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INFLUÊNCIA DA MATÉRIA ORGÂNICA DISSOLVIDA DULCIAQUÍCOLA E MARINHA NA ACUMULAÇÃO E TOXICIDADE DO COBRE NO COPÉPODE *Acartia tonsa*

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SUMÁRIO

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RESUMO

A matéria orgânica dissolvida (MOD) e a salinidade da água podem proteger os animais aquáticos contra a toxicidade do cobre. Assim, o objetivo central desta tese foi avaliar a influência da MOD natural de diferentes origens e em diferentes concentrações na acumulação e toxicidade do cobre no copépode eurialino *Acartia tonsa* em diferentes salinidades. Os experimentos da presente tese foram organizados em dois manuscritos. No primeiro manuscrito, foi determinada a CL50-48 h do cobre para copépodes adultos machos separadamente, para copepoditos e fêmeas adultas em conjunto, e para copepoditos e adultos de ambos os gêneros em conjunto, na ausência de MOD e nas salinidades 5, 15 e 30. Os resultados indicaram que a sensibilidade dos copépodes machos adultos à exposição aguda ao cobre é semelhante à sensibilidade dos copepoditos e fêmeas adultas testados em conjunto. Geralmente a toxicidade para copepoditos e adultos de ambos os gêneros testados em conjunto foi semelhante à toxicidade observada nos outros dois tratamentos, sugerindo que a toxicidade do cobre não é dependente do gênero, e que não há necessidade de distinção entre copepoditos e adultos de *A. tonsa* para a realização dos testes de toxicidade aguda do cobre. Assim, os demais experimentos descritos nesta tese foram realizados com copépodes (adultos e copepoditos) de ambos os gêneros conjuntamente. Foi determinada a CL50-48 h do cobre na ausência e presença de MOD dulciaquícola e marinha (diferentes fontes e concentrações) nas salinidades 5, 15 e 30. O aumento da salinidade protegeu contra a toxicidade aguda do cobre. Em geral, a toxicidade do cobre foi menor na presença do que na ausência de MOD, especialmente nas salinidades 5 e 15. Este efeito protetor da MOD contra a toxicidade do cobre foi também dependente da concentração de carbono orgânico dissolvido (COD), sendo que os maiores efeitos protetores foram observados nas maiores concentrações de COD testadas. Além disso, o efeito protetor da MOD parece ser dependente da fonte de MOD utilizada. Na salinidade 30, baseado nos valores de CL50 calculados a partir das concentrações de cobre livre (estimadas com o programa

VisualMinteq), outras formas químicas, além do cobre livre, ou a formação e assimilação de complexos MOD-cobre podem ter causado toxicidade aos copépodes. Na segunda etapa foi medida a acumulação de cobre corporal, no exoesqueleto e nos tecidos moles dos copépodes expostos às concentrações de cobre correspondentes às CL50-48 h previamente determinadas na ausência e na presença de MOD. A acumulação corporal de cobre foi dependente da salinidade e da concentração e origem da MOD dulciaquícola. Por outro lado, não houve um padrão de acumulação de cobre no exoesqueleto em função destas variáveis, enquanto o conteúdo de cobre nos tecidos moles foi similar entre os tratamentos com e sem adição de MOD dulciaquícola e marinha (34 ng Cu/mg peso seco). Assim, os dados apresentados nesta tese indicam que tanto a toxicidade aguda quanto a acumulação corporal do cobre é dependente da salinidade da água e da MOD (origem e concentração). Além disso, eles sugerem que os tecidos moles podem ser considerados como sendo o ligante biótico onde o cobre se acumula e induz 50% de mortalidade no copépode *A. tonsa*. Portanto, o conteúdo médio de cobre nos tecidos moles (34 ng Cu/mg peso seco) pode ser usado como o valor de acumulação letal para 50% dos organismos (AL50) em uma versão futura do Modelo do ligante biótico (BLM) para ambientes estuarinos e marinhos usando o copépode *Acartia tonsa*.

Palavras-chave: *Acartia tonsa*; bioacumulação; cobre; matéria orgânica dissolvida; salinidade; toxicidade aguda.

ABSTRACT

Dissolved organic matter (DOM) and salinity can protect aquatic animals against copper toxicity. Thus, the main objective of this thesis was to evaluate the influence of natural DOM from different origins and several concentrations on copper accumulation and toxicity in the euryhaline copepod *Acartia tonsa* at different salinitites. Experiments were organized in two manuscripts. In the first manusctript, copper 48-h LC50 was determined for adult males separately, for copepodites and adult females concomitantly, and for copepodites and adults of both genders concomitantly, in the absence of DOM, at salinities 5, 15 and 30. Results indicated that the acute sensibility of adult males to copper was similar to that observed in copepodites and adult females tested concomitantly. Generally copper toxicity was also similar between these two mentioned treatments and that with copepodites and adults of both genders tested concomitantly, suggesting that acute copper toxicity is not dependent on gender, and that there is no need for distinguish between *A. tonsa* copepodites and adults to carrying out acute copper toxicity tests. Then, all remainder experiments in this thesis were made with copepods (adults and copepodites) of both genders concomitantly. Copper 48-h LC50 was determined in the absence and in the presence of freshwater and marine DOM (different sources and concentrations) at salinities 5, 15 and 30. Salinity protected against acute copper toxicity. Generally copper toxicity was smaller in the presence than in the absence of DOM, especially at salinities 5 and 15. This protective effect of DOM against copper toxicity was also dependent on dissolved organic carbon (DOC) concentration (the highest protective effects were observed at the highest DOC concentrations tested). Besides, the protective effect of DOM seems to be dependent on the DOM source. Based on LC50 calculated from free copper concentrations (estimated with the Visual Minteq software) at salinity 30, other copper species, or the formation and assimilation of DOM-copper complexes, could lead to toxicity in copepods. In the second manuscript, copper accumulation was measured in the body, exoskeleton and soft tissues of copepods exposed to copper

concentrations corresponding to the 48-h LC50 previously determined, in the absence and in the presence of DOM. Copper accumulation in the body was dependent on salinity and on concentration and source of freshwater DOM. On the other hand, copper accumulation in exoskeleton did not present a clear pattern, but copper accumulation in soft tissues was similar among all treatments with freshwater and marine DOM and without DOM addition (34 ng Cu/mg dry weight). Therefore, results indicate that acute copper toxicity and accumulation are dependent on both salinity and DOM (origin and cocentration). In addition, results suggest that soft tissues can be considered as the biotic ligand where copper is accumulated and induces 50% of mortality in the copepod *A. tonsa*. Thus, mean copper content in soft tissues (34 ng Cu/mg dry weight) can be used as the lethal accumulation value for 50% of organisms (LA50) in a future version of the Biotic Ligand Model (BLM) for estuarine and marine environments, using the copepod *Acartia tonsa*.

Key-words: *Acartia tonsa*; acute toxicity; bioaccumulation; copper; dissolved organic matter; salinity.

INTRODUÇÃO

A descarga de produtos químicos em rios e estuários, especialmente aqueles localizados próximos a áreas urbanas e industriais, como é o caso do estuário da Lagoa dos Patos (Rio Grande, RS), tem aumentado significativamente a contaminação destes locais com substâncias tóxicas, tais como os metais (Baumgarten e Niencheski, 1990). Porém, processos naturais, como a erosão continental, também geram entrada significativa de metais em áreas costeiras (Niencheski *et al.,* 1994).

O cobre é um micronutriente essencial que participa de diversas funções fisiológicas nos organismos (Morgan, 2000), mas que pode ser tóxico quando presente em elevadas concentrações na água (Salomons *et al.,* 1995). Desta forma, seu lançamento no ambiente deve ser controlado. Em muitos países, inclusive no Brasil, a regulamentação da emissão do cobre geralmente está baseada somente na concentração total (FEPAM, 1995; CONAMA, 2005) ou dissolvida (CONAMA, 2005) do metal presente em efluentes e/ou no ambiente. No entanto, em 1985, a Agência de Proteção Ambiental Americana introduziu o conceito de "Critério de Qualidade de Água" (*Water Quality Criteria*, WQC), reconhecendo que a toxicidade dos metais depende da sua interação com outras substâncias que estão presentes na água (US-EPA, 1985). De fato, foi demonstrado que diversos parâmetros químicos da água, tais como matéria orgânica dissolvida, pH, dureza e composição iônica influenciam a toxicidade aguda do cobre (Erickson *et al.,* 1996).

Neste contexto, foi desenvolvido o Modelo do Ligante Biótico (*Biotic Ligand Model*, BLM), com o objetivo de regular mais corretamente a emissão de metais, incluindo o cobre, em ambientes aquáticos. Este modelo matemático considera a especiação e a complexação do metal dissolvido e a ligação competitiva entre o metal e outros cátions no sítio de ligação (ligante biótico) de um tecido-alvo (Di Toro *et al.,* 2000; Fig. 1). O BLM parte da premissa de que existe uma forte correlação entre a concentração do metal associado ao alvo biológico e sua toxicidade

aguda (Santore *et al.,* 1999). Originalmente, o BLM foi desenvolvido para ambientes dulciaquícolas, com base em dados de toxicidade aguda do cobre em peixes (MacRae *et al.,* 1999) e no cladócero *Daphnia* sp. (Santore *et al.,* 1999). No entanto, uma versão deste modelo para ambientes estuarinos e marinhos ainda não está disponível na literatura. Por isto, atualmente a especiação do cobre tem sido avaliada utilizando-se programas como o Visual MinteQ. Este programa permite a análise da especiação de metais para ambientes de água salgada, porém não avalia a biodisponibilidade e toxicidade dos metais como o BLM. Portanto, estudos que avaliem a toxicidade de metais em organismos estuarinos e marinhos são de grande importância para o futuro desenvolvimento de uma versão do BLM para ambientes estuarinos e marinhos.

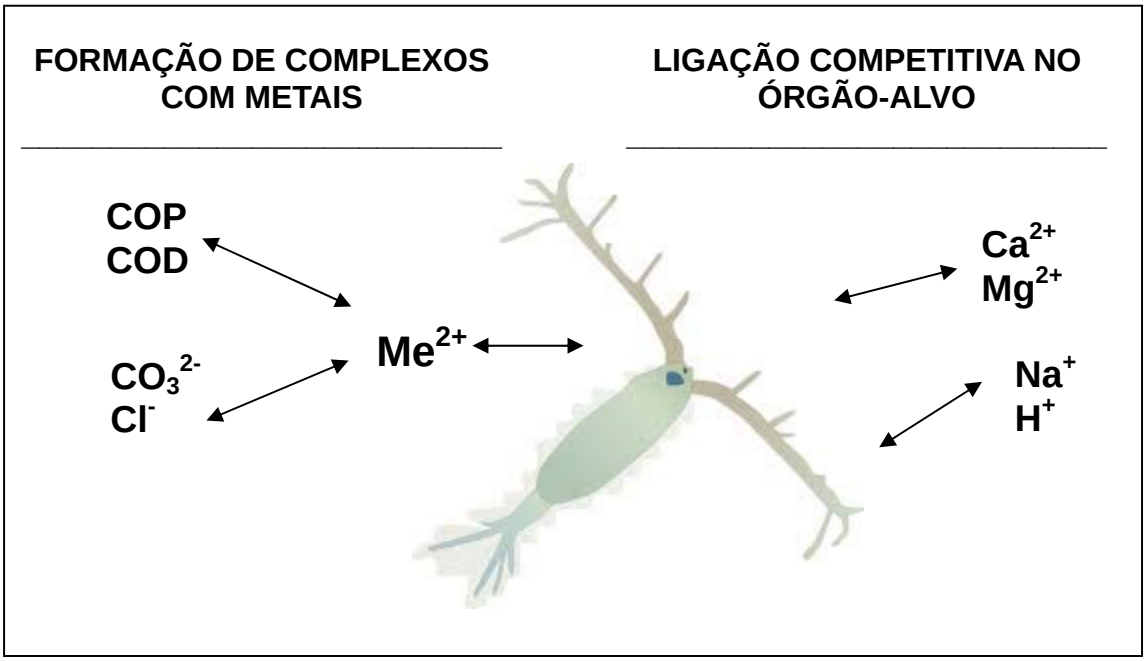

Figura 1. Esquema do modelo do ligante biótico (BLM). As setas indicam a formação de complexos do metal (Me^{2+}) com o carbono orgânico particulado (COP) e dissolvido (COD), e outros compostos presentes na água, bem como a competição do metal pelo sítio ativo de organismos aquáticos (adaptado de www.hydroqual.com).

A matéria orgânica dissolvida (MOD) é um dos principais parâmetros que influenciam a toxicidade dos metais nos organismos aquáticos. A MOD é a fração da matéria orgânica filtrável (<0,45 μm) (Thurman, 1985). Uma vez que a MOD possui uma constituição complexa, sua concentração é expressa em termos de carbono orgânico dissolvido (COD), ou seja, a fração do carbono orgânico total filtrável (<0,45 μm), pois este é um dos principais elementos que a

compõe (Thurman, 1985). No entanto, estudos sobre a influência da MOD sobre a toxicidade dos metais são relativamente recentes, especialmente em animais marinhos (Erickson *et al.* 1996; De Schamphelaere *et al.* 2004; Nadella *et al*., 2009).

Uma vez na água, a MOD forma complexos com os metais, diminuindo assim a disponibilidade e toxicidade destes aos organismos aquáticos (Erickson *et al.* 1996; De Shamphelaere *et al.* 2004, 2005). Geralmente, os estudos sobre os efeitos da MOD são realizados com matéria orgânica comercial, mais frequentemente o ácido húmico da Sigma-Aldrich, extraído de solos (De Shamphelaere *et al.,* 2005). Porém, este composto tem pouca semelhança estrutural com os ácidos húmicos aquáticos (Malcolm e MacCarthy, 1986). A MOD natural pode ser produzida na coluna d'água pelo fitoplâncton, constituindo a MOD autóctone, ou ser proveniente do ambiente adjacente (MOD alóctone). Tipicamente, a MOD autóctone é rica em carboidratos e nitrogênio, possui uma coloração amarela e é composta principalmente de compostos de carbono de cadeia aberta, enquanto a MOD alóctone é rica em substâncias húmicas e fúlvicas aromáticas, possui uma coloração de amarelo a marrom e absorve luz ultravioleta (Buffle 1998 *apud* Richards *et al.* 2001). A fração húmica da matéria orgânica é composta principalmente por ácido húmico e ácido fúlvico.

A MOD natural tem suas características alteradas de acordo com alguns fatores, tais como tipo de água, origem, vegetação e clima (Kramer *et al.*, 2004). Dessa forma, MOD de diferentes locais podem ser compostas por moléculas com características distintas e, portanto, suas características e capacidades de complexação podem variar de um local para outro (Ryan *et al.,* 2004), influenciando assim diferentemente a biodisponibilidade e toxicidade do cobre para os organismos aquáticos habitantes destes locais (Kramer *et al.,* 2004). O modelo para especiação orgânica do cobre (WHAM – Windermere Humic Acid Model) inserido na base de dados do BLM considera valores fixos para as constantes de troca próton-metal (*pK*). Este modelo considera que a *pK* para a ligação de cobre no ácido húmico é 1,5, enquanto para o ácido fúlvico este valor é de 0,8 (Santore *et al.,* 2001). A reação de ligação do cobre com os ácidos húmicos

pode ser descrita, conforme De Schamphelaere *et al.* (2002), como:

$$AH\text{-}H^{+} + Me^{2+} \overset{K}{\rightleftarrows} AH\text{-}Me^{2+} + H^{+}$$

Onde AH é a molécula de ácido húmico e Me^{2+} é um íon metálico, neste caso, o cobre. Portanto, quanto menor o valor de *pK* (maior valor de *K*), mais a reação será desviada para a direita, mais prótons serão substituídos por metais, e assim mais metais se ligarão às moléculas de ácido húmico. Isto, por fim, resulta em uma menor concentração de cobre livre disponível no meio para causar toxicidade. Além disso, foi demonstrado que a incorporação da variabilidade da MOD no BLM aumenta a capacidade preditiva deste modelo para o cladócero de água doce *Daphnia magna* (De Shamphelaere *et al.,* 2004).

Apesar de existirem alguns estudos sobre o efeito de diferentes fontes de MOD na toxicidade e acumulação dos metais, a maioria destes se restringe aos ambientes de água doce. Mesmo nos estudos com organismos estuarinos ou marinhos onde é empregada MOD natural, esta raramente é de origem marinha, predominando as MOD extraídas de água doce. Isto ocorre, em parte, devido à maior dificuldade de extração e concentração de MOD marinha (Rodrigues e Bianchini, 2007). O método ideal para a extração e concentração de MOD de águas naturais deve extrair grandes quantidades de MOD rapidamente, sem causar fracionamento, alterações químicas e outras perdas na MOD (Sun *et al.*, 1995). A água do mar representa, em termos de recuperação de MOD após extração e concentração, uma das fontes de MOD mais desafiadoras, pois a matéria inorgânica pode ser aproximadamente 30.000 vezes mais concentrada do que a matéria orgânica. De fato, a concentração de COD na água do mar varia geralmente entre 0,3 e 2,0 mg C/L (Thurman, 1985). Além disso, os sais presentes na água do mar precipitam ao longo do processo de extração de MOD, representando outra dificuldade na extração de MOD marinha. Portanto, a MOD de água do mar não pode ser extraída através de osmose reversa, um método simples e de baixo custo comumente usado para extrair MOD dulciaquícola. Resinas XAD (Amberlite®) são amplamente utilizadas para extrair MOD dulciaquícola e marinha (Rodrigues e Bianchini, 2007), mas seu processo de limpeza antes de iniciar a extração de MOD é bastante

trabalhoso e lento (Standley e Kaplan, 1998). Um método relativamente fácil e adequado para a extração de MOD marinha, utilizando resinas de polipropileno, foi recentemente desenvolvido (Rodrigues e Bianchini, 2007; Dittmar *et al.*, 2008) e usado nesta tese para extrair e concentrar MOD de águas costeiras e marinhas.

Para a realização desta tese, foram utilizadas três fontes de MOD de água doce e duas fontes de MOD marinha. As fontes das MOD dulciaquícolas foram o ácido fúlvico comercial extraído do rio Suwannee, adquirido junto à International Humic Substances Society (EUA), e MOD extraída por osmose reversa a partir de amostras de água doce coletadas no Arroio Vieira. Neste caso, uma solução concentrada de MOD foi obtida a partir da água coletada antes do ponto de lançamento dos resíduos tratados pela Estação de Tratamento de Esgoto (ETE) Navegantes (Rio Grande, RS), enquanto a outra foi obtida a partir da água coletada após o ponto de lançamento da ETE. As soluções concentradas de MOD de origem marinha foram obtidas a partir de amostras de água do mar coletadas em dois locais distintos da costa brasileira (Rio Grande do Sul e Bahia).

Conforme mencionado anteriormente, a premissa do BLM é que existe uma forte correlação entre a concentração do metal associado ao alvo biológico e sua toxicidade aguda (Santore *et al.,* 1999). Quando a taxa de assimilação do metal é mais rápida que a sua taxa de excreção e detoxificação, a concentração de metal metabolicamente disponível excede um limiar e começa a se ligar a locais onde irá interferir no metabolismo, causando toxicidade (Rainbow, 2007). A acumulação de metais nos tecidos dos animais pode variar em função de diversos fatores, tais como concentração do metal, via e tempo de exposição, bem como pelas taxas de assimilação e excreção do metal que, por sua vez, são influenciadas pela idade, tamanho, sexo e outros fatores (Wang e Fisher, 1999). Hook e Fisher (2001) observaram que metais acumulados em copépodes (*Acartia tonsa* e *Acartia hudsonica*) por transferência trófica estavam presentes nos tecidos internos, enquanto metais acumulados através da fase dissolvida estavam presentes principalmente no exoesqueleto. Neste segundo caso, o metal acumulado não causou toxicidade

ou causou apenas poucos efeitos adversos. Por outro lado, Wang e Fisher (1998) observaram que metais acumulados no copépode *Temora longicornis* por transferência trófica e pela fase dissolvida estavam presentes predominantemente nos tecidos internos, e pouco no exoesqueleto. Portanto, fica evidente que a avaliação somente da concentração de metal corporal pode levar a estimativas erradas da quantidade de metal acumulado que está relacionada à toxicidade. Neste contexto, na presente tese foi analisada a acumulação corporal do cobre, bem como o conteúdo de cobre no exoesqueleto e nos tecidos moles do copépode *A. tonsa* na ausência e na presença de MOD (diferentes origens e concentrações) em uma ampla faixa de salinidade (5-30).

Acartia tonsa é um copépode holoplanctônico, cosmopolita e pertencente à ordem Calanoida (Mauchline, 1998). Os adultos desta espécie toleram uma ampla faixa de salinidade, sendo encontrados no Estuário da Lagoa dos Patos (RS) em salinidades de 0 a 31,5 (Montú e Gloeden, 1986), mas as salinidades ótimas para esta espécie estão entre 15 e 22 (Cervetto *et al*., 1999). É uma espécie onívora, sendo que o fitoplâncton é um importante item de sua dieta (Kleppel *et al.,* 1991). Desta forma, estes copépodes constituem a principal ligação entre o fitoplâncton e os níveis tróficos superiores em muitas cadeias alimentares estuarinas e marinhas (Mauchline, 1998), tornando-os assim importantes no contexto da contaminação ambiental, devido ao seu potencial de acumulação e transferência de contaminantes químicos ao longo da cadeia trófica (Wang e Fisher, 1998; Xu e Wang, 2002). Além disso, por se alimentarem de fitoplâncton, o cultivo de *A. tonsa* em laboratório é relativamente simples e de baixo custo, pois uma ou duas espécies de microalgas podem ser utilizadas como alimento para os copépodes ao longo de todo seu ciclo de vida.

O desenvolvimento das populações de espécies do gênero *Acartia* se caracteriza por um rápido tempo de recrutamento, produção de ovos resistentes em períodos de extrema perturbação ambiental, intervalos de muda tendendo a ser constantes e aumento exponencial em tamanho até a fase adulta (Miller, 1983). Existem diferentes informações a respeito da duração do ciclo de vida da espécie, sendo este dependente de vários fatores, tais como disponibilidade de alimento,

temperatura e salinidade (Gaudy *et al.,* 2000). O copépode *A. tonsa* pode ser tornar adulto aos 7 dias após a eclosão do ovo (Kaminski, 2004), sendo que sua expectativa de vida é de até 80 dias (Sazhina, 1987). O rápido desenvolvimento e ciclo de vida de *A. tonsa* facilitam sua manutenção em cultivos em laboratório e sua utilização em testes de toxicidade aguda e crônica.

Cabe ressaltar que os copépodes são considerados indicadores sensíveis da toxicidade subletal aos metais (Hook e Fisher, 2001), sendo utilizados há bastante tempo em estudos toxicológicos em laboratório (Sosnowski *et al.,* 1979) e em estudos de poluição ambiental (Bianchi *et al.,* 2003). No entanto, a maioria dos estudos realizados com copépodes do estuário da Lagoa dos Patos (RS) é de caráter ecológico. Um dos primeiros trabalhos sobre o zooplâncton do estuário da Lagoa dos Patos foi realizado por Montú (1980). Esta autora analisou amostras de zooplâncton e descreveu as variações temporais e espaciais das espécies amostradas. Desde então, a maioria dos estudos sobre o zooplâncton desse estuário e da região costeira adjacente têm abordado os aspectos taxonômicos e os fatores físicos que influenciam a diversidade, distribuição e abundância das espécies, sendo que *A. tonsa* é uma das espécies predominantes (Montú *et al*., 1998).

Mais recentemente, começaram a ser realizados estudos em laboratório visando investigar, dentre outros aspectos, a influência da salinidade, da temperatura e do tipo de dieta no crescimento e reprodução de copépodes estuarinos e marinhos (Cardozo, 2004; Kaminski e Montú, 2005). Também vêm sendo desenvolvidos experimentos em laboratório com o objetivo de verificar os efeitos de poluentes no copépode *A. tonsa*. Por exemplo, Avila *et al.* (2010) observaram a toxicidade da fração solúvel do petróleo nesta espécie. Pedroso *et al.* (2007) e Pinho *et al.* (2007) observaram efeitos fisiológicos da prata e do cobre, respectivamente Na presente tese, foram observados os efeitos da salinidade e da matéria orgânica dissolvida de diferentes origens na toxicidade aguda e acumulação do cobre em *A. tonsa*.

HIPÓTESES

1. O aumento da salinidade atua como agente protetor contra a acumulação e toxicidade aguda do cobre no copépode *A. tonsa*.

2. O aumento da concentração de carbono orgânico dissolvido atua como agente protetor contra a acumulação e toxicidade aguda do cobre em *A. tonsa*.

3. Matérias orgânicas dissolvidas de diferentes origens agem de forma diferenciada na proteção contra a acumulação e toxicidade aguda do cobre em *A. tonsa*.

4. A acumulação de cobre nos tecidos moles representa melhor a acumulação de cobre no ligante biótico do que a acumulação corporal total.

OBJETIVOS

Objetivo geral

Avaliar a influência da salinidade e da matéria orgânica dissolvida na acumulação e toxicidade aguda do cobre no copépode eurialino *Acartia tonsa.*

Objetivos específicos

1. Avaliar a influência do gênero na sensibilidade do copépode *A. tonsa* à exposição aguda ao cobre na ausência de matéria orgânica dissolvida nas salinidades 5, 15 e 30.
2. Verificar a influência da salinidade (5, 15 e 30) e da matéria orgânica dissolvida de diferentes fontes dulciaquícolas na toxicidade aguda do cobre no copépode *A. tonsa*.
3. Analisar a influência da salinidade (5, 15 e 30) e da matéria orgânica dissolvida de diferentes fontes dulciaquícolas na acumulação corporal do cobre, bem como no conteúdo deste metal no exoesqueleto e tecidos moles do copépode *A. tonsa*.
4. Avaliar a influência da salinidade (5, 15 e 30) e da matéria orgânica dissolvida de diferentes fontes marinhas na toxicidade aguda do cobre no copépode *A. tonsa*.
5. Verificar a influência da salinidade (5, 15 e 30) e da matéria orgânica dissolvida de diferentes fontes marinhas na acumulação corporal do cobre, bem como no conteúdo deste metal no exoesqueleto e nos tecidos moles do copépode *A. tonsa*.

CAPÍTULO 1

MANUSTRITO 1

Acute waterborne copper toxicity in the euryhaline copepod *Acartia tonsa* at different salinities: influence of natural freshwater and marine dissolved organic matter

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Acute waterborne copper toxicity in the euryhaline copepod *Acartia tonsa* at different salinities: influence of natural freshwater and marine dissolved organic matter

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Abstract

The influence of natural dissolved organic matter (DOM) on acute waterborne copper toxicity was evaluated in the euryhaline copepod *Acartia tonsa* at three different water salinities. Three sources of freshwater DOM (extracted by reverse osmosis) and two sources of marine DOM (extracted using a solid-phase technique) were used. Artificial salt water was used to prepare the experimental media. Different combinations of copper concentrations and DOM sources and concentrations were tested at salinities 5, 15 and 30 ppt. Toxicity data (48-h LC50 values) were calculated based on total, dissolved and free copper concentrations. In a broad view, data showed that increasing salinity was protective against the acute waterborne copper toxicity. In general, copper toxicity was also lower in the presence than in the absence of DOM, especially at salinities 5 and 15 ppt. This protective effect of DOM against the acute copper toxicity was also dependent on the DOM concentration, the higher protective effect being observed at the highest DOM concentration tested. Furthermore, it seems to be also dependent on the source of DOM used. In summary, findings reported in the present study clearly indicate that both salinity and DOM (source and concentration) should be taken into account in the development of an estuarine version of the Biotic Ligand Model.

Keywords: *Acartia tonsa*, Acute toxicity, Copper, Natural organic matter, Salinity.

Introduction

Once copper is released into the aquatic environment, a complex set of chemical reactions occurs as a function of the water chemistry, influencing the metal bioavailability and toxicity. For example, dissolved organic matter (DOM), pH, hardness and ionic composition have been shown to protect in some extent against the acute copper toxicity in aquatic animals [1].

There are several studies regarding the influence of dissolved organic matter (DOM), *i.e.* the fraction of natural organic matter (NOM) that is filterable (< 0.45 μm), on metal toxicity [1-11]. DOM concentration is generally measured and expressed in terms of dissolved organic carbon (DOC) concentration (mg C/L) [12]. It has been shown that DOM exerts an important protecting effect against the acute copper toxicity in both freshwater fish and aquatic invertebrates. It forms complexes with copper, thus reducing metal bioavailability and toxicity in fresh water [1,4,8,13].

In general, studies with DOM are performed using commercial organic matter, usually as humic acid (Sigma-Aldrich) from soils [8]. However, the concentration of natural DOM depends primarily on the type of water, origin, vegetation and climate, among other factors [4]. For example, the humic substances constitute 10-30% of the marine DOM and 70-90% of the freshwater marsh DOM [12]. DOM from different places can be composed by molecules with different characteristics because they are formed from different precursors [5]. Therefore, their complexing characteristics and capacities can vary from one place to another [4,5]. Consequently, the DOM source can influence the bioavailability and toxicity of copper to aquatic organisms [4]. However, more studies are needed to better characterize the extension on what the DOM source influences copper toxicity when compared to other factors, especially in a wide range of water salinities.

Models employed to estimate metal bioavailability and toxicity, like the Biotic Ligand

Model (BLM), actually consider the influence of DOM on copper toxicity. However, it characterizes the copper species interaction with the different sources and types of DOM similarly [5]. Therefore, the evaluation of the influence of natural DOM on copper bioavailability and toxicity at different water salinities can help to improve the present BLM version and in the development of a future version of this model for estuarine and marine waters.

Despite the fact that several studies on the effect of different sources of DOM on metals toxicity are available in the literature [3-7,10], most of them are restricted to freshwater species. Only few studies [9-11] have attempted to analyze the effects of DOM on copper toxicity and the consequent implications for saltwater copper criteria. As far as we know, there are no studies reporting the influence of DOM extracted from coastal and marine waters on the acute toxicity of waterborne copper in a wide range of water salinities. There are some studies that evaluated copper toxicity in marine animals exposed to the metal in natural sea water collected in the environment, where DOM concentration and other chemical parameters were measured afterwards and related to metal toxicity [9,11]. The use of concentrated DOM-stock solutions made with natural DOM allows testing different environmentally relevant DOM concentrations besides that present in the natural water. Studies with marine-derived DOM extracted from coastal, and especially from oceanic waters, are lacking because of the methodological difficulties for extracting DOM from salt water [14].

Seawater represents, in terms of DOC recovery yielded after extraction and concentration, one of the most challenging sources of DOM, since inorganic matter can be approximately 30,000 times more concentrated than organic matter. Actually, DOC concentration was estimated to be only between 0.3 and 2.0 mg C/L in sea water [12]. Therefore, DOM from sea water cannot be extracted and concentrated by the simple and inexpensive reverse osmosis method commonly used for extraction of freshwater-derived DOM. A suitable and easy method for extraction of DOM from salt water employing polypropilene (PPL) resins was recently developed [15] and used in the present study to extract and concentrate DOM from coastal and

marine waters.

Therefore, more studies on the possible effect of DOM from different sources on copper toxicity in a wide range of salinity employing copper sensitive species are necessary. In this context, we evaluated in the present study the effect of DOM from five different sources on the acute waterborne copper toxicity in the euryhaline copepod *Acartia tonsa* in a wide range of salinities (5-30 ppt).

The Calanoida copepod *A. tonsa* is cosmopolitan [16]. Adults are tolerant to a wide range of salinities (0-31.5 ppt) [17]. It is a major link between the phytoplankton and the others levels in several food chains both in marine and estuarine waters [16].

It is important to note that marine copepods are considered as sensitive indicators of metal toxicity [18]. However, in studies investigating the toxicity of contaminants, experiments are generally performed with males and females together, thought it is known for many aquatic animals that physiological parameters can be affected differently depending on the gender. In fact, studies with planktonic animals reported differential gender-related sensitivities to different aquatic contaminants [19,20]. Therefore, in the present study, we also investigated a possible differential sensitivity between adult males, copepodite and adult females, and copepodite and adult of both genders of *A. tonsa* to acute waterborne copper toxicity in the absence of DOM.

Materials and Methods

Brackish and sea water

The water at different salinities employed for algae and copepod cultivation were prepared from natural sea water collected at the Cassino Beach (Rio Grande, Rio Grande do Sul state, southern Brazil). However, the different media employed for the acute toxicity tests using copepods were prepared from a stock solution of artificial sea water. This stock solution was prepared diluting artificial sea salts (CoraLife®) in Milli-Q water until reaching the desired

experimental salinities (5, 15 and 30 ppt) [21]. The use of artificial sea water in the present study allowed us to investigate possible effects of different environmentally relevant concentrations of DOM on the acute waterborne copper toxicity in the copepod *A. tonsa*.

Copepod culture and acclimation

The original lot of copepods (*A. tonsa*) was obtained in 2005 from an intensive culture (which was interrupted since 2008) of the Aquaculture Marine Station of the Universidade Federal do Rio Grande (Rio Grande, Rio Grande do Sul state, southern Brazil), maintained as described by Bersano [22]. Briefly, the intensive culture system consists of a conical-cylinder tank in which there is a device with a 150-µm mesh to allow removal of the egg and prevent the passage of adult copepods. It has at the top a glass collector for eggs with a 45-µm mesh. The air inoculated at the base of the central cylinder displaces water upwardly, so that eggs deposited on the bottom of the tank are sucked, pass through the mesh, up through the cylinder and are deposited in the eggs collector cup.

Copepods cultivated at salinity 30 ppt were transferred to the laboratory and kept in 10-L plastic buckets containing water at the desired salinity (5, 15 and 30 ppt). Animals were acclimated at least two weeks before being introduced in the experiments. Room temperature (20°C) and photoperiod (12L:12D) were fixed. Copepods were daily fed with a mixed algal diet composed of *Thalassiosira weissflogii* ($2x10^4$ cells/ml) and *Isochrysis galbana* ($1x10^4$ cells/ml) cultivated in f/2 algae medium [23]. Water at different salinities was prepared by mixing 1-µm mesh filtered sea water collected at the Cassino Beach with distilled water. Media were gently aerated and completely renewed every week.

Freshwater DOM extraction

DOM was extracted from fresh water collected before and after the effluent discharge of the "Navegantes" Sewage Treatment Plant (STP). This STP discharges its treated effluent into

the Vieira Stream, which flows directly into the Patos Lagoon estuary (Rio Grande, southern Brazil; Fig. 1). The first source of DOM was the water collected before the public STP discharge (BSTP-DOM), while the second source was the water collected about 3 m after the discharge of the STP (ASTP-DOM). The third source of NOM, the Suwannee river fulvic acid (SRFA), was purchased from the International Humic Substances Society (SRFA standard I, St. Paul, MN, USA).

Approximately 200 L of water were collected at the two sampling sites and filtered using a sequence of polypropylene filters (nominal pore sizes = 10, 5 and 0.5-µm mesh filters; Polyclean, Cuno, São Paulo, Brazil). The filterable fraction obtained was considered as source of DOM. BSTP-DOM and ASTP-DOM were isolated and concentrated by reverse osmosis [8]. A SRFA stock solution (1,000 mg C/L) was also prepared dissolving the commercial fulvic acid in Milli-Q water.

Saltwater DOM extraction

Marine DOM was extracted from sea water collected in a subtropical region ~20 miles away from Rio Grande coast (southern Brazil). Coastal DOM was extracted from coastal water collected in a tropical region ~5 miles away from the Salvador coast (northeastern Brazil) (Fig. 1).

Water collected at the two sampling sites was filtered using a sequence of polypropylene filters (nominal pore sizes: 1.0 and 0.5-µm mesh filter; Polyclean, Cuno, São Paulo, Brazil). The filtered fraction was considered as source of dissolved DOM, which was extracted and concentrated as previously described [14,15]. DOM powder extracted was diluted in Milli-Q water and DOM stock solutions were stored at 4^{o}C in the dark until their use [13]. DOM stock solutions were filtered again (0.45-µm mesh filter; Sartorius, São Paulo, Brazil) before their use in experiments.

DOM storage and characterization

Dissolved organic carbon (DOC) and copper concentrations in the DOM stock solutions were measured using a total carbon analyzer (TOC 5000, Shimadzu, Japan) and an atomic absorption spectrophotometer (AAS 932 Plus - GBC, IL, USA), respectively. All DOM stock solutions were stored at 4^{o}C in the dark until their use [13,24].

Experimental media

Different experimental media were prepared diluting the DOM with artificial salt water prepared as previously described. Different combinations of salinity (5, 15, and 30 ppt), DOM source (SRFA, BSTP, ASTP, marine and coastal) and environmentally relevant DOC concentrations (measured concentrations; SRFA: 0.6, 1.6 and 4.3 mg C/L; BSTP-DOM: 2.5, 5.4 and 10.5 mg C/L; ASTP-DOM: 1.4, 3.2, and 6.7 mg C/L; marine DOM: 0.5 mg C/L; coastal DOM: 2.2 mg C/L) were tested. Only one concentration of DOC was tested for marine-DOM and coastal-DOM because it was not possible extract and concentrate salt water DOM at high concentrations. The maximum volume of DOM stock solution added to the water to prepare the experimental media (50 mL) was 2 mL.

Different copper concentrations ($CuCl_2.2H_2O$; Vetec, Rio de Janeiro, Brazil) were added to the experimental media from stock solutions (0.02; 0.2; or 2 g/L Cu) acidified with 0.1% nitric acid (SupraPur®, Merck, USA). Copper concentration in DOM stock solutions were previously measured, as described below, and considered in the calculation of the amount of copper needed from the stock solutions to be added to experimental media in order to obtain the desired final concentrations of copper. Experimental media were kept at 20^{o}C in the dark for 24 h before their use in the toxicity tests [13,24]. In fact, it is reported that 24 h is enough to bring solutions to the experimental temperature and to let the complexation between Cu and humic acid reach the equilibrium [24].

Acute toxicity tests: gender- related sensitivity to copper

Toxicity tests were performed in the absence of DOM. Control tests with no copper addition into the experimental media were also performed for each experimental salinity (5, 15 and 30 ppt). Each experimental flask was run in duplicate.

Prior to experiments, copepods (total length = 0.80 ± 0.09 mm; dry weight = 4.5 ± 0.87 μg) were randomly collected from the culture using a 300 μm-mesh net. According to *Acartia* size categories by prosome length and the estimated dry mass [25], and based on the mean prosome lengths of *A. tonsa* copepodites and adult males and females [26], this net mesh size may retain both copepodite and adult copepods of both genders.

Adult males were identified according to antenna conformation [27] and separated under a stereoscopic microscope. Toxicity tests were then run with adult males separately, copepodite and female adults together, or copepodite and adult copepods of both genders together, using a standard static-renew system and in the absence of food. Ten copepods were introduced in each glass flask containing 50 mL of experimental medium prepared as described above.

Flasks were kept under constant rotation (2 rpm) in a incubator with fixed temperature (20^{o}C) and photoperiod (16L:8D). After 24 h, living copepods from each flask were counted and transferred to a fresh experimental medium prepared as described above. Dead copepods were discarded. After 48 h, living copepods from each flask were counted and discarded. Lethal copper concentrations for 50% of the tested organisms (LC50 values) and their corresponding 95% confidence intervals were determined based on the accumulated mortality after 48 h of test, as described below.

Acute toxicity tests: effects of salinity and DOM on acute copper toxicity

Two types of control tests were run in the absence of copper in each experimental salinity: (1) in the absence of DOM; and (2) in the presence of DOM at the desired concentration. Two types of toxicity tests were also performed using different copper

concentrations in each experimental salinity: (1) in the absence of DOM; and (2) in the presence of DOM at the desired concentration. Controls and toxicity tests were run in duplicate.

As a similar sensitivity of adult males, and copepodite and adult females to acute copper exposure was observed, and as in general sensitivity in these two treatments was also similar to that observed in treatments with copepodites and adults of both genders (see the Results section), toxicity tests performed to investigate the effects of different combinations of salinity, DOM (source and concentration) and copper were run with copepodites and adults of both genders together. Prior to the experiments, copepods were randomly collected from the culture media using a 300 μm-mesh net and acutely exposed to DOM, waterborne copper or the combination of both. Toxicity tests were performed following the same procedures described above.

Water chemistry

At the beginning and after 24 h of test, the dissolved oxygen concentration and pH were directly measured in the experimental media using an oxymeter (Digimed, DMO-2, São Paulo, Brazil) and a pH meter (Digimed, DMPH-2, São Paulo, Brazil), respectively.

At the beginning and after 24 h of test, non-filtered and filtered (0.45-μm mesh filter) samples (10 mL) from the experimental media were also collected and acidified (0.5% HNO_3, Suprapur®, Merck, USA) for copper concentration measurements. Non-filtered samples (10 mL) were also collected for water chemistry analysis, as described below.

Copper concentration in filtered (total dissolved copper) and non-filtered (total copper) samples of the experimental media was measured by atomic absorption spectrophotometry in the flame mode (AAS 932 Plus - GBC, IL, USA). Free copper concentrations were calculated based on water chemistry data and the total dissolved copper concentrations determined as described above, using the software Visual MINTEQ version 2.61 (KTH, Department of Land and Water, Resources Engineering, Stockholm, Sweden), as previously described [28].

Cation (Na^+, K^+, Ca^{2+}, and Mg^{2+}) concentrations in non-filtered samples of the experimental media were measured by atomic absorption spectrophotometry in the flame mode (AAS 932 Plus - GBC, IL, USA). Anion (Cl^-) concentration was measured using a commercial reagent kit (Chloride, Doles S.A., Goiânia, Brazil). Absorption readings were performed at 510 nm (B 382, Micronal, Campo Grande, Brazil). Sulphate concentration and alkalinity in the non-filtered samples of the experimental media were measured using a spectrophotometric method [29] and according to the method described by the American Public Health Association [30], respectively. DOC concentration was measured using a total carbon analyzer (TOC-V CPH, Shimadzu, Japan).

Data presentation and statistical evaluation

The 48-h LC50 values were determined by Probit analysis [31] based on total, dissolved, and free copper concentrations. Differences in the 48-h LC50 values were detected by visually comparing their respective 95% confidence intervals. Values were considered different when their 95% confidence intervals did not overlap.

Data from water chemistry parameters were expressed as mean ± standard deviation. Differences in water chemistry between treatments were assessed by one-way analysis of variance (ANOVA) followed by the Tukey test. The significance level adopted was 5% ($\alpha = 0.05$).

Results

Water chemistry

Measured DOC concentrations in the DOM stock solutions were 121.8, 126.3 and 1000, 555.3 and 1119.0 mg C/L for the BSTP-DOM, ASTP-DOM, SRFA, marine and coastal DOM, respectively. Dissolved copper concentrations in BSTP- and ASTP-DOM stock solutions were 848.7, 574.7 µg Cu/L, respectively. It is important to take into account that water samples from

Vieira Stream used to concentrate BSTP- and ASTP-DOM passed successively by the reverse osmosis system until it was reached a small volume of water for each DOM source. Thus BSTP- and ASTP-DOM, as well as other chemicals in the DOM samples, were not concentrated in equal proportions. Dissolved copper concentrations in SRFA, marine and coastal DOM stock solutions were <10 µg Cu/L.

In each water salinity, no significant difference was observed in the artificial salt water composition before and after DOM and/or Cu addition (data not shown). Therefore, only one general mean value was calculated for each water salinity. All water chemistry parameters significantly augmented with the increasing salinities, except the dissolved oxygen content that was similar in salinities 15 and 30 ppt (Table 1).

Gender- and salinity-related sensitivity to acute copper exposure

In the absence of DOM, 48-h LC50 values calculated based on total (Fig. 2A), dissolved (Fig. 2B) or free (Fig. 2C) copper concentrations were similar for adult males and the treatments with copepodites and female adults tested together, at each experimental salinity (5, 15 and 30 ppt). When copepodites and adults of both genders were tested together, 48-h LC50 values calculated based on total or free copper concentrations were similar among the different water salinities. However, the 48-h LC50 value calculated based on dissolved copper concentrations was higher in salinity 30 ppt than in salinity 5 ppt (Fig. 2).

Acute copper toxicity at different combinations of salinity, DOM and copper

In all salinities and for all types of DOM tested in the present study, addition of DOM to the experimental media had no significant effect on mortality of *A. tonsa*, even at the highest DOM concentration tested (data not shown).

Based on total copper concentrations, the 48-h LC50 value was not affected by SRFA addition in the experimental medium, except in salinity 15 ppt, where SRFA at 0.6 mg C/L

increased the 48-h LC50 value (Fig. 3A). Based on dissolved copper concentrations, it was increased at 4.3 mg C/L SRFA in salinity 5 ppt and at 0.6 mg C/L SRFA in salinity 15 ppt. However, the 48-h LC50 was decreased at 0.6 and 1.6 mg C/L in salinity 30 ppt (Fig. 3B). Based on free copper concentrations, the 48-h LC50 decreased as the concentration of SRFA increased in the experimental medium, in all salinities tested, except in the treatment with addition of 0.6 mg C/L SRFA in salinity 5 ppt (Fig. 3C). In the presence of all SRFA concentrations tested, 48-h LC50 values calculated based on total and dissolved copper concentrations were higher in salinity 15 ppt than in salinity 5 ppt (Fig. 3A and 3B). In the presence of 4.3 mg C/L SRFA, 48-h LC50 values calculated based on total and dissolved copper concentrations were also higher in salinity 30 ppt than in salinity 5 ppt (Fig. 3A and 3B). In the absence of DOM, 48-h LC50 value calculated based on free copper concentrations was not affected by water salinity. However, it was lower in salinity 30 ppt than in the other water salinities tested (5 and 15 ppt) when SRFA was added to the experimental medium (Fig. 3C).

Based on total (Fig. 4A) or dissolved (Fig. 4B) copper concentrations, the 48-h LC50 value increased as the concentration of BSTP-DOM augmented in the test medium at salinities 5 and 15 ppt (except at 2.5 mg C/L in salinity 15). In salinity 30 ppt, it was increased only at the highest BSTP-DOM concentration tested (10.5 mg C/L). However, toxicity values based on free copper concentrations were lower in the presence of BSTP-DOM than in the absence of BSTP-DOM in all experimental salinities, especially at 30 ppt, except at 2.5 mg C/L in salinity 5 ppt (Fig. 4C). In the presence of 10.5 mg C/L BSTP-DOM, 48-h LC50 values calculated based on total and dissolved copper concentrations were lower in salinity 5 than in salinities 15 and 30 ppt (Fig. 4A and 4B). 48-h LC50 value calculated based on free copper concentrations was lower in salinity 30 ppt than in the other water salinities tested (5 and 15 ppt) when BSTP-DOM was added to the experimental medium (Fig. 4C).

Based on total copper concentrations, the 48-h LC50 value increased as the concentration of ASTP-DOM augmented in the experimental medium at salinities 15 and 30 ppt (except at 1.4

mg C/L in salinity 30). In salinity 5 ppt, the 48-h LC50 value was increased only at 3.2 mg C/L ASTP-DOM (Fig. 5A). When the 48-h LC50 were calculated based on dissolved copper concentrations, a similar tendency as described above was obtained for salinities 5 and 15 ppt. However, no change in the 48-h LC50 was observed in salinity 30 ppt after addition of ASTP-DOM (Fig. 5B). 48-h LC50 values calculated based on total copper concentrations were higher in salinity 15 than in salinity 5 ppt in the presence of 1.4 and 6.7 mg C/L ASTP-DOM, and were also higher in salinity 30 than in salinity 5 ppt in the presence of 3.2 and 6.7 mg C/L (Fig. 4A and 4B). 48-h LC50 values calculated based on dissolved copper concentrations were higher in the presence of 3.2 and 6.7 mg C/L ASTP-DOM in salinity 15, and 6.7 mg C/L ASTP-DOM in salinity 30, than in salinity 5 ppt. Based on free copper concentrations, the 48-h LC50 decreased as the concentration of ASTP-DOM increased in the experimental medium in all salinities tested, especially at 30 ppt, except at 1.4 mg C/L in salinity 5, 15 and 30 ppt, and at 3.2 mg C/L in salinity 15 ppt (Fig. 5C). 48-h LC50 value calculated based on free copper concentrations was lower in salinity 30 ppt than in the other water salinities tested (5 and 15 ppt) when ASTP-DOM was added to the experimental medium, except at 1.4 mg C/L (Fig. 5C).

In salinities 5 and 15 ppt, 48-h LC50 value calculated based on total (Fig. 6A) or dissolved (Fig. 6B) copper concentrations was higher for copepods exposed to copper in the presence of marine DOM than for those exposed to the metal under control conditions or in the presence of coastal DOM. In salinity 30 ppt, the 48-h LC50 value was similar in all treatments. In the absence of DOM or the presence of coastal DOM, the 48-h LC50 value increased with increasing water salinity. In the presence of marine DOM, no change in the 48-h LC50 value was observed as a function of water salinity. These results were similar when toxicity values were calculated based on total (Fig. 6A) or dissolved (Fig. 6B) copper concentrations. In all salinities, 48-h LC50 value calculated based on free copper concentrations was higher in the presence of marine DOM than in the absence of DOM or the presence of coastal DOM (Fig. 6C). In the absence of DOM, 48-h LC50 value was not affected by water salinity. However, it was lower in

salinity 30 ppt than in the other water salinities tested (5 and 15 ppt) when marine or coastal DOM was added to the experimental medium (Fig. 6C).

Discussion

Some studies reported in the literature indicate that gender can influence the toxicity of chemical contaminants to aquatic invertebrates. For the copepod *A. tonsa*, males were shown to be less tolerant to crude oil than females [19]. However, it is important to note that the majority of toxicological studies performed with small invertebrates does not take this issue into account. In the present study, a possible gender related change in the acute toxicity of waterborne copper was tested using adult males of *A. tonsa,* and comparing them to treatments with copepodites and adult females together, or with copepodites and adults of both genders tested together. Adult males and the treatment with copepodites and adult females showed similar sensitivity to the acute exposure to waterborne copper in a wide range of salinities (5, 15 and 30 ppt). Also, gender was shown to not influence the acute toxicity of tributiltin (TBT) in Caprellidea amphipods [20]. In addition, copepodites and adult copepods seem to have the same sensibility to copper, since no differences were observed in the copper LC50 between adult males and copepodites and adult females tested together. In general, toxicity in these two treatments was similar to that in treatments with copepodites and adult of both genders.

In the present study, the influence of water salinity and DOM on the acute waterborne copper toxicity was also evaluated in *A. tonsa*. In this case, copepodites and adult males and females were tested together, since no marked gender- and age- related influence on copper toxicity was observed, as discussed above. As far as we know, this is the first study to report the influence of natural DOM on acute toxicity of copper in euryhaline copepods in a wide range of water salinities. Waterborne copper was shown to be toxic to *A. tonsa* in all salinities tested (5, 15 and 30 ppt) in either the absence or the presence of DOM. In a broad view, copper toxicity

was mainly associated with total dissolved copper. This statement is based on the fact that virtually no differences were observed between the 48-h LC50 values calculated based on total and dissolved copper concentrations at each experimental condition.

Salinity by itself was protective against the acute waterborne copper toxicity in a concentration dependent manner. In general, 48-h LC50 values at salinity 5 ppt were lower when compared with those found in higher salinities. This finding was also observed when 48-h LC50 values based on dissolved copper concentrations were compared among treatments without DOM addition. It is worth to note that acute toxicity tests performed with filtered-natural sea water have also shown similar results for *A. tonsa* exposed to copper [28]. It is well known that the high levels of ions present in sea water acts as a protecting factor against metal toxicity due to metal complexation with anions, especially chloride, as well as the competition between cations and copper for binding sites on the biotic ligand. Thus, the observed protective effect of water salinity against the acute copper toxicity may be explained by considering the water chemistry. In fact, no significant difference was observed between 48-h LC50 values calculated based on free copper concentrations in treatments without DOM addition at salinities 5, 15 and 30 ppt. Indeed, it is widely reported that free copper is the most toxic copper species to aquatic organisms [32].

Depending on the source and concentration, humic substances can express a xenobiotic-like influence on organisms [33]. However, in all salinities and for all types of DOM tested in the present study, addition of DOM to the experimental media had no significant effect on mortality of *A. tonsa*, even at the highest DOM concentration tested (10.5 mg C/L). Therefore, mortality observed in control copepods cannot be ascribed to possible DOM effects, but likely to stress associated with copepod's handling. This finding is in agreement with results from other studies. For example, the embryogenesis success rate in the sea urchin *Paracentrotus lividus* decreased only at high DOM concentration, with no effect on larval growth [2]. Also, a toxic effect of freshwater DOM from two different sources to developing mussel embryos was observed only at

total levels >10-20 mg C/L [10].

Our data clearly indicate that DOM has a protective effect against the acute copper toxicity in *A. tonsa*. This effect was dependent on the source and concentration of DOM. Overall copper toxicity was lower at higher DOC concentrations. In freshwater invertebrates, it was reported that DOM from three different sources reduced both acute and chronic copper toxicity to the same extent and that an increase in DOC resulted in a linear increase of 21-d NOEC and EC50 values in *Daphnia magna* [3]. The authors also pointed out that DOC concentration was the most important factor in determining copper chronic toxicity in *D. magna*, explaining about 60% of the observed variability [3]. We observed that several concentrations of BSTP- and ASTP-DOM, especially the higher ones, were protective against the acute copper toxicity. A protective effect of NOM against acute waterborne copper toxicity in rainbow trout was also observed, but it was dependent on the NOM source [6]. It was suggested that DOC and humic acid concentrations could better explain the variability in LC50 values in freshwater fish larvae than the detailed considerations or descriptions of the binding between DOM and copper [5], although it was also observed that DOM source had a significant influence on copper toxicity. However, all the studies reported here were performed on freshwater organisms.

In a study with marine mussel embryos, it was observed that, among three different sources of NOM, the one with higher fulvic acid and lower humic acid content (Nordic Reservoir NOM) was the more protective against copper toxicity [10]. The authors believe that Nordic Reservoir NOM, with its high level of fulvic acid, could better represent a marine NOM, because it was reported [34] that fulvic acids comprise the most relevant quantitative fraction of seawater DOC related to protection against copper toxicity rather than humic acids. In the present study, the three sources of freshwater DOM tested were predominantly of terrestrial origin (allochthonous). Based on previous information, it could be expected that, among the freshwater DOM sources tested, SRFA would be the more protective against the acute copper toxicity in the euryhaline copepod *A. tonsa*, as it contains mainly fulvic acid. Based on toxicity

(48-h LC50) values calculated from dissolved copper concentrations, a protective effect of SRFA was only observed at the highest concentration tested (4.3 mg C/L) at salinity 5 ppt. In turn, all concentrations of BSTP-DOM (2.5, 5.4 and 10.5 mg C/L), ASTP-DOM at 3.2 mg C/L, and marine-DOM at 0.5 mg C/L protected against the acute copper toxicity at this salinity. At salinity 15 ppt, SRFA protected against copper toxicity only at the lower concentration tested (0.6 mg C/L), while marine DOM and all concentrations of ASTP- and BSTP-DOM tested (except 2.5 mg C/L BSTP) protected against acute copper toxicity. At salinity 30 ppt, no protection against copper toxicity was observed after SRFA, ASTP-DOM, marine and coastal DOM addition in the experimental medium. An increased copper toxicity was even observed with SRFA at 0.6 and 1.6 mg C/L. Furthermore, a protective effect of DOM against copper toxicity was observed only at the higher concentration of BSTP-DOM tested (10.5 mg C/L). These results clearly indicate that acute copper toxicity may be influenced by DOM, but the degree of DOM protection is dependent on DOM concentration and source, and mainly the water salinity employed in the toxicity test. Indeed, it is known that salinity influences the binding between copper and DOM. Binding between copper and DOM (river humic acid) reached a maximum of 28% at salinity 5 ppt, however, this binding increased to 60% at salinity 15 ppt [35]. Changes in pH and interactions between several other ions present in sea water and humic acid, as well as conformational changes in humic molecules leading to an exposure of more copper-binding sites, could be involved in this process [35].

In one of the few studies regarding the marine DOM influence on metal toxicity for marine animals [11], it was reported a strong linear correlation between DOM concentration and EC50 for copper nitrate in mussel (*Mytilus sp.*) larvae, irrespective the DOM source used (marine and estuarine water samples). Considering this background, one could expect that the coastal DOM tested in the present study would be more protective against the acute toxicity of waterborne copper than the marine DOM, since the concentration of coastal DOM tested was 4.4-fold higher that that employed for marine DOM. However, data from the toxicity test

performed in the present study clearly showed that only marine DOM was protective against the acute toxicity of waterborne copper when compared to coastal DOM or the treatment without DOM addition. Furthermore, this protective effect was dependent on water salinity. In addition, the fluorescent fulvic- and humic-like fractions appeared to be good predictive measures of dissolved copper toxicity in the presence of estuarine and marine DOM [11]. Therefore, considering results from the present study at 5 and 15 ppt salinity, it is possible that the protection against the acute toxicity of waterborne copper showed by the marine DOM tested could be related to a higher fraction of fulvic acid present in this DOM than in the coastal DOM. This could be contradictory, especially when comparing results from toxicity tests with 0.5 mg C/L of marine DOM and 0.6 mg C/L SRFA, once marine-DOM protected more against copper toxicity than SRFA in salinity 5 ppt, and SRFA presented a harmful effect at salinity 30. However, SRFA is a freshwater DOM, thus changes in water chemistry, especially in salt water, may lead to different interactions between DOM and copper, similar to that mentioned for humic acid molecules [35]. Although the measured tryptophan and tyrosine fractions in water samples are likely not able to bind metal, because they are embedded within dissolved proteins as peptide bonds [11], amino acid composition of marine DOM could be considered as an additional explanation for the protective effect of the low concentration of marine DOM tested (0.5 mg C/L), contrasting with no protective effect of coastal DOM (2.2 mg C/L) against copper toxicity.

It is also worth to note that the protective effect of marine DOM against the acute toxicity of waterborne copper was only observed in the lower water salinities tested (5 and 15 ppt). Indeed, both types of salt water DOM did not protected against the acute toxicity of waterborne copper in sea water (salinity 30 ppt). Regarding fulvic acid, a reduction in labile copper concentration with increasing freshwater DOM (Suwannee River fulvic acid) concentrations (8-64 mg C/L) was observed at salinity 34 ppt [36]. However, the magnitude of the reduction was dependent on the fulvic acid and total copper concentrations. In addition, the authors reported that the binding between copper and fulvic acid is weaker than that between copper and humic

acid. However, the lowest DOM concentration used (8 mg C/L) [36] was considerably higher than the environmentally relevant salt water DOM concentrations tested in the present study, i.e., 0.5 mg C/L (marine DOM) or 2.2 mg C/L (coastal DOM). Therefore, it is possible that these DOM concentrations were not high enough to show some protective effect against the acute copper toxicity in sea water (salinity 30 ppt).

Based on the toxicity values calculated from dissolved copper concentrations for the five different DOM sources tested, water salinity seems to be more important than DOM as a protective factor against the acute copper toxicity. This statement is based on the following facts: (1) SRFA was effective as a protective factor against copper toxicity only in low salinity (5 ppt); (2) a higher concentration of BSTP- or ASTP-DOM is needed to reduce copper toxicity as water salinity increases; (3) no protection against copper toxicity was observed in high salinity (30 ppt) even at the highest concentration of SRFA (4.3 mg C/L) and ASTP-DOM (6.7 mg C/L) tested; (4) no protection against copper toxicity was observed in high salinity (30 ppt) in the presence of marine DOM (0.5 mg C/L); and (5) a protective effect of BSTP-DOM against copper toxicity at salinity 30 ppt was only observed at a much higher concentration (10.5 mg C/L BSTP) than the highest ones tested for the other types of DOM. Actually, as salinity increases, chemicals known to mitigate metal toxicity, such as chloride ions, will be at a higher concentration. Therefore, other chemical parameters could be more important in decreasing copper toxicity at higher salinities than DOM. In addition, in the absence of DOM, toxicity (48-h LC50) value calculated based on free copper concentrations was similar in all salinities tested in the present study. This finding clearly indicates that differences in acute toxicity of waterborne copper associated with water salinity in the absence of DOM can be explained only by considering the changes in water chemistry among the different water salinities. Furthermore, findings reported in the present study indicate that the protective effect provided by water salinity is more relevant than that provided by DOM in sea water.

Contrary to the situation discussed for results in the absence of DOM, water chemistry

changes occurring among the different water salinities tested certainly cannot explain the differences observed among the 48-h LC50 values calculated based on free copper concentrations in the presence of marine and coastal DOM at salinities 5, 15 and 30 ppt. In this case, a lower acute lethal toxicity of copper was observed in the presence of marine DOM than in the absence of DOM or in the presence of coastal DOM, in all water salinities tested. This finding suggest that more free copper ion is needed in the presence of marine DOM to induce a toxicity similar to that observed in the absence of DOM or in the presence of coastal DOM. Addition of marine DOM in salt water is probably increasing the copepod tolerance to acute exposure to waterborne copper in a wide range of salinities (5-30 ppt). This effect could be associated to nutritional aspects. However, future studies are needed for a better physicochemical characterization of the marine DOM extracted and used in the present study, as well as its interactive properties with copper in salt water.

On the other hand, for all types of freshwater DOM tested, in general a higher toxicity (48-h LC50) value calculated based on free copper concentrations was observed as DOM concentration increased in the experimental medium. Furthermore, toxicity values observed in the presence of marine and coastal DOM, and almost all toxicity values observed in the presence of freshwater DOM at salinity 30 ppt were significantly smaller than those observed at salinities 5 and 15 ppt. These findings indicate that less free ionic copper is needed to induce an acute toxicity in salinity 30 ppt similar to that observed in lower salinities (5 and 15 ppt). This fact can be explained considering the toxicity of other Cu species, such as $CuCO_3$, $Cu(CO_3)_2^{2-}$, $CuOH^+$ and $Cu(OH)_2$, besides the free ion (Cu^{2+}) [28]. In fact, $CuCO_3$, Cu^{2+}, and $CuOH^+$ were estimated (VisualMinteq software) to be the most abundant Cu species in natural filtered salt water at salinities 5, 15 and 30 ppt, and the 48-h EC_{50} values based on free copper and on hydroxil copper species concentrations did not changed much at salinities 5, 15 and 30 ppt [28]. Another possibility is that copper-DOM complexes might have been ingested by copepods, thus also inducing toxicity to copepods in sea water (salinity 30 ppt), as discussed below.

It was reported that copper-humic acid complexes can be partially available for uptake and therefore lead to an increased toxicity [24]. In addition, the copepod *Acartia spinicauda* was found eating on particles with bound metals [37]. These authors also suggested that fecal pellets associated with metals could also be available for copepods. Approximately 20% of the copper bound to organic matter, as Aldrich humic acid, was available to cause toxicity in fathead minnows (freshwater fish) [1]. However, it is important to note that copepods are able to select food size [16]. Adult individuals of *A. tonsa* eat preferentially particles between 14 and 250 μm [38]. In addition, calanoid copepods, as those from the genus *Acartia*, can eat on particles normally rejected if their preferred food is absent [37]. Also, it is important to stress that experiments in the present study were performed in the absence of food. Therefore, copper-DOM complexes could have reached a size large enough to be selected and ingested by copepods during experiments. It is thus reasonable to consider that *A. tonsa* could be ingesting copper-DOM complexes along the experimental period, although several DOC concentrations tested protected against copper toxicity. In this case, ingestion of copper-DOM complexes should be considered as being significantly harmful to copepods exposed to copper for a long period of time in the absence of food.

In summary, findings reported in the present study indicate that water salinity and DOM source and concentration are important factors to be considered in toxicological modeling aiming to predict the toxicity of waterborne copper in a wide range of salinities, as in the scope of a future BLM version for estuarine and marine waters. This has important implications for humic ion-binding models like WHAM (Windermere humic aqueous model), which is used at the BLM approach to compute organic speciation [39]. Despite the fact that BLM is actually calibrated for several DOM sources, it characterizes the copper species interaction with different sources and types of DOM in the same way [5,32,39], although it is widely reported that DOM properties and complexity vary according to different environments [4,5]. In the present study, we observed that the degree of DOM protection against the acute copper toxicity is actually dependent on both

DOC concentration and source. This is in agreement with a previous study [7], which demonstrated that the incorporation of DOM variability in the BLM as a factor controlling copper speciation, bioavailability, and toxicity, improved the predictive capacity of this model for *D. magna*, a freshwater invertebrate. In this context, more studies regarding the effects of marine-derived NOM on the toxicity of metals are necessary to provide more realistic results for marine and estuarine species in order to improve future speciation models for these organisms.

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Table 1. Physicochemical characteristics of the artificial salt water used in the toxicity tests with the copepod *Acartia tonsa*. Different letters indicate significantly different mean values among salinities for the same parameter.

Parameter	**Salinity**		
	5	**15**	**30**
pH	6.88 ± 0.22^{A}	7.21 ± 0.14^{B}	7.49 ± 0.13^{C}
D.O. (mmol O_2/L)	0.24 ± 0.01^{A}	0.19 ± 0.02^{B}	0.19 ± 0.01^{B}
Na^+ (mmol/L)	60.63 ± 0.93^{A}	243.47 ± 3.75^{B}	490.41 ± 7.55^{C}
K^+ (mmol/L)	1.76 ± 0.03^{A}	7.20 ± 0.11^{B}	14.88 ± 0.23^{C}
Mg^{2+} (mmol/L)	2.73 ± 0.04^{A}	9.07 ± 0.14^{B}	23.32 ± 0.36^{C}
Ca^{2+} (mmol/L)	1.25 ± 0.02^{A}	5.13 ± 0.08^{B}	10.92 ± 0.17^{C}
Cl^- (mmol/L)	107.95 ± 1.66^{A}	355.09 ± 5.47^{B}	639.81 ± 9.85^{C}
SO_4^{2-} (mmol/L)	0.246 ± 0.004^{A}	1.427 ± 0.022^{B}	3.001 ± 0.046^{C}
Alkalinity (mmol $CaCO_3$/L)	0.167 ± 0.003^{A}	0.502 ± 0.008^{B}	1.141 ± 0.018^{C}
DOC (mg/L)	< 0.1	< 0.1	< 0.1
Copper (µg/L)	< 10	< 10	< 10

Figure Legends

Figure 1. Map of South America showing the sampling sites where water was collected to extract the DOM used in the experiments with the copepod *Acartia tonsa*. Freshwater DOM: extracted from fresh water collected in the Vieira Stream (Rio Grande, Rio Grande do Sul state, southern Brazil, 32°06'S 51°10'W). 1 = before the "Navegantes" Public Sewage Treatment Plant (BSTP-DOM); 2 = about 3 m after the "Navegantes" Public Sewage Treatment Plant (ASTP-DOM). Marine DOM: 3 = extracted from marine water collected ~20 miles away from Rio Grande coast (marine DOM; subtropical region, southern Brazil, 32°10'S 51°45'W); 4 = extracted from coastal water collected ~5 miles away from Salvador coast (coastal DOM; tropical region, northeastern Brazil, 13°01'S 38°33'W).

Figure 2. Acute waterborne copper toxicity in the euryhaline copepod *Acartia tonsa* in the absence of DOM at different water salinities. Copepodites and adult females (open bars), adult males (diagonally crossed bars), and copepodites and adult of both genders (diagonally inverted crossed bars) were tested. Data are expressed as 48-h LC50 values and their corresponding 95% confidence intervals. These values were calculated based on total (A), dissolved (B) and free (C) copper concentrations. Copper speciation was performed based on the 48-h LC50 values and their corresponding 95% confidence intervals calculated based on dissolved copper concentrations. Different lowercase letters indicate different 48-h LC50 values among treatments for the same salinity. Different capital letters indicate different 48-h LC50 values among water salinities for the same treatment.

Figure 3. Acute waterborne copper toxicity in the euryhaline copepod *Acartia tonsa* in the presence of SRFA at different water salinities. Different DOM concentrations were tested: control (no DOM addition; open bars); 0.6 mg C/L (diagonally crossed bars); 1.6 mg C/L

(diagonally inverted crossed bars); 4.3 mg C/L (double crossed bars). Copepodites and adult males and females were tested together. Data are expressed as 48-h LC50 values and their corresponding 95% confidence intervals. These values were calculated based on total (A), dissolved (B) and free (C) copper concentrations. Copper speciation was performed based on the 48-h LC50 values and their corresponding 95% confidence intervals calculated based on dissolved copper concentrations. Different lowercase letters indicate different 48-h LC50 values among treatments for the same salinity. Different capital letters indicate different 48-h LC50 values among water salinities for the same treatment.

Figure 4. Acute waterborne copper toxicity in the euryhaline copepod *Acartia tonsa* in the presence of BSTP-DOM at different water salinities. Different DOM concentrations were tested: control (no DOM addition; open bars); 2.5 mg C/L (diagonally crossed bars); 5.4 mg C/L (diagonally inverted crossed bars); 10.5 mg C/L (double crossed bars). Copepodites and adult males and females were tested together. Data are expressed as 48-h LC50 values and their corresponding 95% confidence intervals. These values were calculated based on total (A), dissolved (B) and free (C) copper concentrations. Copper speciation was performed based on the 48-h LC50 values and their corresponding 95% confidence intervals calculated based on dissolved copper concentrations. Different lowercase letters indicate different 48-h LC50 values among treatments for the same salinity. Different capital letters indicate different 48-h LC50 values among water salinities for the same treatment.

Figure 5. Acute waterborne copper toxicity in the euryhaline copepod *Acartia tonsa* in the presence of ASTP-DOM at different water salinities. Different DOM concentrations were tested: control (no DOM addition; open bars); 1.4 mg C/L (diagonally crossed bars); 3.2 mg C/L (diagonally inverted crossed bars); 6.7 mg C/L (double crossed bars). Copepodites and adult males and females were tested together. Data are expressed as 48-h LC50 values and their

corresponding 95% confidence intervals. These values were calculated based on total (A), dissolved (B) and free (C) copper concentrations. Copper speciation was performed based on the 48-h LC50 values and their corresponding 95% confidence intervals calculated based on dissolved copper concentrations. Different lowercase letters indicate different 48-h LC50 values among treatments for the same salinity. Different capital letters indicate different 48-h LC50 values among water salinities for the same treatment.

Figure 6. Acute waterborne copper toxicity in the euryhaline copepod *Acartia tonsa* in the presence of marine and coastal DOM at different water salinities. The following treatments were tested: artificial salt water withouth DOM (open bars); artificial salt water with marine-DOM (0.5 mg C/L) (diagonally crossed bars); and artificial salt water with coastal-DOM (2.2 mg C/L) (diagonally inverted crossed bars). Copepodites and adult males and females were tested together. Data are expressed as 48-h LC50 values and their corresponding 95% confidence intervals. These values were calculated based on total (A), dissolved (B) and free (C) copper concentrations. Copper speciation was performed based on the 48-h LC50 values and their corresponding 95% confidence intervals calculated based on dissolved copper concentrations. Different lowercase letters indicate different 48-h LC50 values among treatments for the same salinity. Different capital letters indicate different 48-h LC50 values among water salinities for the same treatment.

Figure 1

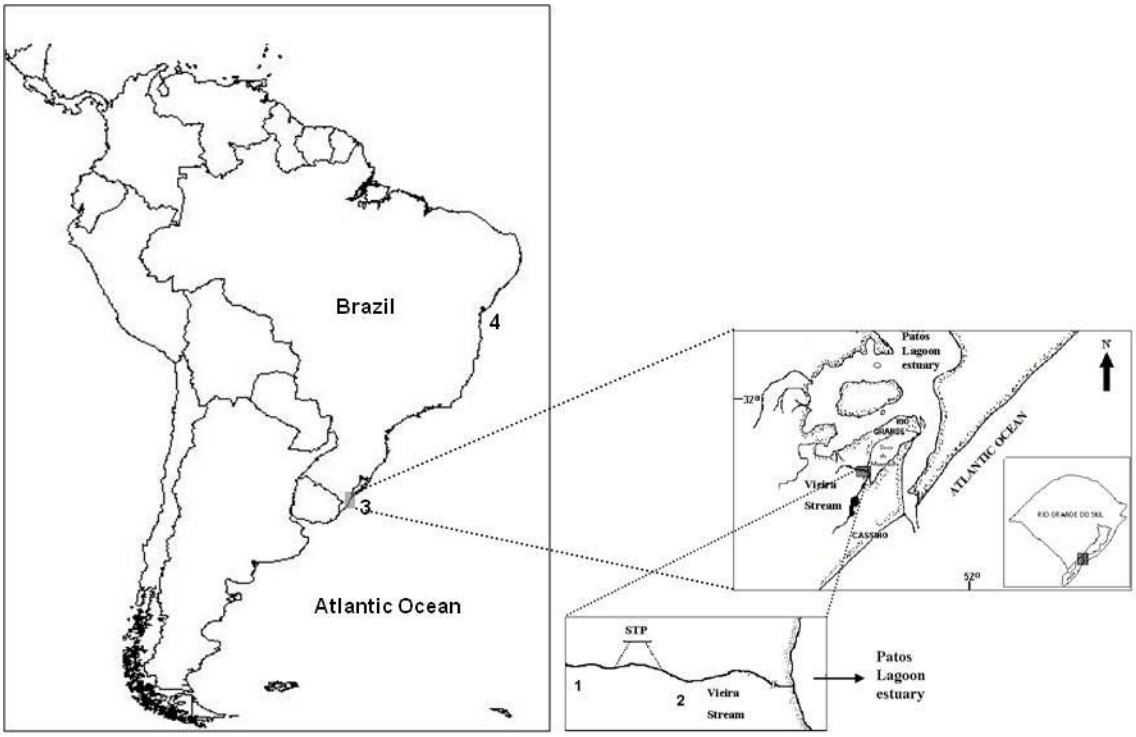

Brazil
4
3
Atlantic Ocean
Patos
Lagoon
estuary
32°
RIO
GRANDE
Vieira
Stream
CASSINO
ATLANTIC OCEAN
N
52°
RIO GRANDE DO SUL
STP
1
2
Vieira
Stream
Patos
Lagoon
estuary

Figure 2

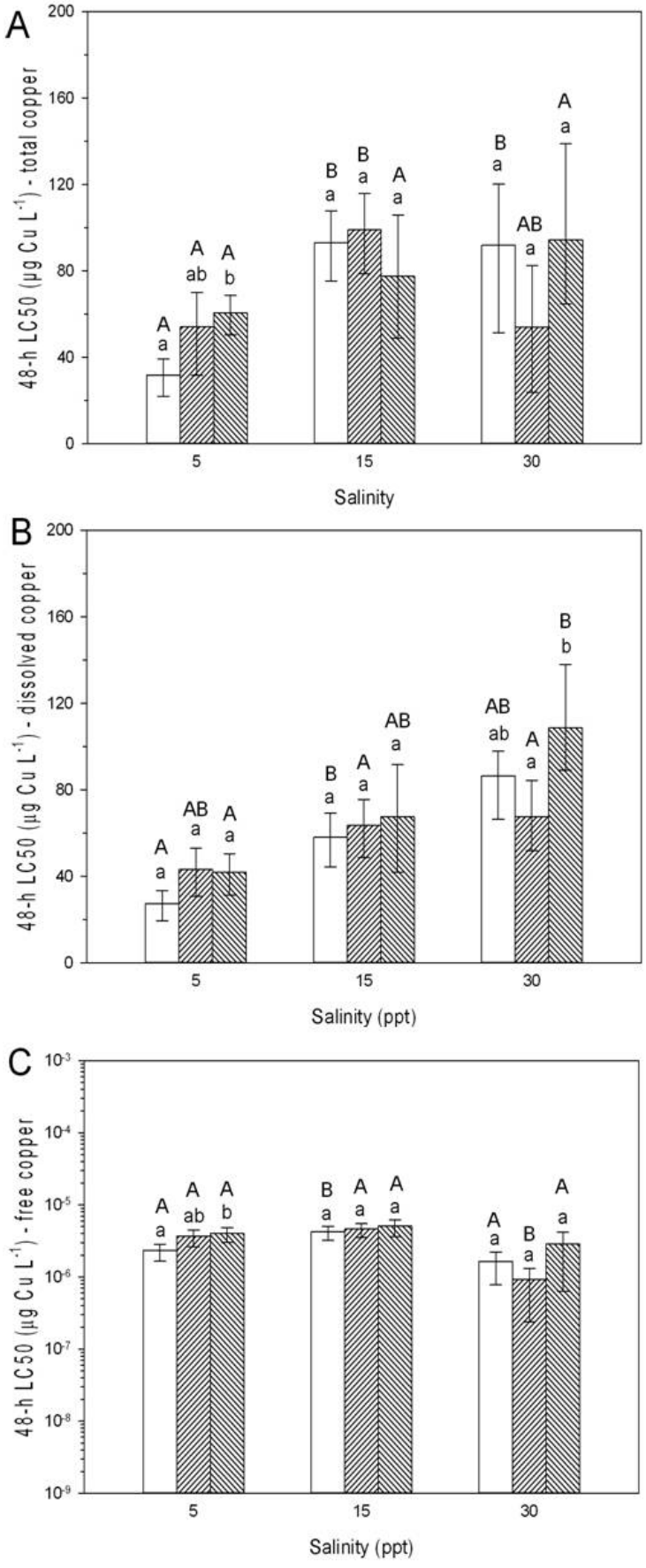

A
48-h LC50 (µg Cu L-1) - total copper
200
160
120
80
40
0
A a
A ab
A b
B a
B a
A a
B a
AB a
A a
5
15
30
Salinity
B
48-h LC50 (µg Cu L-1) - dissolved copper
200
160
120
80
40
0
A a
AB a
A a
B a
A a
AB a
AB ab
A a
B b
5
15
30
Salinity (ppt)
C
48-h LC50 (µg Cu L-1) - free copper
10-3
10-4
10-5
10-6
10-7
10-8
10-9
A a
A ab
A b
B a
A a
A a
A a
B a
A a
5
15
30
Salinity (ppt)

Figure 3

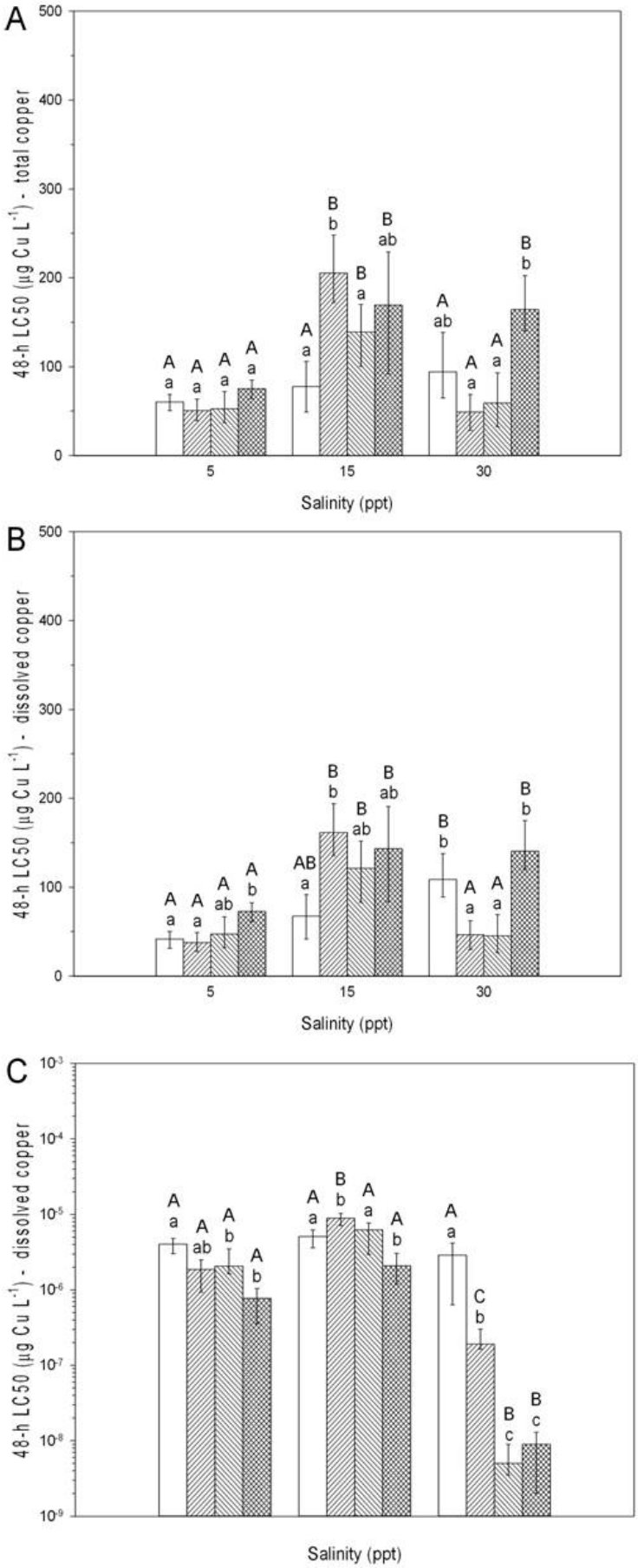

A
48-h LC50 (µg Cu L-1) - total copper
500
400
300
200
100
0
5
15
30
Salinity (ppt)
B
48-h LC50 (µg Cu L-1) - dissolved copper
500
400
300
200
100
0
5
15
30
Salinity (ppt)
C
48-h LC50 (µg Cu L-1) - dissolved copper
10-3
10-4
10-5
10-6
10-7
10-8
10-9
Salinity (ppt)

Figure 4

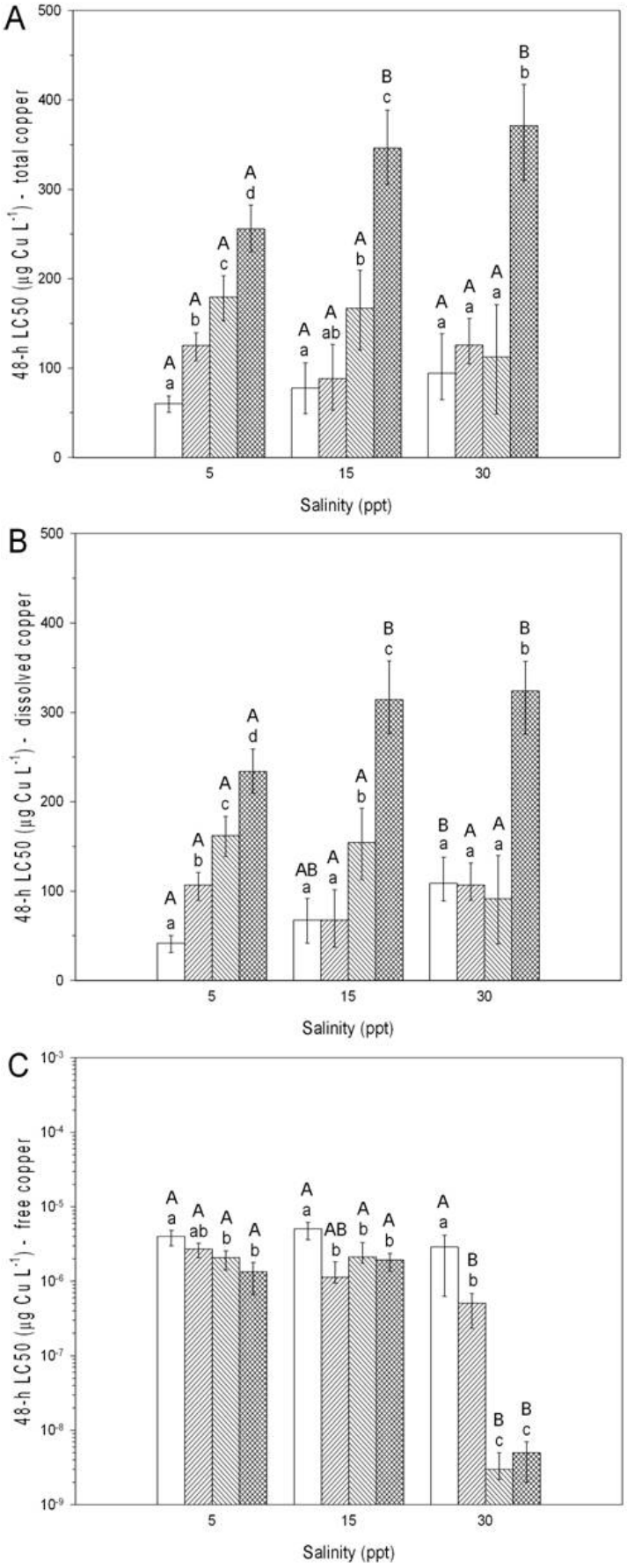

A
48-h LC50 (µg Cu L-1) - total copper
Salinity (ppt)
B
48-h LC50 (µg Cu L-1) - dissolved copper
Salinity (ppt)
C
48-h LC50 (µg Cu L-1) - free copper
Salinity (ppt)

Figure 5

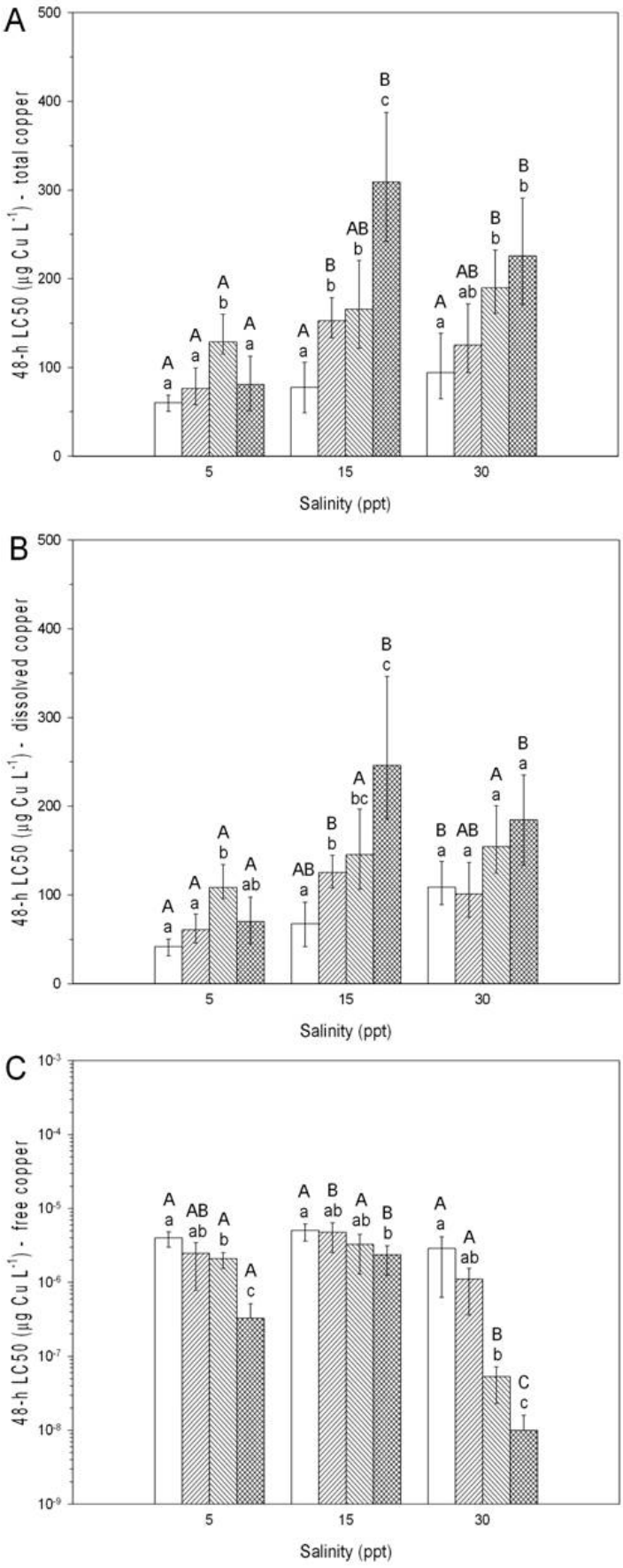
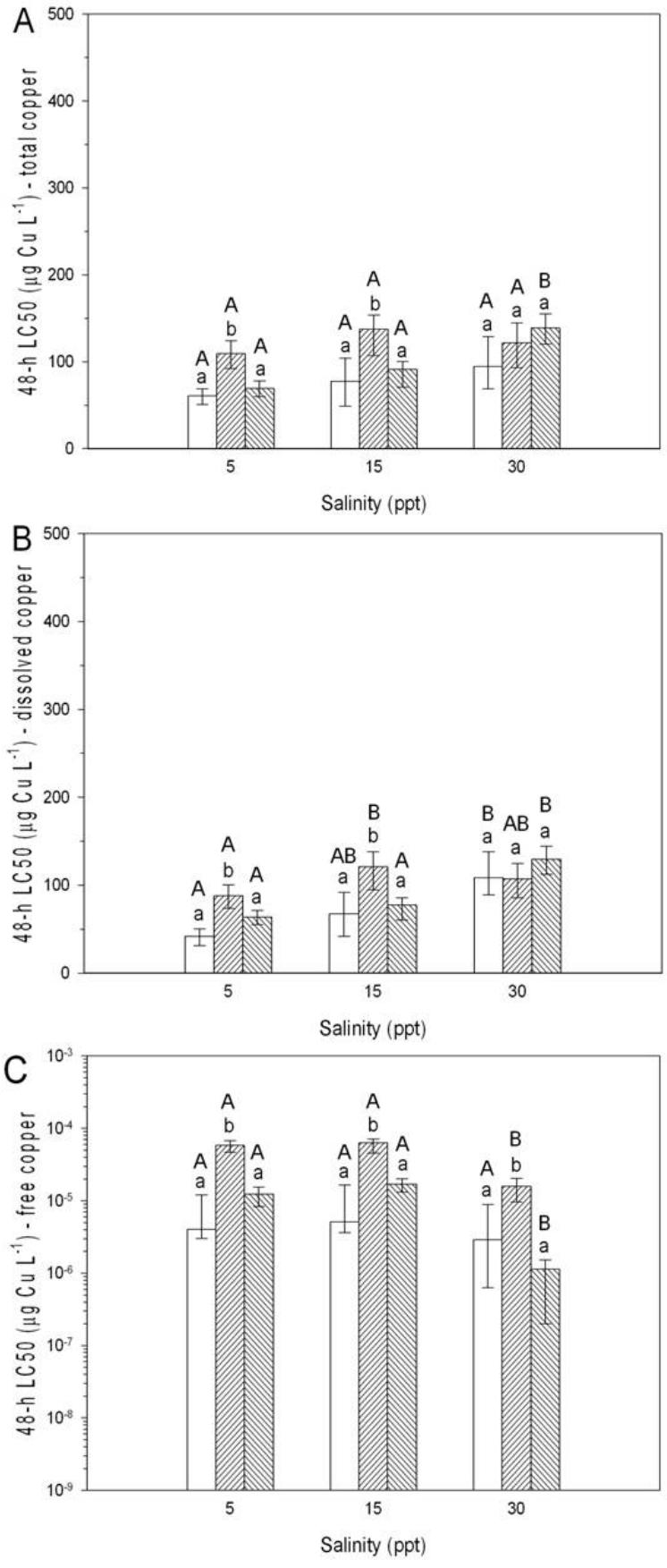

A
48-h LC50 (µg Cu L-1) - total copper
500
400
300
200
100
0
5
15
30
Salinity (ppt)
B
48-h LC50 (µg Cu L-1) - dissolved copper
500
400
300
200
100
0
5
15
30
Salinity (ppt)
C
48-h LC50 (µg Cu L-1) - free copper
10-3
10-4
10-5
10-6
10-7
10-8
10-9
5
15
30
Salinity (ppt)

Figure 6


A
48-h LC50 (µg Cu L-1) - total copper
500
400
300
200
100
0
5
15
30
Salinity (ppt)
B
48-h LC50 (µg Cu L-1) - dissolved copper
500
400
300
200
100
0
5
15
30
Salinity (ppt)
C
48-h LC50 (µg Cu L-1) - free copper
10-3
10-4
10-5
10-6
10-7
10-8
10-9
5
15
30
Salinity (ppt)

CAPÍTULO 2

MANUSCRITO 2

Freshwater and marine dissolved organic matter influence on copper accumulation in the euryhaline copepod *Acartia tonsa:* implications for the Biotic Ligand Model

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Running Head: Influence of DOC on copper accumulation in copepods

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Freshwater and marine dissolved organic matter influence on copper accumulation in the euryhaline copepod *Acartia tonsa:* implications for the Biotic Ligand Model

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Abstract

The influence of dissolved organic matter (DOM) on copper accumulation was evaluated in the euryhaline copepod *Acartia tonsa* at salinities 5, 15 and 30. DOM extracted from three different freshwater sources and from two marine sources was used. Different combinations of copper, salinity and DOM concentrations were tested. Copper accumulation in whole body, exoskeleton and soft tissues were analyzed in copepods exposed (48 h) to the corresponding 48-h LC50 value for copper. In a broad view, whole body copper content was dependent on both water salinity and freshwater DOM concentration and source. Although copper accumulation in the exoskeleton did not show a clear pattern, copper content in soft tissues was quite similar in all treatments analyzed (0.034 μg Cu/mg dry weight), irrespective the water salinity and DOM source (freshwater and seawater DOM) and concentration. These findings clearly indicate that soft tissues can be considered as the biotic ligand for copper in the copepod *A. tonsa* where the metal is exerting its toxicity, since copper concentrations tested corresponded to the 48-h LC50 for each experimental condition. At present, the mean copper content in soft tissues can be used as the lethal accumulation value (LA50) in a future version of the biotic ligand model for estuarine and marine waters using the copepod *A. tonsa* as a model.

Key-words: Accumulation; Biotic ligand model; Copper; Dissolved organic matter; Salinity.

Introduction

Copper is an essential micronutrient, but elevated environmental concentrations of this metal can induce toxicity to aquatic organisms [1]. Once copper is released in the aquatic environment, a set of chemical reactions occur as a function of the water chemistry. Dissolved organic matter (DOM), pH, hardness and ionic composition can influence the acute toxic effects of copper in aquatic animals [2]. Concentration of several anions and cations is higher in estuarine and marine waters than in freshwater. These anions can bind copper, complexing it and reducing its bioavailability and consequent toxicity to aquatic animals [2]. In turn, cations present in the water can compete with metal for binding sites at the biotic ligand, thus influencing acute copper toxicity [3].

Water quality criteria (WQC) is a widely accepted concept introduced by the United States Environmental Protection Agency (US-EPA), which takes into account the interaction of metals with other substances in water, such as ions and dissolved organic matter [4]. In this context, the biotic ligand model (BLM) was developed. This mathematical model aims to regulate metal emission more accurately in aquatic environments. It considers the dissolved metal speciation, taking into account metal complexation in the aquatic environment and the competitive bind between metal and other cations at the binding site (biotic ligand) in a target tissue, such as the gill in a fish [5]. A strong correlation between the metal concentration in the biological target and its acute toxicity constitute the BLM's premise [5-7].

As previously mentioned BLM considers the influence of several chemical parameters of water on copper toxicity to aquatic organisms, including water ion composition and dissolved organic matter (DOM) concentration. However, it characterizes the copper species interaction with the different sources and types of DOM similarly [8], although it is known that DOM source can influence the bioavailability and toxicity of copper to aquatic organisms [9]. The concentration and chemical composition of natural DOM depends primarily on the type of water,

origin, vegetation and climate, among other factors [9]. Humic substances constitute 10-30% of the marine DOM and 70-90% of the freshwater marsh DOM [10]. Therefore, the evaluation of the influence of natural DOM from different sources on copper bioavailability and toxicity can help to improve the present version of BLM.

It is well known that DOM-metal complexes are formed in the aqueous phase, thus lowering metal bioavailability and toxicity to aquatic animals [2]. Nevertheless, DOM *per se* can affect organisms, sometimes in a detrimental way. For example, recently a higher copper toxicity to the marine clam *Mesodesma mactroides* exposed to the metal in the presence of high natural DOM was observed (Marianna Basso Jorge, 2009, Master's thesis, Universidade Federal do Rio Grande, Rio Grande, RS, Brazil). This effect was attributed to DOM-metal complexes formation and absorption by the clams. It is also reported that water soluble humic fractions of DOM with low molecular weight can penetrate into organisms [11]. It can also promote harmful effects linked to oxidative stress, such as lipid peroxidation [12]. Despite these negative effects of DOM, several studies have reported a protective role of DOM against metal toxicity in aquatic animals [2,13-16]. In fact, DOM can change sodium influx rate in daphnids [15] and rainbow trout gill [17] in the presence of metals in the water, leading to a protective effect against metal toxicity. However, most of these studies are restricted to fresh water species and tested natural fresh water DOM or commercial organic matter (usually the Aldrich humic acid, which has little structural similarity with the aquatic humic acids [18]. Studies with marine-derived DOM extracted from coastal, and especially from oceanic waters, are lacking because of the methodological difficulties for extracting DOM from salt water [19]. Therefore, evaluation of the influence of environmentally relevant concentrations of natural DOM on copper toxicity and accumulation in estuarine and marine invertebrates is necessary for a future BLM extension to brackish and sea water.

Aquatic invertebrates take up and accumulate trace metals. When the rate of uptake of a trace metal is faster than the rate of its excretion and detoxification, the concentration of

metabolically available metal exceeds a threshold. In this case, metal will bind to sites of toxicity and interfere with normal metabolic functioning [20]. It is also known that not all invertebrates excrete all trace metals, implicating that the rate of detoxification alone must correspond to or be higher than the rate of metal uptake, in order to avoid toxic effects [20]. Metals accumulate in animal tissues as a function of several factors, such as metal concentration, via and time of exposure, as well as metal assimilation and excretion rates, which in turn are influenced by age, size and sex, among other factors [21]. In addition, metals accumulated in copepods by trophic transfer were present in soft tissues while those accumulated by dissolved phase were present primarily on the copepod's exoskeleton, leading to low or no adverse effects on animals [22].

There are a variety of studies regarding copper accumulation in aquatic invertebrates, both with animals collected in their habitat and with animals submitted to experiments in laboratory. For example, different radiolabelled copper concentrations at different regions of the copepod *Calanus hyperboreus* body (ventral, dorsal and internal region, and urosoma) were observed, and the copper accumulated internally corresponded to 9,3% of total body burden [23]. However, there are no reports in the literature on the tissue fractionation of copper accumulated in copepods exposed to this metal in different combinations of DOM (concentration and source) and water salinities. In the present study, we evaluated the effect of different concentrations of freshwater DOM from three different sources on copper accumulation in the sensitive euryhaline copepod *Acartia tonsa* in a wide range of salinities (5, 15 and 30 ppt). Metal accumulation was evaluated in whole body, exoskeleton and soft tissues of copepods.

The calanoid copepod *A. tonsa* is cosmopolitan and adults are tolerant to a wide range of water salinities (0 – 31.5) [24]. As they are omnivorous [25], they are considered a major link between phytoplankton and the other trophic levels in several food chains in both estuarine and marine waters [26]. It is important to note that marine copepods are for a long time considered as sensitive indicators of metal toxicity [22].

Materials and Methods

Brackish and sea water

The different media employed for algae and copepod cultivation were prepared using natural filtered (1μm mesh filter) sea water collected at the Cassino Beach (Rio Grande, Rio Grande do Sul, Brazil). The different media employed for the acute toxicity tests using copepods were prepared from a stock solution of artificial sea water (prepared with artificial sea salts, CoraLife® and Milli-Q water) at the desired experimental salinities (5, 15 and 30 ppt), as previously described [27].

Copepod culture and acclimation

The original lot of copepods (*A. tonsa*) was obtained in 2005 from an intensive culture (which was interrupted since 2008) of the Aquaculture Marine Station of the Universidade Federal do Rio Grande (Rio Grande, Rio Grande do Sul state, southern Brazil), maintained as described by Bersano [28].

Copepods were transferred to the laboratory and kept in 10-L plastic buckets containing water at the desired salinity (5, 15 and 30 ppt), by adding distilled water to filtered (1-μm mesh filter) sea water collected at the Cassino Beach until the desired salinity was reached. Cultivation media were gently aerated and completely renewed every week. Room temperature (20°C) and photoperiod (12L:12D) were fixed. Copepods were daily fed with *Thalassiosira weissflogii* ($2x10^4$ cells/ml) and *Isochrysis galbana* ($1x10^4$ cells/ml) cultivated in f/2 algae medium [29].

Freshwater DOM extraction

DOM was extracted from fresh water collected before and after the effluent discharge of the "Navegantes" Sewage Treatment Plant (STP), by reverse osmosis. The first source of DOM was the water collected before the public STP discharge (BSTP-DOM), while the second source

was the water collected about 3 m after the discharge of the STP (ASTP-DOM). The third source of NOM, the Suwannee river fulvic acid (SRFA), was purchased from the International Humic Substances Society (SRFA standard I, St. Paul, MN, USA), and afterwards a SRFA stock solution (1,000 mg C/L) was prepared dissolving the commercial fulvic acid in Milli-Q water.

Salt water DOM extraction

Salt water DOM was extracted from two sources.Marine DOM was extracted from sea water in a subtropical region from sea water collected ~20 miles away from Rio Grande coast (southern Brazil). Coastal DOM was extracted from coastal water collected in a tropical region ~5 miles away from the Salvador coast (northeastern Brazil) (Fig. 1).

DOM storage and characterization

DOM stock solutions were stored at 4^{o}C in the dark until their use [30]. DOM stock solutions were filtered again (0.45-μm mesh filter; Sartorius, São Paulo, Brazil) before their use in experiments. Dissolved organic carbon (DOC) and copper concentrations in the DOM stock solutions were measured using a total carbon analyzer (TOC 5000, Shimadzu, Japan) and an atomic absorption spectrophotometer (AAS 932 Plus - GBC, IL, USA), respectively. All DOM stock solutions were stored at 4^{o}C in the dark until their use [30].

Experimental media

Different experimental media were prepared diluting the DOM with artificial salt water prepared as previously described. Copepods were exposed to different combinations of water salinity (5, 15, and 30 ppt), environmentally relevant DOM concentration (SRFA: 0.6, 1.6, and 4.3 mg C/L; BSTP-DOM: 2.5, 5.4, and 10.5 mg C/L; ASTP-DOM: 1.4, 3.2, and 6.7 mg C/L; marine DOM: 0.5 mg C/L, and coastal DOM: 2.2 mg C/L) and DOM source (SRFA, BSTP-DOM, ASTP-DOM, marine-DOM and coastal-DOM). The selected DOM concentrations would

be representative of river (>2.5 mg C/L), shallow sea water (from 0.6 to 1.5 mg C/L), and brackish waters (concentration ranging between those of sea water to those of fresh water DOM) [10]. Copepods from this experiment were collected and prepared for whole-body copper accumulation measurements, as described below. Also, a second exposure was performed under the same experimental conditions, but adding only the highest DOM concentration tested for each type of fresh water DOM (BSTP-DOM: 10.5 mg C/L; ASTP-DOM: 6.7 mg C/L; and SRFA: 4.3 mg C/L) and the DOM concentration tested for each marine DOM (marine DOM: 0.5 mg C/L and coastal DOM: 2.2 mg C/L). Copepods from this second experiment were collected and prepared for measurements of copper accumulation in the exoskeleton and soft tissues, as described below.

In both experiments, different copper concentrations ($CuCl_2$; Vetec, Rio de Janeiro, Brazil) were added to the experimental media from stock solutions (0.02; 0.2; or 2 g Cu/L) acidified with 0.1% HNO_3 (Suprapur, Merck, USA). The maximum volume of DOM stock solution added to the water to prepare the experimental medium (50 mL) was 2 mL. Experimental media were kept at 20^{o}C in the dark for 24 h before their use in the toxicity tests [13,30].

Whole-body copper accumulation

Two types of control tests were run in the absence of copper in each experimental salinity: (1) without addition of DOM and (2) with fresh water DOM (SRFA, BSTP-DOM or ASTP-DOM) at the desired experimental concentration. Two types of acute copper toxicity tests were also performed in each experimental salinity: (1) without addition of DOM and different copper concentrations, and (2) with addition of both fresh water DOM (SRFA, BSTP-DOM or ASTP-DOM) and copper at the desired experimental concentrations. Controls and toxicity tests were run in quadruplicate.

Prior to experiments, copepods (total length = 0.80 ± 0.09 mm; dry weight = 4.5 ± 0.87

µg) were removed from the culture using a 300 µm-mesh net. This net mesh size retains copepodites and adults of both genders [31,32]. Tests were run with copepodites and adult copepods of both genders (introduced in the test media at random) using a standard static-renew system and in the absence of food. Ten copepods were introduced in each glass flask containing 50 mL of experimental medium prepared as described above. Copepods were exposed for 48 h to the corresponding 48-h LC_{50} values at the different experimental conditions, as previously determined (Table 1). Flasks were kept under constant rotation (2 rpm) in a incubator with fixed temperature (20°C) and photoperiod (16L:8D).

After 24 h of exposure, living copepods from each flask were counted and transferred to a fresh experimental medium prepared as described above. Dead copepods were discarded. After 48 h, surviving copepods were individually collected, quickly rinsed (15 s) in Milli-Q water, and transferred to a plastic tube using plastic pipettes. They were pooled (n = 5 up to 10 per tube; three sample tubes) for whole-body copper concentration measurement. Copepods were dried (70°C for 48 h) and digested in 100 µl of 65% HNO_3 (Suprapur, Merck, USA). Copper concentration in digested samples was measured atomic absorption spectrophotometry in the flame mode (AAS 932 Plus - GBC, IL, USA). Dried samples containing 20 copepods each (total length = 0.80 ± 0.09 mm) were previously weighed in an electronic scale (2 µg precision, CHNS/O analyzer, 2400 Series II, Perkin Elmer). Copper accumulation was expressed as µg Cu/mg dry weight.

Copper accumulation in exoskeleton and soft tissues

These tests were performed following the same procedures described for the whole-body copper accumulation experiment. However, the two marine DOM sources were also used in control tests in the absence of copper and in acute copper toxicity tests. After 48 h of exposure, exoskeleton and soft tissues of control and copper-exposed copepods were sequentially digested following procedures previously described [22]. Briefly, surviving copepods were individually

collected, transferred to a polycarbonate filter (10-µm mesh filter), rinsed with an EDTA solution (200 µM) prepared with artificial salt water, and quickly (<15 s) rinsed again with a EDTA solution (200 µM) prepared with MilliQ water. At least ten copepods were transferred to the polycarbonate filter, which was inserted into a Falcon-type plastic tube (three sample tubes). Afterwards, 2 mL of a NaOH solution (0.2 N) were added to the tube, which was maintained at 60°C for 12 h in order to digest the copepod soft tissues. The filter containing the copepod exoskeleton was then rinsed with a NaOH solution (0.2 N) in order to elute any soft tissue remaining in the digestion tube. Finally, the exoskeleton retained in the filter was collected, dried (90^{o}C) and digested with 65% HNO_3 (Suprapur, Merck, EUA) for 24 h. Tubes containing soft tissues were dried (90^{o}C) and digested with 65% HNO_3 (Suprapur, Merck, EUA) for 24 h. Copper concentration was measured as described below. Dried samples containing 20 copepods were previously weighed in an electronic microscale (2 µg precision, CHNS/O analyzer, 2400 Series II, Perkin Elmer, USA). After digestion of the soft tissues, exoskeleton was dried as described above and weighed using the electronic microscale. Dry weight of soft tissues was then calculated based on the difference between the whole-body dry weight and the exoskeleton dry weight. Copper accumulation in exoskeleton and soft tissues was then expressed as µg Cu/mg dry weight.

Water chemistry

At the beginning and after 24 h of test, water pH and dissolved oxygen concentration were directly measured in the experimental media. Also, non-filtered and filtered (0.45-µm mesh filter) samples from the different experimental media were collected and prepared for measurements of total and dissolved copper concentrations, respectively. The following parameters were also analyzed in the non-filtered samples: alkalinity and cation (Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}), anion (Cl^{-}), sulphate and DOC concentration. Measurements were performed as previously described (Monteiro *et al.*, 2012, *in review*).

Data presentation and statistical evaluation

Data of copper accumulation in whole-body, exoskeleton, and soft tissues of copepods were expressed as mean ± standard deviation. Differences in copper accumulation among treatments were assessed by two-way analysis of variance (ANOVA) followed by the Tukey test. The significance level adopted was 5% ($\alpha = 0.05$).

Results

Water chemistry

DOC concentrations in the DOM stock solutions were 121.8, 126.3, 1000, 555.3 and 1119.0 mg C/L for the BSTP-DOM, ASTP-DOM, SRFA, marine and coastal DOM, respectively. Dissolved copper concentrations in BSTP-DOM and ASTP-DOM stock solutions were 848.7, 574.7 µg Cu/L, respectively, and <10 µg Cu/L in SRFA, marine and coastal DOM stock solutions. Data were already reported in a previous study performed under similar conditions (Monteiro *et al.*, 2012, *in review*).

In each water salinity, no significant difference was observed in the artificial salt water composition before and after DOM and/or Cu addition (data not shown). Therefore, only one general mean value was calculated for each water salinity. All water chemistry parameters significantly augmented with the increasing salinities, except the dissolved oxygen content that was similar in salinities 15 and 30 ppt. Data were already reported in a previous study performed under similar conditions (Monteiro *et al.*, 2012, *in review*).

Whole-body copper accumulation

In the absence of copper in the water, whole-body copper content was similar in copepods exposed to the different concentrations of the same DOM at the same water salinity (data not shown). Therefore, only one mean value of whole-body copper content was calculated

for control copepods exposed to each DOM source and water salinity. These values were not different from those observed for control copepods in the absence of copper and DOM (Figs. 2-4). In the absence of DOM, whole-body copper content was significantly higher in copepods exposed to copper than in their respective controls at the same water salinity. Whole-body copper content significantly decreased as salinity increased (Figs. 2-4).

Addition of BSTP-DOM in the water did not affect the whole-body copper content of copepods exposed to the metal in all experimental salinities (Fig. 2). However, addition of ASTP-DOM (Fig. 3) or SRFA (Fig. 4) in the water generally reduced the whole-body copper content of copepods exposed to the metal in salinities 5 and 15 ppt. At the same water salinity, whole-body copper content generally decreased as the DOM concentration increased. As observed for the BSTP-DOM, no significant change in whole-body copper content was observed in copepods exposed to the metal in the presence of ASTP-DOM (Fig. 3) or SRFA (Fig. 4) in salinity 30 ppt (Fig. 4).

When considering all copepods analyzed at the different experimental conditions, a general mean value (± standard deviation) of whole-body copper content in control (in the absence or the presence of DOM) and copper-exposed (in the absence or the presence of DOM) copepods was 0.057 ± 0.013 and 0.838 ± 0.179 μg Cu/mg dry weight, respectively.

Fractionated copper accumulation

Copper content in the exoskeleton of copepods kept under control conditions (no DOM or copper addition in the water) did not change as a function of water salinity. Copper content in copepod exoskeleton did not show a clear pattern as a function of water salinity or DOM addition (data not shown).

As observed for the exoskeleton, copper content in soft tissues of control copepods did not change as a function of water salinity. Copper content in soft tissues of copepods exposed to copper in the absence of DOM was significantly higher than in the respective control copepods

in all water salinities tested. Furthermore, copper content in soft tissues was neither affected by water salinity or addition of DOM (SRFA, BSTP, ASTP, marine or coastal) in the water (Fig. 5).

When considering all copepods analyzed at the different experimental conditions, the general mean value (± standard deviation) of copper content in the soft tissues of control (in the absence of DOM) and copper-exposed (in the absence or the presence of DOM) copepods was 0.0065 ± 0.0005 and 0.034 ± 0.0096 μg Cu/mg dry weight, respectively.

Discussion

There are only few records in the literature regarding the accumulation of metals in copepods in comparison with other invertebrate species, given methodological difficulties associated with body size. In this context, there are even fewer attempts to measure the amount of metals in different fractions (tissues or organs) of the copepod body. The present study is the first one reporting the effects of natural DOM on copper accumulation in whole body, exoskeleton and soft tissues of an euryhaline invertebrate species, the copepod *A. tonsa*, in a wide range of salinities (5, 15 and 30 ppt).

In freshwater invertebrates and fish, several water physicochemical parameters, including water ion composition and DOM concentration, can influence copper bioavailability, accumulation and consequent toxicity [5-7]. In the present study, whole-body copper content of copepods acutely exposed to the 48-h LC50 for copper in the absence of DOM decreased as water salinity increased. In fact, it has been previously reported an equal result for *A. tonsa* under similar conditions, but using natural sea water instead of artificial sea water [33].

The differential whole-body copper accumulation observed in *A. tonsa* according to water salinity in the absence of DOM could be explained considering changes in copper speciation due to the different water chemistry with varying salinity. As water salinity increase, the concentration of copper complexing agents, especially Cl^-, will also increase in water, thus

reducing copper bioavailability. Furthermore, the concentration of copper competing agents for binding sites on the biotic ligand such as Na^+, K^+, Ca^{2+} and Mg^{2+} will also increase as water salinity increases, thus reducing the amount of copper bound at the biotic ligand [5-7]. Therefore, whole-body copper content in copepods would be expected to decrease with increasing water salinity. However, it is important to note that copper concentrations used in all the accumulation experiments at the different water salinities corresponded to the 48-h LC50 values for the respective experimental conditions. Therefore, the amount of toxic copper available would be similar in all experimental salinities, thus compensating the water chemistry effect on copper bioavailability in water and accumulation in copepods. In fact, according to BLM premise, there is a strong correlation between the amount of metal accumulated at the biotic ligand and its acute toxicity [5-7].

Considering the BLM premise, the differential whole-body copper content observed when copepods were exposed to toxicologically equivalent concentrations of copper in the water at different salinities suggests that a more specific target tissue/organ than the whole body would be involved in the accumulation of toxic copper in the euryhaline copepod *A. tonsa*. In turn, it is possible that the differential whole-body copper content observed at different water salinities could be related to the fact that the cuticle of crustaceans can adsorb metals, including copper. Actually, copper adsorption on the cuticle is salinity dependent [34]. Therefore, the accumulation of copper in different fractions (soft tissues/exoskeleton) of copepods was evaluated in the present study in order to better identify the biotic ligand associated with copper toxicity in the copepod *A. tonsa*, as discussed below.

Considering the small size of copepods analyzed (total length = 0.80 ± 0.09 mm; dry weight = 4.5 ± 0.87 μg), only two body fractions were analyzed, the exoskeleton and soft tissues. It is worth to note that several metals can accumulate at different proportions in the exoskeleton and soft organs in marine copepods depending on the metal tested and sometimes on the uptake route, i.e., assimilation from surrounding water or from food. Metals accumulated in marine

copepods by trophic transfer and by the dissolved phase can be present more in the soft tissues (polar components) than in the exoskeleton, except for zinc following uptake from water [34]. On the other hand, in another study metals accumulated in copepods by trophic transfer were shown to be present in soft tissues, while those accumulated from the dissolved phase were present primarily on the copepod's exoskeleton, leading to low or no adverse effects on animals [22]. Therefore, it is clear that total body burden of metals in copepods and other small crustaceans can lead to misinterpretation of results when trying to relate the amount of metal accumulated and its consequent toxicity.

No clear pattern in copper accumulation in the exoskeleton of copepods was observed. This fact could be associated with the use of EDTA to rinse copepods after copper exposure to avoid contamination by the metal in the digested soft tissues. However, the copper content in soft tissues of *A. tonsa* was quite similar in all water salinities tested. As mentioned above, copper concentration used to expose copepods in the accumulation studies corresponded to the 48-h LC_{50} determined at each experimental condition. Taken altogether, these findings corroborate with the idea that acute copper toxicity in the copepod *A. tonsa* is related to the amount of copper accumulated in soft tissues, irrespective water salinity and DOM source and concentration. This fact supports the idea that metal toxicity can be inferred from its concentration in animal's tissues and not from the metal concentration in the environment [35]. In fact, a better prediction of metal toxicity can be obtained based on metal accumulation in sensitive tissues, since it represents the metal available metabolically to exert toxicity [20,36].

Considering the BLM premise, *i.e.*, that exists a strong correlation between the amount of metal accumulated at the biotic ligand and its acute toxicity [5-7], findings from the present study clearly indicate that the acute toxicity of waterborne copper in the absence of DOM in the euryhaline copepod *A. tonsa* is associated with the amount of copper accumulated in soft tissues, and not in the whole body or exoskeleton. Therefore, soft tissues can be considered as an appropriate biotic ligand to calibrate a future version of the BLM for estuarine and marine waters

using the euryhaline copepod *A. tonsa* as a model. In the present study, the mean value of copper accumulated in soft tissues of *A. tonsa* exposed to the 48-LC50 was ~5-fold higher than the copper content in soft tissues of control copepods, irrespective the water salinity and DOM (concentration and source). It corresponded to 0.034 □g Cu/mg dry weight and can be used as the lethal accumulation value (LA50) to calibrate a BLM version for estuarine and marine environments.

In the presence of DOM, an effect of water salinity on whole-body copper content was also observed. However, this effect was more pronounced at the lower concentration of ASTP-DOM and SRFA tested. Higher concentrations of these DOM decreased the water salinity effect on whole-body copper content. For BSTP-DOM, a salinity effect was only observed at the higher concentrations tested. Furthermore, a concentration-dependent reduction in whole-body copper accumulation was not observed with addition of BSTP-DOM in the experimental medium. For ASTP-DOM and SRFA, whole-body copper content was dependent on the DOM concentration in the water, decreasing with increasing DOM concentration. Altogether these findings indicate that whole-body copper content in the euryhaline copepod *A. tonsa* acutely exposed for 48 h to the 48-h LC50 for waterborne copper is dependent on both water ionic composition (salinity) and DOM (concentration and source). Also, they clearly indicate that the effect of water salinity on whole-body copper content is influenced by the DOM concentration and source.

Despite the fact that copepods were only exposed to waterborne copper, accumulation and toxicity associated with copper-DOM complexes cannot be ruled out. ~20% of the copper bound to organic matter, as Aldrich humic acid, was available to cause toxicity in fathead minnows [2]. It was also reported that water soluble DOM humic fractions with low molecular weight can penetrate into organisms [11]. Although metal-DOM complexes are considered too large and too polar to cross biological membranes [37], a higher acute toxicity of waterborne copper in the marine clam *Mesodesma mactroides* exposed to the metal in the presence of high DOM concentrations was observed, and this effect was attributed to DOM-metal complexes

formation and ingestion by clams (Marianna Jorge, 2009, Master's thesis, Universidade Federal do Rio Grande, Rio Grande, RS, Brazil).

In copepods, copper-DOM complexes uptake could be another source of copper contributing to the amount of metal accumulated in the whole body. In this case, copepods might ingest these complexes also as an alternative source of food, especially in experiments performed in the absence of food, as in the present study. In fact, a previous study suggested that copper-DOM complexes could be ingested by *A. tonsa* under similar experimental conditions as those in the present study (Monteiro *et al.*, 2012, *in review*). In addition, differences in silver accumulation at whole body in the daphnid *D. magna* could be attributable to ingestion of silver-DOM complexes, once small colloidal complexes could reach a size (~0.45 μm) large enough to be trapped by the filter mesh of the daphnid feeding apparatus, and treated as food particles 37]. However, copepods are not filter-feeders and they are able to select food size [26]. Adult individuals of *A. tonsa* eat preferentially particles between 14 and 250 μm [39]. It is possible that copper-DOM complexes could have reached a size large enough to be selected and ingested by copepods during experiments, thus *A. tonsa* could be accumulating copper-DOM complexes during copper exposure experiments in the present work.

However, further studies are necessary to evaluate the importance of DOM as a food source for copepods in experiments with and without food addition. Information from these experiments would help to identify the DOM influence on metal assimilation estimates, which depends highly on the metal uptake pathways. In fact, the efflux rate of metals was generally higher following uptake from food than uptake from the dissolved phase in experiments with marine copepods [34]. The authors reported that the relative importance of trace element uptake from the dissolved phase compared to the particulate ingestion in the overall metal uptake depends greatly on the metal assimilation efficiency, the feeding rate of copepods, and the partition coefficient of metals in ingested food particles [34].

Despite the fact that copper-DOM complexes can be contributing for the amount of

copper accumulated in copepods, it is worth to note that the copper content in soft tissues of copepods was similar in all experimental conditions, irrespective the water salinity and DOM source and concentration. This finding clearly indicates that, irrespective the source of copper (dissolved or particulate), the amount of copper accumulated in soft tissues is associated with the acute toxicity of waterborne copper in a wide range of water salinities, as discussed above.

In conclusion, data reported in the present study shows that copper accumulated in whole body of the copepod *A. tonsa* is dependent on water salinity and DOM concentration and source. Also, they show that the influence of water salinity on whole body copper accumulation is dependent on DOM concentration and source. Fractionation of accumulated copper clearly indicated that soft tissues can be considered an adequate biotic ligand for copper in a future BLM version for estuarine and marine waters using the euryhaline copepod *A. tonsa* as a model.

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Table 1. Dissolved copper concentrations corresponding to the 48-h LC_{50} values (μg Cu/L) used to expose (48 h) the euryhaline copepod *Acartia tonsa* in the copper accumulation experiments at different water salinities. Data are based on a previous study (Rodrigues *et al.*, unpublished). SRFA: commercial dissolved organic matter extracted from the Suwannee river; BSTP-DOM: dissolved organic matter extracted from the fresh water collected before the seawage treatment plant; ASTP-DOM : dissolved organic matter extracted from the fresh water collected after the effluent discharge of the seawage treatment plant; marine-DOM: dissolved organic matter extracted from the salt water collected ~20 miles away from Rio Grande coast (subtropical zone, southern Brazil); Coastal-DOM: dissolved organic matter extracted from coastal water collected ~5 miles away from Salvador coast (tropical zone, northeastern Brazil).

Experimental condition	**Salinity (ppt)**		
	5	**15**	**30**
Without DOM addition	41.8	67.4	108.7
SRFA (0.6 mg C/L)	37.6	161.6	46.4
SRFA (1.6 mg C/L)	47.4	121.2	45.2
SRFA (4.3 mg mg C/L)	72.7	143.4	140.5
BSTP-DOM (2.5 mg C/L)	106.6	67.5	106.9
BSTP-DOM (5.4 mg C/L)	162.1	154.3	91.2
BSTP-DOM (10.5 mg C/L)	233.9	314.2	324.0
ASTP-DOM (1.4 mg C/L)	60.6	125.1	101.1
ASTP-DOM (3.2 mg C/L)	108.4	145.4	154.6
ASTP-DOM (6.7 mg C/L)	70.0	246.1	184.5
Marine-DOM (0.5 mg C/L)	87.9	121.1	106.9
Coastal-DOM (2.2 mg C/L)	63.8	77.4	129.4

Figure Legends

Figure 1. Map of South America showing the sampling sites where water was collected to extract the DOM used in the experiments with the copepod *Acartia tonsa*. Freshwater DOM: extracted from fresh water collected in the Vieira Stream (Rio Grande, Rio Grande do Sul state, southern Brazil, 32°06'S 51°10'W). 1 = before the "Navegantes" Public Sewage Treatment Plant (BSTP-DOM); 2 = about 3 m after the "Navegantes" Public Sewage Treatment Plant (ASTP-DOM). Marine DOM: 3 = extracted from marine water collected ~20 miles away from Rio Grande coast (marine-DOM; subtropical zone, southern Brazil, 32°10'S 51°45'W); 4 = extracted from coastal water collected ~5 miles away from Salvador coast (coastal-DOM; tropical zone, northeastern Brazil, 13°01'S 38°33'W).

Figure 2. Whole-body copper content in the euryhaline copepod *Acartia tonsa* kept under control conditions in the absence or the presence of BSTP-DOM, as well as exposed to the 48-h LC_{50} for waterborne copper in the presence of different concentrations of BSTP-DOM at different water salinities for 48 h. Data are expressed as mean ± standard deviation. Different lowercase letters indicate significant different mean values among treatments for the same water salinity ($P<0.05$). Different capital letters indicate significant different mean values among water salinities for control copepods in the absence or the presence of DOM or exposed to copper in the absence of DOM ($P<0.05$).

Figure 3. Whole-body copper content in the euryhaline copepod *Acartia tonsa* kept under control conditions in the absence or the presence of ASTP-DOM, as well as exposed to the 48-h LC_{50} for waterborne copper in the presence of different concentrations of ASTP-DOM at different water salinities for 48 h. Data are expressed as mean ± standard deviation. Different lowercase letters indicate significant different mean values among treatments for the same water

salinity ($P<0.05$). Different capital letters indicate significant different mean values among water salinities for control copepods in the absence or the presence of DOM or exposed to copper in the absence of DOM ($P<0.05$).

Figure 4. Whole-body copper content in the euryhaline copepod *Acartia tonsa* kept under control conditions in the absence or the presence of SRFA, as well as exposed to the 48-h LC_{50} for waterborne copper in the presence of different concentrations of SRFA at different water salinities for 48 h. Data are expressed as mean ± standard deviation. Different lowercase letters indicate significant different mean values among treatments for the same water salinity ($P<0.05$). Different capital letters indicate significant different mean values among water salinities for control copepods in the absence or the presence of DOM or exposed to copper in the absence of DOM ($P<0.05$).

Figure 5. Copper content in soft tissues of the euryhaline copepod *Acartia tonsa* kept under control conditions or exposed (48 h) to the 48-h LC_{50} for waterborne copper in the absence or the presence of DOM (SRFA, BSTP, ASTP, marine and coastal) at different water salinities. Data are expressed as mean ± standard deviation. Different lowercase letters indicate significant different mean values among treatments for the same water salinity ($P<0.05$). Different capital letters indicate significant different mean values among water salinities for control copepods in the absence of DOM or exposed to copper in the absence or the presence of DOM ($P<0.05$).

Figure 1

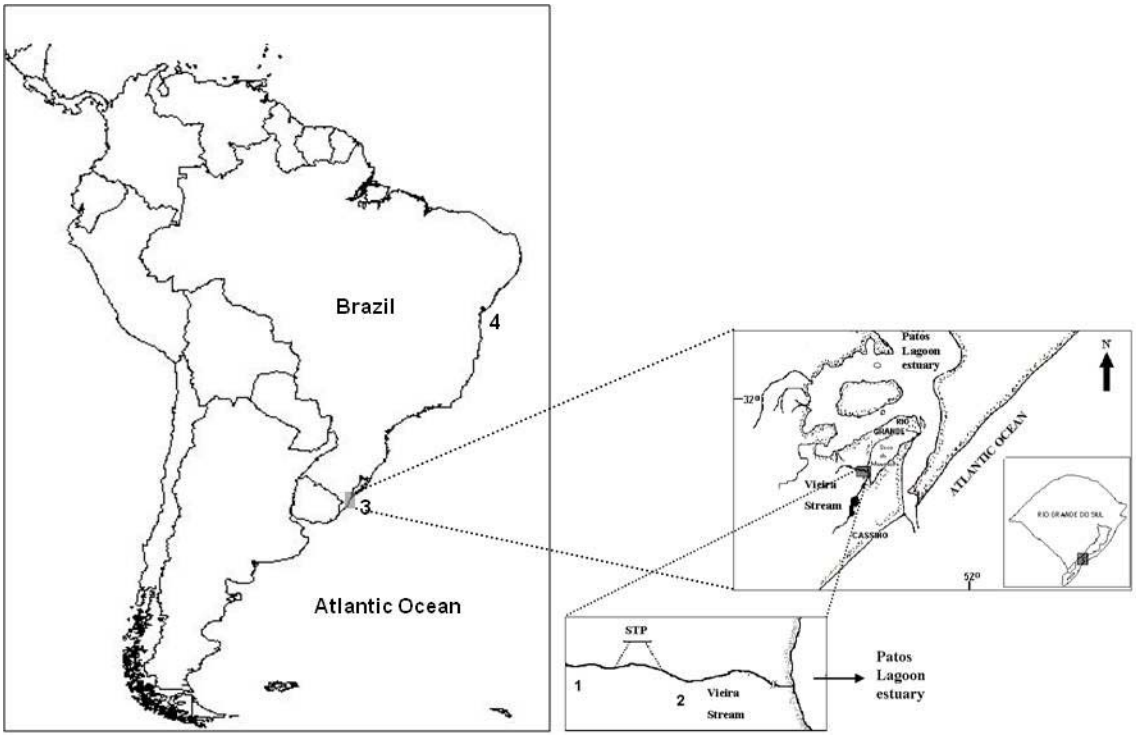

Brazil
4
3
Atlantic Ocean
Patos
Lagoon
estuary
32°
RIO
GRANDE
Vieira
Stream
CASSINO
ATLANTIC OCEAN
N
52°
RIO GRANDE DO SUL
STP
1
2
Vieira
Stream
Patos
Lagoon
estuary

Figure 2

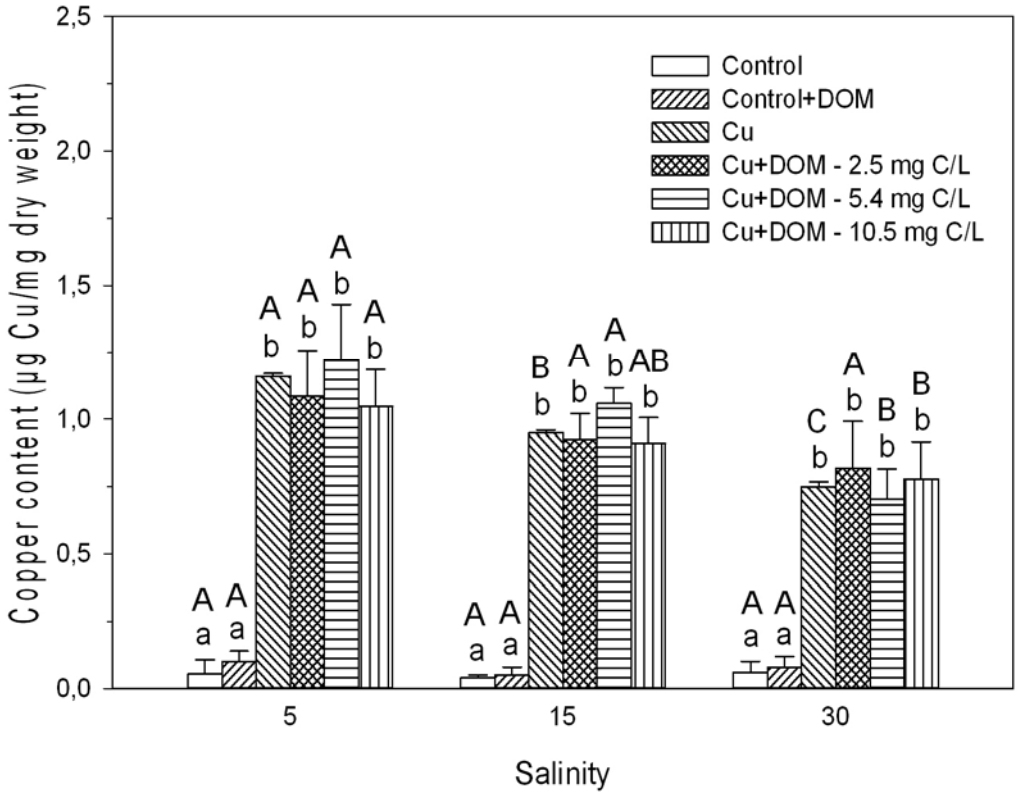

Control
Control+DOM
Cu
Cu+DOM - 2.5 mg C/L
Cu+DOM - 5.4 mg C/L
Cu+DOM - 10.5 mg C/L
Copper content (µg Cu/mg dry weight)
2,5
2,0
1,5
1,0
0,5
0,0
5
15
30
Salinity

Figure 3

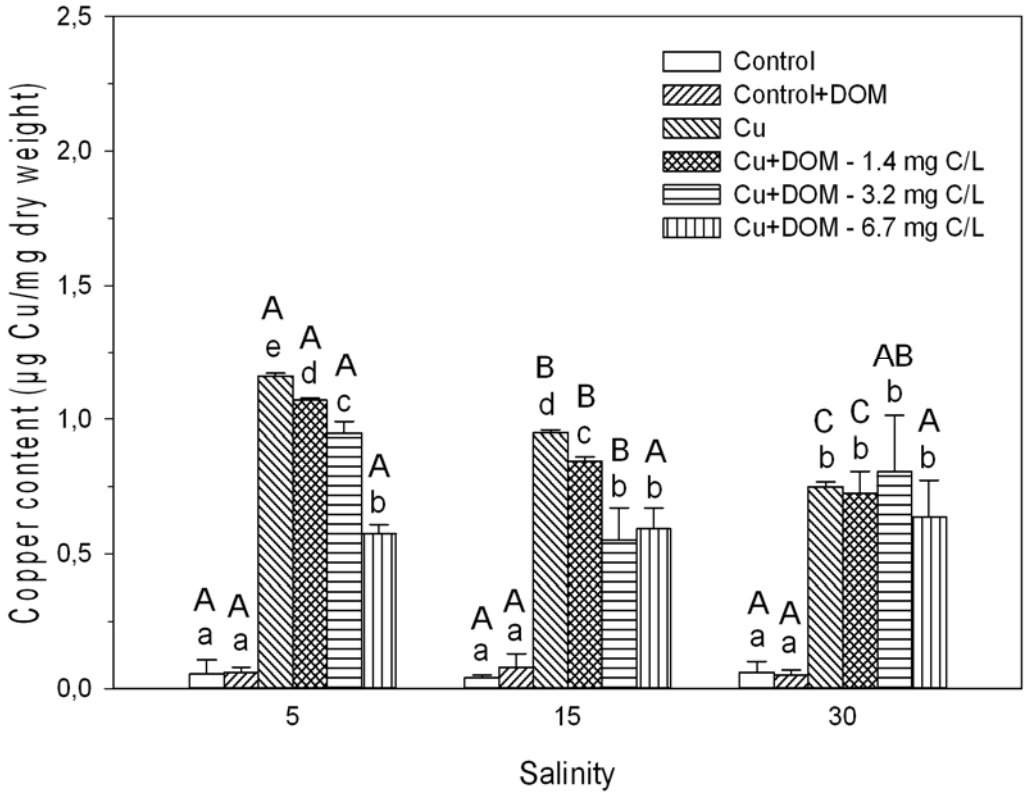

Control
Control+DOM
Cu
Cu+DOM - 1.4 mg C/L
Cu+DOM - 3.2 mg C/L
Cu+DOM - 6.7 mg C/L
Copper content (µg Cu/mg dry weight)
2,5
2,0
1,5
1,0
0,5
0,0
5
15
30
Salinity

Figure 4

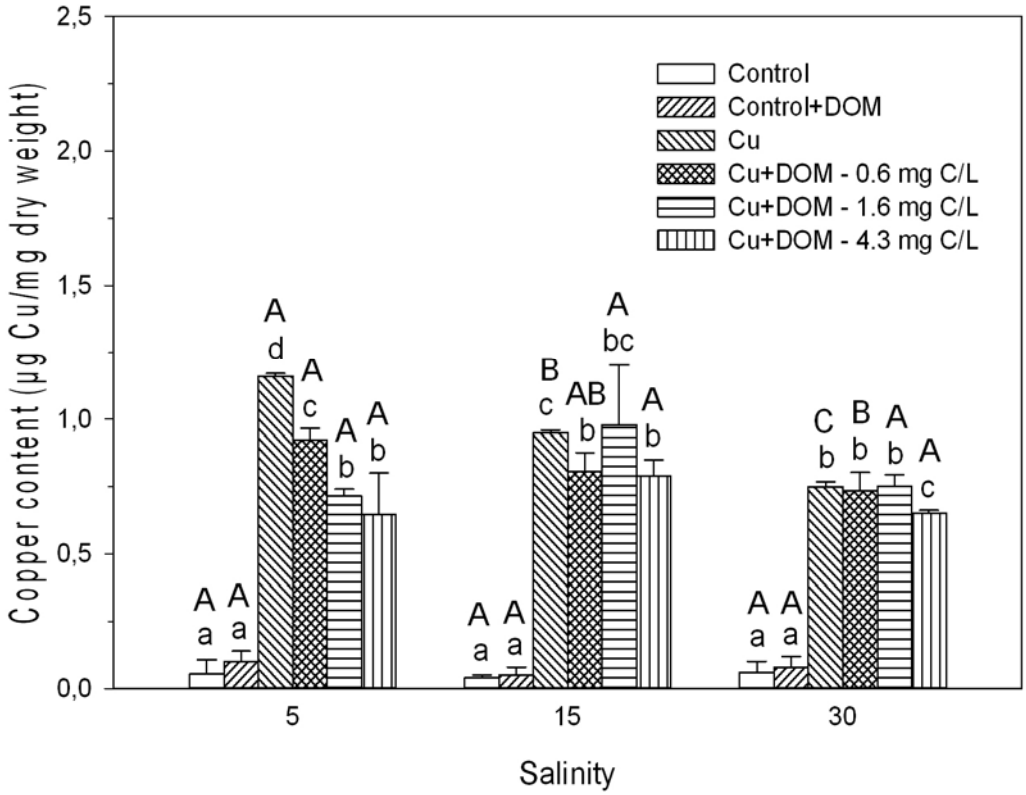
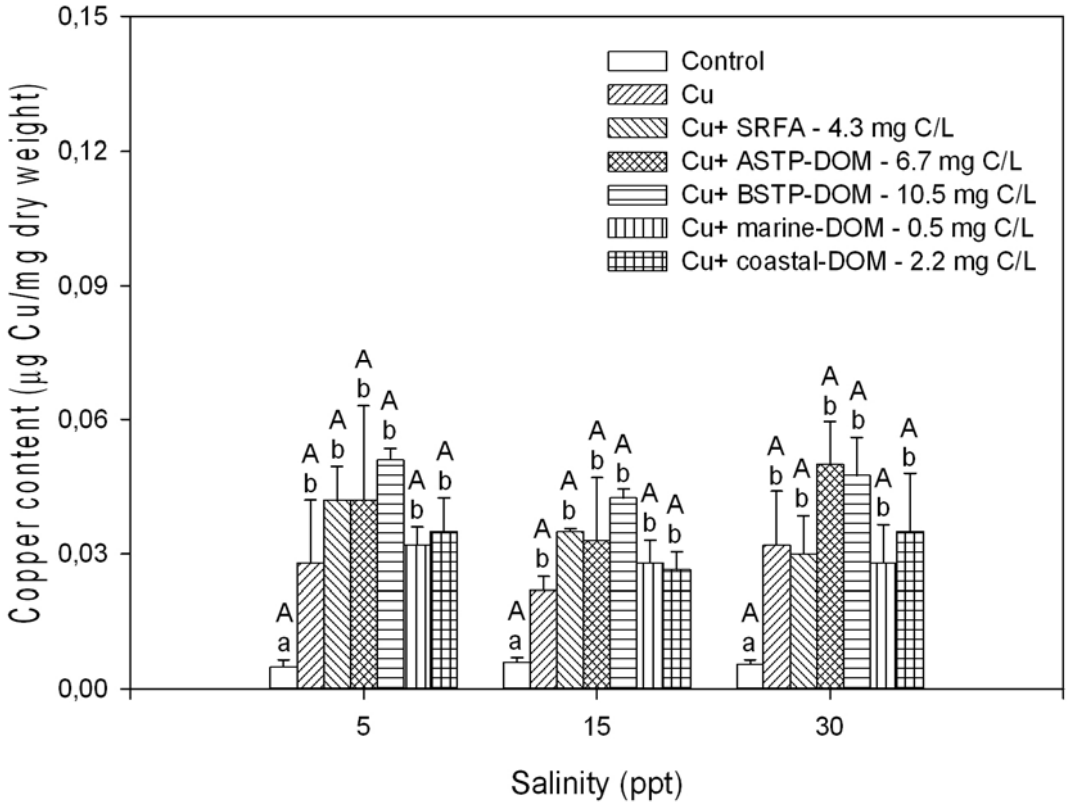

Control
Control+DOM
Cu
Cu+DOM - 0.6 mg C/L
Cu+DOM - 1.6 mg C/L
Cu+DOM - 4.3 mg C/L
Copper content (µg Cu/mg dry weight)
2,5
2,0
1,5
1,0
0,5
0,0
5
15
30
Salinity

Figure 5


Control
Cu
Cu+ SRFA - 4.3 mg C/L
Cu+ ASTP-DOM - 6.7 mg C/L
Cu+ BSTP-DOM - 10.5 mg C/L
Cu+ marine-DOM - 0.5 mg C/L
Cu+ coastal-DOM - 2.2 mg C/L
Copper content (µg Cu/mg dry weight)
0,15
0,12
0,09
0,06
0,03
0,00
5
15
30
Salinity (ppt)

CONCLUSÕES

A partir dos dados obtidos nos experimentos realizados com o copépode eurialino *Acartia tonsa*, pode-se concluir que:

1. machos adultos testados separadamente, bem como copepoditos e fêmeas adultas testados concomitantemente, apresentaram a mesma sensibilidade ao cobre em diferentes salinidades na ausência de matéria orgânica dissolvida (MOD); além disso, a sensibilidade de copepoditos e adultos de ambos os sexos testados concomitantemnete foi geralmente semelhante àquela observada nos dois tratamentos mencionados, indicando que copépodes de diferentes idades (copepoditos e adultos) e gêneros (machos e fêmeas) podem ser utilizados conjuntamente em testes de toxicidade aguda do cobre, facilitando a realização dos experimentos;
2. o aumento da salinidade protegeu contra a toxicidade aguda do cobre nos experimentos realizados com água salgada artificial, e os resultados destes experimentos foram similares aos que já haviam sido observados em testes realizados com água do mar filtrada (1μm) nas mesmas salinidades utilizadas nesta tese (Pinho e Bianchini, 2010), indicando que resultados de toxicidade aguda do cobre em animais expostos à água salgada artificial podem ser extrapolados para água do mar natural filtrada, e que a extensão e calibração de uma versão do modelo do ligante biótico (BLM) para ambientes estuarinos e marinhos deve considerar o efeito da salinidade nos organismos;
3. a toxicidade aguda do cobre está associada principalmente ao cobre dissolvido, uma vez que os valores de cobre dissolvido foram próximos aos valores de cobre total na água, corroborando as informações de trabalhos prévios;
4. diferenças na química da água explicam a toxicidade do cobre nos tratamentos sem adição de MOD, uma vez que as concentrações letais para 50% dos copépodes baseadas nos valores de cobre livre na água foram iguais nas salinidades 5, 15 e 30; no entanto, a concentração de cobre livre não é suficiente para explicar a toxicidade do cobre para os copépodes nos

tratamentos com adição de MOD dulciaquícola e marinha, pois as concentrações letais para 50% dos copépodes baseadas nos valores de cobre livre foram diferentes dependendo da MOD (concentração e fonte) e da salinidade;

5. a MOD *per se* (nas concentrações e fontes testadas nesta tese) não causou efeitos prejudiciais aos copépodes, uma vez que os valores de mortalidade foram semelhantes entre os tratamentos controle com e sem adição de MOD; assim, a mortalidade dos controles pode ser atribuída em grande parte ao estresse de manuseio;
6. a adição de MOD obtidas a partir de diferentes fontes de origem dulciaquícola ou marinha protegeu contra a toxicidade aguda do cobre, sendo que o grau de proteção foi dependente da concentração e da fonte de MOD testada; como a versão atual do BLM (para ambientes dulciaquícolas) considera somente a concentração de COD, os resultados desta tese mostram claramente que a inserção de características químicas de cada fonte de MOD (além da concentração de COD), também deve ser considerada na extensão e calibração de uma versão do BLM para ambientes estuarinos e marinhos e que mais estudos sobre os efeitos da matéria orgânica natural de diferentes fontes na toxicidade de metais em organismos estuarinos e marinhos devem ser realizados;
7. a acumulação de cobre nos animais inteiros e no exoesqueleto não reflete a acumulação do metal no ligante biótico; portanto, estudos que visem investigar a acumulação de cobre relacionada à toxicidade aguda devem quantificar o metal em outros locais (tecidos/órgãos) mais específicos;
8. os tecidos moles podem ser considerados como o ligante biótico para o cobre, sendo que o conteúdo de cobre nestes tecidos pode ser considerado como sendo o valor da acumulação letal de cobre para 50% dos organismos testados (AL50), independente da salinidade ou da MOD (origem e concentração), uma vez que quando expostos aos valores de CL50 os copépodes apresentaram o mesmo nível de cobre nos tecidos internos em todos os experimentos realizados nesta tese; isto corrobora totalmente a premissa do BLM de que

existe uma forte correlação entre o nível de metal acumulado no ligante biótico e sua toxicidade aguda.

CONSIDERAÇÕES FINAIS

1. os animais foram aclimatados em fotoperíodo (12C:12E) e água salgada (filtrada natural) diferentes dos utilizados durante os experimentos (fotoperíodo 16C:8E e água salgada artificial). Portanto, nos próximos estudos, devem ser buscadas condições para a manutenção dos cultivos dos copépodes nas mesmas condições dos experimentos. No entanto, cabe ressaltar que a toxicidade aguda do cobre para os copépodes em água salgada artificial desta tese foi similar à toxicidade aguda do cobre para *A. tonsa* em água do mar filtrada (1μm) para cada salinidade testada (5, 15 e 30) (Pinho e Bianchini, 2010);

2. foi testada somente uma concentração de MOD marinha (0,5 mg C/L) e uma concentração de MOD costeira (2,2 mg C/L), devido às dificuldades do processo de extração de MOD marinha. Em estudos futuros pode ser extraída mais MOD marinha, para serem realizados experimentos com diferentes concentrações de COD, permitindo assim observar o efeito da concentração da MOD, como ocorreu nos experimentos com adição de MOD dulciaquícola;

3. a caracterização química, através da análise da espectroscopia das diferentes fontes de MOD utilizadas no presente estudo (em andamento), provavelmente ajudará a explicar os resultados, contribuindo especialmente para o melhor entendimento da influência de diferentes fontes de MOD na toxicidade e acumulação de cobre nos copépodes. Portanto, é recomendável que em estudos sobre a influência da MOD nos organismos seja realizada sua caracterização espectroscópica ou outra caracterização química que indique os principais compostos presentes na MOD (ácidos húmicos e fúlvicos, aminoácidos, etc);

4. na água do mar, especialmente na salinidade 30, outras formas químicas, além do cobre livre, e/ou a formação e assimilação de complexos MOD-cobre podem ter causado toxicidade aos

copépodes;

5. como existe a possibilidade de assimilação de complexos MOD-cobre pelos copépodes, que influenciaria na acumulação do metal, futuramente podem ser realizados experimentos de acumulação do cobre nos tecidos moles testando diferentes concentrações de COD para a mesma concentração de cobre; além disso, os pellets fecais podem ser contados ao longo do experimento, para estimar se há ingestão de MOD pelos copépodes (conforme Pinho *et al.*, 2007);

6. os organimos foram lavados com EDTA para a determinação de cobre no exoesqueleto. Nis próximos estudos, poderia ser também dosado o cobre no exoesqueleto de copépodes não lavados com EDTA, uma vez que pode ser importante considerar a adsorção de cobre no exoesqueleto de organimos tão pequenos como os copépodes.

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ANEXO 1

Normas para submissão de manuscrito à revista Environmental Toxicology and Chemistry

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letter in the upper left corner of the figure, outside the figure itself (not on the figure). For examples, please see previous issues of *ET&C*.
In addition:

•Ensure that the figure will be legible when reduced to the width of a column of text (80 mm).
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•Place the legend inside the figure or label the lines.
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•Describe what the error bars mean (SE, SD).
•Use minor ticks, especially on log-scales.
•Avoid textures or shading that will not reproduce well or will not be distinguishable in your legend.
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•If the graph is a characterization of correlation, add the coefficient of correlation to the graph.
•Use full words and avoid abbreviations. If you do use an abbreviation, define it in the figure legend.
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•If concentration– or dose–response is being characterized, use an arithmetic or log scale, not a categorical scale.

General Appearance--Halftones (gray scale images) do not reproduce well. Avoid small dotted lines, shading, and stippling. For bar graphs, use black, white, striped, or hatched designs, but only if they are sufficiently wide or separated in order to appear distinct from one another. If no important information will be lost, consider placing fewer numbers on the axes to achieve an uncluttered look. Make lines on maps bold and distinct and eliminate information not pertinent to the subject. Naming too many rivers, towns, and geographical elements results in a cluttered map that is difficult to read.

Size and Proportion--When possible, submit figures in the size they are to appear in the journal. Most illustrations, except some maps and very wide graphs, should be 1-column size (3.5 inches) and a resolution of 300 dpi. If the graph is composed in that size, legibility will be easy to determine. The font size on the *x* and *y* axes should not be larger than that of the title, and the same font (Arial or Times New Roman is preferred) should be used throughout. Numbers on the *x* and *y* axes should be smaller than the descriptive title, which should be 12-point font. Fonts smaller than 12 points are generally not legible when reduced to 1 column size. Use boldface type with care; if illustrations are to be reduced, the letters with open spaces will disappear.

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Abbreviations

Use acronyms and abbreviations *sparingly* to avoid impeding comprehension of the text, and use only those that are well known. Too many acronyms make your manuscript difficult to read. Define each acronym at first introduction at the text, and on each table and figure legend, giving the abbreviation or acronym in parentheses. Spell out acronyms that begin a sentence. Do not use an acronym for words or phrases if that word or phrase is used fewer than five times. Symbols and abbreviations commonly used can be found here: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-8618/homepage/ETCSymbolsandAbbrevs.pdf

Technical Information

Equations, mathematical formulas, flow diagrams

Simple equations should be written as A/B on one line. Decimals are preferred to fractions. Write out and hyphenate simple fractions (two-thirds), except in figures, graphs, legends, and in parentheses. Refer to the document Math to Type.pdf for specific guidelines.

Gases

Express parts per million (ppm) as microliters per liter µl/L) or parts per billion (ppb) as nanoliters per liter (nl/L). Use metric system only.

Ions

Represent ions as follows: Na^+, Mn^{3+}, Br^-, and PO^{3-}

Isotopes

An isotopically labeled compound is indicated by placing the isotopic symbol in square brackets attached to the name or the formula [14C]ethanol; [32P]ATP; [2H]C2H2; [3H]DNA. The specific position of the isotope should be given at the time of first mention; thereafter, it can be abbreviated to the less specific notation. The symbol indicating configuration should precede the isotopic symbol, and the position of isotopic labeling is indicated by Arabic numerals as in D-[14C]lactate;D-[14C]glucose 6-P; sodium D-[14C]acetate; L-[1,2-14 C]alanine.

The term U indicates uniform labeling, as in [U-14C]sucrose, where the isotope is uniformly distributed among all 12 carbons. Preference is given to [14C2] and 32Pi rather than to [14C]CO2 or [14C]CO2 and [32P]Pi.

Numbers

The metric system is standard, and SI units should be used as far as possible. Spell out all numbers or fractions that begin a sentence. If this is awkward, rephrase the sentence to avoid beginning with a numeral. Do not use a hyphen to replace the preposition "to" between numerals: 13 to 22 min, 3 to 10°C. Exception: Use the dash in tables, figures, graphs and in parentheses. Write out numerals one through nine except with units of measure.

Check tabular data, as well as numerical values, reported in the text for the proper number of significant figures. For decimals smaller than one, insert a zero before the decimal point: 0.345.

Powers in tables and figures

Care is needed in tables and figures to avoid numbers with many digits. The unit should be followed by the power of 10 by which the actual quantity was multiplied to give the reported quantity; the unit may be changed by the use of prefixes such as "mM" or "m." For example, an entry "5" under the heading "g x 10-3" means that the value of g is 0.005; and entry "5" under the heading "g x 103" means that the value of g is 5,000. A concentration of 0.0015 M may be expressed as 1.5 under the heading "concn. (mM)" or as 1,500 under the heading "concn. (mM)" as 15 under the heading "10-4 x concn. (M)."

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Mixtures use "to" when general words are used, i. e., "the chloroform to methanol" ratio. Always use a colon with words when numerical ratio is provided, i. e., chloroform:methanol (2:1,v/v). Always use a colon with a number ratio. Use a hyphen with a mixture only if a numerical value is not given, i. e., "used in chloroform-methanol."

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Solutions of common acids and bases should be described in terms of normality (N) , and salts in terms of molarity (M), thus 1 N NaOH, 0.1 N acetic acid and 0.1 M Na2SO4. Fractional concentrations should be expressed in the decimal system: 0.1 N acetic acid and not N/10 acetic acid. The term % must be defined as w/w, w/v, or v/v; 10% (w/v) signifies 10 g/100 ml. Express concentrations as ng/L, mg/L, mg/L, ng/g, mg/g, etc.

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