### Title

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### Publication Date

2020-07-01

### DOI

10.1016/j.aquatox.2020.105481

Peer reviewed

Aquatic Toxicology 224 (2020) 105481

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

# Conventional and nano-copper pesticides are equally toxic to the estuarine amphipod *Leptocheirus plumulosus*

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ARTICLE INFO

*Keywords:*
Ecotoxicity
Nanopesticides
Cu
Estuary
Amphipods
Toxicodynamic model
DEBtox

ABSTRACT

Modern nano-engineered pesticides have great promise for agriculture due to their extended, low dose release profiles that are intended to increase effectiveness but reduce environmental harm. Whether nanopesticides, including copper (Cu) formulations, cause reduced levels of toxicity to non-target aquatic organisms is unclear but important to assess. Predicting how aquatic species respond to incidental exposure to Cu-based nanopesticides is challenging because of the expected very low concentrations in the environment, and the two forms of exposure that may occur, namely to Cu ions and Cu nanoparticles. We conducted Cu speciation, tissue uptake, and 7-day toxicity laboratory experiments to test how a model estuarine organism, the amphipod *Leptocheirus plumulosus,* responded to two popular Cu-based nanopesticides, CuPRO and Kocide, and conventional $CuCl_2$. Exposure concentrations ranged from 0 to 2.5 ppm, which were similar to those found in estuarine water located downstream of agricultural fields. Cu dissolution rates were much slower for the nanopesticides than the ionic formula, and Cu body burden in amphipods increased approximately linearly with the nominal exposure concentration. Amphipod survival declined in a normal dose-response manner with no difference among Cu formulations. Growth and movement rates after 7 days revealed no difference among exposure levels when analyzed with conventional statistical methods. By contrast, analysis of respiration rates, inferred from biomass measurements, with a bioenergetic toxicodynamic model indicated potential for population-level effects of exposure to very low-levels of the two nanopesticides, as well as the control contaminant $CuCl_2$. Our results indicate that toxicity assessment of environmental trace pollutant concentrations may go undetected with traditional ecotoxicological tests. We present a process integrating toxicity test results and toxicodynamic modeling that can improve our capacity to detect and predict environmental impacts of very low levels of nanomaterials released into the environment.

## 1. Introduction

Pollution of estuarine ecosystems is an increasingly serious environmental problem, especially as we introduce new chemicals with relatively little understanding of their potential toxicity. Estuaries are important to society as they provide many ecosystem services, including nutrient cycling, habitat for economically valuable species, and the maintenance of biodiversity (Lenihan et al., 2001; Needles et al., 2015). Estuaries also sequester and harbor microorganisms that degrade anthropogenic contaminants (Boorman, 1999; Kehrig et al., 2003). Thus, the fate, transport, and ecological impacts of emerging pollutants, including nanomaterials, are key concerns in estuarine ecosystem science and management (Klaine et al., 2008; Holden et al., 2013, 2016). To date, many concepts about the ecological implications and impacts of nanomaterials and other emerging contaminants come from traditional ecotoxicological risk assessments that are frequently hampered unavoidably by narrow subsets of relevant species, toxicants, exposure conditions, and levels of impact (Jager et al., 2011; Muller et al., 2015).

Estuaries are major recipients of pesticide-laden runoff from

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https://doi.org/10.1016/j.aquatox.2020.105481
Received 26 September 2019; Received in revised form 3 March 2020; Accepted 24 March 2020
Available online 27 April 2020
0166-445X/ 

agricultural fields and urban landscapes, and therefore represent model systems to assess the effects of incidental exposure to pesticides in downstream ecosystems (Chapman and Wang, 2001; Bernardino et al., 2015). Nano-based products are increasingly used for commercial applications, including an emerging suite of pesticides applied in large-scale agriculture (Kookana et al., 2014), which are generally referred to as nanopesticides (Lin et al., 2015; Kah et al., 2018). One of the most common forms of nanopesticides are Cu-based chemicals, and include two popular brands, CuPRO 2005 (CuPRO) and Kocide 3000 (Kocide) (Hong et al., 2015; Lin et al., 2015). All Cu-based pesticides function primarily by releasing toxic Cu ions. Conventional Cu-based pesticides, including $CuCl_2$ or $CuSO_4$, are very effective at controlling agricultural pests (de Oliveira-Filho et al., 2004) but can also release relatively large amounts of dissolved Cu into natural water bodies that eventually harm non-target organisms (Kiaune and Singhasemanon, 2011). To reduce environmental impacts, Cu-based nanopesticides are engineered (and thus advertised) to be as effective as conventional products but less ecologically harmful because they release affective doses of Cu ions very slowly, thereby repelling pests but exposing non-target organisms to relatively very low levels of Cu (Keller et al., 2017). However, the degree to which the nanopesticides perform to reduce exposure and harm to downstream organisms is poorly understood.

The behavior and toxicity of Cu nanomaterials in natural aqueous solutions (Keller et al., 2010; Adeleye et al., 2014, 2016; Conway et al., 2015), including in seawater (Hanna et al., 2013; Bielmyer-Fraser et al., 2014; Torres-Duarte et al., 2016), have previously been addressed. However, most environmental impact studies of Cu nanopesticides have focused on crop plants and soil organisms (Keller et al., 2017). To the best of our knowledge, only one published study has assessed and compared the toxicity of a commercial Cu-based nanopesticide formulation with that of ionic Cu for an aquatic organism, namely zebrafish, a model freshwater species (Lin et al., 2015). The results of this work indicate that commercial Cu nanopesticides are less toxic to the zebrafish embryo hatching process than ionic Cu, most likely due to the formation of Cu species that are bio-unavailable in the hatching process. Toxicity was also detected at much higher concentrations than those measured thus far in natural waters (Nason et al., 2012). Whether Cu nanopesticides are toxic to non-target estuarine organisms at environmentally relevant concentrations has not yet been adequately tested.

As the volume of nanopesticides production and potential for discharge into the aquatic environment increases, so does potential for wide-ranging ecological impacts (Keller et al., 2017). Amphipods (small arthropod crustaceans) have been used as bioassay organisms to test the ecological impacts of pesticides in aquatic habitats because they live in water and sediment that accumulate contaminants; are sensitive and therefore vulnerable to many pesticides, including those that release Cu and other metal ions (Hanna et al., 2013); and are ecologically valuable in estuarine food webs, mainly as detritivores and/or prey for fish and other predators (US EPA, 1994; Hanna et al., 2013). In fact, a wide variety of ecological risk assessments for marine and estuarine ecosystems have relied in part upon the results of acute and chronic toxicity tests using amphipods (Lenihan et al., 1995; McGee et al., 2004).

Detecting nanopesticide constituents and other nanomaterials in agricultural, urban, and natural ecosystems, and assessing their toxicity at environmentally relevant, usually extremely low concentrations has proven very difficult with conventional ecological risk assessment methodology (Holden et al., 2016). However, with models that combine toxicokinetics and toxicodynamics, such as the Generalized Unified Threshold model for Survival (GUTS), and sublethal toxic effect modules of the Dynamic Energy Budget framework (DEBtox), exposure levels can be related to changes in growth, maintenance, reproduction, and survival (Muller et al., 2010; Nisbet et al., 2010). In turn, impacts on individual test organisms can be used to predict impacts on populations and communities (Jager and Klok, 2010; Muller et al., 2014). The process-oriented structure of these approaches makes toxicity assessment statistics independent of exposure time and the choice of endpoints or experimental conditions. As such, the multitude of impacts of a chemical can be delineated simultaneously, delivering common ecotoxicological parameters to all affected endpoints (Muller et al., 2015; Lecomte-Pradines et al., 2017).

Here, we report the results of a study designed to test whether environmentally relevant concentrations (i.e., those in the range of μg - mg $L^{-1}$ in receiving water) of Cu-based nanopesticides are toxic to a non-target estuarine amphipod *Leptocheirus plumulosus*, a species used frequently in ecotoxicity research and management (US EPA, 1994). We compare the chemical fate, Cu body burden, and relatively toxicity of the two most commonly used Cu-nanopesticides, CuPRO and Kocide, with $CuCl_2$, the major constituent of conventional Cu-based pesticides. Our strategy involved laboratory experiments to determine the dissolution and uptake of Cu and dose-dependent responses in mortality, biomass evolution, and behavior (i.e., motility), followed by the application of toxicodynamic models to assess the impact of Cu pesticide exposure.

## 2. Materials and methods

The copper compounds used in this study were characterized by the Central Materials Library maintained by University of California's Center for Environmental Implications of Nanotechnology (UC CEIN) (Godwin et al., 2009). A detailed summary of the chemicals can be found in the Supporting Information (Table S1). Reagent grade $CuCl_2$ salt was purchased from Sigma Aldrich (St. Louis, Mo), and the nanopesticides ($Cu(OH)_2$) Kocide 3000 from Dupont (Wilmington, DE), and CuPRO 2005 from SePRO (Carmel, IN). The nanopesticide's physicochemical characterizations were analyzed for primary particle size distribution and morphology using a scanning electron microscopy (FEI XL40 Sirion) equipped with an Oxford INCA energy-dispersive X-ray spectroscopy (EDS) probe. The size and surface charge of particles at *pH* 7 (0.5 mM phosphate buffer) were determined by measuring hydrodynamic diameter (HDD) and zeta (ζ) potential using a Zetasizer Nano-ZS90 (Malvern, UK). Purity and copper content (wt %) of particles was assessed via Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Thermo Scientific) (Lin et al., 2015).

Briefly, the main copper phase (XRD) of Kocide and CuPRO was orthorhombic $Cu(OH)_2$ with a primary particle size distribution of $> 10^4$ nm for Kocide (according to Hong et al., 2015), and ~10 nm for CuPRO and a surface charge (mV) of particles at *pH* 7 (0.5 mM phosphate buffer) of -19.9 ± 0.8 for Kocide and -22.9 ± 0.6 for CuPRO. The agglomerate hydrodynamic diameter (nm) was 1172 ± 104 for Kocide and 953 ± 88 for CuPRO. Copper content of particles was 39.9 ± 1.4 (wt %) for Kocide (impurities included C, O, Na, Al, Si, S, and Cl, according to Hong et al., 2015) and 47.1 ± 2.6 (wt %) for CuPRO (impurities included C, O, Na, Al, Si, P, and Ca; Hong et al., 2015). We report the physicochemical kinetics of aggregation, sedimentation, dissolution, speciation, and complex formation of the Cu $(OH)_2$ particles elsewhere (Conway et al., 2015). The exposure concentrations of dissolved, nanoparticle, and bulk forms of Cu (mg $L^{-1}$) in the microcosms are provided in Table S2.

### 2.1. Amphipod culturing and testing

Brood stocks of *Leptocheirus plumulosus* (Family: Aoridae) were obtained from Aquatic Biosystems (Fort Collins, CO, USA). The cultures were maintained in polystyrene bins containing fine quartz and containing 3 L of aerated, filtered seawater (0.5 μm) adjusted to 20 ppt salinity with deionized water at 20.0 °C ± 0.5. Cool fluorescent lights (500 μmol $m^{-2}$ $s^{-1}$) provided illumination with a 14:10 h Light : Dark photoperiod. Approximately 50 % of culture water was removed from culture bins two times per week and replaced with fresh 20 ppt seawater. The amphipods were fed with a suspension of finely ground fish flakes (TetraMin® Blacksburg, VA, USA) after each water change. To

avoid metal contamination, all materials were washed in a 10 % $HNO_3$ acid bath and rinsed thoroughly with deionized water followed by ultrapure water rinse prior to use. Experiments to detect Cu speciation, uptake by amphipods, and the effects of the pesticides on amphipod mortality and motility were conducted in 1 L glass microcosms (see below).

### 2.2. Cu compound preparation and dispersion

Cu-based nanopesticide and ionic pesticide dispersions were prepared 45 min prior to the start of the experiment. A stock suspension of 10 mg $L^{-1}$ was made by diluting each Cu compound in ultrapure water (Barnstead NANOpure Diamond™, 18.2 MV/cm) adjusted to salinity 20 ppt with filtered natural seawater (0.45 μm). To disperse Cu particles, the suspensions were sonicated for 30 min (Branson model 2510 sonic bath; Danbury, CT). The stock solutions were then diluted with additional seawater to the desired final nominal Cu concentrations of 0.1, 0.25, 1.0, and 2.5 mg $L^{-1}$. The final exposure concentrations tested were selected based upon a series of pilot experiments that tested amphipod mortality over a range of $1-1000$ μg $L^{-1}$ in seawater, concentrations of Cu from pesticides observed or expected to occur in the environment (Keller et al., 2017).

### 2.3. Water quality and Cu compound speciation

Water quality parameters in the bins and 1-L microcosms were monitored daily to ensure amphipod viability. A digital Extech DO700 meter was used to measure temperature, dissolved oxygen concentration, and *pH*. Salinity was measured using a refractometer (Hamh Optics&Tools), and the hardness, alkalinity, and ammonia concentrations were determined with water quality test strips (QUANTOFIX®). The three different Cu-based pesticides were introduced into microcosms simulating natural conditions to gain insight into how these chemicals might behave and/or are transformed in estuarine seawater over time. Therefore, we sampled aliquots from microcosms with nominal Cu concentrations of 0.25 and 2.5 mg $L^{-1}$, and seawater controls, after 1, 4, and 7 days of exposure. Immediately after collection, the water samples were analyzed for Cu concentrations (mg $L^{-1}$) in the test media during the experiments to determine physicochemical Cu speciation kinetics. The dissolved fraction (soluble Cu) was quantified after ultrafiltration in Amicon Ultra-4 3 kDa centrifugal filter tubes with maximum pore size ~2 nm (Millipore, Billerica, MA), and centrifuged for 30 min at 4000 × g. The nanoparticulate fraction (nano Cu), in a size range of 2–200 nm, was determined as the fraction of total Cu derived by filtering out particles > 200 nm (Target 0.2 μm PVDF syringe filter, Fisher Scientific) after accounting for the dissolved fraction left in the filtrate. The final filtrates and the total copper bulk solution were placed in metal-free tubes (VWR International, 15 mL), acidified with 2.5 % trace metal grade nitric acid ($HNO_3$, Fisher Scientific, Pittsburgh, PA), and stored at 4 °C until analyzed. Instrumental analyses measured dissolved, nanoparticulate, and the total Cu content via inductively coupled mass atomic emission spectroscopy (ICP-MS, Thermo ICAP 6300, Thermo Fisher Scientific) with a detection limit of 50 μg $L^{-1}$. All analyses were run in triplicate, with standards and blank solutions measured every 15 samples, for quality assurance. In addition, the bulk fraction of Cu (> 200 nm) was accounted for via mass balance as follows: Total Cu – (dissolved Cu + nano Cu).

### 2.4. Cu exposure and body burden in amphipods

Toxicity of the materials was determined by performing 7-day experiments without sediment, following US EPA guidelines (US EPA, 2001; see also Hanna et al., 2013). Amphipod bioassays are conducted with and without sediments (US EPA, 2001): sediment was not used in this experiment because the chemodynamics associated with Cu, sediment, and organic matter are highly complex. Certainly, a next step is to execute a study similar to ours but with sediment. The toxicity endpoints were assessed through assays conducted in the 1-L microcosms (height 20 cm, inner diameter 8 cm), which were fitted with (acid washed) nylon screen bottoms (600 microns) as an artificial benthic substrate. To reduce stress caused from handling, 20 organisms were carefully placed into the microcosms containing only pure seawater for a 48-h acclimation period. After 48 h, toxicity tests were initiated by introducing fresh media containing the Cu compounds test solutions (0.1, 0.25, 1.0, and 2.5 mg $L^{-1}$) or the seawater control, with a total final volume of 700 mL. Male and female amphipods (3–4 weeks old) were used in this study, although differences in the responses of males and females were not recoded (*sensu* US EPA, 2001).

All tests of the different Cu compounds and concentrations were conducted in four replicate microcosms per treatment, each of which were randomly placed together in the same climate-controlled room used for culture preparation. Amphipods were not fed during the experiments to isolate toxicant effects, as Cu is known to react rapidly with organic material and changes in salinity. However, as indicated above, amphipods were fed up until the final exposure periods. Thus, the scenario that we created for the amphipod exposures was analogous to periods of low food availability, for example during strong circulation events or storms, when amphipods are suspended in the water column and transported elsewhere (Lenihan and Micheli, 2000). All microcosms were covered with a petri dish to avoid evaporation and entry of the dust into the test solutions. Gentle aeration was provided to maintain adequate oxygen saturation (> 80 %). Environmental parameters were recorded daily.

To examine potential tissue bioaccumulation of Cu in amphipods, body burden of Cu in whole organism tissues was determined at the end of the experiment by collecting surviving organisms. The organisms were washed in an ethylenediaminetetraacetic acid (EDTA) solution (0.01 M EDTA, 0.1 M $KH_2PO_4$/ $K_2HPO_4$ buffer *pH* 6.0, salinity adjusted to 20 ppt) to remove Cu bound to external surfaces and dried in oven at 60 °C for 3 days. The dried organisms, pooled from each replicate, were then acidified with trace metal grade nitric acid (2.5 % $HNO_3$). Total Cu content of the surviving amphipods was analyzed by ICP-MS. Samples were analyzed in triplicate, and metal standard solutions and blanks were analyzed after every ten samples. Detection limits for metals ≥ 50 μg $L^{-1}$.

### 2.5. Toxicity endpoints

The number of dead organisms in each microcosm was counted and recorded daily. Decomposed and missing organisms (presumed cannibalized) were counted as dead. Corpses were removed, dried, and weighed daily. In addition to mortality, the number of individuals exhibiting any abnormal behavior or appearance was counted and recorded daily. At day 7, live and dead biomass (mg) and individual organism length (mm) were recorded.

To assess effects of the pesticides on behavior, motility was measured at the beginning and at the end of the experiment by placing organisms on 24 well/plates (maximum 5 organisms per well) and recording a series of image sequences (10 images/sec, 10 s in total) with a camera coupled on a stereomicroscope (Olympus SZX12). The images were analyzed by Fiji software, an image processing package (Schindelin et al., 2012), using MTrackJ plugin (Meijering et al., 2012). The motility length parameter was determined and is defined as the total length of the movement track from the starting (first) point to the final point.

### 2.6. Models

For the analysis of survival and biomass data, we used two dose metrics, the nominal concentration and the body burden of Cu. We used the general metric *M* in model derivations and specified the metric for

each application. We assumed that there is potentially a level of the dose metric, $M_{0*}$, below which detrimental effects did not occur (with '*r*' and ┼ substituting * to represent respiration and survival, respectively), and defined the effective dose metric as

$$M_{E*} = \max(0, M - M_{0*}) \quad (1)$$

#### 2.6.1. Biomass dynamics

The amount of living biomass, $W_L$, was reduced due to respiration (at rate *R*) and mortality (at rate *D*) and increased due to cannibalism of corpses (at rate *C*). To retain simplicity, we assumed that the conversion efficiency of biomass recycling was 100 %. Dead biomass was removed daily (at rate *E*). We approximated this removal process by assuming it to be a continuous process that matched the difference between the rates of mortality and cannibalism, i.e., $C = D–E$. Accordingly, the dynamics of living biomass is given by the following balance equation

$$\frac{dW_L}{dt} = -R + E \quad (2)$$

We assumed that the respiration rate was proportional to the amount of living biomass,

$$R = \mu W_L \quad (3)$$

in which $\mu$ is the specific population respiration rate. We assumed that the respiration rate coefficient increased linearly with the dose metric defined in Eq. (1),

$$\mu = \mu_0 \left(1 + \frac{M_{Er}}{M_{Kr}}\right) \quad (4)$$

in which $\mu_0$ and $M_{Kr}$ is the background respiration rate coefficient (i.e., specific population respiration rate in absence of toxic effects) and toxicant scaling parameter, respectively. $EC_x$ values for respiration can be calculated from Eq. (1) and (4) once $M_{Kr}$ and $M_{0r}$ have been estimated. With $EC_x$ defined as the value of the dose metric at which respiration is $(1 - 0.01x)^{-1}$ times that of the control, the result is

$$EC_x = M_{Kr}\left(\frac{x}{100-x}\right) + M_{0r} \quad (5)$$

Accordingly, the $EC_{50}$ is the value of the dose metric at which the respiration rate is twice that of the control and $EC_{50} = M_{Kr} + M_{0r}$.

In the Supporting Information, we show that the rate of manual dead biomass removal was satisfactorily described with the (phenomenological) exponential decay function

$$E = \gamma W_{rm} e^{-\gamma t} \quad (6)$$

in which $W_{rm}$ and $\gamma$ are parameters. Substitution of Eqs. (3) and (6) into 2 gives

$$\frac{dW_L}{dt} = -\mu W_L - \gamma W_{rm} e^{-\gamma t} \quad (7)$$

with $\mu$ specified in Eqs. (4). Eq. (7) can be solved with standard methods to yield

$$W_L = W_{L0} e^{-\mu t} + \frac{\gamma W_{rm}}{\gamma - \mu}(e^{-\gamma t} - e^{-\mu t}) \quad (8)$$

in which $W_{L0}$ is the initial amount of living biomass.

#### 2.6.2. Survival model

The fraction of individuals surviving until time *t*, *S(t)*, is often modeled with the survivor function

$$\frac{dS}{dt} = -S(t)h(t) \quad (9)$$

in which *h(t)* is the hazard rate ('instantaneous probability of dying'). Following Jager et al. (2011), we assumed that the hazard rate was proportional to an abstract damage variable representing the cumulative impacts of ageing and toxic effects on survival potential. We assumed that this damage quantity accumulated at a constant rate in the absence of toxicants and, in addition to this background accumulation, that this damage quantity accumulated at a rate proportional to the dose metric given in Eq. (1). We also assumed that initial damage was negligible. Then, provided the dose metric is invariant, the hazard rate increases linearly in time and

$$h = k_\dagger' \left(1 + \frac{M_{E\dagger}}{M_{K\dagger}}\right) t \quad (10)$$

in which $k_\dagger'$ is the killing rate. Substitution of Eq. (10) into 9 and subsequent solving with all individuals alive at yields

$$S(t) = e^{-0.5k_\dagger'\left(1+\frac{M_E}{M_{K\dagger}}\right)t^2} \quad (11)$$

which describes a Weibull distribution. We defined $LC_x$ as the value of the dose metric at which survival is reduced x% relative to that in the control. From Eqs. (1) and (11),

$$LC_x = \frac{-2M_{K\dagger}}{k_\dagger' t^2} \ln(1 - 0.01x) + M_{0\dagger} \quad (12)$$

### 2.7. Statistical analyses

#### 2.7.1. Experiments

Separate two-way ANOVAs were used to test for differences in the mean total dissolved and nanoparticle Cu concentration (mg $L^{-1}$) in microcosms, Cu body burden in amphipods, and amphipod survival, length, biomass, and motility, all as a function of Cu pesticide type, nominal Cu concentration in the pesticides (mg $L^{-1}$), and their interaction. Prior to ANOVA, all data were square root transformed and tested for heteroscedasticity of variances using Levene's test. Transformed data passed subsequent Levene's tests for homogeneity of variances ($P > 0.05$). Differences between specific treatments were determined with Tukey's honest significant difference (HSD) post hoc tests ($P < 0.05$). All statistical analyses and graphics were conducted in R (R Development Core Team) and Excel.

#### 2.7.2. Models

Parameters of the biomass model were estimated with likelihood methods assuming additive normally distributed measurement error. Parameters of the survival model were estimated by maximizing the log likelihood function of the multinomial distribution (Jager et al., 2011). Fit results were evaluated with 95 % confidence intervals, which were calculated from negative log likelihood profiles.

## 3. Results

### 3.1. Cu speciation

Dissolved Cu was detected in all experimental microcosms, including in trace amounts in the seawater controls (Fig. 1). Mean total dissolved Cu concentration was < 1 mg $L^{-1}$ across treatments, and varied with pesticide type ($CuCl_2$, CuPRO, and Kocide) and nominal Cu concentrations (i.e., 0 mg $L^{-1}$, 0.25 mg $L^{-1}$, and 2.5 mg $L^{-1}$), as indicated by a significant two-way interaction in the ANOVA (2-way ANOVA; Pesticide x Concentration; $F_{2,14} = 48.5$; $P < 0.0001$; Table S3). The total amount of dissolved Cu increased rapidly with increasing nominal Cu concentration across all three Cu pesticides but was much higher for the conventional Cu pesticide ($CuCl_2$) than the two nanopesticides at 0.25 mg $L^{-1}$ and 2.5 mg $L^{-1}$ nominal concentrations (Tukey's HSD test, $P < 0.05$). However, there was no difference in dissolved Cu between the Kocide and CuPRO treatments at 0.25 mg $L^{-1}$ and 2.5 mg $L^{-1}$ nominal concentrations (Tukey's HSD tests, $P > 0.05$).

Mean total nanoparticle Cu concentration also varied with pesticide

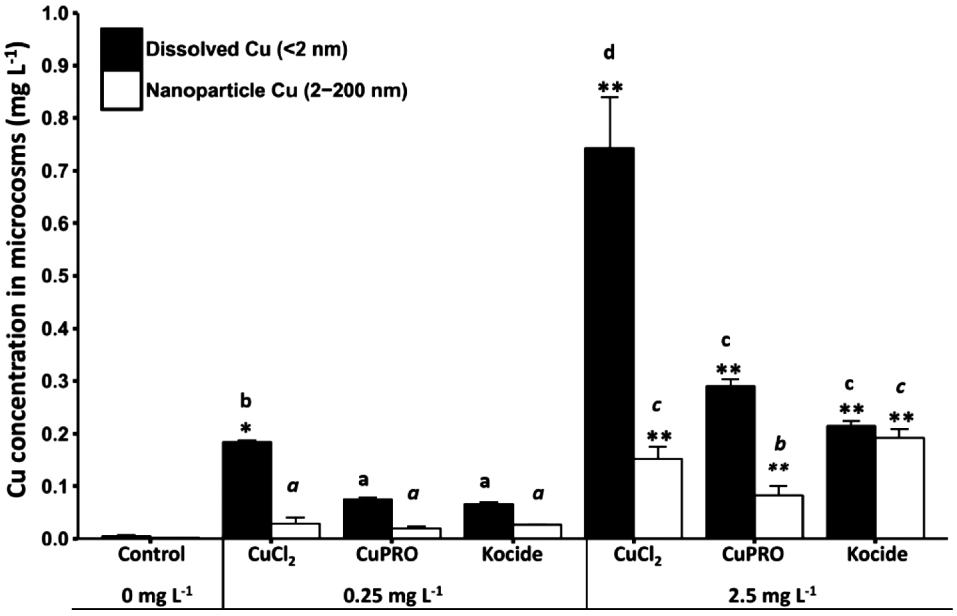


**Fig. 1.** Mean (95 % C.I.) dissolved and nanoparticle Cu concentrations (mg L-1) in amphipod microcosms exposed to conventional Cu pesticide (CuCl2), commercial Cu nanopesticides (Kocide and CuPRO), and seawater controls, as a function of nominal Cu concentrations 0, 0.25 and 2.5 mg L-1. N = 3 replicate microcosms per treatment. Results of a Tukey's honest significant difference (HSD) post hoc test for each analysis (dissolved Cu and Nano-Cu) are provided above each bar (between treatments: a < b < c, at $P < 0.05$; from controls: *, at $P < 0.01$, and **, at $P < 0.001$). Italicized letters represent results for the ANOVA of nanoparticle Cu concentrations.

type and nominal concentrations (2-way ANOVA; Pesticide x Concentration; $F_{2,14} = 18.97$; $P < 0.001$; Table S4) in the microcosms. Low parts-per-million ($0.02 - 0.20$ mg $L^{-1}$) of nanoparticulate Cu were detected in all pesticide treatments but not seawater controls (Fig. 1). Nanoparticles formation in the $CuCl_2$ treatments was expected in seawater because of aggregation, precipitation, binding with organic matter, and various combinations of these processes (Keller et al., 2010). The total amount of nanoparticle Cu increased significantly with increasing nominal Cu concentration across treatments (Tukey's HSD, $P < 0.001$). Total nanoparticle Cu did not vary among nanopesticides at the 0.25 mg $L^{-1}$ nominal concentration (Tukey's HSD, $P > 0.001$) but was significantly lower in the CuPRO than the $CuCl_2$ or Kocide treatments at the 2.5 mg $L^{-1}$ nominal concentration (Tukey's HSD, $P < 0.001$). Bulk forms of Cu, specifically particles > 200 nm in size, were undetectable or in very low concentrations (< 25 µg $L^{-1}$) at the 0.25 mg $L^{-1}$ nominal concentrations for the two nanopesticides. By contrast, bulk Cu particles were the predominant Cu species in the 2.5 mg $L^{-1}$ treatments. No large Cu particles were detected in the controls and $CuCl_2$ treatments. Overall, we recovered and accounted for less total amount Cu than was used in each treatment. We reason that this was the case because Cu was taken up by and excreted by amphipods (*see below*), deposited as organic molecule-Cu aggregates on the bottom of the microcosms, and perhaps even lost to evaporation. A full accounting of the total Cu used in the experiment was not possible.

### 3.2. Cu body burden in amphipods

Copper was present at detectable levels in amphipods from all treatments after 7 days of exposure, including very low concentrations in amphipods from seawater controls (Fig. 2). Body burden of Cu generally increased with nominal concentrations of Cu for all Cu formulations. Concentrations of Cu in amphipod tissues were 140 µg Cu g $DW^{-1}$ in the 0 mg $L^{-1}$ (i.e., control), and 220–266, 203–254, 256–345, and 331–447 µg Cu g $DW^{-1}$ in the 0.1, 0.25, 1, and 2.5 mg $L^{-1}$ treatments, respectively (Fig. 2). There was a significant effect of both main factors 'Pesticide' (2-way ANOVA; Pesticide; $F_{3,39} = 15.00$; $P < 0.0001$; Table S5) and 'Concentration' (2-way ANOVA; Concentration; $F_{3,39} = 26.68$; $P < 0.0001$; Table S5), a pattern driven by the relatively high body burdens of Cu in the Kocide treatment, and increasing body burden as a functioin of nominal concentration.

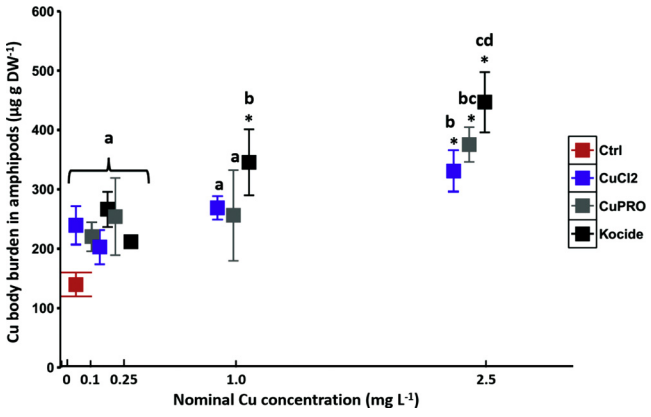


**Fig. 2.** Mean (95 % C.I.) total body burden of copper (µg Cu g DW -1) in estuarine amphipods exposed to conventional Cu pesticide (CuCl2), commercial Cu nanopesticides (Kocide and CuPRO), and seawater controls, as a function of nominal Cu concentrations 0, 0.1, 0.25, 1, and 2.5 mg L-1. N = 4 replicate jars per treatment, each with 20 amphipods. Results of a Tukey's honest significant difference (HSD) post hoc test are provided above each bar (between treatments: a < b < c, at $P < 0.05$; from controls: *, at $P < 0.01$).

### 3.3. Toxicity

Survivorship after 7 days of exposure was relatively high in the controls but generally decreased with increasing nominal concentrations of all three Cu formulations (Fig. 3). The result of ANOVA indicated that mortality was greatest for all pesticides at the two highest nominal concentrations (2-way ANOVA; Concentration; $F_{3,39} = 20.79$; $P < 0.0001$; Table S6). There was no significant difference in survivorship as a function of the type of pesticide or the interaction of pesticides and concentration ($P > 0.05$; Table S6). There was little apparent relationship between Cu body burden and survival.

The total mean biomass (mg) of amphipods at the end of the exposure experimental period of 7 days tended to be lower in all formulations at 1 and 2.5 mg L-1 concentrations than in the seawater control (Table S7). However, the differences were not statistically significant (2-way ANOVA; $P > 0.05$; Table S8). The total mean length (mm) of amphipods increased at the end of the exposure experimental period of 7 days (Table S9), but results of ANOVA indicated that there was no statistically significant effect of either main factors, 'Pesticide'

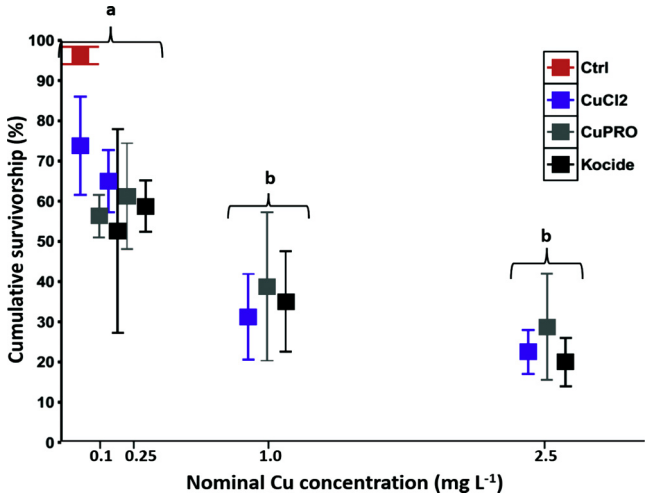


**Fig. 3.** Mean (95 % C.I.) cumulative survivorship (%) of amphipods exposed to conventional Cu pesticide (CuCl2), commercial Cu nanopesticides (Kocide and CuPRO), and seawater controls, as a function of nominal Cu concentrations 0, 0.1, 0.25, 1, and 2.5 mg L-1. N = 4 replicate microcosms per treatment, each with 20 amphipods. Results of a Tukey's honest significant difference (HSD) post hoc test are provided above each bar (between treatments: a > b, at $P < 0.05$).

and 'Concentration', or their interaction (2-way ANOVA; $P > 0.05$; Table S10).

For the total mean motility of amphipods (Table S11), measured as the total distance (mm-cm) that amphipods swam in 10 s, the result of ANOVA indicated that motility was lower for all pesticides at the highest nominal concentration compared with lower concentrations (2-way ANOVA; Concentration; $F_{3,39} = 3.54$; $P < 0.05$; Table S12). There was no significant difference in motility as a function of the type of pesticide (2-way ANOVA; $P > 0.05$; Table S12).

### 3.4. Model analyses

There was a clear dose-response in impacts of each of the Cu-based pesticide formulations on L. *plumulosus*: the amount of biomass left after seven days decreased while mortality increased with exposure concentrations (Figs. 4 and 5). Recall that biomass declined due to respiration and mortality and that corpses were subject to cannibalization before they could be removed, i.e., dead biomass was in part recycled into living biomass (see Section 2.6). Accordingly, at all exposures, the amount of biomass lost due to respiration, as calculated from mass balances, was up to 18 times higher and never less than the amount physically removed from the containers (see Table S13). The biomass decline and survival data were analyzed with simple bioenergetic and survivorship models in order to obtain toxic effect statistics that are independent of exposure time and other specifics of experimental protocols. In addition, the analyses serve to determine differences in toxicity profiles between ionic and nano-copper speciation. For the analysis of survival and biomass data, one can use several dose metrics, including nominal concentrations, scaled or unscaled body burdens, and measures of accumulated damage. To keep the presentation simple, the dose metric in the analyses is either the nominal Cu concentration ($C$) or the body burden of copper ($c$).

The model describing the decline of living biomass contains six parameters (Equations 4 and 8), of which the value of one, the initial amount of living biomass, is fixed at the mean amount of biomass of 20 individuals at the start of the experiment (11.78 mg). The two parameters quantifying the daily removal of corpses have been estimated by fitting the phenomenological function describing the removal process (Equation S1) to the means of the amounts of biomass removed at each treatment (Fig. S1). Accordingly, there are 13 sets of estimated values of the parameters describing biomass removal, one for each of the four exposure levels of the three copper formulations, in addition to that of the control (Table 2 and Table S14). These values have been fixed in the estimation procedure of the background respiration rate and the toxic effect parameters.

The background respiration rate has been estimated by fitting the model describing the decline of living biomass during the experiment (Equations 4 and 8) to the control data (Table 1), which value has been used for the analysis of toxic effects of Cu exposure. With nominal concentration as the dose metric, the toxic effect parameters are the no-effect and tolerance concentration for respiration; with body burden as dose metric, the two effect parameters are the no-effect and tolerance body burden for respiration.

To estimate toxic effect parameters, the biomass decline model was fitted to data from each copper formulation exposure separately, as well as to all data combined. Strikingly, with both the nominal concentration and body burden as the dose metric, the model with parameter values estimated from all data combined predicted the observed decline in biomass with increasing exposure level about equally well, regardless of copper formulation (Fig. 4). In fact, the sums of log likelihoods decreased only 0.03 − 0.68 when the values of the no-effect and tolerance

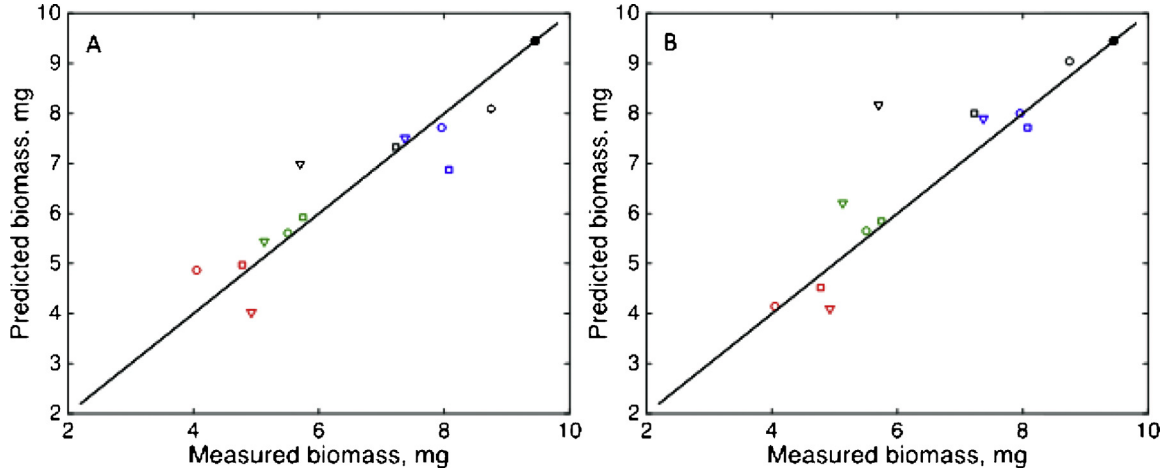


**Fig. 4.** Predicted mean biomass versus mean measured biomass levels after seven days of exposure to CuCl2 (open circles), CuPRO (squares) or Kocide (triangles) using (A) nominal concentrations and (B) body burdens as dose metrics. Closed circle represents controls. Colors represent exposure (nominal) concentrations of 0.1 (mg L-1), (black open symbols), 0.25 (mg L-1), (blue), 1.0 (mg L-1), (green) and 2.5 (mg L-1), (red), respectively. Predictions are based on parameter values estimated from all data combined (Table 1) with Eqs. (4) and (8).

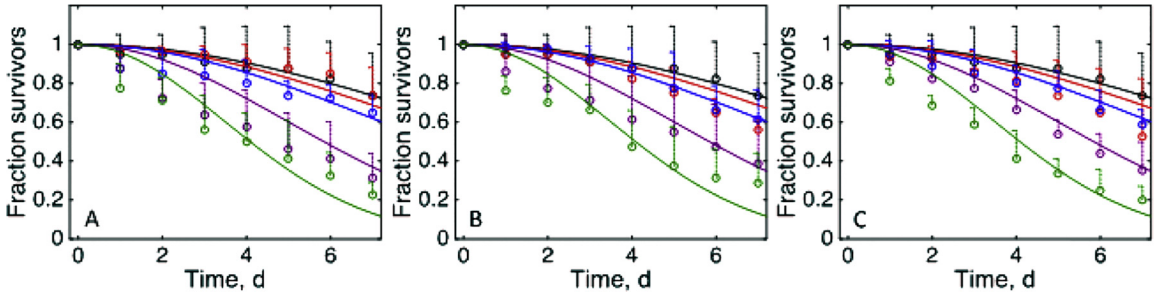


**Fig. 5.** Model fits to survival data with Cnec = 0 and Ck estimated from all Cu species data with nominal Cu concentrations as dose metric; 0 (black), 0.1 (red), 0.25 (blue), 1 (purple) and 2.5 (green) mg $L^{-1}$ nominal Cu concentrations. A: CuCl2; B: CuPRO; C: Kocide. Symbols represent means (n = 4); error bars are standard deviations. Points represent measured data, and curves are model predictions.

**Table 1**
Symbols and parameter estimates from biomass and survival data sets.

| Interpretation | Value [95 % CI] or (SE)[a] | Units |
|---|---|---|
| $c$ Body burden of Cu | Variable | µg Cu g $DW^{-1}$ |
| $c_{Kr}$ Tolerance body burden for effect on respiration | 145.4 [75.7 475.3] | µg Cu g $DW^{-1}$ |
| $c_{K\dagger}$ Tolerance body burden for effect on survival | 45.4 [18.0 184.8] | µg Cu g $DW^{-1}$ |
| $c_{0r}$ No-effect body burden on respiration | 148.6 [-117.1 218.6] | µg Cu g $DW^{-1}$ |
| $c_{0\dagger}$ No-effect body burden for survival | 188.0 [-37.0 251.8] | µg Cu g $DW^{-1}$ |
| $C$ Nominal concentration of Cu | Variable | mg $L^{-1}$ |
| $C_{Kr}$ Tolerance concentration for effect on respiration | 1.27 (0.30) | mg $L^{-1}$ |
| $C_{K\dagger}$ Tolerance concentration for effect on survival | 0.44 (0.13) | mg $L^{-1}$ |
| $C_{0r}$ No-effect concentration on respiration | 0 | mg $L^{-1}$ |
| $C_{0\dagger}$ No-effect concentration for survival | 0 | mg $L^{-1}$ |
| $E$ Biomass removal rate (corpses) | Variable | mg $day^{-1}$ |
| $h$ Hazard rate | Variable | $day^{-1}$ |
| $k_\dagger$ Killing acceleration | 12.5 (12.1) x $10^{-3}$ | $day^{-2}$ |
| $M$ Dose metric, either $c$ or $C$ | Variable | – |
| $M_*$ Dose metric parameter, either $c_*$ or $C_*$ | Variable | – |
| $R$ Respiration rate | Variable | mg $day^{-1}$ |
| $S$ Survival probability | Variable | – |
| $t$ Time | Variable | day |
| $W_L$ Amount of living biomass | Variable | mg |
| $W_{L0}$ Initial amount of living biomass | 11.73 | mg |
| $W_{rm}$ Dead biomass removal parameter | See Table S13[b] | mg |
| $\gamma$ Dead biomass removal parameter | See Table S13[b] | $day^{-1}$ |
| $\mu$ Specific population respiration rate | Variable | $day^{-1}$ |
| $\mu_0$ Background population respiration rate | 28.4 (14.9) x $10^{-3}$ | $day^{-1}$ |

[a] 95 % confidence interval if two values in square brackets are given; standard errors for single values are in parentheses.
[b] Parameter values varied among treatments.

**Table 2**
Parameters estimated from biomass removal data with Equation S1.

| | $CuCl_2$ | | CuPRO | | Kocide | |
|---|---|---|---|---|---|---|
| Nominal [Cu] mg $L^{-1}$ | $\gamma$ $day^{-1}$ | $W_{rm}$ mg | $\gamma$ $day^{-1}$ | $W_{rm}$ mg | $\gamma$ $day^{-1}$ | $W_{rm}$ mg |
| 0 | 0.24 | 0.23 | 0.24 | 0.23 | 0.24 | 0.23 |
| 0.1 | 0.08 | 1.13 | 0.14 | 2.64 | 0.07 | 3.68 |
| 0.25 | 0.38 | 1.57 | 0.02 | 12.77 | 0.03 | 8.65 |
| 1 | 0.49 | 3.43 | 0.09 | 6.10 | 0.03 | 11.21 |
| 2.5 | 0.69 | 3.77 | 0.31 | 3.26 | 0.08 | 7.60 |

metrics were fixed on their respective values estimated from all data combined, instead of estimating them from the data from each copper formulation separately (Table S15). This indicates that there was no significant difference in toxic effects on the metabolism of L. *plumulosus* among copper formulations. The estimate for the no-effect concentration was slightly negative but did not differ significantly from 0 at the 95 % level (results not shown); therefore, we set this value at 0 in subsequent analyses. Although the no-effect body burden also did not differ significantly from 0 (Table 1), its estimated value was substantial and was used in predictions and calculations here, as copper is an essential nutrient. Accordingly, the $EC_{50}$ as calculated from Eq. 5 and the parameter values listed in Table 1 is 1.27 mg $L^{-1}$ and 145.4 µg Cu g $DW^{-1}$ for nominal concentrations and body burdens, respectively.

The approach and results of the analysis of the survival data with the model in Eq. (10 largely paralleled those of the biomass decline data. The parameter quantifying background mortality, the killing acceleration, was estimated by fitting Eq. (10) to the survival data in the control (Table 1). This value was used to estimate the two toxic effect parameters for each of the dose metrics, the no-effect and tolerance concentration or body burden for survival, by fitting the survival model to data from each copper formulation separately and to all data combined. The sum of log likelihoods of the fits to the separate data sets with the no-effect and tolerance levels as free parameters decreased relatively little (0.01 − 0.48; Table S16) when the toxic effect parameters were fixed at their respective values estimated from all data combined. This shows that lethal toxicity of copper in L. *plumulosus* did not differ among copper formulations. The no-effect levels for survival for both nominal concentration and body burden did not significantly differ from 0 at the 95 % level. The estimate for the former is marginally negative, while the latter is substantial. Following the same reasoning as for the no-effect levels for respiration, we set the no-effect nominal concentration at 0 and used the estimated no-effect body burden in subsequent analyses and presentations. The model predictions of survival at each of the copper formulations with nominal concentrations as the dose metric are shown in Fig. 5; predictions with body burdens as the dose metric are comparable (results not shown).

The parameter estimates reveal differences between the sensitivities in lethal and population level metabolic impacts. The estimated value for the no-effect body burden for survival is substantially higher than that for population respiration. The tolerance levels for both metrics indicate that lethal impacts increased more rapidly with increasing exposure levels than effects on population respiration once the corresponding no-effect levels had been exceeded. In other words, the range of exposure levels at which the full spectrum of lethal impacts was observed was narrower than that of toxic effects on population respiration. In contrast to the model parameters quantifying toxic effects, $EC_x$ and $LC_x$ values cannot be easily compared, as the latter, but not the former, depends on exposure time (Eqs. (5) and (12)). The exposure time at which the predicted $LC_x$ equals the predicted $EC_x$ can be solved from Eq. (12). With the parameter estimates in Table 1 and with x = 50, this exposure time was 6.2 days with the nominal concentration and 6.9 days with the body burden as the dose metric; $LC_{50} > EC_{50}$ for shorter exposure times, while $LC_{50} < EC_{50}$ for longer exposure times, implying that lethal impacts appeared somewhat more pronounced than impacts on population respiration in the experiments analyzed in

this study.

## 4. Discussion

The first objective of our study was to estimate whether the ecological risks of exposure to pesticide runoff for non-target aquatic organisms varied between nano-Cu pesticides (CuPRO and Kocide) and the Cu compound ($CuCl_2$) used in conventional pesticides. Our prediction was that Cu-based nanopesticides pose less ecological risk to downstream invertebrate organisms than the conventional compound. We executed our test using an estuarine amphipod as a model organism, exposing them to environmentally relevant (i.e., relatively low) concentrations of the materials, and then quantifying Cu body burden as well as several toxic responses. Overall, we found support for the general claim by industry that Cu nanopesticides release relatively low levels of Cu into the environment per unit time (Kah et al., 2018), at least in comparison with the conventional pesticide compound $CuCl_2$ used in the experiment. We also observed the highest Cu body burdens in amphipods exposed to the nanomaterials but found no biologically significant differences among the nano- and conventional forms in terms of sublethal and lethal toxicity using conventional toxicity assays. This information can be used in risk assessment modeling designed to compare the overall ecological impacts and impacts of Cu-based nano- and conventional pesticides.

Our results then motivated our second objective, which was to apply a toxicodynamic modeling approach to test whether the Cu materials vary in subtle biological effects that might directly or indirectly influence demographic performance. Results of our model analyses revealed no differences in toxic effects among the Cu materials that we examined but provided important insights for developing new approaches to the ecological risk assessment for trace concentrations of Cu and other toxic materials. In addition to mortality, growth (i.e., changes in length and biomass) and motility, effects were also quantified in our study as the impact of ionic copper ($CuCl_2$) and nano-copper (Kocide and CuPRO) on population-level respiration, which was indirectly assessed through changes in amphipod biomass. The results give rise to the following conclusions. First, in all cases, we observed a normal dose-response, meaning that mortality and respiration increased with exposure level. Second, the toxicodynamic models describe the effects of copper exposure on survival and population respiration about equally well with either the nominal concentration or body burden (of copper) as dose metric (Figs. 4 and 5). This is in agreement with the observation that body burdens increase approximately linearly with nominal concentrations (Fig. 4) and indicates that body burdens equilibrated relatively rapidly. Third, the no-effect body burden for survival and respiration of copper was substantial, which is not surprising, since copper is an essential micronutrient. Fourth, and strikingly, the estimated model parameters quantifying effects on survival and population respiration do not differ significantly among treatments (Table 1, S15 and S16). This indicates that ionic and nano-copper have similar toxic effects on amphipods. We speculate that this was due to the fact that amphipods are detritus feeders and thereby ionic as well as aggregates of copper compounds are bioavailable to them. This is corroborated by Fig. 2, which suggest there was little difference among copper formulation with respect to bioaccumulation potential. Accordingly, copper speciation does not appear to affect bioavailability of copper for this species. Fifth, the estimated no-effect body burden for survival was lower than that for population respiration, although the 95 % confidence intervals are relatively wide. This appears unremarkable, as sublethal effects are often observed in individuals before survival is impacted. However, it should be noted that the impact of copper exposure on survival was mitigated by cannibalism, which occurred especially at the higher exposure levels. Survivors likely benefitted from their cannibalistic activity through an increase in life expectancy. This assumed benefit was concealed in the analysis of data here, as calculated respiration rates were normalized to population biomass content. Cannibalism is a process that emerges at the population level, which signifies that the extrapolation from individual to population level impacts should be done with care (Gergs et al., 2014).

Lethal impacts were quantified with the Stochastic Death (SD) variant of the General Unified Threshold Model of Survival (GUTS), which assumes that the instantaneous probability that an individual dies at a certain moment is proportional to the amount of damage due to stress it has accumulated in its body (Jager et al., 2011; Ashauer et al., 2015). The amount of damage is an abstract additive quantity that gauges the impairment of components of biochemical machinery due to ageing, starvation, and toxicant exposure, among other potential stress factors. Thus, a benefit of the damage concept is that it integrates impacts of multiple stress factors (in this study: starvation and toxic effects due to copper exposure). It also provides a process-based description that translates the exposure level of a chemical, which may interact with the biochemical machinery via multiple unknown molecular initiating events, into an adverse outcome, in this case death (Murphy et al., 2018). Due to the paucity of available data, we neither considered the Individual Threshold variant of GUTS (Jager et al., 2006), nor included the possibility of damage repair (Klanjscek et al., 2016) in our analysis. Doing so requires information from recovery experiments, but it is unlikely that such information would change our calculation of $LC_x$ values (Ashauer et al., 2015). A major strength of toxicodynamic models is that the toxic effect parameters that can be estimated from data, namely a no-effect level and a stress coefficient scaling the magnitude of toxic impact, do not depend on exposure time (Jager et al., 2006). Summary statistics, such as the $EC_x$ and $LC_x$, can then be calculated for any chosen exposure time (Eqs. (5) and (12)) (Jager et al., 2006; Miller et al., 2017). This means that the toxicity parameters estimated in this study can be used to predict the impacts of ionic and nano copper on the respiration and survival in amphipods at other exposure scenarios, including those with alternate exposure times and concentrations, as well feeding regimes.

As we have shown here with regards to the differences in toxicological effects observed in the laboratory and modeling, additional efforts should be made in developing toxicity studies and toxicokinetic – toxicodynamic models that contribute to the environmental regulation of nanomaterials (Grillo et al., 2018). For instance, we found nano-Cu pesticides released less Cu in ionic form than $CuCl_2$, thus indicating the nano form may decrease adverse ecological effects by releasing less of the active ingredient (AI) in the environment (Kah et al., 2013). Future research on the ecological implications of Cu-based nanopesticides for long-term exposure periods should consider that Cu-based nanopesticides with slow release times have the potential for runoff for several months or more after being applied to cultivated land (Certis_USA, 2018), and their behavior will depend on ENM composition and environmental parameters (Keller et al., 2017).

## Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

## CRediT authorship contribution statement

**Caroline P. Vignardi:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Erik B. Muller:** Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Funding acquisition. **Kelly Tran:** Validation, Investigation, Data curation. **Jessica L. Couture:** Methodology. **Jay C. Means:** Methodology, Validation. **Jill L.S. Murray:** Conceptualization, Resources, Funding acquisition. **Cruz Ortiz:** Investigation. **Arturo A. Keller:** Methodology, Resources. **Nicolas Smith Sanchez:** Investigation, Data curation. **Hunter S. Lenihan:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Supervision, Project administration,

Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

This material is based upon work supported by the California Sea Grant under Cooperative Agreement Number R/HCME-24. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of Sea Grant. We thank the Patricia Holden and her Lab (Bren-UCSB) for the use of their analytical instruments, and Sage Davis (Bren-UCSB) for his assistance in carrying out these experiments.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105481.

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