-ICP-MS (Element XR, Thermo Scientific).

2.3. Statistical analysis

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

2.3.1. Free metal ions

Generalized mixed models were fitted to test the possible effects of exposure time, *E. coli* presence/absence, treatments and their interactions on the free metal (Cu, Cd) ion concentration and percentage. In all models, exposure time (days) was entered as a continuous variable. *E. coli* presence/absence and treatments, plus their interactions, were included as fixed effects. Because the free metal ion concentration was repeatedly measured within the same wells over time, observations from the same well were not independent. To account for this non-independence, a random intercept term for well was added to the model. Measurements of the control group and Zn group were omitted from the analysis, since the free metal ion concentration was always (close to) zero. Subsequently, for each metal treatment both with and without *E.coli*, a one-way ANCOVA analysis was fitted to determine the slope of the regression line and thus analyse the effect of time on the free metal ion concentration and percentage. When time did not have an influence on free metal ions, a one-way analysis of covariance (ANCOVA) was fitted for each treatment to analyse if the slopes of the regression lines of *E. coli* presence and absence differed. Likewise, the main effect of metal treatment on the slopes of the regression lines of the free metal ion concentration was analysed when - in both treatments involved - *E. coli* was present or absent. Thus, free metal concentration was compared for each treatment in the presence and absence of *E. coli*. For each *E. coli* condition the following comparisons between treatments were made: Cd vs. ZnCd, Cu LC5 vs. CuLC20, CuLC20 vs. ZnCu, CuLC20 vs. CuCd, CuLC20 vs. ZnCuCd, ZnCu vs. CuCd, ZnCu vs. ZnCuCd and CuCd vs. ZnCuCd.

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

2.3.2. Body burden

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

3. RESULTS

3.1. Metal speciation in the exposure medium

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

For all Cd and Cu treatments, both the free metal ion concentration and percentage remained stable over time, except for CuLC5 where *E. coli* presence caused a gradual decrease over time in the free Cu ion concentration and percentage, reaching an 88.7% reduction after 12 days ($P<0.001$) (Fig. 1).

3.1.1. Metal speciation in *E. coli* absence

In all treatments in the absence of *E. coli*, the ISE measurements indicate that practically all of the Cd and Cu was found to be in the free ion form (average 94.5% and 92%, respectively), (Fig. 1).

3.1.2. Metal speciation in *E. coli* presence

The presence of *E. coli* affected the speciation of both Cu and Cd (Fig. 1 and 2). The bacterial influence was greater for Cu than for Cd treatments, resulting in an average of 39.0% for the free Cu ion percentage, while the mean free Cd ion percentage was still 85.0%. The presence of *E. coli* caused a mean decrease of 10.0% in free Cd ion concentration and percentage in both Cd and ZnCd exposure, as compared to in the absence of bacteria (P<0.001). In the case of Cu, the presence of *E. coli* led to an even greater decrease in free ion concentration and percentage in all treatments (P<0.001) by, on average, 88.7% for CuLC5, 54.4% for CuLC20, 45.9% for ZnCu, 52.3% for CuCd, and 44.2% for ZnCuCd. Again, the effect was more pronounced for the CuLC5 exposure.

Similar trends were found for dissolved metal concentrations, especially for Cu (Fig. .2). The concentrations of dissolved Cd did not differ between *E. coli* presence or absence, while presence of *E. coli*, in percentage terms, caused a significantly slightly lower concentration of dissolved Cd in the Cd treatment (4%, P<0.05) compared to *E. coli* absence (Fig. 2). Similarly, for Zn treatments no difference between *E. coli* conditions could be found, except for a smaller dissolved Zn concentration with *E. coli* than without bacteria (17%, P<0.05) (Fig. 3). This difference reflects the amount of Zn that is associated with the *E. coli*. In contrast, the presence of *E. coli* bacteria caused a reduction of the dissolved Cu concentration in all treatments; again the largest percentage decrease was noted for CuLC5 (Fig. 2). Compared to *E. coli* absence, the dissolved Cu concentration in the presence of *E. coli* decreased by 71.2% for CuLC5, 51.3% for CuLC20, 39.7% for ZnCu, 48.5% for CuCd and 44.7% for ZnCuCd (P<0.001). Slightly smaller reductions between *E. coli* conditions were observed for the percentage of dissolved Cu: 61% for CuLC5 (P<0.001), 34.8% for CuLC20 (P<0.01), 27.7% for ZnCu (P<0.01), 31.8% for CuCd (P<0.01) and 29.6% for ZnCuCd (P<0.001).

3.1.3. Concentration dependence of Cu speciation

Although the presence of *E. coli* had a significant effect on the free metal ion concentration of Cu, differences between LC5 and LC20 treatments were smaller. Independent of the presence of *E. coli*, dissolved Cu concentration differed between CuLC5 and CuLC20 (P<0.001), while dissolved Cu percentage in each case only differed in *E. coli* presence (P<0.05) (Fig. 2). In the absence of *E. coli*, CuLC20 had on average a 6.2% lower percentage of free Cu ions than CuLC5 (P<0.01) (Fig. 1). However, in that condition free Cu concentration of CuLC20 was 5 times higher than of CuLC5 (P<0.001), which was expected from the higher total Cu exposure concentration. In *E. coli* presence, CuLC20 had a 20.1 times higher free Cu ion concentration (P<0.001), while its percentage of free Cu ions was on average 3.8 times higher than for CuLC5 (P<0.001) (Fig. 1). The lowest Cu concentration (CuLC5) showed to have the strongest reduction in free Cu over time and at the end of the experiment almost all Cu was bound in *E. coli* presence (97%), while at CuLC20 exposure 54% of Cu was bound to *E. coli* bacteria (Fig. 1).

3.1.4. Metal speciation in mixtures

Differences in metal speciation between mixtures and single metals were smaller than those between individual metals (Fig. 1, 2, and 3). ZnCd had a 4.0% higher free Cd ion concentration and percentage than Cd exposure, both with and without *E. coli* (P<0.001) (Fig.

1). No difference in dissolved Cd concentration and percentage was noted between Cd and ZnCd in the absence of *E. coli*, while in *E. coli* presence a lower dissolved Cd concentration (7.5%) and percentage (3.9%) was observed for Cd than for ZnCd ($P<0.05$) (Fig. 2). Treatments with equal total Cu concentration did not differ in the absence of *E. coli*, but in *E. coli* presence, free Cu ion concentration and percentage of CuLC20 was slightly lower than in the case of ZnCu and ZnCuCd (15.9%, $P<0.001$) (Fig. 1). Moreover, a 12.6% higher free Cu ion concentration and percentage of ZnCu and ZnCuCd than in the case of CuCd was observed ($P<0.01$). Furthermore, in *E. coli* presence, the concentration and percentage of free Cu ions of CuCd was similar to that of CuLC20, while free Cu percentage of ZnCu was similar to that of ZnCuCd. In contrast, no difference in concentration or percentage of dissolved Cu was measured between mixtures and corresponding single metals (Fig. 2). Also for dissolved Zn concentration and percentage no difference between treatments was noted (Fig. 3).

3.2. Body burden

Nematodes accumulated significant amounts of metals in their bodies under all LC20 exposure conditions considered. As compared to the control, internal Cu concentrations in Cu (20.2x, $P<0.001$), ZnCu (16.4x, $P<0.001$), CuCd (20.4x, $P<0.001$) and ZnCuCd (12.2x, $P<0.01$) exposed nematodes was significantly higher (Fig. 4). Also Cd accumulation in Cd (24.7x, $P<0.001$), ZnCd (26.9x, $P<0.001$), CuCd (12.7x, $P<0.01$) and ZnCuCd (12.7x, $P<0.01$) exposed nematodes was significantly greater than that of the control. For Zn, the difference in body burden between metal exposed groups and the control was not significant due to the large standard deviation. Furthermore, no significant differences were found for accumulation of the major elements Na, K, Ca, Mg and Fe, under any of the exposure conditions.

4. DISCUSSION

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

4.1 Metal speciation in the exposure medium

In the absence of *E. coli* practically all of the Cd and Cu is found to be in the free ion form (Fig. 1). Using Visual MINTEQ, others have reported the percentage of free Cu^{2+} and Zn^{2+} to be 92% in K-medium (Freeman et al., 1998), in good agreement with our data, whilst the major Cd species are predicted to be chloro-complexes ($CdCl^-$) (64%) and free ions (20%) (Cressman III and Williams, 1997). The apparent discrepancy between our ISE measurements of Cd and the predictions of Visual MINTEQ are likely due to uncertainties in the stability constants used in the model. The stability of metal ion complexes with chloride is rather low, and the computed speciation is sensitive to the magnitude of the stability constant, K, employed. Visual MINTEQ uses a log K value of 0.3 for CuCl and 1.98 for CdCl. However, in aqueous media, log K values as high as *ca.* 1 have been reported for CuCl (Sato and Kato, 1977) and the IUPAC recommended value is 0.83 (Powell et al., 2007), whilst for CdCl, log K values as low as 0.5 have been reported (Simoes et al., 1981). Since some reports of log K values for ZnCl are of order 0.5, it is possible that the majority of this metal is in the free ion form in the exposure media (Aparicio and Elizalde, 1996; de Robertis and de Stefano, 1998).

In the presence of *E. coli*, the Cu and Cd speciation depends on the metal-to-bacteria ratio. Fig. 1 shows that at low Cu concentration, all Cu is bound to *E. coli*, while at higher Cu concentration a greater proportion of Cu is present in the form of free ions. Our results are in broad agreement with literature data on Cd-*E. coli* (Höss et al., 2011) and Cu-*E. coli* binding (Mullen et al., 1989; Fang et al., 2009), interpolated to the same metal-to-bacteria ratio.

4.2 Body burden

Our results indicate that, although less total Cu in the exposure medium was required to cause the same lethality of 20% as Cd and Zn (Moyson et al., 2018), this is not reflected in a higher total body burden. Rather, in contrast, the total internal concentration of Cu was 40.7% lower compared to Cd, and 61.4% lower than Zn accumulation after single metal exposure (Fig. 5). Per liter of exposure medium, one gram of worms took up 0.8% of total Cd, 2.5% of total Cu and 0.9% of total Zn from the metal exposure medium (i.e. in presence of *E. coli*).

The amount of metals accumulated by *C. elegans*, coupled with information on metal speciation in the exposure medium, provides insights into the relative contributions of waterborne and dietborne metals to the body burden. Considering the waterborne free metal ions, in the case of Cu (LC20) the concentration of free Cu^{2+} is 9×10^{-6} mol dm^{-3} and the surface area of the biointerface is approximately 6.6×10^{-7} m^2 (external and internal surface). The steady-state limiting diffusive supply flux of the free Cu^{2+} is given by Dc/δ (van Leeuwen et al., 2005) where D is the diffusion coefficient of Cu (*ca.* 7×10^{-10} m^2 s^{-1}; Li and Gregory, 1974), c is the concentration of Cu^{2+}, and δ is the thickness of the aqueous diffusion layer (*ca.* 5×10^{-4} m in unstirred solution; Levich, 1962), which yields a value of 1.3×10^{-8} mol m^{-2} s^{-1}, i.e. a total of 7.4×10^{-10} mol per worm after 24 h of exposure. If the entire supply flux of Cu^{2+} was accumulated by *C. elegans*, the ensuing body burden would be 4700 µg/g wet weight. The measured body burden is more than two orders of magnitude lower than this, i.e. the free metal ion concentration alone is well able to satisfy the uptake demand of the organism. A parallel computation for Cd shows the same picture: the measured body burden in this case is approximately 1000 times lower than that computed on the basis of the supply flux of the free ion being the determinant of bioaccumulation. For assessment of the dietborne exposure the ingestion rate was estimated to be 10^5 *E. coli* per worm per day (Gomez-Amaro et al., 2015). For the case of Cu, the concentration of Cu that is associated with *E. coli* is 1.1×10^{-5} mol dm^{-3} at an *E. coli* concentration of 1.6 g (wet weight) dm^{-3}. The wet weight of an *E. coli* cell is *ca.* 10^{-12} g, and thus each *E. coli* contains 4.4×10^{-16} g Cu. Accordingly, ingestion of 10^5 *E. coli* by an individual nematode (with a wet weight of *ca.* 10^{-5} g) corresponds to an intake of 4.4×10^{-11} g of Cu, which is equivalent to 4.4 µg/g wet weight. This is a factor *ca*. 10 *lower* than the measured body burden. For the case of Cd, a parallel analysis also yields 4.4 µg/g wet weight based on ingestion of bacteria, which is a factor *ca*. 15 times lower than the measured body burden. These results imply that the free metal ion is the predominant contributor to bioaccumulation of both Cu and Cd.

In agreement with our results, Höss et al. (2011) suggested that the main Cd uptake route is from “aqueous Cd”, taken up together with the bacteria, rather than from bacterial-bound Cd concentrations. The “aqueous Cd” corresponded to all forms of Cd remaining in solution after bacteria removal. Nevertheless, the outcome should be regarded with some caution because the pharyngeal pumping rate is sensitive to the presence of metal ions, albeit that typically a *lower* rate is observed (Jones and Candido, 1999), i.e. if anything, our calculations are an overestimation of the dietborne contribution to the body burden. The bioaccumulation pattern of Cu and Cd in the present study was also noticed in earlier studies. In these studies, a concentration dependent metal content was established for Cu and Cd, which was also time dependent for Cd (Offerman et al., 2009; Chun et al., 2017). In the case of Zn, the body burden of *C. elegans* is reported to be largely proportional to the dietary Zn and can be moderately changed in response to that, although the uptake route has not been established (Davis et al., 2009). Nematodes exposed to 500 µM Zn in the presence of bacteria had a 109%

higher total Zn content, compared to control nematodes (Kumar et al., 2016). Baseline Zn content increased from 0.09 µg/mg in L3 stage to 0.1 µg/mg for 1-day-old adults and to 0.14 µg/mg for 5-day-old adults, indicating a moderate increase in zinc content with age (Kumar et al., 2016).

4.3 Relationship between exposure conditions, bioaccumulation, and toxicological effects

Models for prediction of metal biouptake, e.g. the biotic ligand model and the free ion activity model, assume that only free metal ions are available for biouptake (Campbell, 1995; Brown and Markich, 2000; Paquin et al., 2002; Slaveykova and Wilkinson, 2005; Jakob et al., 2017), and attempts have been made to use metal ion characteristics as predictors of toxic effects (Renner, 1997; Tatara et al., 1997, 1998). Recent work with *C. elegans* showed that toxicological responses to metal ions were strongly time dependent (Moyson et al., submitted), yet the results reported herein show that the metal speciation in the exposure medium is largely invariant with time (Fig. 1). In agreement with our results, others have assumed that Cd speciation of a 48 h exposure would be comparable to that of 24 h (Cressman III and Williams, 1997; Freeman et al., 1998). Accordingly, the nature and time dependence of adverse effects also involves the nature and time dependence of biological processes, e.g. bio-uptake, bioaccumulation and defence mechanisms.

The differentiated role played by bacteria in metal toxicity is another factor to consider in the case of *C. elegans*. Bacteria are necessary as a food source to prevent effects of starvation, but their presence affects the rate of pharyngeal pumping and influences metal speciation. For example, our results show that the presence of *E. coli* has a larger influence on Cu speciation than on Cd speciation. The influence of bacteria on the results of toxicity testing has been observed in several studies (Spraque, 1985; Williams and Dusenbery, 1988; Donkin and Dusenbery, 1993; Donkin and Williams 1995). The presence and density of bacteria (Höss et al., 2011; Win et al., 2013) as well as the bacterial species (Venette and Ferris, 1998) are reported to have an influence on the metal toxicity. For example, it was proposed that Cd availability decreases with increasing bacterial density, resulting in a lower Cd toxicity (Boyd et al., 2003; Offermann et al., 2009; Höss et al., 2011). However, no consensus exists about the interpretation of their data, so many hypotheses have been proposed. For example, the reduced Cd toxicity at higher bacterial densities might be explained by the Cd induced feeding inhibition (Höss et al., 2011). Metals may affect feeding behaviour by blocking pharyngeal pumping and affecting or damaging gut structure (Popham and Webster, 1978). For Cu, Zn and Cd, the EC50 for feeding was 3.32, 12.6 and 5.2 mg/L respectively (Jiang et al., 2016), which are comparable to the concentrations used in the present study. Reduced metal toxicity with increasing food densities may also be due to the fact that more metals are bound to the bacteria, reducing their environmental availability (Boyd et al., 2003). Because a similar toxicity was observed if *C. elegans* was fed with live or dead bacteria, the potential binding of Cd to bacteria is thought to be passive (Anderson et al., 2001). Blériot and co-workers (2014) also determined the presence of a binding protein for Cu in *E. coli*. It was suggested that *E. coli* functions both as a food organism and as a vector for contamination uptake (Höss et al., 2001). Nevertheless, under the conditions used herein, metals bound to *E. coli* are not the major contributor to the body burden (see §4.4.2). Consistent with our findings, for the case of single metal ion exposure of *C. elegans*, data published by Offerman et al. (2009) reveal a consistent link between Cd body burden and toxic effect, irrespective of the composition of the exposure medium (different total Cd concentrations and different *E. coli* concentrations) and different exposure times (Fig. 6).

This was in contrast with the findings for other organisms, e.g. *Daphnia magna* (De Schampelaere et al, 2004), where, body burden alone did not appear to be a good indicator of metal toxicity. Knowing the uptake mechanism can help us to better understand the earlier observed toxicological effects. A couple of other studies have attempted to identify the relationships between waterborne vs. dietborne metals and toxicological endpoints for single metal exposures (Höss et al., 2011; Yu et al., 2012). Höss and co-workers (2011) performed 48 h - 96 h exposures to Cd concentrations in K-medium between 0 and 8 mg/L combined with *E. coli* at concentrations of 0-2000 formazin absorption units (FAU). By analysis of % inhibition of reproduction as a function of total, “aqueous”, and bacterial-bound Cd concentrations, Höss et al. (2011) found that the aqueous Cd was the best predictor of toxicity. We performed a similar analysis of the data published by Yu et al. (2012) on Cu toxicity to *C. elegans* under a range of waterborne and foodborne concentrations in K-medium. The results for the endpoint of growth status clearly show that the waterborne Cu is the best predictor of the eventual toxicity (Fig. 7). The results discussed in the preceding sections imply that the majority of the waterborne Cu is in the form of the free metal ion.

In the case of metal mixtures, body burden alone is not a straightforward indicator of toxic effects. Earlier described differences in toxicity between mixtures and their corresponding single metals (Moyson et al., 2018; Moyson et al., submitted) cannot be explained by the competition to enter the *C. elegans* body since their internal concentrations were of similar value (Fig. 4). Furthermore, there were no differences in metal speciation found between mixtures and their corresponding single metals. That is, mixture toxicity effects can be unambiguously ascribed to biotic handling differences and not to differences in the exposure medium. It seems likely that the metal toxicity is a consequence of their different modes of action, as well as potential differences in subcellular compartmentalisation. Body accumulation in combination with intracellular speciation, i.e. the distribution of internalised metals over the tissues (e.g. gut, vesicles) in which metals are stored or detoxified, could provide important insights into metal toxicity. For example, transmembrane Zn transporters such as cdf-proteins may influence Zn homeostasis by mobilizing Zn. Cdf-2 is involved in storage of Zn in vesicles of intestinal cells, while cdf-1 may transport Zn from these cells to the body cavity or intestinal lumen to promote excretion (Davis et al., 2009; Dietrich et al., 2016). In our earlier study (Moyson et al., submitted) the potential role of cdf-2 in mitigating Cd toxicity was identified, and thus it is possible that Cd is also stored in the gut granules, thereby rendering it biologically inactive. The same detoxification mechanism has been proposed for Cu (Chun et al., 2016), nevertheless, our observation that at comparable body burdens Cu is more toxic than Zn or Cd suggests that this process is less effective in case of Cu. In contrast to Cd and Zn, Cu is observed to be homogeneously distributed throughout the body of the nematodes (Jackson et al., 2005).

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

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

5. CONCLUSIONS

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

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Tables and figures

Fig. 1: Free Cd ion concentration (mg/L (A); % (C)) of Cd and ZnCd treatment in the presence and absence of *E.coli*. Free Cu ion concentration ((mg/L) B; % (D)) of CuLC5, CuLC20, ZnCu, CuCd and ZnCuCd treatment in the presence and absence of *E.coli*. Data are shown as mean ± standard deviation.

Fig. 2: Dissolved Cd concentration (µg/L (A); % (C)) of Cd and ZnCd treatment in the presence and absence of *E.coli*. Dissolved Cu concentration (mg/L (B); % (D)) of CuLC5, CuLC20, ZnCu, CuCd and ZnCuCd treatment in the presence and absence of *E.coli*. Data are shown as mean ± standard deviation. Symbols denote significant differences between metal treatment (*) and between *E. coli* conditions (+).

Fig. 3: Dissolved Zn concentration (µg/L (A); % (B)) of Zn, ZnCu, ZnCd and ZnCuCd treatment in the presence and absence of *E.coli*. Data are shown as mean ± standard deviation. Symbols denote significant differences between *E. coli* conditions (+).

Fig. 4. Internal concentration of Cu, Cd, Zn, Na, K, Ca, Mg and Fe of nematodes exposed for 24 h to LC20 concentrations of metals and their mixtures. Replicates are shown as well as the average. The body burden refers to the wet weight of the nematodes. Asterisks denote significant differences (*P<0.05; **P<0.01; ***P<0.001) compared to control.

Fig. 5. Dissolved and free metal ions (%) of Cu, Cd and Zn after exposure to their LC20 concentrations (right-hand axis) and internal metal concentration of nematodes exposed to these LC20 concentrations (left-hand axis). The body burden refers to the wet weight of the nematodes.

Fig. 6: Relationship between *C. elegans* body burden and cdr-1 expression. The nematodes were exposed to different *E. coli* and Cd concentrations for different exposure times as indicated in the legend. (Data are obtained from Offerman et al., 2009).

Fig. 7: Growth status of *C. elegans* after exposure to Cu under a range of conditions. The various Cu concentrations (mg/L) correspond to the total concentration (black circles), the amount associated with the *E. coli* food (red squares) and the amount in the aqueous phase (blue triangles). (Data are obtained from Yu et al., 2012).

Table 1: LC5 and LC20 values of Zn, Cu and Cd after 24 h of exposure (NA = not applicable).

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

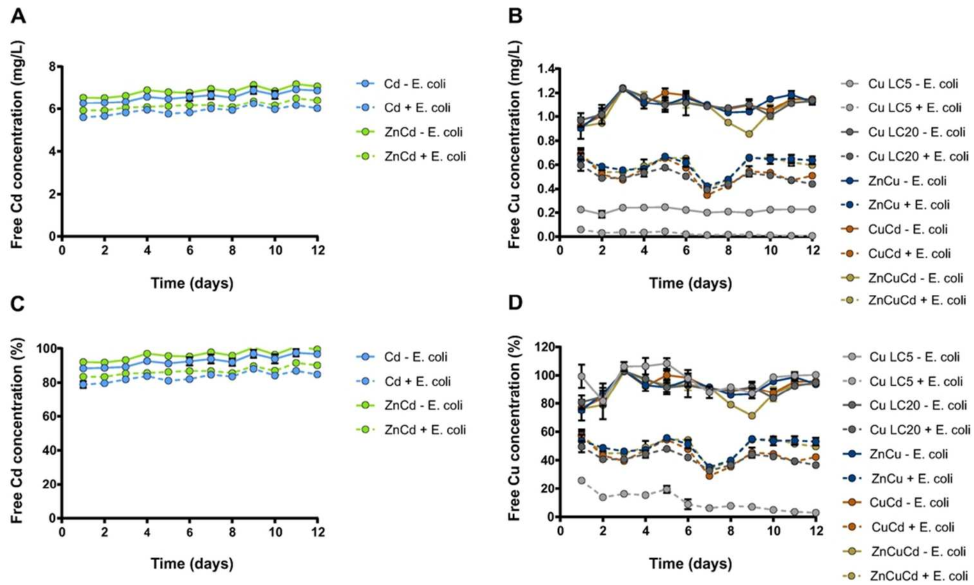

Fig. 1: Free Cd ion concentration (mg/L (A); % (C)) of Cd and ZnCd treatment in the presence and absence of E.coli. Free Cu ion concentration ((mg/L) B; % (D)) of CuLC5, CuLC20, ZnCu, CuCd and ZnCuCd treatment in the presence and absence of E.coli. Data are shown as mean ± standard deviation.

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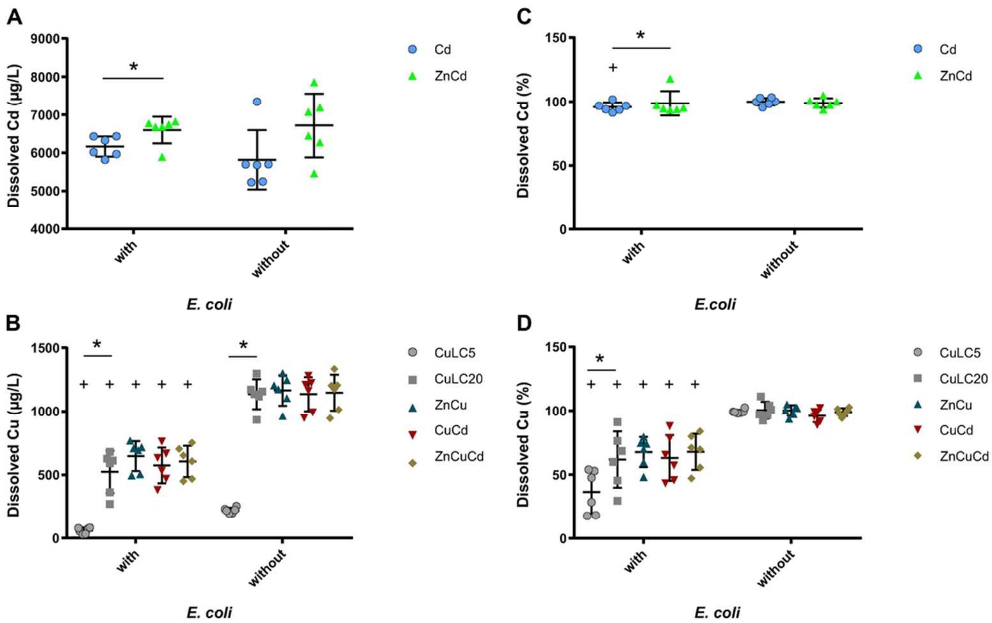

Fig. 2: Dissolved Cd concentration (µg/L (A); % (C)) of Cd and ZnCd treatment in the presence and absence of E.coli. Dissolved Cu concentration (mg/L (B); % (D)) of CuLC5, CuLC20, ZnCu, CuCd and ZnCuCd treatment in the presence and absence of E.coli. Data are shown as mean ± standard deviation. Symbols denote significant differences between metal treatment (*) and between E. coli conditions (+).

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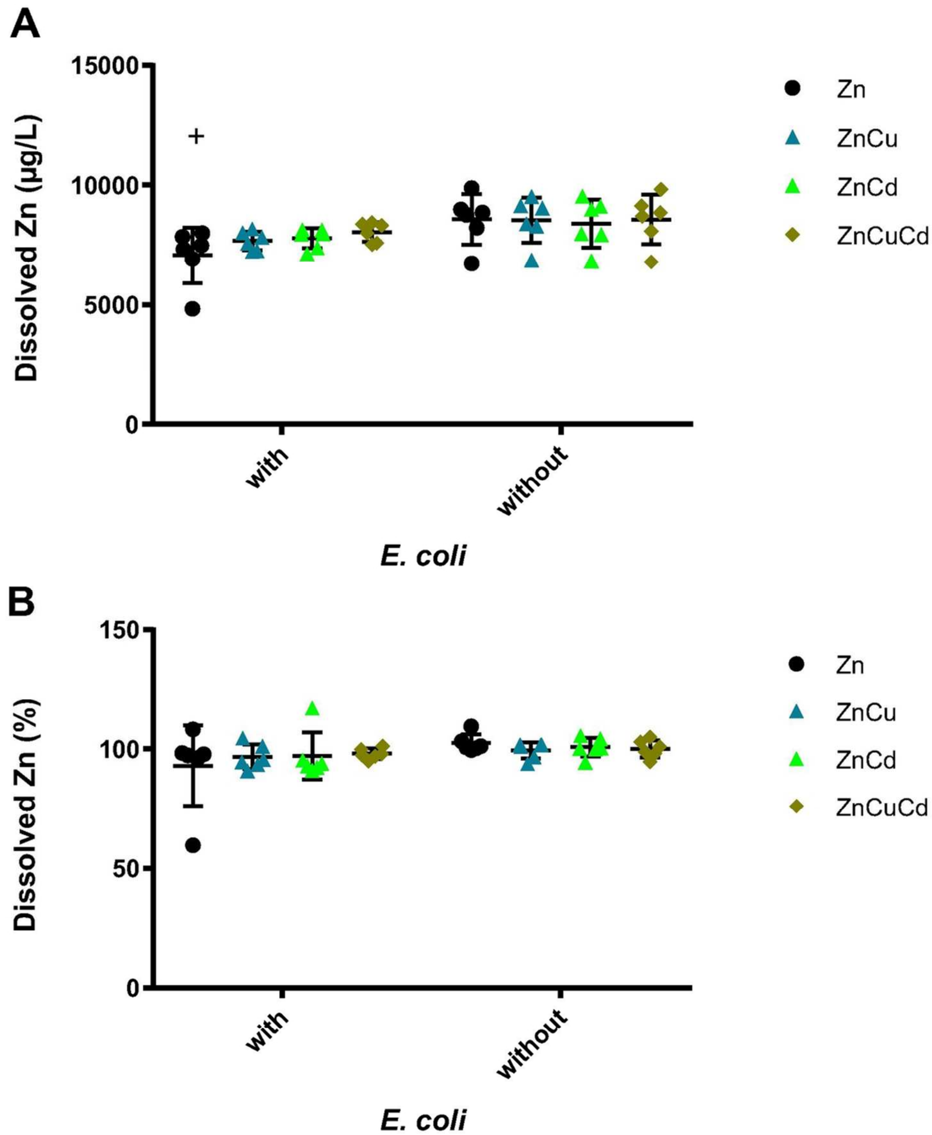

Fig. 3: Dissolved Zn concentration (µg/L (A); % (B)) of Zn, ZnCu, ZnCd and ZnCuCd treatment in the presence and absence of E.coli. Data are shown as mean ± standard deviation. Symbols denote significant differences between E. coli conditions (+).

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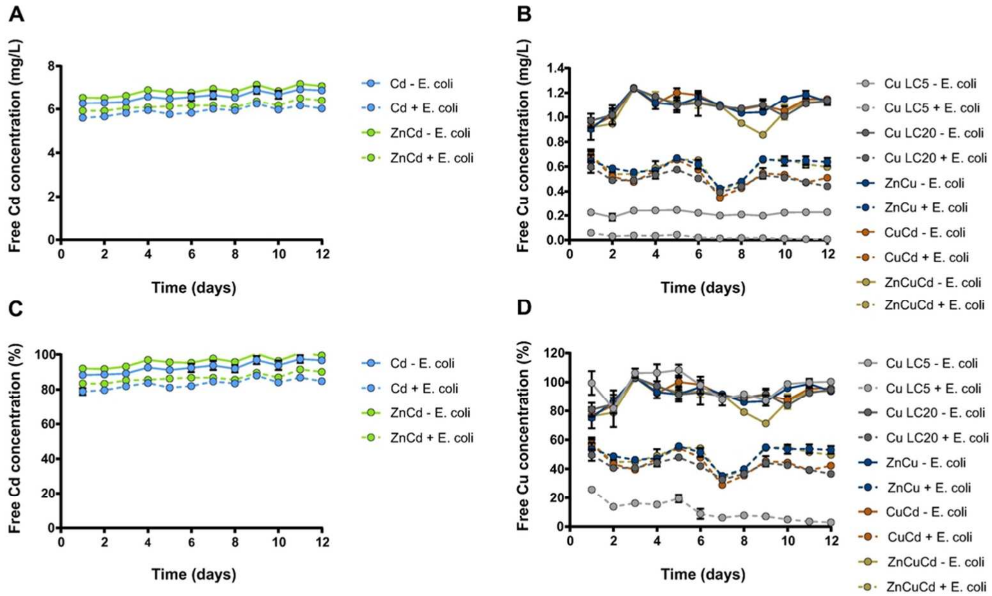

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94x55mm (300 x 300 DPI)

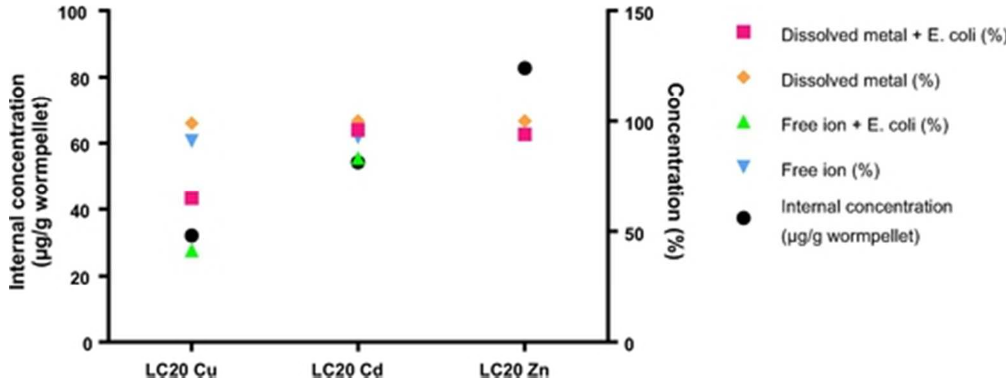

Fig. 5. Dissolved and free metal ions (%) of Cu, Cd and Zn after exposure to their LC20 concentrations (right-hand axis) and internal metal concentration of nematodes exposed to these LC20 concentrations (left-hand axis). The body burden refers to the wet weight of the nematodes.

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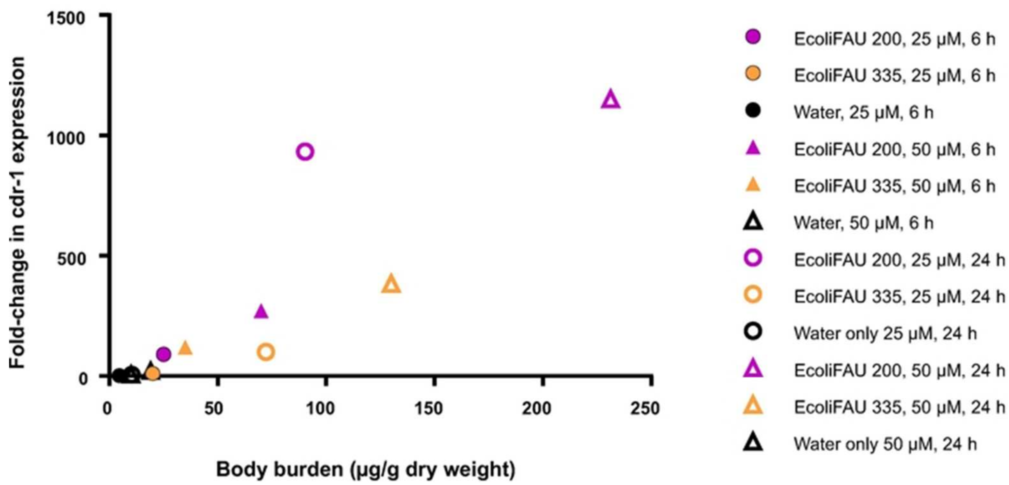

Fig. 6: Relationship between C. elegans body burden and cdr-1 expression. The nematodes were exposed to different E. coli and Cd concentrations for different exposure times as indicated in the legend. (Data are obtained from Offerman et al., 2009).

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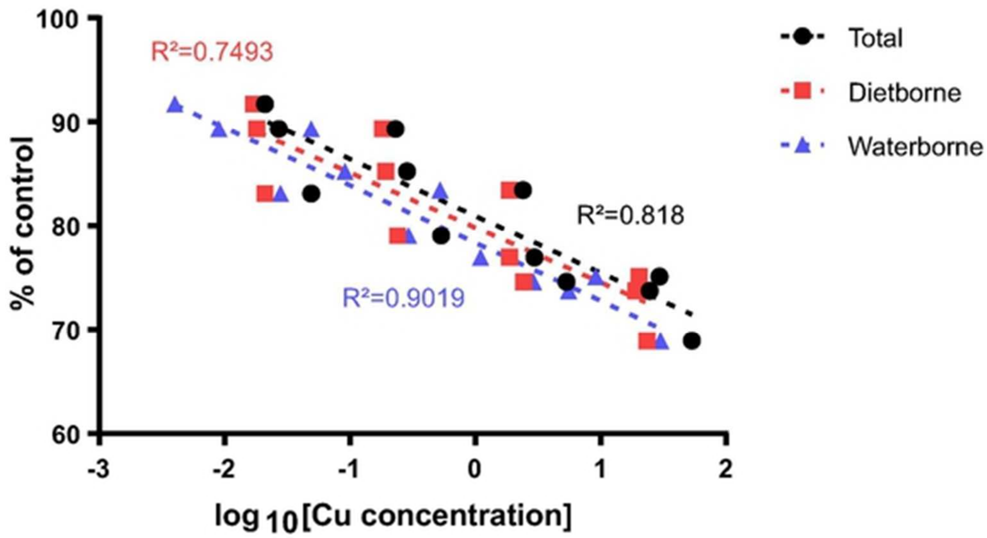

Fig. 7: Growth status of C. elegans after exposure to Cu under a range of conditions. The various Cu concentrations (mg/L) correspond to the total concentration (black circles), the amount associated with the E. coli food (red squares) and the amount in the aqueous phase (blue triangles). (Data are obtained from Yu et al., 2012).

49x30mm (300 x 300 DPI)

	LC5		LC
	(mg/L)	(mM)	(mg/L)
Zn	NA	NA	9.501 ± 2.841
Cu	0.226 ± 0.104	0.004 ± 0.002	1.299 ± 0.409
Cd	NA	NA	7.110 ± 2.315

20
(mM)
0.145 ± 0.043
0.020 ± 0.006
0.063 ± 0.021