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ACUTE COPPER TOXICITY IN THE EURYHALINE COPEPOD *ACARTIA TONSA*: IMPLICATIONS FOR THE DEVELOPMENT OF AN ESTUARINE AND MARINE BIOTIC LIGAND MODEL

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Abstract—Copepods (*Acartia tonsa*) were exposed (48 h) to waterborne, diet-borne (non-Cu-equilibrated and Cu-equilibrated food), and waterborne plus diet-borne Cu in either the absence or the presence of food (diatom *Thalassiosira weissflogii*). Toxicity tests were run in different salinities (5, 15, and 30 ppt) together with measurements of physicochemical parameters and total and dissolved Cu concentrations in the experimental media. Results show that most of the toxic Cu fraction was in the dissolved phase. In general, Cu toxicity was higher in low (5 ppt) than in high salinity (30 ppt), regardless of the pathway of Cu exposure tested. In the absence of food, data clearly indicate that differences in waterborne Cu toxicity can be explained by changes in water chemistry. However, addition of food (either non-Cu-equilibrated or Cu-equilibrated) to the experimental media protected against acute Cu toxicity in salinities 5 and 15 ppt, suggesting that *A. tonsa* requires extra energy to cope with the stressful condition imposed by Cu exposure associated with the ionoregulatory requirements in low salinities. For diet-borne exposure, a very high Cu concentration was necessary to precontaminate the diatoms to a level resulting in copepod mortality. Therefore, availability of food exerted a more important positive impact in protecting against acute Cu toxicity than its potential negative impact via contamination resulting in toxicity. Findings indicate the need for incorporation of both salinity and food in a future biotic ligand model (BLM) version for Cu in estuarine and marine waters. In this context, the euryhaline copepod *A. tonsa* would be a suitable model species with which to perform experiments to validate and calibrate any future saltwater BLM. Environ. Toxicol. Chem. 2010;29:1834–1840. © 2010 SETAC

Keywords—*Acartia tonsa* Acute toxicity Biotic ligand model Copper Salinity

INTRODUCTION

Copper, like other metals, is in constant movement from soils to aquatic environments. Natural runoff is the main source of Cu in water, as a result of mining activities. Despite the existence of natural sources, industrial, agricultural, and harbor activities are continuously contributing to the increased input of Cu in estuarine and marine environments [1–6]. In Southern Brazil, Cu is the most relevant contaminant in coastal areas close to anthropogenic sources [2–4]. A marked 17-fold increase (2–34 μg/L) in dissolved Cu concentration has been reported over an approximately 20-y period in the Patos Lagoon estuary (Southern Brazil), the largest coastal lagoon in South America [1–4].

The presence of Cu at low concentrations in the aquatic media is very important because of its role in a number of physiological processes. However, when a threshold concentration is reached, normally associated with anthropogenic activities, Cu can be toxic to many aquatic organisms. Thus, Cu discharge in the environment has to be regulated [2].

In many countries, Cu emission is regulated based only on the total metal concentration in either effluents or in the environment. In both cases, water chemistry and the potential biological effects of metal exposure are not considered. However, it is well known that the chemistry of trace metals in water and, consequently, their toxicity are determined typically by complexation, biological uptake, and sorption on suspended solids [7,8]. Thus, to predict metal toxicity in aquatic systems, it is important to consider not only the metal concentration in the water but also the potential influence of many physicochemical parameters of the water on metal bioavailability. In 1985, the U.S. Environment Protection Agency (U.S. EPA), acknowledging the interaction between metals and different substances in water, adjusted the maximum allowed concentrations of metals in water by considering the water hardness [9]. However, this re-evaluation, though providing more environmentally relevant values than the former regulations, was still incomplete, insofar as it overlooked other key factors affecting metal toxicity. For example, water parameters such as pH [10,11], cation and sulfide concentrations [7], dissolved organic matter [11–13], hardness [10,12], suspended particulate matter [8], salinity [14], and ion composition [15] have been shown to influence Cu bioavailability and toxicity. Thus, it is clear that the environmental risk assessment of metals is not a simple task.

Protective effects of various water chemistry parameters were then incorporated into the biotic ligand model (BLM) for Cu [16]. This model simultaneously accounts for the speciation and complexation of dissolved metal and competitive binding of metal and other cations at the site of action. The fundamental premise of the BLM is that metal concentration in/on the target is correlated with the acute toxicity of the metal [17,18]. This model has now been adopted by the U.S. and Australia/New Zealand Environmental Protection Agencies as a legal tool for managing environmental regulatory issues concerning metal release in the aquatic environment. It is also in the process of being adopted by Environment Canada and the European Union.

Another important factor to be considered is the influence of the route of exposure on metal toxicity. Because organisms can

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be exposed to contaminants both directly (dissolved metal in the water) and indirectly (trophic transfer), toxicological testing has to address the importance of the different routes of exposure. For example, food can influence metal toxicity, not only by altering water chemistry but also by altering the amount of metal accumulated. Furthermore, it has been previously demonstrated that nutrition can have an influence on the response of field populations of calanoid copepods to Cu exposure [19]. More recently, it has been determined that metal contamination via food, including Cu, has toxic effects on copepods [20–22].

Therefore, it is clear that water chemistry and the presence of food in the experimental medium could alter Cu toxicity to aquatic animals. Unfortunately, most of the information and knowledge on Cu toxicity in invertebrates reported so far is available only for freshwater environments after acute exposure, a situation that normally does not involve the presence of food in water.

Based on this background, the main goal of the present study was to determine the effects of both salinity and the pathway of exposure on acute Cu toxicity in a copepod species in a wide range of salinities. Copepods are considered sensitive indicators of metal toxicity [21], being employed in toxicological studies both in the laboratory [19] and in the field [23]. The species selected to conduct the present investigation was the euryhaline copepod *Acartia tonsa,* because it is an euryhaline species tolerating a wide range of salinities [24,25]. Furthermore, it is cosmopolitan and a key species in the food web of estuaries and coastal waters [26].

MATERIALS AND METHODS

Copepods culture and acclimation

Copepods were cultivated according to the method described by Bersano [27]. The original lot of copepods (*Acartia tonsa*) was obtained from a permanent intensive culture at the University Aquaculture Marine Station (Rio Grande, RS, Southern Brazil). Copepods cultivated at salinity 30 ppt were transferred to our laboratory and kept in 10-L plastic buckets containing water and acclimated, for at least one week, to the desired experimental salinity (5, 15, or 30 ppt). Water at different salinities was prepared by mixing filtered (1-μm mesh) seawater collected at the Cassino Beach (Rio Grande, RS, Southern Brazil) with distilled water. Seawater used in the present study was previously analyzed and considered free of major contaminants, such as metals and organics (pesticides and hydrocarbons), being used for many years in the aquaculture facilities of the Federal University of Rio Grande (Rio Grande, Rio Grande do Sul, Brazil). Copepods were fed daily with a mixed algal diet composed of the diatoms *Thalassiosira weissflogii* (2×10^4 cells/ml) and *Isochrysis galbana* (1×10^4 cells/ml). Media were gently aerated and completely renewed every week. Room temperature was fixed at 20°C.

Acute toxicity tests

Toxicity tests (48-h median effective concentration value [EC50]) were performed using adult copepods. Prior to experiments, adult male and female copepods (total length 0.80 ± 0.09 mm, dry wt 9.0 ± 1.7 μg) were randomly collected from the culture using a 300-μm mesh net. Standard static-renewal procedures, as used for daphnid tests [28], were used for copepods, together with direct measurements of dissolved oxygen, pH, ion concentration (Na^+, Cl^-, K^+, SO_4^{2-}, Mg^{2+}, and Ca^{2+}), alkalinity, dissolved organic carbon, and total and dissolved (0.45-μm mesh filter) Cu concentrations in the experimental media.

For waterborne exposure, Cu as $CuCl_2$ (Merck) was added to water at the desired concentration from stock solutions (0.2, 2.0, or 20 g Cu/L) prepared with MilliQ® water acidified with 1% HNO_3 (Suprapur®; Merck). For waterborne exposure in the presence of non-Cu-equilibrated food, Cu was added to the water as described above for waterborne exposure. However, non-Cu-equilibrated food (*T. weissflogii*) was added to the experimental medium at a final concentration of 2×10^4 cells/ml. This concentration was selected based on the fact that saturation in *A. tonsa* egg production was observed under this feeding condition, indicating a maximum long-term physiological performance of copepods at this algae concentration [29]. Food was added to the experimental media 3 h prior to copepod introduction. Food was prepared by growing algae (*T. weissflogii*) for 3 d in f/2 medium. This is a common and widely used, general, enriched seawater medium designed for growing coastal marine algae, especially diatoms. The concentration of the original formulation, f medium, was reduced by half [30]. Growing media was prepared with filtered (1-μm mesh) and autoclaved saltwater at the desired experimental salinity (5, 15, or 30 ppt). After growth, log-phase cells were counted and added to the experimental medium to achieve the desired final algae cells concentrations (2×10^4 cells/ml).

For waterborne plus diet-borne exposure, Cu was added to the experimental medium as described above for waterborne exposure. However, Cu-equilibrated food was added to the experimental medium at a final concentration of 2×10^4 cells/ml. Food was added to the experimental media 3 h prior to copepod introduction. Copper-equilibrated food was prepared as previously described. It was shown that algae (*T. weissflogii*) were saturated with Cu after 24 h of exposure under similar experimental conditions [31]. Therefore, *T. weissflogii* was exposed for 24 h to Cu dissolved in saltwater at the desired experimental salinity. Copper concentrations used were the same as those employed for the copepod exposure to waterborne Cu. After 24 h of exposure, Cu-equilibrated algae cells were collected and added to fresh solutions of the experimental media, which were prepared as for the algal cells contamination. The final concentration of algal cells in the experimental media was 2×10^4 cells/ml.

For the dietborne exposure, Cu-equilibrated cells were prepared as described above and centrifuged (10,000 *g*). The supernatant was discarded, and the pellet of algae cells obtained was resuspended in freshly prepared saltwater at the desired experimental salinity (5, 15, or 30 ppt), and added (2×10^4 cells/ml final concentration) to the saltwater used for copepods exposure, without Cu addition. Measurements of Cu (total and dissolved) concentration in the experimental medium showed no significant metal leaching from the algae into the waterborne phase (data not shown).

For each salinity and different pathway of exposure, control tests were also run under the same experimental conditions as described above. However, no Cu or Cu-equilibrated food was added to the experimental media.

All experimental media were prepared 3 h prior to copepod introduction. All experiments were performed in duplicate using 50-ml glass flasks properly sealed, containing 50 ml experimental medium and 10 organisms in each. Flasks were maintained in an incubator and under constant rotation (2 rpm) to ensure food suspension and medium homogeneity. Temperature and photoperiod in the incubator were fixed at 20°C and 16:8 h light:dark, respectively. Every 24 h, surviving copepods

were counted and transferred using plastic pipettes to a new set of test solutions prepared 3 h before copepod introduction, as described above.

All materials used in the experiments had previously been acid washed in 1% HNO_3 (Merck) and rinsed thoroughly with distilled water before their use.

Water chemistry

At the start and after 24 h of exposure, nonfiltered and filtered (0.45-μm mesh) samples (10 ml) from the different experimental media were collected for Cu concentration measurements and water chemistry analyses, as described below. Those employed for Cu concentration measurements were immediately acidified (1% HNO_3).

Total and dissolved Cu concentrations in the experimental media were measured by atomic absorption spectrophotometry (AAS 932 Avanta-Plus; GBC), as previously described [31]. A Cu standard solution (Standard Reference Material® 3114) from the National Institute of Standards and Technology (Gaithersburg, MD, USA) was used to check the accuracy of measurements. The detection limit of the measurement was 10 μg /L. Na^+, K^+, and Ca^{2+} concentrations were measured by flame photometry (Micronal). Mg^{2+} and Cl^- concentrations were measured by spectrophometric methods using commercial reagent kits (Doles). Absorbance readings (490 nm) were made using a microplate reader (Bio-Tek Elx-800). Water pH and dissolved oxygen were measured using a pH meter (Digimed DM 20) and an oximeter (Digimed DMO-2), respectively. Sulfate concentration in the experimental media was measured by using the spectrophometric method described by Tabatabai [32]. Water alkalinity was determined according to the method described by the American Public Health Association [33]. Dissolved organic carbon was measured in filtered water samples using a total organic carbon analyzer (V_{CPN} series; Shimadzu).

Statistical analysis

Water chemistry data were expressed as mean ± standard deviation and were analyzed by one-way analysis of variance to detect possible differences between treatments. The significance level adopted was 95%.

Accumulated mortality data after 48 h of test were used to calculate the 48-h EC50 values, and their corresponding 95% confidence intervals were calculated by using Probit analysis. The EC50 values were calculated based on total and dissolved Cu concentrations as well as on the concentration and activity of the major Cu species. Copper speciation was performed with the physicochemical parameters and the dissolved Cu concentration measured in the absence of food (waterborne exposure) in Visual MINTEQ software version 2.61 (KTH, Department of Land and Water, Resources Engineering, Stockholm, Sweden). It is important to note that modeling results presented are tentative, insofar as modeled speciation is strongly dependent on the quality of the speciation model used. In this context, Visual MINTEQ is a Windows version of MINTEQA2 version 4.0, which was released by the U.S. EPA in 1999. MINTEQA2 is a chemical equilibrium model for the calculation of metal speciation, solubility equilibriums, etc., for natural waters. It is probably the most widespread model used for these purposes today, and it is renowned for its stability. In all cases, the EC50 values were considered different when the 95% confidence intervals did not overlap.

RESULTS

Water chemistry data for the different experimental media are shown in Table 1. Because no significant differences were observed between data in the absence and the presence of food in the water, only one mean value for each parameter was calculated in each salinity tested. For all parameters analyzed, the mean values significantly were augmented with increasing salinity (Table 1).

The overall mean concentration of total Cu among all the toxicity tests performed represented 87.8 ± 13.7% of the nominal concentrations of Cu added to the experimental media. In all experimental media, virtually all Cu in the water was present in the dissolved form, representing from 92.4 to 114.0% of the total measured Cu.

In all control treatments, copepod mortality was equal to or lower than 10% over the 48-h period of testing. In Cu-exposed copepods, acute Cu toxicity was salinity dependent, the highest toxicity being observed at the lowest salinity tested (5 ppt) in almost all treatments.

In the absence of food (waterborne exposure), the 48-h EC50 values calculated based on total Cu concentrations were 2.7- and 3.3-fold lower in salinity 5 than 15 and 30 ppt, respectively (Fig. 1A). Based on dissolved Cu concentrations, they were 2.1- and 2.7-fold lower in salinity 5 than 15 and 30 ppt, respectively (Fig. 1B). In all salinities, the most abundant Cu species were $CuCO_3$ and the free ion (Cu^{2+}), followed by $CuOH^+$. At salinity 30 ppt, an important amount of $Cu(CO_3)_2^{2-}$ was also observed (Fig. 2). In general, the 48-h EC50 values based on the free Cu (and Cu activity), $CuOH^+$, and $Cu(OH)_2$ concentrations did not show marked changes at the different experimental salinities. However, toxicity values based on $CuCO_3$, and $Cu(CO_3)_2^{2-}$ concentrations significantly increased with increasing salinities (Fig. 2).

Table 1. Water chemistry for the different experimental media employed to perform experiments with the copepod *Acartia tonsa*[a]

	Salinity (ppt)		
Parameter	5	15	30
pH	7.25 ± 0.05A	7.28 ± 0.03A	7.72 ± 0.03B
Dissolved oxygen (mmol O_2/L)	0.26 ± 0.002A	0.25 ± 0.001A	0.19 ± 0.002B
Na^+ (mmol/L)	70.5 ± 1.3A	240.0 ± 11.2B	462.0 ± 4.5C
Cl^- (mmol/L)	88.4 ± 1.0A	296.0 ± 13.8B	512.3 ± 15.6C
K^+ (mmol/L)	1.37 ± 0.07A	4.04 ± 0.15B	8.60 ± 0.72C
Ca^{2+} (mmol/L)	1.73 ± 0.00A	4.65 ± 0.31B	8.50 ± 0.58C
Mg^{2+} (mmol/L)	5.80 ± 0.02A	22.4 ± 0.70B	39.16 ± 3.17C
SO_4^{2-} (mmol/L)	1.11 ± 0.04A	4.40 ± 0.08B	7.68 ± 0.09C
Alkalinity (mmol $CaCO_3$/L)	0.38 ± 0.002A	1.06 ± 0.003B	2.41 ± 0.002C
Dissolved organic carbon (mg C/L)	0.129 ± 0.068A	0.736 ± 0.113B	1.172 ± 0.098C

[a] Different letters represent significant different mean values within salinities for each parameter.

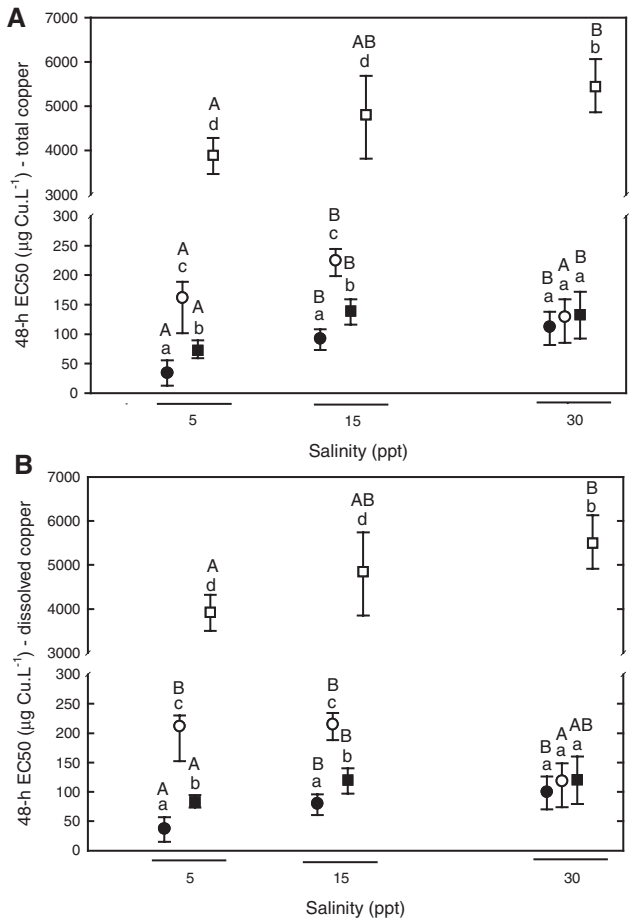

Fig. 1. Acute copper toxicity in the euryhaline copepod *Acartia tonsa* in a wide range of salinities. Four different pathways of Cu exposure were tested: waterborne Cu (Cu added to the water without food; solid circles), waterborne plus food (Cu and non-Cu-equilibrated food added to the water; open circles), waterborne plus diet-borne (Cu and Cu-equilibrated food added to the water; solid squares), and diet-borne (Cu-equilibrated food added to clean water; open squares). Data are expressed as 48-h median effective concentration values (EC50) and their corresponding 95% confidence intervals. Values were calculated based on total (**A**) and dissolved (**B**) Cu concentrations. Different lowercase letters indicate different 48-h EC50 values between treatments for the same salinity. Different capital letters indicate different 48-h EC50 values between salinities for the same treatment.

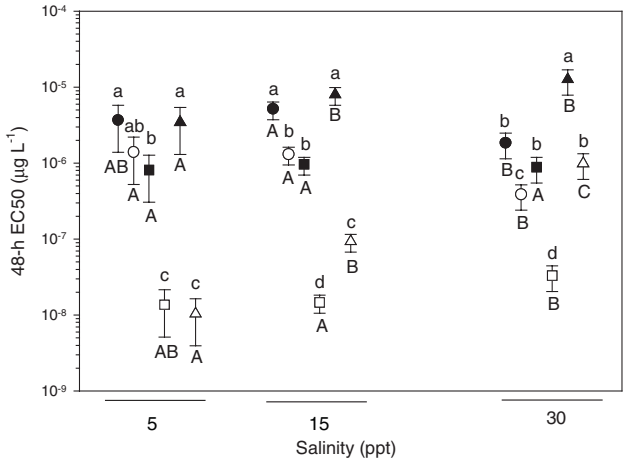

Fig. 2. Acute toxicity of different copper species in the euryhaline copepod *Acartia tonsa* in the absence of food in a wide range of salinities. Free Cu (solid circles), free Cu activity (open circles), $CuOH^+$ (solid squares), $Cu(OH)_2$ (open squares), $CuCO_3$ (solid triangles), and $Cu(CO_3)_2^{2-}$ (open triangles). Copper speciation was performed based on the 48-h median effective concentration values (EC50) and their corresponding 95% confidence intervals calculated based on dissolved Cu concentrations in the absence of food (waterborne Cu exposure). Data are expressed as 48-h EC50 values and their corresponding 95% confidence intervals. Different lowercase letters indicate different 48-h EC50 values between treatments for the same salinity. Different capital letters indicate different 48-h EC50 values between salinities for the same treatment.

In the presence of non-Cu-equilibrated food in the water (waterborne plus food), the influence of salinity on acute Cu toxicity was less marked, and a different pattern was observed when 48-h EC50 values were calculated using total and dissolved Cu concentrations. Based on total Cu concentrations, the 48-h EC50 value was 1.4-fold lower in salinity 5 than 15 ppt (Fig. 1A). However, no significant difference was observed when the 48-h EC50 values were calculated based on dissolved Cu concentrations. Based on total Cu concentrations, no significant difference was observed between the 48-h EC50 values in salinities 5 and 30 ppt, but the acute Cu toxicity was 1.6-fold lower in salinity 15 ppt (Fig. 1A). When dissolved Cu concentrations were considered, acute Cu toxicity was 1.8-fold lower in salinities 5 and 15 than 30 ppt (Fig. 1B).

When Cu-equilibrated food was added to the contaminated water (waterborne plus diet-borne exposure), 48-h EC50 values calculated based on total measured Cu concentrations were 1.9- and 1.6-fold lower in salinity 5 than 15 and 30 ppt, respectively (Fig. 1A). Based on total dissolved Cu concentration concentrations, acute Cu toxicity was 1.4-fold lower in salinity 5 than 15 ppt. However, no significant difference was observed between the 48-h EC50 values at salinities 5 and 30 ppt (Fig. 1B).

In the experiments in which copepods were exposed to Cu only via food (diet-borne exposure), the 48-h EC50 value calculated based on total Cu concentrations was 1.4-fold lower in salinity 5 than 30 ppt. No significant difference was observed between the 48-h EC50 values in salinities 5 and 15 ppt (Fig. 1A). Based on dissolved Cu concentrations, they were 1.2- and 1.4-fold lower in salinity 5 than 15 and 30 ppt, respectively (Fig. 1B).

The 48-h EC50 values were significantly higher in the presence of food (non-Cu-equilibrated or Cu-equilibrated) than in the absence of food in the water in salinities 5 and 15 ppt. However, food did not significantly alter the acute waterborne Cu toxicity in salinity 30 ppt (Fig. 1A,B).

DISCUSSION

In the present study, the influence of salinity and route of exposure on acute Cu toxicity was analyzed in the euryhaline copepod *A. tonsa* acclimated to different salinities (5, 15, and 30 ppt) together with measurements of water chemistry and Cu concentrations in the experimental media. Toxicity data (48-h EC50 values) clearly show a protective effect of salinity against the acute Cu toxicity both in the absence and in the presence of food. This protective effect is very marked in the range of salinities from 5 to 15 ppt, being less important in the range of salinities from 15 to 30 ppt. A protective effect of salinity against the acute Cu toxicity was previously reported in the literature for several euryhaline crustacean species, including *A. tonsa*. However, those studies were always performed in the absence of food [31,34]. Considering a possible future extension of the BLM for Cu to estuarine and marine conditions, our results combined with those reported in the literature for other euryhaline crustacean species (isopods and crabs) under similar

experimental conditions [34] clearly indicate that the copepod *A. tonsa* would be much more suitable to validate and calibrate an ''estuarine and marine BLM.'' This statement is based on the fact that copepods were much more sensitive to acute waterborne Cu exposure than isopods and crabs. Despite the fact that they are less sensitive to Cu than other organisms in seawater, such as developing embryos of blue mussels [35], they have great advantages for development of a saltwater BLM because they are truly euryhaline.

The protective effect of salinity against the acute Cu toxicity can be explained by considering the water chemistry of the experimental media employed for waterborne Cu exposure. For example, Cu complexation with dissolved organic matter and inorganic ions (anions) is augmented as salinity increases because of an increasing higher dissolved organic carbon and ion concentration. In this situation, less unbound Cu is available, decreasing its accumulation and consequent toxicity [36]. Furthermore, the cationic competition from Na^{+}, Ca^{2+}, Mg^{2+}, K^{+}, and Sr^{2+} for Cu^{2+} binding sites is stronger in saltwater than in freshwater, thus reducing the toxic effect of Cu on the biotic ligand. In summary, the salinity-dependent pattern of the acute waterborne Cu toxicity observed in the present study and reported in the literature for other crustacean species can be attributable to changes in water chemistry according to the environmental salinity. This idea is strongly supported by the fact that the 48-h EC50 values calculated based on the most toxic Cu species (the free ion) and its activity did not show marked changes over a wide range of salinities (5–30 ppt).

In addition to the salinity effect on Cu speciation and availability salinity can also affect the physiology of invertebrates through the demands of ionic regulation. Some aquatic invertebrates (e.g., estuarine invertebrates) respond mechanistically to salinity changes (i.e., osmoregulation) in such a way that their physiological responses interact with changes in free metal ion availabilities to control trace metal uptake rates [36]. Depending on the osmoregulatory physiology of the species, water and ions (including metals) are exchanged between the organism and the external medium at various rates in relation to the environmental salinity. An example of the possible control of trace metal uptake during osmoregulation has been demonstrated in the hyperbenthic mysid *Neomysis integer*. In this species, Cd toxicity was reduced at the salinity corresponding to the isosmotic point [37]. The authors concluded that reduced ionic exchange associated with osmoregulation led to a decreased uptake of metal from solution, suggesting that metal uptake may be mediated by normal cation transport mechanisms.

For the copepod *A. tonsa*, there is some evidence that metal uptake may indeed be mediated by osmoregulatory mechanisms. This hypothesis is based on the facts that the isosmotic point of this copepod is about salinity 36 ppt (3.2% of NaCl) [20] and that Cu uptake is reduced at salinity 36 ppt in this species [36]. In accordance with these findings, the lowest acute waterborne Cu toxicity was observed in salinity 30 ppt in the present study, the highest salinity tested and the nearest to the isosmotic point of the species.

In addition to the salinity protection against acute Cu toxicity discussed above, data reported in the present study indicate that the presence of food markedly decreased the acute Cu toxicity in low salinities (5 and 15 ppt). This protective effect at lower salinities was observed in the two treatments in which algae cells were added to the water as non-Cu-equilibrated food and Cu-equilibrated food. However, the protective effect was more effective when non-Cu-equilibrated food was added to the water. These findings suggest that the protection against acute waterborne Cu toxicity by food could be associated with water chemistry changes, with algae cells acting as a biotic ligand taking Cu from the medium, thus reducing the amount of metal available for copepods. However, no significant changes in water chemistry were observed among the various routes of Cu exposure employed in the present study for each salinity. Furthermore, a study reporting the effects of chronic dietary Cu exposure on growth and reproduction of the freshwater invertebrate *Daphnia magna* [38] demonstrated that both pathways (waterborne and waterborne plus diet-borne) of metal exposure were toxic to reproduction in the highest Cu concentration tested. However, with Cu concentrations below the highest one tested in that study, diet-borne and waterborne plus diet-borne exposures were actually beneficial to reproduction in *D. magna*, whereas only waterborne Cu did not present this favorable effect. Except in the highest Cu concentration tested, dietary Cu clearly resulted in an increased Cu uptake, but without toxic effects. Therefore, other aspects must be involved in the protective role of food against the acute waterborne Cu toxicity. Some physiological aspects are considered and discussed below.

An important physiological aspect directly associated with the stress susceptibility in aquatic invertebrates is the nourishment condition of the affected animals. For instance, it has been shown that copepods are more tolerant to salinity variations, especially in low salinities, when fed than when starved [26]. We have reported that *A. tonsa* show higher rates of food (algae cells) consumption in low salinities than in seawater [31]. Also, the metabolism of *A. tonsa* has been reported to be higher in low salinities than in seawater [39]. Finally, it has been shown that *A. tonsa* is a hyperosmoregulator in low salinities but an osmoconformer in seawater [24,25]. Taken together, these findings clearly indicate that *A. tonsa* requires extra ions and energy in low salinities to counteract actively the ion losses from the animal to the diluted media at energy expenses. In this case, the presence of food in the experimental medium would be providing the extra ions and energy required by copepods to cope with the stressful condition imposed by the osmotic gradient and the acute waterborne Cu exposure in low salinities. In fact, we have demonstrated that the continuous presence of food in the water effectively blocks the ionic disturbance induced by waterborne Cu in *A. tonsa* exposed in high salinities (15 and 30 ppt) and reduces the Cu effect in low salinity (5 ppt) [31]. Furthermore, we have demonstrated that the continuous presence of food in the water also completely blocks the chronic inhibitory effect of Cu on *A. tonsa* reproduction (Pinho et al., unpublished data). The fact that food markedly protected against the waterborne Cu toxicity in low salinity (5 ppt) and did not alter this toxicity in seawater (salinity 30 ppt) in the present study is also in complete agreement with the findings discussed above.

For marine herbivores, including the copepod *A. tonsa*, it has been demonstrated that Cu can be accumulated from both waterborne and diet-borne exposure. Thus, it would be expected that simultaneous Cu accumulation from both dissolved phase and food would increase the whole-body Cu accumulation and consequently the acute Cu toxicity in the copepod *A. tonsa*. However, an opposite response was observed, insofar as acute Cu toxicity was lower in the presence than in the absence of Cu-equilibrated food at low salinities (5 and 15 ppt). Furthermore, no significant changes were observed at the highest salinity (30 ppt). This lack of increased toxicity in the presence of food could be related to the differential metal distribution in internal

organs after exposure via different pathways, resulting in different degrees of toxicity [40]. Thus, if more Cu is accumulated after the waterborne plus diet-borne Cu exposure, this metal is either binding on molecules associated with metal detoxification (e.g., metallothioneins, glutathione, etc.) or on sites not directly related to acute Cu toxicity. Finally, the extremely high Cu concentrations needed to contaminate food with a Cu level high enough to induce toxicity in *A. tonsa* after diet-borne exposure clearly indicate that food is acting much more as a protective factor against the acute Cu toxicity than as a vector of Cu contamination and toxicity in *A. tonsa.* In this context, the protective influence of food could be incorporated in a future saltwater BLM version considering the amount of energy and/or carbon in the natural food source needed for the tested species to achieve a maximum physiological performance (osmoregulation, growth, and reproduction) at different ambient salinities. For example, the concentration of 20×10^3 algae cells $\cdot$ ml^{-1} ($\sim$500 mg C $\cdot$ L^{-1}) corresponded to the amount of energy/carbon needed for copepods *A. tonsa* to cope with osmotic stress, to block acute and chronic disturbances induced by waterborne Cu, and to achieve maximum egg production and hatching [29,31], as discussed above.

In conclusion, we report in this paper, for the first time, data on acute Cu toxicity resulting from different methods of metal exposure in a euryhaline invertebrate in a wide range of salinities. Data obtained strongly indicate that water chemistry (salinity) has an important protective role against the acute waterborne Cu toxicity in the euryhaline copepod *A. tonsa*. Moreover, we show that food is a protective factor against the acute waterborne Cu toxicity rather than an important vector of Cu contamination and toxicity in *A. tonsa*.

The findings described in the present paper will be valuable for a better understanding of the relationship among salinity, food, and Cu speciation, bioaccumulation, and toxicity in euryhaline invertebrates. Thus, they will be useful for the extension of a future version of the Cu BLM for estuarine and marine conditions, and the consequent generation of BLM-derived water quality criteria for Cu to be applied in estuarine and marine waters. Our data strongly suggest that the euryhaline copepod *A. tonsa* would be a suitable species with which to validate and calibrate this future BLM version.

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