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INFLUÊNCIA DA MATÉRIA ORGÂNICA DISSOLVIDA NA TOXICIDADE AGUDA E ACUMULAÇÃO DO COBRE NO COPÉPODE EURIALINO *Acartia tonsa*: IMPLICAÇÕES PARA O MODELO DO LIGANTE BIÓTICO

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RESUMO

O objetivo deste trabalho foi analisar os possíveis efeitos de matérias orgânicas dissolvidas (MODs) de diferentes origens sobre a toxicidade aguda do cobre dissolvido na água e a acumulação corporal do cobre no copépode eurialino *Acartia tonsa*, em diferentes salinidades (5, 15 e 30 ppt). MODs de três diferentes fontes foram utilizadas: ácido fúlvico comercial extraído do rio Suwannee (AFRS) e MODs extraídas do arroio Vieira antes (AETE) e depois (DETE) da liberação do efluente da estação de tratamento de esgoto "Navegantes" (ETE). A salinidade *per se* protegeu contra a toxicidade aguda do cobre. Em todas as salinidades, a toxicidade aguda do cobre foi geralmente menor na presença do que na ausência de MOD. No entanto, este efeito protetor dependeu da concentração e fonte de MOD, sendo que as MODs AETE e DETE foram mais efetivas do que o AFRS. Em relação à acumulação corporal de cobre, esta foi, de forma geral, semelhante em todos os tratamentos experimentais, indicando que o nível de acumulação corporal de cobre que induz 50% de mortalidade (0.877 mg Cu/g peso seco) não varia em função da salinidade e MOD (concentração e fonte), corroborando assim com a premissa do Modelo do Ligante Biótico (BLM). Os resultados aqui apresentados indicam que tanto a salinidade quanto a MOD (origem e concentração) devem ser consideradas para fins de regulação da emissão de cobre no ambiente aquático.

Palavras-chave: *Acartia tonsa*, acumulação, cobre, matéria orgânica dissolvida, salinidade, toxicidade.

ABSTRACT

The aim of this work was to evaluate possible effects of dissolved organic matters (DOMs) from different origins on the acute waterborne copper toxicity and whole body copper accumulation in the euryhaline copepod *Acartia tonsa* in different salinities (5, 15 e 30 ppt). DOMs from three different sources were employed: commercial fulvid acid extracted from the Suwannee River (SRFA) and DOMs extracted from the Vieira stream before (BSTP) and after (ASTP) the effluent discharge of the “Navegantes” seawage treatment plant (STP). Salinity by itself protected against the acute copper toxicity. In all salinities, acute copper toxicity was generally lower in the presence than in the absence of DOM. However, this effect was dependent on the concentration and source of DOM, BSTP and ASTP

DOMs were more effective than the SRFA. Regarding whole body copper accumulation, it was generally similar in all experimental conditions, indicating that the lethal accumulation level of copper that induces 50% mortality (0.877 mg Cu/g dry weight) does not change as a function of salinity and DOM (concentration and source), corroborating with the Biotic Ligand Model (BLM) premise. Results reported here indicate that both salinity and DOM (origin and concentration) must be taken into account to regulate copper emission in aquatic environments.

Key-words: *Acartia tonsa*, accumulation, copper, dissolved organic matter, salinity, toxicity.

1. INTRODUÇÃO

O ambiente aquático é afetado por processos de contaminação causados por uma enorme variedade e quantidade de substâncias químicas que são lançadas em função de atividades antrópicas. A descarga de poluentes em rios e estuários, especialmente aqueles localizados próximos a áreas urbanas e industriais, como o estuário da Lagoa dos Patos (RS), tem aumentado significativamente a contaminação destes locais com substâncias tóxicas, tais como hidrocarbonetos, pesticidas e metais (Forstner & Wittmann 1983; Baumgartem & Niencheski 1990). No entanto, sabe-se que processos naturais, como erosão continental, também geram entrada significativa de metais em áreas costeiras (Niencheski *et al.* 1994). De fato, tem sido estimado que fenômenos naturais sejam responsáveis pela entrada de cerca de quatro vezes mais cobre nos oceanos do que por fontes antropogênicas (Landner & Lindestrom 1999). Logo, a ocorrência de íons metálicos no ambiente aquático não é somente o resultado de atividades humanas, mas pode ser também considerado como um fenômeno natural, onde diversos destes íons exercem funções de grande importância nos ciclos biogeoquímicos.

O cobre é um micronutriente essencial que participa de diversas funções fisiológicas nos organismos (Morgan 2000). Entretanto, em elevadas concentrações, alguns elementos essenciais podem ser tóxicos (Salomons *et al.* 1995). Desta forma, apesar do cobre ser um oligoelemento vital, seu lançamento para o ambiente deve ser controlado (Baumgarten & Niencheski 1998). Em muitos países, inclusive no Brasil (FEPAM 1995, CONAMA 2005), a regulamentação da emissão do cobre geralmente está baseada somente na

concentração total ou dissolvida do metal presente em efluentes e/ou no ambiente. No entanto, há duas décadas, a Agência de Proteção Ambiental Americana introduziu o conceito de “Critério de Qualidade de Água” (WQC), reconhecendo que a toxicidade dos metais depende da sua interação com outras substâncias que estão presentes na água (US-EPA 1985). De fato, diversos fatores químicos da água, tais como carbono orgânico dissolvido, pH, dureza e composição iônica, fornecem proteção contra os efeitos tóxicos agudos do cobre (Erickson *et al.* 1996).

Com o objetivo de regular de forma mais correta a emissão de cobre em ambientes aquáticos, foi desenvolvido o Modelo do Ligante Biótico (“Biotic Ligand Model” - BLM). 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 biológico) de um tecido-alvo (Di Toro *et al.* 2000) (Figura 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 dulciaqüícolas, com base em dados de toxicidade aguda do cobre em peixes (MacRae *et al.* 1999). Com base em dados obtidos para *Daphnia sp.*, este modelo também foi calibrado para invertebrados (Santore *et al.* 1999).

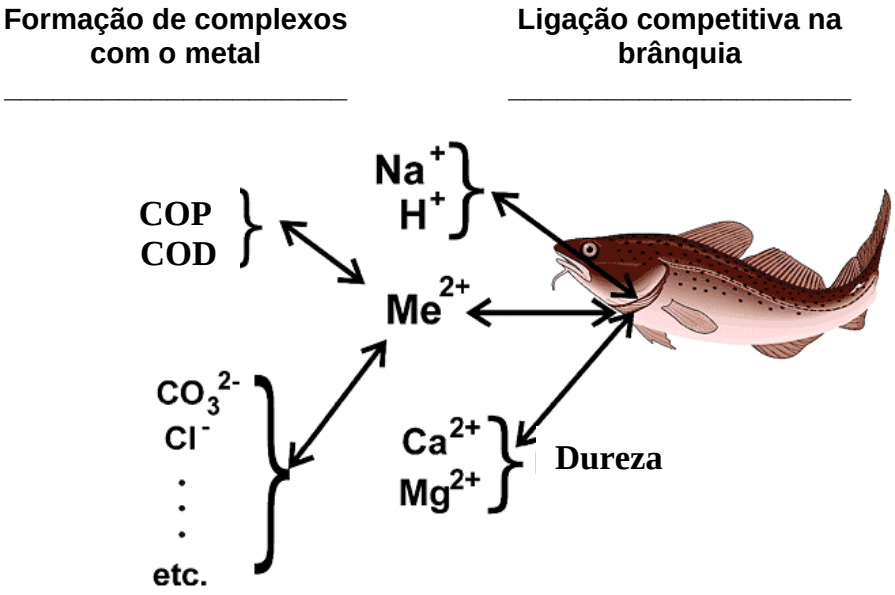

Figura 1 – Esquema do modelo do ligante biótico. As setas indicam a formação de complexos do metal (Me^{2+}) com o carbono orgânico particulado (COP) e dissolvido (COD), e outros compostos presentes na água, bem como a competição do metal pelo sítio ativo na brânquia de organismos aquáticos. (Fonte: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

Um dos principais parâmetros que influenciam a toxicidade e acumulação dos metais nos organismos aquáticos é a matéria orgânica dissolvida (MOD). A MOD é a fração da matéria orgânica filtrável (<0,45 µm). De fato, foi demonstrado que a incorporação da variabilidade da MOD ao BLM aumenta a capacidade preditiva deste modelo para *Daphnia magna*, um cladócero dulciaqüícola (De Shamphelaere *et al.* 2004).

Como a matéria orgânica dissolvida tem uma constituição complexa, ainda não bem elucidada, 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 compõem (Thurman 1985). No entanto, estudos sobre a influência da MOD sobre a toxicidade dos metais são relativamente recentes (Erickson *et al.* 1996, De Shamphelaere *et*

al. 2004, Kramer *et al.* 2004, Ryan *et al.* 2004). O COD, cuja concentração é determinada em mg/L, forma complexos com os metais, diminuindo assim a biodisponibilidade e toxicidade destes aos organismos aquáticos (Erickson *et al.* 1996, Ma *et al.* 2001, De Shamphelaere *et al.* 2004, 2005). Geralmente, os estudos sobre os efeitos do COD são realizados com matéria orgânica comercial, mais freqüentemente o ácido húmico da Aldrich (De Shamphelaere *et al.* 2005). Porém, este composto tem pouca semelhança estrutural com os ácidos húmicos aquáticos (Malcolm & MacCarthy 1986). Além disso, MODs 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).

A matéria orgânica natural (MON) pode ser produzida na coluna d'água pelo fitoplâncton (MON autóctone) ou ser proveniente do ambiente adjacente (MON alóctone). Tipicamente, a MON autóctone é rica em carboidratos e nitrogênio, possui uma coloração amarela e é composta principalmente de compostos de carbono de cadeia aberta, enquanto a MON 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 fração de ácido húmico, que na maioria das águas naturais está entre 10 e 15% da MOD, forma agregados e precipita facilmente no estuário (Sholkovitz 1976, Thurman 1985). Portanto, para experimentos que

utilizam MOD comercial em água salgada é preferencial a escolha de ácido fúlvico, pois este permanece solúvel em qualquer salinidade e pH (Thurman 1985).

Apesar de existirem alguns estudos sobre o efeito de diferentes fontes de MOD na toxicidade dos metais, a maioria está restrita a ambientes de água doce. Portanto, estudos sobre os possíveis efeitos da MOD de diferentes origens na toxicidade do cobre em uma ampla faixa de salinidade, utilizando espécies eurialinas sensíveis ao metal, como é o caso do copépode *Acartia tonsa*, são necessários para a futura extensão do BLM para ambientes estuarinos e marinhos. No presente estudo, foram utilizadas três fontes de MOD: ácido fúlvico comercial extraído do rio Suwannee e MODs extraídas da água coletada no Arroio Vieira antes e após lançamento dos efluentes da Estação de Tratamento de Esgoto “Navegantes” (Rio Grande, RS).

A. tonsa (Figura 2) é um copépode Calanoida cosmopolita. Os adultos toleram uma ampla faixa de salinidade, sendo encontrados no Estuário da Lagoa dos Patos (Rio Grande, RS) em salinidades de 0 a 31,5 (Montú & Goeden 1986). *A. tonsa* é uma espécie onívora, sendo o fitoplâncton um importante item de sua dieta (Gifford & Dagg 1988, 1991, Kleppel *et al.* 1991). Desta forma, constituem a principal ligação entre o fitoplâncton e os níveis tróficos superiores em muitas cadeias alimentares marinhas. 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). *A. tonsa* pode tornar-se adulta 7 dias após a eclosão do ovo (Kaminski 2004), sendo que a expectativa de vida é de até 80 dias (Sazhina 1987). Quanto à produção de ovos, diferentes valores também são encontrados na literatura, sendo esta muito influenciada pela qualidade do alimento. Cabe ressaltar que copépodes marinhos são considerados indicadores sensíveis da toxicidade subletal dos metais (Hook & Fisher 2001), sendo utilizados há um tempo razoável em estudos toxicológicos em laboratório (Sosnowski *et al.* 1979), bem como mais recentemente em estudos de poluição ambiental (Bianchi *et al.* 2003).

Figura 2 – Fotografia de um copépode *Acartia tonsa* adulto.

2. OBJETIVOS

Objetivo Geral:

Avaliar os possíveis efeitos da matéria orgânica dissolvida (MOD) de diferentes fontes sobre a toxicidade aguda do cobre dissolvido na água e na acumulação corporal do cobre no copépodo eurialino *A. tonsa,* em uma ampla faixa de salinidade.

Objetivos Específicos:

(1) Verificar a influência da salinidade e da MOD na toxicidade aguda e acumulação corporal do cobre no copépode *A. tonsa*, em diferentes salinidades (5, 15 e 30 ppt).

(2) Analisar a variabilidade da toxicidade aguda do cobre e da acumulação corporal de cobre em *A. tonsa*, em função da origem da MOD em diferentes salinidades (5, 15 e 30 ppt).

3. MATERIAL E MÉTODOS

3.1. Cultivos de manutenção

3.1.1. Meios de cultivo

Os meios de cultivo do fitoplâncton e dos copépodes utilizados nos experimentos foram preparados com água do mar coletada na Praia do Cassino (Rio Grande, RS). Porém, os meios experimentais utilizados nos testes de toxicidade aguda e acumulação do cobre foram preparados a partir de uma solução-estoque de água do mar artificial, a qual foi preparada através da dissolução de sal marinho comercial (CoraLife®) em água Milli-Q (Bielmyer *et al.* 2004). Assim, foi possível estudar os efeitos de diferentes concentrações e origens de COD na toxicidade aguda e acumulação do cobre em *A. tonsa*.

3.1.2. Cultivo de fitoplâncton

Células algais das espécies *Thalassiosira weissflogii* e *Isochrysis galbana* foram inicialmente obtidas junto à Estação Marinha de Aquacultura (EMA-FURG). Os cultivos de manutenção foram realizados no Departamento de Ciências Fisiológicas (DCF-FURG) em garrafas plásticas de 5 L contendo água nas salinidades 5, 15 e 30 ppt, em uma incubadora do tipo DBO (20°C), sob aeração e iluminação constantes. Os meios de cultura das algas foram do tipo f/2 de Guillard (1975). Para *T. weissflogii*, o meio de cultura foi produzido com água (salinidade 5, 15 ou 30 ppt) enriquecida com silicato, fosfato e nitrato na proporção de 1000, 650 e 650 µL para cada litro de água, respectivamente.

No caso de *I. galbana*, foram utilizadas as mesmas concentrações de nitrato e fosfato. As algas foram oferecidas como alimento aos copépodes durante o período de aclimatação à salinidade, mas não durante os testes de toxicidade.

3.1.3. Cultivo de *Acartia tonsa*

Os copépodes foram obtidos junto à Estação Marinha de Aqüicultura da FURG e transferidos para o DCF, onde foram mantidos em baldes plásticos com capacidade de 10 ou 20 L, em uma sala com temperatura controlada (20°C), sob aeração suave constante e fotoperíodo 12C:12E. Os copépodes foram aclimatados por pelo menos 2 dias nas diferentes salinidades experimentais (5, 15 e 30 ppt), e foram alimentados diariamente com fitoplâncton nas seguintes concentrações: 2×10^4 células/mL de *T. weissflogii* e 10×10^4 células/mL de *I. galbana*. Para estimar o número de células de *T. weissflogii* e *I. galbana* nos seus respectivos meios de cultivo, foi retirada uma alíquota de cada meio, e foi contado o número de células em uma câmara de Neubauer. Dessa forma, foi possível calcular os volumes de meios de cultivo de algas a serem adicionados nos baldes com copépodes.

3.2. Matéria orgânica dissolvida (MOD)

3.2.1. Extração, preparação, manutenção e caracterização da MOD

Um tipo de MON foi extraído a partir da água doce coletada antes do lançamento dos efluentes da Estação de Tratamento de Esgoto “Navegantes” (ETE) da Companhia Riograndense de Saneamento em Rio Grande (RS), a qual libera seu efluente tratado diretamente no Arroio Vieira, que por sua vez deságua no estuário da Lagoa dos Patos (Rio Grande, RS). Outro tipo de MON também foi extraído a partir da água doce coletada no Arroio Vieira, porém após o lançamento dos efluentes da ETE (figura 3).

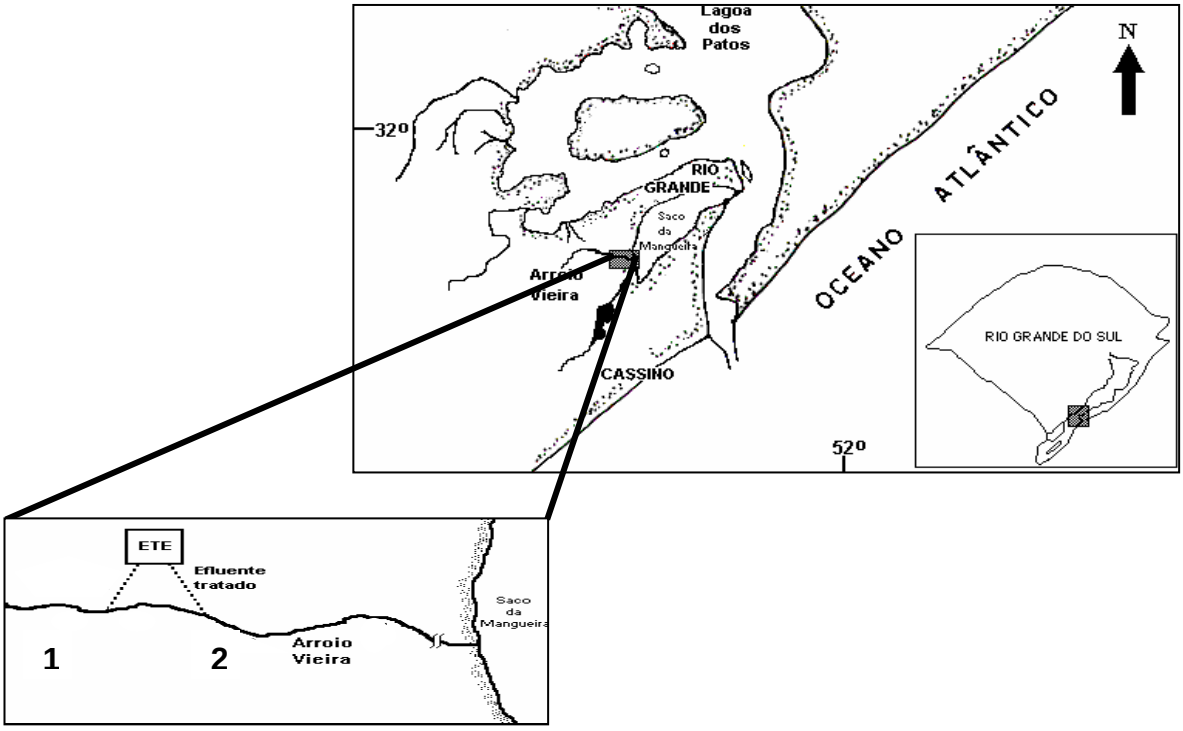

Figura 3 – Mapa com a localização dos pontos de coleta de água no Arroio Vieira para extração de MON, antes (1) e após (2) o lançamento dos efluentes da Estação de Tratamento de Esgoto “Navegantes” (Rio Grande, RS) (Fonte: Gilberto Fillmann, Laboratório de Microcontaminantes Orgânicos e Ecotoxicologia Aquática, FURG).

Aproximadamente 200 L de água doce foram coletados em cada um dos dois pontos amostrais, transportados ao laboratório e filtrados (filtros de polipropileno 10, 1 e 0,5 µm, respectivamente; Polyclean®, Cuno). Após a

filtragem, a água passou por uma resina trocadora de íons tipo sódio. Tanto a MON derivada da água doce coletada antes da ETE quanto aquela derivada da água doce coletada após a ETE, foram isoladas e concentradas por osmose reversa (Figura 4) (De Schamphelaere *et al.* 2005).

A terceira MON utilizada foi o ácido fúlvico ("fulvic acid Standard I"), o qual é extraído do Rio Suwannee e comercializado pela International Humic Substances Society (IHSS, EUA).

As soluções-estoque de MOD foram mantidas a 4ºC no escuro, antes de serem usadas nos testes de toxicidade e acumulação de cobre. Seguindo recomendações de De Schamphelaere *et al.* (2005), as concentrações de carbono orgânico dissolvido, Mg, Ca, Na, K, Cl^-, SO_4^{2-} e Cu foram medidas para caracterizar os três diferentes tipos de MOD. Estas medidas foram realizadas conforme descrito no item 3.6.

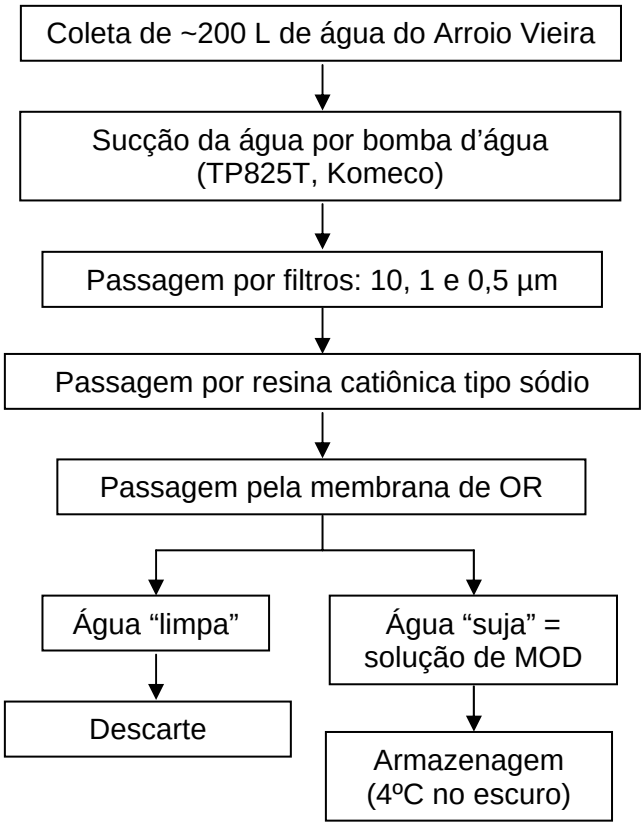

Figura 4 - Procedimentos para a extração, preparação e manutenção da MOD natural derivada de água coletada no Arroio Vieira antes e depois da ETE "Navegantes". OR = osmose reversa.

3.3. Meios experimentais

Diferentes meios experimentais foram preparados utilizando as MODs obtidas conforme descrito acima. Cada MOD foi adicionada aos meios experimentais tendo-se como referência a sua concentração de COD, previamente medida, o que permitiu o cálculo dos volumes de soluções-estoque de MOD a adicionar nos meios experimentais. A MOD foi diluída em água salgada artificial, a qual foi preparada conforme descrito anteriormente. Diferentes combinações de salinidade (5, 15 e 30 ppt), concentrações de COD (1,0; 2,5 e 5,0 mg C/L; Bielmyer *et al.* 2004, Rosen *et al.* 2005) e fontes de

COD (ácido fúlvico e MODs antes e após a ETE) foram testadas. Foram adicionadas aos meios experimentais entre cinco e sete diferentes concentrações de cobre ($CuCl_2$; Vetec, Brasil), a partir de soluções-estoque de cobre (0,02; 0,2 ou 2 g Cu/L). As concentrações de cobre presentes nas soluções-estoque de MOD (medido conforme item 3.6.) foram consideradas nos cálculos dos volumes de soluções-estoque de cobre a serem adicionados nos meios experimentais. Depois de preparados, os meios experimentais foram mantidos a 20^{o}C por 24 h antes de seu uso nos testes de toxicidade (Lorenzo *et al*. 2005).

3.4. Testes de toxicidade aguda

Dois tipos de testes controles foram realizados sem adição de cobre: (1) sem adição de COD; e (2) com adição de COD. Dois tipos de testes de toxicidade aguda foram realizados com adição de cobre nos meios experimentais: (1) sem adição de COD; e (2) com adição de COD. Estes testes foram realizados nas três salinidades experimentais (5, 15 e 30 ppt). Tanto os testes controles quanto os de toxicidade aguda foram feitos em duplicata.

Para a colocação dos copépodes nos meios experimentais, estes foram primeiramente retirados do meio de cultivo com uma rede (malha de 300 µm) e transferidos para um frasco de plástico, através de lavagem cuidadosa da rede com água salgada artificial na mesma salinidade do cultivo. Este frasco foi depois mantido sobre a superfície de vidro de uma caixa construída para a visualização dos copépodes, contendo em sua face inferior uma lâmpada

fluorescente. Assim, foi possível visualizar e selecionar os copépodes através do fundo translúcido do frasco de plástico. Em seguida, os copépodes foram cuidadosamente coletados, individualmente, com uma pipeta plástica, e transferidos para o frasco com meio experimental.

Dez copépodes adultos de ambos os sexos foram testados em cada frasco de vidro contendo 50 mL do meio experimental. Os frascos foram mantidos sob rotação constante (2 rpm) em uma incubadora do tipo DBO (20^{o}C; fotoperíodo 16C:8E). Após 24 h de teste, os copépodes sobreviventes em cada frasco foram contados e transferidos para um meio experimental novo, preparado como descrito anteriormente. Após 48 h, os copépodes vivos em cada frasco foram contados e descartados (Figura 5). A diferenciação entre animais mortos e vivos foi feita através de microscópio estereoscópico (animais sem movimentação aparente de tecidos e/ou órgãos foram considerados mortos). Os valores de concentração letal para 50% dos organismos testados (CL_{50}) e seus respectivos intervalos de confiança (95%) foram determinados com base nos dados de mortalidade acumulada após 48 h de teste, considerando as concentrações de cobre total medido, filtrado medido e livre estimado. As concentrações de cobre livre foram estimadas somente para os meios experimentais sem COD, tendo em vista que a concentração real de COD não foi medida nos meios experimentais. As concentrações de cobre livre foram obtidas conforme descrito abaixo (item 3.6).

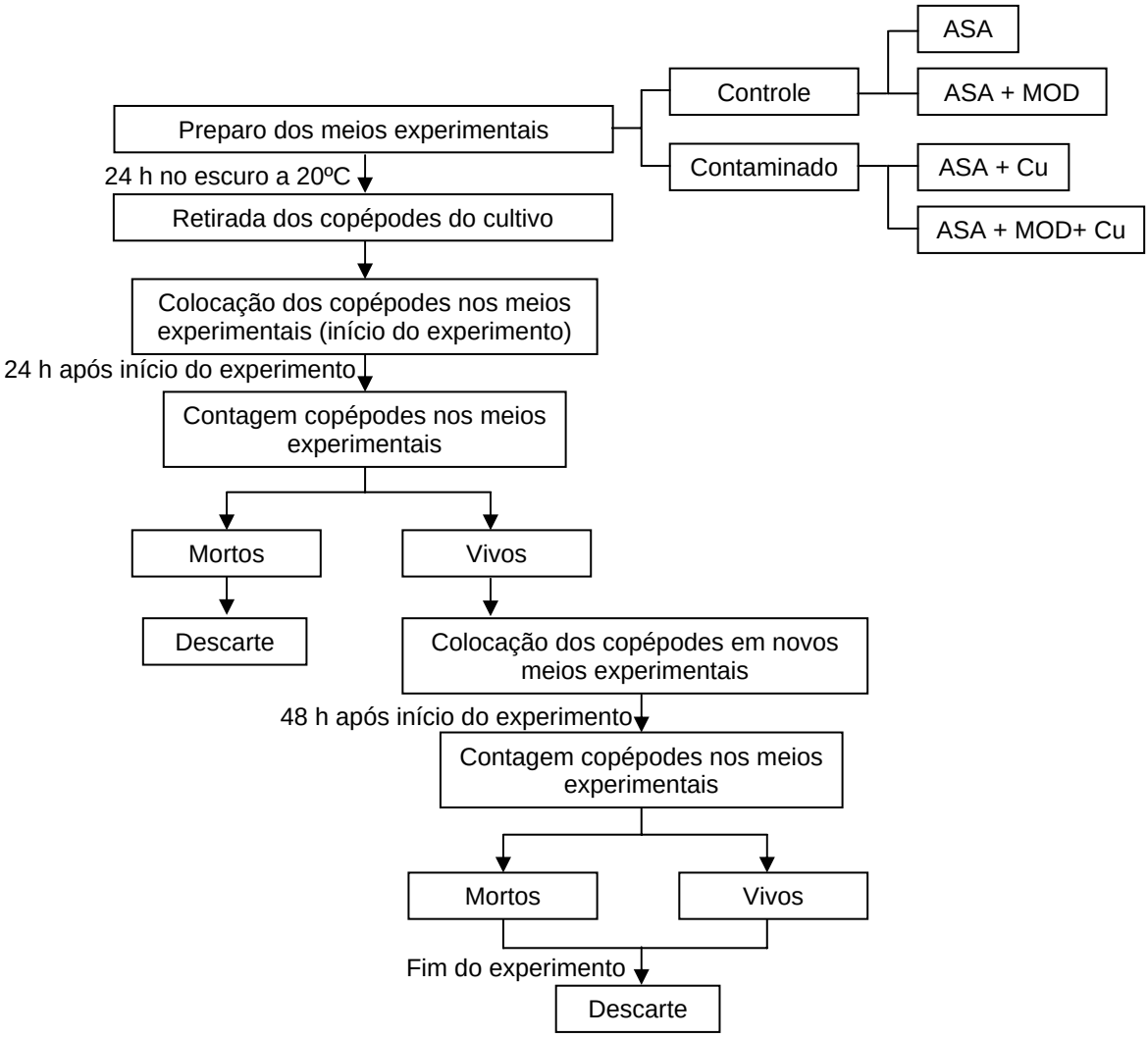

Figura 5 – Procedimentos para os testes de toxicidade aguda do cobre no copépode *A. tonsa*, na presença ou ausência de matéria orgânica dissolvida (MOD). ASA = água salgada artificial.

Para a análise da composição química dos meios experimentais, foram coletados 10 mL de água filtrada (filtros de acetato-celulose; 0,45 µm, Sartorius) e não filtradas dos meios experimentais, imediatamente antes e 24 h após a introdução dos copépodes. Conforme descrito abaixo (item 3.6), as seguintes medições foram realizadas na água: pH, O_2 dissolvido, temperatura, alcalinidade e concentrações de COD, Cu, Ca, Mg, Cl^-, Na, K, SO_4^{2-} e alcalinidade.

3.5. Testes de acumulação corporal de cobre

Os testes de acumulação corporal de cobre foram realizados em quadruplicata utilizando-se diferentes combinações de salinidade (5, 15 e 30 ppt) e fontes de COD (AFRS, AETE e DETE), na ausência (controle) ou presença de cobre, seguindo-se os mesmos procedimentos descritos para os testes de toxicidade aguda. Com base nos resultados dos testes de toxicidade aguda, os copépodes foram expostos por 48 h às diferentes combinações de salinidade e de concentrações de COD e cobre. Porém neste caso, a concentração de cobre utilizada foi aquela correspondente à CL_{50} determinada em cada uma das combinações de salinidade e concentração de COD.

Ao final de 48 h de exposição, os copépodes sobreviventes foram coletados, rapidamente enxaguados (15 s) em água Milli-Q, acondicionados em tubos tipo Eppendorf (n = 5 ou 10 por tubo), secos em estufa (70^{o}C) por 48 h e digeridos em 100 µl de HNO_3 65% (Suprapur®, Merck, EUA). A concentração de cobre foi medida conforme descrito abaixo (item 3.6) e expressa em mg Cu/g de peso seco. Previamente, amostras secas de 20 copépodes haviam sido pesadas na balança do analisador de CHNS/O (2400 Series II, Perkin Elmer, precisão de 2 µg). O peso seco obtido (4,5 ± 0,87 µg) foi utilizado nos cálculos da concentração corporal de cobre nos copépodes.

3.6. Análises químicas

As concentrações de COD nas soluções-estoques de MOD foram medidas utilizando-se um analisador de carbono total (TOC 5000, Shimadzu). As concentrações de cobre nas soluções-estoques de MOD, nos meios experimentais (amostras filtradas e não filtradas) e nas amostras de copépodes digeridos, bem como as concentrações de cátions (Na, K, Ca e Mg) nos meios experimentais foram medidas por espectrofotometria de absorção atômica com chama (AAS 932 Plus, GBC, IL, EUA). A concentração de cobre livre foi calculada com base nos parâmetros físico-químicos da água e nas concentrações de cobre filtrado, usando-se o pacote computacional BLM (Hydroqual 2002). A concentração de Cl^- nos meios experimentais foi determinada por espectrofotometria (510 nm, B 382, Micronal, Campo Grande, MS) utilizando-se um kit de reagentes (Doles S.A., Goiânia, GO). A concentração de SO_4^{2-} e a alcalinidade nos meios experimentais foram medidas por espectrofotometria utilizando-se os métodos descritos por Tabatabai (1974) e pela APHA (1989), respectivamente. O teor de O_2 dissolvido e o pH foram medidos nos meios experimentais utilizando-se um oxímetro (DMO 2, Digimed, São Paulo, SP) e um medidor de pH (DMPH-2, Digimed, São Paulo, SP), respectivamente.

3.7. Limpeza dos materiais

Todo o material utilizado nos testes de toxicidade aguda e acumulação do cobre, bem como para coleta de água do Arroio Vieira e armazenagem de MOD (bombonas plásticas e frascos de vidro), foi previamente lavado com

HNO_3 1% por no mínimo 24 h. Para retirar o HNO_3, o material foi enxaguado diversas vezes com água destilada e seco em estufa. Os frascos de vidro utilizados para colocação dos meios experimentais controles e contaminados nos testes de toxicidade aguda e acumulação do cobre foram lavados separadamente.

3.8. Análise estatística

Os valores de CL_{50} e seus respectivos intervalos de confiança (95%) foram determinados pela análise dos Probitos (Finney 1971). A acumulação corporal de cobre foi avaliada através de análise de variância (ANOVA) de três vias (concentração e fonte de COD e salinidade), seguida do teste *a posteriori* de Tukey (α=0,05), utilizando-se o pacote estatístico Statistica 5.1 (StatSoft Inc., EUA).

4. RESULTADOS

4.1. Fontes de MOD

Ao longo do presente estudo foi possível extrair, caracterizar e manter sob condições adequadas em laboratório MODs de diferentes origens, a saber, de água doce coletada no Arroio Vieira antes e após a liberação dos efluentes da Estação de Tratamento de Esgoto “Navegantes” (Rio Grande, RS). A quantidade de MOD extraída foi suficiente para a realização dos experimentos de toxicidade aguda do cobre dissolvido na água com o copépode eurialino *Acartia tonsa* (vide Anexo I).

4.2. Parâmetros físico-químicos da água

Os valores dos parâmetros físico-químicos dos meios experimentais aumentaram significativamente com o aumento de salinidade, exceto a concentração de oxigênio dissolvido que foi semelhante nas salinidades 15 e 30 ppt. Porém, não foram observadas diferenças significativas na composição química dos meios experimentais antes e após a adição de COD, em todas as salinidades testadas (vide Anexo II – Tabela 2).

4.3. Mortalidades nos copépodes controles

A mortalidade dos copépodes nos tratamentos controles (com e sem adição de COD) foi semelhante ($p>0,05$), tendo variado entre 8.7 e 30% (vide Anexo II – Figura 2).

4.4. Toxicidade aguda do cobre

O aumento da salinidade *per se* apresentou um efeito protetor contra a toxicidade aguda do cobre no copépode *A. tonsa*. Este efeito pode ser atribuído exclusivamente à química da água, uma vez que não houve diferença significativa entre os valores de CL_{50} calculados com base nas concentrações de cobre livre nas diferentes salinidades (vide Anexo II – Figura 3).

Em todas as salinidades testadas, a toxicidade aguda do cobre foi geralmente menor na presença do que na ausência de MOD. No entanto, este efeito protetor foi dependente da concentração e fonte de MOD. Efeitos protetores maiores foram observados para a maior concentração de COD testada. De maneira geral, as MODs derivadas do Arroio Vieira (antes e após a ETE) foram mais efetivas na proteção contra a toxicidade aguda do cobre do que o ácido fúlvico extraído do rio Suwannee (vide Anexo II – Figuras 4, 5 e 6).

Em todos os tratamentos experimentais, a maior parte da toxicidade aguda do cobre foi devida à fração solúvel do metal, uma vez que não houve diferença significativa entre os valores de CL_{50} calculados com base nas concentrações de cobre total medido e filtrado para cada tratamento experimental (vide Anexo II – Figuras 3, 4, 5 e 6).

4.5. Acumulação corporal de cobre

Na ausência de COD, a acumulação corporal de cobre diminuiu significativamente com o aumento da salinidade. Na presença de COD, a acumulação corporal de cobre foi, geralmente, semelhante nas diferentes condições experimentais, independente da concentração e fonte de COD e da salinidade, indicando que a quantidade de cobre corporal acumulado capaz de induzir 50% de mortalidade não varia em função da salinidade, bem como da concentração e fonte de COD (vide Anexo II – Figuras 7, 8, 9 e 10).

5. CONCLUSÕES

A partir dos resultados descritos no presente estudo, conclui-se que:

- A toxicidade aguda do cobre para o copépode eurialino *A. tonsa* está associada principalmente ao cobre dissolvido;
- A salinidade apresenta um efeito protetor contra a toxicidade aguda do cobre dissolvido na água;
- A presença de MOD diminui a toxicidade aguda do cobre dissolvido na água, sendo este efeito dependente da concentração e fonte de COD;
- A quantidade de cobre corporal acumulado que induz 50% de mortalidade do copépode eurialino *A. tonsa* não é, de forma geral, afetada pela salinidade, bem como pela concentração e fonte de COD.
- Não somente a concentração, mas também a fonte da MOD deve ser levada em consideração em futuras versões do BLM, a fim de aumentar a capacidade de previsão deste modelo.

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ANEXO I

Extraction and concentration of freshwater- and sea water-derived dissolved organic matter for use in aquatic toxicology studies

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Extraction and concentration of freshwater- and sea water-derived dissolved organic matter for use in aquatic toxicology studies

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Abstract

Dissolved organic matter (DOM) is defined as the organic matter that passes through a 0.45-μm mesh filter. Recent studies have shown the DOM importance in mitigating trace metals and organic pollutants toxicity. In general, studies with DOM are performed using commercial organic matter, usually as humic acid (Aldrich). However, it has been demonstrated that this humic acid has little structural similarity with the aquatic humic acids. Furthermore, a natural DOM is composed by different fractions, which can exhibit different complexing properties with metals. Thus, it is important to evaluate the effect of different sources of natural DOM on pollutants toxicity. To use natural DOM in aquatic toxicity tests, it is necessary to extract and provide suitable storage for samples in the laboratory. The ideal process for DOM isolation from natural waters should be capable of rapidly and effectively extracting large quantities of DOM from water without fractionation, chemical alteration and/or other losses. Therefore, in the present paper we describe the methodological approaches used for extraction and concentration of both freshwater and sea water-derived DOM using XAD as adsorption resins, and compare them with the reverse osmosis (freshwater) and solid phase extraction (sea water) techniques currently in use in our laboratory.

Key-words: freshwater; organic matter; PPL resin; reverse osmosis; sea water; sewage; solid phase extraction; XAD resin.

Introduction

Dissolved organic matter (DOM) is defined as the organic matter that passes through a 0.45-µm mesh filter. It is expressed in $mg.L^{-1}$ as dissolved organic carbon (DOC), since carbon is its major component, and because its structure is complicated and not well defined. Furthermore, DOM composition can vary according to origin, season, and physicochemical characteristics of the surrounding environment. Carbon accounts for about 50% of the natural organic matter (NOM) weight. The term "total organic carbon" (TOC) refers to all organic carbon species found in organic structures, present in the water, from methane with a molecular weight of 16 Da to the large and complex humic substances (500-100,000 Da). DOM is composed mainly of humic substances, which are described as heterogeneous polyfunctional polymers formed through the breakdown of plant and animal tissues by chemical and biological processes, and generally comprise one-third to one-half of the DOC present in natural waters (Thurman, 1985).

DOM has various functions and plays important roles in aquatic ecosystems. For instance, it interacts with trace metals and controls their dynamics. Furthermore, it fuels the microbial loop, generates gases and nutrients with biological and photochemical reactions, absorbs and extinguishes light, and affects satellite images (Ogawa & Tanoue, 2003). Several studies have also shown the DOM importance in mitigating the toxicity of trace metals and organic pollutants (*e.g.* Erickson *et al*., 1996; Fliedner, 1997). DOM forms complexes with metals, thus reducing their bioavailability and toxicity (Erickson *et al.,* 1996; Ma *et al.,* 2001; Kramer *et al*., 2004; De Shamphelaere *et al.,* 2004). In general, studies regarding the DOM influence on metals toxicity are

performed using commercial organic matter, usually as humic acid (Aldrich) from soils (De Shamphelaere *et al*., 2005). However, it has been demonstrated that this humic acid has little structural similarity with the aquatic humic acids (Malcolm & MacCarthy, 1986). Furthermore, a natural DOM is composed by different fractions, as the humic and fulvic acids, which can exhibit different complexing properties with metals (Ma *et al.,* 2001).

Natural DOM concentrations depend primarily on the type of water, origin, vegetation and climate, among other factors (Kramer *et al.,* 2004). For example, the humic substances constitute 10-30% of the marine DOM and 70-90% of the freshwater marsh DOM (Thurman, 1985). DOMs from different sources can be composed by molecules with different characteristics, since they are formed from different precursors (Ryan *et al.,* 2004). Thus, their complexing characteristics and capacities can vary from one place to another (Kramer *et al*., 2004; Ryan *et al*., 2004). Consequently, the DOM source can influence the bioavailability and toxicity of metals to aquatic organisms (Kramer *et al.,* 2004).

To use natural DOM in toxicological tests, is necessary to extract and provide suitable storage for samples in the laboratory. The ideal process for DOM isolation from natural waters should be capable of rapidly and effectively extracting large quantities of DOM from water without fractionation, chemical alteration and/or other losses (Sun *et al*., 1995). DOM has been isolated from water by various methods, including precipitation, vacuum evaporation, ultrafiltration, solvent extraction, freeze-drying, freeze-concentration, charcoal, strong anion-exchange resins, and by adsorption chromatography with macroporous resins, such as XAD resins (for review, see Malcolm, 1989). However, there are clear differences in the practicality, extraction

efficiency, and characteristics and amount of DOM extracted using these different techniques. In this context, XAD resins are widely used by hydrology researchers since early 1970's, because they overcame some of the limitations of older used methods (Thurman & Malcolm, 1981; Malcolm, 1989). XAD-2, XAD-4, and XAD-8 (Amberlite®) are the most common adsorbers used for extraction and concentration of DOM from both freshwater and sea water (Peuravuori *et al.*, 1997; Maurice *et al.*, 2002; Engbrodt, 2001; Engbrodt & Kattner, 2005).

In light of the above, the present work aims to show the methodological approaches for extraction and concentration of natural DOM for use in aquatic toxicology studies using the solid phase extraction with XAD resins and those ongoing in our laboratory: reverse osmosis and PPL resin for freshwater- and sea water-derived DOM extraction, respectively.

Experimental section

Water collection for DOM extraction

Approximately 200 L of water from freshwater or sea water are collected in the field and transported to the laboratory in polyethylene containers previously cleaned with 1% HNO_3. Freshwater is then filtered using a sequence of polypropylene filters (nominal pore sizes = 10, 5, and 0.5-µm mesh filters; Cuno, Polyclean®, Brazil). For sea water, only the 0.5 µm-mesh filter can be used.

If it is not possible to extract and concentrate DOM immediately, collected water must be kept as cold as possible and in the dark to avoid photochemical transformation (Bertilsson & Tranvik, 2000; Ma *et al.*, 2001).

DOM extraction and concentration using solid phase extraction with XAD resins

For freshwater, DOM extraction and concentration can be performed following the procedures described by Maurice *et al.* (2002). In this case, DOM is isolated and concentrated using Amberlite® XAD-8 resin (commercial Amberlite®, practical grade quality, ROHM & HASS Corp., Philadelphia). However, XAD-8 is specifically designed to isolate the humic fraction of DOM. Procedures are summarized in Figure 1.

XAD-8 resin should be cleaned prior to use. For resin cleaning, procedures described by Standley & Kaplan (1998). Briefly, resin can be prepared for cleaning by first stirring continuously with 2-3 bed volumes of 0.1 N NaOH for 1 h and decanting the rinsate (this step is repeated until DOC concentration in the rinsate is <1 mg $C.L^{-1}$). Then, resin is cleaned sequentially with methanol, acetonitrile, methanol, acetonitrile, and methanol, in a Soxhlet extractor. Each extraction takes about 24 h for each solvent. Standley & Kaplan (1998) also reported an easier and faster alternative method for XAD-8 resin cleaning, which consist in stirring with solvents (1 h each solvent), and stirring repeatedly with several bed volumes of deionized water, instead of using the Soxhlet extractor for 24 h. After cleaning, resin is rinsed with deionized water, and 0.1 N HCl (about three cycles of 2 bed volumes each rinse) (Standley & Kaplan, 1998) or glass low-pressure chromatography columns are filled with resin and cleaned further using three successive 0.1 N Na OH – 0.1 N HCl rinses just prior to the extraction

(Maurice *et al.*, 2002). Therefore, DOC can be determined in a final sample (10 mL) of the acid solution used to clean the resin to check for possible remaining contamination from the packed column.

According to Maurice *et al.* (2002), 2 L of resin are used for every 70 L of water sample. After water sampling and filtration through the series of three filters, water is acidified (pH 2) with HCl. Then, sample is pumped through the XAD-8 resin. DOM adsorbed on the resin is eluted with 0.1 N NaOH and immediately reacidified (pH 2) with HCl. This eluate is brought again to XAD-8 resin, to be reconcentrated, followed by rinsing with distilled water to desalt, and back eluted with 0.1 N NaOH. The eluate is immediately passed through H^+-saturated cation exchange resin, to H^+ saturate the sample, remove sodium, and further decrease the concentration of other metals. Finally, the cleaned, concentrated, and fractionated material is lyophilized. Therefore, a stock solution with Milli-Q water can be made.

For sea water, DOM extraction and concentration can be performed following the procedures described by Malcolm (1989) and modified by Engbrodt (2001), which are summarized in Figure 2. In this case, DOM is isolated and concentrated using Amberlite® XAD-2 and XAD-4 adsorption resins (commercial Amberlite®, practical grade quality, ROHM & HASS Corp., Philadelphia) following the procedures and cautions described by Engbrodt (2001) and by Engbrodt & Kattner (2005). Two resins are used to maximize the adsorption efficiency for DOM, since XAD-4 has a higher adsorption capability for smaller molecules than XAD-2, which extracts preferentially larger molecules. The XAD resins are cleaned successively with dichloromethane and acetonitrile followed by methanol in a Soxhlet extractor. Each extraction takes about 24 h and is performed 5 times. To control the cleaning process, a sub-sample of 10 mL of

the resin is taken out of the extractor after the last extraction with methanol. In an acid-rinsed glass chromatography column, the resin sample is washed with 1,000 mL of Milli-Q water, 100 mL of 0.1 M sodium hydroxide and 100 mL of 0.1 M hydrochloric acid solution (Suprapur® grade, Merck, Darmstadt) with a flow rate of 10 $mL.min^{-1}$. DOC is determined in the final 10 mL of the acid. If the sample is DOC-free, the resin is carefully transferred into acid-rinsed 30 mL chromatography columns (300 mm x 14 mm inner diameter) with a P2-frit and a Teflon stopcock. Several pairs of XAD-2 and XAD–4 columns are prepared. These columns are carefully topped and sealed with parafilm until utilization. The resin is kept in methanol to avoid gas bubble formation and to maintain sterile resins. For DOC extraction, methanol is completely removed: the columns are mounted under a dropping funnel and washed with 2 L of Milli-Q water, followed by acidic and alkaline solutions (100 mL of 2 M hydrochloric acid and 100 mL of 0.1 M sodium hydroxide solution, with a flow rate of 2 $mL.min^{-1}$). The two resins are sequentially introduced in the column. To avoid blocking of the resin pores by macromolecules, which would lead to a reduced adsorption capacity, the sample will pass first the coarser XAD-2 and then the XAD-4. Samples are acidified to pH = 2 prior to extraction with concentrated hydrochloric acid solution to protonate acidic groups and reduce polarity, leading to an increased adsorption efficiency. At a flow rate of 1 drop per second, the extraction of a 20 L sample takes between 21 and 24 h. After the sample has completely passed the columns, they are rinsed with 250 mL of 0.1 M hydrochloric acid to remove the rest of the saline sample from the resin. The resins are then eluted separately. Polar molecules are eluted with 100 mL sodium hydroxide solution. Less polar substances are eluted with 100 mL of methanol. The fractions are collected in acid-rinsed polyethylene bottles and stored at –20°C until its use. Prior to

use, the methanol is removed in a vacuum rotary evaporator. To avoid DOM destruction, water bath temperature should never exceed 40^{o}C and methanol not evaporated until dryness. Complete removal of methanol is ensured by triple addition of 50 mL Milli-Q water and repeated evaporation. The sample volume is then adjusted to 100 mL with Milli-Q water. The two fractions of DOM are then mixed and stored (4^{o}C in the dark) until its use (Ma *et al.*, 2001; De Schamphelaere *et al.*, 2004). To assure that no carbon is released from the resins during the DOM extraction, 20 L of acidified Milli-Q water are passed through the resin columns in blank experiments. These experiments are performed as described above.

DOC concentration in the DOM solutions extracted from freshwater and sea water can be determined using a Total Organic Carbon (TOC) analyzer. These solutions are then stored at 4^{o}C in the dark (Ma *et al*., 2001; De Schamphelaere *et al.*, 2004) until its use in toxicological experiments.

Freshwater-derived DOM extraction and concentration using reverse osmosis

Approximately 200 L of freshwater are collected in the field and transported to the laboratory in polyethylene containers previously cleaned with 1% HNO_3. Water is then filtered using a sequence of polypropylene filters (nominal pore sizes = 10, 5, and 0.5-µm mesh filters; Cuno, Polyclean®, Brazil). The filterable fraction obtained is considered as the DOM source. DOM can be then isolated and concentrated by reverse osmosis (RO) as currently in use in our laboratory. The experimental procedures were based on the methodology described by De Schamphelaere *et al.* (2005).

After water collection, procedures for DOM extraction and concentration must be done as quickly as possible. These procedures are summarized in Figure 3. Briefly, the permeate solution from the RO system, *i.e.* the purified water, is discarded, while the concentrated solution is recycled into the sample reservoir and mixed with additional water from the sampling site. The concentrated solution, which would be discarded from the RO system, is the concentrated DOM. RO extraction is performed until DOM is concentrated as desired. DOC concentration in the concentrated solution can be determined using a TOC analyzer. This solution is then stored at 4^{o}C in the dark (Ma *et al*., 2001; De Schamphelaere *et al.,* 2004) until its use in toxicological experiments.

Sea water-derived DOM extraction and concentration using solid phase extraction with PPL cartridges

Approximately 200 L of seawater are collected as previously described for freshwater DOM, and filtered using a 0.5-µm mesh filter (Cuno, Polyclean®, Brazil). The filterable fraction obtained is considered as source of DOM. Marine-derived DOM is then concentrated using PPL cartridges (Mega Bond Elut PPL, 5 GM 60 mL, 16/PK, Varian), as recommended by Koch (Boris Koch, Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany, personal communication). Bond Elut PPL is a functionalized styrene-divinylbenzene polymer, which has been optimized for the extraction of highly polar species from large-volume water samples. Featuring a proprietary high-purity spherical polymer with an extremely nonpolar surface, Bond Elut PPL retains even the most polar compounds, such as phenols, and achieves high

recoveries and fast extraction speeds (Varian Inc., Palo Alto, CA, USA). PPL cartridges are activated using methanol (Merck) and Milli-Q water before its use for DOM extraction. Two successively fillings of each solution are performed to activate the PPL cartridges. Filtered sea water is then acidified (pH 2) with HCl, and placed in 10-L glass flasks previously cleaned with 1% HNO_3. Each flask is connected to one PPL cartridge through a plastic tube and sealed with a plastic cap. Flow rate should not exceed 20 mL min^{-1}, and up to 60 L of seawater can be allowed to pass through each cartridge.

After all sea water passed through cartridges, the next step is to remove salts from the resin, before DOM elution. Thus, cartridges are rinsed with 100 mL of Milli-Q water acidified (pH 2) with HCl. Then, DOM is eluted with 120 mL of methanol at a maximum flow rate of 10 mL min^{-1}. Methanol eluate is dried at natural temperature (~20ºC) or at a maximum temperature of 40ºC. Freeze-drying can be also used to obtain the final DOM extract. Afterwards, DOM powder extracted is stored in waterproof dark-flasks, until its use in toxicological tests. DOC concentration in the DOM powder can be determined using a TOC analyzer, and a DOM stock-solution prepared using Milli-Q water. This solution is then stored at 4ºC in the dark until its use in toxicological tests.

Any pumping device needs to be used to accelerate the methanol and Milli-Q water flows during cartridge activation or the seawater flow during the marine-DOM concentration and elution. All procedures for sea water-derived DOM extraction and concentration are summarized in Figure 3.

Results and discussion

In our laboratory, freshwater-derived DOM was extracted from water collected from the Vieira Stream (Rio Grande, RS, Southern Brazil) (Fig. 4) in March 2006. The first source of DOM was the water collected before the "Navegantes" Public Sewage Treatment Plant (BSTP). The second one was the water collected about 2 m after the effluent discharge from the Treatment Plant (ASTP). The effluent from the Treatment Plant goes directly into the Vieira Stream, which flows into the Patos Lagoon Estuary (Rio Grande, RS, Southern Brazil). Water collection and DOM extraction and concentration were performed as described in the "Experimental section". DOM extraction and concentration were done by reverse osmosis (RO).

The third source of DOM was the water collected approximately 20 miles away from the Cassino Beach (Rio Grande, RS, Southern Brazil), representing a more autochthonous source of DOM (Fig. 4). Water was collected in December 2006, following the procedures described in the "Experimental section". DOM extraction and concentration were performed as described in the "Experimental section" using the solid phase extraction technique with PPL cartridges.

Total organic carbon (TOC) concentration in DOM stock solutions was measured before toxicological tests using a TOC analyzer (TOC 5000, Shimadzu). DOM stock solutions were filtered again prior to TOC measurements using 0.45-µm mesh acetate-cellulose filters (Sartorius). In this case, we considered that the DOM was completely dissolved, being TOC considered as DOC.

Water samples collected for freshwater-derived DOM extraction had their volume reduced by ~15-fold. The DOC concentration in the water from the Vieira stream was 18 mg $C.L^{-1}$ at both sampling sites, and increased to 121.8 and 126.3 mg $C.L^{-1}$ in the DOM stock solutions prepared with water collected before (BSTP) and after

(ASTP) the public sewage treatment plant, respectively. Therefore, DOC concentration increased ~7-fold for both DOM stock solutions, and the recovery yielded was ~50%. This result is similar to that obtained by Maurice *et al.* (2002) using XAD-8 resins to concentrate DOM from a freshwater fen in a pine region. However, other studies using RO observed higher recoveries (between 80 and 100%) (Serkiz & Perdue, 1990; Sun *et al*., 1995; Maurice *et al.*, 2002; De Shamphelaere *et al.*, 2005). Although RO isolation gives, in general, higher recoveries of carbon than XAD-8, it was reported that it gives also higher ash content, especially Si and S, and it can promotes condensation and coagulation (Maurice *et al.*, 2002). On the other hand, XAD-8 resins, as previously mentioned, is designed to isolate only the humic fraction of DOM, and it apparently isolates more hydrophobic compounds and causes ester hydrolysis, thus leading to DOM chemical alterations (Maurice *et al.*, 2002).

Although high recovery yield is an important reason for the successful use of RO for DOM extraction, there are other factors to be considered. Among the main advantages of this sampling technique are the low probability of chemical alteration, and the relatively quickness of concentration of large quantities of DOM from natural surface waters (Serkiz & Perdue, 1990; Sun *et al*., 1995). In fact, research with natural DOM is greatly facilitated if significant quantities of DOM can be extracted and concentrated relatively fast, providing samples to prepare, for months after collection, large volumes of experimental media with various nominal DOC concentrations, if necessary. Thus, the use of RO for freshwater-derived DOM extraction is highly recommended for this purpose in spite of some problems can occur during DOM extraction. Some of these problems are discussed below.

A possible problem associated with the use of the RO extraction is the increasing intermolecular interactions at higher DOM concentrations (Zsolnay, 2003). In addition, pressure can also influence DOM solubility during concentration procedures in the RO system. Pressure changes can result in cavitation with the formation of small gas bubbles. Surface-active DOM can then adsorb on the bubble surfaces, and once bubbles collapse they adsorb DOM as particulate organic matter (Zsolnay, 2003). To ensure that DOM concentrated by RO was really dissolved, DOM solutions can be filtered again using 0.45-µm mesh filters prior to use in toxicological experiments. If filters can release organic materials, a blank test must be performed filtering Milli-Q water and measuring DOC, as previously described. In our study with DOM derived from freshwater collected at the Vieira Stream, DOC in Milli-Q water was only 3.27 mg $C.L^{-1}$, being attributable to a DOC releasing from the acetate-cellulose filters used. In this case, the contribution of the acetate-cellulose membrane to the total DOC in the stock solutions was only of 2.7 and 2.6% for the BSTP and ASTP DOMs. Despite these adversities, De Shamphelaere *et al*. (2005) showed that DOM extraction and concentration using the RO technique does not affect the physicochemical characteristics of the experimental media prepared with the DOM extracted and concentrated, as well as the protective effects of the DOM against metal toxicity in freshwater organisms.

To ensure the quality of the DOM extracted, some precautions should be taken into account when using the RO technique. One of them is related to how often the RO membrane should be changed. In our laboratory, we used one RO membrane for each extraction. However, the fact that only ~50% recovery was obtained suggests that the RO membrane should be changed more frequently along the DOM extraction, to avoid

membrane saturation. As previously mentioned, cavitation can lead to particulate organic matter formation, thus increasing probability for membrane saturation, although water was passed through a sodium type cation exchange resin before to reach the RO membrane.

Another precaution is related to the complete cleaning of the RO system before and after each new extraction. For example, Sun *et al.* (1995) described a thorough cleaning procedure conducted in their laboratory. The process consisted in recirculating a 0.5 g. L^{-1} solution of a detergent (Alconox) at low operating pressure, followed by a thorough rinse with pure water. In our case, after the extraction of BSTP-derived DOM, we let tap water followed by ASTP water flows through the RO equipment before ASTP extraction. No problems associated with the cleaning procedure adopted would be expected, since BSTP water should contain less suspended solids and other particulate materials than the ASTP water, and also because both sources of DOM were collected in the same stream (Vieira Stream).

Despite the clear advantages showed by the RO system for extraction of freshwater-derived DOM, this equipment cannot be used for DOM extraction from sea water, since salts start to precipitate right away (e.g., calcium is nearly its solubility limit in sea water). In addition, sea water represents, in terms of DOC recovery yielded after extraction and concentration, one of the most challenger sources of DOM, since inorganic matter can be approximately 30,000 times more concentrated than organic matter, and DOC was estimated to be only between 0.3 and 2.0 mg $C.L^{-1}$ in sea water (Thurman, 1985). Despite these difficulties, some different adsorbers for extraction of large amounts of DOM from seawater were recently tested (adsorbers with varying hydrocarbon chains bonded to a silica structure and styrene divinil benzene polymer

type adsorbers). Solid phase extraction through PPL cartridges was the most efficient adsorber among those tested (Boris Koch, Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, German, personal communication).

In the present study, we used solid phase extraction with PPL resins and ~93.4 mg of DOM was extracted from 60 L of sea water. Considering that 50% of DOM is DOC, then this volume of water yielded ~46.7 mg DOC. It is reported that the mean DOC concentration in sea water in the euphotic zone or near coastal regions is 1.5 mg $C.L^{-1}$ (Malcolm, 1989), although it can vary between 0.3 and 2.0 mg $C.L^{-1}$, depending on several factors (Thurman, 1985). Thus, recovery yielded in our laboratory was ~52%, which could be considered as a good recovery percentage. This statement is based on the fact that extraction efficiency of PPL cartridge are generally around 50% (Boris Koch, Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, German, personal communication). On the other hand, in experiments with sea water, 99% of the initial DOC content was recovered in the hydrophilic and hydrophobic fractions of the fractionation process using XAD-2 and XAD-4 resins, and hence no irreversible adsorption at the resin was observed (Engbrodt, 2001). However, solid phase extraction of DOM with XAD resins show some disadvantages compared to PPL resins. For example, careful XAD resin cleaning is required in order to guarantee that possible incorporation of organic matter from the adsorbent does not occurs (Thurman & Malcolm, 1981; Daignault *et al*., 1988; Malcolm, 1989; Standley & Kaplan, 1998). The equipment required for the cleaning procedure is expensive (high volume Soxhlet units and organic solvents) and the method is time consuming (the original method takes 5 consecutive days to be completed), demanding much effort (Daignault *et al*., 1988; Malcolm, 1989). Solvents used for cleaning are in general hazardous (methanol,

dichloromethane, acetonitrile, diethyl ether, and acetone), demanding an efficient exhaustion system in the laboratory. Besides, alterations of the natural extracted DOM may occur associated with extreme variations in pH during the isolation process, irreversible interactions with resins, contamination from resin bleed, and size-exclusion effects (De Shamphelaere *et al*., 2005). Therefore, it is clear that, although solid phase extraction has lower recovery yielded than the method described by Engbrodt (2001) using XAD-2 and XAD-4 resins, it has more advantages than disadvantages. It is faster and cartridges are easy to manipulate than XAD resins. For example, there is no need for the thorough resin cleaning procedures described here, and solid phase extraction demands the use of a few reagents (methanol and HCl) during extraction procedures.

Based on the information revised here, and the experience developed in our laboratory, we believe that RO extraction and solid phase extraction with PPL cartridges are suitable techniques for extraction of freshwater- and sea water-derived DOM to be employed in toxicology studies, when compared to other available techniques using XAD resins. They clearly showed more advantages than disadvantages.

Acknowledgments

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CAPTIONS FOR FIGURES

Figure 1. Procedures for collection, concentration and storage of freshwater-derived DOM by solid phase extraction using XAD-8 resin.

Figure 2. Procedures for collection, concentration and storage of sea water-derived DOM by solid phase extraction using XAD-2 and XAD-4 resins.

Figure 3. Procedures for collection, concentration and storage of freshwater- and sea water-derived DOM using reverse osmosis (RO) and PPL cartridges techniques, respectively.

Figure 4. Sampling sites where water was collected to extract natural organic matter from both the Vieira Stream and Cassino Beach (Rio Grande, RS, Southern Brazil). 1 = before the "Navegantes" Public Sewage Treatment Plant (BSTP); 2 = about 2 m after the "Navegantes" Public Sewage Treatment Plant (ASTP); 3 = about 20 miles away from the Cassino Beach. Source: Gilberto Fillmann (Fundação Universidade Federal do Rio Grande, RS, Brazil).

Figure 1

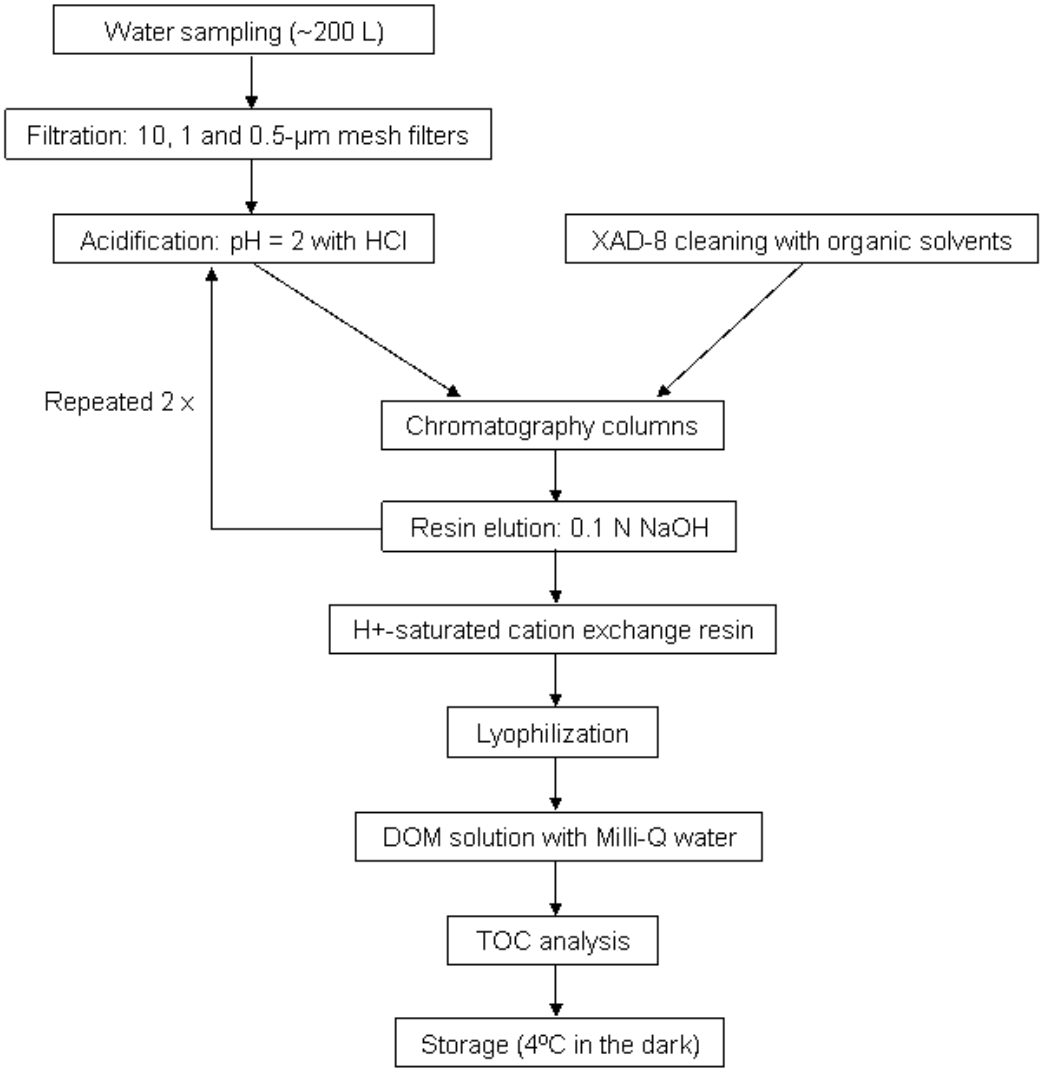

Water sampling (~200 L)
Filtration: 10, 1 and 0.5-µm mesh filters
Acidification: pH = 2 with HCl
XAD-8 cleaning with organic solvents
Repeated 2 x
Chromatography columns
Resin elution: 0.1 N NaOH
H+-saturated cation exchange resin
Lyophilization
DOM solution with Milli-Q water
TOC analysis
Storage (4ºC in the dark)

Figure 2

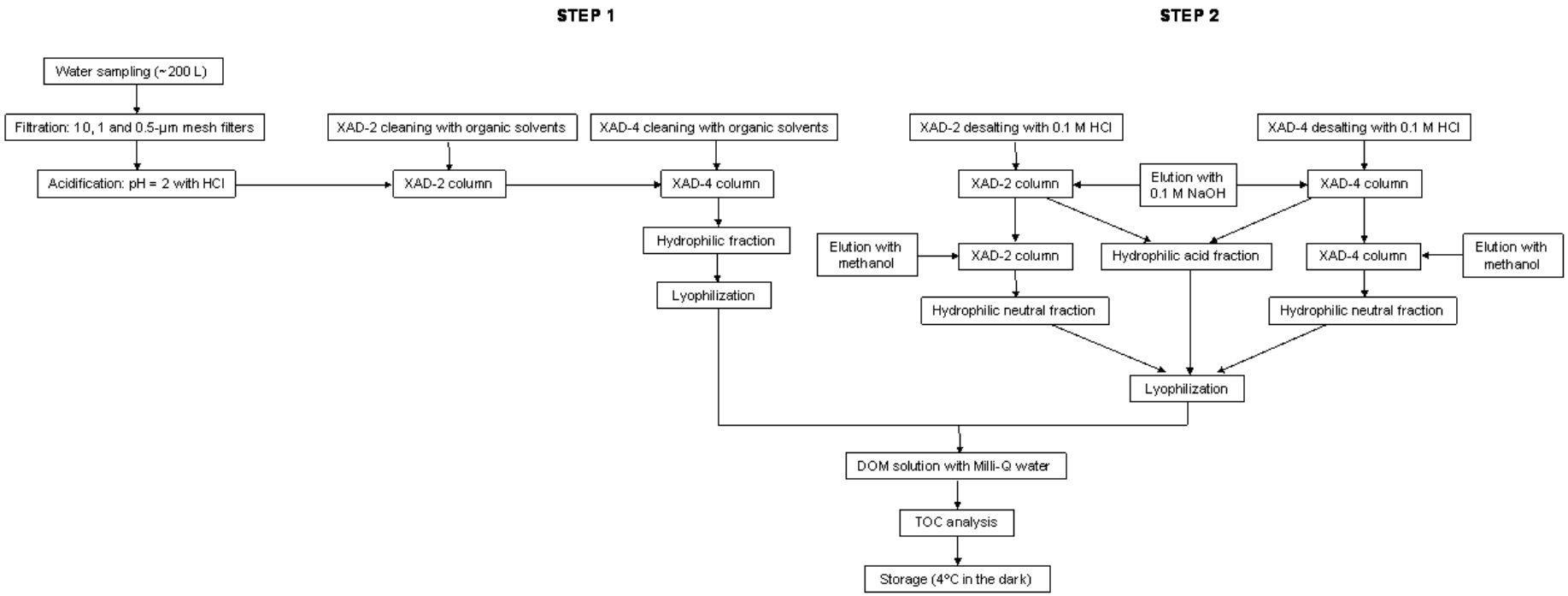

STEP 1
STEP 2
Water sampling (~200 L)
Filtration: 10, 1 and 0.5-µm mesh filters
Acidification: pH = 2 with HCl
XAD-2 cleaning with organic solvents
XAD-2 column
XAD-4 cleaning with organic solvents
XAD-4 column
Hydrophilic fraction
Lyophilization
XAD-2 desalting with 0.1 M HCl
XAD-2 column
Elution with 0.1 M NaOH
XAD-4 desalting with 0.1 M HCl
XAD-4 column
Elution with methanol
XAD-2 column
Hydrophilic acid fraction
XAD-4 column
Elution with methanol
Hydrophilic neutral fraction
Hydrophilic neutral fraction
Lyophilization
DOM solution with Milli-Q water
TOC analysis
Storage (4°C in the dark)

Figure 3

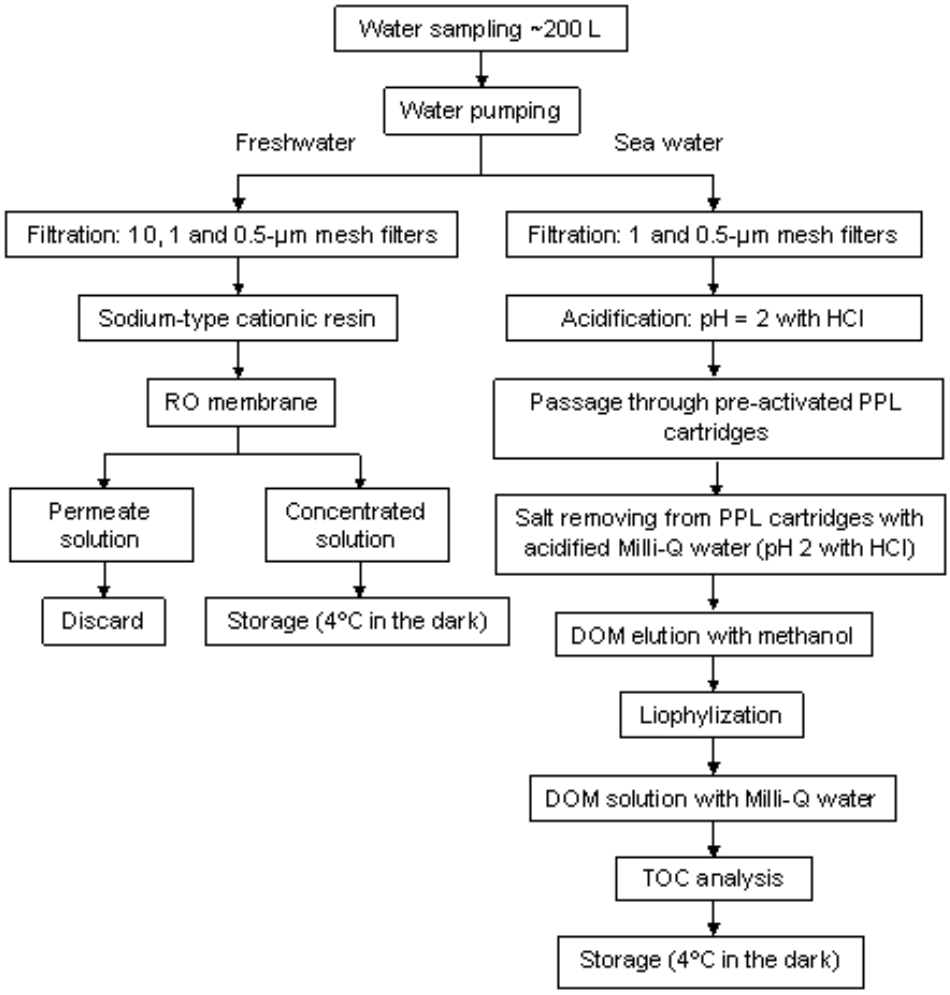
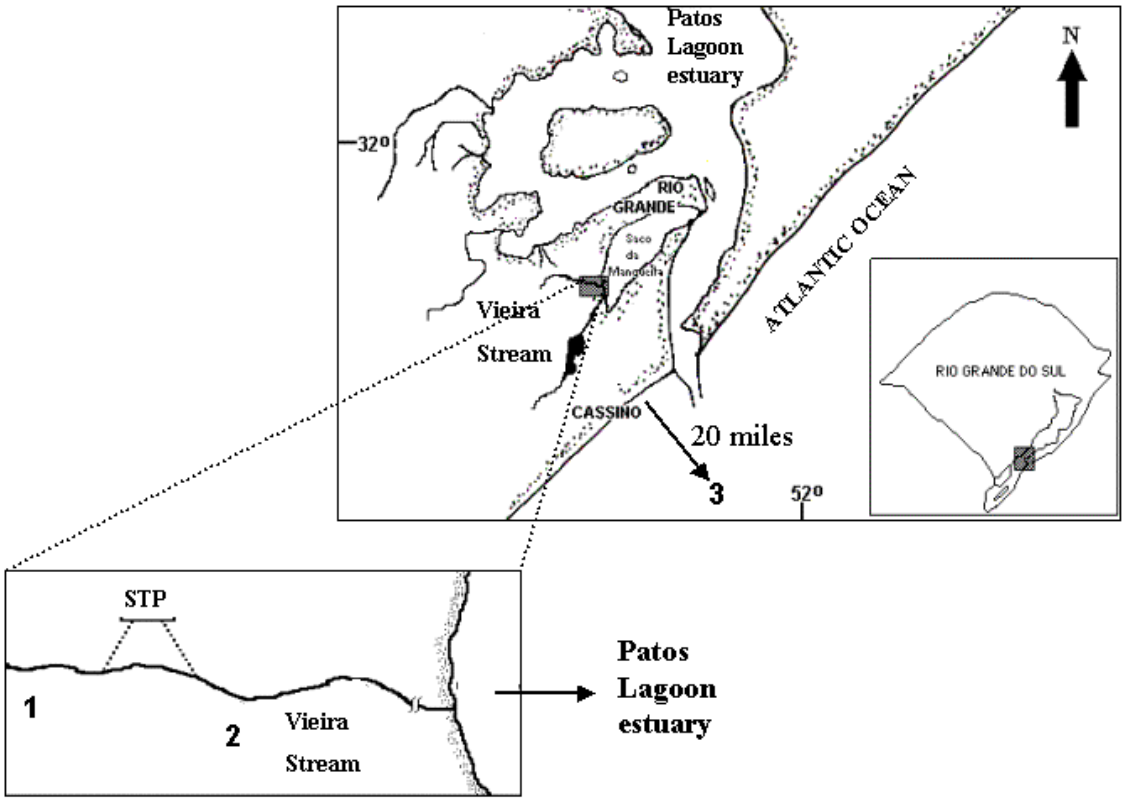

Water sampling ~200 L
Water pumping
Freshwater
Sea water
Filtration: 10, 1 and 0.5-µm mesh filters
Sodium-type cationic resin
RO membrane
Permeate solution
Concentrated solution
Discard
Storage (4°C in the dark)
Filtration: 1 and 0.5-µm mesh filters
Acidification: pH = 2 with HCl
Passage through pre-activated PPL cartridges
Salt removing from PPL cartridges with acidified Milli-Q water (pH 2 with HCl)
DOM elution with methanol
Liophylization
DOM solution with Milli-Q water
TOC analysis
Storage (4°C in the dark)

Figure 4


Patos
Lagoon
estuary
N
32º
RIO
GRANDE
Saco
da
Mangueira
ATLANTIC OCEAN
Vieira
Stream
RIO GRANDE DO SUL
CASSINO
20 miles
3
52º
STP
1
2
Vieira
Stream
Patos
Lagoon
estuary

ANEXO II

Dissolved organic matter effects on acute waterborne copper toxicity and accumulation in the euryhaline copepod *Acartia tonsa*: implications for the Biotic Ligand Model

Revista escolhida para submissão do artigo: Aquatic Toxicology

Dissolved organic matter effects on acute waterborne copper toxicity and accumulation in the euryhaline copepod *Acartia tonsa*: implications for the Biotic Ligand Model

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Abstract

The main goal of the present study was to analyze the possible influence of natural organic matter (NOM) on acute waterborne copper toxicity and whole body copper accumulation in *Acartia tonsa*. Three NOMs were used: freshwater-derived DOM from water collected before (BSTP) and after (ASTP) a seawage treatment plant (STP) and commercial fulvic acid extracted from the Suwannee River (SRFA). The first two DOMs were extracted by reverse osmosis. Different combinations of copper and DOC concentrations were tested at salinities 5, 15 and 30 ppt. 48-h LC_{50} values were calculated based on both total measured and filtered (0.45-µm mesh) copper concentrations. In each experimental condition, whole body copper accumulation was analysed in copepods exposed for 48 h to the corresponding 48-h LC_{50} value. Increasing salinity was protective against the acute waterborne copper toxicity. In all salinities, copper toxicity was generally lower in the presence than in the absence of DOC. However, the protective effect was dependent on the concentration and source of DOC. Higher protective effect was observed at the highest DOC concentration. Overall, BSTP and ASTP DOC were more protective than SRFA. In a broad view, whole body copper accumulation was similar in all experimental conditions, being close to 0.9 mg Cu/g dry weight. These findings clearly indicate that both salinity and DOC (source and concentration) should be taken into account for regulatory purposes. Also, they indicate that the amount of copper acummulated at the biotic ligand inducing 50% mortality is not dependent on salinity and DOC source and concentration. Thus, an LA_{50} value of 0.9 mg Cu/g dry weight is suggested to calibrate a future Biotic Ligand Model version for estuarine and marine conditions using the euryhaline copepod *A. tonsa*.

Key-words: *Acartia tonsa*, acute toxicity, Biotic Ligand Model, copper accumulation, dissolved organic matter, salinity.

Introduction

Copper is an essential micronutrient involved in several biological functions, being part of several enzymes involved in the defense against free radicals and cellular respiration (Dameron and Howe, 1998). However, it can be toxic to aquatic animals when present in elevated concentrations (Salomons *et al.*, 1995). 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 carbon, pH, hardness and ionic composition have been shown to protect in some extent against the acute copper toxicity in aquatic animals (Pagenkopf, 1983; Erickson *et al.*, 1996).

Studies regarding the influence of dissolved organic matter (DOM), *i.e.* the fraction of the natural organic matter (NOM) that is filterable (< 0.45 μm), on metal toxicity are relatively recent (Erickson *et al.*, 1996; Kim *et al.*, 1999; Lorenzo *et al.*, 2002; VanGenderen *et al.*, 2003; De Shamphelaere and Janssen, 2004; De Shamphelaere *et al.*, 2004; Glover and Wood, 2004; Kramer *et al.*, 2004; Ryan *et al.*, 2004; Schwartz *et al.*, 2004; Glover *et al.*, 2005a, b). DOM, which concentration is measured in terms of DOC (mg/L), exerts an important protecting effect against the acute copper toxicity in both fish and aquatic invertebrates. It forms complexes with copper, thus reducing metal bioavailability and toxicity (Erickson *et al.*, 1996; Ma *et al.*, 2001; Kramer *et al.*, 2004; De Shamphelaere *et al.*, 2004, 2005). Furthermore, it was observed that DOM can induce beneficial changes in the sodium balance – main parameter affected by dissolved copper – in gills of rainbow trout (Matsuo *et al.*, 2004).

In general, studies with DOM are performed using commercial organic matter, usually as humic acid (Aldrich) from soils (De Shamphelaere *et al.*, 2005). However, it has been demonstrated that this humic acid has little structural similarity with the aquatic humic acids (Malcolm and MacCarthy, 1986). Furthermore, a natural DOM is composed by different fractions, as the humic, fulvic and hydrophilic acids, which can exhibit different complexing properties with copper (Ma *et al.*, 2001). The concentration of natural DOM depends primarily on the type of water, origin, vegetation and climate, among other factors (Kramer *et al.*, 2004). For example, the humic substances constitute 10-30% of the marine DOM and 70-90% of the freshwater marsh DOM (Thurman, 1985). DOMs from different places can be composed by molecules with different characteristics because they are formed from different precursors (Ryan *et al.*, 2004). Thus, their complexing characteristics and capacities can vary from one place to another (Kramer *et al.*, 2004; Ryan *et al.*, 2004). Consequently, the DOM source can influence the bioavailability and toxicity of copper to aquatic organisms (Kramer *et al.*, 2004). However, the extension on what the DOM source influences copper toxicity when compared to that observed for other factors remains still to be tested.

Recent 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 (Ryan *et al.*, 2004). Therefore, the evaluation of the influence of natural DOM on copper bioavailability and toxicity can help to improve the present version of this model. For example, Ryan *et al.* (2004) studied the influence of DOM on the acute copper toxicity in fish larvae. These authors reported that DOC and humic acid concentrations

could better explain the variability of the LC_{50} values than the detailed considerations or descriptions of the binding between DOM and copper, although they recognized that DOM source had a significant influence on copper toxicity. In fact, it was recently demonstrated that the incorporation of the DOM variability in the BLM as a factor controlling copper speciation, bioavailability and toxicity, improved in a minor way the predictive capacity of this model for *Daphnia magna* (De Shamphelaere *et al.*, 2004).

Despite the fact that several studies on the effect of different sources of DOM on metals toxicity are available in the literature, most of them are restricted to freshwater species. Only few studies have attempted to analyze the effects of DOM on copper toxicity and the consequent implications for saltwater copper criteria. 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 for a future extension of the BLM for brackish and marine environments. In this context, we evaluated the effect of three different DOM sources on acute copper toxicity and accumulation in the sensitive euryhaline copepod *Acartia tonsa* in a wide range of salinities (5, 15 and 30 ppt).

A. tonsa is a cosmopolitan Calanoida copepod and adults are tolerant to a wide range of salinities (0 – 31.5 ppt) (Montú and Goeden, 1986). Although phytoplankton can be important in *A. tonsa* diet, it is an omnivorous species (Gifford and Dagg, 1988, 1991; Kleppel *et al.*, 1991). Thus, they are a major link between the phytoplankton and the others levels in several food chains in marine and estuarine waters. The development of populations of *Acartia* are characterized by a short time for recruitment, constant molting periods, and exponential growth in size up to the adult stage (Miller, 1983). It is important

to note that marine copepods are for long time considered as sensitive indicators of metal toxicity (Hook and Fisher, 2001).

Materials and Methods

Brackish and sea water

The different media employed for algae and copepod cultivation were prepared from natural seawater collected at the Cassino Beach (Rio Grande, RS, 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 to reach the desired experimental salinities (5, 15 and 30 ppt), as described by Bielmyer *et al.* (2004). The use of artificial seawater in the present study allowed us to investigate possible effects of different environmentally relevant concentrations of dissolved organic carbon on the acute copper toxicity and accumulation in the copepod *A. tonsa*.

Copepods culture and acclimation

The method used for copepod cultivation was that described by Bersano (2003). The original lot of copepods (*A. tonsa*) was obtained from a permanent intensive culture of the Aquaculture Marine Station of the Fundação Universidade Federal do Rio Grande (Southern Brazil). 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).

Room temperature was fixed at 20°C and photoperiod was 12C:12E. Copepods were daily fed with a mixed algal diet composed of *Thalassiosira weissflogii* (2 x 10^4 cells/ml) and *Isochrysis galbana* (1 x 10^4 cells/ml) (f/2 algae medium, according to Guillard, 1975). Water at different salinities was prepared by mixing 50-µm mesh filtered seawater collected at the Cassino Beach (Rio Grande, RS, Southern Brazil) with distilled water. Media were gently aerated and completely renewed every week.

NOM collection

NOM was extracted from freshwater collected before and after the effluent discharge of the "Navegantes" Sewage Treatment Plant (STP) (Rio Grande, RS, Southern Brazil; Fig. 1). The first source of NOM was the water collected in the Vieira Stream before the public STP discharge (BSTP). The second source was the water collected about 2 m after the discharge from the STP (ASTP). The "Navegantes" STP discharges its treated effluent into the Vieira Stream, which flows directly into the Patos Lagoon estuary (Rio Grande, RS, Southern Brazil). 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).

DOM preparation, storage, and characterization

Approximately 200 L of water were collected at the two sampling sites (Fig. 1), and filtered using a sequence of polypropylene filters (nominal pore sizes = 10, 5 and 0.5-µm mesh; Polyclean®, Cuno, Brazil). The filterable fraction obtained was considered as

source of DOM. BSTP- and ASTP-derived NOMs were isolated and concentrated by reverse osmosis (De Schamphelaere *et al.*, 2005). 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. A SRFA stock solution (1000 mg C/L) was also prepared dissolving the commercial fulvic acid in Milli-Q water. All DOM stock solutions were stored at 4^{o}C in the dark until their use (Ma *et al.*, 2001; De Schamphelaere *et al.*, 2004).

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), environmentally relevant DOC concentration (1.0, 2.5, and 5.0 mg C/L; Bielmyer *et al.*, 2004; Rosen *et al.*, 2005) and DOM source (BSTP- and ASTP-derived DOM and SRFA) were tested. According to Thurman (1985), the selected DOC concentrations would be representative of river (5 mg C/L), brackish (2.5 mg C/L) and shallow sea (1 mg C/L) waters. The maximum volume of DOM stock solution added to the water to prepare experimental medium (50 mL) was 2 mL.

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). Copper concentration in DOM stock solutions were considered in the calculation of copper stock solutions volumes to be added to experimental media. Experimental media were kept at 20^{o}C in the dark for 24 h before their use in the toxicity tests (Kim *et al.*, 1999; Ma *et al.*, 2001). 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 (Lorenzo *et al.*, 2005).

Acute toxicity tests

In each experimental salinity, two types of control tests were run in the absence of copper: (1) without addition of DOC and (2) with DOC at the desired concentration. In each salinity, two types of acute copper toxicity tests were also performed: (1) without addition of DOC and different copper concentrations, and (2) with addition of both DOC and copper at the desired concentrations. Controls and tests were run in duplicate.

Prior to experiments, adult 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. Toxicity tests were run with adult copepods of both sexes 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. LC_{50} values and their corresponding 95% confidence intervals were determined based on the accumulated mortality after 48 h of test, as described below.

Copper accumulation tests

Experiments were run in quadruplicate using the same experimental conditions described for the toxicity tests. Copepods were exposed for 48 h to the corresponding 48-h LC_{50} values at the different experimental conditions (Table 1). After copper exposure, 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) for whole body copper concentration measurement. Copepods were dried (70°C for 48 h), weighed using an electronic microscale, and digested in 100 µl of 65% HNO_3 (Suprapur®, Merck, USA). Total copper concentration in digested samples was measured by atomic absorption spectrophotometry by flame (AAS 932 Plus - GBC, IL, USA).

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, SP, Brazil) and a pH meter (Digimed, DMPH-2, São Paulo, SP, 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 different experimental media were collected and acidified (0.5% HNO_3, Suprapur®, Merck) for copper concentration measurements. Non-filtered samples (10 mL) were also collected for water chemistry analysis, as described below.

Copper concentration in the filtered (total dissolved copper) and non-filtered (total copper) samples of the experimental media was measured by atomic absorption spectrophotometry by flame (AAS 932 Plus - GBC, IL, USA). Free copper concentrations were calculated based on water chemistry data and total dissolved copper concentrations

determined as described above, using the software BLM (Hydroqual, 2002). Free copper concentrations were calculated only in treatments without DOM addition because DOC concentrations in the experimental media were not measured.

Cation (Na, K, Ca, and Mg) concentrations in the non-filtered samples of the experimental media were measured by atomic absorption spectrophotometry by flame (AAS 932 Plus - GBC, IL, USA). Anion (Cl^-) concentration was measured using a commercial reagent kit (Chloride, Doles S.A., Goiânia, GO, Brazil). Absorption readings were done at 510 nm (B 382, Micronal, Campo Grande, MS, Brazil). Sulphate concentration and alkalinity in the non-filtered samples of the experimental media were measured using the spectrophotometric method described by Tabatabai (1974) and according to the method described by the American Public Health Association (1989), respectively.

Data presentation and statistical evaluation

The 48-h LC_{50} values were determined by Probit analysis (Finney, 1971) based on total measured, total dissolved, and free copper concentrations. Differences in LC_{50} values were detected by comparing their respective 95% confidence intervals.

Data from water chemistry parameters and whole body copper accumulation were expressed as mean ± standard deviation. Differences in water chemistry and whole body copper accumulation between treatments were assessed by two-way analysis of variance (ANOVA) followed by the Tukey's test. The significance level adopted was 95% ($\alpha = 0.05$).

Results

Water chemistry

Measured DOC concentrations in the DOM stock solutions were 121.8, 126.3 and 1000 mg C/L for the BSTP- and ASTP-derived DOM and SRFA, respectively. Total dissolved copper concentrations were 848.7, 574.7 and <10 µg Cu/L, respectively.

In each 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 salinity. All water chemistry parameters significantly increased with the increasing salinities, except the dissolved oxygen content that was similar in salinities 15 and 30 ppt (Table 2).

Acute copper toxicity

In all salinities tested, mean mortality values in control copepods were similar ($p > 0.05$) in the absence and the presence of DOM. They ranged from 8.7 to 30% (Fig. 2).

In the absence of DOC, no significant difference was observed between the 48-h LC_{50} values calculated based on total measured or free copper concentrations in different salinities. However, 48-h LC_{50} values calculated based on total dissolved copper concentrations significantly augmented with increasing salinities (Fig. 3).

In all salinities, 48-h LC_{50} values were higher in the presence than in the absence of DOM. However, differences were dependent on the concentration and source of DOM. Higher 48-h LC_{50} values were observed at the highest DOC concentration tested (5 mg/L

DOC). Overall, BSTP- (Fig. 4) and ASTP-derived (Fig. 5) DOMs were more protective than SRFA (Fig. 6).

For each treatment, no significant difference was observed between the LC_{50} values calculated based on total measured and total dissolved copper concentrations (Figs. 4-6).

Whole body copper accumulation

In the absence of DOM, whole body copper accumulation was significantly higher in copper-exposed copepods than in their respective controls. Copper accumulation significantly decreased as salinity increased (Fig. 7).

In the presence of DOM, copper accumulation was similar in copepods exposed to the different DOC concentrations of the same DOM source at the same salinity (data not shown). Thus, only one mean concentration of whole body copper accumulation was calculated for control copepods for the same DOM source and salinity. Significantly higher whole body copper accumulation was observed in copper-exposed copepods than in their respective controls, irrespective the DOM source and DOC concentration (Figs. 8-10). In general, copper accumulation was similar among all treatments, except in that with 1 mg C/L of BSTP-derived DOM at salinity 5 ppt (Fig. 8). A general mean of 0.077 ± 0.040 and 0.877 ± 0.316 mg Cu/g dry weight was calculated for control and copper-exposed copepods, respectively.

Discussion

In the present study, the possible acute effects of DOM on waterborne copper toxicity and whole body copper accumulation were analyzed in the euryhaline copepod *A.*

tonsa in a wide range of salinities (5, 15 and 30 ppt), using environmentally relevant DOC concentrations. As far as we know, this is the first time that the effects of natural DOM on acute toxicity and accumulation of copper were evaluated in an invertebrate species in a wide range of salinities.

Waterborne copper was toxic to the euryhaline copepod *A tonsa*, toxicity being mainly associated with the total dissolved copper. This statement is based on the fact that no significant differences were observed between the 48-h LC_{50} values calculated based on total measured and total dissolved copper concentrations at each experimental condition.

Salinity by itself was protective against the acute waterborne copper toxicity in a concentration dependent manner. This finding was observed when 48-h LC_{50} values based on filtered copper concentrations were compared. Toxicity tests performed previously in our laboratory with filtered-natural sea water have also shown similar results for *A. tonsa* (Pinho and Bianchini, 2007). It is well known that the high ions levels present in seawater acts as a protecting factor against metal toxicity due to complexation with anions, especially Cl^-, as well as the competition between cations and copper for binding sites on the biotic ligand, *i.e.*, the copepod's body surface. Thus, the observed salinity protection against copper toxicity may be explained only by considering the water chemistry. In fact, no significant difference was observed between 48-h LC_{50} values calculated based on free copper concentrations. It is widely reported that free copper is the most toxic copper species to aquatic organisms (for review: Paquin *et al*., 2002).

Depending on the source and concentration, humic substances can express a xenobiotic-like influence on organisms (Meems *et al.*, 2004). However, in all salinities and for all types of DOM tested, addition of DOM to the experimental media had no significant

effect on mortality of *A. tonsa*. In the present study, the highest DOC concentration tested was 5 mg C/L. Therefore, mortality observed in control copepods would not be attributable to possible DOM effects by itself, but likely to stress associated with handling. Our results are in agreement with other reported in the literature. For example, Lorenzo *et al.* (2002) demonstrated that the embryogenesis success rates in the sea urchin *Paracentrotus lividus* decreased only at high DOC concentration, without effect on the larval growth. Furthermore, Kim *et al.* (1999) also did not observe any detrimental effect of DOM in the daphnid *Ceriodaphnia dubia*.

Our data clearly indicate that DOM has a protective effect against the acute copper toxicity in *A. tonsa*. This effect was dependent on both concentration and source of DOM. Overall, copper toxicity was lower at higher DOC concentrations. In freshwater invertebrates, De Schamphelaere and Janssen (2004) reported that all three DOM sources tested in *D. magna* 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. They 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. In freshwater fish larvae, Ryan *et al.* (2004) suggested that DOC and humic acid concentrations could better explain the variability in LC_{50} values than the detailed considerations or descriptions of the binding between DOM and copper, although they also observed that DOM source had a significant influence on copper toxicity. Richards *et al.* (2001) observed that increasing concentrations of NOM from different sources increased rainbow trout survival after exposure to a six metals mixture. However, they found that NOM having the most autochthonous properties increased fish survival least. Schwartz *et*

al. (2004) also observed a protective effect of NOM against acute waterborne copper toxicity in raibow trout, which was dependent on the NOM source. Therefore, results from the present study agree with the idea that is important to consider not only the DOM concentration, but also the DOM source when analysing acute copper toxicity in *A. tonsa*.

In the present study, the general protective tendency observed was as follow: BSTP-derived DOM > ASTP-derived DOM ≥ SRFA. 48-h LC_{50} values based on total copper without and with addition of BSTP-derived DOM (5 mg C/L) were 60.46 and 255.60 µg Cu/L for salinity 5; 77.44 and 346.35 µg Cu/L for salinity 15; and 94.37 and 371.47 µg Cu/L for salinity 30, respectively. Hence, addition of BSTP-derived DOM (5 mg/L) into the experimental medium protected up to ~ 4-fold against the acute waterborne copper toxicity, in all salinities tested. For ASTP-derived DOM, the same pattern of protection was observed only at salinity 15. In salinity 30, DOM protection was only ~2-fold. An ~2 fold protection was also observed with SRFA, but only at salinity 15. In fathead minnows, a similar 4-fold increase in total copper LC_{50} was observed with DOM addition, as Aldrich humic acid, respect to the treatment without DOM (Erickson *et al.*,1996). These authors reported a 90% copper complexation at 5 mg/L humic acid. Even so, it is important to consider that not only the humic acid fraction can bind metals, but other fractions such as fulvic acid, can also form complexes with metals (Ryan *et al.*, 2004). Therefore, a possible explanation for the different protective effects of DOMs against acute copper toxicity in *A. tonsa* could be associated with differences in their molecule content. In turn, the observed differences in the 48-h LC_{50} values among salinities for each DOM type could be related to the influence of other water chemistry parameters on DOM properties. It is believed that NOM sites characterized as phenolic, not carboxyl sites, account for the majority of copper

complexation under natural water conditions, and Cu-NOM complexation is mainly through the replacement of H^+ by Cu^{2+} at the phenolic binding sites (Lu and Allen, 2002). Nevertheless, this behavior was observed in freshwater, and it can be different in salt water. In fact, it is known that salinity influences the binding between copper and DOM. Lores and Pennock (1998) showed that at salinity 5, binding between copper and DOM (river humic acid) reached a maximum of 28%. However, this binding increased to 60% at salinity 15. 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 (Lores and Pennock, 1998).

Regarding whole body copper accumulation in *A. tonsa*, it was generally similar among all DOC concentrations and salinities tested, irrespective the DOC source employed. It is important to note that copper concentrations used in the accumulation experiments corresponded to the 48-h LC_{50} value for the respective experimental condition, indicating that the level of copper inducing 50% mortality in copepods is similar in all treatments. Therefore, these data corroborate with the BLM premise, *i.e.*, that exists a strong correlation between the amount of metal accumulated at the biotic lingand and its acute toxicity (Santore *et al.* 1999). In the euryhaline copepod *A. tonsa*, a whole body copper accumulation corresponding to 0.877 mg Cu/g dry weight was found when 50% of the tested individuals were dead. This value is ~11-fold higher than that found in non copper-exposed copepods. Therefore, we suggest to apply this value as the lethal accumulation value (LA_{50}) to calibrate a future BLM version for estuarine and marine environments using the euryhaline copepod *A. tonsa* as a model species.

It is known that metals can 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 (Wang and Fisher, 1999). In addition, Hook and Fisher (2001) observed that metals accumulated in copepods by trophic transfer were present in internal tissues. On the other hand, those accumulated by dissolved phase were present primarily on the copepod's exoeskeleton, leading to low or no adverse effects on animals. Despite the fact that copepods were only exposed to waterborne copper, toxicity linked to copper-DOM complexes cannot be ruled out. In fact, Erickson *et al.* (1996) found that ~20% of the copper bound to organic matter, as Aldrich humic acid, was available to cause toxicity in fathead minnows. Despite that metal-NOM complexes are considered too large and too polar to cross biological membranes (Richards *et al.*, 2001), they could be ingested by copepods. For example, the copepod *Acartia spinicauda* was found eating on particles with bound metals (Xu and Wang, 2002). These authors also suggested that fecal pellets associated with metals could also be available for copepods. Glover and Wood (2005) also suggested that observed differences in silver accumulation in the Cladocera *D. magna* could be attributable to ingestion of NOM-silver complexes. These authors reported that even colloidal silver-NOM complexes could potentially reach a size (~0.45 μm) whereby they would be trapped by the filter mesh of the daphnid feeding apparatus, and treated as food particles. However, it is important to take into account that copepods are not filter feeders, and they are able to select food size (Mauchline, 1998). Adult individuals of *A. tonsa* eat preferentially particles between 14 and 250 μm (Berggreen *et al.,* 1988). In addition, calanoid copepods, as those from the genus *Acartia*, can eat on particles normally

rejected if their preferred food is absent. It is important to note that experiments in the present study were performed in the absence of food. Copper-DOM complexes could have reached a size large enough to be selected and ingested by copepods during experiments. Thus, it is reasonable to consider that *A. tonsa* could be ingesting and accumulating copper-DOM complexes along the experimental period.

Findings reported in the present study have important implications for humic ion-binding models like WHAM (Windermere humic aqueous model), which is used at the BLM approach to compute organic speciation (Di Toro *et al.*, 2001). 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 (Di Toro *et al.*, 2001; Paquin *et al.*, 2002; Lu and Allen, 2002; Ryan *et al.*, 2004), although it is widely reported that DOM properties and complexity vary according to different environments (Kramer *et al*., 2004; Ryan *et al.,* 2004). In fact, it was recently demonstrated that the incorporation of the DOM variability in the BLM as a factor controlling copper speciation, bioavailability, and toxicity, improved in a minor way the predictive capacity of this model for *D. magna* (De Shamphelaere *et al*., 2004). In this context, results from toxicity tests performed in the present study clearly showed that, in a broad view, acute waterborne copper toxicity decreased in the presence of DOC. However, the degree of DOC protection was dependent on both DOM source and concentration, highlighting the importance to consider not only the DOM concentration but also the DOM source in a future BLM version to increase its predictive capacity.

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Table 1. 48-h LC_{50} values (µg Cu/L) used to expose copepods for 48 h in the whole body copper accumulation studies with the euryhaline copepod *Acartia tonsa*. BSTP DOM = dissolved organic matter derived from the water collected before the seawage treatment plant ; ASTP = dissolved organic matter derived from the water collected after the effluent discharge of the seawage treatment plant ; SRFA = commercial dissolved organic matter extracted from the Suwannee river.

Experimental condition	**Salinity (ppt)**		
	5	**15**	**30**
Without DOM	41.8	67.4	108.7
BSTP DOM (1.0 mg C/L)	106.6	67.5	106.9
BSTP DOM (2.5 mg C/L)	162.1	154.3	91.2
BSTP DOM (5.0 mg C/L)	233.9	314.2	324.0
ASTP DOM (1.0 mg C/L)	60.6	125.1	101.1
ASTP DOM (2.5 mg C/L)	108.4	145.4	154.6
ASTP DOM (5.0 mg C/L)	70.0	246.1	184.5
SRFA (1.0 mg C/L)	37.6	161.6	46.4
SRFA (2.5 mg C/L)	47.4	121.2	45.2
SRFA (5.0 mg C/L)	72.7	143.4	140.5

Table 2. Water chemistry for the experimental media employed to perform experiments with the euryhaline copepod *Acartia tonsa*. Data are mean ± standard deviation. D.O. = dissolved oxygen. Different letters indicate significant different values ($P<0.05$) among salinities at each parameter.

Parameter	**Salinity (ppt)**		
	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 (mmol/L)	2.73 ± 0.04^{A}	9.07 ± 0.14^{B}	23.32 ± 0.36^{C}
Ca (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}

Figure Legends

Figure 1. Sampling sites where water was collected to extract natural organic matter from the Vieira Stream (Rio Grande, RS, Southern Brazil). 1 = before the "Navegantes" Public Sewage Treatment Plant (BSTP); 2 = about 2 m after the "Navegantes" Public Sewage Treatment Plant (ASTP).

Figure 2. Mortality rates in the euryhaline copepod *Acartia tonsa* in the absence or in the presence of DOM at different salinities. Data are expressed as mean ± standard deviation. Since no significant difference was observed between the different DOM concentrations tested (1.0, 2.5, and 5 mg C/L), only one mean was calculated for each DOM. See text for the different types of DOM tested (BSTP, ASTP, and SRFA).

Figure 3. 48-h LC_{50} values and their corresponding 95% confidence intervals for waterborne copper in the euryhaline copepod *Acartia tonsa* in the absence of DOM at different salinities. Values were calculated based on total measured (A), filtered (B) and free (C) copper concentrations. Different letters indicate significant different values ($P<0.05$).

Figure 4. 48-h LC_{50} values and their corresponding 95% confidence intervals for waterborne copper in the euryhaline copepod *Acartia tonsa* in the presence of BSTP-derived DOM at different salinities. Values were calculated based on total measured (A)

and filtered (B) copper concentrations. Different letters indicate significant different values ($P<0.05$) for each salinity.

Figure 5. 48-h LC_{50} values and their corresponding 95% confidence intervals for waterborne copper in the euryhaline copepod *Acartia tonsa* in the presence of ASTP-derived DOM at different salinities. Values were calculated based on total measured (A) and filtered (B) copper concentrations. Different letters indicate significant different values ($P<0.05$) for each salinity.

Figure 6. 48-h LC_{50} values and their corresponding 95% confidence intervals for waterborne copper in the euryhaline copepod *Acartia tonsa* in the presence of SRFA at different salinities. Values were calculated based on total measured (A) and filtered (B) copper concentrations. Different letters indicate significant different values ($P<0.05$) for each salinity.

Figure 7. Whole body copper concentration in the euryhaline copepod *Acartia tonsa* exposed (48 h) to the 48-h LC_{50} for waterborne copper in the absence of DOM at different salinities. Data are expressed as mean ± standard deviation. Different letters indicate significant different values ($P<0.05$). DW = dry weight.

Figure 8. Whole body copper concentration in the euryhaline copepod *Acartia tonsa* exposed (48 h) to the 48-h LC_{50} for waterborne copper in the presence of BSTP-derived DOM at different salinities. Data are expressed as mean ± standard deviation. Different

letters indicate significant different values ($P<0.05$) among DOM concentrations for each salinity. DW = dry weight.

Figure 9. Whole body copper concentration in the euryhaline copepod *Acartia tonsa* exposed (48 h) to the 48-h LC_{50} for waterborne copper in the presence of ASTP-derived DOM at different salinities. Data are expressed as mean ± standard deviation. Different letters indicate significant different values ($P<0.05$) among DOM concentrations for each salinity. DW = dry weight.

Figure 10. Whole body copper concentration in the euryhaline copepod *Acartia tonsa* exposed (48 h) to the 48-h LC_{50} for waterborne copper in the presence of SRFA at different salinities. Data are expressed as mean ± standard deviation. Different letters indicate significant different values ($P<0.05$) among SRFA concentrations for each salinity. DW = dry weight.

Figure 1

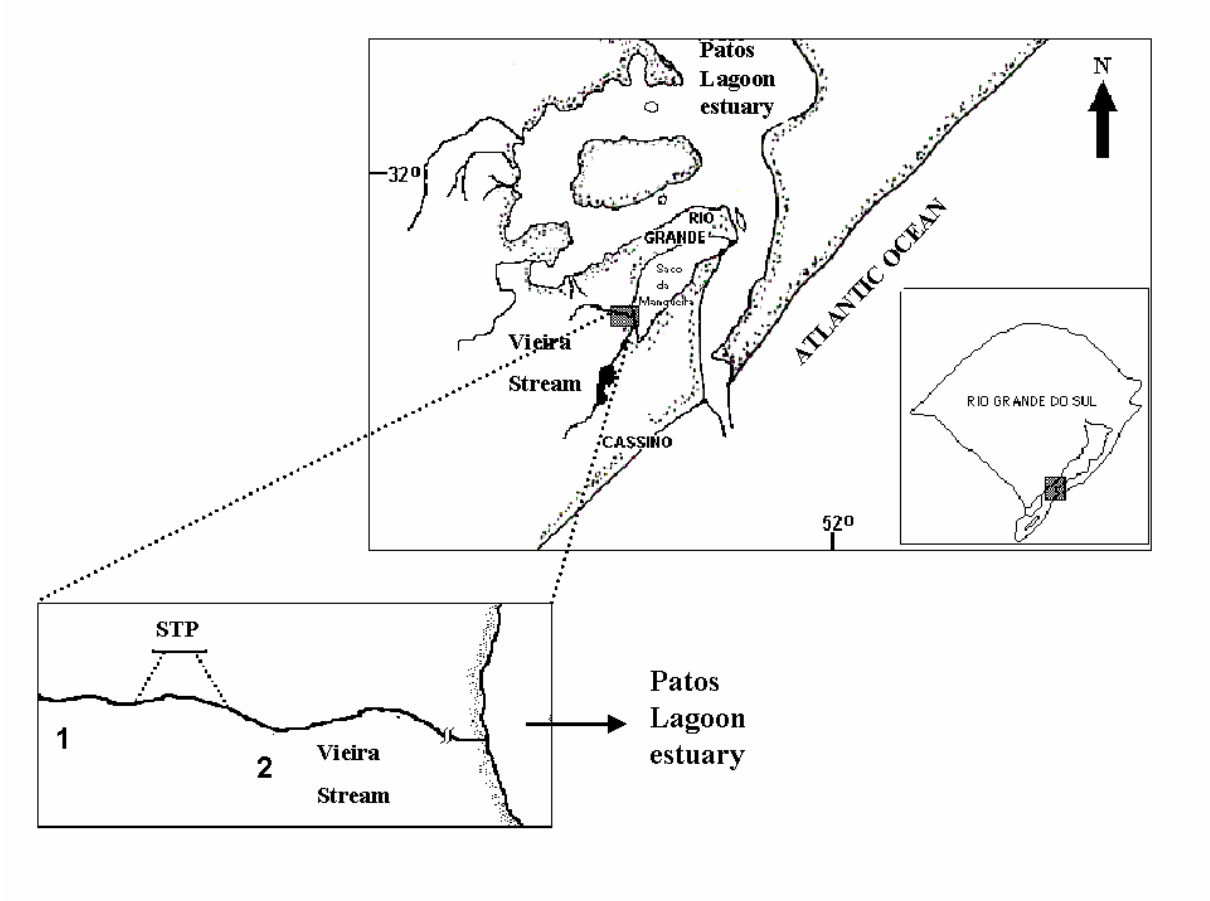

Patos
Lagoon
estuary
N
32º
RIO
GRANDE
Saco
da
Mangueira
ATLANTIC OCEAN
Vieira
Stream
CASSINO
RIO GRANDE DO SUL
52º
STP
1
2
Vieira
Stream
Patos
Lagoon
estuary

Figure 2

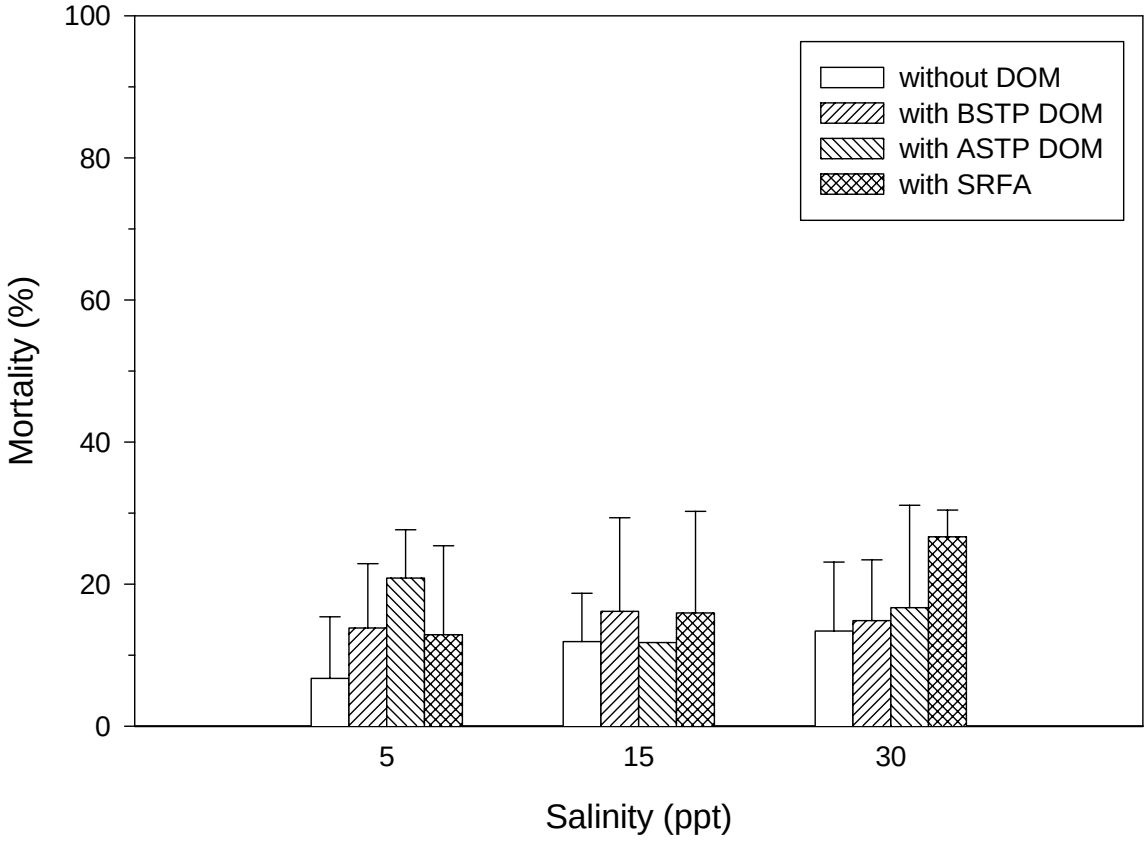

100
80
60
40
20
0
Mortality (%)
without DOM
with BSTP DOM
with ASTP DOM
with SRFA
5
15
30
Salinity (ppt)

Figure 3

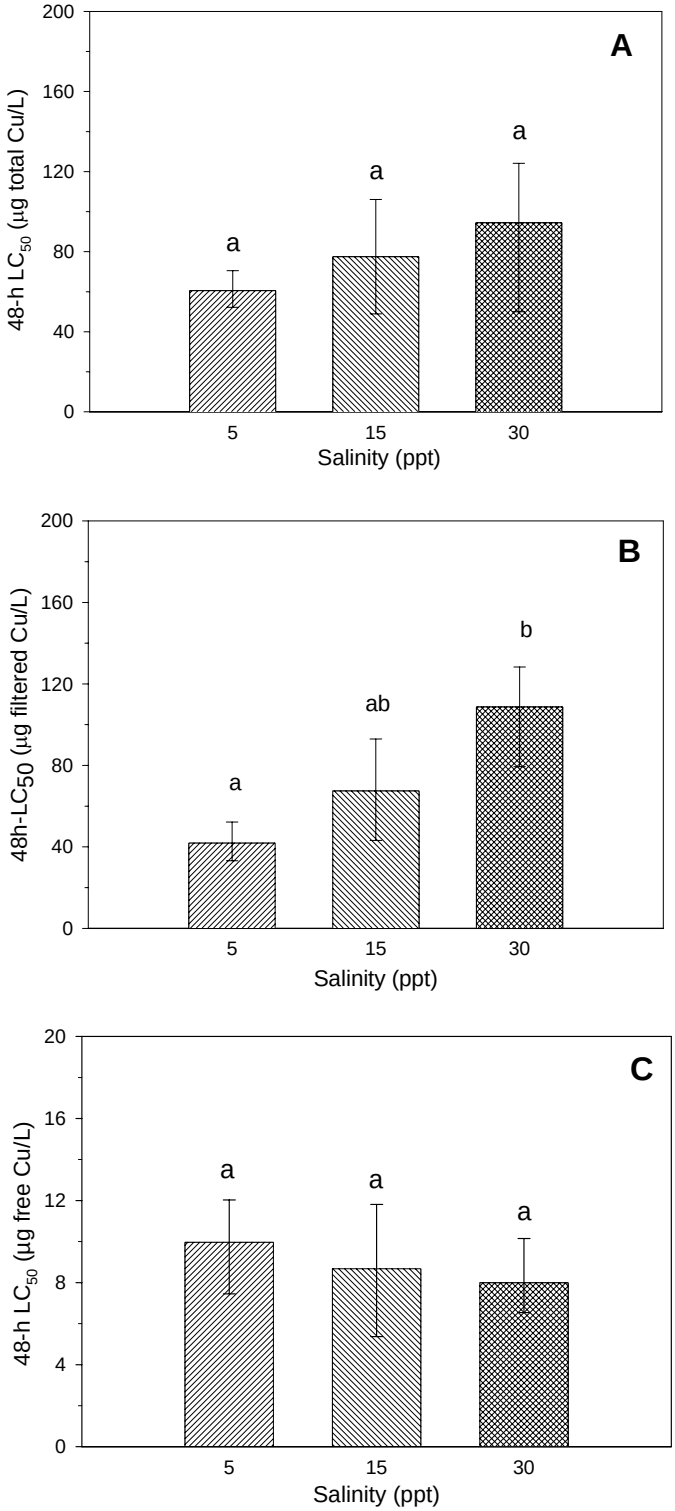
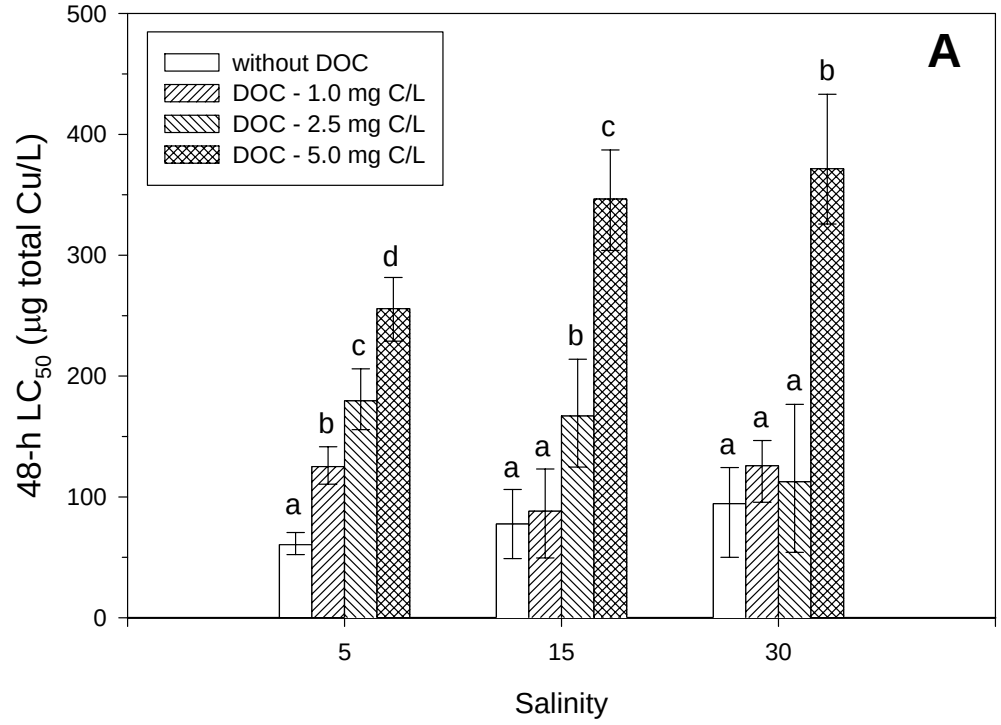

A
48-h LC50 (µg total Cu/L)
200
160
120
80
40
0
a
a
a
5
15
30
Salinity (ppt)
B
48h-LC50 (µg filtered Cu/L)
200
160
120
80
40
0
a
ab
b
5
15
30
Salinity (ppt)
C
48-h LC50 (µg free Cu/L)
20
16
12
8
4
0
a
a
a
5
15
30
Salinity (ppt)

Figure 4


A
without DOC
DOC - 1.0 mg C/L
DOC - 2.5 mg C/L
DOC - 5.0 mg C/L
48-h LC50 (µg total Cu/L)
500
400
300
200
100
0
a
b
c
d
a
a
b
c
a
a
a
b
5
15
30
Salinity

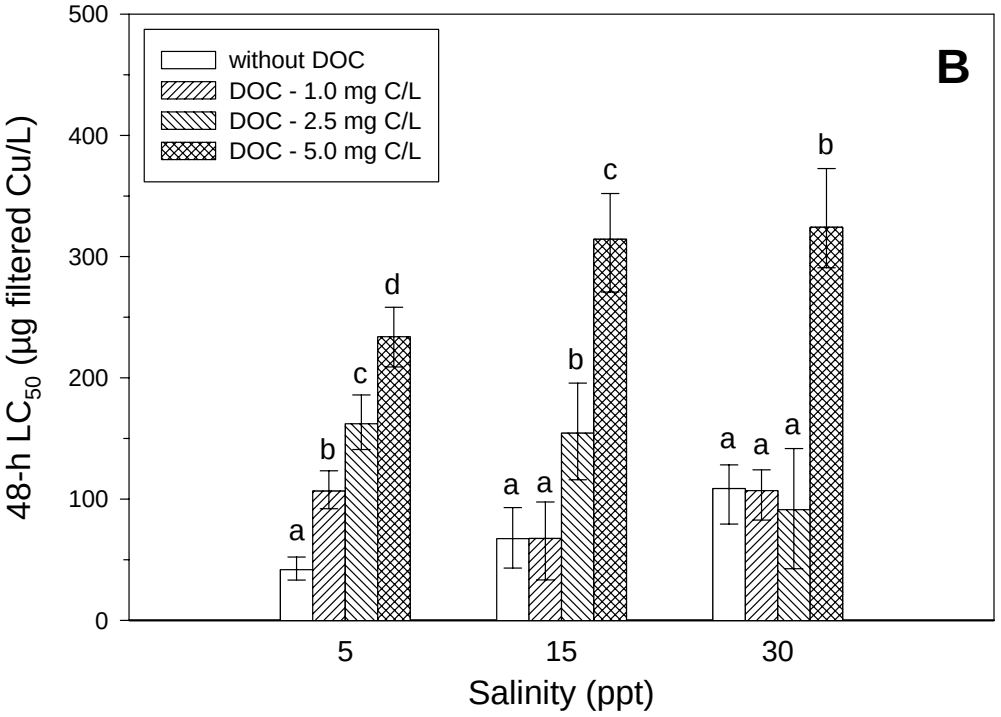

B
without DOC
DOC - 1.0 mg C/L
DOC - 2.5 mg C/L
DOC - 5.0 mg C/L
48-h LC50 (µg filtered Cu/L)
500
400
300
200
100
0
a
b
c
d
a
a
b
c
a
a
a
b
5
15
30
Salinity (ppt)

Figure 5

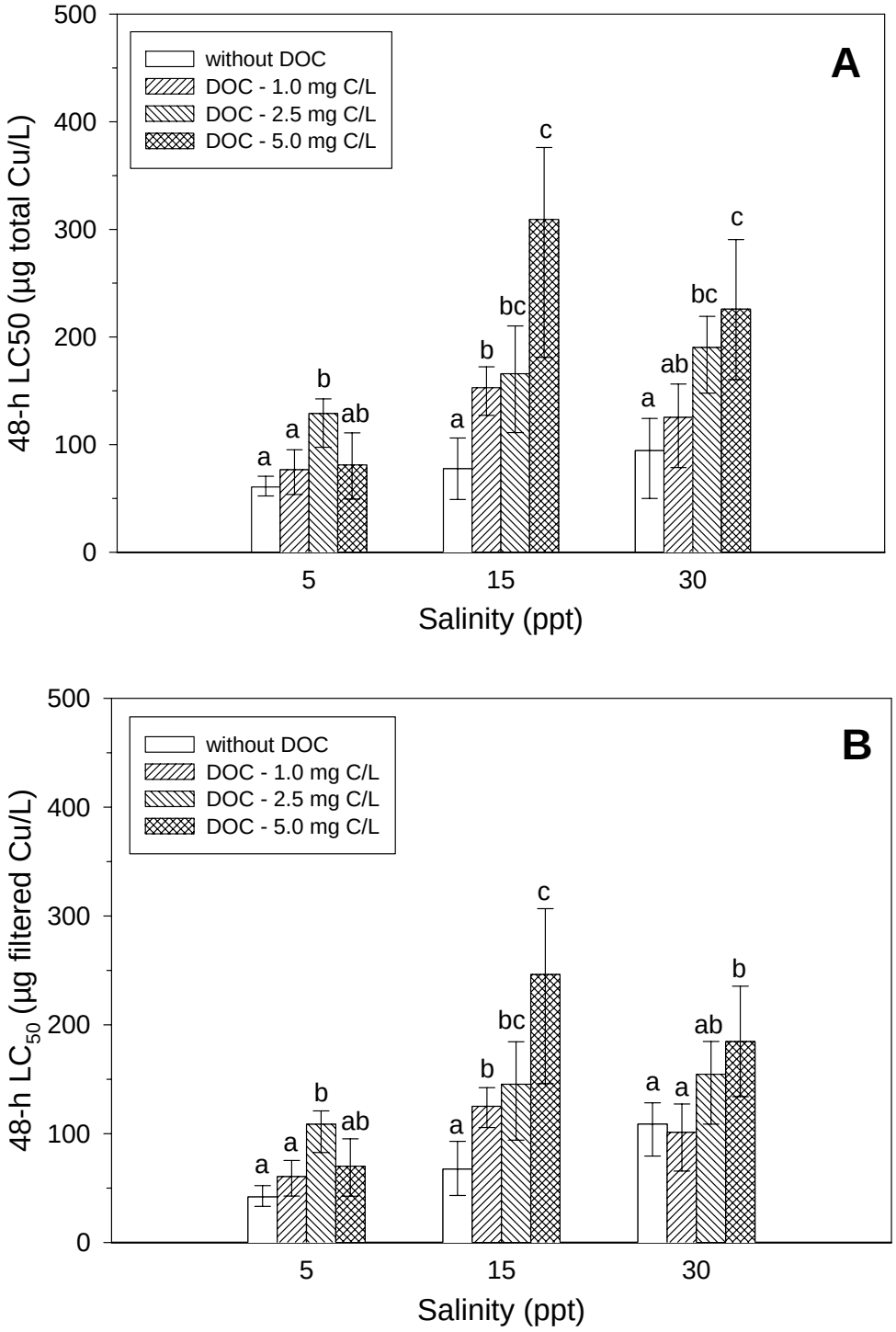
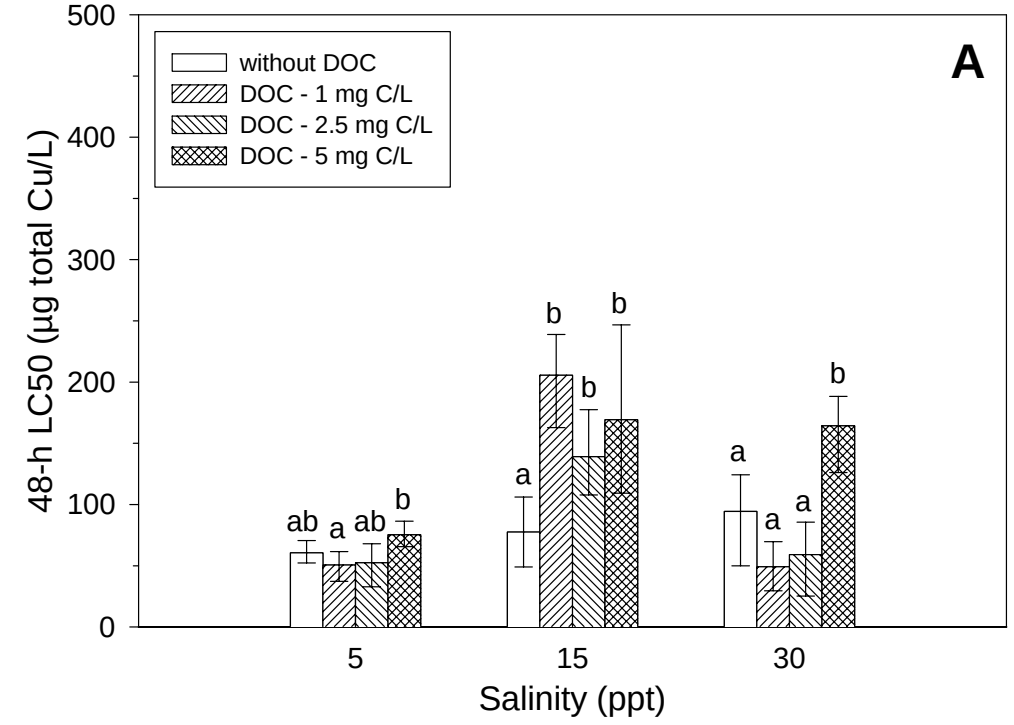

A
without DOC
DOC - 1.0 mg C/L
DOC - 2.5 mg C/L
DOC - 5.0 mg C/L
48-h LC50 (µg total Cu/L)
Salinity (ppt)
B
48-h LC50 (µg filtered Cu/L)
Salinity (ppt)

Figure 6


A
without DOC
DOC - 1 mg C/L
DOC - 2.5 mg C/L
DOC - 5 mg C/L
48-h LC50 (µg total Cu/L)
500
400
300
200
100
0
ab a ab b
a b b
a a b
5
15
30
Salinity (ppt)

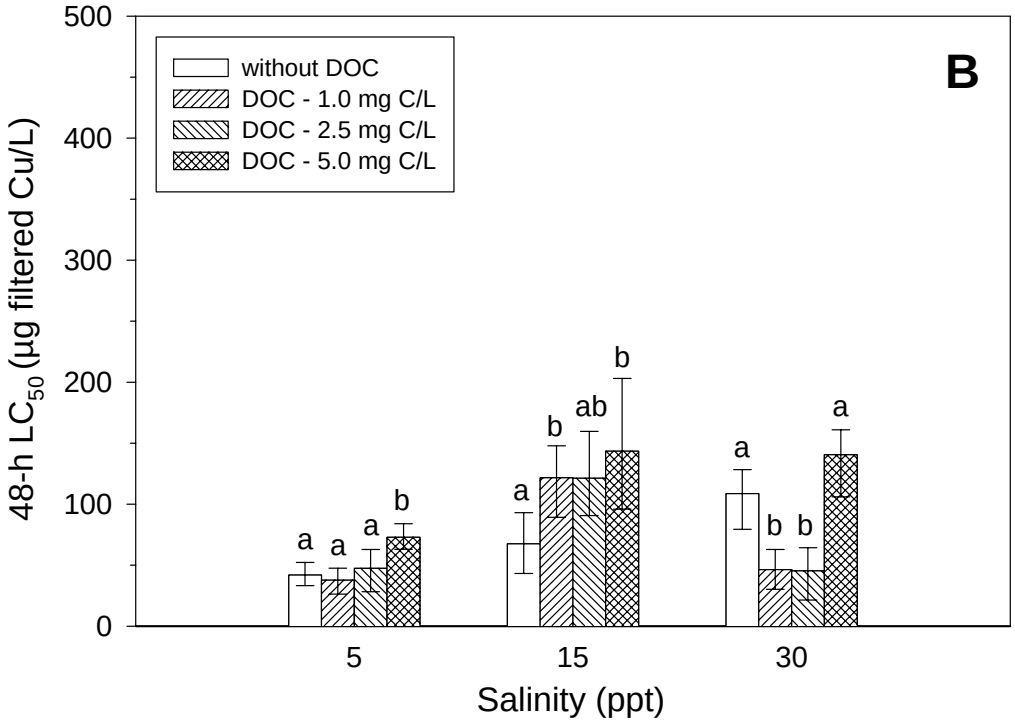

B
without DOC
DOC - 1.0 mg C/L
DOC - 2.5 mg C/L
DOC - 5.0 mg C/L
48-h LC50 (µg filtered Cu/L)
500
400
300
200
100
0
a a a b
a b ab b
a b b a
5
15
30
Salinity (ppt)

Figure 7

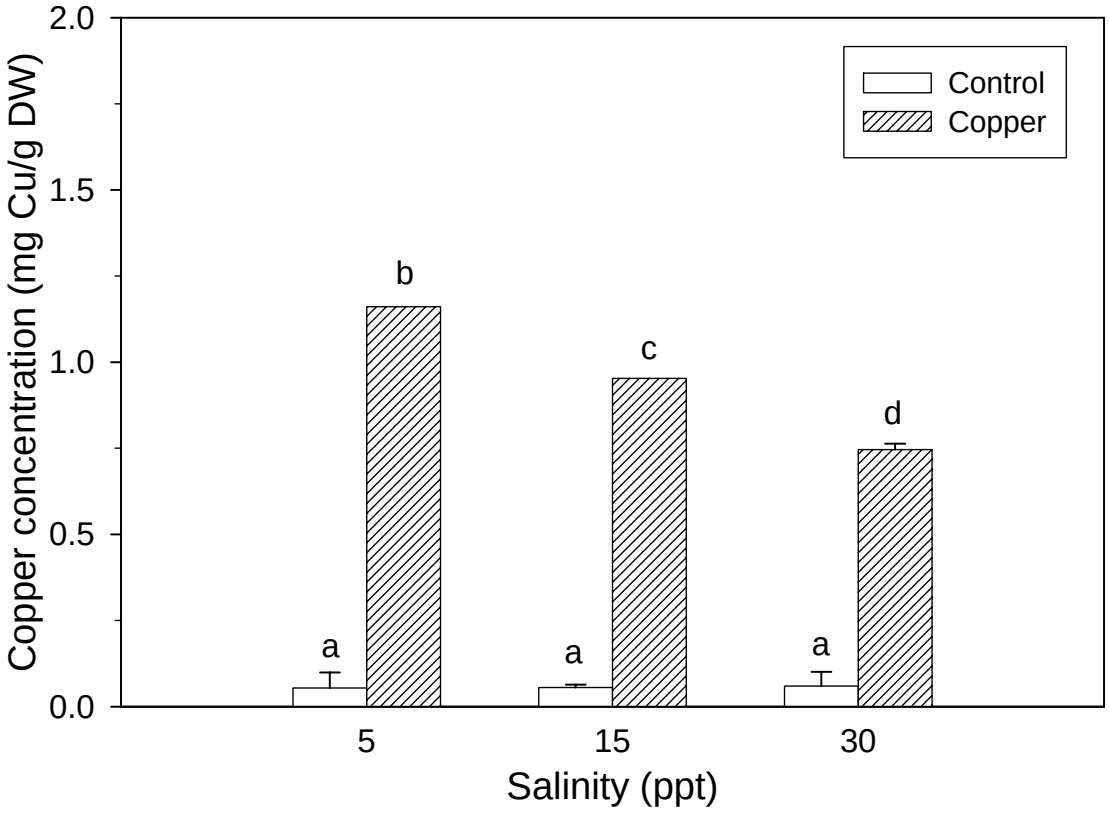

2.0
1.5
1.0
0.5
0.0
Copper concentration (mg Cu/g DW)
Control
Copper
a
b
a
c
a
d
5
15
30
Salinity (ppt)

Figure 8

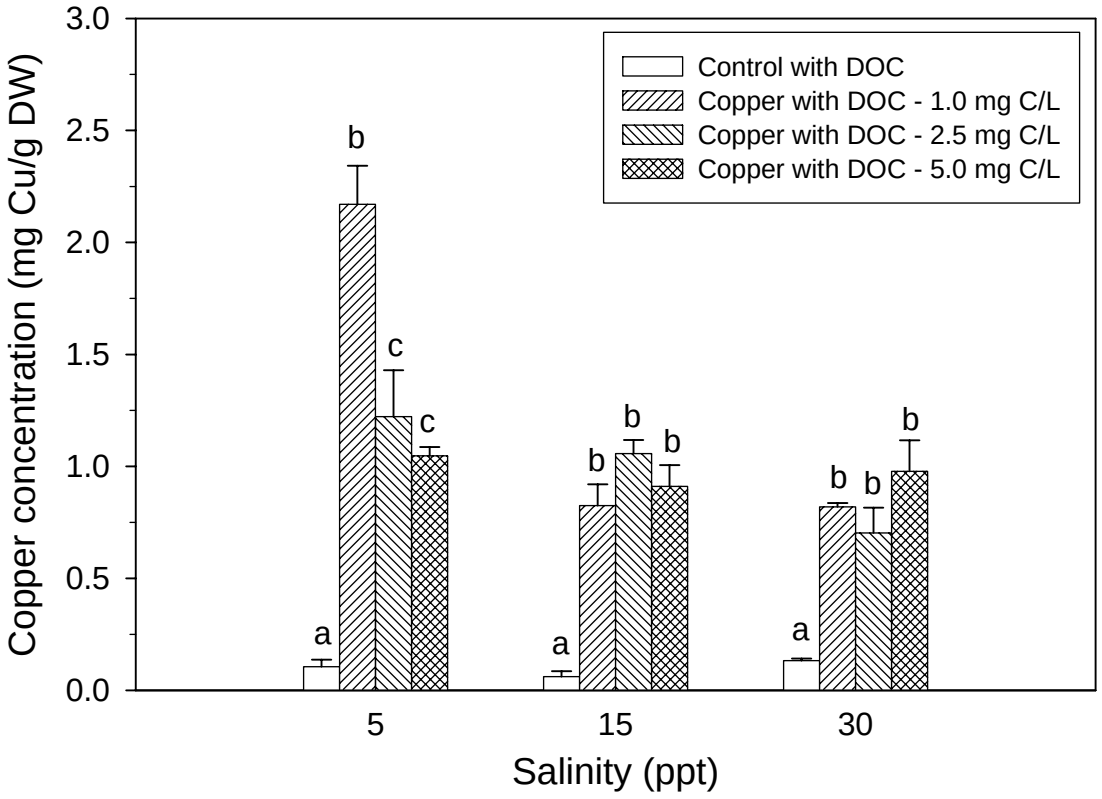

Control with DOC
Copper with DOC - 1.0 mg C/L
Copper with DOC - 2.5 mg C/L
Copper with DOC - 5.0 mg C/L
Copper concentration (mg Cu/g DW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
c
a
b
b
b
a
b
b
b
5
15
30
Salinity (ppt)

Figure 9

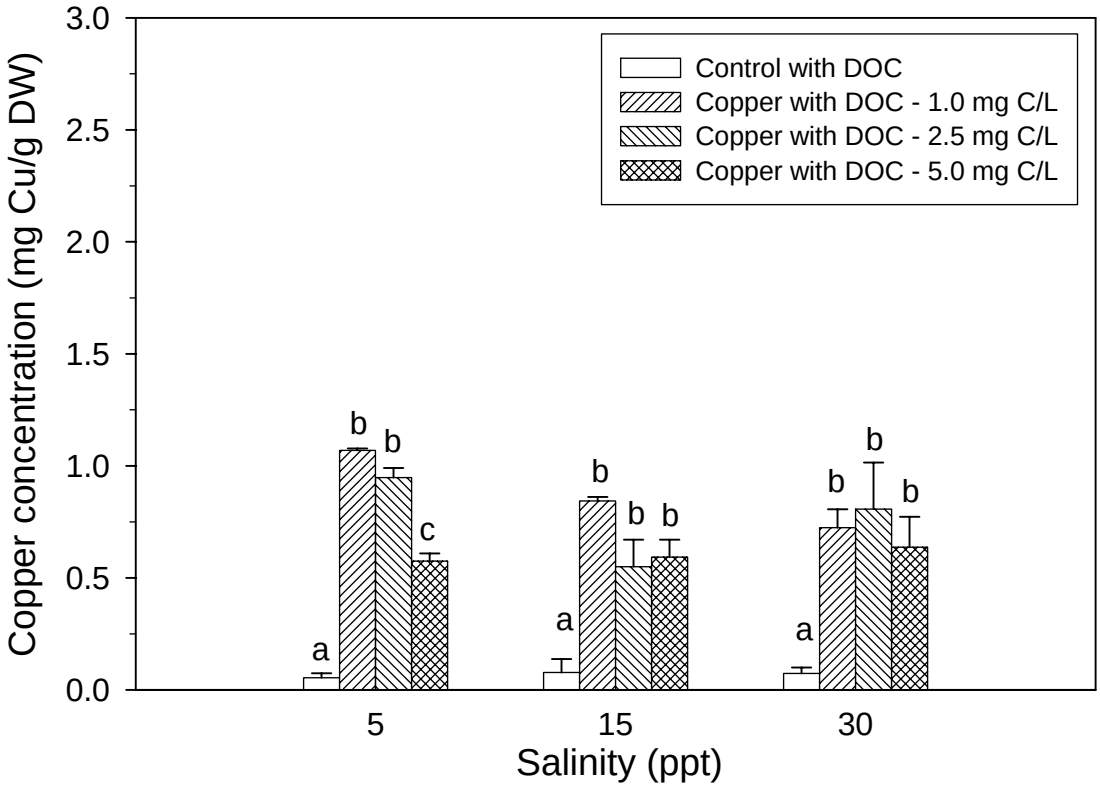
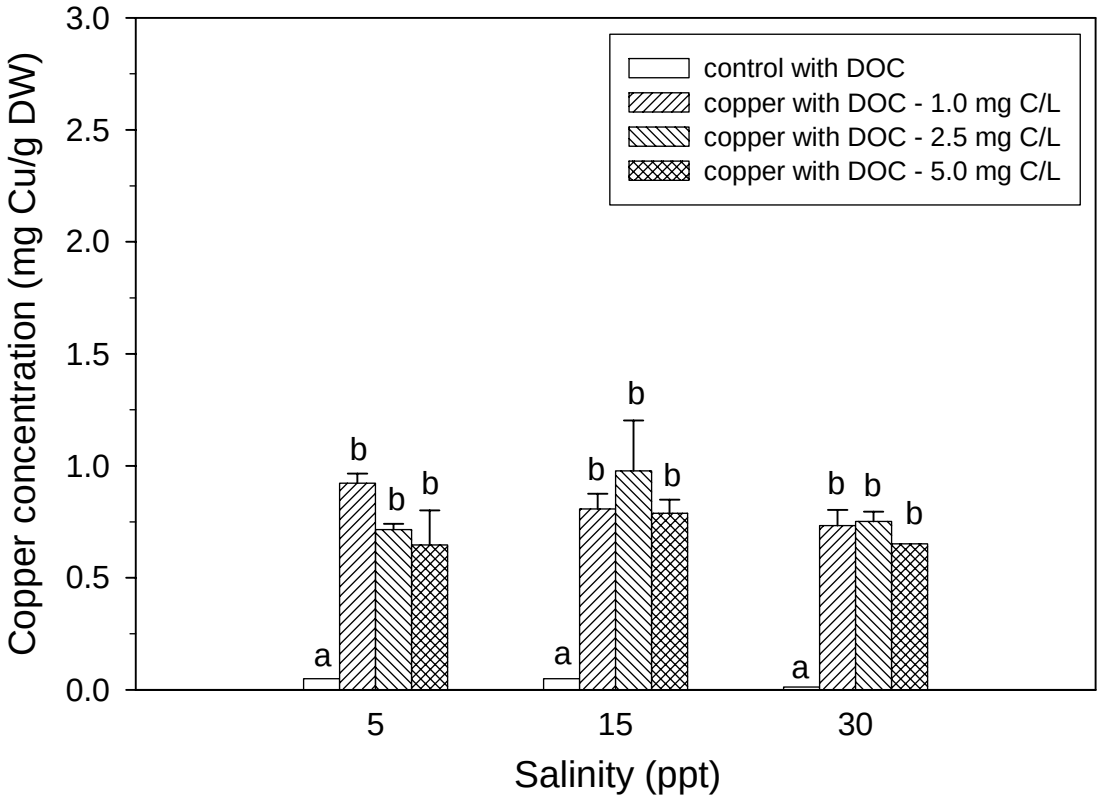

Control with DOC
Copper with DOC - 1.0 mg C/L
Copper with DOC - 2.5 mg C/L
Copper with DOC - 5.0 mg C/L
Copper concentration (mg Cu/g DW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
c
a
b
b
b
a
b
b
b
5
15
30
Salinity (ppt)

Figure 10


Copper concentration (mg Cu/g DW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control with DOC
copper with DOC - 1.0 mg C/L
copper with DOC - 2.5 mg C/L
copper with DOC - 5.0 mg C/L
a
b
b
b
a
b
b
b
a
b
b
b
5
15
30
Salinity (ppt)

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