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CHRONIC COPPER TOXICITY IN THE ESTUARINE COPEPOD *ACARTIA TONSA* AT DIFFERENT SALINITIES

MARIANA M. LAUER and ADALTO BIANCHINI*
Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Campus Carreiros, Rio Grande do Sul, Brazil

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Abstract—Chronic Cu toxicity was evaluated in the euryhaline copepod *Acartia tonsa*. Male and female copepods were exposed (6 d) separately to different combinations of Cu concentration and water salinity (5, 15, and 30 ppt) using different routes of exposure (waterborne, waterborne plus dietborne, and dietborne). After exposure, groups of one male and three female copepods were allowed to reproduce for 24 h. In control copepods, egg production augmented with increasing water salinity. However, egg hatching rate did not change. Copper exposure reduced egg production and hatching rate in all water salinities tested, but the reproductive response was dependent on the route of Cu exposure. Median effective concentration (EC50) values for egg production after waterborne exposure were 9.9, 36.8, and 48.8 μg/L dissolved Cu at water salinities of 5, 15, and 30 ppt, respectively. For waterborne plus dietborne exposure, they were significantly higher (40.1, 63.7, and 109.9 μg /L, respectively). After dietborne exposure, approximately 40% decrease in egg production was observed, independently of Cu concentration and water salinity tested. At water salinities of 5 and 30 ppt, egg hatching rate reduced after waterborne exposure, together or not with the dietborne exposure. At water salinity of 15 ppt, Cu toxicity was only observed after dietborne exposure. Data indicate that egg production is a more reliable reproductive endpoint to measure chronic Cu toxicity in copepods than egg hatching rate in a wide range of water salinities. They also suggest that both water salinity and route of Cu exposure should be taken into account in the development of a chronic biotic ligand model version for estuarine and marine environments. Environ. Toxicol. Chem. 2010;29:2297–2303. © 2010 SETAC

Keywords—*Acartia tonsa* Biotic ligand model Copper Salinity Reproduction

INTRODUCTION

Copper is an essential metal to aquatic animals, including crustaceans. However, it can be toxic when in elevated concentrations in water or food. Several models were developed to predict its bioavailability and toxicity in freshwater. The biotic ligand model approach takes into account the competition of cations, such as H^+, Ca^{2+} and Na^+, with the free metal ion for binding at the hypothesized biotic ligand, which is the site of action of the metal [1–3]. The biotic ligand model also considers the free metal ion complexation with organic ligands, such as the dissolved organic carbon, or inorganic ones, such as OH^-, CO_3^-, and Cl^-.

Because water chemistry varies between freshwater, brackish water, and saltwater, changes in Cu toxicity according to water salinity would be expected. In fact, studies performed to determine the influence of salinity on acute Cu toxicity showed higher Cu sensitivity in lower water salinities [4]. It was also reported that increasing water salinity is protective against the acute Ag toxicity in the euryhaline copepod *Acartia tonsa* [5]. It is important to note that waterborne Cu and Ag show a similar mechanism of toxicity in freshwater animals [6].

Even if the present freshwater biotic ligand model version could be used to predict copper bioavailability and toxicity in a wide range of salinities, it is important to consider that this model only takes into account metal uptake from the dissolved phase and after acute exposure. Therefore, this valuable model needs to be adapted to incorporate important parameters associated with sublethal responses to Cu exposure, such as those related to reproductive impairments after chronic exposure to waterborne and dietborne Cu. In this context, it was reported that zooplankton exposed to dietary metals (Ag, Hg, and Cd) showed an approximately 50% decrease in egg production and hatching rate [7,8]. Total reproduction and brood size of the saltwater cladoceran *Moina monogolica* were also shown to be affected by chronic exposure to dietary Cu [9]. Although dietary exposure is important, effects induced by Cu accumulated from the dissolved phase after chronic exposure cannot be ruled out. In fact, it has been suggested that herbivorous marine zooplankton accumulate Cu mainly by trophic transfer, but almost 60% of the total Cu accumulated in these animals has shown to be assimilated from the dissolved phase [10]. Usually, accumulation from the dissolved phase occurs by direct absorption through body surfaces, while particulate metals can be accumulated following ingestion and digestion of food [11].

Copepods have a great capacity to accumulate trace metals from contaminated waters, with bioconcentration factors ranging from 4 to 7. Therefore, they can serve as bioindicators to assess metal contamination in the aquatic environment [12]. These organisms are the dominant marine zooplankton in pelagic systems, because they are the primary consumers in food webs in estuarine and marine systems. The copepod *A. tonsa* is a cosmopolitan species found in temperate regions and shows a high degree of tolerance to environmental changes, especially water salinity [13].

In light of these facts, the main goal of the present study was to evaluate the Cu toxicity on the reproduction of the euryhaline copepod *A. tonsa* in a wide range of water salinities after chronic exposure to waterborne, dietborne, and waterborne plus dietborne Cu.

* To whom correspondence may be addressed (adaltobianchini@furg.br).
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MATERIALS AND METHODS

Algae culture

Cultures of the diatom *Thalassiosira weissflogii* were held at the desired experimental water salinities (5, 15, and 30 ppt) to feed adult copepods during rearing and toxicity tests. The diatom *Isochrysis galbana* was also cultured, but it was used only as a food source for nauplii and small copepodits. The algal medium consisted of F/2 medium [14] prepared in filtered (1-μm mesh filter) saltwater at the desired salinity. Water at different salinities (5, 15, and 30 ppt) was prepared by diluting the filtered seawater with distilled water. Seawater used was collected at Cassino Beach (Rio Grande, RS, Southern Brazil). It was previously analyzed and considered free of major contaminants, such as metals and organics (pesticides and hydrocarbons). Physicochemical parameters of this seawater were previously reported [5,15–17]. Algae were kept under constant white fluorescent lights at 20°C with mild continuous aeration, for no more than 7 d. Algal density was measured using a 0.1-mm depth hemocytometer (Neubauer chamber; Laboroptik).

Algae copper exposure

For dietborne and waterborne plus dietborne exposures, copper (as $CuCl_2$) was added to *T. weissflogii* culture (40×10^4 cells/ml) at different concentrations and allowed to equilibrate for 24 h. Procedures for algae exposure and analysis were performed as previously described [17]. Copper concentrations used to expose algae used in the waterborne plus dietborne exposure were the same employed to contaminate the experimental media where copepods were exposed (Table 1). Those used to expose algae employed in the dietborne exposure were 0 (control: no addition of Cu to the water), 40, 80, and 160 μg Cu/L for all water salinity tested. These concentrations were selected based on the concentrations of Cu that kill 50% of tested copepods after 48 h of exposure (48-h LC50), which previously had been determined under the same experimental conditions used in the present study [15,17].

Algae used in the dietborne Cu exposure were previously centrifuged (5 min; 1,000 *g*) and suspended with 3 ml of clean saltwater at the corresponding salinity to remove the excess of Cu adsorbed onto the algal surfaces, ensuring that metal effects observed were only associated with Cu assimilated from food.

Copepod cultivation and acclimation

Adult copepods (*A. tonsa*) were obtained from cultures of the Laboratory of Zooplankton of the Universidade Federal do Rio Grande (Rio Grande, RS, Southern Brazil) and cultivated as previously described [5,15–17]. Briefly, copepods were held in 20-L plastic buckets containing water at salinity of 15 or 30 ppt under mild continuous aeration. Temperature and photoperiod were fixed at 20°C and 16:8 h light:dark, respectively. Copepods were daily fed a mix of the diatoms *T. weissflogii* (2×10^4 cells/ml) and *I. galbana* (1×10^4 cells/ml). For tests at water salinity of 5 ppt, copepods from cultures developed in water at salinity of 15 ppt were gradually acclimated to the experimental salinities for at least two weeks.

Copepod exposure to copper

Male and female copepods (*A. tonsa*) were separately exposed to Cu for 6 d through different routes of exposure (waterborne, waterborne plus dietborne, and dietborne). Every day, copepods were exposed to Cu for 12 h, and then transferred and kept in clean water for a further 12 h. For waterborne Cu exposure, they were exposed (12 h) to Cu-contaminated water in the absence of food, and then transferred and kept (12 h) in clean water in the presence of noncontaminated food. For waterborne plus dietborne Cu exposure, copepods were exposed (12 h) to both water and food previously contaminated with Cu, and then transferred and kept (12 h) in clean water in the absence of food. For dietborne exposure, copepods were kept (12 h) in clean water in the presence of food previously contaminated with Cu, and then transferred and kept (12 h) in clean water in the absence of food.

In all treatments, three different Cu concentrations were tested at each experimental water salinity (5, 15, or 30 ppt) along with the respective control (water and food not contaminated with Cu). The 12-h feeding protocol was also used to perform the control tests. This feeding protocol was selected as a way to standardize the feeding regime between Cu exposure pathways and to insure a good reproductive performance of copepods. It is important to note that the 12-h feeding period with change of the experimental media was adopted for all experimental conditions because copepods exposed to waterborne Cu only should be fed with clean food to avoid mortality over the 6-d experimental period. Introduction of clean food in

Table 1. Copper concentrations (μg Cu/L) in the experimental media employed to evaluate the effect of copper on reproduction endpoints in the euryhaline copepod *Acartia tonsa* in different water salinities using different routes of exposure[a]

Water salinity	Route of exposure							
	Waterborne copper concentration				Waterborne plus dietborne copper concentration			
	Control	C1	C2	C3	Control	C1	C2	C3
5 ppt								
Nominal	0	5	10	20	0	20	40	60
Total	7.1 (±0.2)	12.2 (±0.3)	15.9 (±0.3)	28.3 (±0.5)	7.7 (±0.4)	26.6 (±0.2)	32.1 (±0.1)	47.4 (±0.6)
Dissolved	6.8 (±0.1)	10.3 (±0.4)	13.6 (±0.3)	27.9 (±0.7)	6.5 (±0.2)	24.4 (±0.5)	30.0 (±0.4)	41.6 (±0.4)
15 ppt								
Nominal	0	20	40	100	0	20	40	100
Total	12.7 (±0.3)	30.3 (±0.2)	36.1 (±0.5)	71.7 (±1.2)	12.0 (±0.5)	33.3 (±1.0)	35.2 (±0.8)	76.9 (±1.6)
Dissolved	11.5 (±0.4)	30.1 (±0.5)	33.9 (±0.6)	69.4 (±0.9)	11.3 (±0.3)	32.8 (±0.7)	34.9 (±1.1)	69.9 (±1.3)
30 ppt								
Nominal	0	40	80	160	0	40	80	160
Total	14.2 (±0.8)	39.0 (±1.3)	66.9 (±1.9)	127.4 (±3.4)	14.9 (±0.7)	35.0 (±0.6)	64.8 (±1.4)	118.7 (±2.3)
Dissolved	12.9 (±0.6)	36.6 (±1.7)	64.9 (±1.5)	116.4 (±2.7)	14.5 (±0.6)	34.1 (±0.9)	62.9 (±1.9)	101.3 (±2.8)

[a] For all salinities, water copper concentrations used to pre-expose algae employed in the dietborne exposure were 0 (Control), 40 (C1), 80 (C2), and 160 (C3) μg Cu/L. No copper leakage from algae was observed in the dietborne exposure. Data for total and dissolved copper concentrations are expressed as mean ± standard deviation ($n = 10$).

the water contaminated with Cu could alter the metal concentration in the experimental media, an effect associated with both adsorption and absorption of Cu by algae. Food was provided concomitantly in all treatments. Furthermore, the experimental factors were the same in the two 12-h exposure periods for all the tests. These procedures were adopted to avoid possible changes in feeding behavior, and consequently in the development, growth, and reproduction of the tested copepod.

Different Cu concentrations were used according to the water salinity because a higher toxicity was expected in low water salinity (Table 1). As previously mentioned, Cu concentrations were selected based on the acute and chronic 48-h LC50 values for Cu previously determined under the same experimental conditions used in the present study [15,17].

Experiments were run as previously described [15,17]. Briefly, they were performed using glass flasks containing 50 ml of the experimental medium with 10 copepods in each flask. For waterborne or waterborne plus dietborne copper exposures, copper (as $CuCl_2$) was added to the experimental media at least 3 h prior to copepod's introduction. For waterborne plus dietborne or dietborne Cu exposures, algae were previously contaminated with Cu, as described above, and added to the experimental media at least 3 h prior to copepod's introduction. For each combination of Cu concentration, water salinity, and route of exposure, seven and three replicates were run for females and males, respectively. Flasks were kept under constant rotation (2 rpm) to avoid food deposition. Flasks were kept in an incubator with fixed temperature (20°C) and photoperiod (16:8 light:dark). Every 12 h, surviving copepods were collected using plastic pipettes and transferred to a fresh experimental medium, prepared as described above, considering the route of exposure being tested.

After 6 d of exposure, five groups of one male and three females were formed. Copepods were then allowed to reproduce in noncontaminated medium at the respective experimental salinity. Copepods were kept in glass flasks under constant rotation (2 rpm), as described above. During the reproductive period, they fed noncontaminated food (*T. weissflogii*; 2×10^4 cells/ml). Nauplii and eggs produced over 24 h were counted using a stereoscopic microscope. Results were expressed as the number of eggs produced per female per day.

Eggs ($n = 10$–20) from each reproduction group were collected and individually kept in a culture plate well containing clean water at the respective experimental salinity. After 24 h, hatched eggs in each plate were counted. Egg hatching rate was determined considering the total number of eggs tested. Results were expressed as percentages.

Water samples

Before and after Cu exposures, nonfiltered and filtered (0.45-μm mesh filter) samples (10 ml) of each experimental medium were collected and acidified (1% HNO_3) for total and dissolved Cu concentration measurements, respectively. Copper concentration was measured by atomic absorption spectrophotometry (AAS 932 Avanta-Plus; GBC), as previously reported [15,17].

Statistical analysis

Copper accumulation data in algae exposed to different concentrations of Cu in the dissolved phase were expressed as mean ± standard error (SE) ($n = 5$). Data was analyzed by linear regression analysis for each salinity.

Egg production data were expressed as mean ± SE ($n = 5$). Differences between mean values for control and Cu-exposed copepods at the different water salinities were assessed by one-way ANOVA followed by the Tukey's test. Comparisons of mean values were performed only between Cu concentrations for the same water salinity and route of Cu exposure because different Cu concentrations were tested at different water salinities and routes of exposure. Percentage of inhibition in egg production was calculated considering the mean number of eggs produced per female per day under control conditions as 100%. The percentage values were used to determine the Cu concentration inducing 50% decrease in copepod reproduction after 6 d of exposure (6-d EC50) and its respective 95% confidence interval for each experimental condition. Once all mean inhibition values were lower than 50% after the dietborne Cu exposure, 6-d EC50 values were calculated only for the waterborne and the waterborne plus dietborne Cu exposures. The EC50 values and their corresponding 95% confidence intervals were calculated based on total measured and dissolved Cu concentrations using Probit analysis [18]. Differences between EC50 values were analyzed by visually comparing their 95% confidence intervals. Differences between mean values of egg production after dietborne Cu exposure were assessed by two-way ANOVA followed by the Tukey's test.

For each treatment, egg hatching rate data were expressed as mean ± SE ($n = 5$). No EC50 values were calculated for this endpoint because in most cases the observed effect was lower than 50%. Therefore, differences between mean values were assessed by one-way ANOVA followed by Tukey's test. Percentage values were mathematically transformed (arcsine square root transformation) before ANOVA. As for egg production, comparisons of mean values were performed only between Cu concentrations for the same water salinity and route of Cu exposure because different Cu concentrations were tested at different water salinities and routes of exposure. In all cases, the significance level adopted was 95% ($\alpha = 0.05$).

RESULTS

Copper accumulation in algae cells followed a positive linear relationship as a function of the dissolved Cu concentration in the experimental medium. Increase in algae Cu concentration was higher in intermediate water salinity (15 ppt) than in low (5 ppt) and high (30 ppt) water salinities. The lower Cu accumulation was observed in water salinity of 30 ppt (Fig. 1).

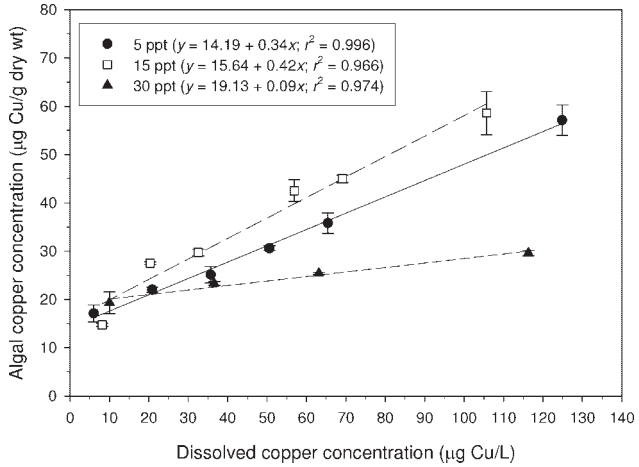

Fig. 1. Copper accumulation in the diatom *Thalassiosira weissflogii* after 24 h of exposure in different water salinities. Data are expressed as mean ± standard deviation ($n = 5$).

In control copepods, the number of eggs produced per female per day was dependent on the water salinity, augmenting with increasing salinity. Egg production was significantly higher (1.62-fold) in water salinity of 30 ppt than in water salinity of 5 ppt (Fig. 2).

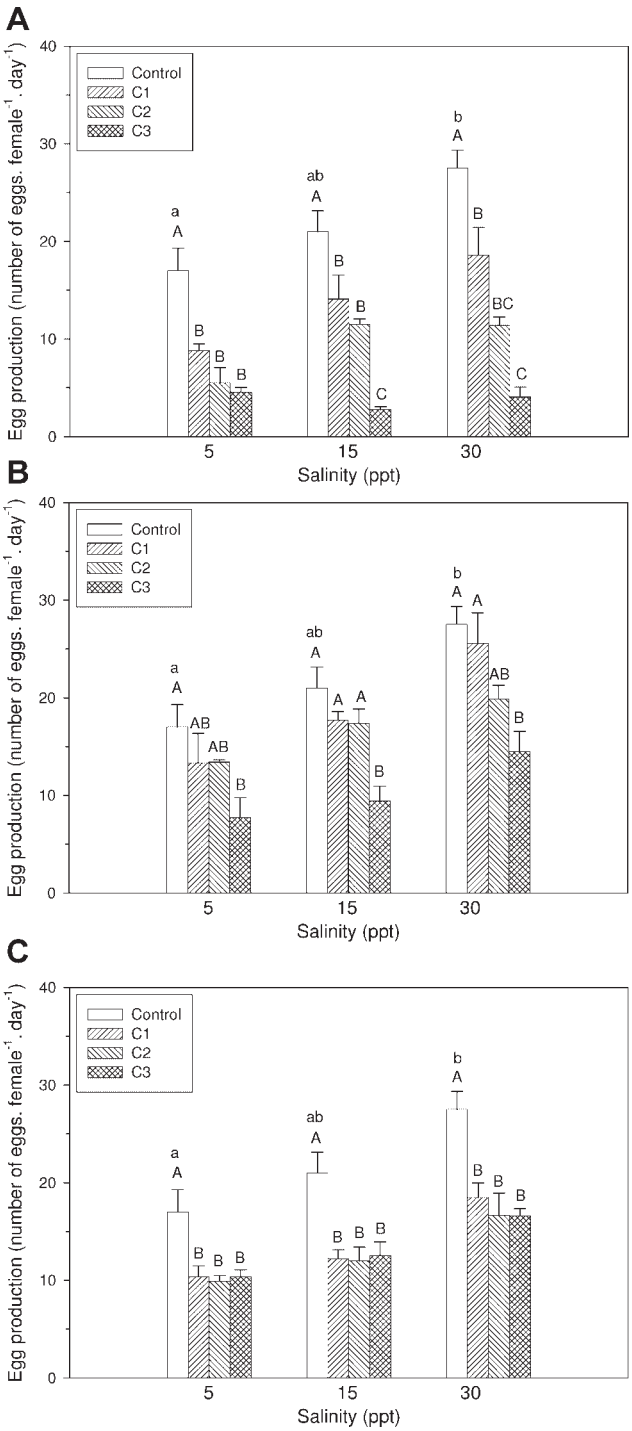

Fig. 2. Egg production in the copepod *Acartia tonsa* kept in clean water or exposed to copper through different routes of exposure in different water salinities: waterborne copper exposure (**A**); waterborne plus dietborne copper exposure (**B**); and dietborne copper (**C**). Copper concentrations tested (Control, C1, C2, and C3) as defined in Table 1. Different small letters indicate significantly different mean values between salinities for control copepods. Different capital letters indicate significantly different mean values between copper concentrations for the same route of exposure and water salinity.

After waterborne Cu exposure (Fig. 2A), egg production by copepods was significantly reduced in all concentrations and salinities tested. In general, inhibition in egg production was inversely related to the Cu concentration in water. At water salinity of 5 ppt, 48, 68, and 73% inhibition was observed when copepods were exposed to nominal Cu concentrations of 5, 10, and 20 μg Cu/L, respectively. At water salinity of 15 ppt, these values were 33, 45, and 87% when copepods were exposed to nominal concentrations of 20, 40, and 100 μg Cu/L, respectively. At water salinity of 30 ppt, they were 32, 59, and 85% when copepods were exposed to nominal Cu concentrations of 40, 80, and 160 μg Cu/L, respectively.

After waterborne plus dietborne Cu exposure (Fig. 2B), egg production by copepods was inversely related to Cu concentration in all salinities tested. At water salinity of 5 ppt, 22, 21, and 55% inhibition was observed when copepods were exposed to nominal Cu concentrations of 20, 40, and 60 μg Cu/L, respectively. At water salinity of 15 ppt, these values were 16, 17, and 55% when copepods were exposed to nominal concentrations of 20, 40, and 100 μg Cu/L, respectively. At water salinity of 30 ppt, they were 7, 28, and 47% when copepods were exposed to nominal Cu concentrations of 40, 80, and 160 μg Cu/L, respectively.

After dietborne Cu exposure, copepod egg production was significantly reduced in all concentrations and salinities tested. However, no significant differences were observed between copepods exposed to the different Cu concentrations at the same water salinity (Fig. 2C). At water salinity of 5 ppt, 39, 42, and 39% inhibition was observed when copepods were fed with algae precontaminated at nominal Cu concentrations of 40, 80, and 160 μg Cu/L, respectively. At water salinity of 15 ppt, these values were 42, 43, and 40%, respectively. At water salinity of 30 ppt, they were 33, 40, and 40%, respectively.

Six-day EC50 values for waterborne Cu exposure were always significantly lower than those for waterborne plus dietborne exposure. In both cases, toxicity decreased (increased 6-d EC50 value) with increasing water salinity. A similar result was observed when 6-d EC50 values were calculated based on total or dissolved Cu concentrations. For each treatment (waterborne Cu and waterborne plus dietborne Cu exposure), no significant difference was observed between the 6-d EC50 value estimated based on total measured Cu concentrations and that estimated based on dissolved Cu concentrations (Table 2). For copepods exposed to dietborne Cu, it was not possible to calculate the 6-d EC50 values, because egg production inhibition was not significantly different at the different Cu concentrations tested in each water salinity. Furthermore, this inhibition was never higher than 43%.

Figure 3 shows the egg hatching rates for all treatments. In control copepods, no significant difference was observed between water salinities. After the waterborne Cu exposure in water of salinity 5 ppt, significant decreases of 50 and 64% in egg hatching rate were observed for copepods exposed to 10 and 20 μg Cu/L, respectively (Fig. 3A). After the waterborne plus dietborne Cu exposure in water of salinity 5 ppt, significant decreases of 45 and 65% in egg hatching rate were observed for copepods exposed to 40 and 60 μg Cu/L, respectively (Fig. 3B). After the dietborne exposure in water of salinity 15 ppt, significant decreases of 60, 70, and 55% in egg hatching rate were observed for copepods fed with algae exposed to 40, 80, and 160 μg Cu/L, respectively (Fig. 3C).

Table 2. Concentration of copper (μg Cu/L) inducing 50% inhibition in egg production in the copepod *Acartia tonsa* after 6 days of exposure (6-d EC50) to the metal through different routes of exposure in different water salinities[a]

	Water salinity (ppt)		
Route of exposure	5	15	30
Waterborne			
Total copper	12.0 (9.3–13.3)A[b]	38.4 (35.0–41.8)B[b]	51.9 (44.9–58.8)C[b]
Dissolved copper	9.9 (6.9–11.3)A[b]	36.8 (33.6–40.1)B[b]	48.8 (42.3–55.0)C[b]
Waterborne plus dietborne			
Total copper	45.2 (40.5–54.7)A	69.2 (60.1–85.6)B	125.6 (101.4–176.9)C
Dissolved copper	40.1 (36.3–47.9)A	63.7 (56.5–76.4)B	109.9 (90.3–150.9)C

[a] Values are calculated based on total measured and dissolved copper concentrations. Numbers in parenthesis represent the 95% confidence interval. Letters A–C indicate significant different 6-d EC50 values between water salinities for the same route of exposure and copper fraction. No significant difference was observed between values calculated based on total and dissolved copper concentrations for the same salinity and route of exposure.
[b] Indicates significant difference between routes of exposure for the same water salinity and copper fraction.

DISCUSSION

Data from the chronic tests performed in the present study clearly indicate that both salinity and Cu exposure affect the reproductive performance of the euryhaline copepod *A. tonsa*. Also, data obtained showed that chronic Cu effects on reproductive endpoints are dependent on the metal exposure pathway, because differences in toxicity were observed between the different routes of exposure.

Regarding salinity, no significant effect was observed on egg hatching rate. Only few studies have examined the effect of salinity on egg hatching rate, and it still remains unclear. In contrast to the present study, egg hatching rate in *A. tonsa* from a Baltic population increased with increasing salinity, being maximal at salinity 25 ppt [19]. Egg hatching rates of 55 and 78% were observed at salinity 15 ppt for North Sea [20] and Baltic Sea populations [19], respectively. In the present study, the egg hatching rate was similar to that found for the Baltic Sea populations, being 77 and 84% in water of salinities 15 and 30 ppt, respectively.

Copepods kept under control conditions (no addition of Cu in water or food) showed a higher egg production at water of salinity 30 ppt than at water of salinity 5 ppt. Castro-Longoria [21] reported that species from the *Acartia* genus produced fewer eggs in low salinity. Furthermore, *A. tonsa* spawning rate decreased when water salinity was reduced from 30 to 10 ppt [22]. Other copepod species also showed a similar pattern. For example, *Pseudodiaptomus annandalei* showed a higher egg production in water of salinities 15 and 20 ppt than in water of salinities 5 and 10 ppt [23]. *Nitocra affinis* also produced more eggs in high salinities (30 and 35 ppt) than in intermediate (15, 20, and 25 ppt) and low (5 and 10 ppt) salinities [24]. An explanation for this decreased egg production in reduced salinities could be an increased metabolic rate associated with the ionic and osmoregulatory challenges imposed by the diluted media [25,26]. Because egg production in copepods represents the difference between energy inputs and metabolic costs [27], it appears that less energy was available for reproduction in the copepod *A. tonsa* in low salinities.

Considering that salinity affected reproduction, Cu effects were determined in a wide range of salinities (5 to 30 ppt) to evaluate a possible combined effect of both parameters (salinity and Cu) on the reproduction of the euryhaline copepod *A. tonsa* after chronic exposure (6 d). Furthermore, three different ways of exposure to Cu (waterborne, waterborne plus dietborne, and dietborne exposure) were analyzed.

A decrease in egg production was observed after Cu exposure, disregarding the route of exposure adopted. However, waterborne Cu was more toxic than dietborne Cu. This general picture was observed in the whole range of salinities tested, with Cu being more toxic in water of salinity 5 ppt and less toxic in water of salinity 30 ppt, after waterborne or waterborne plus dietborne Cu exposure. Nevertheless, Cu toxicity after dietborne exposure was not affected by water salinity or Cu concentration. In all experimental salinities, egg production was inhibited by approximately 40%, disregarding the Cu concentration tested. Because Cu concentrations measured in water from the dietborne exposure were similar to those from water collected at the control treatments (Table 2), it can be assumed that no Cu leaching from food to water occurred, indicating that the toxicity observed was only due to the dietary Cu.

Regarding egg hatching rate, Cu exposure also affected this reproductive parameter. In water of salinities 5 and 30 ppt, waterborne and waterborne plus dietborne exposures significantly decreased the egg hatching rate. In water of salinity 15 ppt, a significant inhibition was observed after the dietborne Cu exposure.

Many studies showed that dietary metals affect the reproductive success of invertebrates, but only a few have investigated the possible influence of the different ways of exposure on the chronic toxicity. In the present study, dietborne Cu exposure induced a significant inhibition (~40%) in egg production, disregarding the Cu concentration and water salinity tested. Similar results were reported after continuous metal exposure through food in *A. tonsa*. A 50% inhibition of reproduction was observed at approximately 1.5 μg Cu/L, with no substantial increasing effects at concentrations up to 5.6 μg Cu/L [28]. In *Daphnia magna*, total reproduction was reduced to approximately 50% after dietary exposure to Cu [29]. Taking all these findings together, it appears that planktonic crustaceans' reproduction is affected by metal exposure up to 50% and no further effects are observed even at high concentrations, as previously suggested [30]. A plausible explanation for the highly unusual dose–response relationship observed after the dietborne exposure could be related to the fact that no evidence of metal desorption from the diatom *T. weissfloggii* was found even after the algal cells were disrupted by sonication, subjected to pH 2.0, or treated with digestive enzymes. Therefore, it is unlikely that Cu, once attached to the algae cell wall, can be released by digestion in herbivorous invertebrates like the copepod *A. tonsa*, as previously discussed for dietborne silver exposure [5]. Another possible explanation would be associated with a saturation of algae with Cu after exposure to elevated concentrations of the metal. In the present study, we expected that waterborne plus dietborne exposure would result

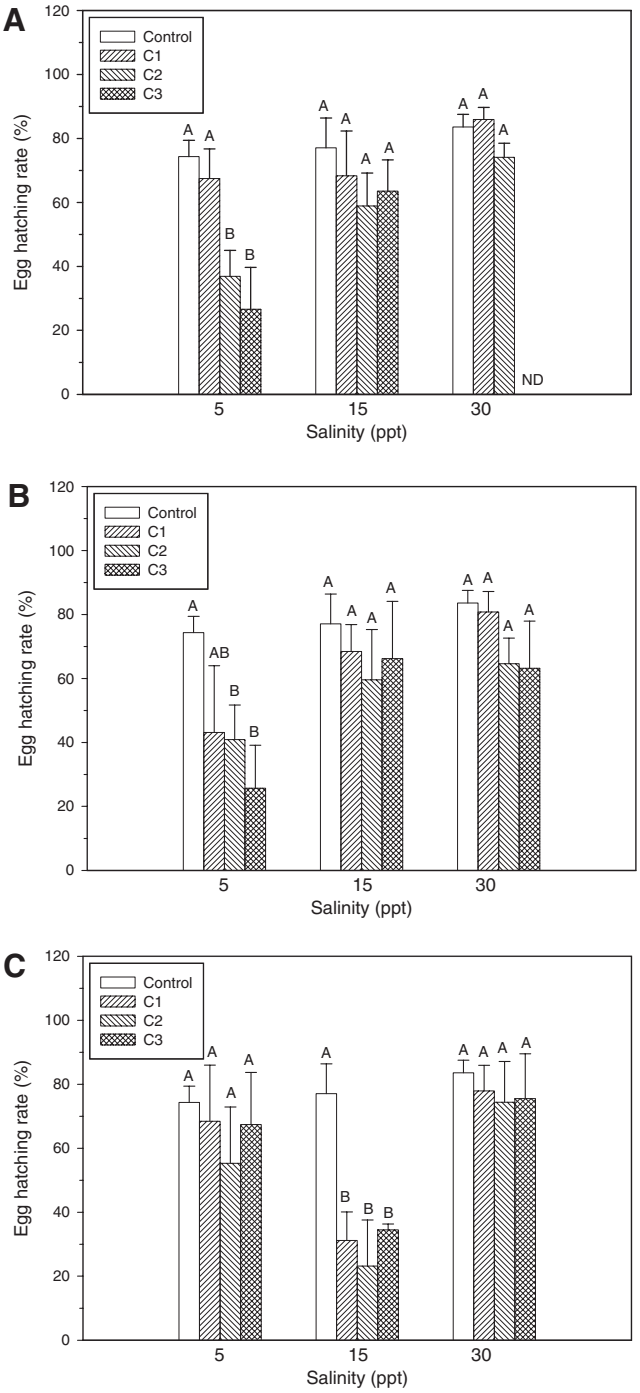

Fig. 3. Hatching rate of eggs produced by the copepod *Acartia tonsa* kept in clean water or exposed to copper through different routes of exposure in different water salinities: waterborne copper exposure (**A**); waterborne plus dietborne copper exposure (**B**); and dietborne copper (**C**). Copper concentrations tested (Control, C1, C2, and C3) as defined in Table 1. No significant difference was observed between water salinities for control copepods. Different letters indicate significantly different mean values between copper concentrations for the same route of exposure and water salinity. ND = not determined.

in more important reproductive impairment than the waterborne exposure in the copepod *A. tonsa*. However, results obtained showed that the waterborne Cu was more toxic than the dietary one. In fact, dietary Cu was shown to enhance reproduction in *D. magna*, but effects of exposure to waterborne and waterborne plus dietborne Cu were related to the concentrations used. In this case, reproduction was enhanced up to 70 μg Cu/L and ceased at higher concentrations [31].

Based on the information discussed above, it is clear that the thresholds for chronic Cu toxicity are different following exposure to dietborne and waterborne Cu. Furthermore, effects of waterborne plus dietborne Cu seem to be more related to waterborne exposure than to dietborne exposure. In fact, effects of dietary metal appear to be more on the reproductive system, whereas those of waterborne metal seem to be more general effects. It was suggested that dietary toxicity to egg production in copepods is caused by an inhibition of vitellogenesis [7], through inhibition of vitellogenin production or inhibition of processing vitellogenin to lipovitellin [32].

Aquatic toxicants can reduce energy acquisition by decreasing the feeding rate, food assimilation efficiency, or a combination of both [33,34]. *Acartia tonsa*, the species tested in the present study, is an opportunistic copepod that does not build up energy reserves. It survives starvation for only 3 d, and uses all energy taken in reproduction and other metabolic expenditures, because adult copepods do not molt [27]. Thus, changes in food quality and quantity can affect egg production.

As mentioned above, effects of dissolved Cu in the aqueous phase appear to be related to increased energy consumption and reduced energy intake. Exposure to dissolved Cu for 24 h reduced the grazing activity in copepods [35]. In fact, acute effects of waterborne Cu on *A. tonsa* physiology (feeding rate and ion regulation) are associated with a combined effect of Cu and food restriction [17]. Therefore, the reproductive impairment observed in the present study after waterborne Cu exposure could be explained by a metal effect on energy metabolism. Copepods exposed to waterborne Cu would show a higher energy expenditure rate, than those nonexposed to Cu, to face the stress induced by the metal exposure. Thus, even if these copepods were allowed to feed on clean food for 12 h in clean saltwater, they would have their feeding ability impaired and would not able to recover to their best nutritional status. At the end of 6 d of waterborne Cu exposure, their egg production rate would be then affected, probably because of a negative energetic imbalance induced by waterborne Cu exposure.

Waterborne plus dietborne Cu exposure, however, induced lower toxicity than the waterborne Cu exposure, probably because, in this treatment, waterborne copper was simultaneously offered with the dietary Cu. In this case, food could have acted as a protecting factor against Cu toxicity, even if it was contaminated. In fact, a protecting effect of food against acute Cu toxicity in *A. tonsa* physiology was previously shown [17]. Therefore, copepods exposed to waterborne plus dietborne Cu would have enough time and/or ability to obtain the energy necessary to face the Cu-induced stress before the effects of waterborne Cu would take place. However, studies on feeding rate, oxygen consumption, and total energy expenditure and storing are necessary to confirm this hypothesis.

Besides the differences in the reproductive performance of copepods observed in relation to the route of Cu exposure, changes associated with the water salinity were also observed. In fact, salinity was shown to alter Cu toxicity, especially when the metal is present in the dissolved phase [4]. In the present study, reproductive Cu toxicity was inversely related to water salinity, disregarding the route of exposure tested.

In summary, data reported in the present study indicate that Cu affects *A. tonsa* reproduction with waterborne Cu exposure producing more significant reproductive impairment than the dietary Cu exposure. They also show that egg production is an endpoint more sensitive to chronic Cu exposure than egg

hatching rate. Regarding salinity, our data show that the water chemistry plays an important role controlling chronic Cu toxicity in the copepod *A. tonsa*. In this case, salinity acts as an important protecting factor against the chronic effects induced by copepod exposure to waterborne or waterborne plus dietborne Cu. Therefore, results presented here clearly suggest that both water salinity and route of Cu exposure should be taken into account in the development of a future chronic version of the biotic ligand model for estuarine and marine environments.

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