Toxicidade do cobre na reprodução do copépode eurialino *Acartia tonsa* em diferentes salinidades.

Mariana Machado Lauer

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Orientador: Dr. Adalto Bianchini

SUMÁRIO

1. AGRADECIMENTOS

Em primeiro lugar, agradeço ao meu orientador, Adalto Bianchini, mais conhecido como chefe, por mais dois anos de orientação. No total, são 5 anos! Que venham os próximos 4, "né", chefe? Agradeço aos professores do curso que compartilharam o seu conhecimento e me ensinaram a gostar do maravilhoso mundo da fisiologia animal comparada. Nem por um segundo me arrependi da escolha que fiz, apesar de ter tido só um ano de bolsa. Falando em bolsa, agradeço ao CNPq pela minha! Agradeço também aos colegas, em especial aos das salinhas 3 e 4, pelos momentos de descontração e, claro, pelos de estudo, "né, grupo"! À Grasi, que me ensinou a lidar com os copépodes, e à Sandrinha pela compreensão na hora de dividir potinhos, animais e salsicheiro, um muito obrigada! Não posso deixar de citar aquelas que não participaram diretamente do mestrado, mas estão presentes em cada milímetro de mim. Minha avó Áurea, que por onde passa diz cheia de orgulho que eu sou a neta que faz mestrado e mora em Rio Grande, como se isso fosse algo muito importante. Meu vô Willy, que sempre diz que eu se eu não fosse neta dele, ele me comprava, e minha vó Teresa, que sempre pergunta dos meus experimentos, mesmo sem entender o que realmente faço. Aos meus pais, obrigada pelo universo! Aproveito para pedir desculpas por todos os momentos em que fui ausente e eles não foram poucos durante este mestrado. Por fim, agradeço à minha teimosia imensa, que não me deixou desistir apesar do cansaço, de ter que acordar cedo, dormir pouco, trabalhar muito e, algumas vezes, perder um experimento inteiro, de ter que ficar sozinha durante horas no departamento, ter que encontrar maneiras mirabolantes de contar copépodes sem energia elétrica, ter que trabalhar em finais de semana, feriados, durante 13 dias consecutivos e logo em seguida começar tudo de novo. Confesso que pensei em

procurar emprego e largar de vez essa vida de pesquisa. Graças à teimosia, persisti. Sigo agora rumo ao doutorado. Sei que ainda tenho um longo caminho para percorrer neste mundo científico. Também sei que não estarei sozinha.

2. RESUMO GERAL

No presente estudo foi avaliado o efeito do cobre na reprodução do copépode *Acartia tonsa*. Machos e fêmeas adultos foram expostos (6 dias) separadamente a diferentes combinações de concentração de cobre, salinidade (5, 15 e 30) e via de exposição (fase dissolvida, fase dissolvida mais transferência trófica e transferência trófica). Após exposição, foram determinadas a produção de ovos e a taxa de eclosão dos ovos. Na ausência de cobre, a produção de ovos foi diretamente dependente da salinidade, porém esta não afetou a taxa de eclosão dos ovos. Por sua vez, o cobre afetou tanto a produção de ovos quanto a taxa de eclosão. Com base nas concentrações de cobre dissolvido, os valores de CE_{50} (IC 95%) para produção de ovos foram 9,9 (6,9-11,3), 36,8 (33,6-40,1) e 48,8 (42,3-55,0) µg Cu/L para a exposição via fase dissolvida nas salinidades 5, 15 e 30. Para a exposição via fase dissolvida mais transferência trófica, estes valores foram de 40,1 (36,3-47,9), 63,7 (56,5-76,4) e 109,9 (90,3-150,9) µg Cu/L, respectivamente. Após exposição via alimento, o cobre reduziu a produção de ovos em cerca de 40% em relação ao número de ovos produzidos pelos organismos controle, independentemente da concentração de cobre e da salinidade testada. A exposição ao cobre dissolvido na água diminuiu significativamente a taxa de eclosão dos ovos nas salinidades extremas (5 e 30), enquanto que na salinidade intermediária (15), apenas a exposição via alimento causou toxicidade. Os resultados obtidos indicam que os efeitos do cobre na reprodução do copépode *A. tonsa* dependem tanto da salinidade quanto da via de exposição ao metal, sugerindo, portanto, que ambos parâmetros devam ser considerados em uma futura versão do Modelo do Ligante Biótico para ambientes estuarinos e marinhos.

Palavras-chave: *Acartia tonsa*, cobre, copépode, Modelo do Ligante Biótico, reprodução, salinidade.

3. INTRODUÇÃO

A zona costeira é habitada por cerca de metade da população mundial e está sujeita a diversos impactos antrópicos, tais como a introdução de efluentes domésticos e industriais, de resíduos da mineração e das indústrias naval e petrolífera. Dentre os contaminantes mais comumente liberados, encontram-se os metais. Alguns destes, como o cobre, são considerados essenciais, por participarem de funções fisiológicas nos organismos. Apesar de sua essencialidade, o cobre em altas concentrações no ambiente pode ser danoso aos organismos, em especial os aquáticos. Visando diminuir os impactos dos metais nestes ambientes, diversos modelos foram criados com o intuito de predizer a sua toxicidade, sempre levando em consideração a composição química da água, já que esta pode alterar a toxicidade dos metais (Di Toro et al., 2001). Dentre os modelos desenvolvidos, o Modelo do Ligante Biótico (Biotic Ligand Model; BLM) é aquele que leva em consideração a especiação e a complexação do metal dissolvido em solução e a competição entre o íon metálico livre e outros cátions pelos sítios de ligação no ligante biótico (Paquin et al., 2002a, 2002b).

Apesar de bem desenvolvido e aceito, o BLM está calibrado apenas para organismos de água doce e considera apenas situações de exposição aguda na fase dissolvida do metal. Estas situações não são as que melhor representam o que ocorre com um organismo em seu ambiente natural, já que situações de exposição aguda na ausência de alimento são muito raras. Além disso, a presença de alimento no meio pode influenciar a toxicidade dos metais, por causar modificações tanto na química da água quanto nas rotas de acumulação. Estudos que levam em consideração situações de exposição crônica e na presença de alimento são escassos, apesar de serem de grande importância e de estarem intimamente associados. Além disso, estudos com espécies

estuarinas e marinhas também são raros, apesar destas também estarem sujeitas aos efeitos biológicos da contaminação por metais. Por sua vez, a salinidade é um fator importante na química da água, sendo que diversos estudos mostram que ela atua como protetora na exposição ao cobre na fase dissolvida. Geralmente, quanto menor a salinidade, maior é a toxicidade do metal (Grosell et al., 2007).

A exposição crônica aos metais não afeta apenas o organismo, mas coloca em risco as populações em geral, já que estes podem afetar também os processos de alimentação, crescimento e reprodução (Fisher & Hook, 2002). Chang e Reinfelder (2002) sugerem que organismos herbívoros do zooplâncton marinho acumulam cobre principalmente via transferência trófica, mas a absorção do metal a partir da fase dissolvida pode ser importante em águas contaminadas. Em copépodes, os fatores de bioconcentração de metais (Al, Cr, Cu, Fe, Mn, Pb, Zn) variam de 4 a 7, sugerindo que estes animais possuem uma grande capacidade de acumulação de metais traços e podem ser utilizados como bioindicadores na avaliação da contaminação e impacto de metais no ambiente marinho (Fang et al., 2006).

Hook e Fisher (2001a) mostraram que a exposição ao Hg (1 nM) e ao Cd (5 nM) via alimento (alga *Thalassiosira pseudonana*) causou um decréscimo de cerca de 50% na produção e na taxa de eclosão dos ovos e de cerca de 75% no sucesso reprodutivo dos copépodes *Acartia hudsonica* e *Acartia tonsa*. Quando expostos à concentrações similares destes metais na fase dissolvida, a produção e a taxa de eclosão de ovos não foram afetadas pelo Cd, mas diminuiram nas concentrações de Hg superiores a 0,25 nM. O desenvolvimento do ovário das fêmeas também foi afetado pelos metais. Estes efeitos ocorreram em concentrações de 2 a 3 ordens de magnitude abaixo das concentrações letais para estes metais. Este mesmo estudo mostrou que diferentes rotas de exposição alteram a acumulação destes metais nos copépodes. Quando a exposição ocorreu via

alimento contaminado, os metais acumularam principalmente nos tecidos internos, enquanto que a exposição via fase dissolvida foi responsável pelo acúmulo no exoesqueleto. Um outro estudo de Hook e Fisher (2001b) mostrou que a exposição de *Acartia sp*. a Ag (1 nM) via alimento causou uma redução de 50% na produção e na eclosão de ovos, enquanto que nenhum efeito foi observado quando a exposição ocorreu via fase dissolvida do metal. Para *A. tonsa*, a exposição via alimento contaminado com cobre foi responsável por um decréscimo de cerca de 50% na reprodução (Bielmyer et al., 2006).

Cladóceros também apresentam decréscimo na reprodução quando expostos ao cobre via alimento. Quando *Daphnia magna* ingeriu alga contaminada com alta concentração de cobre (500 μg/L ou 3053 μg/g de peso seco algal), o número de juvenis produzidos foi reduzido em 50% (De Schamphelaere et al., 2007). Por sua vez, *Ceriodaphnia dubia* também teve sua reprodução inibida, mas em concentrações bem menores de cobre, ou seja, a partir de 15,9 μg/g de peso seco algal (Sofyan et al., 2006). No cladócero de alga salgada *Moina monogolica*, a reprodução foi inibida em concentrações a partir de 45 μg Cu/L no meio algal (Wang et al., 2007).

Estes estudos ressaltam a importância da transferência trófica na acumulação e toxicidade do cobre em invertebrados zooplanctônicos, que por sua vez são ecologicamente de grande importância e ao mesmo tempo sensíveis à exposição ao metal. Para organismos suspensívoros, como os copépodes, tanto a fase dissolvida do metal quanto a transferência trófica são importantes vias de acumulação (Wang & Fisher, 1999). Além disto, não há registro de estudo que avalie o efeito do cobre em uma espécie do zooplâncton marinho após exposição ao metal por diferentes vias utilizando um regime alimentar padronizado.

4. OBJETIVO GERAL

Levando-se em conta o exposto anteriormente, o objetivo principal do presente estudo foi determinar os efeitos da exposição ao cobre dissolvido na água e via alimento sobre a reprodução do copépode eurialino *Acartia tonsa*, em uma ampla faixa de salinidade. Esta espécie foi escolhida por ser um animal cosmopolita, possuir uma alta tolerância a variações ambientais, incluindo a salinidade, e apresentar um curto ciclo de vida e alto potencial reprodutivo. Por sua vez, a alga *Thalassiosira weissflogii* foi utilizada para avaliar o efeito do alimento contaminado, uma vez que esta é um importante item alimentar do copépode em estudo.

5. OBJETIVOS ESPECÍFICOS

1) Avaliar o efeito do cobre dissolvido sobre a reprodução (número de ovos produzidos e viabilidade dos ovos) do copépode *A. tonsa*, em diferentes salinidades.
2) Avaliar o efeito do cobre via transferência trófica sobre a reprodução (número de ovos produzidos e viabilidade dos ovos) do copépode *A. tonsa*, em diferentes salinidades.
3) Avaliar o efeito do cobre dissolvido mais via transferência trófica sobre a reprodução (número de ovos produzidos e viabilidade dos ovos) do copépode *A. tonsa*, em diferentes salinidades.

6. ARTIGO

Copper toxicity on the reproduction of the euryhaline copepod *Acartia tonsa* at different salinities

Mariana Machado Lauer[a], Adalto Bianchini[ab,*]

[a] Programa de Pós-Graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, Fundação Universidade Federal do Rio Grande, Av. Itália km 8, Campus Carreiros, 96201-900, Rio Grande, RS, Brazil.

[b] Departamento de Ciências Fisiológicas, Fundação Universidade Federal do Rio Grande, Av. Itália km 8, Campus Carreiros, 96201-900, Rio Grande, RS, Brazil.

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Mariana Machado Lauer[a], Adalto Bianchini[ab,*]

[a] Programa de Pós-Graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, Fundação Universidade Federal do Rio Grande, Av. Itália km 8, Campus Carreiros, 96201-900, Rio Grande, RS, Brazil.

[b] Departamento de Ciências Fisiológicas, Fundação Universidade Federal do Rio Grande, Av. Itália km 8, Campus Carreiros, 96201-900, Rio Grande, RS, Brazil.

[*] Corresponding author: Adalto Bianchini

Fundação Universidade Federal do Rio Grande

Departamento de Ciências Fisiológicas

Av. Itália, km 8 – Campus Carreiros

96201-900 - Rio Grande, RS, Brazil.

Phone: +55 53 32336853

FAX: +55 53 32336848

E-mail: adalto@octopus.furg.br

Abstract

In the present study, copper toxicity on the reproduction of the euryhaline copepod *Acartia tonsa* was evaluated. Male and female copepods were exposed (6 days) separately to different combinations of copper concentration, salinity (5, 15 and 30 ppt) and via of exposure (waterborne, waterborne plus dietborne and dietborne). After exposure, egg production and egg hatching rate were determined. In control copepods (no copper addition to water or food), egg production was positively related with salinity. However, egg hatching rate was not significantly affected. On the other hand, copper exposure affected both egg production and egg hatching rate. Based on dissolved copper concentrations, EC_{50} (95% CI) values for egg production after waterborne exposure were 9.9 (6.9-11.3), 36.8 (33.6-40.1) and 48.8 (42.3-55.0) µg Cu/L at salinities 5, 15 and 30 ppt, respectively. For waterborne plus dietborne exposure, they were 40.1 (36.3-47.9), 63.7 (56.5-76.4) and 109.9 (90.3-150.9) µg Cu/L, respectively. After dietborne exposure, a decrease in egg production about 40% was observed, disregarding the copper concentration and salinity tested. Waterborne copper exposure significantly reduced the egg's hatching rate in the extreme salinities (5 e 30 ppt), while in the intermediate (15 ppt) salinity only the dietborne exposure induced toxicity. These results indicate that the copper effects on the reproduction of the copepod *A. tonsa* are dependent on salinity and via of exposure, suggesting that both parameters should be taken into account in the development of a future version of the Biotic Ligand Model for estuarine and marine environments.

Keywords: *Acartia tonsa*, Biotic Ligand Model, copper, salinity, reproduction.

Introduction

Copper is an essential metal to aquatic animals, especially crustaceans. However, it can be toxic when in elevated concentrations in water or food. To predict the bioavailability and toxicity of metals in the aquatic environment, including copper, several models were developed. Among them, the Biotic Ligand Model (BLM) is a model that takes into account the competition of cations, such as H^+, Ca^{2+} and Na^+, with the free metal ion for binding at the biotic ligand, which is the site of action of the metal [1-3]. This 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^-.

Since water chemistry varies between freshwater, brackish water and saltwater, changes in metal toxicity according to salinity would be expected, including for copper. Grosell et al. [4] reviewed the literature and found that only eight studies were conducted to determine the influence of salinity on acute copper toxicity. Results from these studies showed that the highest sensitivity to copper occurred at the lowest salinity. Pedroso et al. [5] also reported that increasing salinity is protective against the acute silver toxicity in the euryhaline copepod *Acartia tonsa*.

Even if the BLM can accurate predict copper bioavailability and toxicity in a wide range of salinities, it only considers the metal uptake from the dissolved phase and after acute exposure. Therefore, this model still miss a lack of important sublethal responses, as reproductive impairments, that could be affected by chronic metal exposure and contaminated food. Hook and Fisher [6, 7] showed that zooplankton exposed to dietary Ag, Hg and Cd had a decrease of 50% on egg production and egg hatching rate. Total reproduction and brood size of the saltwater cladoceran *Moina monogolica* were also affected during 21 days of exposure to dietary copper [8]. Although dietary exposure is

important, effects of chronic exposure induced by copper accumulated from the dissolved phase cannot be ruled out, as shown by Chang and Reinfelder [9]. These authors suggested that herbivorous marine zooplankton accumulate copper mainly by trophic transfer, but they also showed that copper uptake from the dissolved phase accounted for almost 60% of the total copper accumulated in these animals. Usually, accumulation from the dissolved phase occurs by direct adsorption to body surfaces, while particulate metals can be accumulated following ingestion and digestion of food [10].

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 [11]. These organisms are the dominant marine zooplankton in pelagic systems, being the primary consumers in trophic webs of estuarine and marine systems [12, 13]. The copepod *A. tonsa* is a cosmopolitan species found in temperate regions, showing a high degree of tolerance to environmental changes, especially salinity [14].

In light of the above, the main goal of the present study was to evaluate the effect of copper on the reproduction of the euryhaline copepod *A. tonsa* in a wide range of salinities, after metal exposure through different pathways.

Materials and Methods

Algae culture

Cultures of the diatom *Thalassiosira weissflogii* were held at desired experimental salinities (5, 15 and 30 ppt) to feed adult copepods during rearing and toxicity tests. *Isochrysis galbana*, another diatom, was also cultured, but was used only as food source

for nauplii and small copepodits. The algal medium consisted of F/2 medium [15] in filtered (1 μm) seawater (salinity 30) or seawater diluted with distilled water (salinities 5 and 15 ppt). Seawater was collected at Cassino Beach (Rio Grande, RS, Southern Brazil). Algae were incubated under constant white fluorescent lights at 20ºC with mild continuous aeration for no more than 7 days. Algal density was measured using a haemocytometer (Neubauer chamber, 0.1 mm depth).

Algae copper exposure

For dietborne and waterborne plus dietborne exposures, copper was added to *T. weissflogii* algal medium ($40x10^4$ cells/mL) at different concentrations and equilibrated for 24 h (Table 1). Concentrations used to expose algae for waterborne plus dietborne exposure were the same used to contaminate water. Before feeding the copepods, algae for dietborne exposure was centrifuged (5 min; 1000 x g) and suspended with 3 mL of seawater without copper to ensure that metal exposure was only due to uptake from food.

Copepod cultivation

Adult copepods (*A. tonsa*) were obtained from cultures of the Laboratory of Zooplankton of the Fundação Universidade Federal do Rio Grande (Rio Grande, RS, Southern Brazil) and cultivated at the Laboratory as previously reported [5, 16]. Briefly, copepods were held in 20-L plastic buckets containing water at the salinity 15 or 30 ppt under mild continuous aeration. Temperature and photoperiod were fixed at 20ºC and 12L:12D, respectively. Copepods were daily fed a mix of the diatoms *T. weissflogii* ($2x10^4$ cells/mL) and *I. galbana* ($1x10^4$ cells/mL). For tests at salinity 5, copepods were acclimated gradually from cultures at salinity 15 ppt, for at least 2 weeks.

Copper exposure

Male and female copepods (*A. tonsa*) were separately exposed to copper for 6 days through different via of exposure. This period is considered as chronic exposure, since the species life cycle is short. Every day, copepods were exposed for 12 h to copper and maintained 12 h in clean water. For waterborne copper exposure, they were exposed for 12 h in contaminated water without food and maintained for 12 h in clean water in the presence of non-contaminated food. For waterborne plus dietborne copper exposure, copepods were exposed for 12 h to both water and food previously contaminated with copper and kept 12 h in a clean water without food. For the dietborne exposure, copepods were exposed for 12 h in clean water and fed with algae previously contaminated with copper. After copper exposure, they were kept in clean water without food for 12 h. In all cases, three different copper concentrations were tested at each experimental salinity (5, 15 or 30 ppt). The respective control treatment was also tested, keeping the same schedule of 12h. This schedule was chosen as a way to standardize the feeding regime between the ways of copper exposure and to guarantee that the food didn't interfere in the dissolved copper toxicity. Different copper concentrations were used between salinities, because a higher toxicity was expected in low salinity (Table 2).

Experiments 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 copepod introduction. For waterborne plus dietborne or dietborne copper exposures, algae previously contaminated with copper, as described above, were added to the experimental media at least 3 h prior copepod introduction.

For each combination of copper concentration, salinity and via of exposure, seven and three replicates were run for females and males, respectively. Flasks were kept under constant rotation (2 rpm) in an incubator with fixed temperature (20ºC) and photoperiod (16L:8D) to avoid food deposition. Every 12 h, surviving copepods were collected using plastic pipettes and transferred to a fresh experimental medium, prepared as described above, considering the via of exposure being tested.

After 6 days of exposure, groups of 1 male and 3 females were formed and copepods were allowed to mate in a non-contaminated medium of same salinity and with non-contaminated food (*T. weissflogii*, $2x10^4$ cells/mL). Nauplii and eggs produced over 24 h were counted using a stereoscopic microscope and results were expressed as eggs per female per day. Aliquots of eggs were collected and kept in cell culture plates for 24 h to determine the egg hatching rate.

Water samples

After 12h of copper exposure, non-filtered and filtered (0,45 μm) samples (10 mL) of each experimental medium were collected and acidified (1% HNO_3) to measure total and dissolved copper concentration using an atomic absorption spectrophotometer (AAS 932 Avanta-Plus, GBC, Hampshire, IL, USA). Water samples from control treatments with and without food were also collected and analyzed.

Statistical analysis

The effect of copper on reproduction was analyzed comparing the mean number (± SD) of eggs produced per female per day or egg hatching rate in copepods kept under control conditions (no copper addition to water or food) and those exposed to copper.

Significant differences between mean values were assessed by one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test at a significance level of 5%.

Percentage of inhibition of egg production in respect to control values were calculated and used to determine the copper concentration causing 50% of effect after 6 days of exposure (6-d EC_{50}) and its respective 95% confidence interval. Once inhibition values higher than 50% were obtained only after waterborne or waterborne plus dietborne exposure, EC_{50} values were calculated for these experimental conditions. EC_{50} values and their corresponding 95% confidence intervals were calculated based on total measured and dissolved copper concentrations using Probit analysis [17]. Differences between EC_{50} values were analyzed by comparison of the 95% confidence intervals. Statistical differences between mean values of egg production and egg hatching rate after dietborne exposure were also determined by ANOVA.

Results

In control copepods (without addition of copper in the water or food), the number of eggs produced per female per day was dependent of water salinity, augmenting as salinity increased. The mean values (± SD) were 17.0 ± 4.3, 21.0 ± 4.8 and 27.5 ± 4.1 at salinities 5, 15 and 30 ppt, respectively. Egg production was significantly higher in salinity 30 ppt than in salinity 5 ppt.

After dietborne exposure, copper induced an inhibition of egg production in all concentrations and salinities tested. At salinity 5 ppt, 39, 42 and 39% of inhibition in relation to control value was observed for nominal copper concentrations of 40, 80 and 160 μg Cu/L, respectively. At salinity 15 ppt, inhibition was of 42, 43 and 41%, respectively. At salinity 30, it was 33, 40 and 41%, respectively. Significant differences

were observed between the control and the copper exposed copepods in each salinity tested. However, no significant differences were observed between copepods exposed to the different copper concentrations at the same salinity.

Table 3 contains the 6-d EC_{50} (95% CI) values for waterborne and waterborne plus dietborne copper exposure. EC_{50} values for waterborne exposure were always significantly lower than those for waterborne plus dietborne exposure. The lower was the water salinity the lower was the EC_{50} value, i.e., the toxicity was higher. No significant differences were observed between the 6-d EC_{50} values estimated based on total measured copper concentrations and those estimated based on dissolved copper concentrations.

Table 4 shows the egg hatching rates for all treatments. No significant difference was observed between salinities. In copper exposed copepods, no statistical comparison was done between salinities and treatments, since copper concentrations used in each salinity for each treatment were different. However, it was performed between the concentrations tested and the control condition at the same salinity.

Discussion

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

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. As opposed to found in the present study, Holste and Peck [18] reported that egg hatching rate in *A. tonsa* from a Baltic population increased with increasing salinity, being maximal at salinity 25 ppt. Egg hatching rates of 55% and 78% were observed at salinity 15 ppt for North Sea [19] and Baltic Sea populations [18], respectively. In the present study, the egg hatching rate was similar to that found for the Baltic Sea populations, being 77 and 84% in salinities 15 and 30 ppt, respectively.

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

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

A decrease in egg production was observed after copper exposure, disregarding the way of exposure adopted. However, copper showed to be more toxic after waterborne exposure than after the exposures involving diet. This general picture was observed in the whole range of salinities tested, with copper being more toxic at salinity 5 ppt and less toxic at salinity 30 ppt after waterborne or waterborne plus dietborne copper exposure. On the other hand, copper toxicity after dietborne exposure was not affected by salinity or copper concentration, because it was observed a ~40% inhibition in egg production in respect to the control, in all experimental salinities, for all copper concentrations tested. Since copper concentration 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 copper leaching from food to water occurred, indicating that the toxicity observed was only due to the dietary copper.

Regarding egg hatching rate, copper exposure also affected this reproductive parameter. At salinities 5 and 30 ppt, waterborne and waterborne plus dietborne exposures significantly decreased the egg hatching rate. At salinity 15 ppt, significant inhibition was found only after dietborne copper 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. For example, it was shown that copepods from the *Acartia* genus had their egg production and egg hatching rate reduced by at least 50% after exposure to Ag, Hg and Cd via food, but no effect was observed after exposure to Ag and Cd via dissolved phase [6, 7]. Also, waterborne Hg exposure decreased egg production. Hook and Fisher [6] also noted that metals accumulated from ingested food were distributed primarily in internal tissues, whereas metals taken from dissolved phase accumulated in the exoskeleton. In the present study, dietborne copper

exposure significantly reduced egg production by ~40% in all concentrations and salinities tested. Similar results were reported by Bielmyer et al. [27] after continuous metal exposure through food in *A. tonsa*. These authors reported a 50% inhibition of reproduction with no substantial increasing effects when concentrations were higher than 5.6 μg Cu/L and 3.8 μg Zn/L. In *Daphnia magna*, total reproduction was also shown to be reduced to about 40% and 50% after dietary exposure to Zn and Cu [28, 29]. Taken all these findings together, it appears that microcrustacean reproduction is affected by metal exposure up to 50% and no further effects are observed even at high concentrations, as suggested by Hook and Fisher [30].

Sofyan et al. [31] observed that reproduction of *Ceriodaphnia dubia* was reduced after Cd exposure through all different ways tested. However, these authors showed that the combined waterborne and dietborne exposure was more toxic than only aqueous or dietary exposure. In the present study, we also expected that waterborne plus dietborne exposure would result in more important reproductive impairment in the copepod *A. tonsa*, as reported by Sofyan et al. [31]. However, results obtained showed that the waterborne copper was more toxic than the dietary one. In fact, dietary copper was shown to enhance reproduction in *D. magna*, but effects of exposure to waterborne and waterborne plus dietborne copper were related to the concentrations used. In this case, reproduction was enhanced up to 70 μg Cu/L and ceased at higher concentrations [32]. Canli [33] also observed differences in Zn tolerance in *D. magna* according to the pathway of exposure, with waterborne plus dietborne exposure being less toxic than other ways of exposure, and waterborne exposure being more toxic.

Based on the information given above, it is clear that the mechanisms involved in the reproductive impairment are different following exposure to dietborne and waterborne copper. Furthermore, effects of waterborne plus dietborne copper seem to be

more related to waterborne exposure than to dietborne exposure. Goulet et al. [34] observed that dietary Cd did not affect neonate production in *D. magna*, while in waterborne and waterborne plus dietborne exposure it was not even possible to calculate an EC_{50} because there was limited reproduction. This finding corroborates our idea that the effects observed on *A. tonsa* reproduction after waterborne plus dietborne exposure were mostly associated with the copper uptake from the dissolved phase.

Effects of dietary metal appear to be more on the reproductive system while those of waterborne metal seems to be more associated to general effects. For example, Hook and Fisher [6] suggested that dietary toxicity to egg production in copepods is caused by an inhibition of vitellogenesis, through inhibition of vitellogenin production or inhibition of processing vitellogenin to lipovitellin, as shown by Lee and Noone [35].

Aquatic toxicants can reduce energy acquisition by decreasing the feeding rate, food assimilation efficiency or a combination of both [36, 37]. *Acartia tonsa*, the species tested in the present study, is an opportunistic copepod that does not build up energy reserves. This copepod survives starvation for 3 days only, and uses all energy taken in reproduction and other metabolic expenditures, since adult copepods do not molt [26]. Thus, changes in food quality and quantity can affect egg production.

As mentioned above, effects of dissolved copper in the aqueous phase appear to be related to increased energy consumption and reduced energy intake. Sharp and Stearns [12] observed that exposure to dissolved copper for 24 h reduced the grazing activity in copepods. In fact, Pinho et al. [16] reported that acute effects of waterborne copper on *A. tonsa* physiology (feeding rate and ion regulation) are associated with a combined effect of copper and food restriction. Therefore, the reproductive impairment observed in the present study after waterborne copper exposure could be explained by a metal effect on energy metabolism. Copepods exposed to waterborne copper would show a

higher energy expenditure rate than those non-exposed to copper 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 had already their feeding ability impaired and were not able to recover to their best nutritional status. At the end of 6 days of waterborne copper exposure, their egg production rate was then affected, probably because of a negative energetic imbalance induced by waterborne copper exposure.

On the other hand, waterborne plus dietborne copper exposure induced lower toxicity than the waterborne copper exposure probably because in this treatment waterborne copper was simultaneously offered with the dietary copper. In this case, food could have acted as a protecting factor against copper toxicity, even if it was contaminated. A protecting effect of food against acute copper toxicity in *A. tonsa* physiology was previously showed by Pinho et al. [16]. Therefore, copepods exposed to waterborne plus dietborne copper would have enough time and/or ability to obtain the energy necessary to face the copper-induced stress before the effects of waterborne copper would take place. To confirm this hypothesis, studies on feeding rate, oxygen consumption and total energy expenditure and storing are necessary.

Besides differences in the reproductive performance of copepods observed in relation to the way of copper exposure, changes associated with the water salinity were also observed. It is known that salinity can alter metal toxicity, including copper, especially when it is present in the dissolved phase [4]. Although the protecting effect of salinity against metal bioaccumulation and toxicity is generally observed for the waterborne metal exposure, Widmeyer and Bendell-Young [38] reported that high salinity diminished the dietary Cd uptake by *Mytilus trossulus*. In the present study, reproductive copper toxicity was inversely related to salinity, disregarding the way of exposure.

Conclusions

Data reported in this study indicate that *A. tonsa* reproduction is affected by copper through different via of exposure, with waterborne copper producing more significant reproductive impairment than the dietary copper. They also show that egg production is an endpoint more sensitive to chronic copper exposure than egg hatching rate.

Regarding salinity, our data show that the water chemistry plays an important role controlling chronic copper 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 copper. Therefore, results presented here clearly suggest that both salinity and way of copper exposure should be taken into account in the development of a future version of the Biotic Ligand Model.

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Table 1. Copper concentrations (µg Cu/g dry weight) accumulated in algae employed in treatments involving trophic transfer.

Salinity	Nominal copper concentration	Cooper accumulated
5 ppt	0 µg/L	17,1 ± 3,9
	20 µg/L	22,0 ± 1,2
	40 µg/L	25,1 ± 3,8
	60 µg/L	30,6 ± 1,1
	80 µg/L	35,8 ± 4,7
	160 µg/L	57,1 ± 7,0
15 ppt	0 µg/L	14,7 ± 0,7
	20 µg/L	27,5 ± 0,6
	40 µg/L	29,7 ± 1,6
	80 µg/L	42,5 ± 5,0
	100 µg/L	45,0 ± 1,8
	160 µg/L	48,6 ± 10
30 ppt	0 µg/L	19,3 ± 5,0
	40 µg/L	23,3 ± 0,9
	80 µg/L	27,4 ± 0,5
	160 µg/L	29,6 ± 1,2

Table 2. Copper concentrations (µg Cu/L) in seawater employed to test the metal effect on reproduction of the copepod *Acartia tonsa* at different salinities and through different treatments.

Treatment	5 ppt	15 ppt	30 ppt
Control without food			
Nominal	0	0	0
Total measured	10	26	3
Dissolved	7	24	ND
Control with food			
Nominal	0	0	0
Total measured	16	23	ND
Dissolved	10	22	ND
Waterborne exposure			
Nominal	5	20	40
	10	40	80
	20	100	160
Total measured	12	30	39
	15	36	56
	18	71	127
Dissolved	10	30	36
	13	33	44
	17	69	116
Waterborne plus dietborne exposure			
Nominal	20	20	40
	40	40	80
	60	100	160
Total measured	26	33	25
	32	35	64
	47	76	118
Dissolved	24	32	24
	30	34	62
	41	69	101
Dietborne exposure			

Nominal	40	40	40
	80	80	80
	160	160	160
Total measured	9	26	ND
	7	26	ND
	11	26	ND
Dissolved	9	26	ND
	9	25	ND
	9	26	ND

Table 3. Concentrations inducing 50% reduction of egg production in the copepod *Acartia tonsa* after 6 days of exposure (6-d EC_{50}) to waterborne and waterborne plus dietborne copper based on total measured copper or dissolved copper, at different salinities. Numbers between parenthesis indicate the 95% of confidence interval. * indicates significant difference between waterborne and waterborne plus dietborne exposure at each salinity tested. Small and capital letters indicate significant difference between salinities for waterborne and waterborne plus dietborne copper exposure, respectively.

Treatment	5 ppt	15 ppt	30 ppt
Waterborne			
Total copper (µg Cu/L)	12.0 (9.3-13.3)^{a*}	38.4 (35.0-41.8)^{b*}	51.9 (44.9-58.8)^{c*}
Dissolved copper (µg Cu/L)	9.9 (6.9-11.3)^{a*}	36.8 (33.6-40.1)^{b*}	48.8 (42.3-55.0)^{c*}
Waterborne plus dietborne			
Total copper (µg Cu/L)	45.2 (40.5-54.7)^{A}	69.2 (60.1-85.6)^{B}	125.6 (101.4-176.9)^{C}
Dissolved copper (µg Cu/L)	40.1 (36.3-47.9)^{A}	63.7 (56.5-76.4)^{B}	109.9 (90.3-150.9)^{C}

Table 4. Egg hatching rate (%) of the copepod *Acartia tonsa* after exposure to copper through different vias at different salinities and control. * indicates statistical difference between copper concentrations and control in each salinity.

Treatment	5 ppt		15 ppt		30 ppt	
	µg Cu/L (nominal)	Hatching rate (%)	µg Cu/L (nominal)	Hatching rate (%)	µg Cu/L (nominal)	Hatching rate (%)
Control						
	0	74.3 ± 5.1	0	77.1 ± 9.3	0	83.6 ± 3.9
Waterborne						
	5	67.47 ± 9.2	20	68.3 ± 14.0	40	85.9 ± 3.8
	10	36.9 ± 8.1*	40	58.9 ± 10.3	80	74.1 ± 4.4*
	20	26.6 ± 13.1*	100	63.5 ± 9.8	160	ND
Waterborne plus dietborne						
	20	43.1 ± 20.9*	20	68.4 ± 8.4	40	80.8 ± 6.4
	40	40.9 ± 10.8*	40	59.6 ± 15.7	80	64.6 ± 8.0*
	60	25.7 ± 13.4*	100	66.2 ± 17.9	160	63.2 ± 4.7*
Dietborne						
	40	68.4 ± 17.6	40	31.2 ± 8.9*	40	77.9 ± 8.0
	80	55.3 ± 17.6	80	23.2 ± 14.4*	80	74.4 ± 12.7
	160	67.4 ± 16.3	160	34.5 ± 11.8*	160	75.5 ± 14.0

7. CONCLUSÕES

Com base nos resultados apresentados no presente estudo, pode-se concluir que:

1) A salinidade afeta a reprodução do copépode *Acartia tonsa*, sendo que o número de ovos produzidos é menor em salinidade 5 do que em salinidade 30.
2) Nas três salinidades testadas (5, 15 e 30), a reprodução do copépode *A. tonsa* é afetada pelo cobre, tanto após exposição ao metal dissolvido na água quanto via alimento, sendo que a maior toxicidade é observada quando o metal encontra-se dissolvido na água.
3) A transferência trófica do metal foi responsável por um decréscimo máximo de cerca de 40% no número de ovos produzidos, sendo, portanto, uma via de exposição ao metal menos importante que aquela na fase dissolvida.
4) Considerando-se os resultados obtidos no presente estudo, a incorporação dos parâmetros salinidade e via de exposição ao metal deve ser considerada no desenvolvimento de futuras versões do BLM visando sua aplicação em ambientes estuarinos e marinhos.

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