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

Environmental
Toxicology and Chemistry

Environmental Toxicology

MIXTURE EFFECTS OF COPPER, CADMIUM AND ZINC ON MORTALITY AND BEHAVIOUR OF *C. ELEGANS*

SOFIE MOYSON, KRIS VISSENBERG, ERIK FRANSEN, RONNY BLUST, and STEVEN J. HUSSON

Environ Toxicol Chem., **Accepted Article** • DOI: 10.1002/etc.3937

Environmental Toxicology **Environmental Toxicology and Chemistry**
DOI 10.1002/etc.3937

MIXTURE EFFECTS OF COPPER, CADMIUM AND ZINC ON MORTALITY AND BEHAVIOUR OF *C. ELEGANS*

SOFIE MOYSON,[a,*] KRIS VISSENBERG,[b,c] ERIK FRANSEN,[d] RONNY BLUST,[a] and STEVEN J. HUSSON[a]

[a]Systemic Physiological and Ecotoxicological Research, Department of Biology, University of Antwerp, Antwerp, Belgium

[b]Integrated Molecular Plant Physiology Research, Department of Biology, University of Antwerp, Antwerp, Belgium

[c]Plant Biochemistry & Biotechnology Lab, Department of Agriculture, School of Agriculture, Food & Nutrition, University of Applied Sciences Crete – Technological Educational Institute, Stavromenos, Heraklion, Crete, Greece

[d]StatUa Center for Statistics, University of Antwerp, Antwerp, Belgium

* Address correspondence to: Sofie.Moyson@uantwerpen.be

This article contains online-only Supplemental Data

Submitted 17 April 2017; Returned for Revision 2 June 2017; Accepted 7 August 2017

Abstract: In this study toxicity effects of zinc, copper and cadmium, both as single metals and in combination, were examined for the nematode *Caenorhabditis elegans*. Metal effects on lethality were analysed in a time-dependent manner using different concentrations in K-medium. LC20 concentrations were used to investigate the effects on locomotion and chemosensation. The results showed that copper toxicity was higher compared to cadmium and zinc, resulting in higher mortality rates and a more reduced locomotion. For all metals lethality increased over time. When cadmium concentrations were added to copper, and vice versa, significant increases in toxicity were noted. Different interaction effects were observed for the mixtures ZnCd, ZnCu, CuCd and ZnCuCd. Zinc seemed to have a neutral toxic effect on cadmium, while in combination with copper, a similar additive effect was seen as for the CuCd combination. Binary and tertiary metal mixtures caused a strong decrease in locomotion, except for the ZnCd combination, where zinc seems to have a neutral effect. After $LC20_{24\,h}$ exposure, a reduced crawling speed, except for zinc, and a reduced thrashing behaviour, except for zinc and ZnCd mixture were observed. Almost no significant effects were observed on chemosensation. Since the same trend of mixture effects was noted in locomotion and in lethality tests, locomotion could probably be considered as a sensitive endpoint for metal toxicities. This article is protected by copyright. All rights reserved

Keywords: Mixtures, *C. elegans*, Metals, Behavioural toxicology, Lethality

INTRODUCTION

Metal accumulation in soil is caused by both industrial and natural sources. Moreover, the number of anthropogenic sources of heavy metals in soil increased during several past decades, leading to higher metal concentrations in aquatic environments, especially in mining and industrial areas [1]. Exposure to these metals can lead to serious health hazards for diverse animals, including humans, and results in a persistent (eco) toxicological concern. It is well known that metals can cause neurotoxicity, alter neuronal excitability and impair chemosensation, and that they are involved in neurodegenerative diseases [2, 3]. Furthermore, free radicals and reactive oxygen species generated by metals can induce protein and DNA oxidation and lipid peroxidation [4]. They also disrupt cellular homeostasis by impairing DNA repair, inhibiting enzyme activity and affecting protein binding [5]. In general, cadmium replaces zinc in various proteins and causes e.g. cellular lesions and mitochondrial dysfunction, while an overload of the essential metal copper can lead to protein damage, cellular injury and structural impairment of essential metal binding sites [6-9]. An excess of zinc, another essential element, can cause multiple biological defects affecting life span, reproduction, development, etc. [10, 11].

Soil nematodes, living within the interstitial waters of soil particles, are in direct contact with dissolved contaminants and play a major role in nutrient cycling and dynamics by feeding on bacteria and fungi. In these soil nematodes, a reduction in movement [e.g. 14, 15], feeding [15, 16], reproduction [10, 17, 12], bioluminescence [18], growth and a delay in egg laying [7, 19] and an increased generation time [20] was noted after zinc, copper and/or cadmium exposure. Compared to the nematodes *Pristionchus pacificus* and *Panagrellus redivivus*, *C. elegans* shows an intermediate toxicity response to copper in aquatic and soil tests, making *C.*

elegans a representative of other rhabditid species for toxicity testing [16]. However, it is not yet clear if the main uptake route for metals is from dietborne exposure or from waterborne exposure. Höss and co-workers [12] suggested that the toxic effects are caused by "aqueous cadmium", taken up together with the bacteria, rather than by bacterial-bound or total cadmium concentrations. Bacteria may facilitate uptake of dissolved cadmium by stimulating pharyngeal pumping; however, they may also adsorb significant amounts of cadmium, thereby potentially lowering the cadmium toxicity. Höss et al. [12] could not exclude that food associated cadmium also had a partly toxic effect. Likewise, Offermann et al. [13] found no straightforward relationship between internal response and cadmium bioaccumulation. In contrast to Höss et al. [12], dietary exposure was found to play the major role in cadmium bioaccumulation and internal availability, whereas aqueous exposure was a less important contributor.

The bacterivorous nematode *C. elegans*, ubiquitous in soil, has a considerable ecological value and is of great importance in laboratory toxicity tests. Its rapid growth, small body size and fast reproduction enhance high throughput screening [21]. Since mixture toxicity tests generate a lot of samples in a semi factorial design, a rapid and simple manipulation of test organisms is needed. Furthermore, *C. elegans* can respond to aversive stimuli such as certain odorants (e.g. repellent 1-octanol), touch sensation, and a variety of toxic compounds, such as metals [22] via a number of sensory neurons in its head and tail and via mechanosensory neurons distributed along its body [23]. Due to its centralised nervous system consisting of only 302 neurons, *C. elegans* can be used for assessing behavioural changes. Although the *C. elegans* nervous system is simple, it shares all basic features with those of higher animals, including humans [24].

Metal toxicity research with *C. elegans* has been performed in soil, on agar plates, in sediment samples and in liquid medium, representing the interstitial pore water within the soil

[e.g. 7, 24, 25, 26]. However, in contrast to the increasing understanding of the toxic effects of single metals, much less is known about their effects upon interaction, which frequently occurs in the natural environment. Since each metal may affect a variety of metabolic pathways causing specific toxic effects [27], interactive effects of metals in mixtures can be additive, antagonistic or synergistic, all resulting in different toxicity responses. Unfortunately, due to their complex relationships in biological systems, no consistent explanation for the effect of metals in interactions exists. Metal mixture toxicity studies with soil invertebrates are still scarce, although a few studies have been performed with mixtures of copper, cadmium or zinc with earthworms [28], *Enchytraeus species* [e.g. 29], *Folsomia candida* [30] and *C. elegans* [e.g. 17, 31]. Furthermore, unlike lethality, reproduction, body length, egg production and stress protein production, locomotion has been less evaluated as a toxicological endpoint for both single metals and mixtures [e.g. 16, 18, 24, 32, 33].

The aim of this study is to gain insight into the sensitivity of *C. elegans* to the selected metals (copper, cadmium, zinc) and to investigate whether and how these sensitivities are affected by each other in mixture exposure scenarios. To assess different endpoints, we fully exploited the benefits of *Caenorhabditis elegans* as a unique model for both fundamental neuroscience and (eco) toxicology [34]. Young fed adult nematodes were exposed during 2 h, 8 h, 24 h and 48 h to different concentrations of copper, cadmium and zinc (as single metals and in combination) to analyse lethality effects over time, based on dose-response curves, and to gain insight into mixture toxicity. Locomotory behaviour was evaluated by measuring crawling speed on agar plates and thrashing behaviour in liquid medium. Furthermore, the degree of modulating or impairing (chemo) sensory capacities as a result of metal exposure was investigated. For the above locomotory and chemosensation tests, the effects of single metals and mixtures were

assessed using the LC20 of single metals. Behavioural responses were compared to lethality and finally these behavioural responses were determined as an endpoint for assessing toxicological effects on *C. elegans,* exposed to single metals and their mixtures.

MATERIALS AND METHODS

Caenorhabditis elegans culture

The wild type N2 strain of the nematode *Caenorhabditis elegans* was obtained from the Caenorhabditis Genetic Centre, Minneapolis, USA. The nematodes were maintained at 20°C on nematode growth medium (NGM) agar plates, seeded with *Escherichia coli* (OP50 strain) as food source. Synchronisation of the nematodes was achieved by bleaching: treatment of mixed-stage nematodes with a hypochlorite solution (5 N NaOH, 8% sodium hypochlorite) kills the nematodes that are not protected by an egg shell. Eggs were raised on OP50-seeded NGM plates. Young L4 nematodes were transferred to a 24-well plate filled with 900 µL K-medium (52 mM NaCl, 32 mM KCl, 5 µg/mL cholesterol, pH 5.1) [35], supplemented with *E. coli* OP50 (1.5 g/L), and under gentle shaking conditions (160 revolutions per minute (rpm)) incubated at 20°C for 24 h.

Test media and experimental set-up

Dilution series of cadmium (0, 2.5, 5, 10, 100, 400, 800 and 1200 mg/L), copper (0, 0.05, 0.5, 2.5, 25, 50, 100, 200 mg/L) and zinc (0, 5, 10, 25, 50, 100, 200, 400 mg/L) were made from $CdCl_2.2.5H_2O$ (Alfa Aesar, Karlsruhe, Germany), $CuCl_2.2H_2O$ (Merck A.G., Darmstadt, Germany) and $ZnCl_2$ (Alfa Aesar, Karlsruhe, Germany) in K-medium. Metal concentrations were verified (typically 93 - 108 % recovery) by High Resolution Inductively Coupled Plasma Mass Spectrometer ICP-MS (Element XR, Thermofisher Scientific, Bremen, Germany). The metal loading in our experiments, expressed in terms of mg per g of bacteria, is in line with

reported metal contents of polluted soils that have been used for toxicity studies with *C. elegans* [36]. Prior to toxicity testing, metal solutions were incubated with the bacterial suspensions for 12 h at 4°C to allow metal partitioning between the aqueous phase and the bacteria. After the incubation period, 100 µL of test medium (K-medium containing metal concentration) or control (K-medium) was added to the 24-well plate, bringing the total volume of each well up to 1 mL. Three replicates were made for each concentration. For each sample of each experiment, the determined pH before (5.1 ± 0.1) and after the experiment (5.2 ± 0.3), was within an acceptable pH range for *C. elegans*, excluding its potential effect on the measured parameters.

Lethality tests

Approximately 10 nematodes per replicate were used for each of the following lethality tests.

Single metal. After 0 h, 2 h, 8 h, 24 h and 48 h of exposure to either copper, cadmium or zinc, the number of living nematodes was counted in each well using a stereomicroscope (Nikon AZ100, Tokyo, Japan). As mentioned above, 8 different concentrations of each metal were used. Nematodes that were not moving or did not respond to gentle plate shaking, were considered to be dead. The critical metal concentration from which survival was influenced significantly, was determined as well as the strength of the decrease of survival. Furthermore, LC10, LC20 and LC50 were analysed for each metal.

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

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

To examine if metal mixtures lead to higher toxicities compared to individual compounds and which interactive effects of metals occur in mixtures, the LC20 of metal A was added to LC20 of metal B. This was determined for both LC20 of 24 h ($LC20_{24\ h}$) and for LC20 of 48 h ($LC20_{48\ h}$) of copper, cadmium and zinc (Table1). A mortality rate of around 40% for binary mixtures and around 60% for tertiary mixtures was expected if the metals would have a simple additive effect. Deviations of these values may be the result of interactions between the two metals due to common cellular targets or uptake mechanisms. We defined a synergistic interaction as a combination of two metals having a significantly 25% higher mortality rate than expected [27]. An antagonistic effect is suggested if the mortality rate was significantly 25% lower than predicted. Mortality rates of mixtures of $LC20_{24\ h}$ after 48 h of exposure were compared to the sum of the mortality rates of the corresponding single metals.

Locomotion

For the locomotory analyses, nematodes were exposed during 24 h to $LC20_{24\ h}$ and during 48 h to $LC20_{48\ h}$ of the three metals (Table1). Mixtures CuCd, ZnCu, ZnCd and ZnCuCd, made by combinations of the LC20 concentrations of the single metals, were tested. For each treatment three replicates were used.

Average crawling speed on solid medium. After 24 h and 48 h, approximately 20 nematodes were washed with K-medium and transferred to small NGM plates, seeded with *E. coli* (OP50 strain). After 30 min, nematode locomotion was recorded for 1 minute (15 frames per second) by video tracking with a camera (Nikon DS-Ri1, Tokyo, Japan), attached to a stereomicroscope (Nikon, AZ100, Tokyo, Japan). Using the plugin wrMTrck in the image

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

Thrashing behaviour in liquid medium. A thrash is defined as a change in the direction of midbody bending [11]. After 24 h and 48 h of metal exposure, the number of thrashes in 30 seconds was counted for each nematode in test medium. Therefore, their movement was recorded for 30 seconds (15 frames per second) by video tracking with a camera (Nikon, AZ100, Tokyo, Japan), attached to a stereomicroscope (Nikon, AZ100, Tokyo, Japan). The video was manually analysed. The number of full body bends was also quantified for each nematode exposed during 48 h to $LC20_{24\ h,}$

Chemosensation

For the chemosensory analyses, nematodes were exposed for 24 h to $LC20_{24\ h}$ and for 48 h to $LC20_{48\ h}$ of copper and cadmium (Table1). The CuCd mixture, made by the combination of the LC20 concentrations of the single metals, was also tested.

Chemotactic index. For this test, nematodes were exposed in falcon tubes instead of 24-well plates. After metal exposure, nematodes were washed with K-medium and approximately 30 nematodes were transferred to a 5 cm agar plate seeded with OP50 *E.coli*. The plate was divided in quadrants. Opposite quadrants contained the test solution (1-octanol) or the control solution (K-medium). Nematodes and solutions were added according to the protocol of Margie et al. [37]. After 60 minutes, the number of nematodes in each quadrant was counted under the microscope (Leica, S8APO, Heerbrugg, Switzerland) and the chemotactic index (CI) was calculated as

$$CI = (\sum(T1 + T2) - \sum(C1 + C2)) / \sum(T1 + T2 + C1 + C2)$$

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

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

Statistical analysis

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

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

Linear mixed models were fitted to test the possible effects of exposure time and metal concentrations and their interaction on the fraction of surviving animals. In all models, the survival fraction was entered as dependent variable. Exposure time and metal concentration, plus their interaction, were included as fixed effects. Observations with 100% survival (time=0h and control group) were omitted from the analysis. Since the survival fraction was repeatedly measured over time within the same wells, 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. Significance of the interaction term was calculated using an F-test with a Kenward-Roger correction for degrees of freedom. When the interaction between exposure time and metal concentration was significant, separate regression models were fitted for the different exposure times, and the slopes of the regression lines with 95% confidence intervals were calculated at different exposure times. Finally, LC10, LC20 and LC50 concentrations (24 h and 48 h) of the single metals were determined by fitting dose-response curves. The main effects of metal exposure, exposure time and their interaction on LC values were analysed by a two-way ANOVA. Subsequently, pairwise differences between the groups were calculated, with a Tukey HSD correction for multiple hypothesis testing. To test for the interaction between copper and cadmium, linear regression models were fitted. At each exposure time multiple linear regression models were fitted to investigate if the decrease in survival fraction with increasing $\log_{10}$ [copper concentration], was altered by the cadmium concentration and vice versa. First, linear regression models were fitted with the $\log_{10}$ [copper concentration] and the cadmium concentration as independent variables, plus their interaction. The interaction term in these models tests if the effect of $\log_{10}$[copper concentration] is the same across all cadmium concentrations. If this term was significant, separate models were fitted with $\log_{10}$[copper concentration] as independent

variable, at each separate concentration of Cd. Above calculations were repeated swapping Cd and Cu concentration, specifying Cd concentration as independent variable at a fixed value of the $\log_{10}$[Cu concentration].

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

Finally, dose-response curves were fitted using two-parameter log-logistic model to estimate LC10, LC20 and LC50 values. These curves were fitted using the drm() function [38] in the drc package [39] in R. A non-parametric ANOVA, Kruskal-Wallis test, followed by pairwise comparison testing with Bonferroni correction was performed to determine if the mixtures of LC20 concentrations led to significant higher mortalities than the individual metals. To test which interactive effects occur in the mixtures, the observed mortality rates were compared with the expected values using the Wilcoxon test.

Locomotion. The main effects of metal exposure, exposure time and their interaction on the average speed of the nematodes were analysed by a two-way ANOVA. Comparisons within treatment were carried out by a one-way ANOVA. Subsequently, a Tukey HSD test was used to determine the differences between groups.

For thrashing behaviour, the effect of metal exposure to $LC20_{24\ h}$ (measured at 24 h and 48 h) and $LC20_{48\ h}$ were analysed separately. For the $LC20_{24\ h}$ values, the main effects of metal exposure and exposure time were analysed by two-way generalised linear model procedures (quasi poisson distribution). One-way generalised linear model procedures were used for $LC20_{48\ h}$ concentrations (quasi poisson distribution). Subsequently, a Tukey HSD test was used to determine the differences between groups.

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

RESULTS

Lethality

Single metals. For all metal concentrations mortality rate increased along with the length of the exposure period (Fig. S1). Zinc exposed nematodes showed a stepwise decrease of surviving fraction, which was significant for concentrations starting from 200 mg/L at 2 h, 50 mg/L at 8 h, 10 mg/L at 24 h and 5 mg/L at 48 h (Fig. 1A). Nematodes exposed to copper for 2 h and 8 h had a significant lower survival from 25 mg/L onwards, except for one measurement (50 mg/L after 2 h). After 24 h, survival was already declining from 2.5 mg/L, while after 48 h of exposure all copper exposed nematodes showed a lower survival fraction (Fig. 1B). Cadmium did not alter survival after 2 h of exposure, except for the 10 mg/L concentration. For the other time points, survival was significantly lower than control starting from 2.5 mg/L (Fig. 1C).

For each metal, the interaction between exposure time and concentration was highly significant for the regression slopes ($P<0.001$). All regression coefficients were negative, meaning that the survival fraction declined with increasing concentration. Except for cadmium at exposure times 2 h and 8 h and for copper at 2 h, a highly significant effect of metal exposure on the survival fraction was noted ($P<0.001$). For zinc, a highly significant effect of metal exposure on the fraction of survival was observed for each exposure time ($P<0.001$). The regression slope

of zinc was always the steepest, although not significantly different from copper at 24 h and from cadmium from 24 h onwards. Cadmium had the least steep regression slope, but not significantly different from copper (and from zinc from 24 h onwards); at 24 h copper had a similar slope as cadmium (Fig. 2; Table S1).

Compared to cadmium, LC50 concentration of copper was 91.5% lower after 24 h of exposure and even 95.8% lower after 48 h of exposure ($P<0.001$) (Table 1). Compared to zinc, copper had a LC50 which was 88.1% lower after 24 h and 94.6% lower after 48 h ($P<0.001$). LC50 concentration of zinc was 28.7% (24 h) and 21.1% (48 h) lower than LC50 of cadmium, which was not significant. The lethal concentrations of the metals zinc, copper and cadmium significantly decreased over time by 68.3%, 85.7% and 71.3% respectively (Table 1).

Metal mixtures. For the CuCd mixture, all regression coefficients were negative, meaning that the fraction of survival declined with increasing metal concentrations. When cadmium was added to copper, a significant increase in toxicity was noticed 2 h after 10 mg/L cadmium was added ($P<0.05$) and 8 h after 0 mg/L ($P<0.05$), 2.5 mg/L ($P<0.01$), 10 mg/L ($P<0.05$) and 400mg/L ($P<0.001$) cadmium was added to copper. After 24 h and 48 h, every cadmium concentration that was combined with copper induced a highly significant decrease in survival rate ($P<0.001$; 400 mg/$L_{48\ h}$ $P<0.01$). The interaction between cadmium concentration and $\log_{10}$ [copper concentration] was significant after 8 h ($P<0.01$) and 48 h ($P<0.001$) (Fig. 3, Fig. S2, Table S2). Similarly, the decrease in survival fraction with increasing $\log_{10}$ [cadmium concentration], was altered by the copper concentration (Fig. 4, Fig. S3, Table S3). A significant effect on the regression slope was noticed 2 h after 0.5 mg/L copper ($P<0.01$) and 8 h after 25 mg/L copper ($P<0.001$) was added to cadmium. After 24 h of exposure, all combinations of cadmium with 0 ($P<0.001$), 0.05 ($P<0.01$), 0.5 ($P<0.001$), 2.5 ($P<0.001$), 25 ($P<0.01$), or 50

(P<0.05) mg/L copper caused a decrease in survival. When concentrations of 0 mg/L to 2.5 mg/L copper were combined with cadmium, an increase in mortality was observed after 48 h of exposure (P<0.001). A significant interaction between copper concentration and $\log_{10}$ [cadmium concentration] was seen after an exposure period of 8 h (P<0.05), 24 h (P<0.05) and 48 h (P<0.001). Curiously, the intercept on the Y-axis was very different for the various regression lines. This means that in the absence of one metal, the presence of the other metal already had a strong effect on survival. All the regression slopes were similar when copper or cadmium was added to the other metal. Except for the combinations of the two highest concentrations of one metal with the other, a trend was seen where the regression slopes became steeper over time (Table S2, S3).

When zinc was added to copper, mortality increased significantly (P<0.01) (Fig. 5). The ZnCu mixture had a higher mortality compared to copper ($LC20_{24\ h}$ 24 h) and to both individual metals ($LC20_{48\ h}$ and $LC20_{24\ h}$ 48 h) (P<0.05). However, zinc seemed to have a neutral effect on cadmium, since no obvious increase in mortality was seen (Fig. 5). For both $LC20_{24\ h}$ (24 h and 48 h) and $LC20_{48\ h}$, the observed mortality was respectively 58.7%, 45.3%, 56.3% lower than the expected mortality of 40% (P<0.05). In the CuCd mixture, a significant higher mortality compared to the single metals was observed (P<0.05) ($LC20_{24\ h}$ 24 h and 48h) (Fig. 5), although only copper had a significant lower mortality than the mixture of $LC20_{48\ h}$ of copper and cadmium (P<0.05). The lethality of the ZnCu and CuCd mixtures did not differ significantly from the expected value: mortality in these metal combinations suggests additive effects. The mortality rate of the tertiary ZnCuCd mixture was significantly higher compared to the 3 single metals (P<0.05) ($LC20_{24\ h}$ 24 h and 48 h) and to copper and zinc ($LC20_{48\ h}$) (P<0.05). When the

metals were combined in a tertiary mixture, an additive effect was seen after 24 h, but after 48 h (for both $LC20_{24\,h}$ and $LC20_{48\,h}$) this effect became antagonistic ($P<0.05$) (Fig. 5).

Locomotion

Metal exposure had a significant effect on the average speed and thrashing behaviour of *C. elegans* ($P<0.001$). A 24 h exposure to $LC20_{24\,h}$, induced a significant decrease in average crawling speed with 33.6%, 80.9%, 49.7%, 72.1%, 85.8% and 83.3% of nematodes exposed to copper ($P<0.05$), cadmium ($P<0.001$) and to the mixtures ZnCu ($P<0.001$), ZnCd ($P<0.001$), CuCd ($P<0.001$), and ZnCuCd ($P<0.001$) respectively (Fig. 6A). Exposure to zinc seemed to have no effect. Furthermore, the average crawling speed of nematodes exposed to the ZnCu and ZnCd mixture was lower than the ones treated with zinc ($P<0.001$), while exposure to the CuCd mixture caused a significantly lower speed than exposure to only copper ($P<0.001$). *C. elegans* exposed to the CuCd mixture made from $LC20_{48\,h}$ concentrations had a 84.3%, 84.6% and 83.3% lower average speed than respectively non-exposed ($P<0.01$), cadmium ($P<0.05$) and copper exposed nematodes ($P<0.01$) (Fig. 6B).

Also in liquid medium, exposure to $LC20_{24\,h}$ (at 24 h and 48 h) of copper ($P<0.001$), cadmium ($P<0.01$) and to the mixtures ZnCu ($P<0.01$), CuCd ($P<0.001$), and ZnCuCd ($P<0.05$) caused a decrease in locomotion of *C. elegans* (Fig. 7A). The nematodes showed 86.3%, 58.9%, 96.7%, 81.3% and 90.4% less thrashes, respectively, compared to the control. Exposure to ZnCu and ZnCuCd mixtures led to diminished thrashing behaviour compared to the zinc exposure ($P<0.05$). Thrashing behaviour of exposed nematodes to $LC20_{48\,h}$ concentrations of the mixtures ZnCu ($P<0.01$), CuCd ($P<0.05$) and ZnCuCd ($P<0.01$) decreased significantly with 84.1%, 97.7%, 86.9% respectively, compared to the control (Fig. 7B). Both CuCd ($P<0.05$) and ZnCuCd ($P<0.01$) exposure caused less thrashes than cadmium exposure alone.

Chemosensation

No significant effect of metal treatment on the chemotactic index was observed. However, a (non-significant) trend was seen, where copper exposure slightly impaired the avoidance reaction to the repellent (Fig. 8). The error bars suggest very heterogeneous reactions of the population of nematodes studied.

For the drop tests, metal exposure only had a significant effect ($P<0.01$) in the group exposed to $LC20_{48\ h}$, where copper exposed nematodes had a longer reaction time ($P<0.01$) compared to control (Fig. 9).

DISCUSSION

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

Based on toxicant mass units of mg/L, cadmium was the least toxic and copper the most toxic to *C. elegans*, which is consistent with prior studies [24, 27, 35, 40]. When the data are converted to micromolar, the order of lethality was copper > cadmium > zinc. However, LC values of cadmium and zinc were not significantly different. The LC50 values corresponded to earlier findings [27, 35]. Toxicological differences with previous studies may be due to differences in methodology, nature of chemicals, age of tested nematodes, *E.coli* density, number and density of the animals, exposure time, life stage, temperature, etc. Furthermore, for all

metals, the survival fraction decreased with increasing concentrations. At each time point, the regression slope of zinc was the most negative, although the 95 % confidence intervals slightly overlapped with copper after 24 h of exposure and with cadmium from 24 h onwards. 2 h and 8 h after zinc addition, mortality seemed to increase faster than when copper or cadmium was added. Cadmium had the least steep regression slope till 8 h of exposure, but at 24 h the slope was similar to that of copper and at 48 h the slope was intermediary between copper and zinc. However, the 95 % confidence intervals of cadmium overlapped with those of copper from 2 h onwards. This suggests that a longer exposure time is needed for Cd to become even or more toxic than the other metals, which is confirmed in the study of Williams and Dusenbery [35], where cadmium became more toxic than copper and zinc when the exposure period was extended to 96 h; LC50s were respectively 0.06, 0.26 and 1 mg/L. Moreover, in comparison with $LC50_{24\ h}$, $LC50_{48\ h}$ strongly decreased for all metals, representing the increased toxicity of metals for longer exposure periods. This decrease was also seen in earlier studies [35, 41, 42]. In our study, zinc exposed nematodes showed a stepwise decrease in survival over time. Copper exposure already caused an increasing lethality after 2 h, but after 24 h a huge drop in survival was noted. For cadmium exposed nematodes the decline in survival started after 8 h, but became more prominent after 24 h of exposure. The difference in declining rates suggests that uptake and elimination rates differ between metals [41]. Again, 48 h of cadmium exposure seemed less toxic for *C. elegans* than the other metals. However, for most aquatic organisms cadmium is the most toxic compound [24]. When $LC50_{96\ h}$ of *C. elegans* was compared with *Daphnia* species, the average of invertebrates and benthos (e.g. bristle worm, caddis fly), *C. elegans* was more sensitive than the average of invertebrates and more sensitive to zinc and cadmium than benthic organisms. For copper, this was the opposite, *C. elegans* was less sensitive than benthos. It

showed less sensitivity to both copper and zinc than *Daphnia sp.*, but sensitivity for cadmium was similar [35]. In another study [41], 12 nematode species were compared in terms of sensitivity to cadmium and pentachlorophenol. After an aquatic exposure of 72 h, *C. elegans* seemed to perceive only an intermediate effect of cadmium and was insensitive to pentachlorophenol. Fast colonizers (e.g. *Diplogasteritus sp.*) were relatively more sensitive to cadmium than slow colonizing species (e.g. *A. obtusicaudatus*). It was suggested that the variety in ectodermal tissue among nematodes plays an important role in explaining the observed differences in acute toxicity data. The high level of cadmium insensitivity of *C. elegans* suggests that these nematodes possess efficient defence mechanisms preventing cadmium-related damage. Cellular detoxification systems, including glutathione, methallothioneins, heat shock proteins, pumps and transporters, regulate intracellular metal levels by detoxifying and excretion of metals [5]. This implies that a sensitive molecular response may contribute to resistance at the organismal level, resulting in relatively high LC50 concentrations. It was also seen that *C. elegans* was less sensitive to cadmium in K-medium (3000 mg/L NaCl, 2360 mg/L KCl, pH 5.5-6.0, total alkalinity of 0.1 mg/L as $CaCO_3$), as compared to Recon (EPA moderately hard reconstituted reference water: 96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4.2H_2O$, 60 mg/L $MgSO_4$, 4 mg/L, pH 7.5-8.1, total alkalinity of 80 mg/L as $CaCO_3$) and moderately hard mineral water [42]. Furthermore, cadmium is reported to be predominantly present as chloro-complexes in K-medium (64%), whilst the concentration of the more biologically active free-form was much lower. In Recon media, chloro-complexes are reported to be a minor fraction of the total Cd concentration (< 1%) [42]. Copper on the other hand, is mostly present in the free-ion form (92%), which may explain the lower LC50 values [40]. The metal speciation and ensuing toxic effects are likely dependent on the composition and/or pH of soil or aquatic system. LC values

reported for Cu, Cd and Zn in soil systems were much lower than those in aquatic solutions, which was explained by the presence of organic complexants [25]. These organic complexants confound interpretation of data for soil systems: complexation of metal ions by organic matter lowers the free metal ion concentration in solution and can reduce metal mobility. It was suggested that *E. coli* functions both as a food organism and as a vector for contamination uptake [25]. This might also be the case in our study.

Since all observed regression coefficients of metal mixtures were negative, the fraction of survival declined with increasing metal concentrations. When cadmium was added to copper and vice versa, significant increases in toxicity were observed, indicating a significant interaction where one metal caused a mortality increase when it was added to the other. All the regression slopes were similar, meaning that the toxicity in terms of mortality changed at similar rates when copper or cadmium were added to each other. However, when considering reproduction as endpoint, more cadmium in the mixture caused a decrease in the toxicity, while more copper increased the toxicological effect on reproduction [17]. However, it is difficult to relate these observations to underlying physiological mechanisms. It is possible that defence mechanisms play a role. All the regression slopes became steeper over time, unless the 2 highest concentrations of one metal were combined with each other. For these concentrations, the intercept on the y-axis was already much lower compared to the other concentrations, where, nevertheless, a 48 h exposure (almost) caused 100% mortality.

The CuCd combination with $LC20_{24\,h}$ concentrations led to a significant higher mortality (after 24 h and 48 h) compared to the single metals, while copper had a significant lower mortality than the CuCd mixture of $LC20_{48\,h}$. Zinc seemed to have a neutral effect on cadmium, since no obvious increase in toxicity was noticed, which is supported by earlier studies [e.g. 27].

For the ZnCu combination, a higher mortality compared to copper ($LC20_{24}$ 24 h) and to both metals ($LC20_{48}$ and $LC20_{24}$ 48 h) was noticed. The CuCd and ZnCu mixtures did not differ significantly from the expected mortality, indicating that these metals appear to have an additive effect in combination. This corresponds to the findings of a 6-month soil test with different nematode communities [26], where an additive or less than additive effect of copper and zinc was observed. However, in another study [27] the interaction between copper and cadmium and between copper and zinc appeared synergistic. In that study the combination of LC20 of copper and cadmium even led to 100% mortality after 48 h. Furthermore, the mortality rate of the ZnCuCd mixture was significantly higher compared to the single compounds ($LC20_{24\ h}$ 24 h and 48 h) and to copper and zinc ($LC20_{48\ h}$). In this tertiary mixture, an additive effect was seen at 24 h, but after 48 h (for both $LC20_{24\ h}$ and $LC20_{48\ h}$) this effect became antagonistic. These interactive effects were also observed in a soil toxicity test, using a transgenic strain of *C. elegans*, carrying a stress-inducible β-galactosidase reporter, where the combination of copper and cadmium led to a larger response than cadmium alone, while the ZnCd mixture caused a lower β-galactosidase activity than cadmium alone [19]. They also tested for the internal metal content of *C. elegans* tissues, which was only fraction of the total metal content. Differences in uptake route, assimilation efficiency, bioavailability, etc. may result in various metal toxicities [12, 43]. Zinc, copper and cadmium may compete for the entry into nematodes, eliciting different responses than for the individual metals. Another hypothetical explanation for the interactions is based on the stability indexes of metal ions and their binding sites [44], the covalent index and ionic index. Metals with a high ionic index are being displaced by metals with high covalent indexes for the binding sites. Copper and cadmium have a similar ionic index, while copper has the highest covalent index followed by cadmium and zinc. This sequence corresponded to the

observed toxicity in that study, when metal concentrations were expressed in mmol/L. Metals with higher covalent index tend to have a synergistic effect, while metals with a low covalent index seem to have a variable impact [27]. Furthermore, it was seen that zinc tends to neutralize the toxic effect of other metals with a low covalent index, e.g. cadmium. Finally, it is possible that deviations from addivity are only seen under certain conditions: in the study of Jonker et al. [31] synergism was only observed at high dose levels, i.e. higher than LC50, while we only considered the combinations of LC20 concentrations in this study. Furthermore, for the ZnCu and CuCd mixtures additive effects were observed after 24 h and 48 h of exposure, while the effects of the tertiary mixture changed from additive to antagonistic after 48 h of exposure, suggesting that a longer exposure period can induce other interactive effects. The differences in toxicity indicate the importance of testing mixtures and their individual metals simultaneously to obtain a reliable insight in the combined actions.

Many *C. elegans* behaviours, such as locomotion and chemosensation are modulated by the presence of food [45]. When nematodes are fed on a bacterial lawn, they switch between two different behaviour patterns. Around 80% of the time, the nematodes are moving slowly and staying in a restricted area ("dwelling"), but they can suddenly switch into rapid locomotion across the lawn ("roaming"). Besides speed lowering, the presence of a food source enhances avoidance responses to soluble repellents.

Like in many animals, copper and cadmium have been found to affect movement in *C. elegans* [e.g. 14, 24, 46]. In the study of Dhawan and co-workers [24], fed nematodes exposed to copper and cadmium for 24 h showed a change in movement at much lower concentrations than the concentration at which they showed lethality. The behavioural EC50 values for nematodes were between 20 and 50 times lower than LC50 values. Behaviour was much more sensitive to

cadmium and copper than lethality, while for nematodes exposed to zinc, the opposite was true. This was also observed in our study: nematodes exposed to $LC20_{24\,h}$ of copper and cadmium had a significant lower crawling speed and showed a diminished thrashing behaviour compared to control, in contrast to nematodes exposed to zinc. The number of thrashes corresponds to the control finding of Li et al. [47] and a similar effect for copper (150µM, 12 h) was observed in the study of Xing et al. [48]. However, in an earlier study using 2.5, 75 and 200 µM zinc (without food), *C. elegans* showed less body bends after 72 h compared to control [10], suggesting that a starvation period can severely increase the toxic effect of zinc on thrashing behaviour. The shorter exposure period and/or presence of food can be an explanation why we did not see an effect of zinc on locomotion (yet).

In our study both locomotory responses of nematodes decreased more in binary and tertiary mixtures, which was in line with the findings of the lethality tests. After 24 h of exposure the average speed in the ZnCu and ZnCd mixture, was lower compared to zinc alone, while the average speed of the nematodes exposed to the CuCd mixture was significantly different from nematodes exposed to only copper. *C. elegans* exposed to copper had a slightly higher speed than in the ZnCu mixture, while cadmium induced a similar speed as the CuCd mixture, but a slightly lower speed than in the ZnCd mixture. For thrashing behaviour, a non-significant trend was observed, when nematode exposed to ZnCu and CuCd mixture showed less thrashes compared to the single metals, while for nematodes exposed to the ZnCd mixture thrashing behaviour was similar to that of nematodes exposed to the single metals. This again suggests that zinc has a neutral effect on cadmium and that copper and zinc or cadmium have additive effects on locomotion.

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

C. elegans uses a sinusoidal-like locomotion to crawl forward in an unobstructed environment, which is modulated by a number of processes including omega-turns, pirouettes and gentle turns. This locomotion pattern is controlled by interneurons, which receive and integrate input signals from various chemo- and mechanosensory neurons [49]. When *C. elegans* encounters a potentially harmful chemical, it avoids this compound by reversing its movement, which is mediated by sensory amphid neurons. These reversals are suppressed by antagonistic inputs from phasmid neurons [50]. Hilliard and co-workers [50] suggested that *C. elegans* utilizes a simple head-tail chemical sensory map for avoiding toxic compounds, whereas a more flexible temporal strategy that can locate signals in any direction is used for chemotaxis to food sources and mates. Chemosensation represents thus a complex and important response of the animal to its environment. In the present study, copper exposed nematodes showed a slightly altered chemotactic index, although this was not significant. In the study of Moore et al. [51], the chemotactic index showed a huge variation. Furthermore, control nematodes started a backward motion within 1 s of the delivery of the drop, which corresponds to the findings of Hilliard et al. [50]. All animals reacted within 4s, causing positive responses to each drop. The non-significant trend, observed for the chemotactic index, was significant in the drop test. Nematodes exposed to $LC20_{48\ h}$ of copper needed significantly more time to start a backward movement, compared to control, although the reaction was still very fast and positive. This result suggests that an exposure to LC20 of copper slightly affects the neurons. A longer exposure period, absence of

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

CONCLUSION

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

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

Acknowledgment—We would like to thank V. Kayawe Mubiana and S. Joosen for analysing metal concentrations. This study was funded by the University Research Fund (BOF) and the Interuniversity Attraction Poles Programme – Belgian State – Belgian Science Policy [IUAP VI/33].

Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (Sofie.Moyson@uantwerpen.be).

REFERENCES

1. Peredney CL, Williams PL. 2000. Utility of *Caenorhabditis elegans* for assessing heavy metal contamination in artificial soil. *Arch Environ Contam Toxicol* 39: 113-118.

2. Wright RO, Baccarelli A. 2007. Metals and neurotoxicology. *J Nutr* 137: 2809-2813.

3. Mathie A, Sutton GL, Clarke CE, Veale EL. 2006. Zinc and copper: pharmacological probes and endogenous modulators of neuronal excitability. *Pharmacol. Ther.* 111: 567-583.

4. Caito S, Fretham S, Martinez-Finley E, Chakraborty S, Avila D, Chen P, Aschner M. 2012. Genome-wide analyses of metal responsive genes in *Caenorhabditis elegans*. *Front Genet* 3(52): 1-9.

5. Martinez-Finley EJ, Aschner M. 2011. Revelations from the nematode *Caenorhabditis elegans* on the complex interplay of metal toxicological mechanisms. *J Toxicol* Article ID 895236, 10 pages, 2011. doi:10.1155/2011/895236.

6. Vallee BL, Ulmer DD. 1972. Biochemical effects of mercury, cadmium, and lead. *Ann. Rev. Biochem.* 41: 91-128.

7. Popham JD, Webster JM. 1979. Cadmium toxicity in the free living nematode, *Caenorhabditis elegans*. *Environ Res* 20: 183-191.

8. Goldstein S, Czapski G. 1986. The role and mechanism of metal ions and their complexes in enhancing damage in biological systems or in protecting these systems from the toxicity of O_2^-. *J Free Radic Biol Med* 2: 3-11.

9. Alt ER, Sternlieb I, Goldfisher S. 1990. The cytopathology of metal overload. *Int Rev Exp Pathol* 31: 165-188.

10. Wang D, Shen L, Wang Y. 2007. The phenotypic and behavioural defects can be transferred from zinc-exposed nematodes to their progeny. *Environ Toxicol Pharmacol* 24: 223-230.

11. Chen P, Martinez-Finley EJ, Bornhorst J, Chakraborty S, Aschner M. 2013. Metal induces neurodegeneration in *C. elegans*. *Front Aging Neurosci* 5: 1-11.

12. Höss S, Schlottmann K, Traunspruger W. 2011. Toxicity of ingested cadmium to the nematode *Caenorhabditis elegans*. *Environ Sci Technol* 45: 10219-10225.

13. Offermann, K., Matthäi, A., Ahlf, W. 2009. Assessing the importance of dietborne cadmium and particle characteristics on bioavailability and bioaccumulation in the nematode *Caenorhabditis elegans*. *Environ Sci Technol* 28 (6): 1149-1158.

14. Anderson GL, Cole RD, Williams PL. 2003. Assessing behavioural toxicity with *Caenorhabditis elegans*. *Environ Toxicol Chem* 23(5): 1235-1240.

15. Boyd WA, Cole RD, Anderson GI, Williams PI. 2003. The effects of metals and food availability on behaviour of *Caenorhabditis elegans*. *Environ Toxicol Chem* 22: 3049-3055.

16. Boyd WA, Williams PL. 2003. Comparison of the sensitivity of three nematode species to copper and their utility in aquatic and soil toxicity tests. *Environ Toxicol Chem* 22: 2768-2774.

17. Jonker MJ, Piskiewicz AM, Catsella NI, Kammenga JE. 2004. Toxicity of binary mixtures of cadmium-copper and carbendazim-copper to the nematode *Caenorhabditis elegans*. *Environ Toxicol Chem* 23 (6): 1529-1537.

18. Lagido C, McLaggan D, Flett A, Pettitt J, Glover LA. 2009. Rapid sublethal toxicity assessment using bioluminescent *Caenorhabditis elegans*, a novel whole-animal metabolic biosensor. Toxicol Sci 109(1): 88-95.

19. Power RS, de Pomerai DI. 1999. Effects of single and paired metal inputs on a soil in stress-inducible transgenic nematode. *Arch. Environ. Contam. Toxicol.* 37: 503-511.

20. Calafato S, Swain S, Hughes S, Kille P, Stürzenbaum SR. 2008. Knock down of *Caenorhabditis elegans* cutc-1 exacerbates the sensitivity toward high levels of copper. *Toxicol Sci* 106 (2): 384-391.

21. Leung MCK, Williams PL, Benedetto A, Au C, Helmcke KJ, Aschner M, Meyer JN. 2008. REVIEW: *Caenorhabditis elegans*: An Emerging Model in Biomedical and Environmental Toxicology. *Toxicol Sci* 106(1): 5–28.

22. Tobin DM, Bargmann CI. 2004. Invertebrate nociception: behaviors, neurons and molecules. *J Neurobiol* 61: 161-174.

23. Ward S, Thomson N, White JG, Brenner S. 1975. Electron microscopical reconstruction of the anterior sensory anatomy of the nematode *Caenorhabditis elegans*. *J Comp Neuron* 160: 313-338.

24. Dhawan R, Dusenbery DB, Williams PL. 2000. A comparison of metal-induced lethality and behavioural responses in the nematode *Caenorhabditis elegans*. *Environ Toxicol Chem* 19: 3061-3067.

25. Höss S, Henschel T, Haitzer M., Traunspruger W. 2001. Toxicity of cadmium to *Caenorhabditis elegans* (nematode) in whole sediment and pore water-the ambiguous role of organic matter. *Environ Toxicol Chem* 20 (12): 2794-2801.

26. Korthals GW, Bongers M, Fokkema A, Dueck TA, Lexmond TM. 2000. Joint toxicity of copper and zinc to a terrestrial nematode community in an acid sandy soil. *Ecotoxicology* 9: 219-228.

27. Chu KW, Chow KL. 2002. Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. *Aquat Toxicol* 61: 53-64.

28. Weltje L. 1998. Mixture toxicity and tissue interactions of Cd, Cu, Pb, and Zn in earthworms (Oligochaeta) in laboratory and field sources: A critical evaluation of data. *Chemosphere* 36: 2643-2660.

29. Lock K, Janssen CR. 2002. Mixture toxicity of zinc, cadmium, copper, and lead to the potworm *Enchytraeus albidus*. *Ecotoxicol Environ Saf* 52: 1-7.

30. Van Gestel CAM, Hensbergen PJ. 1997. Interactions of Cd and Zn toxicity for *Folsomia candida* Willem (Collembola: Isotomidae) in relation to bioavailability in soil. *Environ Toxicol Chem* 16: 1177-1186.

31. Jonker MJ, Sweijen RAJC, Kammenga JE. 2004. Toxicity of simple mixtures to the nematode *Caenorhabditis elegans* in relation to soil sorption. *Environ Toxicol Chem* 23 (2): 480-488.

32. Dhawan R, Dusenbery DB, Williams PL. 1999. Comparison of lethality, reproduction, and behaviour as toxicological endpoints in the nematode *Caenorhabditis elegans*. *Toxicol Environ Health* 58: 451-462.

33. Roh JY, Lee J, Choi J. 2006. Assessment of stress-related gene-expression in the heavy metal-exposed nematode *Caenorhabditis elegans*: a potential biomarker for metal-induced toxicity monitoring and environmental risk assessment. *Environ Toxicol Chem*, 25(11): 2964-2956.

34. Brenner S. 1973. The genetics of *Caenorhabditis elegans*. *Genetics* 77: 71-94.

35. Williams PL, Dusenbery DB. 1990. Aquatic toxicity testing using the nematode, *Caenorhabditis elegans*. *Environ Toxicol Chem* 9: 1285-1290.

36. Höss S, Jänsch S, Moser, T, Junker, T, Römbke, J. 2009. Assessing the toxicity of contaminated soils using the nematode *Caenorhabditis elegans* as test organism. *Ecotoxicol Environ Saf* 72: 1811-1818.

37. Margie O, Palmer C, Chin-Sang I. 2013. *C. elegans* Chemotaxis Assay. *J Vis Exp* 74: e50069.

38. R Core Team. 2014. R: A Language and Environment for Statistical Computing. Vienna, Austria: the R Foundation for Statistical Computing. ISBN: 3-900051-07-0. [cited 2017 June 13]. Available online at http://www.R-project.org/.

39. Ritz, C, Streibig, JC. 2005. Bioassay Analysis using R. *J. Statist. Software* 12 (5): 1-22.

40. Freeman, MN, Marse, TJ, Williams, PL. 1998. Establishment of quality assurance procedures for aquatic toxicity testing with the nematode *Caenorhabditis elegans*. In Little EE, DeLonay AJ, and Greenberg BM, eds., Environmental Toxicology and Risk Assessment, Seventh Volume, ASTM STP 1333. American Society for Testing and Materials, West Conshohocken, Pennsylvania, USA, pp 45-59.

41. Kammenga JE, Van Gestel CAM, Bakker J. 1994. Patterns of sensitivity to cadmium and pentachlorophenol among nematode species from different taxonomic and ecological groups. *Arch Environ Contam Toxicol* 27: 88-94.

42. Cressman III CP, Williams PL. 1997. Reference toxicants for toxicity testing using *Caenorhabditis elegans* in aquatic media. In James Dwyer F., Doane TR, and Hinman ML, eds., Environmental Toxicology and Risk Assessment: Modeling and Risk Assessment (Vol 6), ASTM STP 1317. American Society for Testing and Materials, Philadelphia, USA, pp 518-532.

43. Rainbow PS. 2007. Trace metal bioaccumulation: Models, metabolic availability and toxicity. *Environ Int* 33: 576-582.

44. Nieboer E, Fletcher GG. 1996. Effect of single and paired metal inputs in soil on a stress-inducible transgenic nematode. *Arch Environ Contam Toxicol* 37: 503-511.

45. Hart AC, Chao MY. 2010. Chapter 1: From odors to behaviors in *Caenorhabditis elegans*. Boca Raton Florida, USA: In Menini A, eds, The neurobiology of olfactation. CRC Press/Taylor & Francis. [cited 2016 December 10]. Available form: https://www.ncbi.nlm.nih.gov/books/NBK55983/

46. De Boeck G, Van der Ven K., Hattink J, Blust R. 2006. Swimming performance and energy metabolism of rainbow trout, common carp and gibel carp respond differently to sublethal copper exposure. *Aquat Toxicol* 80: 92–100.

47. Li W-H, Shi Y-C, Tseng I-L, Liao VH-C. 2013. Protective efficacy of selenite against lead-induced neurotoxicity in *Caenorhabditis elegans*. PLoS ONE 8(4): e62387.

48. Xing X, Du M, Zhang Y, Wang D. 2009. Adverse effects of metal exposure on chemotaxis towards water-soluble attractants regulated mainly by ASE sensory neuron in nematode *Caenorhabditis elegans*. *J Environ Sci* 21: 1684-1694.

49. Bargmann C.I. 2006. Chemosensation in *C. elegans*. USA: WormBook ed. *The C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.123.1, [cited 2017 January 06]. Available from: http://www.wormbook.org/chapters/www_chemosensation/chemosensation.pdf

50. Hilliard MA, Bargmann CI, Bazzicalupo P. 2002. *C. elegans* responds to chemical repellents by integrating sensory inputs from the head and the tail. *Curr Biol* 12: 730-734.

51. Moore CE, Lein PJ, Pushner B. 2014. Microcystins Alter chemotactic behavior in *Caenorhabditis elegans* by selectively targeting the AWA sensory neuron. *Toxins* 6: 1813-1836.

Figure 1. Concentration-response relationship for exposure to zinc (A), copper (B) and cadmium (C) at different exposure times. Dotted line denotes 95% survival. Survival proportions are shown with their 95% confidence intervals represented as error bars.

Figure 2. Scatterplots of the fraction of survival plotted against the log10 [metal concentration]. Solid lines represent the linear regression slopes of zinc (A), copper (B) and cadmium (C) at different exposure times.

Figure 3. Regression slopes of the CuCd mixture at different exposure times. Graphs represent combinations when cadmium was added to log10 [Cu concentrations].

Figure 4. Regression slopes of the CuCd mixture at different exposure times. Graphs show the mixtures when copper was added to log10 [Cd concentrations].

Figure 5. Mortality (%) of nematodes exposed to LC20 concentrations of metals and their mixtures. Nematodes were exposed to the $LC20_{24\ h}$ concentrations for 24 h (left), 48 h (middle) or to $LC20_{48\ h}$ for 48 h (right). Data are shown as mean ± SD. Asterisks (*) denote significant differences (P<0.05) compared to the mixture.

Figure 6. Average speed (mm/s) of nematodes exposed to LC20 concentrations of metals and their mixtures. Nematodes were exposed to LC2024 h (A) or to LC2048 h (B) concentrations. Individual data are shown as well as the median and interquartile range. Asterisks denote significant differences (*P<0.05; **P<0.01; ***P<0.001) compared to the corresponding control (a) and to the corresponding single metals Zn (b), Cu (c), Cd (d).

Figure 7. Thrashing behaviour of nematodes exposed to LC20 concentrations of metals and their mixtures. Nematodes were exposed to LC2024 h (A) or to LC2048 h (B) concentrations. Individual data are shown as well as the median and interquartile range. Asterisks denote

significant differences (*P<0.05; **P<0.01; ***P<0.001) compared to the corresponding control (a) and to the single metals Zn (b) and Cu (c).

Figure 8. Chemotactic index of nematodes exposed to LC20 concentrations of Cu and Cd and their mixture. Left: exposure to the LC2024 h concentrations at 24 h. Right: Exposure to the LC2048 h concentration at 48 h. Values are mean ± SD. No significant differences were observed.

Figure 9. Reaction time (s) of nematodes exposed to LC20 concentrations of metals and their mixtures. Exposure to the LC2024 h concentrations (left) and to the LC2048 h concentration (right). Individual data are shown as well as the median and 95% confidence intervals. Asterisks denote significant differences (*P<0.05; **P<0.01; ***P<0.001) compared to the corresponding control.

Table 1: LC10, LC20 and LC50 of zinc, copper and cadmium after 24 h and 48 h of exposure.**Error! Not a valid link.**

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

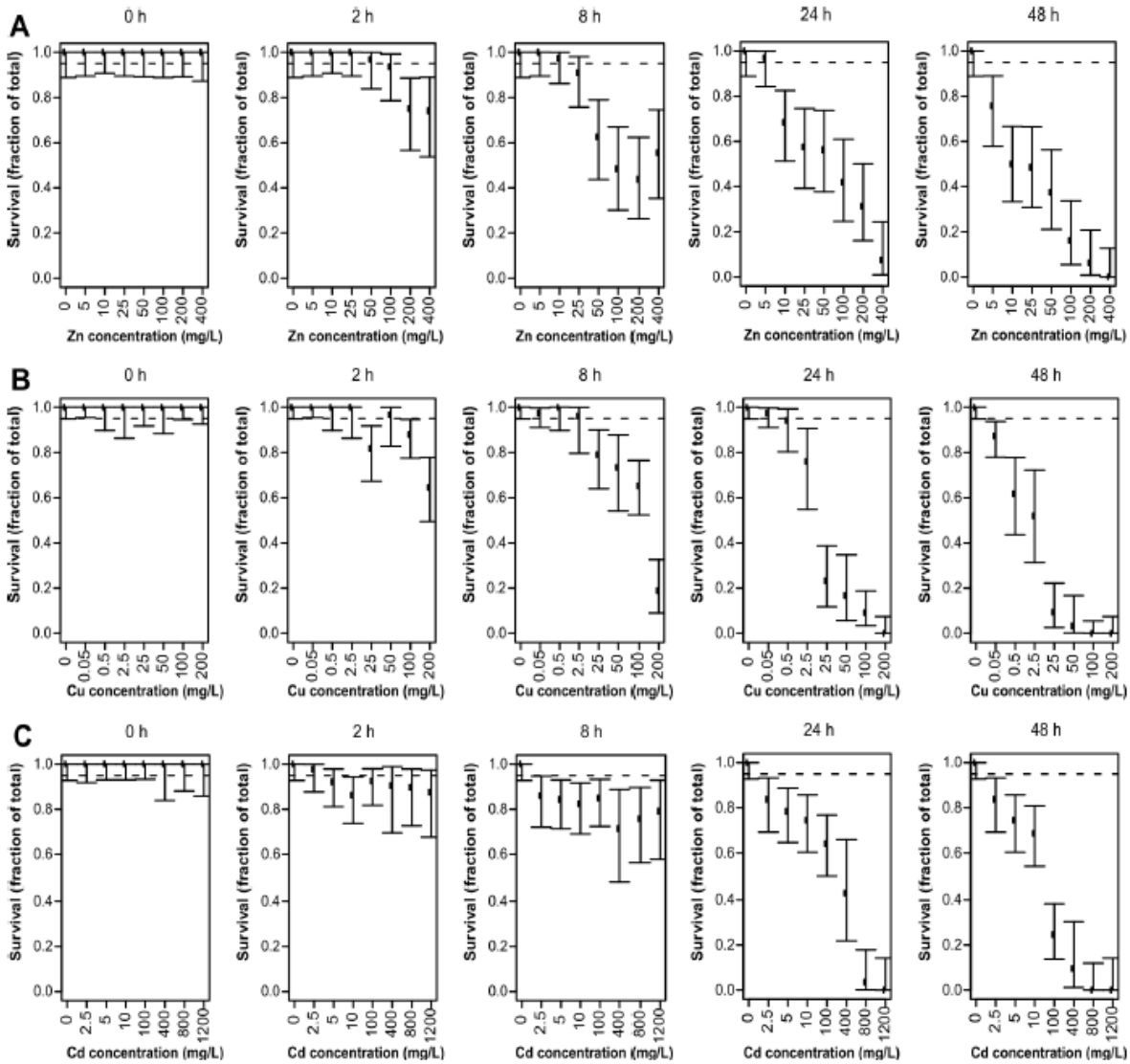

Fig. 1. Concentration-response relationship for exposure to zinc (A), copper (B) and cadmium (C) at different exposure times. Dotted line denotes 95% survival. Survival proportions are shown with their 95% confidence intervals represented as error bars.

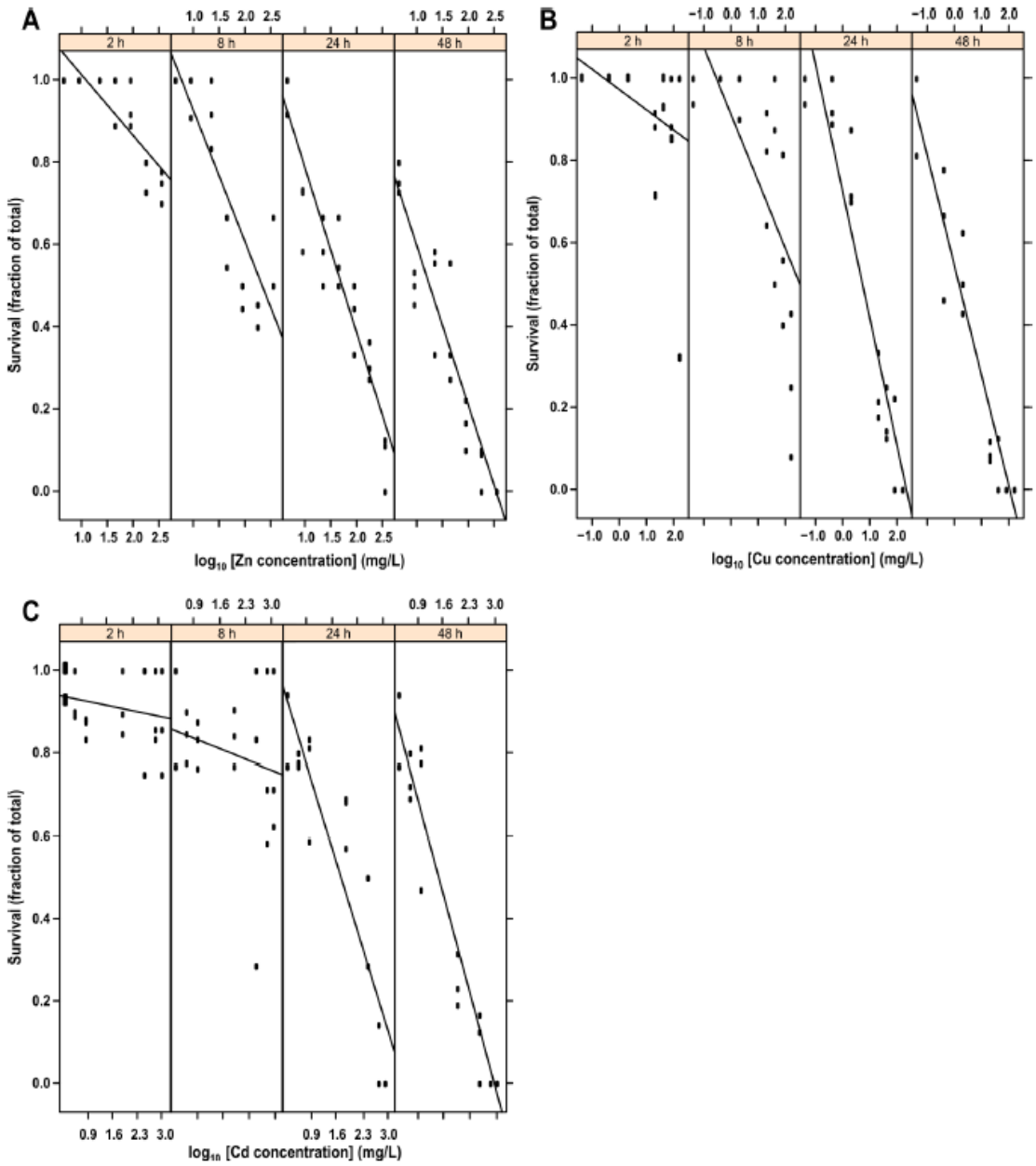

Fig. 2. Scatterplots of the fraction of survival plotted against the $\log_{10}$ [metal concentration]. Solid lines represent the linear regression slopes of zinc (A), copper (B) and cadmium (C) at different exposure times.

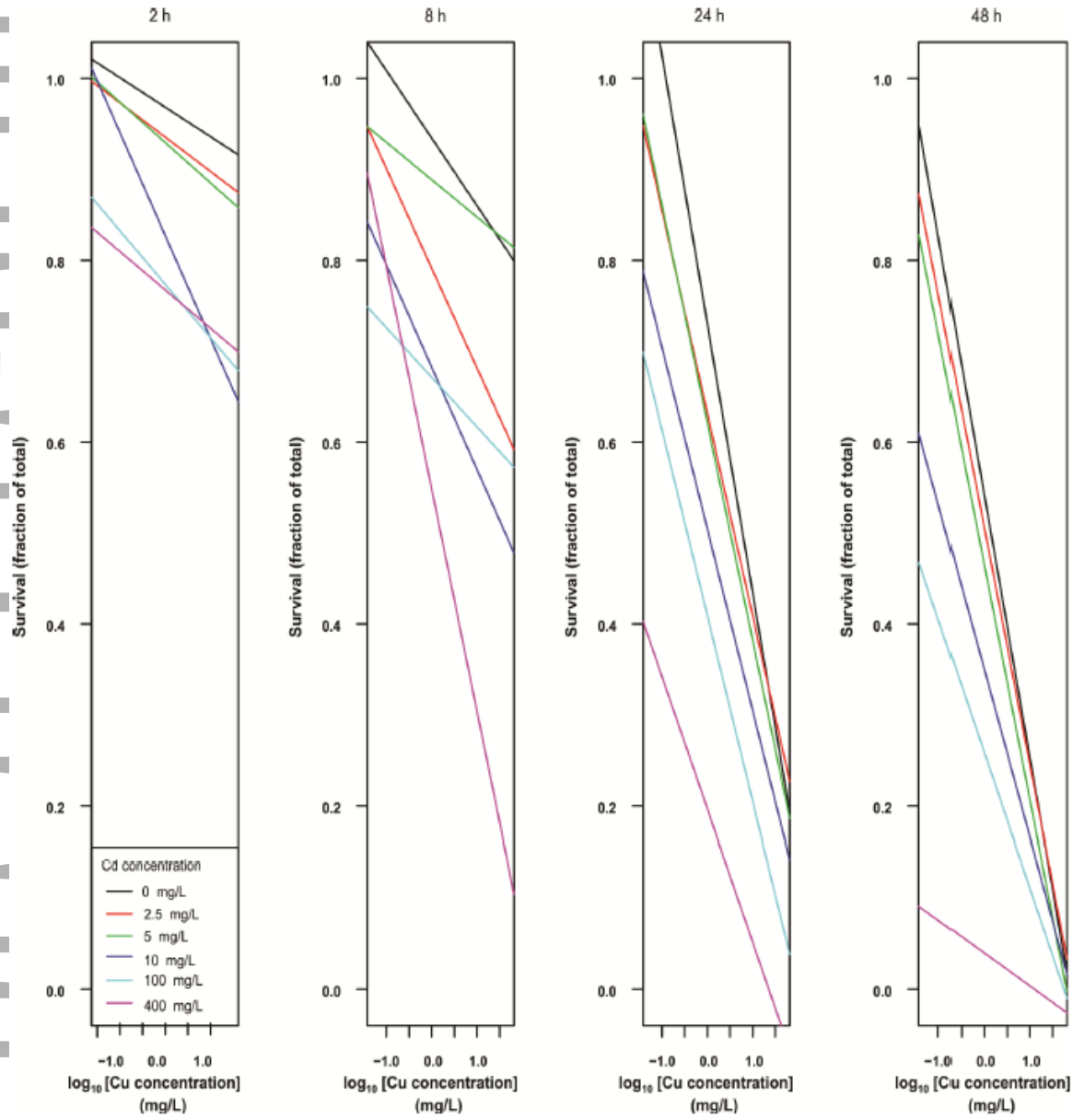

Fig. 3. Regression slopes of the CuCd mixture at different exposure times. Graphs represent combinations when cadmium was added to $\log_{10}$ [Cu concentrations].

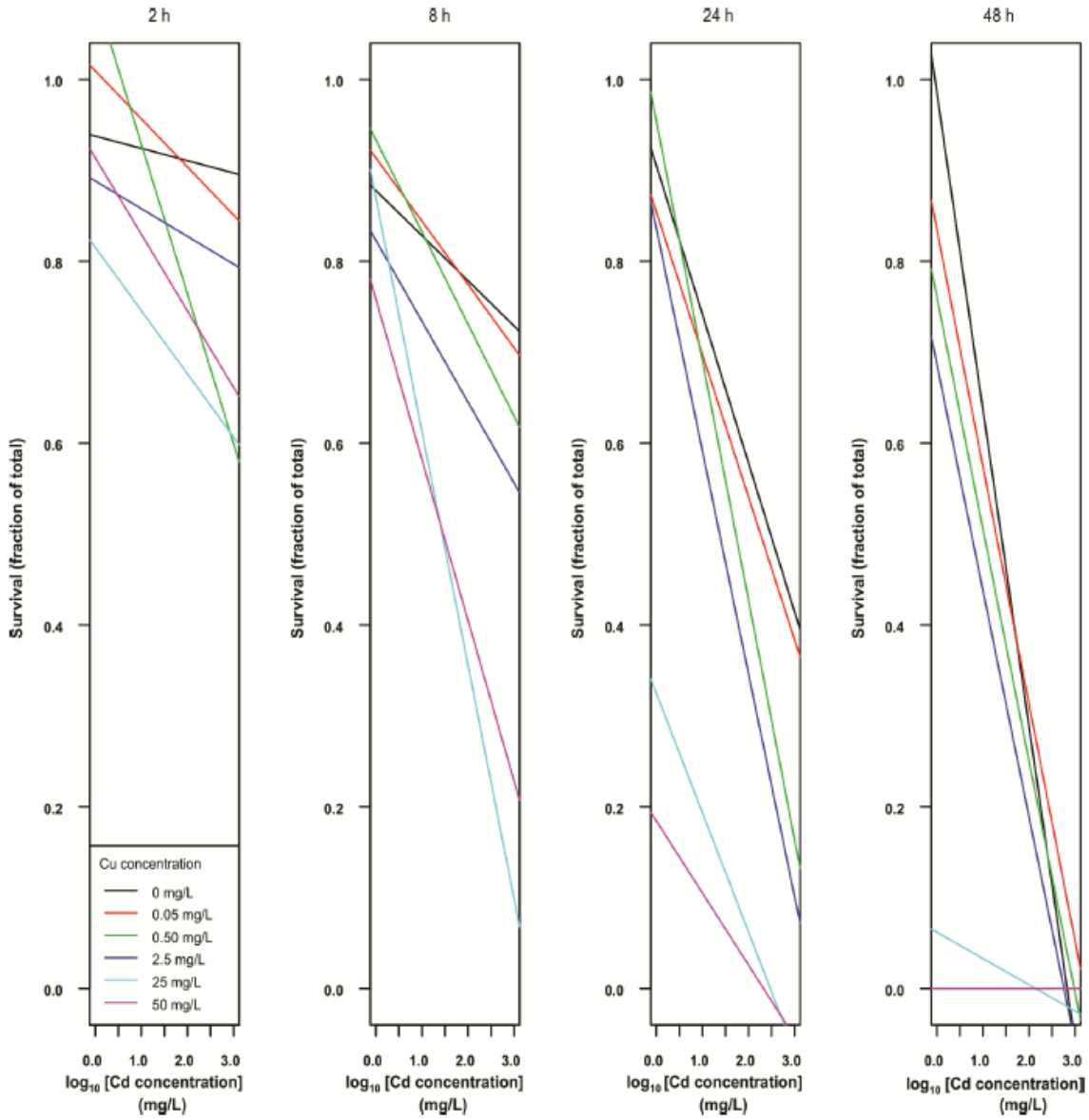

Fig. 4. Regression slopes of the CuCd mixture at different exposure times. Graphs show the mixtures when copper was added to $\log_{10}$ [Cd concentrations].

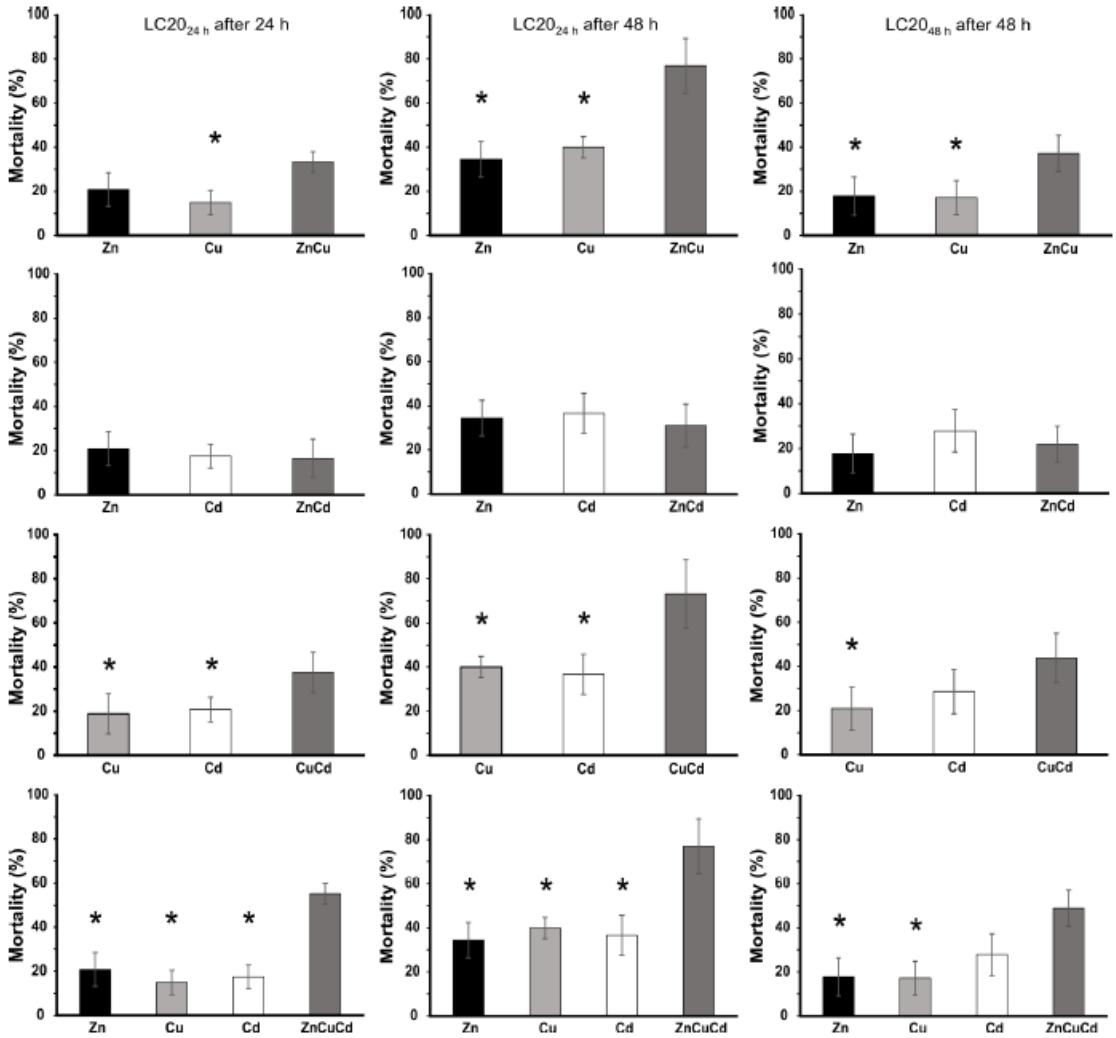

Fig. 5. Mortality (%) of nematodes exposed to LC20 concentrations of metals and their mixtures. Nematodes were exposed to the $LC20_{24\,h}$ concentrations for 24 h (left), 48 h (middle) or to $LC20_{48\,h}$ for 48 h (right). Data are shown as mean ± SD. Asterisks (*) denote significant differences ($P<0.05$) compared to the mixture.

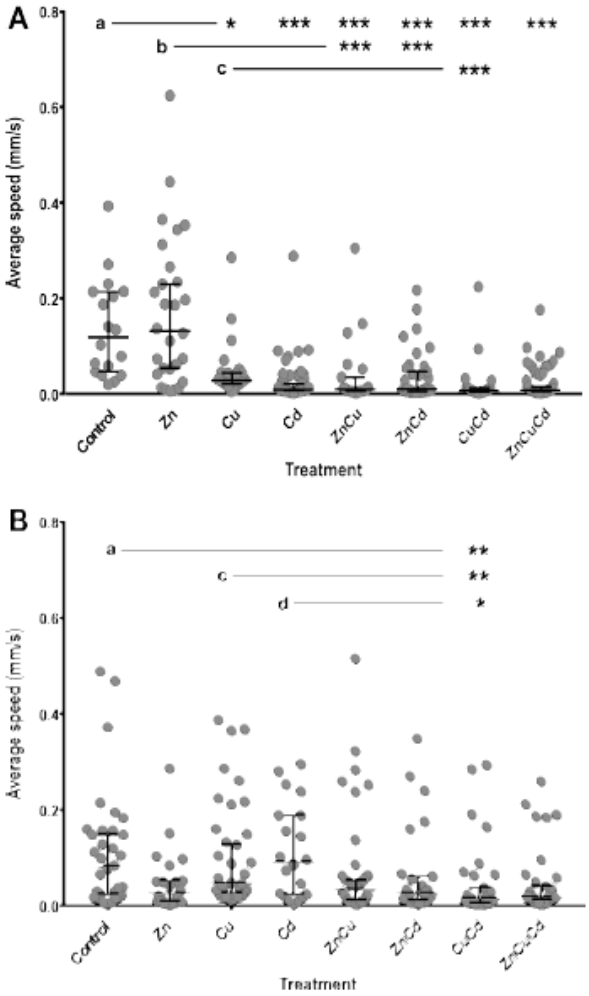

Fig. 6. Average speed (mm/s) of nematodes exposed to LC20 concentrations of metals and their mixtures. Nematodes were exposed to $LC20_{24\,h}$ (A) or to $LC20_{48\,h}$ (B) concentrations. Individual data are shown as well as the median and interquartile range. Asterisks denote significant differences (*P<0.05; **P<0.01; ***P<0.001) compared to the corresponding control (a) and to the corresponding single metals Zn (b), Cu (c), Cd (d).

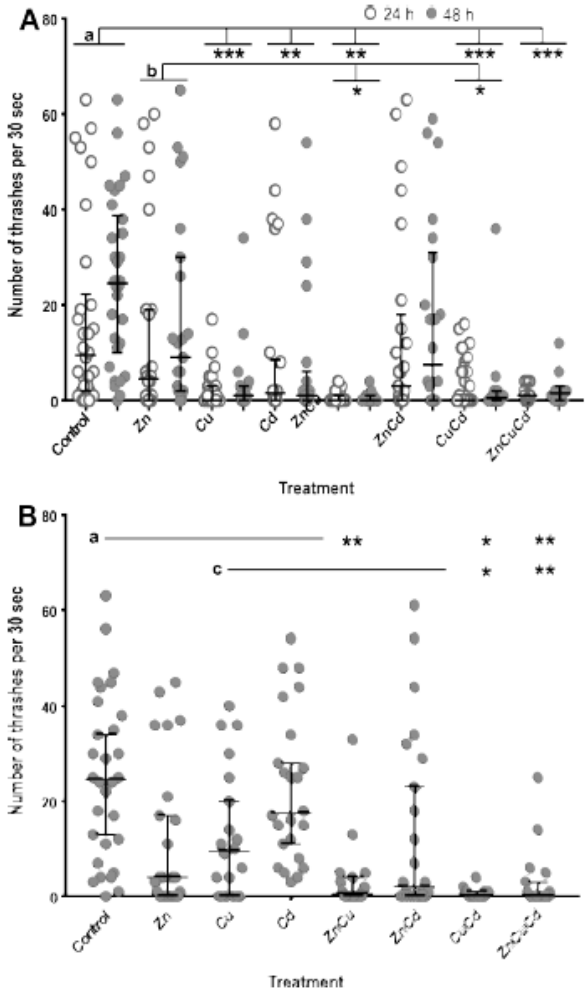

Fig. 7. Thrashing behaviour of nematodes exposed to LC20 concentrations of metals and their mixtures. Nematodes were exposed to $LC20_{24\,h}$ (A) or to $LC20_{48\,h}$ (B) concentrations. Individual data are shown as well as the median and interquartile range. Asterisks denote significant differences (*P<0.05; **P<0.01; ***P<0.001) compared to the corresponding control (a) and to the single metals Zn (b) and Cu (c).

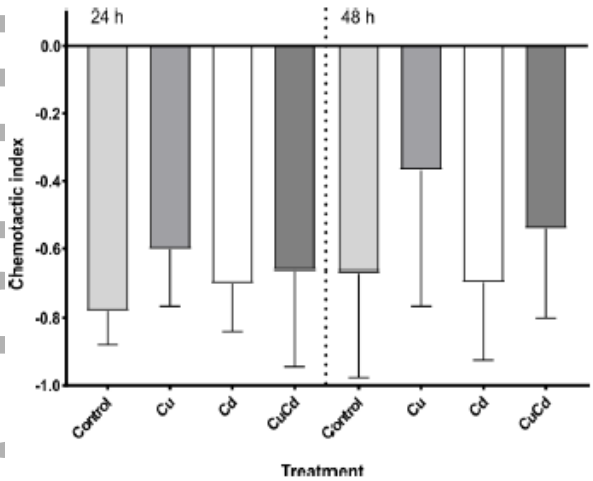

Fig. 8. Chemotactic index of nematodes exposed to LC20 concentrations of Cu and Cd and their mixture. Left: exposure to the $LC20_{24\,h}$ concentrations at 24 h. Right: Exposure to the $LC20_{48\,h}$ concentration at 48 h. Values are mean ± SD. No significant differences were observed.

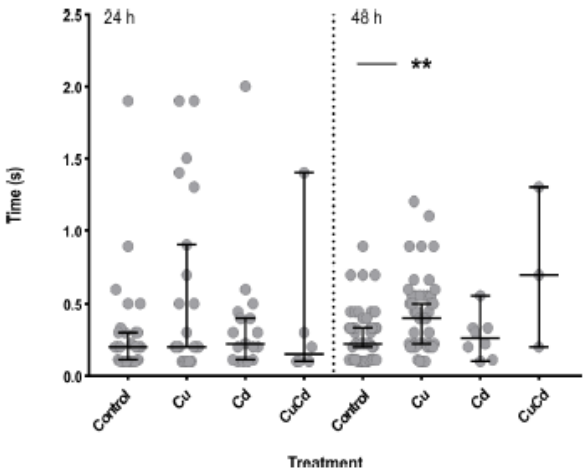

Fig. 9. Reaction time (s) of nematodes exposed to LC20 concentrations of metals and their mixtures. Exposure to the $LC20_{24\,h}$ concentrations (left) and to the $LC20_{48\,h}$ concentration (right). Individual data are shown as well as the median and 95% confidence intervals. Asterisks denote significant differences (*P<0.05; **P<0.01; ***P<0.001) compared to the corresponding control.